Investigation of Factors Contributing to Appetite Control and Body Composition in Fully Breastfed Term Infants

Zoya Gridneva (BSc)

This thesis is presented for the degree of Doctor of Philosophy at

The University of Western Australia

School of Molecular Sciences

2017
I, Zoya Gridneva, certify that:

This thesis has been substantially accomplished during enrolment in the degree.

This thesis does not contain material, which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution.

No part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of The University of Western Australia and where applicable, any partner institution responsible for the joint-award of this degree.

This thesis does not contain any material previously published or written by another person, except where due reference has been made in the text.

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The research involving human data reported in this thesis was assessed and approved by The University of Western Australia Human Research Ethics Committee. Approval #: RA/1/4253, RA/4/1/2639.

The work described in this thesis was funded by an unrestricted research grant from Medela AG (Switzerland).

Technical assistance was kindly provided by Ms Wan J. Tie for human milk components analyses that are described in Chapters 6 and 7. Statistical assistance was provided by Ms Anna R. Hepworth for statistical analyses that are described in Chapters 2, 3, 5, 6 and 7.

This thesis contains published work and/or work prepared for publication, some of which has been co-authored.

Signature:

Date: 19 September 2017
This thesis was supervised by Associate Professor Donna Geddes, Emeritus Professor Peter Hartmann, and Professor Mark Spackman (School of Chemistry and Biochemistry, The University of Western Australia, UWA). My candidature was financially supported by a University Postgraduate Award (UPA), UWA safety net Top Up Scholarship and Medela Ad Hoc Scholarship.

The work presented in this thesis is my own work, except where stated. All data collection was carried out in the laboratory of Hartmann Human Lactation Research Group at the King Edward Memorial Hospital for Women and Children. All experimental work was carried out in the laboratory of Hartmann Human Lactation Research Group in the School of Molecular Sciences, Faculty of Science, UWA. The material presented in this thesis has not been presented for any other degree.

This thesis is presented as a series of scientific papers; five of which are published (Chapters 2, 3, 4, 5 and 6), and one is in preparation for publication (Chapter 8). Permission has been granted by co-authors for inclusion of their work in this thesis, and their contribution is outlined in the Authorship Declaration: Co-authored Publications (pages 16-18). Chapters 2 - 6 are identical to the published/submitted work. The work of this thesis has also been presented at scientific conferences in both oral and poster formats, as outlined in Publications and Awards (pages 9-15).
ABSTRACT

Background

There is clear evidence that early nutrition plays a role in developmental programming of the infant affecting risk of metabolic disease later in life. Human milk (HM) provides a degree of protection from obesity despite radical changes in the human diet in the past 60 years. Research suggests this is mediated by factors such as the less rapid growth of the breastfed infant, HM composition and development of neural pathways involved in appetite control. As in utero where adipogenesis is influenced by maternal diet it is plausible that adipogenesis is further impacted by nutrition in early infancy. Information on effect of HM components on infant appetite control and body composition (BC) is extremely limited, mostly due to the lack of well-designed studies.

Aims

In this study we aimed to investigate mechanisms involved in regulation of appetite control and development of BC of breastfed infants in a series of cross-sectional studies and longitudinal using cost-effective, non-invasive techniques adapted for the use in infants in our laboratory, to determine relationship between HM composition, feeding frequency, gastric emptying (GE) and BC as well as the influence of maternal BC on HM composition. We also investigated the effects of the feed volume on resistance measures in breastfed infants to establish the feasibility of using these values interchangeably during data collection, and evaluated and compared two methods of measuring infant body composition: ultrasound skinfolds (US) and bioelectrical impedance spectroscopy (BIS).

Methods

Accurate, non-invasive and inexpensive methods are needed for monitoring of infant BC. BIS is predominantly used in adults and prior to use in pediatric population method standardization and evaluation are required. To assess the effect of the feed volume, feed duration, and the volume of the infant’s stomach and bladder on the resistance values pre-/post-feed, 48 breastfeeding infants were measured with BIS at 2, 5, 9 and/or 12 months (n=62 sessions) before the start and after the end of breastfeed. Resistance measurements and impedance indices at key frequencies were analyzed. Feed volume was measured by test weights. Free-water volumes and free-water change were determined from stomach and bladder volumes calculated from ultrasound images.

To evaluate the performance of US and BIS in determining percentage fat mass (%FM) in term infants, 58 breastfed infants were measured at of 2, 5, 9 and 12 months,
ABSTRACT

using BIS-derived total body water equations and skinfold equations %FM was calculated and compared to reference models. Skinfolds were measured with ultrasound at two and four sites (biceps, suprailiac and/or triceps, subscapular).

To ensure that the physiological levels of HM leptin are analyzed, we optimized an enzyme-linked immunosorbent assay for leptin measurement in both whole and skim HM and compared leptin levels between both HM preparations of milk samples collected from 61 lactating mothers at the 2nd, 5th, 9th and/or 12th month of lactation, in which concentrations of adiponectin, fat, lactose, total carbohydrate, lysozyme, total, casein and whey protein were also measured for assessment of the effect of maternal BC (%FM measured using BIS) on HM composition.

Next we analysed the effect of HM appetite hormones and macronutrients and demographics, anthropometrics and BC of term fully breastfed infants (n=41, 2 and/or 5mo) on GE of a single breastfeed. Stomach volumes were calculated from ultrasound scans measured pre-/post-feed then repeatedly until the next feed. Feed volume was measured by test-weigh method.

Finally, BC of breastfeeding dyads (n=20) and 24-h feed volume and feeding frequency were measured at 2, 5, 9 and/or 12 months after birth with US (infants) and BIS (infants and mothers) and associations between maternal and breastfed term infant anthropometrics and BC and feeding parameters, concentrations and CDI of HM components in first 12 months of life were established.

Results

No significant effect of infant feed volume or free-water change was detected. Small pre- to post-feed resistance changes were not significantly different from zero (p>0.097), ensuring flexibility in the timing of measurements of infants in the research setting.

Both BIS and US are practical for predicting %FM in infants. While %FM values calculated from ultrasound and BIS were not significantly different (p=0.35), %FM differed widely within and between methods, with the degree of variation affected by infant age/sex. No one method/equation of determining %FM was consistent with the distributions of appropriate reference values for all age/sex groups. Moderate number of matches with references values was seen for both types of equations and high number of matches was seen for US-based equations, while BIS calculations were highly dependent upon an appropriate set of validated age-matched equations.
ABSTRACT

No association was found between whole HM leptin and fat content \((p=0.17)\), and no difference in whole or skim HM leptin concentration was found between pre- and post-feed samples \((p>0.29)\). Whole HM contained, on average, 0.24±0.01 ng/mL more leptin than skim HM \((p<0.001)\), highlighting the importance of optimizing HM leptin measurement and assaying it in whole HM to accurately examine the amount of leptin received by the infant during breastfeeding.

Higher maternal %FM was associated with higher leptin concentrations in both whole \((p=0.008)\) and skim HM \((p=0.007)\), and protein \((p=0.028)\) concentrations in cross-sectional cohort \((n=59)\); while higher weight, fat-free mass and fat mass were associated with higher whole milk leptin \((p<0.044)\), weight and fat-free mass were also associated with whey protein \((p<0.040)\) in the longitudinal cohort \((n=20)\). Adiponectin and other measured components concentrations were not associated with maternal BC \((p>0.054)\). Further whole milk concentrations of adiponectin and leptin did not differ significantly over the first year of lactation.

In the longitudinal study of infant GE higher feed volumes were associated with faster GE rate \((p<0.001)\), higher post-feed stomach volumes \((p<0.001)\) and longer GE times \((p=0.033)\). Higher whey protein concentration was associated with higher post-feed stomach volumes \((p=0.023)\). Longer GE time was associated with higher adiponectin concentration and dose \((p<0.005)\) and lower casein:whey ratio \((p=0.003)\), while the casein:whey ratio and lactose concentration modified the GE curve depending on feed volume \((p<0.037)\).

Lastly, in the longitudinal study of infant BC higher 24-h feed volume was associated with higher infant fat mass (FM) \((p=0.014)\) and %FM \((p=0.033)\) and higher feeding frequency \((p<0.001)\). Higher feeding frequency was associated with higher %FM \((p=0.045)\) and CDI of whole milk leptin, total and whey protein, total carbohydrates, lactose and lysozyme \((p<0.020)\).

Higher concentrations of adiponectin were associated with lower infant fat-free mass (FFM) \((p=0.040)\) while higher CDI were associated with lower FFM \((p=0.016)\) and higher FM \((p=0.049)\) and skinfolds \((p<0.042)\). Higher whole milk leptin concentrations were associated with lower infant biceps thicknesses \((p=0.021)\).

Higher concentrations of casein were associated with larger skinfolds \((p<0.039)\) while higher CDI were associated with lower length \((p=0.049)\) and FFM \((p<0.038)\) and higher FM \((p=0.024)\) and %FM \((p=0.044)\).
ABSTRACT

Higher total carbohydrate concentrations were associated with lower %FM ($p=0.014$) and longer ($p<0.001$), heavier ($p=0.005$) infants with higher FFM ($p=0.034$) while higher CDI were associated with higher %FM ($p=0.011$) and FM ($p=0.030$), with calculated concentrations of oligosaccharides and CDI of lactose and oligosaccharides following the same patterns.

Higher CDI of lysozyme were associated with higher infant BMI ($p=0.025$), %FM ($p=0.046$) and FM ($p=0.023$).

Conclusions

The findings of this thesis suggest that the level of maternal adiposity during lactation may influence concentrations of HM components, which in turn potentially impact early appetite programming of term breastfed infants by modulating GE and breastfeeding patterns (milk intake, feeding frequency).

Concentrations and doses of HM components in combination with breastfeeding patterns and GE influence the development of infant BC in the first year of life. This time is a critical window of infant programming and HM potentially influences risk of later disease via modulation of BC. This thesis elucidated the multiple ways of the lactocrine programming in breastfed infant, indicating that several possibilities for intervention exist in this important period of life to improve the infant outcomes.
ACKNOWLEDGMENTS

“Choose a job you love, and you will never have to work a day in your life.”
Confucius

This thesis is a result of many collaborations and tremendous support from many people around me.

First I would like to acknowledge my supervisors. I am very grateful to Emeritus Professor Peter Hartmann, who decisively persuaded me to enter into PhD game at the later stage of my life, complicated by the presence of children. He guided me, personally, professionally, and with great degree of humour, through my time at UWA, and reminded at all times that greatest achievements are possible. I am enormously thankful to Associate Professor Donna Geddes who made this research possible and led me through any hiccups, personal or professional, giving the kind of support every human should receive, not just students lucky enough to have Donna as a supervisor. Also special thank you to Winthrop Professor Mark Spackman for his expertise and advice and ensuring that my PhD run smoothly. Huge thank you to Emeritus Professor Leigh Ward for showing us “the ropes” and ensuring that we are on the right path in the tricky field of infant body composition.

Big thank you to The University of Western Australia for providing financial support (APA) and to Medela AG (Switzerland) for Ad Hoc Scholarship and unrestricted research grant; these resources made this study possible.

Thank you to all the members of the Hartmann Human Lactation Research group, past and present, for professional and personal support and friendship. Huge thank you to Ching-Tat Lai for always “can do” attitude and all the technical magic. Enormous thank you to Sambavi Kugananthan, Wan Jun Tie and Anna Cannon for making it possible by processing and analysing gigantic amounts of samples and helping with writing of the manuscripts. Thank you to Anna Hepworth for your statistical expertise and education, making sure that I will not be lost in the dark. Thank you to Jackie Kent for mentoring and timely advice, and to Jacki McEachran and Ruth Abbot for all the support and assistance. Thank you to Jian Du and Melvin Gay for authoring two papers with me, giving an extra edge to my research. Thank you to Hazel Gardner, Alecia-Jane Twigger, Sharon Perrella, Gwendoline Kueffer, Donna Savigni, Mohammed Alsaweed, Vanessa Sakalidis, Alex George and all of you already mentioned above for your help, friendly advise and much needed debriefing sessions.
AKNOWLEDGEMENTS

Many thanks are extended to all the mothers and members of the Australian Breastfeeding Association and their infants; your valuable time, milk, enthusiasm and excitement towards human milk research are the foundation of this thesis.

I want to say a big thank you to all my friends, but in particular to Kate Concanen and Helen Mountain, who have decided that I should not waste my talent and nudged me out of the comfort of motherhood, changing my life to becoming extremely interesting, and killing domestic goddess in the process. Also a huge thank you to my friend Olga Sidorenko, who was so supportive and took care of me during this candidature, making sure that I am resting and/or shopping enough.

Lastly I want to thank my family, for giving me all the support, love, hugs and kisses I needed on this journey. Thank you to my daughters Vera and Audrey Ptolemy for inspiring me. To Vera, for always believing in me and showing respect and admiration towards my little and big successes, and to Audrey, for being near almost at each step of my PhD, since she never knew me not studying. To my dog Cherry, for always being happy to see me when I am coming home late and for being a “hot water bottle” on my lap during long and cold writing or statistical sessions. And, of course, enormous thank you to my partner David Ptolemy, who also made this research possible, practically, emotionally and financially. David has an understanding of importance of breastfeeding, which every partner should have, and he showed it, firstly, during my lengthy breastfeeding career through the support and care we received, and secondly, in the years and particularly last months of my PhD, when he almost totally replaced me at home and let me to concentrate on finishing this amazing journey. I dedicate this thesis to you, David.

Lots of love and gratitude,

Zoya Gridneva
PUBLICATIONS AND AWARDS

The articles, conference papers and seminars that have arisen from and awards received for work done during and under the scope of this thesis are presented below:

JOURNAL PAPERS


PUBLICATIONS AND AWARDS

ADDITIONAL JOURNAL PAPERS


CONFERENCE PAPERS


PUBLICATIONS AND AWARDS


PUBLICATIONS AND AWARDS


SEMINARS


PUBLICATIONS AND AWARDS

AWARDS

1. Centre for Neonatal Research and Education Student Award at the Perinatal Society of Australia and New Zealand Student Symposium ($200), 2016

2. The Nestle Nutrition Institute Prize for “making a difference through translational research that is aimed at improving early life outcomes” at the Developmental Origins of Health and Disease Society of Australia and New Zealand Annual Conference ($2,500), 2016

3. Medela Top Up Award ($2,500 per annum), January 2014 – February 2016

4. Graduate Research School Travel Award ($1,850), 2014
AUTHORSHIP DECLARATION: CO-AUTHORED PUBLICATIONS

This thesis contains work that has been published or prepared for publication. The author, Zoya Gridneva, completed the majority of the work presented in this thesis. However other individuals require acknowledgement for their contributions to each chapter and the publications arising from this thesis. All co-authors critically reviewed all of the manuscripts. Details of the work:

Chapter 1

Extent of contribution by the author is 90%. This Chapter was written by Zoya Gridneva and edited by Associate Professor Donna T. Geddes and Emeritus Professor Peter E. Hartmann.

Chapter 2

Extent of contribution by the author is 60%. The author conceived and designed of the study, collected, visually examined and summarised the data, performed the statistical analyses, wrote the manuscript; Ms Anna R. Hepworth was involved in research design, data analysis and interpretation; Emeritus Professor Leigh C. Ward provided technical and educational support and contributed to design of the study; Assistant Professor Ching-Tat Lai provided technical support; Emeritus Professor Peter E. Hartmann substantially contributed to conception and design of the study and contributed materials/analysis tools for research; Associate Professor Donna T. Geddes conceived and designed of the study, carried out the ultrasonography component of the study and provided the ultrasound images.

Chapter 3

Extent of contribution by the author is 60%. The author conceived and designed of the study, collected, visually examined and summarised the data, performed the statistical analyses, wrote the manuscript; Ms Anna R. Hepworth was involved in research design, data analysis and interpretation; Emeritus Professor Leigh C. Ward provided technical and educational support and contributed to design of the study; Assistant Professor Ching-Tat Lai provided technical support; Emeritus Professor Peter E. Hartmann substantially contributed to conception and design of the study and contributed materials/analysis tools for research; Associate Professor Donna T. Geddes conceived and designed of the study and carried out the ultrasonography component of the study, provided the ultrasound images.
AUTHORSHIP DECLARATION: CO-AUTHORED PUBLICATIONS

Chapter 4
Extent of contribution by the author is 40%. The author conducted experiments, collected the data, assisted with design of the study, biochemical analysis and writing of the manuscript; Ms Sambavi Kugananthan designed the study, conducted experiments and data analyses, interpreted results, and wrote the manuscript; Assistant Professor Ching-Tat Lai conducted experiments and data analyses; Dr Peter J. Mark designed the study and interpreted results; Associate Professor Donna T. Geddes designed of the study and interpreted results. Assistant Professor Foteini Kakulas designed the study and interpreted results.

Chapter 5
Extent of contribution by the author is 40%. The author conceived and designed the study, conducted experiments, collected the data, conducted data analysis and interpretation, and wrote the manuscript; Ms Sambavi Kugananthan designed the study, conducted experiments and data analyses, interpreted results, and wrote the manuscript; Assistant Professor Ching-Tat Lai conducted experiments; Ms Anna R. Hepworth provided consultation for research design and conducted data analysis and interpretation; Dr Peter J. Mark designed the study and interpreted results; Associate Professor Donna T. Geddes designed of the study, collected the data and interpreted results. Assistant Professor Foteini Kakulas designed the study and interpreted results.

Chapter 6
Extent of contribution by the author is 70%. The author conceived and designed of the study, collected the data, performed biochemical analysis, performed the statistical analyses, wrote the manuscript; Ms Sambavi Kugananthan performed the biochemical analysis; Ms Anna R. Hepworth was involved in research design, data analysis and interpretation; Ms Wan J. Tie performed biochemical analysis; Assistant Professor Ching-Tat Lai performed biochemical analysis and provided technical support; Emeritus Professor Leigh C. Ward provided technical and educational support and reagents/materials/analysis tools for research; Associate Professor Donna T. Geddes conceived and designed of the study, carried out the ultrasonography component of the study and provided the ultrasound images.
AUTHORSHIP DECLARATION: CO-AUTHORED PUBLICATIONS

Chapter 7

Extent of contribution by the author is 70%. The author conceived and designed of the study, collected the data, performed biochemical analysis, performed the statistical analyses, wrote the manuscript; Ms Sambavi Kugananthan performed the biochemical analysis; Ms Anna R. Hepworth was involved in research design, data analysis and interpretation; Emeritus Professor Leigh C. Ward provided technical and educational support and contributed to design of the study; Ms Wan J. Tie performed biochemical analysis; Assistant Professor Ching-Tat Lai performed biochemical analysis and provided technical support; Emeritus Professor Peter E. Hartmann substantially contributed to conception and design of the study and contributed reagents/materials/analysis tools for research; Associate Professor Donna T. Geddes conceived and designed of the study, carried out the ultrasonography component of the study and provided the ultrasound images.

Chapter 8

Extent of contribution by the author is 90%. This Chapter was written by Zoya Gridneva and edited by Associate Professor Donna T. Geddes and Emeritus Professor Peter E. Hartmann.

Student signature:

Zoya Gridneva
Date: 19 September 2017

I, Donna T. Geddes certify that the student statements regarding their contribution to each of the works listed above are correct.

Coordinating supervisor signature:

Date: 19 September 2017
**ABBREVIATIONS AND UNITS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ACSA</td>
<td>antral cross sectional area</td>
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<tr>
<td>ADP</td>
<td>whole-body air-displacement plethysmography</td>
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<td>AgRP</td>
<td>agouti-related peptide</td>
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<td>APT</td>
<td>applied potential tomography</td>
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<td>BC</td>
<td>body composition</td>
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<td>BDNF</td>
<td>brain-derived neurotrophic factor</td>
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<td>BCM</td>
<td>body cell mass</td>
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<td>BD</td>
<td>body density</td>
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<td>BIA</td>
<td>bioelectrical impedance analysis</td>
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<td>BIS</td>
<td>bioelectrical impedance spectroscopy</td>
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<td>bone mineral content</td>
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<td>body mass index</td>
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<td>BMI for age</td>
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<td>CCK</td>
<td>Cholecystokinin</td>
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<td>CDI</td>
<td>calculated daily intake</td>
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<td>CI</td>
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<td>cm</td>
<td>centimeter</td>
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<td>CV</td>
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<td>DPA</td>
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<td>dual-energy x-ray absorptiometry</td>
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<td>extracellular fluid</td>
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<td>epidermal growth factor</td>
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<td>ELISA</td>
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<td>glial cell line-derived neurotrophic factor</td>
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<tr>
<td>HGF</td>
<td>hepatocyte growth factor</td>
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<td>NCD</td>
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<td>NEFA</td>
<td>nonesterified fatty acids</td>
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<td>ng</td>
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<td>NPY</td>
<td>neuropeptide Y</td>
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<td>ohm</td>
<td>resistance value, Ω</td>
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<td>Proopiomelanocortin</td>
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<td>R</td>
<td>Resistance</td>
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<td>Radioimmunoassay</td>
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<td>secretory immunoglobulin A</td>
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<td>$T_{1/2}$</td>
<td>gastric emptying half time</td>
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<td>TBK</td>
<td>total body potassium</td>
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<td>TBW</td>
<td>total body water</td>
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<td>TE</td>
<td>technical error</td>
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<td>TGFβ</td>
<td>transforming growth factors beta</td>
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TNF-α  tumor necrosis factor alpha
TOBEC  total body electrical conductivity
US     Ultrasound
VEGF   vascular endothelial growth factor
WAZ    weight for age
WHO    World Health Organization
WLZ    weight for length
Z      Impedance
α      Alpha
β      Beta
κ      Kappa
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*Part 1.3 “Appetite hormones and appetite control” includes material also presented in an
human milk—what is their role. *Australian Biochemist*, April 2017, 5-7]
1.1 INTRODUCTION

An epidemic of childhood obesity and non-communicable diseases (NCD) associated with it are of increasing international concern (Wells et al. 2016). The lifelong risk of NCD could be modified through early nutrition and other environmental cues during a period from conception to early childhood (Koletzko et al. 2014). Scientists from many countries, including Australia, are working together to investigate how nutrition, during this sensitive period of developmental plasticity, impacts cytogenesis, organogenesis, metabolic and endocrine responses and gene expression and affects health later in life (Figure 1.1).

![Figure 1.1 Nutritional and metabolic factors acting during sensitive time periods of developmental plasticity before and after childbirth modulate cytogenesis, organogenesis, metabolic and endocrine responses, and epigenetic regulation of gene expression and thereby can induce metabolic programming of lifelong health and disease risk. Reproduced by permission from S. Karger AG, Basel (Koletzko et al. 2014).](image)

A major research focus seeks to elucidate the developmental origins of adiposity and obesity and their health outcomes later in life, since convincing evidence exists of early programming effects on obesity and adiposity (Koletzko et al. 2014). The likely causes of the early life origins of adiposity and health problems associated with obesity hypothesized are: the fuel-mediated in utero hypothesis, the accelerated postnatal
growth hypothesis and the mismatch of pre- and postnatal growth trajectories hypothesis (Figure 1.2).

Figure 1.2 The current key hypotheses on early metabolic programming of adiposity and related diseases explored by the EarlyNutrition project include the fuel-mediated in utero hypothesis, the accelerated postnatal growth hypothesis, and the mismatch of pre- and postnatal growth trajectories hypothesis. Reproduced by permission from S. Karger AG, Basel (Koletzko et al. 2014).

Postnatal feeding choices offer window of opportunity to prevent obesity. It is evident that breastfed infants are at 15–20% reduced risk of obesity and obesity related disease later in life (Weng et al. 2012; Koletzko et al. 2009b), however the protective mechanisms of breastfeeding are not fully understood (Thompson 2012). The suggestion that a deviation from the optimal growth trajectory in early infancy may have a significant effect on health of adults later in life is widely hypothesized (Hypponen et al. 1999; Andersson et al. 2001; Stettler et al. 2003; Stettler et al. 2005) and the obesity preventive effect has been linked to the different growth pattern of breastfed infants which results in reduced weight gain in the first 2 years of life compared to formula fed infants. Rapid weight gain has been associated with both an increased risk of obesity (Druet et al. 2011) and the elevated protein content in infant formula (Luque et al. 2015; Koletzko et al. 2009a), thus the avoidance of infant foods that provide excessive protein intake could be an effective strategy in reduction of
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childhood obesity and has resulted in the emergence of lower protein formulas (Koletzko et al. 2009a).

Breastfed infants also feed on demand yet little is known about development of the infant appetite control mechanism, which in itself is a major regulator of energy intake. Breastfed infants display a variety of feeding patterns and feed volumes throughout the day thus self-regulating their energy intake (Arthur et al. 1987) yet why there is such variation is unclear, but factors such as milk composition as well as fetal exposure and infant genetics all likely play a role.

Human milk (HM) is recognized as the best form of nutrition for optimal growth and development of the human infant and as such is species specific in composition, particularly in comparison to bovine milk, the basis for most infant formula. HM is multifunctional fluid shaped by many thousands years of evolution, it contains essential nutrients as well as immunological and bioactive components that provide nutrition, protection against infectious deceases, and developmental factors and, most recently discovered, a host of appetite control factors such as leptin and adiponectin (Hennet et al. 2016). Since research has shown that fully breastfed infants control their food (energy) intake, it is likely the alterations of appetite control is related to weaning, where infants transition from full breastfeeding to solid foods (Cohen et al. 1994). This transitional period is a critical window for potential intervention and may have enormous impact on the prevention of obesity, yet the majority of the studies concentrate on the early post-partum period, due in most part to the methodological issues and resources. Whilst breastfeeding suggests a protective effect for NCD, there is still ongoing debate about effects of the type of nutrition on infant body composition (BC). These are largely fuelled by the lack of comprehensive studies relating to infant feeding patterns, volumes and HM composition with respect to growth and BC.

An extensive study to investigate appetite control that combines analysis of HM composition and appetite factors along with accurate measures of milk production, feeding patterns, infant and mother BC and infant gastric emptying (GE) would determine relationships between breastfeeding and infant growth, BC and appetite control. Such study has not yet been attempted for the first year of life in breastfed infants but promises to provide the basis for the potential to develop early obesity prevention measures that could have an enormous impact on national health (NCD Risk Factor Collaboration (NCD-RisC) 2016). In addition, clinical guidelines may be formulated for the assessment of the nutrition and feeding patterns of breastfed infants, allowing more accurate monitoring of infant health and development.
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1.2 HUMAN MILK COMPOSITION

HM is a complex biological liquid comprised of 87% water, 7% lactose, 3.8% fat and 1% protein (Guo 2014). It is the optimal form of nutrition for healthy term infants and contains not only a mixture of proteins, fats, carbohydrates, minerals, vitamins, trace elements, but also different cell types along with bioactive immunological, developmental and programming components, such as growth factors and hormones that regulate metabolism and BC (Ballard et al. 2013). These components provide nutrition and protection, regulate growth and development of the infant and present in milk in concentrations according to the needs of growing infant, leading to health and development that is impossible to achieve with any other diet (Lonnerdal 2000). Some of the components of HM are synthesised by mammary gland and others are dietary in origin or originate from maternal stores (fat tissue, serum) (Ballard et al. 2013).

The composition of HM has gained increased attention in the last decade with the discovery of new components, such as leptin and ghrelin, as well as the explanation of some mechanisms of the effects of maternal health and nutritional status, such as level of adiposity (BMI) and smoking, on HM composition. Many components of HM such as proteins, lipids, oligosaccharides, vitamins and cells have been isolated, characterised and linked to beneficial effects of breastfeeding on infant health later in life. Nevertheless, the area of metabolic programming by HM as a continuation of in utero programming has not been extensively examined. Those studies examining composition and infant outcomes rarely report dose in terms of the volume of milk ingested by the infants (Grote et al. 2016; Kon et al. 2014), nor take into account changes of HM composition over time.

HM components are often classified according to their nutritive function however, many have dual functions, most often an immune function which is also conducive to better health and hence better long term outcomes.

1.2.1 Macronutrients

The macronutrient composition of HM is remarkably conserved across populations appearing to be largely independent of geographic, ethnic, dietary factors (Garcia-Rodenas et al. 2016) or maternal nutritional status (Prentice 1995). While term milk is estimated to be approximately 8–12 g/L for protein, 32–41 g/L for fat, and 60–78 g/L for lactose (Ballard et al. 2013; Mitoulas et al. 2002), macronutrient concentrations of HM are associated with number of maternal factors: adiposity, parity, protein intake and return of menstruation (Nommsen et al. 1991). Levels of milk production also appear to
influence macronutrient concentrations, with higher volumes associated with lower concentrations of fat and protein and higher of lactose (Nommsen et al. 1991).

1.2.1.1 Proteins

The majority of proteins in HM are synthesised by mammary gland (Neville et al. 1983) and some are taken up from maternal circulation (Mather et al. 1983). HM protein concentration is not affected by maternal diet, but is higher when maternal weight for height is higher and decreases when produced milk volumes are higher (Nommsen et al. 1991). The research in our group identified 415 proteins in skim HM, with 261 of them being found for the first time (Molinari et al. 2012). The remarkable array of HM proteins and peptides is divided into 4 main categories: whey (60–70%) and casein fractions (30–40%), peptones (peptides with low molecular weight) and milk fat globular membrane (MFGM) proteins or mucins (Lonnerdal 2003; D'Alessandro et al. 2010), with the most abundant proteins being casein, α-lactalbumin, lactoferrin, secretory immunoglobulin slgA, lysozyme and serum albumin (Lonnerdal 2004; Jensen 1995). The cell fraction also contains a small proportion of proteins together with milk fat globular membrane proteins accounting for approximately 4% of total protein content (Czank et al. 2007b). The biological roles of the HM proteins is not limited to providing nutrients and defence molecules against pathogens, but also includes direct stimulation of the growth of neonatal organs and tissues, and the development of an independent immune system, both through the induction of a number of molecular cascades associated with cell differentiation and proliferation (D'Alessandro et al. 2010).

The whey/casein ratio in HM ranges from 80/20 to 70/30 in early lactation and slowly decreases to 50/50 in the later stages (Lonnerdal 2003) with considerable variations between individuals and casein subunits (Kunz et al. 1992). The whey fraction contains proteins that remain soluble in the liquid portion after isoelectric precipitation of caseins by acid in presence of calcium (Kunz et al. 1989) and is easier for the infant to digest than casein fraction that curds in the acidic environment of the stomach. HM whey fraction is proportionally greater compared to other mammals and differs in protein content from widely used bovine whey (Artym et al. 2013) (Figure 1.3). The whey proteins include α-lactalbumin, lactoferrin, immunoglobulins, lysozyme, various hormones, enzymes and binding proteins. Serum albumin and α-lactalbumin are major nutritive proteins and are a source of amino acids for the infant. Lysozyme, lactoferrin and slgA not only have nutritive value, but are major contributors to
bacteriostatic, bactericidal and antiviral properties of HM (Hamosh 1998). The whey fraction hosts a variety of bioactive proteins and peptides with non-nutritive functions.

**Whey proteins, as a percentage of total whey proteins and caseins, as a percentage of total caseins:**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Percentage</th>
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<tr>
<td>β-lactoglobulin</td>
<td>52%</td>
</tr>
<tr>
<td>α-lactalbumin</td>
<td>17%</td>
</tr>
<tr>
<td>Immunoglobulins [IgG, IgA, IgM]</td>
<td>10%</td>
</tr>
<tr>
<td>Serum albumin</td>
<td>5%</td>
</tr>
<tr>
<td>Lactoferrin</td>
<td>1.5%</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>&lt;0.1%</td>
</tr>
<tr>
<td>Lactoperoxidase</td>
<td>0.5%</td>
</tr>
<tr>
<td>Glycomacropeptide</td>
<td>1.2%</td>
</tr>
<tr>
<td>Proline rich polypeptides</td>
<td>&lt;0.1%</td>
</tr>
<tr>
<td>Other proteins and peptides</td>
<td>2%</td>
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**Figure 1.3** Typical content of milk proteins in mature bovine and human milk. Shown are the major milk proteins, as well as selected additional proteins. The contents of proteins are expressed as percentage (%) of whey proteins and caseins in the total milk proteins and also as a percentage within the groups. ND – no data. Reproduced by permission from *Postępy Higieny i Medycyny Doświadczalnej* (PHMD, Advances in Hygiene and Experimental Medicine) (Artym et al. 2013).

Casein in HM is present in the lowest concentration, compared with other species. The casein fraction consists of 30–40 kDa phosphoproteins with several subunits (α, β and κ-casein), which form supramolecular structures known as micelles containing Ca²⁺ and PO₄³⁻ that give milk its typical white appearance (Lonnerdal et al. 1985). HM casein micelles (30–75 nm) are considerably smaller in size than micelles from bovine milk (600 nm), and probably form looser, softer and more easily digested curds in the acidic environment of the infant’s stomach (Lonnerdal et al. 1985). Casein micelles are
not only a source of amino acids and trace elements (calcium and phosphorus) for the infant (Czank et al. 2007b) but also breakdown to bioactive peptides that have an array of functions including gastrointestinal (GI), antimicrobial, antithrombotic, immunomodulating, antihypertensive and opioid effects (Clare et al. 2000). It is possible that the casein fraction or particular casein species of HM could therefore influence GE and digestion and potentially impact infant growth and development of BC, explaining the observed difference between breastfed and formula-fed infants (Poth et al. 2008).

### 1.2.1.2 Carbohydrates

HM carbohydrates are a major macronutrient and contribute to approximately 44% of energy of the HM (Casadio et al. 2010). HM carbohydrates include lactose, the principal carbohydrate, monosaccharides (mainly glucose and galactose), and HM oligosaccharides (HMO). Concentrations of glucose and galactose in HM are small, 0.25–0.27 g/L for glucose and 0.11–2.7 g/L for galactose (Newburg et al. 1995; Grote et al. 2016).

Lactose is a disaccharide and consists of glucose and galactose, linked together by a 1,4β-glycosidic bond and is synthesised by lactocytes (Neville et al. 1983). In contrast to fat, lactose content is relatively constant in mature HM, which is important for maintaining a constant osmotic pressure (Martin et al. 2016). The synthesis of lactose in the Golgi secretory vesicle system of lactocytes results in water being drawn into the milk, therefore, the rate of lactose synthesis is a major controlling factor of milk production (Arthur et al. 1989). Lactose is hydrolysed by the brush border enzyme lactase in the small intestine, and lactose malabsorption and intolerance syndrome can be observed in the infant if the small intestine does not produce enough of the enzyme (Martin et al. 2016; Woolridge et al. 1988).

HMO are complex carbohydrate polymers formed from a small number of different monosaccharides. More than 200 unique oligosaccharides structures that vary from 3 to 22 sugars have been identified (German et al. 2008). HMO are the third most plentiful component of HM and make up 8% of total nutrient intake of HM however, they are not digested in small intestine and enter the colon, where colonic bacteria ferment them, breaking HMO down to short-chain fatty acids. HMO act as prebiotic in the development of a diverse and balanced infant gut microbiome, thereby improving mucosal immune system and defense responses (Walker 2013). *Bifidobacteria longum* biovar *infantis*, which dominates breastfed infant microbiome (up to 90%) before weaning (Gura 2014), has been shown to thrive on HMO as the sole carbon source.
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(German et al. 2008) emphasizing the importance of these unique components of HM. This domination of infant microbiome results in a low bacterial diversity and plays an important role in maturation of the immune system (Laursen et al. 2017). HMO possess anti-infective, bacteriostatic and bactericidal properties and protects the infant GI tract against bacteria such as Salmonella, Listeria, and Campylobacter, acting as decoys that bind the pathogens isolating them from the intestinal wall (Gura 2014).

The composition and proportions of HMO are highly variable between mothers (Bode 2015) and may exert different effects in the infant gut. The compositional differences of gut microbiome have been implicated in obesity and infant weight gain (Turnbaugh et al. 2009; Koleva et al. 2015; Chakraborti 2015) (Figure 1.4) and in this context a number of individual HMO have displayed differing associations with infant BC (Alderete et al. 2015) however, the total concentration of HMO was not analyzed.

![Figure 1.4](image) Pre and postnatal exposures demonstrated to modify the infant gut microbiota (A) and hypothesized to impact childhood obesity risk through microbial induced mechanisms (B). Reproduced by permission from MDPI (Koleva et al. 2015). HMO - human milk oligosaccharides; LPS - lipopolysaccharides; SCFA - short chain fatty acids.

1.2.1.3 Fat

HM fat has multiple functions; it is a major energy supply (approximately 50%) (Czank et al. 2007a), is essential for maturation and development of the central nervous system (Dean III et al. 2013), regulates immune function and inflammatory responses (Jakaitis et al. 2014) and carries soluble vitamins (Riordan 1993), taste and aroma to the infant (Beauchamp et al. 2011). The fat fraction of HM consists of several different classes: mainly triacylglycerols (98%) with the reminder being di- and monoacylglycerols, phospholipids, nonesterified fatty acids (NEFA), cholesterol and cholesterol esters. The triacylglycerols are hydrolyzed to free fatty acids and glycerol by lipase present both in
infant gut and HM. The fatty acids needed for the synthesis of triacylglycerols are either produced in the mammary gland or taken up from the blood, derived from the diet, maternal adipose tissue or synthesized by the liver. Synthesized triacylglycerols form the core of the milk fat globules (MFG) of around 4 μm. The core is coated with milk fat globule membrane (MFGM), which originates from the plasma membrane of lactocytes during secretion of the MFG and consists of proteins, phospholipids, enzymes, cholesterols and mucopolysaccharides (Jensen 1995).

The HM fat content is positively related to maternal weight gain during pregnancy (Martin et al. 2016; Jensen 1995) and varies significantly with maternal diet, particularly with fatty acids composition (Anderson et al. 2005) and protein intake (Nommsen et al. 1991). The wide variation in HM medium-chain fatty acids associated with maternal carbohydrate intake may have relevance to infant growth and development, particularly to the neurological development (Innis 2014).

Fat is the most highly variable macronutrient in HM and ranges from 3.5% (24.6 g/L) to 4.5% (30.5 g/L) during lactation (Guo 2014), while more dramatic changes are seen during a breastfeeding with fat content changing on average from 27.2 g/L pre-feed to 69.1 g/L 30 min after the end of the feed (Hassiotou et al. 2013b), which makes it difficult to confidently measure and analyse this component in HM particularly if only one sample is taken. The best approach is to measure fat concentration in pre- and post-feed HM samples over the 24-h period, which takes into account changes in fat content during a feed, circadian changes and differences between the breasts, as well as ensures minimum interference with infant breastfeeding behaviour as shown in our group (Mitoulas et al. 2002).

1.2.2 Micronutrients

HM provides the normative standard for infant nutrition and usually has an adequate amounts of micronutrients including vitamins, minerals and other bioactive components. It is possible that regulation of nutrient concentration in HM does not rely on maternal diet as many components could be produced by the mammary gland. A good example is that women with severe iron deficiency anaemia are producing milk with normal iron content (Lonnerdal 1986). Calcium and phosphate in HM remain stable (250 mg/L and 150 mg/L respectively) independently of the maternal diet and vitamin D (Kent et al. 2009). Nevertheless, many micronutrients vary in HM depending on maternal body stores and diet, thus supplementation is often recommended. Components considered to be depended on maternal diet include vitamins A, B1, B2,
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B6, B12, D, iodine (Valentine et al. 2013) and, independent of maternal diet, vitamins D and K and iron are regarded as low in HM (Martin et al. 2016; Scott et al. 2016).

1.2.3 Bioactive components

Bioactive components of food are elements that “affect biological processes or substrates and hence have an impact on body function or condition and ultimately health” (Schrezenmeir et al. 2000). Bioactive components in HM come from different sources, some secreted by mammary gland, some produced by HM cells and some transported from maternal serum. They vary between the mothers and between lactation stages, representing responsiveness to the changing needs of the infant, often acting synergistically with each other. With few exceptions, such as HMO, some fatty acids and nucleotides, most of bioactive components in HM are proteins (Lonnerdal 2010) and could be grouped as growth and immunological factors, considering that bioactive components provide efficient nutrient transfer from mother to infant and immunological protection form bacteria and viruses (Newburg 2001).

Bioactive components can enter into infant’s circulation via six potential mechanisms, which have been previously described (Wada et al. 2014; Alsaweed et al. 2015). First, HM has high concentrations of α1-antitrypsin which inhibits protease; second, infant GI tract has high pH that supports the absorption of bioactive compounds and proteins; third, immature pancreatic enzymatic activity favours absorption by resistance to proteolysis (Lonnerdal 2010). Forth, adipokine receptors for specific bioactive compounds, such as leptin and adiponectin, have been found in GI tract (Savino et al. 2009). Fifth, paracellular diffusion is an important no-digestive pathway for absorption of bioactive compounds and plays a major role in infancy (Wada et al. 2014). Sixth, peptides and other bioactive compounds, such as microRNA, can be transferred into infant circulation via exosomes or live HM cells as shown in our group (Alsaweed et al. 2015; Hassiotou et al. 2015). These factors demonstrate that intact non-nutritive bioactive compounds can enter infant’s circulation and may influence infant development and health outcomes.

1.2.3.1 Growth factors

Various growth factors and cell signalling proteins cytokines such as epidermal growth factors (EGF), neuronal growth factors (BDNF, GDNF), insulin-like growth factors (IGF), vascular endothelial growth factors (VEGF), hepatocyte growth factors (HGF), transforming growth factors beta (TGFβ)1,2,3, tumour necrosis factor alpha (TNF-α) and interleukins (IL-6,8) are present in HM and have effects on several cellular
processes, such as cell differentiation and proliferation via affecting B-cells, T-cells, NK-cells, dendritic cells, epithelial, endothelial and hematopoietic cells and macrophages (Lonnerdal 2010). Although all of these factors are immunomodulatory, they also have pro- and anti-inflammatory functions, and levels for most of them are highest in colostrum and decline with duration of lactation probably due to dilution factor (Munblit et al. 2016; Affolter et al. 2016). Few growth factors have been studied recently in regards to infant BC and it was found that HM EGF is higher in milk of mothers with normal-weight infants than in milk of mothers with obese infants (n=80) (Khodabakhshi et al. 2015). Furthermore, IGF-1 is associated with higher weight gain (Kon et al. 2014); IL-6 is associated with reduced growth and adiposity in infants; while TNF-α is associated with lower fat-free mass (FFM), but not fat mass (FM) (Fields et al. 2012). TNF-α is also implicated in bone mineral content (BMC), where results are differing depending on either sex of the infants or time postpartum (Casazza et al. 2014), and may have a role in bone health and modelling process, thus affecting infant BC through bone accretion rate or through immune system pathways.

Hormones are also present in HM, including growth-regulating hormones calcitonin and stomatostatin, and hormones that regulate metabolism and BC, appetite hormones leptin and adiponectin. These adipokines may contribute to the development of infant BC and appetite control, regulation of infant food intake, weight and adiposity, and prevention of obesity (section 1.3) (Savino et al. 2013). Further, hormones such as resistin, ghrelin, obestatin, motilin, insulin and many others appear to play an important role in the regulation of energy conversion, infant BC and appetite control (Savino et al. 2012c; Savino et al. 2007; Savino et al. 2012a; Dundar et al. 2010).

1.2.3.2 Immunological factors

HM protects infant against infection and inflammation (Goldman 2000) with particularly early milk being especially rich in immune factors that ensure the survival of the infant (Gao et al. 2012). Both the whey and casein fractions contain immunological factors that are typically highest in colostrum and decrease throughout the lactation (Ballard et al. 2013).

Lactoferrin is an iron binding glycoprotein of the whey fraction with molecular mass of 78-80 kDa. It facilitates absorption of iron and other trace elements and exerts bacteriostatic activity by withholding iron from bacteria (Lonnerdal 2003). Lactoferrin is also involved in cell growth regulation and has anti-inflammatory, antitumor
response, antiviral and antifungal effects (Beljaars et al. 2004; Kuipers et al. 1999) as well as some enzymatic activity (Hamosh 1998).

Peptides derived from the digestion of α-lactalbumin appear to have antibacterial and immuno-stimulatory functions (Lonnerdal 2003). α-lactalbumin may be related to lysozyme because of the striking similarities in their genetic features (40% homology) and molecular structure, but unlike lysozyme, which appears widely between species and fluids, α-lactalbumin occurs only in mammalian milk and colostrum (Artym et al. 2013; McKenzie 1996; Qasba et al. 1997).

Lysozyme is a 15 kDa glycoprotein which catalyses the hydrolysis of β-1,4 linkages between N-acetylmuramic acid and N-acetyl glucosamine in bacterial cell walls and lyses Gram-positive and some Gram-negative bacteria in combination with lactoferrin. HM lysozyme is present in whey at relatively high concentrations (6%) compared with bovine milk (<0.1%) and has multiple roles, such as influencing the stomach microbiome and speeding up the digestion of microbial protein (Wang et al. 2012; Artym et al. 2013; Montagne et al. 2001; Qasba et al. 1997). In adults, lysozyme was found to be up-regulated to control a bacterial population linked to inflammatory conditions of GI tract, protecting individual mucosal segments from chronic inflammation (Rubio 2014). In a clinical study in preterm infants, donor HM or dry adapted milk formula enriched in lysozyme (50 mg/L) given to preterm infants with inflammatory or infectious diseases (n=29) resulted in increase in body weight, reduction of the infectious inflammatory foci, normalization of the stool, with stabilization of lysozyme levels in the coprofiltrates and in the blood serum compared with control group (n=35) (Bol'shakova et al. 1984). All of this suggests that HM lysozyme could potentially have effect on GE, digestion and BC of breastfed infants.

Peptides α- and β-casomorphins, fragments of α- and β-caseins that are also presented in human and bovine milk in different concentrations (Figure 1.3), are the result of hydrolysis of HM caseins in GI tract. They are called “opioid peptides” since they show pharmacological effects similar to morphine. Casomorphins induce an analgesic and sedative effects along with slowing of GI motility due to their action on the nervous system (Artym et al. 2013). However, HM κ-casein fragments (casoxin) behave as opioid antagonists. Further, κ-casein has been shown to inhibit the binding of Helicobacter pylori to human mucosa in vitro (Stromqvist et al. 1995; Hamosh 1998; Lonnerdal 2010). H. pylori have been shown to down-regulate levels of appetite
hormones, ghrelin and leptin in the stomach (Francois et al. 2011), which in infant may significantly affect GE and breastfeeding patterns.

Secretory IgA is the predominant immunoglobulin (Ig) in HM and together with IgM and IgG it provides passive immunity to the newborn. HM α-lactalbumin, IgA, IgM are in their highest concentrations in early lactation and then decrease, remaining stable in mature milk, while IgG levels are stable throughout the lactation (Affolter et al. 2016). Both lactoferrin and lysozyme decrease from colostrum to transitional milk and then increase again as lactation progresses to 12th weeks (Montagne et al. 2001). While concentration of lactoferrin stabilises, lysozyme concentration remains elevated after the 12 weeks (Goldman et al. 1982).

1.2.4 Human milk cells
HM contains a variety of cells, including white blood cells leukocytes (T cells, macrophages, lymphocytes), mammary epithelial cells (lactocytes and myoepithelial cells), and progenitor and stem cells (Hassiotou et al. 2013a). As the majority (13–70%) of colostrum cells are leukocytes, the number of them in mature milk drops dramatically (0–2%), but increases if either mother or infant have an infection (Hassiotou et al. 2015). HM leukocytes via phagocytosis and secretion of antimicrobial factors provide active immunity and promote development of immunocompetence in the breastfed infant and possibly protect mammary gland from infection. Stem cells found in HM appear to be non-tumorigenic and therefore have potential uses in regenerative medicine. A small proportion of HM stem cells enter the offspring’s bloodstream and migrate to different organs and tissues (microchimerism), where they may potentially provide immunity or boost infant development early in life (Hassiotou et al. 2014b).

The cellular fraction of HM can represent a significant portion of milk, comparable to the skim and fat fractions. HM cells, which are predominantly of epithelial origin in mature HM of healthy mother/infant dyads (Hassiotou et al. 2013a; Hassiotou et al. 2014b), are also thought to contribute to the concentration of appetite hormones, particularly leptin, in whole HM (Hassiotou et al. 2014c). Thus measurements of these appetite hormones in whole HM, not just skim milk fraction, are warranted. Whilst it is not known if cellular leptin is bioavailable, the process of digestion may release these hormones from HM cells. It is also possible that these cells could be absorbed by the GI tract after ingestion and enter the circulation, as has been confirmed with HM stem cells and leukocytes (Hassiotou et al. 2012a; Weiler et al. 1983), thus delivering the hormones to organs and tissues.
1.2.5 Compositional changes of human milk

HM composition is dynamic; it varies greatly both between and within mothers adapting to the needs of the growing infant and changing during both a breastfeed and entire lactation (Hassiotou et al. 2013a; Hassiotou et al. 2013b; Khan et al. 2013b), and also diurnally/circadianly (Gidrewicz et al. 2014) and between populations (Koletzko et al. 1992). HM composition can be affected by many interconnecting factors, including infant or maternal factors (Figure 1.5) (Fields et al. 2016).

Figure 1.5 Interconnecting maternal, physiological, and behavioural factors that impact human milk composition. Modified from (Fields et al. 2016). Reproduced by permission from John Wiley & Sons, Inc.

After the dramatic shift in HM composition during establishment of lactation from colostrum to transitional and mature milk, which is completed by six weeks postpartum, HM remains relatively stable in composition (Gidrewicz et al. 2014). But subtle changes in total and whey protein content of HM can be seen, with a decrease during
the first year (Mitoulas et al. 2002; Grote et al. 2016), whereas casein-to-whey ratio increases, while different casein subunits vary throughout the lactation duration (Kunz et al. 1992; Affolter et al. 2016), indicating that synthesis and/or secretion of caseins and whey proteins is regulated by different mechanisms. HM curd is softer in colostrum and more solid in mature milk (Sanchez-Pozo et al. 1986) but is not clear how changes in casein-to-whey ratio affect consistency of the curd during the entire period of exclusive breastfeeding and after weaning, and how the differences in casein-to-whey ratio may affect infant GE, growth and BC.

HMO profiles also change over the course of lactation while the total proportion of HMO declines, with the mean concentration at 1 year postpartum being less than half of that in the first few weeks (Chaturvedi et al. 2001; Thurl et al. 2010). Longitudinal changes are also observed in mean fat content, which decreases at 2 months and increases again at 9 months of lactation (Mitoulas et al. 2002).

Weaning affects HM composition dramatically, with concentrations of lactose decreasing and protein, sodium, chloride and magnesium increasing in the milk of weaning mothers, where it stays stable in non-weaning mothers at the same time postpartum. Independently of weaning, concentrations of calcium, free phosphate, citrate and glucose decrease, while concentrations of potassium and lipids increase after the first 6 months (Neville et al. 1991).

The reports in literature in regards to circadian variations in protein concentrations are conflicting (Mitoulas et al. 2002; Lammi-Keefe et al. 1990). More recently Khan et al. (Khan et al. 2013a) showed that the concentration of protein (total protein, whey protein and casein) remained the same over 24 hours. Lactose tends to be the least variable component of HM, showing no diurnal or within feed changes (Mitoulas et al. 2002; Khan et al. 2013a) however, recent study showed a small decrease in post-feed concentration (1.0 g/L) (Cannon et al. 2015). On the contrary, one of the most drastic changes in concentration is seen for milk fat where fluctuations occur over a 24-h period (higher fat content during the day and lower at night) and also within a breastfeed (Kent et al. 2006; Khan et al. 2013a). Concentration of fat increases across the duration of the feed (Neville et al. 1984; Kent et al. 2006), and peaks approximately 30 min after the end of the feed (Hassiotou et al. 2013b). Fat content also differs between the women throughout the day (Mitoulas et al. 2002).

Apart from macronutrients, circadian variations in HM were found in leptin, with leptin levels gradually decreasing between 00:00 h, with a plateau between 06:00 and 17:00 and an increase until 24:00 with leptin levels being significantly higher from
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22:00 to 04:00 compared to other times (Cannon et al. 2015). These variations in fat and leptin may translate into differences in feeding frequency (FFQ), energy intake and infant growth and development, including development of BC between infants.

Furthermore, preterm HM in the first week is significantly higher in carbohydrate, fat, energy content and sodium levels compared to term HM (Bauer et al. 2011). Differences also exist in HM between mothers of male and female infants, although studies results are contrasting, with some studies reporting richer in energy HM being provided for males (Thakkar et al. 2013; Powe et al. 2010) while mothers from lower social-economic status were found to provide richer milk for females (Fujita et al. 2012), suggesting that milk synthesis has more plasticity than previously believed. HM composition is also reported to be affected by parity with multiparous mothers producing a more lipid rich milk (Bachour et al. 2012) and contributing to differences in development of offspring. Although Powe et al. have controlled for the time since last feeding and Fujita et al. standardised time of collection, in these four studies no 24-h milk productions were conducted and only single sample of milk was analysed, thus the results could be misleading and need further clarification.

Studies, investigating relationships between HM composition and maternal factors are infrequent. While HM protein concentration is not affected by maternal diet, it is higher when maternal adiposity is higher and decreases with increase of produced milk volumes (Nommsen et al. 1991). Other factors that have been reported to influence milk composition include high body mass index (BMI) and smoking, which are associated with lower protein content of HM and lower proportion of lipid content (Bachour et al. 2012). Recent studies also have found that maternal BMI is positively related to protein and lipid content of HM (Chang et al. 2015; Grote et al. 2016; Innis 2014) and galactose content and energy (Grote et al. 2016), yet negatively to lactose content (Chang et al. 2015), but again, 24-h milk productions, which would give more reliable results were not conducted.

Although discovery of appetite hormones in HM generated a number of studies, the majority of them have concentrated on associations between maternal BMI and HM leptin and recently adiponectin (Andreas et al. 2014), which are described in sections 1.3.2.2 and 1.3.3.1. 24-h milk intake (MI) profiles and more precise measurements of maternal BC are needed to confirm these relationships as well as establish which maternal BC compartments are responsible for HM compositional changes. The establishment of which HM components are specifically influenced by the changes in maternal body weight or diet could contribute to the implementation of measures to
simultaneously improve both the programming of the infant long-term health during the perinatal period and the long-term outcomes for mothers (Demmelmaier et al. 2017).

1.2.6 Lactocrine programming
HM is a living mixture of biomolecules with overlapping biological, chemical and physical properties, with qualities that cannot not be replicated by HM substitutes. Proteomic analysis shows that there are thematic distinctions in protein composition of HM at different lactation stages (Liao et al. 2011) and between transitional and mature milk (Gao et al. 2012), indicating the need to match donor milk to the developmental stage of the infant where possible. The plasticity of HM composition, particularly the maternal influence, could explain in part programming of infant growth and development. Extensive evidence has shown that HM bioactive agents such as hormones, growth factors, neuropeptides, and anti-inflammatory and immune-modulating agents influence the growth, development and the function of the GI tract and other organs and tissues during early infancy (Goldman 2000). HM likely enhances infant programming, reduces obesity and results in lower incidence of late metabolic diseases, such as type 2 diabetes (Savino et al. 2013; Yan et al. 2014; Woo et al. 2015), however, there is much to be learned about the spectrum of HM programming and the components that involved in it, how their patterns change throughout the lactation period and their short term effect on breastfeeding behaviour, GE rate, gut microbiome and BC of the breastfed infants.

It is hypothesized that infant behaviour varies as a function of the mother’s milk composition, directly interacting with infant’s neurobiology and indirectly through the gut bacteria resulting in a milk-microbiota-brain-behaviour dynamic, suggesting potential implications for current and emerging methods of intervention (Allen-Blevins et al. 2015) (Figure 1.6). In the breastfed infant during first year of life this dynamic may be manifested in infant appetite regulation and feeding patterns. Indeed, the gut-brain axis has multiple pathways through which microbiota can affect neurobiology and behaviour (Figure 1.7), including activation of immune cells and release of the hormones. A holistic approach is required to understand multiple levels of HM programing and regulatory effects.
Figure 1.6 Conceptual models of bioactives in milk mediating maternal-offspring conflict and coordination. Bioactives in milk affect gut microbiota in the infant, impacting the development of neurobiology and subsequently behavior. Reproduced by permission from Oxford University Press (Allen-Blevins et al. 2015).

Figure 1.7 Conceptual The gut–brain axis pathways by which gut microbiota can affect neurobiology and subsequently behaviour. Bacteria (blue rods and olive green circles) can produce neurotransmitters (yellow circles) or extract them from the gut lumen. Neurotransmitters can then interact with nerve cells of the vagus nerve or be released into portal circulation and possibly interact with other nerve cells. Microbiota can induce immune cell (red circles) activation or release hormones (purple circles). Bacterial species can also competitively inhibit other species, effectively selecting the metabolites able to be produced in the gut. Reproduced by permission from Oxford University Press (Allen-Blevins et al. 2015). HMO - human milk oligosaccharides.
1.2.6.1 Breastfeeding and obesity

Unlike the other health benefits of HM over infant formula, such as protection against infectious diseases, higher IQ, lower rate of some NCD later in adulthood, protection against increased adiposity is still debated in the literature. While observational studies and multiple meta-analyses suggest that breastfeeding is protective against development of obesity during childhood and later in life (Woo et al. 2015; Victora et al. 2016), these findings are considered to be controversial since suggested pathways that connect HM and infant physiology involve complex interactions between infant and mother, include both biologic and non-biologic factors, and may have both direct and indirect effects on obesity risk later in life. A recent review (Woo et al. 2015) of over 80 studies highlighted these issues specific to breastfeeding, and concluded that breastfeeding, and especially exclusive and/or for longer duration is associated with 10—20% reduction in obesity prevalence in children. This review also urged researchers to conduct studies that can identify specific biological pathways in order to elucidate the role of HM in prevention of obesity. Furthermore, the review of 113 studies by The Lancet 2016 Breastfeeding Series (Victora et al. 2016) pointed out that longer duration of breastfeeding was associated with 26% reduction in overweight and obesity across income classification (13% in a selection of 23 high-quality studies that had more than 1500 participants and accounted for multiple confounders).

There is a need to state the reasons for doubts in protective role of HM and breastfeeding. The opinion exists that a negative study is less likely to be published (Woo et al. 2015) and may be met with more criticism. However, the Promotion of Breastfeeding Intervention Trial (PROBIT study, n=17,046) is often given as the example of evidence against a direct role of breastfeeding in protection against obesity, showing that prolonged exclusive breastfeeding in the group with intervention did not result in reduced BMI and triceps thickness at 6.5 years, or BMI, FMI (fat mass index) and percentage fat mass (%FM) at 11.5 years (Kramer et al. 2007; Kramer et al. 2009; Martin et al. 2013). Interestingly, this study set up an intervention in a Belarussian population with a very low prevalence of obesity and compared the outcomes in two groups, with breastfeeding being present in both to some degree for a short duration (51.9% vs 28.3% and 10.6% vs 1.6% predominantly breastfed in the intervention and control groups at 3 and 6 months, respectively). Authors also suggested that PROBIT mothers from the interventional group could have deliberately increased frequency and duration of feeding, leading to the faster weight and length gain observed in first 3 months of life and reported one of the participating centres being disqualified for
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falsifying outcome data (Martin *et al.* 2013). Another study that reported mostly negative results (moderate protective effect only in case of prolonged breastfeeding) is a study of middle-class Brazilian 18 year old men (Victora *et al.* 2003), but breastfeeding status and duration in this study population was recalled at the interview and breastfeeding itself lacked exclusivity in the first months, as infants also received herbal teas or water from the first week after birth and fruit juice from age of 2—3 months.

To complicate the matter further, possible benefits of breastfeeding could be results of both, HM and breastfeeding itself. Breastfeeding is a variable human behaviour, and differences between populations could be attributed to the population itself and confounding factors specific for this population rather than the study quality. Attempts have been made to account for population specific confounding structures by comparing cohorts from high-income with low- or middle-income countries (Brion *et al.* 2011), suggesting that protection by breastfeeding from obesity seen in the high-income countries with obesogenic lifestyle is likely to be explained by residual confounding and reverse causality. Unfortunately, as with many studies, BMI was used as a measure of adiposity and breastfeeding data was collected at the interview during follow-up appointments and not measured.

Disagreement on this subject could also be a reflection of the humans’ adaptive mechanisms to the food availability. To advance the research, an integrated approach for studying of breastfeeding is needed, that will include a wide range of populations and go beyond epidemiologic studies to answer the question if breastfeeding and particularly the dose of HM components that infant receives rather than concentration are directly associated with reduced adiposity. Cross-disciplinary research will be critical for improving our understanding of how multiple factors interrelate to impact the development of adiposity in infancy, childhood and beyond.

1.3 APPETITE HORMONES AND APPETITE CONTROL

HM is a complex mixture consisting not only of nutrients but also bioactive molecules including hormones, growth factors, neuropeptides, immunomodulating and anti-inflammatory agents (Goldman 2000). An increasing number of appetite hormones have been recently identified in HM along with studies aiming to elucidate their relationships with maternal adiposity and effects on infant growth and development. These appetite hormones, mainly derived from maternal serum and also synthesised by the breast, may be involved in regulation of satiety and influence growth and development of the child.
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(Savino et al. 2013), since they act as mediators between the infants adipose tissue, GI tract and brain.

1.3.1 Appetite control

The emergence of obesity has focused research on establishing how human appetite and weight are controlled. The research clearly indicates the existence of complex neuroendocrine system, in which circulating hormones deliver information about energy balance to areas of the brain that control energy expenditure and eating (Schwartz et al. 2002). Hormones that regulate appetite and food intake either act rapidly and influence individual meals or act more slowly and regulate the deposition of fat.

The long-term acting hormones, such as insulin and leptin, are released into circulation according to the amount of body fat (Bagdade et al. 1967) and increase energy expenditure while having an inhibitory effect on food intake. Declining levels of these hormones are sensed by the brain, and both metabolic efficiency and appetite are increased to recover the lost body fat.

Short-term regulators or meal-related “satiety” signalers, such as cholecystokinin (CCK) and ghrelin, are released from GI tract during eating (Harvey 1983). Together with stomach distension, CCK promotes sense of “fullness”, encouraging stopping the meal consumption. In contrast, circulating ghrelin levels rise before meals thus stimulating appetite and fall quickly after the meal (Cummings et al. 2001). Both hormones clearly participate in the “meal-to-meal” control system that adjusts the frequency and the size of the meals according to the body fat deposits and is also affected by the changes in other hormones, such as insulin and leptin.

The arcuate nucleus of the brain contains two groups of neurons, which regulate food intake. The “brake” neurons produce melanocortin peptides to inhibit food intake, while the “accelerator” neurons produce neuropeptide Y (NPY) that stimulates eating (Fan et al. 1997). Activation of one group of neurons causes the inhibition of other group: NPY-expressing neurons also release agouti-related peptide (AgRP), which blocks melanocortin receptors (Schwartz 1997). As result, food intake is regulated via two pathways, through increasing/decreasing the release of the appetite-stimulating peptides, and by blocking the appetite-suppressing receptors.

Hormones such as leptin and insulin are communicating to the brain and deliver the information about positive changes in body weight, thus inhibiting the NPY/AgPR-expressing neurons (Figure 1.8), and, in case of leptin or insulin deficiency, partially
activating them. Ghrelin also activates NPY/AgPR-expressing neurons, stimulating food intake (Nakazato et al. 2001).

**Figure 1.8** Hormones that control eating. Leptin and insulin (lower part of the figure) circulate in the blood concentrations proportionate to body-fat mass. They decrease appetite by inhibiting neurons (centre) that produce the molecules NPY and AgRP, while stimulating melanocortin-producing neurons in the arcuate-nucleus region of the hypothalamus, near the third ventricle of the brain. NPY and AgRP stimulate eating, and melanocortins inhibit eating, via other neurons (top). Activation of NPY/AgRP-expressing neurons inhibits melanocortin-producing neurons. The gastric hormone ghrelin stimulates appetite by activating the NPY/AgRP-expressing neurons. Adapted from (Schwartz et al. 2002). Reproduced by permission from Nature Publishing Group.

While this brief explanation may not provide deep insight into mechanisms and pathways of developing the obesity, it provides a basic framework for understanding how obesity occurs and highlights possible intervention points. A deeper understanding of the factors that influence levels of circulating appetite hormones particularly in the breastfed infant that represents the developmental norm, will provide insight into regulation of food intake/energy expenditure with respect to infant GE and BC.
The satiety hormone leptin and the appetite stimulating hormone adiponectin are also present in HM. Although not transferred to the infant circulation in direct manner, levels of HM leptin and adiponectin have been found to correlate with levels of these hormones in infant serum (Savino et al. 2016; Wang et al. 2011) and are known to affect both appetite control and BC (Miralles et al. 2006; Woo et al. 2009), but are yet to be investigated in relation to GE and feeding patterns in the term breastfed infant.

1.3.2 Leptin

Leptin is a 16-k Da polypeptide hormone synthesized by the white adipose tissue and is the most widely studied of all appetite hormones. It is involved in the regulation of adipose tissue, food intake and body weight. Leptin causes weight loss by suppressing appetite via signalling satiety, decreasing the sensation of hunger and increasing metabolic rate (de Graaf et al. 2004).

1.3.2.1 Leptin analyses in human milk

Historically leptin in HM has been predominately measured by an enzyme-linked immunoassay (ELISA) in skim milk (Tables 1.1 and 1.2), which does not contain the cellular and fat components (Schuster et al. 2011; Miralles et al. 2006; Weyermann et al. 2006). Considering the leptin peptide is capable of lipophilic interactions (Xie et al. 1991; Kline et al. 1997), it is plausible it may associate with the fat globule in whole HM. Moreover, HM cells, which are predominantly of epithelial origin in mature HM of healthy mother/infant dyads (Hassiotou et al. 2014b; Hassiotou et al. 2013a), are also thought to contribute to the leptin concentration of whole HM (Hassiotou et al. 2014c).

A few previous studies have measured leptin in whole HM using radioimmunoassay (RIA) (Houseknecht et al. 1997; Smith-Kirwin et al. 1998). However, RIA is not considered appropriate for measuring leptin in a lipid-rich medium, such as whole HM, due to the interference of triacylglycerols with the binding of radioactive-labeled antigens to antibodies, which compromises the sensitivity of the assay (Resto et al. 2001; Scott M. Grundy et al. 1979). Only one study used ELISA in whole vortexed HM, diluted 1:1 (Miralles et al. 2006), although they have not provided the validation details and the sample size. Given the lack of an optimized assays to detect leptin in whole HM and the absence of reliable comparisons of leptin concentrations between whole and skim HM, a new method of detection utilizing ELISA in whole milk is required to ensure more accurate measures of leptin in HM.
1.3.2.2 Leptin and maternal adiposity

Leptin in HM is sourced both endogenously from the mammary gland epithelium and from the maternal serum, following secretion from white adipocytes and gastric chief cells into the bloodstream (Pico et al. 2007; Hassiotou et al. 2014c). Leptin concentrations in HM have been shown to display a circadian rhythm (Cannon et al. 2015) and are influenced by maternal adiposity, ethnicity and even years of education (Weyermann et al. 2007), although the effect of maternal diet on HM leptin has not been elucidated and studies in rats show no effect (Bautista et al. 2008), suggesting possible correlation between maternal education and adiposity.

A recent meta-analysis by Andreas et al. has established that 11 well-designed studies reported a positive association between leptin and maternal BMI, while 4 found no association (Andreas et al. 2014). Overall, out of 23 studies (15 from meta-review and 8 new studies) presented in Table 1.1, 18 reported some association of maternal BMI or adiposity with leptin concentration in HM at some time points during lactation, with the rest showing no associations. The majority of these studies analysed leptin in skim HM in cross-sectional cohorts, and most have focused on the 1\textsuperscript{st} and 3\textsuperscript{rd} months postpartum. There are very few longitudinal studies, and they also have reported the association between HM leptin and BMI (Schuster et al. 2011; Miralles et al. 2006), with one study reporting an association between BMI and skim milk leptin measured only in pre-feed, but not in post-feed samples (Andreas et al. 2016), despite no significant difference between pre- and post-feed concentrations. Methodologies used in these studies could likely explain discrepancies in the results, in particular those that collected data at multiple points did not attempt to adjust for month of lactation or failed to match time of sample collection to the time of maternal BMI measurement (Andreas et al. 2014). Only 4 studies (Quinn et al. 2015; Schueler et al. 2013; Khodabakhshi et al. 2015; Quinn et al. 2016) have measured and analyzed maternal %FM using either a “stand-on” BC bioelectrical impedance analyzer, skinfold thickness (ST) or dual-energy x-ray absorptiometry (DXA) (Table 1.1).

While some studies have reported decline in leptin concentration during first 6 months of lactation (Karatas et al. 2011; Ilcol et al. 2006; Fields et al. 2017), others observed no significant change (Andreas et al. 2016) or reported significant fluctuations, such that leptin concentrations decreased in the first 3 months, and then increased, with higher concentrations measured at 12 months compared with 3 and 6 months (Bronsky et al. 2011; Schuster et al. 2011).
# CHAPTER 1: APPETITE CONTROL AND BODY COMPOSITION

Table 1.1 *Studies investigating associations between human milk leptin and maternal adiposity*

<table>
<thead>
<tr>
<th>Author, year</th>
<th>Study type</th>
<th>Time postpartum</th>
<th>Sample Size</th>
<th>Analyses, sample type</th>
<th>HM leptin (ng/mL) *</th>
<th>Maternal BMI (kg/m²)/%FM</th>
<th>Association with BMI/adiposity</th>
<th>B (SE) or correlation</th>
<th>P - value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fields, 2017</td>
<td>LS</td>
<td>1 month 6 months</td>
<td>37</td>
<td>ELISA, SHM</td>
<td>1m=0.59 (0.35–1.45) 6m=0.43 (0.15–0.94)</td>
<td>Pre-pregnancy (19 – 47)</td>
<td>Overweight, Obese vs Normal BMI</td>
<td>96.5% higher 315.1% higher</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Andreas, 2016</td>
<td>LS</td>
<td>1 week</td>
<td>105</td>
<td>MIA, SHM</td>
<td>1wpre=541.7 (161.2) 1wpost=614.4 (172.1) 3mpre=684.8 (117.4) 3mpost=464.3 (125.1)</td>
<td>1w=25.4±4.5 3m=23.9±3.6</td>
<td>Positive association with pre-feed concentrations only</td>
<td>0.31 0.30 &lt;0.001</td>
<td>0.02 0.01</td>
</tr>
<tr>
<td>De Luca, 2016</td>
<td>CS</td>
<td>1 month</td>
<td>50 obese,</td>
<td>RIA, WHM</td>
<td>Obese = 4.8±2.7 Normal = 2.5±1.5</td>
<td>Obese BMI=35.3 (95%CI: 34.4-36.2) Normal BMI=24.0 (95%CI: 23.1-25.0)</td>
<td>Leptin concentrations higher in obese mothers</td>
<td>0.33 &lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Savino, 2016</td>
<td>CS</td>
<td>10 days to 6 months</td>
<td>58</td>
<td>RIA, SHM</td>
<td>&lt;2m=0.83±1.17 2-4m=1.18±1.29 4-6m=0.87±3.55</td>
<td>24.0±4.5</td>
<td>Positive association</td>
<td>0.37 0.004</td>
<td></td>
</tr>
<tr>
<td>Quinn, 2016</td>
<td>CS</td>
<td>0 days to 36 months</td>
<td>116, 66 from Kathmandu, 50 from Nubri</td>
<td>ELISA, SHM</td>
<td>Overall: 0.27±0.25 Kathmandu: 0.35±0.33 Nubri: 0.20±0.13</td>
<td>%FM with ST Kathmandu: 27.4±4.4 %FM with ST Nubri: 22.8±4.4</td>
<td>Kathmandu unadjusted adjusted Nubri unadjusted adjusted</td>
<td>0.045 (0.011) 0.034 (0.011) 0.021 (0.007) 0.023 (0.007)</td>
<td>&lt;0.001 &lt;0.006 &lt;0.003 &lt;0.003</td>
</tr>
<tr>
<td>Khodabakhshi, 2015</td>
<td>CS</td>
<td>2 month 4 months 6 months</td>
<td>Mothers of 40 obese, 40 normal infants</td>
<td>ELISA, SHM</td>
<td>Obese*=1.78 (1.67–1.94) Normal*=1.81 (1.65–1.94)</td>
<td>Obese*=27.2±4.5 Normal*=26.0±3.8</td>
<td>Obese* BMI Normal’ BMI Obese* %FM with BIA Normal’ %FM with BIA (Details provided by authors)</td>
<td>0.48 &lt;0.01 0.07 &gt;0.05 0.41 &lt;0.05 0.07 &gt;0.05</td>
<td></td>
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<tr>
<td>Quinn, 2015</td>
<td>CS</td>
<td>10 days to 36 months</td>
<td>113</td>
<td>ELISA, SHM</td>
<td>0.30±0.29 (0.05–2.1)</td>
<td>BMI=20.1±3.3 %FM with ST=24.8±3.5 Weight=45.7±8.2</td>
<td>Positive association (log)</td>
<td>1.59 (0.45) 2.25 (0.48) 1.27 (0.39)</td>
<td>0.001 0.001 0.002</td>
</tr>
<tr>
<td>Brunner, 2014</td>
<td>LS</td>
<td>6 weeks 4 months</td>
<td>151</td>
<td>RIA, SHM</td>
<td>6w=0.11 (0.19) 4m=0.09 (0.18)</td>
<td>Pre-/during pregnancy, values NA</td>
<td>6w leptin and pre-pregnancy BMI, at 15w gestation at 32w gestation</td>
<td>0.49 &lt;0.001 0.52 &lt;0.001 0.57 &lt;0.001</td>
<td></td>
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</tbody>
</table>
### Table 1.1  Continued

<table>
<thead>
<tr>
<th>Author, year</th>
<th>Study type</th>
<th>Time postpartum</th>
<th>Sample size</th>
<th>Analyses, sample type</th>
<th>HM leptin (ng/mL) *</th>
<th>Maternal BMI (kg/m²)/%FM Association with BMI/adiposity</th>
<th>Association with BMI/adiposity</th>
<th>B (SE) or correlation</th>
<th>P - value</th>
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<tbody>
<tr>
<td>Kon, 2014</td>
<td>LS</td>
<td>1 month</td>
<td>103</td>
<td>ELISA, SHM</td>
<td>Low WG: 1m=1.63±0.27 3m=1.35±0.31 Normal WG: 1m=1.55±0.17 2m=1.83±0.23 3m=3.29±0.70 High WG: 1m=1.53±0.29 2m=2.20±0.28 3m=3.57±1.37</td>
<td>Maternal BMI and weight values NA</td>
<td>No association</td>
<td>NA</td>
<td>NA</td>
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<td>2 months</td>
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<td>3 months</td>
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<tr>
<td>Schueler, 2013</td>
<td>CS</td>
<td>4-5 weeks</td>
<td>13</td>
<td>RIA, SHM</td>
<td>1.0±0.7 (0.2-2.6)</td>
<td>BMI=26.0±4.2 (20.4-33.0) %FM=39.9±7.6 (26.5-53.0) Weight=72.0±11.8 (52.8-92.3)</td>
<td>BMI</td>
<td>0.82</td>
<td>0.001</td>
</tr>
<tr>
<td>Savino, 2012</td>
<td>CS</td>
<td>87±40 days</td>
<td>23</td>
<td>RIA, SHM</td>
<td>2.34±5.73</td>
<td>27.4±4.9</td>
<td>Time not reported</td>
<td>No association</td>
<td>NA</td>
</tr>
<tr>
<td>Fields, 2012</td>
<td>CS</td>
<td>1 month</td>
<td>19</td>
<td>ELISA, SHM</td>
<td>0.09±0.05</td>
<td>Pre-pregnancy 26.6±6.6</td>
<td>Positive association</td>
<td>0.78</td>
<td>&lt;0.001</td>
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<td>(40±4 days)</td>
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<tr>
<td>Bronsky, 2011</td>
<td>LS</td>
<td>0 days</td>
<td>72</td>
<td>ELISA, SHM</td>
<td>0d=0.30±0.04 1m=0.20±0.03 3m=0.10±0.01 6m=0.10±0.02 12m=0.20±0.04</td>
<td>Pre-pregnancy 21.9±0.4</td>
<td>No association</td>
<td>NA</td>
<td>NA</td>
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<td>1 month</td>
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<td>12 months</td>
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<tr>
<td>Eilers, 2011</td>
<td>LS</td>
<td>3 days</td>
<td>40 term</td>
<td>RIA, SHM</td>
<td>Term: 3d=0.65±0.67 28d=0.50±0.50 Preterm: 3d=0.70±0.79 28d=0.50±0.40</td>
<td>Pre-pregnancy 23.0±3.5</td>
<td>All infants: 3d 28d</td>
<td>0.28</td>
<td>&lt;0.01</td>
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<tr>
<td></td>
<td></td>
<td>28 days</td>
<td>37 preterm</td>
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</table>
Table 1.1 Continued

<table>
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<tr>
<th>Author, year</th>
<th>Study type</th>
<th>Time postpartum</th>
<th>Sample size</th>
<th>Analyses, sample type</th>
<th>HM leptin (ng/mL) *</th>
<th>Maternal BMI (kg/m^2)/%FM</th>
<th>Association with BMI/adiposity</th>
<th>β (SE) or correlation</th>
<th>P - value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Schuster, 2011</td>
<td>LS</td>
<td>1 week 6 months</td>
<td>23</td>
<td>RIA, SHM</td>
<td>1w=0.21±0.19 6m=0.18±0.15</td>
<td>Pre-pregnancy 21.4±2.6 20.9 (19.3; 22.6)</td>
<td>Positive association with BMI; No association with pre-pregnancy BMI</td>
<td>0.30</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Weyermann, 2007</td>
<td>LS</td>
<td>6 weeks 12 months 24 months</td>
<td>674</td>
<td>ELISA, SHM</td>
<td>6w=0.18(0.4.12) BMI: &lt;20=0.12±0.13 20-24.9=0.23±0.26 25-29.9=0.39±0.34 &gt;30=0.81±0.89</td>
<td>Pre-pregnancy 23.6±4.0 (16.7-45.7)</td>
<td>Strong positive association</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Bronsky, 2006</td>
<td>CS</td>
<td>48 hours after the initiation of lactation</td>
<td>59</td>
<td>ELISA, SHM</td>
<td>0.5±0.05 (0-1.37)</td>
<td>Pre-pregnancy 21.4±0.4 At delivery 26.8±0.4</td>
<td>Pre-pregnancy BMI Pre-pregnancy weight BMI at delivery Weight at delivery</td>
<td>0.40</td>
<td>0.003</td>
</tr>
<tr>
<td>Miralles, 2006</td>
<td>LS</td>
<td>1 month 3 months 6 months 9 months</td>
<td>28</td>
<td>ELISA, WHM</td>
<td>1m=0.16±0.08 (0-0.85)</td>
<td>Pre-pregnancy 21.6±0.5 (16.3-27.3)</td>
<td>Positive associations at each time point Stronger when log transformed</td>
<td>0.39</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Dundar, 2005</td>
<td>LS</td>
<td>15 days 1 month 2 months 3 months</td>
<td>11 SGA 22 AGA 14 LGA</td>
<td>RIA, WHM</td>
<td>SGA: 15d=13.4±2.2 1m=17.0±3.4 2m=11.4±2.3 3m=9.1±1.8 AGA: 15d=15.2±2.0 1m=19.4±1.7 2m=18.3±2.4 3m=11.8±1.8 LGA: 15d=18.5±4.4 1m=15.5±4.9 2m=15.1±2.7 3m=17.4±3.4</td>
<td>Time not reported 23.8±0.8</td>
<td>No association</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Author, year</td>
<td>Study type</td>
<td>Time postpartum</td>
<td>Sample size</td>
<td>Analyses, sample type</td>
<td>HM leptin (ng/mL) *</td>
<td>Maternal BMI (kg/m²)/%FM</td>
<td>Association with BMI/adiposity</td>
<td>β (SE) or correlation</td>
<td>P - value</td>
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<tr>
<td>Bielicki, 2004</td>
<td>LS</td>
<td>2-3 days 4-5 days 4-6 weeks</td>
<td>33 24 term 9 preterm</td>
<td>RIA, WHM</td>
<td>Term=1.34±0.14 Preterm=0.63±0.18 2-3d=1.15±0.12 4-5d=0.79±0.10 4-6w=NA</td>
<td>Term2-3d=25.7±0.8 Preterm4-6w=24.4±0.9 Preterm2-3d=23.8±1.2 Preterm4-6w=21.8±0.8</td>
<td>BMI at 2-3d</td>
<td>0.15</td>
<td>0.02</td>
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<td></td>
<td></td>
<td>No association at 4-6w</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Uysal, 2002</td>
<td>CS</td>
<td>3 months</td>
<td>17 obese 33 normal infants</td>
<td>RIA, SHM</td>
<td>Obese <em>=0.27±0.2 Normal</em>=0.37±0.4</td>
<td>BMI at sample collection 25.9±4.4</td>
<td>Positive association</td>
<td>0.62</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Ucar, 2000</td>
<td>CS</td>
<td>3-120 days</td>
<td>18</td>
<td>RIA, SHM</td>
<td>3.36±11.0</td>
<td>25.9±0.7</td>
<td>No association</td>
<td>NA</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Houseknecht, 1997</td>
<td>CS</td>
<td>Not reported</td>
<td>14</td>
<td>RIA, SHM WHM</td>
<td>SHM=1.5±0.9 WHM=10.1±2.6</td>
<td>Not reported</td>
<td>BMI - SHM</td>
<td>0.66</td>
<td>0.008</td>
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<td></td>
<td>BMI - WHM</td>
<td>0.50</td>
<td>&lt;0.06</td>
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<td></td>
<td>Triceps - SHM</td>
<td>0.84</td>
<td>&lt;0.001</td>
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<td></td>
<td></td>
<td>Triceps – WHM</td>
<td>0.55</td>
<td>&lt;0.05</td>
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<td></td>
<td></td>
<td>Weight - SHM</td>
<td>0.69</td>
<td>&lt;0.04</td>
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<td></td>
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<td></td>
<td></td>
<td></td>
<td>Weight - WHM</td>
<td>0.56</td>
<td>&lt;0.04</td>
</tr>
</tbody>
</table>

AGA – average for gestational age; β - parameter estimate; BIA – bioelectrical impedance analysis; BMI – body mass index; CS – cross-sectional study; DXA – dual-energy X-ray absorptiometry; ELISA – enzyme-linked immunosorbent assay; %FM – percentage fat mass; HM – human milk; LGA – large for gestational age; LS – longitudinal study; MIA – mixed immunoassay; NA – not available/reported; RIA – radioimmunoassay; SE – standard error of estimate; SGA – small for gestational age; SHM – skim human milk; ST – skinfold thickness; WHM – whole human milk. * Indicate mothers of obese or non-obese (normal) infants. Leptin concentration in human milk is presented as mean±SD or as median (IQR). Effect of adiposity on leptin concentration is presented as parameter estimate (SE) or as correlation coefficient.
1.3.2.3 Leptin and infant gastric emptying

The positive associations between maternal percentage FM (%FM)/BMI and HM leptin suggest that the greater amount of adipose tissue a mother has, the more leptin is secreted into her circulation and thus transferred into her milk. In infants, HM is believed to be a major source of leptin early in life, with the endogenous leptin-synthesising mechanisms being still immature (Oliver et al. 2002). Higher serum leptin levels are observed in breastfed infants compared to formula-fed infants, likely due either to leptin being rendered inactive during the processing of bovine milk for formula (Savino et al. 2005a) or to human leptin antibodies not binding optimally to bovine leptin during assay. Leptin and its receptor were initially detected in the gastric mucosa of adult humans (Sobhani et al. 2000) and animals (Goiot et al. 2001), then later it was found that all mucosae in human fetus starts expressing the leptin Ob-Rb receptor between 7th and 9th week of gestation, and the leptin protein around the 11th week, suggesting an important role of leptin in the developmental process and maturation of the human GI tract (Aparicio et al. 2005).

Indeed, leptin is known to have multiple complex roles in the metabolism and rapid growth of the newborn infant, including developing the areas of the hypothalamus that control and regulate energy balance (Stocker et al. 2008), modulating several important functions of the GI tract through the vagal afferent nerve including motility of the small intestine and intestinal inflammatory response, and potentially controlling the assimilation of macronutrients, fats and proteins (Yarandi et al. 2011; Prescott 2016). Leptin in HM has been hypothesised to be involved both in the short-term control of appetite and in developmental programming of appetite and energy signalling pathways, promoting efficient energy control and storage throughout life (Pico et al. 2007; Bouret et al. 2004b; Schwartz et al. 2002). In support of these roles, leptin administered during the first 14 days of life acts as a neurotrophic agent, promoting neural growth from the arcuate nucleus of the hypothalamus to multiple appetite control centres located in the central nervous system (Proulx et al. 2002). However, the influence of HM leptin on GE, a key regulator of appetite, has not been well studied. Studies in murine models have confirmed that ingested maternal milk leptin is absorbed in the stomach and transferred to the circulation remaining biologically active (Sanchez et al. 2005) and that injection of leptin into fourth ventricle delays GE of a glucose solution (Smedh et al. 1998) and oral administration reduces food intake (Sanchez et al. 2005). In the rat model central administration of leptin slowed GE of a solid meal and intraperitoneal
injections reduced food intake but did not affect gastric transit (Martinez et al. 1999). Also rat pups that received leptin orally during lactation period were better protected against fat accumulation and more sensitive to the short- and long-term regulation of food intake by the leptin (Pico et al. 2007).

In contrast, in our group it was shown that in term breastfed infants skim HM leptin is not associated with the time between the feeds across a 24-h period (3–21 weeks; \( n=19 \)) (Cannon et al. 2015) or GE, time between two consecutive feeds and pre-feed gastric residual (GR) volume (6-32 weeks; \( n=20 \)) (Cannon et al. 2017). Firstly, this emphasizes the need for studies analysing whole milk leptin, where we have shown levels of leptin to be higher (Kugananthan et al. 2016). Secondly, this suggests that the action of HM leptin on short-term appetite control may be mediated by upregulation of circulating melanocortins, potent anorexigenic agents that promote satiety (Miralles et al. 2006). Thus actions other than regulation of GE or downstream effects of HM leptin not yet measured are likely responsible for the beneficial effects of HM in protection against obesity later in life. Scientific investigation is yet to refine our understanding of the intricacies of gastric function particularly in relation to bioactive molecules such as leptin, whose actions are influenced by CCK (Yarandi et al. 2011) and ghrelin in HM (Inui et al. 2004).

### 1.3.2.4 Leptin and infant body composition

Leptin from rat adipocytes and osteoblasts is a known bone controller and both, supresses and stimulates bone growth, and might control bone growth in humans (Whitfield et al. 2002) and affect infant BC. While limited research has been conducted to investigate the link between infant adiposity and HM leptin levels, the reported results, although not uniform, suggest a possible role of leptin in regulating infant growth and BC. Out of the 18 studies conducted in breastfed infants at various time points between birth and 5 years of age (Table 1.2), 7 studies found no associations with infant anthropometry/BC. Three studies found positive associations with weight and/or adiposity (Kon et al. 2014; Bielicki et al. 2004; Weyermann et al. 2007). Six studies found negative associations (Miralles et al. 2006; Schuster et al. 2011; Brunner et al. 2014; Quinn et al. 2015; Fields et al. 2017; Quinn et al. 2016), with one of these studies (Brunner et al. 2014) loosing the associations seen in the early months of lactation at 2 years of age and at at 3, 4 and 5 years follow up (Meyer et al. 2016). To add to the confusion, two studies reported both: positive associations with either weight (Dundar ...
et al. 2005) or weight gain (Fields et al. 2012), and negative with either weight gain (Dundar et al. 2005) or BMI z-scores (Fields et al. 2012).

With exception of Miralles et al., who measured leptin in whole HM, these studies have measured leptin in skim milk using predominantly ELISA. There are also studies that found positive associations with infant weight gain using ELISA in skim HM (Kon et al. 2014) and infant birth weight and BMI using RIA in whole HM (Bielicki et al. 2004) respectively.

As with studies of maternal adiposity and HM leptin (Andreas et al. 2014), there are very few longitudinal studies of breastfed infants. Those that have been conducted are fraught with failure to match the time of sample collection to the time of the infant BC measurement and focus almost exclusively on the earlier months of lactation. Crude infant measurements of BC such as weight, weight gain and BMI, weight for age (WAZ), weight for length (WLZ), BMI z-scores are often made. Only 4 studies have used either DXA (Fields et al. 2012; Fields et al. 2017) or skinfolds (Brunner et al. 2014; Quinn et al. 2016) to predict infant BC, and one of them only reported WAZ scores but not the skinfolds findings (Quinn et al. 2016). Unfortunately, these studies analyzed leptin in skim HM.

The relationship between HM leptin and infant BC is also complicated by the fact that maternal body weight and BC predicts infant anthropometric outcomes, thus correlation between infant growth and adiposity parameters and HM leptin could be a result of maternal BMI determining both infant BC (Castillo-Laura et al. 2015) and HM leptin (Andreas et al. 2014) without a direct relevance between them (Demmelmair et al. 2017).
### Table 1.2 Studies investigating associations between human milk leptin and infant anthropometrics and adiposity

<table>
<thead>
<tr>
<th>Author, year</th>
<th>Study type</th>
<th>Time postpartum</th>
<th>Sample size</th>
<th>Analyses, sample type</th>
<th>HM leptin (ng/mL) *</th>
<th>Effect on infant body composition/anthropometry</th>
<th>β (SE) or correlation</th>
<th>P -value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fields, 2017</strong></td>
<td>LS</td>
<td>1 month 6 months</td>
<td>37 30</td>
<td>ELISA, SHM</td>
<td>1m=0.59 (0.35–1.45) 6m=0.43 (0.15–0.94)</td>
<td>1m leptin and: 6m weight length %FM with DXA FM with DXA trunk FM with DXA</td>
<td>-805 (-1688, 78) -2.01 (-3.75, -0.27) -4.41 (-8.13, -0.69) -614 (-1134, -94) -335 (-586, -84)</td>
<td>0.072 0.026 0.022 0.023 0.011</td>
</tr>
<tr>
<td><strong>Andreas, 2016</strong></td>
<td>LS</td>
<td>1 week 3 months</td>
<td>105 92</td>
<td>MIA, SHM</td>
<td>1wpre=541.7(161.2) 1wpost=614.4(172.1) 3mpre=684.8(117.4) 3mpost=464.3(125.1)</td>
<td>No association with infant anthropometrics</td>
<td>NA NA</td>
<td></td>
</tr>
<tr>
<td><strong>De Luca, 2016</strong></td>
<td>CS</td>
<td>1 month</td>
<td>50 obese, 50 normal mothers</td>
<td>RIA, WHM</td>
<td>Obese mothers = 4.8±2.7 Normal mothers = 2.5±1.5</td>
<td>No association with infant weight, length, head circumference, weight gain, weight gain velocity at birth and at 1m</td>
<td>NA &gt;0.17</td>
<td></td>
</tr>
<tr>
<td><strong>Quinn, 2016</strong></td>
<td>CS</td>
<td>0 days to 36 months</td>
<td>116, 66 from Kathmandu, 50 from Nubri</td>
<td>ELISA, SHM</td>
<td>Overall: 0.27±0.25 Kathmandu: 0.35±0.33 Nubri: 0.20±0.13</td>
<td>WAZ z-scores Kathmandu: unadjusted adjusted Nubri: unadjusted adjusted</td>
<td>-0.001 (0.0004) -0.001 (0.0005) 0.001 (0.001) 0.001 (0.001)</td>
<td>0.035 0.020 0.25 0.26</td>
</tr>
<tr>
<td><strong>Khodabakhshi, 2015</strong></td>
<td>CS</td>
<td>2 month 4 months 6 months</td>
<td>40 obese 40 normal infants</td>
<td>ELISA, SHM</td>
<td>Obese= 1.78 (1.67–1.94) Normal= 1.81 (1.65–1.94)</td>
<td>No association with infant weight (Details provided by the authors)</td>
<td>-0.007 – 0.187</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td><strong>Quinn, 2015</strong></td>
<td>CS</td>
<td>10 days to 36 months</td>
<td>113 71 &lt;1y 42 &gt;1y</td>
<td>ELISA, SHM</td>
<td>0.30±0.29 (0.05-2.1)</td>
<td>WAZ z-scores 10d-36m univariate 10d-36m adjusted &lt;1y all &lt;1y females &lt;1y males Similar for BMI Z-scores</td>
<td>-0.08 (0.04) -0.12 (0.14) -0.39 (0.16) -0.57 (0.19) -0.28 (0.25) NA</td>
<td>0.032 0.40 0.017 0.006 0.28 NA</td>
</tr>
</tbody>
</table>
## Table 1.2 Continued

<table>
<thead>
<tr>
<th>Author, year</th>
<th>Study type</th>
<th>Time postpartum</th>
<th>Sample size</th>
<th>Analyses, sample type</th>
<th>HM leptin (ng/mL) *</th>
<th>Effect on infant body composition/anthropometry</th>
<th>B (SE) or correlation</th>
<th>P -value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brunner, 2014</td>
<td>LS</td>
<td>6 weeks 4 months</td>
<td>151 120</td>
<td>RIA, SHM</td>
<td>6w=0.11 (0.19) 4m=0.09 (0.18)</td>
<td>6w leptin - no associations 4m leptin: 4m weight (kg) 4m FFM with ST (kg) No association at 2y</td>
<td>NA -0.61 (-1.17; -0.04) -0.40 (-0.78; -0.02) NA</td>
<td>NA 0.037 0.039 NA &gt;0.11</td>
</tr>
<tr>
<td>(follow up) Meyer, 2016</td>
<td></td>
<td>2 years 3 years 4 years 5 years</td>
<td>118 139 137 132</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kon, 2014</td>
<td>LS</td>
<td>1 month 2 months 3 months</td>
<td>103 Low, normal, high WG</td>
<td>ELISA, SHM</td>
<td>Low WG: 1m=1.63±0.27 3m=1.35±0.31 Normal WG: 1m=1.55±0.17 2m=1.83±0.23 3m=3.29±0.70 High WG: 1m=1.53±0.29 2m=2.20±0.28 3m=3.57±1.37</td>
<td>At 3m leptin is higher in milk of mothers of infants with normal and high WG</td>
<td>NA 0.02 (0.0; 0.04)^ NA</td>
<td>NA &lt;0.05</td>
</tr>
<tr>
<td>Schueler, 2013</td>
<td>CS</td>
<td>4-5 weeks</td>
<td>13</td>
<td>RIA, SHM</td>
<td>1.0±0.7 (0.2-2.6)</td>
<td>No association with infant anthropometrics</td>
<td>NA NA</td>
<td></td>
</tr>
<tr>
<td>Fields, 2012</td>
<td>CS</td>
<td>1 month (40±4 days)</td>
<td>19</td>
<td>ELISA, SHM</td>
<td>0.09±0.05</td>
<td>Univariate: WG Adjusted: BMI Z-scores WLZ Z-scores No associations for weight, length, %FM, FM, FFM with DXA</td>
<td>0.52 -0.54 -0.44 -0.37 -0.28 &gt;0.05</td>
<td>0.03 0.03 0.08 0.05</td>
</tr>
<tr>
<td>Bronsky, 2011</td>
<td>LS</td>
<td>0 days 1 month 3 months 6 months 12 months</td>
<td>72</td>
<td>ELISA, SHM</td>
<td>0d=0.30±0.04 1m=0.20±0.03 3m=0.10±0.01 6m=0.10±0.02 12m=0.20±0.04</td>
<td>0d - body length No associations for weight and WG</td>
<td>NA NA</td>
<td></td>
</tr>
<tr>
<td>Author, year</td>
<td>Study type</td>
<td>Time postpartum</td>
<td>Sample size</td>
<td>Analyses, sample type</td>
<td>HM leptin (ng/mL) *</td>
<td>Effect on infant body composition/anthropometry</td>
<td>β (SE) or correlation</td>
<td>P - value</td>
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</tr>
<tr>
<td>Schuster, 2011</td>
<td>LS</td>
<td>1 week 6 months</td>
<td>23</td>
<td>RIA, SHM</td>
<td>1w=0.21±0.19 6m=0.18±0.15</td>
<td>No association with weight 1w leptin - WG at 4w 1w leptin – WG 1-6m</td>
<td>NA</td>
<td>0.15</td>
</tr>
<tr>
<td>Weyermann, 2007</td>
<td>LS</td>
<td>6 weeks 12 months 2 years</td>
<td>674</td>
<td>ELISA, SHM</td>
<td>6w=0.18(0-4.12) Maternal BMI: &lt;20=0.12±0.13 20-24.9=0.23±0.26 25-29.9=0.39±0.34 &gt;30=0.81±0.89 Higher leptin associated with higher odds ratio for being overweight at 2 years: ever breastfed breastfed for 6 months</td>
<td>1.1 (0.8, 1.5) NA</td>
<td>0.15</td>
<td>0.007</td>
</tr>
<tr>
<td>Bronsky, 2006</td>
<td>CS</td>
<td>48 h after initiation of lactation</td>
<td>59</td>
<td>ELISA, SHM</td>
<td>0.5±0.05 (0-1.37) Birth weight - borderline association Ponderal index - no association</td>
<td>0.24</td>
<td>0.072</td>
<td></td>
</tr>
<tr>
<td>Miralles, 2006</td>
<td>LS</td>
<td>1 month 3 months 6 months 9 months 2 years</td>
<td>28</td>
<td>ELISA, WHM</td>
<td>1m=0.16±0.04 (0-0.85) 1m leptin - BMI at 18m BMI at 2y Log leptin and BMI: 1m leptin – 12m 18m 24m 3m leptin – 12m 18m 24m No association with weight and WG in full data Excluding 2 outliers 1m leptin - weight and WG at all ages</td>
<td>-0.49 &lt;0.05</td>
<td>-0.46 &lt;0.05</td>
<td>-0.43 &lt;0.05</td>
</tr>
</tbody>
</table>
### Table 1.2 Continued

<table>
<thead>
<tr>
<th>Author, year</th>
<th>Study type</th>
<th>Time postpartum</th>
<th>Sample size</th>
<th>Analyses, sample type</th>
<th>HM leptin (ng/mL) *</th>
<th>Effect on infant body composition/anthropometry</th>
<th>β (SE) or correlation</th>
<th>P - value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dundar, 2005</td>
<td>LS</td>
<td>15 days 1 month 2 months 3 months</td>
<td>11 SGA 22 AGA 14 LGA</td>
<td>RIA, WHM</td>
<td>SGA: 15d=13.4±2.2 1m=17.0±3.4 2m=11.4±2.3 3m=9.1±1.8 AGA: 15d=18.2±2.0 1m=19.4±1.7 2m=18.3±2.4 3m=11.8±1.8 LGA: 15d=28.5±4.4 1m=15.5±4.9 2m=15.1±2.7 3m=17.4±3.4</td>
<td>15d leptin - birth weight 15d leptin - 15d WG 15d leptin - 1m WG</td>
<td>0.47 -0.44 -0.40</td>
<td>0.001 0.002 0.005</td>
</tr>
<tr>
<td>Bielicki, 2004</td>
<td>LS</td>
<td>2-3 days 4-5 days 4-6 weeks</td>
<td>33 24 term 9 preterm</td>
<td>RIA, WHM</td>
<td>Term=1.34±0.14 Preterm=0.63±0.18 2-3d=1.15±0.12 4-5d=0.79±0.10 4-6w=NA</td>
<td>2-3d leptin - birth weight and BMI</td>
<td>0.16 &lt;0.05</td>
<td></td>
</tr>
<tr>
<td>Ucar, 2000</td>
<td>CS</td>
<td>3-120 days</td>
<td>18</td>
<td>RIA, SHM</td>
<td>3.36±11.0</td>
<td>No association with BMI</td>
<td>NA</td>
<td>&gt;0.05</td>
</tr>
</tbody>
</table>

*β - parameter estimate; BMI – body mass index; BMZ – BMI-for-age z-scores; CI – confidence intervals; CS – cross-sectional study; ELISA – enzyme-linked immunosorbent assay; FFM – fat-free mass; FM – fat mass; %FM – percentage fat mass; HM – human milk; LAZ – length-for-age z-scores; LGA – low for gestational age; LS – longitudinal study; MIA – mixed immunoassay; NA – not available/reported; RIA - radioimmunoassay; SE – standard error of estimate; SHM – skim human milk; ST – skinfold thickness; WAZ – weight-for-age z-scores; WG – weight gain; WHM – whole human milk; WLZ – weight-for-length z-score. Leptin concentration in human milk is presented as mean±SD or as median (IQR). Effect of leptin on infant adiposity is presented as parameter estimate (SE) or as correlation coefficient. ^ Unadjusted for confounders.
1.3.3 Adiponectin

Adiponectin is the appetite hormone present in the highest concentrations in HM and is more than 40 times higher than that of ghrelin and leptin (Martin et al. 2006; Savino et al. 2012b; Scherer et al. 1995). Amongst its various functions adiponectin has anti-inflammatory properties, breaks down fatty acids and heightens sensitivity to insulin (Nigro et al. 2014). Adiponectin is secreted by adipose tissue and also synthesised by the breast (Hassiotou et al. 2014c), and circulates as oligomers of different sizes, from low-molecular-weight trimer to the most common form in HM a high-molecular-weight octodecamer (Pajvani et al. 2003). In contrast the most common bovine milk form of adiponectin is a middle-molecular-weight form (Heinz 2014). Adiponectin is present in HM in a biologically active form, which is resistant to digestion and is able to reach the infant GI tract populated with adiponectin receptors (Newburg et al. 2010).

1.3.3.1 Adiponectin and maternal adiposity

HM adiponectin concentrations are positively associated with maternal serum levels (Woo et al. 2012), however in contrast to leptin, generally maternal serum concentrations of adiponectin are lower if body weight and BMI are higher (Arita et al. 1999; Savino et al. 2012b). Counter-intuitively, the majority of studies evaluated in the meta-review (Andreas et al. 2014) and some recent studies show no association, with only two out of 12 studies reporting positive associations (Martin et al. 2006; Woo et al. 2009) (Table 1.3). Thus it is unlikely that maternal adiposity plays a major role in influencing HM adiponectin levels and the majority of HM adiponectin may be synthesized and controlled by the mammary gland (Anderson et al. 2016) highlighting the importance of this HM hormone for the infant.

As with leptin, the use of BMI as a measure of maternal adiposity may contribute to these conflicting findings, although %FM predicted with ST measurements has also shown no association (Anderson et al. 2016). Furthermore, different trends for adiponectin concentrations across the lactation period are reported with adiponectin concentration either declining gradually in the first 6-7 months (Martin et al. 2006; Wang et al. 2011; Savino et al. 2012b; Woo et al. 2009) or increasing during (Ozarda et al. 2012) or after first 6 months of lactation (Bronsky et al. 2011). Interestingly, reduced adiponectin concentrations are observed in the milk of smoking mothers, with lowest concentrations in those that smoked higher numbers of cigarettes per day (Weyermann et al. 2007), while higher adiponectin concentrations in colostrum are associated with higher maternal protein intake during third trimester and lower weight gain during
pregnancy (Luoto et al. 2012). As such, investigations into other factors that may affect adiponectin concentrations in HM and its effect on infant growth and BC development are warranted.

1.3.3.2 Adiponectin and infant gastric emptying

Strong correlations between adiponectin levels in HM and infant serum levels (Wang et al. 2011; Newburg et al. 2010) along with the presence of adiponectin receptors in infant’s intestinal tract (Newburg et al. 2010) imply a pathway for maternal hormonal signals in the developing infant. The fact that circulating adiponectin levels are generally higher in newborns and infants (Kotani et al. 2004) than in adults and children, emphasize its importance for the infant and raises questions regarding it’s biological role. Indeed, higher levels of circulating plasma adiponectin in adults are related to lower BMI and healthier metabolism, and in infants, are known to associate with weight and BC (Woo et al. 2009; Brunner et al. 2014; Woo et al. 2012), but are yet to be investigated in relation to appetite control and GE in the term breastfed infant.

In animal models (rat, mouse) it has been shown that gastric epithelium and glands are populated with adiponectin receptors, which down-regulate gastric motility (Kentish et al. 2015; Gonzalez et al. 2010). Also adiponectin inhibits tension sensitive gastric vagal afferent mechanosensitivity, modulating satiety signals in both lean and obese animals, while simultaneously increasing the mechanosensitivity of mucosal gastric vagal afferent in an obesity-induced model (Kentish et al. 2015). Conversely in humans, elevated serum levels of adiponectin are associated with more rapid GE in diabetic patients (Iwase et al. 2009), while in 3 months old infants HM adiponectin was not associated with FFQ (Anderson et al. 2016). It is not known whether HM adiponectin levels impact MI, time between the feeds or GE in the infant and this warrants further investigation.
Table 1.3 *Studies investigating associations between human milk adiponectin and maternal adiposity*

<table>
<thead>
<tr>
<th>Author, year</th>
<th>Study type</th>
<th>Time postpartum</th>
<th>Sample size</th>
<th>Analyses, sample type</th>
<th>HM adiponectin (ng/mL) *</th>
<th>Maternal BMI (kg/m²)/%FM</th>
<th>Association with BMI/adiposity</th>
<th>β (SE) or correlation</th>
<th>P - value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Anderson, 2016</strong></td>
<td>CS</td>
<td>0 to 24 months</td>
<td>117</td>
<td>ELISA, SHM</td>
<td>7.47±5.75 (1.37-19.11)</td>
<td>BMI: 19.9±3.1</td>
<td>%FM: 24.9±3.6</td>
<td>BMI: no association</td>
<td>0.004 (0.023)</td>
</tr>
<tr>
<td><strong>Quinn, 2016</strong></td>
<td>CS</td>
<td>0 days to 36 months</td>
<td>116, 66 from Kathmandu, 50 from Nubri</td>
<td>ELISA, SHM</td>
<td>Overall: 4.1±2.13</td>
<td>%FM Kathmandu: 27.4±4.4</td>
<td>No association</td>
<td>0.064 (0.088)</td>
<td>&lt;0.44</td>
</tr>
<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
<td>Kathmandu: 3.60±1.89</td>
<td>Nubri: 4.39±2.25</td>
<td>Kathmandu unadjusted</td>
<td>0.057 (0.096)</td>
<td>&lt;0.55</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>adjusted</td>
<td></td>
<td>0.034 (0.121)</td>
<td>&lt;0.78</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Nubri unadjusted</td>
<td>0.066 (0.16)</td>
<td>&lt;0.60</td>
<td></td>
</tr>
<tr>
<td><strong>Khodabakhshi, 2015</strong></td>
<td>CS</td>
<td>2 months</td>
<td>Mothers of 40 obese, 40 normal infants</td>
<td>ELISA, SHM</td>
<td>Obese*: 323.48 (281.14-350.89)</td>
<td>No association</td>
<td>Obese* BMI</td>
<td>-0.08</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Normal*: 330.05 (298.33-376.81)</td>
<td>Normal* BMI</td>
<td>-0.007</td>
<td>&gt;0.05</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Obese* %FM with BIA</td>
<td>-0.10</td>
<td>&gt;0.05</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Normal* %FM with BIA (Details provided by the authors)</td>
<td>0.05</td>
<td>&gt;0.05</td>
<td></td>
</tr>
<tr>
<td><strong>Brunner, 2014</strong></td>
<td>LS</td>
<td>6 weeks</td>
<td>151</td>
<td>RIA, SHM</td>
<td>6w=10.93 (8.34)</td>
<td>Pre-/during pregnancy, values NA</td>
<td>No association</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>4m=10.36 (9.40)</td>
<td></td>
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<tr>
<td><strong>Ley, 2012</strong></td>
<td>LS</td>
<td>2 days</td>
<td>170</td>
<td>RIA, SHM</td>
<td>2d=50.0 (21.9/104.6)</td>
<td>Pre-pregnancy 24.4±2.9</td>
<td>No association</td>
<td>0.003 (0.014)</td>
<td>0.81</td>
</tr>
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<td>3m=12.3 (9.9/17.2)</td>
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<tr>
<td><strong>Luoto, 2012</strong></td>
<td>CS</td>
<td>0-3 days</td>
<td>181</td>
<td>FIA, NA</td>
<td>0-3d=18.4 (2.9-317.0)</td>
<td>Pre-pregnancy 23.8±3.7</td>
<td>No association</td>
<td>0.023</td>
<td>0.76</td>
</tr>
<tr>
<td><strong>Bronsky, 2011</strong></td>
<td>LS</td>
<td>0 days</td>
<td>72</td>
<td>ELISA, WHM</td>
<td>0d=22.8±0.8</td>
<td>Pre-pregnancy 21.9±0.4</td>
<td>No association</td>
<td>NA</td>
<td>NA</td>
</tr>
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<td></td>
<td></td>
<td>1 month</td>
<td></td>
<td></td>
<td>1m=22.0±0.6</td>
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<td></td>
<td>3 months</td>
<td></td>
<td></td>
<td>3m=20.5±0.6</td>
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<td>6 months</td>
<td></td>
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<td>6m=21.4±0.8</td>
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<td>12 months</td>
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<td>12m=25.7±1.4</td>
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<td><strong>Luoto, 2011</strong></td>
<td>CS</td>
<td>1-3 days</td>
<td>30</td>
<td>FIA, NA</td>
<td>1-3d=10.5 (3.1-98.9)</td>
<td>Pre-pregnancy 23.0 (18.4-28.4)</td>
<td>No association</td>
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<td><strong>Dundar, 2010</strong></td>
<td>CS</td>
<td>1 day</td>
<td>25</td>
<td>ELISA, WHM</td>
<td>1d=29.5±6.4 (1.26-77.1)</td>
<td>Pre-pregnancy 23.1±0.8</td>
<td>No association</td>
<td>NA</td>
<td>NA</td>
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<tr>
<td>Author, year</td>
<td>Study type</td>
<td>Time postpartum</td>
<td>Sample size</td>
<td>Analyses, sample type</td>
<td>HM adiponectin (ng/mL) *</td>
<td>Maternal BMI (kg/m²)</td>
<td>Association with BMI/adiposity</td>
<td>β (SE) or correlation</td>
<td>P -value</td>
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<td>Woo, 2009</td>
<td>LS</td>
<td>1 week</td>
<td>45</td>
<td>RIA, SHM</td>
<td>25.6±8.4</td>
<td>1m postpartum 25.4±3.5</td>
<td>Positive association</td>
<td>0.67 (0.30)</td>
<td>0.02</td>
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<td>2 weeks</td>
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<td>No association after adjusting for month postpartum</td>
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<td>3 weeks</td>
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<tr>
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<td></td>
<td>1-6 months</td>
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<tr>
<td>Weyermann, 2007</td>
<td>CS</td>
<td>33-71 days</td>
<td>767</td>
<td>ELISA, SHM</td>
<td>6w=10.9 (0.8-110.0)</td>
<td>Pre-pregnancy 23.6±4.0 (16.7-45.7)</td>
<td>No association</td>
<td>NA</td>
<td>NA</td>
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<td></td>
<td>43 (39/48)</td>
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<tr>
<td>Martin, 2006</td>
<td>LS</td>
<td>2-242 days</td>
<td>199</td>
<td>RIA, SHM</td>
<td>17.7 (4.2-87.9)</td>
<td>Pre-pregnancy 24.5 (19.5-34.2)</td>
<td>Positive association</td>
<td>0.08 (0.02)</td>
<td>&lt;0.001</td>
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</tbody>
</table>

β - parameter estimate; BMI – body mass index; CS – cross-sectional study; ELISA – enzyme-linked immunosorbent assay; %FM – percentage fat mass; FIA – dissociation enhanced lanthanide fluoro-immunoassay; HM – human milk; LS – longitudinal study; NA – not available/reported; RIA - radioimmunoassay; SE – standard error of estimate; SHM – skim human milk; WHM – whole human milk. * Indicate mothers of obese or non-obese (normal) infants. Adiponectin concentration in human milk is presented as mean±SD or as median (IQR). Effect of adiponectin on infant adiposity is presented as parameter estimate (SE) or as correlation coefficient.
Evidence suggests that HM adiponectin levels may regulate infant growth (Table 1.4). Out of 14 studies conducted on breastfed infants at various time points between birth and 10 years of age (Table 1.4), 4 studies found no associations of HM adiponectin with infant anthropology/BC (Kon et al. 2014; Cesur et al. 2012; Bronsky et al. 2011) (Dundar et al. 2010). Two studies found positive associations with either, WAZ and BMI z-scores (Anderson et al. 2016) or being overweight at 10 years of age (Luoto et al. 2011). Six studies found negative associations (Khodabakhshi et al. 2015; Weyermann et al. 2006; Weyermann et al. 2007; Woo et al. 2009; Wang et al. 2011; Quinn et al. 2016). One study showed negative associations with WAZ and WLZ z-scores in the first 6 months, none at 12 months, and positive at 2 years (Woo et al. 2012), when infants displayed more rapid growth and accretion of lean body mass and in a sense did ‘catch up’ to infants that received lower levels of adiponectin. These results were also supported by another study (Brunner et al. 2014), which found that higher concentrations of HM adiponectin up to 4 months were associated with lower infant FFM and anthropology at 4 months, but with greater weight and adiposity up to 2 years of age. The follow up at 3, 4 and 5 years of age has not shown any relationship with the exception of the positive association between HM adiponectin levels at 4 months postpartum and FM at 4 years (Meyer et al. 2016).

This reversal of the initial trend in early life is speculated to be related to the cessation of breastfeeding (Woo et al. 2012). High HM adiponectin levels may initially down-regulate infant growth, and later promote adipogenesis and adipocyte hypertrophy (Bieswal et al. 2006). Conversely, lean populations with lower concentrations of HM adiponectin demonstrate a positive association with the infant WAZ z-scores. This suggests, that the association between HM adiponectin and infant growth may in fact be parabolic, further highlighting the pleiotropic effects of adiponectin during development and the adaptive mechanisms that humans display depending on food availability (Anderson et al. 2016). On the other hand, elevated concentrations of adiponectin (35 ng/mL) and protein (12.5 g/L) have been observed at 1 year postpartum in a case study of excessive weight gain during the breastfeeding period, although no HM composition analyses were performed in the earlier months (Grunewald et al. 2014).

These findings support the notion of differential age related effects such that adiponectin modulates growth in early development and this growth pattern is thought to be responsible for the reduced or increased incidence of adult obesity.
## Table 1.4  Studies investigating associations between human milk adiponectin and infant anthropometrics and adiposity

<table>
<thead>
<tr>
<th>Author, year</th>
<th>Study type</th>
<th>Time postpartum</th>
<th>Sample size</th>
<th>Analyses, sample type</th>
<th>HM adiponectin (ng/mL) *</th>
<th>Effect on infant body composition/anthropometry</th>
<th>B (SE/95%CI) or correlation</th>
<th>P - value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anderson, 2016</td>
<td>CS</td>
<td>0 to 24 months</td>
<td>117</td>
<td>ELISA, SHM</td>
<td>7.47±5.75 (1.37-19.11)</td>
<td>Positive association WAZ z-scores</td>
<td>0.26 (0.11)</td>
<td>0.022</td>
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<td></td>
<td>WAZ z-scores</td>
<td>0.46 (0.21)</td>
<td>0.028</td>
</tr>
<tr>
<td>Quinn, 2016</td>
<td>CS</td>
<td>0 days to 36 months</td>
<td>116, 66 from Kathmandu, 50 from Nubri</td>
<td>ELISA, SHM</td>
<td>Overall: 4.1±2.13</td>
<td>WAZ z-scores</td>
<td>Kathmandu: unadjusted adjusted</td>
<td>-0.0002 (0.0001)</td>
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<td></td>
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<td></td>
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<td></td>
<td></td>
<td>Kathmandu: unadjusted adjusted</td>
<td>-0.0003 (0.0001)</td>
<td>0.003</td>
</tr>
<tr>
<td></td>
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<td></td>
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<td></td>
<td>Nubri: unadjusted adjusted</td>
<td>-0.006 (0.064)</td>
<td>0.93</td>
</tr>
<tr>
<td></td>
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<td></td>
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<td></td>
<td></td>
<td>Nubri: unadjusted adjusted</td>
<td>-0.019 (0.052)</td>
<td>0.77</td>
</tr>
<tr>
<td>Khodabakhshi, 2015</td>
<td>CS</td>
<td>2 months 4 months 6 months</td>
<td>40 obese 40 normal infants</td>
<td>ELISA, SHM</td>
<td>Obese=323.48 (281.14-350.89) Normal=330.05 (298.33-376.81)</td>
<td>Weight at 4m in obese infants only</td>
<td>-0.354</td>
<td>0.01</td>
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<td>(Details provided by the authors)</td>
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</tr>
<tr>
<td>Brunner, 2014</td>
<td>LS</td>
<td>6 weeks</td>
<td>151</td>
<td>RIA, SHM</td>
<td>6w=10.93 (8.34)</td>
<td>6w: FFM at 4m</td>
<td>-11.43 (-20.51; -2.34)</td>
<td>0.015</td>
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<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
<td>Sum 4 ST at 1y</td>
<td>0.09 (0.01; 0.18)</td>
<td>0.037</td>
</tr>
<tr>
<td></td>
<td></td>
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<td></td>
<td></td>
<td>%FM at 1y</td>
<td>0.06 (0.12)</td>
<td>0.037</td>
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<td></td>
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<td></td>
<td>FM at 1y</td>
<td>8.86 (0.25; 17.47)</td>
<td>0.046</td>
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<td></td>
<td>WG at 1y</td>
<td>23.70 (1.23; 46.17)</td>
<td>0.041</td>
</tr>
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<td></td>
<td>WG at 2y</td>
<td>27.01 (3.34; 50.69)</td>
<td>0.027</td>
</tr>
<tr>
<td>Meyer, 2016 (follow up)</td>
<td>LS</td>
<td>4 months 2 years 3 years 4 years 5 years</td>
<td>120 118 139 137 132</td>
<td>RIA, SHM</td>
<td>4m=10.36 (9.40)</td>
<td>4m: Sum 4 ST at 2y</td>
<td>0.14 (0.02; 0.25)</td>
<td>0.023</td>
</tr>
<tr>
<td></td>
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<td></td>
<td></td>
<td>%FM at 2y</td>
<td>0.10 (0.02; 0.18)</td>
<td>0.020</td>
</tr>
<tr>
<td></td>
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<td>FM at 2y</td>
<td>17.42 (0.43; 34.41)</td>
<td>0.048</td>
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<td></td>
<td>WG at 2y</td>
<td>29.48 (3.29; 55.05)^</td>
<td>0.026</td>
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<td>No associations at 3, 4, 5y</td>
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<td></td>
<td>4m: Sum 4 ST at 2y</td>
<td>0.02 (0.00; 0.04)^</td>
<td>0.042</td>
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<td>%FM at 2y</td>
<td>0.10 (0.02; 0.18)</td>
<td>0.020</td>
</tr>
<tr>
<td>Author, year</td>
<td>Study type</td>
<td>Time postpartum</td>
<td>Sample size</td>
<td>Analyses, sample type</td>
<td>HM adiponectin (ng/mL) *</td>
<td>Effect on infant body composition/anthropometry</td>
<td>β (SE/95% CI) or correlation</td>
<td>P-value</td>
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<td>Kon, 2014</td>
<td>LS</td>
<td>1 month</td>
<td>103</td>
<td>ELISA, SHM</td>
<td>Normal WG: 1m=1.14±0.09 2m=1.04±0.09 3m=1.14±0.08</td>
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<td>NA</td>
<td>NA</td>
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<td>Cesur, 2012</td>
<td>LS</td>
<td>1st month</td>
<td>25</td>
<td>ELISA, SHM</td>
<td>1m=23.61±32.95</td>
<td>No associations: Weight at 1m</td>
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<td>4th month</td>
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<td>ELISA, SHM</td>
<td>4m=6.66±9.48</td>
<td>Weight at 1m; BMI at 1m; Weight at 4m</td>
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<td>Woo, 2012</td>
<td>LS</td>
<td>Monthly up to 12 months</td>
<td>192</td>
<td>RIA, SHM</td>
<td>21.57±5.12</td>
<td>WAZ z-scores: 0m 3m 6m 12m</td>
<td>No association with infant weight</td>
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<td>0.21 (0.10) ^</td>
<td>0.04</td>
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<td>Bronsky, 2011</td>
<td>LS</td>
<td>0 days</td>
<td>72</td>
<td>ELISA, WHM</td>
<td>0d=22.8±0.8 1m=22.0±0.6 3m=20.5±0.6 6m=21.4±0.8 12m=25.7±1.4</td>
<td>No association with infant weight</td>
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<td>NA</td>
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<td>Luoto, 2011</td>
<td>LS</td>
<td>1-3 days</td>
<td>15 obese</td>
<td>FIA, NA</td>
<td>1-3d=10.5 (3.1-98.9)</td>
<td>Children overweight at 10 y had lower colostrum A</td>
<td>NA</td>
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<td></td>
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<td></td>
<td>15 normal</td>
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<td>Normal=15.2 (23.4) Obese=5.9 (2.1)</td>
<td>BMI at 10 years</td>
<td>NA</td>
<td>0.009</td>
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<td>10 years</td>
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### Table 1.4 Continued

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<th>Sample size</th>
<th>Analyses, sample type</th>
<th>HM adiponectin (ng/mL) *</th>
<th>Effect on infant body composition/anthropometry</th>
<th>β (SE/95% CI) or correlation</th>
<th>P-value</th>
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<tbody>
<tr>
<td>Wang, 2011</td>
<td>LS</td>
<td>2 weeks</td>
<td>73</td>
<td>ELISA, NA</td>
<td>2w=14.62 (5.93-140.40)</td>
<td>Higher A at 2w - lower WAZ at 13, 26, 52w</td>
<td>NA</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4 weeks</td>
<td></td>
<td></td>
<td>4w=7.32 (2.04-29.35)</td>
<td>Higher A at 4w - lower WAZ, WLZ, zBMI at 13, 26, 52w</td>
<td>NA</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td>13 weeks</td>
<td></td>
<td></td>
<td>13w=6.84 (2.72-15.65)</td>
<td>Higher A at 13w - lower WAZ, WLZ, zBMI at 13, 26, 52w</td>
<td>NA</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td>26 weeks</td>
<td></td>
<td></td>
<td>26w=4.88 (1.12-13.38)</td>
<td>Higher A at 26w - lower WAZ, WLZ, zBMI at 52w</td>
<td>NA</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td>52 weeks</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>Dundar, 2010</td>
<td>CS</td>
<td>1 day</td>
<td>25</td>
<td>ELISA, WHM</td>
<td>1d=29.5±6.4 (1.26-77.1)</td>
<td>No association with birth weight or BMI</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Woo, 2009</td>
<td>LS</td>
<td>1 week</td>
<td>45</td>
<td>RIA, SHM</td>
<td>25.6±8.4</td>
<td>Over first 6m: WAZ z-scores</td>
<td>-0.20 (0.04)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 weeks</td>
<td></td>
<td></td>
<td></td>
<td>WLZ z-scores</td>
<td>-0.29 (0.08)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3 weeks</td>
<td></td>
<td></td>
<td></td>
<td>LAZ z-scores</td>
<td>0.09 (0.06)</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1-6 months</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>Weyermann, 2007</td>
<td>CS</td>
<td>6 weeks</td>
<td>767</td>
<td>ELISA, SHM</td>
<td>6w=10.9 (0.8-110.0)</td>
<td>Higher A associated with higher odds ratio for being overweight at 2y: ever breastfed for 6 months</td>
<td>1.6 (1.0, 2.6)</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>12 months</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2.1 (1.1, 4.2)</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 years</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>Weyermann, 2006</td>
<td>CS</td>
<td>6 weeks</td>
<td>766</td>
<td>ELISA, SHM</td>
<td>12.8±10.1 (0.8-110.0)</td>
<td>Concentrations of A higher in mothers of LGA infants</td>
<td>NA</td>
<td>0.004</td>
</tr>
</tbody>
</table>

A – adiponectin; β - parameter estimate; BMI – body mass index; CI – confidence intervals; CS – cross-sectional study; ELISA – enzyme-linked immunosorbent assay; FFM – fat-free mass; FM – fat mass; %FM – percentage fat mass; FIA – dissociation enhanced lanthanide fluoro-immunoassay; HM – human milk; LAZ – length-for-age z-scores; LGA – low for gestational age; LS – longitudinal study; NA – not available/reported; RIA - radioimmunoassay; SE – standard error of estimate; SHM – skim human milk; ST – skinfold thickness; WAZ – weight –for-age z-scores; WG – weight gain; WHM – whole human milk; WLZ – weight –for-length z-scores; ^ Unadjusted for confounders. Adiponectin concentration in human milk is presented as mean±SD or as median (IQR). Effect of adiponectin on infant adiposity is presented as parameter estimate (SE) or as correlation coefficient.
1.3.4 Other appetite hormones

1.3.4.1 Ghrelin

Ghrelin is a less studied orexigenic 28 amino-acid peptide produced primarily in the stomach, but also in the lactating breast resulting in HM having a higher ghrelin concentration than maternal serum (Kierson et al. 2006). Ghrelin is an antagonist to leptin and stimulates appetite, gastric motility, acid secretion and food intake and is involved in long-term regulation of growth, weight and energy metabolism, thus heightened concentrations in HM could be vital for the infant drive to feed. Only few studies have investigated the association between ghrelin in HM and maternal BMI, reporting no association (Kon et al. 2014; Aydin et al. 2005; Aydin et al. 2007; Aydin 2010; Khodabakhshi et al. 2015). Ghrelin levels in HM increase during lactation (Ilcol et al. 2007; Aydin et al. 2005) and therefore, may influence infant feeding patterns and BC, although results of studies of the effect of ghrelin are sparse and contradictory.

The greatest activation of ghrelin receptors has been identified in the stomach rather than the hypothalamus as previously assumed, suggesting an important role for ghrelin in the regulation of GE (Inui et al. 2004). Ghrelin stimulates appetite and food intake and increases GE in rats (Asakawa et al. 2001) and humans (Levin et al. 2006) via vagal nerve and afferent activity.

High infant serum ghrelin levels are associated with increased age, length and weight in both formula-fed and breastfed infants (Savino et al. 2011; Savino et al. 2005b), with formula-fed infants having higher serum ghrelin levels than breastfed due to higher levels in formula (Savino et al. 2005a; Savino et al. 2011). Higher ghrelin serum levels in breastfed infants are associated with lower weight gains (Savino et al. 2005b) and in formula-fed infants with lower BMI (Savino et al. 2005a), indicating a role for ghrelin in the regulation of body weight in infants. Higher ghrelin levels in colostrum and mature HM are associated with lower birth weight, BMI (Dundar et al. 2010) and weight (Kon et al. 2014; Khodabakhshi et al. 2015) but with higher weight gain in breastfed infants (Kon et al. 2014; Cesur et al. 2012). These somewhat contradictory results may be due to the combined effects of other HM hormones that were not measured in previous studies. Also it is not clear if changes in ghrelin levels precede changes in appetite (Savino et al. 2006b) and body weight (Philipps; Savino et al. 2005b) or follow them. It has been established however, that both HM total and active ghrelin concentrations decreases from pre- to post-feed sample during established lactation period (measured at 2 and 5 months) (Karatas et al. 2011), indicating that
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ghrelin may contribute to the development of satiation at the end of breastfeeding and suggesting that HM ghrelin is synthesized or regulated in the mammary gland during breastfeeding.

Ghrelin influences novelty seeking behaviour in rodents and humans (Hansson et al. 2012), which may translate into an advantage of locating new sources of food. In humans, levels of ghrelin are blamed for impulsive shopping behaviour, as images of food become more desirable (Malik et al. 2008). These behaviour modulations mean that raised ghrelin levels in the infant will translate into hunger cues to gain attention of the mother, while levels of other hormones may indicate infant satiety cues to the mother, both enabling dynamic interaction between mother and infant and influencing milk composition and transfer, and infant growth (J. Wells 2016).

Further, longer fasting times are associated with higher serum levels of ghrelin in formula-fed infants in the first 6 months of life (Savino et al. 2006b). Inverse correlations between ghrelin and both leptin (Savino et al. 2005a) and insulin (Savino et al. 2006a) in infant serum indicate that these HM hormones may play a role in lowering ghrelin levels. Since ghrelin and leptin actions are mediated by NPY, reduced leptin levels allow ghrelin NPY stimulation and increase in food intake (Savino et al. 2005a). Indeed, more comprehensive studies are required to clarify potential interactions between these appetite hormones.

1.3.4.2 Insulin

Insulin is a major hormone involved in glucose metabolism and is produced by the pancreas. Studies in rats show that dietary insulin is not degraded in the stomach and plays an important role in the maturation and development of small intestine (Buts et al. 1988), thus HM insulin could play a similar role in the development of human intestinal epithelium. Concentrations of insulin are higher in colostrum than in mature milk (Shehadeh et al. 2003; Ley et al. 2012) and higher (almost 4 times) in human compared to bovine milk and infant formula where it is barely detectable (Shehadeh et al. 2001). Insulin is actively transported into HM in concentrations similar to maternal circulating levels (Whitmore et al. 2012). A recent systematic review (Andreas et al. 2014) identified four studies that examined effect of maternal (predominantly pre-pregnancy) BMI on HM insulin with two studies reporting no association (Fields et al. 2012; Shehadeh et al. 2003) and two reporting positive association (Ley et al. 2012; Ahuja et al. 2011) along with the two most recent studies (Koletzko et al. 2016; Fields et al. 2012).
2017), with Fields et al. study showing interaction between maternal BMI and infant sex with higher insulin levels detected in HM of obese mothers of girls.

No differences between insulin serum concentration, fasting time and anthropometrics of 1-18 months-old breastfed (n = 37) and formula-fed (n=25) infants were seen in one study (Savino et al. 2006a), although insulin in HM was not measured and analysed. Study that analysed HM found that higher concentrations of HM insulin were related to lower weight, WLZ, BMIZ z-scores and FFM but not the FM in 1 month-old breastfed infants (n=19) (Fields et al. 2012), reflecting insulin’s role as a regulator of energy balance, food intake and adiposity. However, the longitudinal study (n=37, 1 and 6 months), which also included the same infants, showed no associations with infant weight or adiposity (Fields et al. 2017). Recently HM insulin has been linked to microbial diversity of the infant gut, with both insulin and leptin associated with beneficial microbial metabolic pathways able to reduce inflammation (Lemas et al. 2016), further highlighting the diverse effects of these hormones.

1.3.4.3 Obestatin

Obestatin is an appetite-suppressing derivative of the ghrelin peptide precursor further emphasizing the complexity of the appetite hormone system. Obestatin is produced by the cell lining of the stomach and small intestine and salivary glands (Tang et al. 2008). Early studies reported that obestatin opposes ghrelin’s effect on body weight, food intake and GE (Zhang et al. 2005). However, later obestatin was implicated in inhibition of thirst and anxiety, regulation of sleep, memory improvement, induction of cell proliferation and increasing the secretion of the pancreatic juice enzymes (Tang et al. 2008). Obestatin has been identified in HM and while the source of obestatin has still not been established, it is likely actively produced by the mammary gland as concentrations are more than twice that of corresponding circulating maternal levels (Aydin et al. 2008). The relationship between obestatin, infant development and BC clearly requires further investigation.

1.3.4.4 Other hormones

Already a myriad of hormones have been identified in HM and there are likely still many more to be discovered. Hormones such as apelin, resistin, IGF-I, motilin, oxytocin and CCK also play significant role in metabolic development of infants and could be of interest in further research. Interestingly CCK, a peptide hormone of the GI system responsible for stimulating the digestion of fat and protein, appears to be involved in a feedback loop with leptin and is also involved in satiety. The effects of
leptin have also been shown to differ depending on either the presence or absence of CCK. Leptin regulates feeding behavior and appetite in the absence of CCK and increases GI tract motility if CCK is present, and CCK itself increases the release of leptin, suggesting that these two hormones form a positive feedback loop (Yarandi et al. 2011). Further, CCK has similar relationship with oxytocin, which has dual effect on GE, inhibiting GE via the CCK release and CCK-receptors, and stimulating gastric contractions via oxytocin receptors (Borg et al. 2012). Furthermore, other HM hormones such as ghrelin and insulin may counteract or interact synergistically with leptin (Perry et al. 2012; Chaudhri et al. 2006) and adiponectin.

In a summary, HM appetite hormones are associated with early infant growth and may influence subsequent health later in life. There is a serious need for innovative, well-designed studies to investigate the effects of HM appetite hormones on breastfeeding patterns, GE and development of BC in term breastfed infants.

1.4 BREASTFEEDING BEHAVIOUR OF HUMAN INFANTS

Human milk has evolved to provide nutrition specifically for human infants with an optimal form of delivery – on demand. Each breastfeeding dyad is unique and has different breastfeeding patterns and behaviour, suited to the dyad, such that there is a wide variation in both FFQ and volume of milk consumed by the infant between dyads (Butte et al. 1984; Kent et al. 2006; Dewey et al. 1983; Arthur et al. 1987). These variations may in part be influenced by milk composition in particular the minimally researched recently discovered appetite hormones in HM. Out of very few studies that have attempted to investigate the impact of daily intakes of HM components on infant growth one has been limited to a few components and the first 3 months of life (Kon et al. 2014), and second, while having an extensive list of fatty and linoleic acids analysed, has measured them in single daily samples, not in 24-h profile (Grote et al. 2016; Kon et al. 2014).

1.4.1 Milk intake

Breastfeeding rates in Australia show 96% of mothers initiating breastfeeding, with a rapid decline to 39% of infants still being exclusively breastfed at 3 and 15% at 5 months (Australian Institute of Health and Welfare 2011). Thereafter, 28% of infants are still breastfed at 12, 9% at 18, and 5% at 24 months (Australian Institute of Family Studies (AIFS) 2008). Feeding patterns and feed volumes differ between breastfed and formula-fed infants. From 6th week of life onwards formula-fed infants receive higher
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Feed volumes, approximately 60 mL per feed by the 17th week (Sievers et al. 2002). MI also varies enormously within and between breastfed infants. 24-h volumes are highest from 1 to 6 months of age (Kent et al. 2013; Rattigan et al. 1981) with breastfed infants taking an average of 788±169 g (range: 478–1356 g) over a 24-h period with an average meal (a feed from one breast; 2 feeds from both breasts within 30 minutes; 3 clustered feeds within 30 min) volume of 101.4±15.6 g (range: 0–350 g) (Kent et al. 2006).

There is strong evidence that breastfed infants regulate their MI and that MI is not restricted by capacity of the breast to produce milk, as infants consume on average 67% of milk available in the breast (range: 0–100%) (Kent et al. 2006). Further, when the milk production is increased healthy exclusively breastfed infant does not consume extra milk, indicating superb appetite control (Dewey et al. 1986). Infants have also been observed terminating the breastfeed during a milk ejection when milk is flowing freely, which again suggests precise appetite control (Ramsay et al. 2004).

From 4 to 6 months milk production declines with the introduction of complementary feeding, thus leading to a reduction of energy and protein intake from milk (Figure 1.9) (Grote et al. 2016). Despite this trend, volumes reported at later stages of lactation are still substantial (9 months: 41±215 mL, n=74 (Grote et al. 2016); 750±252 mL, n=28 (Nommsen et al. 1991); 720±280 mL, n=5 (Kent et al. 1999); 12 months: 43±132 mL, n=77 (Grote et al. 2016); 516±232 mL, n=21 (Nommsen et al. 1991); 534±296 mL, n=5 (Kent et al. 1999)). Lower values in Grote et al. data could be explained by a short exclusive breastfeeding period (3 months) as an inclusion criterion, which was reflected in the decrease in HM intake from 4 months onwards, and possibly by the cultural differences in complimentary feeding in the Italian population. HM was shown to contribute up to 28% of total energy and 16% of infant protein intake at 12 months of age (n=219) (Scott et al. 2016), although these figures could be underestimated or misjudged, as non-direct method of MI measurement and estimate of nutrient intake by summing and averaging of three days of intake were used.

Complementing solid food with HM appears to be important in the first few years of life most likely for regulation of infant growth and development and modulation of infant GI tract (Goldman 2000) however, likely other effects exist. Little is known how HM during weaning contributes to the development and programming of infant BC. Emerging data suggests that introduction of solid foods before 4 months is associated with childhood overweight, yet interestingly, this relationship was not significant in exclusively breastfed infants (Weng et al. 2012). Dietary patterns established during this period are also strongly influenced by maternal socio-demographic factors, such as
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ethnicity and age (Lim et al. 2016) and could be responsible in part for the high variability of infant BC.

Figure 1.9 Energy and protein intake from all foods and from breast milk during the first year of life. Boxplots with median and interquartile ranges, upper and lower adjacent values and outliers. Reproduced by permission from Nature Publishing Group (Grote et al. 2016).

Previous research shows that infant growth rate is not related to the amounts or concentrations of lactose, fat and protein and also energy intake, but is associated with the volume of milk consumed (Mitoulas et al. 2002; Dewey et al. 1991; Kent et al. 1999). The relationship between the average daily MI and the average growth rate of the infants over the first 6 months suggests that milk production of the mother is either a limiting growth factor, or a response to infant growth rate and appetite, or both (Kent et al. 1999). In the rare cases of extreme weight gain in breastfed infants, one case report (Perrella 2015a) measured a higher than average MI (1127 mL/24-h) and subsequently higher daily intakes of the macronutrients protein and fat, while second study reported elevated concentrations of protein and adiponectin (Grunewald et al. 2014). Unfortunately, there have been no measures of BC in these infants; therefore, it is unclear wether that infants have had increased FM or FFM or both, making it difficult to clinically manage these infants.
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While it is possible that daily intakes of HM components will increase with higher milk volumes consumed, this is dependent on the actual concentration of the component, which can vary enormously between women. Other potential factors apart from HM composition governing MI include infant stomach capacity, GE rate, infant BC and the gut microbiome. Thus a more comprehensive investigation of both concentration of multiple milk components and MI in relation to infant growth and development is warranted.

1.4.2 Feeding frequency

The current recommendation for mothers of breastfed infants is to breastfeed “on demand,” according to their appetite (Kent 2007). The number of meals per day is highly variable between breastfeeding dyads with studies reporting on average 7 to 8 meals per day (n=52) between 1 and 6 months of infant age (Kent et al. 2013; Kent et al. 2006). The majority of infants feed at night time, particularly before 9 weeks of age, which could be due to the smaller breast storage capacity and/or volume of milk taken at one feed and faster GE time or rate (Kent et al. 2006), since night feeds do not result in significantly higher 24-h MI (Butte et al. 1985). Further, lower MI are more common in the evening and larger MI in the morning when FFQ is lower compared to other times during 24-h (Butte et al. 1985; Kent et al. 2006; Cannon et al. 2015), probably due to increased GE time.

It is not fully understood what influences FFQ in breastfed infants. It is assumed that infants feed when stomach is empty (Lawrence et al. 2011). That implies that there should be an inverse relationship between FFQ and MI. Associations of FFQ and 24-h MI during period of exclusive breastfeeding have not been consistently found, as in Butte et al. study (Butte et al. 1985), although MI was higher if time preceding the feed was longer (n=45). The absence of association between FFQ and MI in this study could be explained by the fact that some participants did not feed on demand, particularly at 1 month postpartum. Similarly, De Carvalho seen no relationship at 1 month postpartum (n=46) (De Carvalho et al. 1982), although detected significant positive correlation at 2 weeks in the group that was encouraged to breastfeed more frequently (experimental: n=20, control: n=24) (De Carvalho et al. 1983). Others have reported that increased FFQ is associated with greater 24-h MI (p<0.05, n=27) (Rattigan et al. 1981) however, this study analysed milk productions at 1, 3, 6, 9, 12 and 15 months of lactation and allowed “partial breastfeeding”. FFQ declines with the duration of lactation (Butte et al. 1985; Kent et al. 2013) and is associated with a decrease in MI, which between 1 and 6
months of age is 10 mL for each additional feeding on average (Kent et al. 2013). Further work is needed to clarify whether milk production in mothers is controlled by FFQ and/or infant’s appetite during the period of exclusive breastfeeding.

FFQ and MI are not associated with feed duration (Butte et al. 1985), which is also varies greatly in breastfed infants. This is further supported by the knowledge that the healthy term infants remove 50% of the milk in the first 2 min and 80% of the milk in the first 4–5 min of a breastfeed (Lucas et al. 1979) as determined by the test weighing. Research conducted in our group confirms these findings, showing that the first milk ejection occurs within 2 min from the start of either a breastfeed or pumping, and the second in the following 2 min (Cannon et al. 2016; Ramsay et al. 2006; Gardner et al. 2015), with the first and second milk ejections delivering 45% and 29% of total milk volume respectively (Kent et al. 2008; Ramsay et al. 2006). Higher FFQ, shorter feeding duration and smaller MI during a breastfeed are common in traditional societies (Hale et al. 2007), so short feeding times do not imply a problem unless infant growth is compromised, while higher frequency and smaller feeds could be beneficial for infant development and appetite control regulation. On a contrary, some researchers state that higher than average FFQ have been reported among populations at relative risk of under-nutrition (Butte et al. 1985), but modern studies conducted in Western societies still display a wide range of FFQ (Kent et al. 2013).

Unlike 24-h MI, which is associated with the infant growth rate (Mitoulas et al. 2002; Dewey et al. 1991; Kent et al. 1999), MI per feed, FFQ and feed duration were found to be not associated with infant weight gain (Butte et al. 1985). Infants that were heavier at birth demanded significantly more feeds and nursed longer during the first 14 days of life (De Carvalho et al. 1982), and those that were encouraged to feed more frequently took more milk (725 vs 502 mL/24-h) and had gained more weight from birth (561 vs 347 g), although this relationship was not seen at 1 month of age, despite continuation of frequent nursing (De Carvalho et al. 1983). With majority of the studies investigating FFQ during first few months of lactation and hardly any study looking at associations between FFQ and infant characteristics such as anthropometry, BC or GE rate, it would be an advantage to find if FFQ and MI are related to infant growth parameters and could be the tool for adjustment of infant growth rate.

**1.4.3 Composition of human milk and feeding frequency**

HM consumption can be affected by many interconnecting factors, including infant (e.g. appetite and/or GE rate) or maternal factors (Figure 1.7) (Fields et al. 2016). Khan et al.
studied short-term (weekly) variations in HM and found that breastfeeding patterns and milk composition vary greatly between individuals, and milk protein intake may play a major role in infant appetite control, as greater mean 24-h intakes of total protein, casein and whey protein were associated with fewer breastfeeds per day (Khan et al. 2013a). Further, higher lactose concentrations were associated with the more breastfeeds per day, with fat having no effect.

Appetite hormones are present in HM (Savino et al. 2013) and could influence FFQ and patterns in infants, by either signalling the end of the meal or increasing time between the breastfeeds. In animal studies administration of the satiety hormone leptin was shown to inhibit GE of a glucose solution in mice (Yarandi et al. 2011) and a solid meal in rats (Martinez et al. 1999), while elevated serum levels in diabetic patients are associated with faster GE (Iwase et al. 2009). It is therefore conceivable that appetite hormones may influence GE rates and times and thus FFQ in breastfed infants. Higher nocturnal concentrations of leptin and fat in HM coincide with decline in FFQ of breastfed infants during 24-h (Cannon et al. 2015), although relationships between both FFQ and these components’ concentrations have not been analysed.

Further, longitudinal investigation of relationship between array of HM composition (fat, carbohydrates, protein, and appetite hormones) and infant FFQ, MI, 24-h MI and GE is required to acquire better understanding of the drivers of the breastfed infants’ growth patterns and development.

1.5 GASTRIC EMPTYING IN BREASTFED INFANTS

Longer duration of breastfeeding is associated with reduced risks of developing obesity and other NCD later in life, the incidence of which are dramatically rising (Geddes et al. 2013; Marseglia et al. 2015; Prescott 2016; Ejlerskov et al. 2015). The early programming of infant appetite control is a complex concept in the need of extensive research in order to fully understand how infant feeding patterns contribute to the development of obesity and other NCD (Sievers et al. 2002; Bartok 2011). Infants breastfed on demand are known to self-regulate their nutrient intake (Kent et al. 2006) and to develop better appetite control when introduced to solid food as opposed to their formula-fed counterparts (Savino et al. 2009; Hassiotou et al. 2014a). It is assumed these effects are in part mediated by the composition of the HM, which has the pleiotropic role, providing immune and anti-inflammatory protection (Le Huërrou-Luron et al. 2016; Manti et al. 2016) and endocrine, developmental, neural and psychological
benefits (Marseglia et al. 2015). Non-nutritive HM components such as appetite hormones (Savino et al. 2009), growth factors, neuropeptides, and anti-inflammatory and immune-modulating agents also influence the growth, development and function of the GI tract during early infancy (Goldman 2000), whilst some micronutrients act as nutritional antioxidants, improving GI functions (Hanson et al. 2016).

GE is a key regulator of appetite (Hellstrom et al. 2006), however, there is much to be learned about the spectrum of HM programming agents, how their patterns change throughout lactation period and their short term effect on GE rate of the breastfed infants. Despite numerous investigations into the different effects of HM and formula, few components, including major macronutrients, have been studied in connection with the regulation of MI, feeding patterns, pre-feed GR volumes prior to cuing for the feed and GE of fully breasted term infants.

1.5.1 Gastric emptying as a mechanism of appetite control

GE can be defined as the process in which food is emptied, by means of tonic contractions, from the stomach into the small intestine for further digestion and absorption (Hellstrom et al. 2006; Janssen et al. 2011). It is one of the key regulators of appetite such that rapid GE leads to an earlier sensation of hunger (Hunt 1980). GE rate and patterns depend on the nature and macronutrient composition of the ingested meal as well as the age and sex of the person. In adults, liquid meals empty in curvilinear fashion while solid meals have initial lag time and then empty in a linear fashion (Urbain et al. 1995) (Figure 1.10).

GE is controlled by metabolic (blood sugar), neuronal and hormonal signals, mainly by slowing down the emptying process after the food intake. It is also regulated by gastro-duodenal motor activity, which matures during gestation and early infancy and enables the movement of nutrients from the stomach to small intestine (Berseth 1996; Hellstrom et al. 2006). Gastric mechanosensation, particularly distension, is an important factor in the regulation of satiation during food intake. In healthy adults, gastric distension is shown to trigger stretch and tension mechanosensitive receptors that transfer the information via vagal and splanchnic nerves to the brain (Marciani et al. 2001). The vagal nuclei and the vagal nerve innervate most of the GI tract. While meal volume is a main determinant of the rate of GE of liquids, vagal afferents are also directly stimulated by changes in viscus tension caused by food passing through the GI tract. As the stomach empties distension decreases and plays a smaller role in satiety signalling with the emphasis shifting towards regulation of satiety via intestinal
exposure of nutrients, which itself is controlled by the rate of GE (Janssen et al. 2011) (Figure 1.11 (c) and (d)).

Figure 1.10 Gastric emptying curves for solids and liquids. In normal subjects, solid emptying is sigmoidal in shape with an initial lag phase with no or little emptying, followed by a linear phase with constant emptying rate and a much slower late phase. Liquid curve follows a single exponential course. Reproduced (modified) from (Urbain et al. 1995) by permission from Elsevier.

Although the intestines are also sensitive to distension, the chemical stimuli from protein, carbohydrates, acids, fatty acids and osmolytes activates taste receptors and releases peptides from mucosal enteroendocrine cells along the small bowel (Janssen et al. 2011). The peptides such as CCK, peptide YY (PYY) or gastric inhibitory polypeptide (GIP) activate negative feedback mechanisms acting locally or entering the circulation and working as hormones (ileal brake; Figure 1.12) (Urbain et al. 1995; Camilleri 2015; Maljaars et al. 2008). Some of these peptides, such as orexigenic ghrelin, or anorexigenic CCK and gastric leptin, act on vagal or other pathways to induce appetite or satiety (Camilleri 2015). The ileal brake regulates the rate of delivery and optimizes digestion and absorption of nutrients (Hellstrom et al. 2006) and is partially responsible for the reduction of food intake and inhibition of hunger as shown in animal studies (Maljaars et al. 2008). Activation of the ileal brake leads to decrease in in the frequency of antral and duodenal peristaltic pressure waves and induces an increase in pyloric sphincter pressure contributing to the delay in GE (Fone et al. 1990) and as well as a decrease in the frequency of jejunal contractions (Welch et al. 1988).
Delayed GE, in turn, is responsible for larger post-feed intragastric volumes and increased distension resulting in an increased feeling of satiety and delay of hunger return, thus reduction of gastric distension contributes to the development of hunger after a meal (Wisen et al. 1995; Geliebter 1988; Sepple et al. 1989).

![Diagram](image)

**Figure 1.11** A hypothetical model of the regulation of satiation, satiety and hunger by gastric motility-controlled processes. All graphs represent a period during (grey area) and after food intake. (a) Satiation and satiety during and after food intake respectively, (b) hunger, (c) gastric (dis)tension, (d) intestinal exposure of nutrients and (e) phase III contractions. In this model gastric (dis)tension, intestinal exposure and phase III contractions have an equal maximal contribution. Summation of graph c and d result in graph a, whereas graph e deduced with graph c and d result in graph b. Modified from (Janssen et al. 2011). Reproduced by permission from John Wiley and Sons.
Figure 1.12 Integrated neurohormonal response to the ingestion of food. Sensing of different nutrients by enteroendocrine cells results in the release of diverse hormones and peptides that result in gastric accommodation to the meal, stimulation of gastric contractions that lead to emptying, and, when the nutrients reach different levels of the small intestine, the release of substances that provide generally negative feedback that delays gastric emptying (e.g., CCK in the duodenum, GLP-1 and PYY in the more distal small intestine and colon). Reproduced from (Camilleri 2015) by permission from Elsevier.

The interaction between appetite, GE and gastric distension was studied in detail by a series of experiments, which combined ultrasound imaging and scintigraphy. In a summary, satiation and satiety are both inversely correlated to GE measured by antral area and presumably therefore, antral distension (Strum et al. 2004; Hveem et al. 1996). However, satiation and satiety are dependent on clearance of fat from the stomach and intestinal exposure to the nutrients (Carney et al. 1995). Interestingly, animal studies suggest that while the effects of fatty acids on GE both concentration and dose-dependent, their suppressive effect on energy intake is only dose-dependent. This has been also confirmed in humans, in which dose but not concentration of lauric acid
delivered via intraduodenal infusions is associated with plasma CCK and PPY, energy intake and antropyloroduodenal pressure waves in adults (Feltrin et al. 2007). This highlights that the component dose, which is currently not measured needs to be understood as a critical component of GE and appetite regulation.

1.5.1.1 Gastric emptying in infancy

In infants, patterns of GE vary depending on postnatal age and the type of milk they receive (artificial or HM). In preterm infants the pattern changes from linear to curvilinear between 28 and 32 weeks of gestation as the GI tract and gastro-duodenal motor activity matures (Carlos et al. 1997; Cavell 1981) and GE is considered mature from 34 weeks (Riezzo et al. 2009). While well studied in the preterm population (Gomez et al. 2003; Carlos et al. 1997; S. Perrella et al. 2015), in healthy term fully breastfed infants GE rate and its relationship with breastfeeding patterns and appetite control are not fully understood.

GE rate and patterns in infants are known to depend on the nature and macronutrient composition of the ingested meal as well as the MI. Milk in the infant stomach separates into two phases, a liquid phase (water, whey proteins, lactose etc.) and a semi-solid phase consisting of curd formed by casein and lipids. The semi solid phase typically empties more slowly, than the liquid phase. Different proportions of these phases in part explain the difference between linear GE patterns of formula-fed and the curvilinear pattern of breastfed infants (Cavell 1979; Gomez et al. 2003; Cavell 1981). HM and formulas with different casein to whey ratios have also exhibited different GE rates, with casein-dominant formulas emptying more slowly than whey-dominant in term infants less than 12 months of age (Meyer et al. 2015). GE rates of breastfed infants reported in literature are faster than in formula-fed with gastric emptying half time ($T_{1/2}$), which is to be between 36 and 48 min for HM and 65 to 78 min for formula (Cavell 1981; Van Den Driessche et al. 1999; Ewer et al. 1994).

This evidence suggests that differences in HM composition may influence GE rates, with the potential to impact both feeding patterns and infant growth (Meyer et al. 2015; S. Perrella et al. 2015).

1.5.2 Methods for monitoring gastric emptying in infancy

The numerous methods that have been developed for assessment of GE in humans such as MRI, stable isotope breath test, radiolabeled scintigraphy, dye dilution technique and ultrasonography indicate that GE is complex process, which can not be measured or
visualized with a single technique. The methods used to study GE can be characterized as intubation, indirect and imaging techniques (Vantrappen 1994).

1.5.2.1 Intubation techniques

Intubation techniques include gastric aspiration and dilution marker methods. These techniques involve the repeated withdrawal, measurement or residual volume and reinsertion of gastric content following the delivery of a liquid feed. Residual volumes are reported as proportions of the delivered feed volume, gastric curves are constructed and T_{1/2} are estimated (Husband et al. 1969; Cavell 1981). The advantages of these techniques are the relatively low cost and the ability to be performed at the bedside, so they have been used in preterm infants for HM or formula test feeds (Cavell 1979; Cavell 1981; Siegel et al. 1985). On the other hand they are still invasive and may interfere with the microbiome and protective acidic environment of the stomach (Milisavljevic 2013) as well as with GE itself, confounding results. Intubation techniques present particular problems when studying GE as a mechanism of appetite control in term healthy breastfed infants – these techniques are not able to show the uninterrupted pattern of GE for an individual meal and will unnecessarily stress infants and mothers and would likely result in more frequent feeding for comfort, thereby producing biased results.

1.5.2.2 Indirect techniques

GE of the meal can also be evaluated by measuring the amount of the ingested marker appearing in the blood or an expired air sample (Vantrappen 1994). The paracetamol absorption test and stable isotope breath test are examples of indirect tests. Paracetamol is completely absorbed in the small intestine and serial blood assays reflect the increasing proportion of the meal in small intestine over time, and GE curves and T_{1/2} are reported (Willems et al. 2001). Similarly, stable isotopes such as $^{13}$C, cleaved from the substrate ingested with a meal and oxidized to produce $^{13}$CO_{2}, are eliminated via the lungs and are collected and analysed (Ghoos et al. 1993; Vantrappen 1994). While the stable isotope test has been validated against scintigraphy in infants (Barbosa et al. 2005), use and validation of paracetamol absorption test in infants has not been reported, possibly due to the limited data for paracetamol use in this population (Cuzzolin et al. 2012). Both methods are invasive and absorption, metabolism and excretion of both markers are dependent on degree of maturation of the organs in the infant, all of which will likely to contribute to the variation between participants.
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There are less invasive methods that can assess GE in infants, such as applied potential tomography (APT) and epigastric impedance (EI) that are based on electrical conductivity and resistivity of the gastric content (Vantrappen 1994). When the measured resistivity is a half of the immediate post-feed value, it indicates that $T_{1/2}$ has been reached (Nour et al. 1995). While both methods are safe, non-invasive, inexpensive and have been used in infants (Nour et al. 1995; Savino et al. 2004; Smith et al. 1993), application of these methods in pediatric population is challenging, given the fact that movement may affect the measurements, requiring sedation and that milk is not suitable for testing, needing an addition of a conductive solution, all of which is not desirable in the appetite control studies.

1.5.2.3 Imaging techniques

Several imaging techniques are available for monitoring GE with scintigraphy being the gold standard for clinical practice and research. Although a radioactive marker is used in this method, tracking is conducted using a gamma camera, with the measurements of amounts of radioactivity emitted by the stomach used to construct GE curves and to report proportions emptied (Donohoe et al. 2009). One of the advantages of this technique is the ability to follow the emptying of liquids and solids separately and simultaneously by using specific markers (Vantrappen 1994). However, the use of scintigraphy in infants presents problems, such as exposure to radiation and the inability to account for the initial rapid emptying of a liquid meal during delivery resulting in overestimation of gastric residuals and misdiagnosis of normal GE as delayed (Lin et al. 2000).

Magnetic resonance imaging (MRI) of the gastric region using a liquid phase marker provides data for construction of three-dimensional images of the meal and the stomach and GE curves of liquid meals resulting from this technique have good correlations with scintigraphic curves (Vantrappen 1994), however method has not been validated in infants. Furthermore, MRI is expensive, time-consuming and not readily available and it is also likely to require infant sedation, thus is not suitable for the appetite control studies.

Ultrasonography is an appealing technique for studying GE, since it is safe and non-invasive, and offers the possibility not only to measure the GE rate and pattern, but also to visualize contractions of the stomach wall and the movement of the stomach content. The ultrasound (US) image is created by high frequency sound waves, which are attenuated during travel through tissue and reflected back to transducer, producing a
real time 2-dimentional image (Chan et al. 2011; Wagner 2013). The volume of the stomach can be calculated from the measurements of the areas of cross-sectional images (Bateman et al. 1982). While this method has existed for more than 30 years, the major disadvantage of ultrasound in assessing GE of liquids was the fact that it only measured volume changes of the gastric antrum (Vantrappen 1994) from which the antral cross sectional area (ACSA) as a proxy measure of gastric volume was calculated. Curves created with ACSA measurements are assumed to resemble patterns of GE and ACSA reduction time to 50% is an estimation of $T_{1/2}$ (Newell et al. 1993). This method has been validated in preterm infants demonstrating linear relationship between the feed volume and ACSA ($r = 0.78$) (Newell et al. 1993), but while it is a better option than intubation techniques, it measures a restricted area of the stomach gaining only limited information and is easily influenced by the body positioning and intragastric gas (Tomomasa et al. 1996).

The direct US method, which involves scanning of entire stomach for estimation of the stomach volume (Figure 1.13), is based on assumption that infants’ stomach is spheroid in shape and has been trialed in young healthy infants ($n=6$; mean age: 36 days; range 14–49) and those with gastric emptying disorders ($n=14$; mean age: 55 days; range 15–95) (Lambrecht et al. 1988) and validated in infants and children ($n=10$; 6 months–3 years) (Gomez et al. 2003).

The technique has been further validated for measurement of GE in preterm infants (Perrella et al. 2013) demonstrating high levels of intra- and interrater measurement agreement and indicating that the direct stomach volume calculation method has the potential to be used as a clinical diagnostic tool. In our group Khan et al. have trialed direct US method for monitoring of GE in term infants (Khan et al. 2012b; Khan 2012) and established an approach to determining the relationships between GE and the volume and macronutrient content of a breastfeed as well as curd characterization following milk ingestion. This approach was further refined in our recently conducted pilot study of GE in term breastfed infants ($n=20$) (Cannon et al. 2017). These studies showed that direct US could be successfully used in many populations, providing the use of population-specific algorithms for analysis of GE in infants and for fitting a time curve to the sequential post-feed stomach volumes using linear mixed effect models.
1.5.3 Effect of volume on gastric emptying

1.5.3.1 Effect of milk intake on gastric emptying

Gastric distention is a major determinant of GE and an important factor in the regulation of satiation during food intake (Hellstrom et al. 2001) with volume-related suppression of GE rate reported in animal models (rats) where the volume of milk ingested affects the rate of GE as well as suppression of the following feed volume (Lorenz 1985). This mechanism is expected to be a major appetite regulator in breastfed infants since ingested feed volumes differ between the feeds and individuals (Kent et al. 2006), but there is limited evidence for the effect of feed volume on GE in term and preterm populations, not to mention exclusively breastfed infants.

In preterm infants an increase in feed volume (from 10 mL/kg to 20 mL/kg) alone has been reported to have no effect on GE rate unless osmolality (controlled by the
addition of sorbitol) is simultaneously reduced; this combination accelerates the GE (Ramirez et al. 2006). However, in this study $T_{1/2}$ was measured and the fact that two different feed volumes attain equivalent $T_{1/2}$ indicates that initial GE is faster with larger feed volumes and that $T_{1/2}$ does not reflect GE across the postprandial period, thus is not suitable for studying the effects of feed volume on GE. Indeed, some other studies suggested that feeds of larger volumes empty more rapidly (mL/min) in the early postprandial period (Riezzo et al. 2009; Chen et al. 2013), although no study has specifically examined for this effect. While this seems trivial since it did not affect time between the feeds, which are usually scheduled in preterm population, it could be very important factor to consider in handling infants with feed intolerance or other GI tract issues and in the studies of the effect of HM composition on GE rate in infants.

Unlike preterm infants, where volumes and frequency of feed (usually every 2 or 3 hours) are determined according to body weight (Ayde 2011), breastfed infants control their MI, which differs greatly from feed to feed and between breastfed infants (Kent et al. 2006). In our pilot study of term breastfed infants larger MI were associated with larger post-feed stomach volumes (Cannon et al. 2017) and logically, longer GE time, although that was not analysed.

The available evidence indicates that volumes affect GE time and rate, especially early postprandially, and this effect together with the influence of HM components on infant GE should be further investigated.

1.5.3.2 Pre-feed residual stomach volumes

It is a common and misleading assumption that an infant cuing for a feed is hungry and has an empty stomach (Lawrence et al. 2011). In preterm infants, delayed GE diagnosed by the presence of GR volume is regarded as a risk factor for feed intolerance and necrotizing enterocolitis in conjunction with other symptoms (Moore et al. 2011; Caple et al. 2004). Some studies have shown that GR does not correlate with the preterm infants ability to reach full-feeding volumes and feeding intolerance, and that different care providers use different GR before stopping feeds. The most agreed upon GR that raises concern is 50% of prior feed and blood- or bile-tinged GR (Shulman et al. 2011; Sankar et al. 2008; Carter 2012; Parker et al. 2015). No clear information exists in literature on the prevalence and ranges of GR in preterm population. Study in our group showed that GR were present in 66% of preterm infants, with some infants having a considerable amount (Perrella et al. 2013).
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Presence of GR in preterm infants indicate that it is not uncommon for breastfed infants to have HM in the stomach prior to feeding, which was recently confirmed in our study of term breastfed infants (n=20) where more than half of the infants cued to feed with various GR present (median [IQR]: 14.5[2.6, 32.2], range 0.0–87.2 mL) (Cannon et al. 2017). Further, it may be beneficial if not vital to the developing infant to have the gastric mucosa exposed to HM anti-inflammatory components such as lysozyme or immunomodulatory agents and growth factors all of which contribute to protection and maturation of the GI tract (Goldman 2000; Yarandi et al. 2011). Thus it may be potentially detrimental to extend times between feeds in breastfed infants increasing the likelihood of an empty stomach devoid of HM components.

Research into whether GR play a role in MI in dyads breastfeeding on demand, and whether they are related to GE or composition of the HM is needed to further understand appetite control in healthy term infants during the exclusive breastfeeding period.

1.5.4 Effect of milk composition on gastric emptying

1.5.4.1 Effect of macronutrients on gastric emptying

HM is both a unique and dynamic fluid with superior composition, components of which vary between feeds within women as well as between women (Mitoulas et al. 2002; Khan et al. 2013a; Cannon et al. 2015). When infants breastfeed on demand and thereby control their nutrient intake (Kent et al. 2006) it is plausible that their GE pattern could be influenced by milk composition. Indeed, it has been shown that frequency of breastfeeding is reduced with increased total 24-hour protein intake, and increased with increased lactose concentration (Khan et al. 2013a). This suggests that variations in macronutrients, as well as appetite hormones such as leptin, may potentially influence GE patterns and at least in part explain the wide variation in feeding patterns displayed by breastfed infants (Kent et al. 2006; Khan et al. 2013a).

Unfortunately, the majority of the studies that investigate the effect of macronutrient composition of the feed on GE in infants are conducted using bovine-based formula milk for which the structure of molecules differ from HM and are usually carried on with a high level of control over variables such as feed volume or GE time. These studies report that higher energy density, osmolality and fat, protein and carbohydrate content result in delayed GE (Salvia et al. 2001; Seigel et al. 1985). However, there is very limited information regarding these relationships between HM components and GE of breastfed infants. GE studies show that HM empties faster than
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formula (~ 20 min faster), which indicates that HM is more easily digested and absorbed by the infant (Van Den Driessche et al. 1999; Tomomasa et al. 1987).

Given that HM is a much more complex and diverse fluid containing live cells, probiotics and proteins that are non-existent in formula the gastric response may be different in breastfed infants compared to formula fed infants.

1.5.4.1.1 Energy density

In adults, liquid meals of higher energy density empty slower than those of lower density irrespective of the biochemical composition of the meal (Calbet et al. 1997; Kwiatek et al. 2009). In infants, the reports are inconclusive. Preterm formula of various densities showed that intra-individual retained feed proportions (measured each 20 min by aspiration technique) were higher with each incremental increase in energy density (0 (distilled water), 6.5, 13, 20 and 24 kcal/30mL) with the exception of the two highest density dilutions (Siegel et al. 1984). Despite the inhibition of GE, higher density feeds were associated with emptying of more calories over comparable periods thus regulation of GE by energy density in preterm infants was considered qualitatively to be similar to that of adults.

Another study, which compared formula and HM using $^{13}$C-octanoic acid breath test reported no significant difference in T$_{1/2}$ for formula and HM of various densities (5, 10, and 20 kcal/30mL, volume 10mL/kg) (Ramirez et al. 2006). In this study HM energy density was assumed to be constant (20kcal/30mL) when in fact it varies in term HM (21 kcal/30mL; range 19–27.6) and is higher in preterm HM (25.7 kcal/30mL; range 21.3–29.7) (Ballard et al. 2013), thus possible variations could confound the results.

Surprisingly, in a study of term 1–6 months old breastfed infants (n=15) the energy content of HM was not associated with intervals between feeds, feed duration, MI, 24-h MI from each breast or number of the breastfeeds per day. Nor was the total energy delivered to the infant associated with total 24-h MI (Khan et al. 2013a).

1.5.4.1.2 Osmolality and carbohydrates

Higher osmolality and carbohydrate (glucose) content are known to decrease the rate of GE in adults with carbohydrate content having a stronger effect than osmolality (Vist et al. 1995), however in infants, the results are dependent on the type of carbohydrate.

In preterm infants, osmolality (controlled by the addition of sorbitol) had no effect on T$_{1/2}$ on its own but reduced osmolality has been reported to accelerate GE (shorten T$_{1/2}$) of larger feed volumes (Ramirez et al. 2006). Further, studies in our group found
that higher HM lactose concentrations are associated with faster GE during enteral feed delivery but not postprandially (S. Perrella et al. 2015; S.L. Perrella et al. 2015).

In normal newborns, GE was studied using the double marker technique showing that addition of xylose to glucose solution to increase in osmolality did not result in a difference in the volumes of solutions emptied in 30 min (Hunt et al. 1982). On the contrary, test meals of water and 5% and 10% glucose solutions given to newborns \((n=13)\) showed results comparable to those in adults: the higher the concentration of glucose, the slower the GE, but with more calories delivered to the small intestine per unit time in the case of higher concentrations (Husband et al. 1969). Furthermore, unlike in adults, isocaloric starch was found to empty faster in newborns than glucose due to a deficiency of pancreatic amylase (Husband et al. 1970).

In term breastfed infants \((1–6\text{ months}; \ n=15)\), greater FFQ is associated with higher lactose concentrations (Khan et al. 2013a) but no effect of this major carbohydrate was seen on GE in our recent study \((6–32\text{ weeks}; \ n=20)\) (Cannon et al. 2017).

These findings indicate that HM carbohydrates may influence GE in the breastfed infants but methods allowing for testing for multiple HM components are preferred to elucidate the direction of the effect.

1.5.4.1.3 Protein

Studies in dogs indicate that all three major macronutrients activate the ileal brake, resulting in a reduction of GE; limited human studies support the findings for fat and carbohydrates while associations with protein are not so straightforward (Maljaars et al. 2008; Van Citters et al. 2006). In adults, protein, the most satiating macronutrient, influences GE but different protein structures \(\text{e.g., casein and whey proteins, or peptides created during their hydrolysis}\) result in varied gastroenterone response and subsequent GE rate and patterns (Hall et al. 2003; Calbet et al. 2004).

Proteins from different HM fractions, such as whey and casein are resistant to proteolysis in infant stomach (Lonnerdal 2010). Bovine and HM casein and whey differ in their digestion (Meyer et al. 2015). Bovine caseins form firm curd, while curd formed by HM caseins is soft and flocculent, thus is likely digested more easily and emptied faster (Van Den Driessche et al. 1999; Ewer et al. 1994). This may be one of the reasons why infant formula empties more slowly than HM in term infants (Carlos et al. 1997; Cavell 1981; Ewer et al. 1994).
Further, both whey protein and the casein:whey protein ratio have been shown to influence infant GE in formula studies (Cavell 1979; Khoshoo et al. 2002). In term infants, casein-predominant formulas have been shown to reduce GE while both whey-predominant formulas and hydrolyzed casein increased GE (Billeaud et al. 1990; Meyer et al. 2015). Thus the variable casein:whey ratio demonstrated in HM (Kunz et al. 1992), which is known to change during the course of lactation (Lonnerdal 2003; Lonnerdal et al. 1985), as well as the presence of other active proteins in HM may in part explain differences in GE rates and the FFQ in term breastfed infants.

In preterm infants, findings for HM caseins effect on GE are contradictory to the formula studies. Our studies demonstrated that higher HM casein and whey protein concentrations are associated with faster GE during enteral feed delivery (S. Perrella et al. 2015; S.L. Perrella et al. 2015), while only higher casein concentrations are associated with faster postprandial GE (Perrella 2015b).

In term breastfed infants (1–6 months; n=15), greater FFQ is associated with lower total 24-h protein intakes (Khan et al. 2013a), but no direct effect of HM total protein is seen on GE (6–32 weeks; n=20) (Cannon et al. 2017). Interestingly, significantly higher curd volume and density are exhibited later in the emptying, indicating that curd formation and resistance to emptying may actually play important role in controlling GE rate (Khan 2012).

Furthermore, the casein subunit k-casein has been shown to inhibit the binding of *Helicobacter pylori* to human mucosa in vitro (Stromqvist et al. 1995). *H. pylori* are Gram-negative bacteria present in the stomach, and are known to down-regulate levels of ghrelin and leptin in the stomach (Francois et al. 2011), which may significantly affect GE. The protective action of HM k-casein is reinforced by lactoferrin and also by lysozyme, which is present in whey in a relatively high concentration (6% of whey protein), catalyzes the hydrolysis of specific bonds in Gram-negative bacteria cell walls and plays multiple roles in digestion. These functions include controlling the microbiome in the stomach (Montagne et al. 2001; Rubio 2014) and speeding up the digestion of microbial protein which may affect gastric motility and GE rate (Wang et al. 2012; Artym et al. 2013). In a clinical study of preterm infants lysozyme added to donor HM or formula was associated with increased body weight, normalization of the stool and improved feed tolerance (Bol'shakova et al. 1984). All of these studies suggest that HM proteome could potentially affect GE in term breastfed infants.
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1.5.4.1.4 Fat

Fat plays an important role in nutrition of exclusively breastfed infants providing approximately 50% of total energy intake (Jensen 1995) with more than 98% of fat in HM presented in the form of triacylglycerols (Czank et al. 2007a). In bovine-based formula, fat is largely replaced with vegetable oils blends to achieve a fatty acid composition similar to HM. The dietary triacylglycerols structures in formula are different to that of HM and formula milk fat globules are less accessible to gastric lipase resulting in 1.7-2.5-fold lower level of lipolysis and overall fat digestion in formula-fed infants (Armand et al. 1996).

Studies in animals and humans indicate that lipids initiate the ileal brake when they reach the ileum via hydrolysis of triacylglycerol into fatty acids thereby producing a delay in GE through the release of GIP, PPY and CCK (Maljaars et al. 2008; Van Citters et al. 2006). There are limited studies of the effect of fat content and composition on GE in infants.

In a study conducted in preterm infants (HM: n=11; premature infant formula: n=9), fatty acids profiles were not associated with GE rate (Armand et al. 1996), while another study reports faster GE for medium-chain compared with long-chain triacylglycerols (n=11) (Seigel et al. 1985).

In term infants, results are also conflicting with faster GE attributed to the fat and protein component of feeds with similar lactose concentration and osmolality in one study (HM: n=8; formula: n=9) (Cavell 1981), although no statistical analyses was performed. Whereas other studies showed no direct effect of fat on FFQ (1-6 months; n=15) (Khan et al. 2013a), (1–6 months; n=71) (Kent et al. 2006) and GE (6–32 weeks; n=20) (Cannon et al. 2017) in breastfed infants.

1.5.4.2 Effect of appetite hormones on gastric emptying

The effect of HM appetite hormones leptin, adiponectin and ghrelin on GE in animal and human models has been described in sections 1.3.2.3, 1.3.3.2 and 1.3.4.1.

1.5.5 Effect of infants’ characteristics on gastric emptying

Little is known about relationship between GE rate and BC, although this could conceivably be an avenue of regulation of appetite and food intake. An inverse linear relationship between GE rate of solids and body weight and body surface area is established in healthy adults (Lavigne et al. 1978). This is also confirmed in dogs where larger dogs have slower GE than smaller ones (Bourreau et al. 2004). On the other hand, delayed GE has been observed in patients with anorexia nervosa (Dubois et al. 2005).
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1979), whereas enhanced GE has been reported in the clinically obese population (Johansson et al. 1976), suggesting link with leptin resistance and other hormonal abnormalities and stressing importance of further studies needed in the rapidly developing research field of metabolism and BC.

In healthy adults, post-lag GE and colonic transfer is reported to be faster in men than in women (Degen et al. 1996), while in infants maximum, but not average, breastfeeding volume is higher in males than females, and so is the milk production by the mothers (1–6 months old, n=71; males: 831±187 g; females: 755±151 g) (Kent et al. 2006). In preterm infants, increasing postnatal age and feed (ml/kg) were associated with faster GE during enteral feed delivery, while increasing infant weight was associated with slower postprandial GE, although the magnitudes of these effects were small (S. Perrella et al. 2015). Differences in term infant characteristics (age, sex, anthropometrics and BC) may also explain the variability in the time between the feeds for infants over a 24-h period (Kent et al. 2006; Cannon et al. 2015). FFQ decreases between 1 and 3 months of lactation while milk intake during each breastfeeding session increases, with both parameters then remaining constant to 6 months (Kent et al. 2013; Butte et al. 1984). This is attributed to the fact that as infants mature they become able to consume larger feed volumes (Kent et al. 2006) resulting in longer time between feeds, although relationships between FFQ and MI and infant anthropometrics and BC are yet to be evaluated. If such differences exist then this may have implications in the management of feeding as well as sleep issues.

In conclusion, existing studies of the normal physiological emptying of the stomach in the term infant have either analysed formula or carbohydrate solutions, restricted monitoring time and/or controlled infants’ volume intakes, employed scintigraphy or US of antral area and reported gastric T1/2 in very small cohorts, often including children or unwell infants. There is a need for a comprehensive study of GE of a single (on demand) breastfeed in term fully breastfed infants to elucidate possible relationships between GE, breastfeeding patterns, MI, HM composition and infant anthropometrics and BC.

1.6 BODY COMPOSITION IN INFANCY

Nutrition adequacy in infants is a major global concern. In the past and currently simple anthropometric measurements such as weight, length, head circumference and BMI are widely used, but recently it is apparent that not only quantity but also quality of growth
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is important. This is particularly relevant to childhood obesity and the increased survival rate of preterm infants since BC in early life appears to play an important role in programming long-term health outcomes including obesity, type II diabetes, stroke, hypertension and other NCD (Wells et al. 2007).

The majority of large-scale epidemiological studies associating infant development with disease later in life have been limited to birth weight as an index of fetal growth and serial measurements of weight, length and head circumference, and BMI to monitor growth due to the limited access to sophisticated BC measurement techniques. BMI fails to reflect body shape, fat distribution and density and therefore is a very limited index of adiposity (Prentice et al. 2001; Wells et al. 2007; Wells et al. 2016). For example two infants, one with high muscle mass and the other with high fat mass, could have same BMI. Evaluations of adiposity using the waist:hip ratio or the triceps:subscapular skinfold thickness are problematic in analyses of early growth due to poor statistical validity (Wells et al. 2007). BC is a good indication of the growth and development of the infant, nutritional adequacy, and predictor of health outcome, allowing medical professionals to tailor the infant diet for optimum infant health. Unfortunately, all BC measurement methods are originally developed for use in adults and with a few techniques. As these techniques are not directly applicable to pediatric population they require adaptation and validation due to differences in physiology in the early life. As such an understanding of theoretical backgrounds, assumptions and limitations of different methodologies and models upon BC measurements are based is necessary.

1.6.1 Body composition

Maintaining a healthy body weight and level of adiposity is a prerequisite of health and longevity. The term BC describes the various components that make up a human body. The absolute and relative amounts and distribution of these components differ between the subjects and are relevant to body functions and influence health and susceptibility to disease. BC research consists of three overlapping areas: BC levels and their organization, BC measuring techniques and biological factors that influence BC. Wang et al. proposed a commonly used multicomponent BC model (Wang et al. 1992) with five levels of interrelated and increasing complexity: from atomic to molecular to cellular to tissue/organ to the whole body. At each level body mass (BM) is viewed as the sum of all its components and can be described by the equations (Table 1.5) (Wang et al. 1992). Beyond this five-level model, approaches to BC analysis can be organized
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according to the number of compartments (Figure 1.14). Both complex and simple models of BC, based on chemical and molecular content, physiological function and anatomy, have been developed (Ellis 2002). Typically, BC measures are based on weight divided into two or more compartments (Heyward et al. 2004b).

Understanding of the theoretical models that underlay the measurement of human BC is required when assessing BC techniques. Cadaver analysis is considered the gold standard of BC analyses, and currently in vivo techniques are not equivalent to this accuracy. On the other hand, limited numbers of studies of cadavers have been conducted, particularly in neonate and infants, to provide reference data for the development of BC models (Heyward et al. 2004b).

Table 1.5 Multicomponent models representing the five levels of body composition and components they include

<table>
<thead>
<tr>
<th>Level</th>
<th>Body Composition Model</th>
<th>Number of Components</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atomic</td>
<td>BM = H+O+N+C+Na+K+Cl+P+Ca+Mg+S+R1</td>
<td>11</td>
</tr>
<tr>
<td>Molecular</td>
<td>BM = FM+TBW+Pr+M+Gly+R2</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>BM = FM+TBW+Pr+M</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>BM = FM+TBW+Pr+M_B+M_S+Gly</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>BM = FM+TBW+Pr+M_B+M_S</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>BM = FM+FFM</td>
<td>2</td>
</tr>
<tr>
<td>Cellular</td>
<td>BM = FM+BCM+ECF+ECS</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>BM = Cells+ECF+ECS</td>
<td>3</td>
</tr>
<tr>
<td>Tissue-organ</td>
<td>BM = AT+SM+Bone+VO+Blood+R3</td>
<td>5</td>
</tr>
<tr>
<td>Whole-body</td>
<td>BM = Head+Neck+Trunk+LE+UE</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>BM = Head+Trunk+Limbs</td>
<td>3</td>
</tr>
</tbody>
</table>

Abbreviations: AT – adipose tissue; BCM – body cell mass; BM – body mass; ECF – extracellular fluid; ECS – extracellular solids; FM – fat mass; Gly – glycogen; LE – lower extremity weight; M – mineral; M_B – bone or osseous mineral; M_S – soft-tissue or non-osseous mineral; NFS – non-fat solids; Pr – protein; R1 – residual mass of element present in amounts <0.2% of body weight; R2 – residual chemical compounds present in amount of <1% of body weight; R3 – remaining 15% of body mass; SM – skeletal muscle; SLT – soft lean tissue; TBW – total body water; UE – upper extremity weight; VO – visceral organs. Adapted from (Wang et al. 1992).

The simplest two-compartment (2-C) model, on which densitometry methods are based, divides body weight into FM and FFM (Figure 1.14). The methods include anthropometry, densitometry, bioelectrical impedance and isotope dilution for total
body water (TBW). The estimate of FFM is first derived and FM is defined as the difference between body weight and FFM. To classify levels of adiposity relative body fat, or %FM is used. %FM is expressed as a percentage of total body weight:

\[
\%FM = \frac{FM}{\text{Body Weight}} \times 100
\]

2-C methods suffer from the assumption that the density of both compartments usually is constant, which may be appropriate for FM defined by the ether-extractable lipid fraction of the human body (Forbes 1987). However, FFM is a complex tissue compartment including muscle, bone, organs and supporting tissue, which may be affected by hydration levels, introducing uncertainty in the estimation of FFM, particularly in children, where compartments change with growth and development and disease states (Weber et al. 2012). Since FFM is considerably larger than FM, the error in estimation of FFM is then fully transferred to smaller FM, thus increasing the error dramatically.

**Figure 1.14** The classic two-compartment (2-C) model with illustration of the fat-free compartment content (body water, bone and non-bone mineral, protein and glycogen) is used in densitometry methods.

More complex models (multicompartment models) further describe components of the FFM such as mineral, protein and water, or functional components such as body cell mass (BCM). For example DXA, which is considered the reference method, is a 3-compartment (3-C) model, in which BC constitutes FM, non-osseous lean tissue mass
(LTM) and BMC, all of which have different tissue densities and differentially attenuate energy beams, allowing for accurate quantification of each tissue (Ellis 2002).

4-compartment (4-C) BC models consist of FM, TBW, mineral and protein. Thus measures of protein and mineral compartments are required in addition to TBW measurements, a total of 3 separate measures. Thus the 4-C model is the most accurate estimate of %FM as a reference for development and validation of new BC models, methods or prediction equations.

With additional measurements it may be possible to extend number of compartments in the BC model, however the new measure should be compositionally independent of other measures, although the use of the overlapping methods could be beneficial in the field of measuring BC, particularly in pediatric population, to avoid the single method technical or model limitations and to confirm the normality or abnormality status (Ellis 2000).

1.6.2 Methods for measuring body composition in infancy

The number of techniques available to assess infant BC in addition to anthropometrics is increasing and include bioelectrical impedance analysis (BIA), DXA, isotope dilution, MRI, US, whole-body air-displacement plethysmography (ADP), computed tomography (CT) and others (Ward et al. 2013). These techniques have better accuracy and reliability than anthropometric measurements, including BMI, and should be employed to investigate BC in relation to nutrition, particularly to HM, as the existing evidence of links between HM and BC is sparse, contradictive and inconsistent. Unfortunately, utilization of these techniques in infants and children presents multiple challenges. Some of these techniques are invasive, others present concerns regarding exposure to low levels of radiation (DXA and CT), some require the infant to be restrained during scans or involve repeated blood sampling, further both isotope dilution and MRI are expensive and time-consuming to analyze.

In general, low-cost, simple-to-use techniques usually exhibit the lowest precision and accuracy (Tables 1.6 and 1.7). All techniques have inherent limitations however; a combination of existing methods may offer relatively accurate, safe and non-invasive measurements of infant BC making them an attractive research tool for the infant population.
### Table 1.6 Accuracy, precision and minimum detectable change of different methods for measurement of infant body composition

<table>
<thead>
<tr>
<th>Author, year</th>
<th>Body Composition Compartment</th>
<th>Method</th>
<th>Accuracy $^a$</th>
<th>Precision $^b$</th>
<th>Minimum Detectable Change $^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ellis, 2002 (i)</td>
<td>TBW</td>
<td>D$_2$O dilution</td>
<td>2-4%</td>
<td>1-2%</td>
<td>100 mL (5%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>BIA/BIS</td>
<td>3-7%</td>
<td>2-4%</td>
<td>200 mL (10%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TOBEC</td>
<td>4-6%</td>
<td>2-3%</td>
<td>150 mL (8%)</td>
</tr>
<tr>
<td></td>
<td>BCM</td>
<td>TBK</td>
<td>3-5%</td>
<td>2-3%</td>
<td>17 mEq (5%)</td>
</tr>
<tr>
<td></td>
<td>FFM</td>
<td>DXA</td>
<td>1-4%</td>
<td>1.5%</td>
<td>125 g (5%)</td>
</tr>
<tr>
<td></td>
<td>FM</td>
<td>DXA</td>
<td>3-5%</td>
<td>2-3%</td>
<td>50 g (9%)</td>
</tr>
<tr>
<td>Ellis, 2007 (ii)</td>
<td>%FM</td>
<td>ADP</td>
<td>3.7%</td>
<td>0.4±1.3%</td>
<td>NA</td>
</tr>
<tr>
<td>Pineau, 2007 (iii)</td>
<td>FM</td>
<td>Ultrasound</td>
<td>1%</td>
<td>2%</td>
<td>NA</td>
</tr>
<tr>
<td>Reilly, 1995 (iv)</td>
<td>%FM</td>
<td>Skinfolds</td>
<td>8-10% (v, vi)</td>
<td>2% (iv)</td>
<td>NA</td>
</tr>
<tr>
<td>Wells, 1999 (v)</td>
<td>%FM</td>
<td>Skinfolds</td>
<td>8-10% (v, vi)</td>
<td>2% (iv)</td>
<td>NA</td>
</tr>
<tr>
<td>Wong, 2000 (vi)</td>
<td>%FM</td>
<td>Skinfolds</td>
<td>8-10% (v, vi)</td>
<td>2% (iv)</td>
<td>NA</td>
</tr>
</tbody>
</table>

$^a$ Accuracy error for absolute mass; $^b$ reproducibility for repeat measures; $^c$ percentage change based on 3.5 kg infant with 15% fat. Abbreviations: BCM – body cell mass; BIA – bioelectrical impedance analysis; BIS – bioelectrical impedance spectroscopy; DXA – dual-energy X-ray absorptiometry; FFM – fat-free mass; FM – fat mass; %FM – percentage fat mass; TBK – total body potassium; TOBEC – total body electrical conductivity. Adapted from (i) infants (Ellis 2002); (ii) 2-23 weeks old infants (Ellis et al. 2007); (ii) adults (Pineau et al. 2007); (iv) 7-11 year old children (Reilly et al. 1995); (v) 8-12 years old children (Wells et al. 1999); (vi) girls aged 11-15 years (Wong et al. 2000).
Table 1.7 Considerations of commonly used technologies for measurement of infant body composition

<table>
<thead>
<tr>
<th>Technique</th>
<th>Cost</th>
<th>Infra-structure</th>
<th>Precision</th>
<th>Portability</th>
<th>Ease of use</th>
<th>Time involved</th>
<th>Patient convenience</th>
<th>Patient compliance</th>
<th>Safety</th>
<th>Operator skills</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skinfolds</td>
<td>L</td>
<td>L</td>
<td>M</td>
<td>VH</td>
<td>H</td>
<td>L</td>
<td>H</td>
<td>M</td>
<td>H</td>
<td>M</td>
</tr>
<tr>
<td>ADP</td>
<td>H</td>
<td>M</td>
<td>M-H</td>
<td>L</td>
<td>H</td>
<td>M</td>
<td>H</td>
<td>M</td>
<td>H</td>
<td>M</td>
</tr>
<tr>
<td>Isotope dilution</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>M</td>
<td>H</td>
<td>H</td>
<td>M</td>
<td>L</td>
</tr>
<tr>
<td>DXA</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>None</td>
<td>M</td>
<td>H</td>
<td>L</td>
<td>M</td>
<td>M</td>
<td>H</td>
</tr>
<tr>
<td>MRI</td>
<td>VH</td>
<td>VH</td>
<td>H</td>
<td>None</td>
<td>M</td>
<td>H</td>
<td>L</td>
<td>L</td>
<td>M</td>
<td>H</td>
</tr>
<tr>
<td>CT</td>
<td>VH</td>
<td>VH</td>
<td>H</td>
<td>L-H</td>
<td>M</td>
<td>M-H</td>
<td>L-M</td>
<td>L-M</td>
<td>M</td>
<td>H</td>
</tr>
<tr>
<td>US</td>
<td>L-M</td>
<td>L</td>
<td>M</td>
<td>L-H</td>
<td>H</td>
<td>L</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>M</td>
</tr>
<tr>
<td>BIA</td>
<td>L-M</td>
<td>L</td>
<td>M-H</td>
<td>H</td>
<td>H</td>
<td>L</td>
<td>H</td>
<td>M</td>
<td>H</td>
<td>M</td>
</tr>
</tbody>
</table>

H - high; L – low; L-H - low to high; L-M - low to medium; M – medium; M-H - medium to high; VH – very high. The advantages for research translation are highlighted. Abbreviations: ADP - air-displacement plethysmography; BIA – bioelectrical impedance analysis; CT – computed tomography; DXA – dual X-ray absorptiometry; MRI - magnetic resonance imaging; US – ultrasound. Adapted from (Ward et al. 2013; Shepherd et al. 2016).
1.6.2.1 Body density and volume measurements

1.6.2.1.1 Underwater Weighing (Hydrodensitometry)

Densitometry refers to measurement of body density (BD), which is calculated by dividing body weight by body volume:

\[ BD = \frac{\text{Body Weight}}{\text{Body volume}} \]

Hydrodensitometry, a classic 2-C model, provides an estimate of total body volume from the water displaced by the body when it is fully submerged. This method is widely used in adults and, providing a residual lung volume is measured, gives a good measure of body volume from which BD and further %FM can be calculated. The density of lean tissue is higher than that of fat tissue therefore, the higher the density reading the higher the proportion of lean tissue. Clearly, this method is not suitable for children and infants, particularly those with health issues. An alternative more user-friendly technique based on air displacement has recently been developed to overcome these issues (ADP; described below) (Wells 2006; Heyward et al. 2004d).

1.6.2.1.2 Whole Body Air-displacement Plethysmography

ADP technology is considered the most advanced technique available for assessing infant BC. ADP directly measures body weight and air pressure in a chamber and calculates the volume of the subject by comparing with volume of the empty chamber. Since the volume of air close to the body surface is overestimated by 40% due to isothermal behavior surface, area artifact is automatically calculated to correct the subject’s body volume. Thoracic gas volume is also measured, as it is not a component of body volume and must be accounted for (Urlando et al. 2003; Ellis et al. 2007).

This method is safe, non-invasive, rapid (only takes few minutes) and suited for frequent testing. It has been validated for use in adults, children (McCready et al. 1995; Fields et al. 2000), term (Ma et al. 2004; Ellis et al. 2007) and preterm infants (Ramel et al. 2014) with published reference models for calculations available (Fomon et al. 1982; Butte et al. 2000a). ADP provides rapid FM and FFM measurements and is accommodative to most infant behaviors (movement, vocalization, etc.) (Rosendale et al. 2012). There are two systems: the PEA POD and BOD POD (Life Measurement Inc., Concord, CA). The PEA POD has been developed for measurement of BC in infants from birth to 6 months of age (1-8 kg as approved by FDA) and the BOD POD is designed to accommodate older children and adults (12-165 kg).
The PEA POD is still relatively new and although it is a viable tool for measuring BC, the error attributable to imprecision in measuring small volumes exists in pediatric population (Rosendale et al. 2012), and further investigation of the sources of variability are needed to improve the accuracy of this technology. Also, more studies using multi-compartment models as a reference standard are needed (Baracos et al. 2012). Despite commercial availability of the PEA POD for more than decade, the number of publications is still very limited, although the available data supports the conclusion that this method provides good accuracy and reproducibility (Table 1.7) (Li et al. 2013). However, the only published study that compared PEA POD data with that acquired using 4-C model (Ellis et al. 2007) employed the FFM density values of Butte in calculating BC.

The default density model used in the PEA POD utilizes age specific FFM density values of Fomon et al. (Fomon et al. 2002), while the alternative density model uses Butte et al. (Butte et al. 2000a) density values. There is still an unresolved query about large discrepancy in fat content (particularly in the earlier months) of the reference infants of Butte and Fomon, with Butte data showing higher %FM values. Additional determinations of total body potassium (TBK) are required to resolve these differences (Fomon et al. 2002; Carberry et al. 2010). While recent studies seem to favour the Fomon model for earlier weeks of life (Fields et al. 2011; Roggero et al. 2010), the International Atomic Energy Agency lists both Fomon and Butte and considers Butte model a better choice for the older infants (International Atomic Energy Agency 2010). Less data is available on infant composition from middle- and low-income countries, where both growth impairment and physiology of BC deposition should be studied, and, interestingly, density values of Fomon have been found to be appropriate in the recent study conducted in Ethiopia (Andersen et al. 2013) in which reference FM and FFM data has been collected and validated against a stable isotope method.

The major limitations of PEA POD are the inability to estimate distribution of the body fat and to measure infants larger than 8 kg. Over 50% of males and 20% of females will exceed 8 kg limit by 6 months of age (World Health Organization 2009a) thereby reducing the applicability of this method for longitudinal studies. Longitudinal studies currently require a change to the BOD POD (which represents some challenges with small children) or the use of an alternative technique. However, one study received an exception for use of the PEA POD in infants up to 10 kg, allowing to capture BC data on all infants during the first 6 months of life (Bartok 2011) although it is not clear how many infants were heavier than 8 kg. Another study measured infants aged 6–48
months ($n=69$) with the BOD POD (Rosendale et al. 2012) however several modifications of the BOD POD were required to enable testing of young children.

### 1.6.2.2 Dilution methods (Hydrometry)

Measurement of TBW by stable isotope dilution allows estimation of FFM, since it has been shown that a proportion of lean body mass is composed of water. In healthy adults, TBW represents approximately 73% of FFM and 60% of body weight (Kotler et al. 1999). The content however is not constant across the life span (Fomon et al. 1982) and variant depending on diseases (Kotler et al. 1999). The TBW of healthy term infant is typically 80–83% of FFM at birth, and decreases rapidly over the first few months and then the next 3–5 years until the hydration fraction reaches that seen on adults, reflecting the change in the ratio of water between the extracellular and intracellular compartments (Fomon et al. 1982).

A dose of deuterium labeled water is given and after equilibration enrichment of the body water pool is measured using samples or blood, urine or saliva by isotope ratio mass spectrometry or spectrophotometric techniques (Davies et al. 1994; Wells 2006). A modification of dilution method can also be used to measure extracellular water (ECW) pools, in this case the most commonly used sample is plasma (Ellis 2000). Following the measurement intracellular water (ICW) volume can be calculated as the difference between TBW and ECW.

This technique is simple to carry out and needs minimal cooperation from participants and can easily be used in field studies. However, used alone as two-compartment method it is subjected to error due to variations in the infant FFM composition, especially in case of under- or over-hydration.

### 1.6.2.3 Whole body counting (TBK)

The methodology for TBK count originated from the whole body counters built for nuclear research and weapons facilities to monitor for internal contamination. During the monitoring it was noted that a constant peak exists independent of the exposure and an association between naturally radioactive potassium ($^{40}$K) and FFM was established leading to the development of a BC measurement method (Ellis 2000). TBK became the first $in vivo$ chemical analysis of human body and is considered to be the best BC index for assessing BCM, as it has a good accuracy in adults (1–5%), which decreases slightly for term (Table 1.6) and preterm infants (Ellis et al. 1993). While a great addition to 2-C models, this method requires a considerable time (15 min) to record a signal,
expensive and the number of counters available is limited, making this method impractical for routine non-clinical use.

1.6.2.4 Dual X-ray Absorptiometry

DXA has replaced a method called dual-photon absorptiometry (DPA) used in the early 1980s to measure bone mineral density and total body bone mineral. Instead of a radioactive isotope, DXA uses an X-ray source, which allows for better precision and accuracy (Mazess et al. 1990). Not only does it provide gold standard measurements of bone mineral content, it also estimates BC by the attenuation of X-rays, which is dependent on the thickness, density and chemical composition of the tissue (Pietrobelli et al. 1996).

DXA is relatively simple and rapid to use in children and infants the major drawback being movement therefore, infants are often swaddled. The accuracy of DXA has been demonstrated in piglets (Brunton et al. 1993) and good reliability has been shown in newborns (Table 2.3), thus it has also been suggested as reference technique (Godang et al. 2010). DXA however does suffer difficulties as different machines give slightly different results making it difficult to compare published values. Furthermore, it is considered that accuracy has not been sufficiently confirmed (Wells 2006). Bias in FM, FFM, %FM and DXA-measured body weight varies depending on age, sex, size, fatness and health status (Williams et al. 2006), thus caution should be exercised while interpreting DXA results particularly in longitudinal studies.

DXA uses ionizing radiation, and while the effective dose is below background levels, it is less attractive for longitudinal participants, especially for the rapidly developing infant who is more sensitive to radiation.

1.6.2.5 Magnetic Resonance Imaging and Computed Tomography

1.6.2.5.1 Magnetic Resonance Imaging

MRI is an imaging technique that estimates the volume, not the mass of adipose tissue. The technique produces images based on variations in both frequency and the phase of absorbed and emitted energy. A series of cross-sectional images with required slice and interslice thickness are produced and are summed to calculate regional tissue volumes. While the distribution and quantity of fat can be estimated using MRI, the image analysis algorithms that distinguish specific fat compartments are lacking. It is difficult to compare results of MRI with other techniques, as the method requires the assumption
that the fat content of adipose tissue is constant when in fact it is variable, and MRI cannot measure fat in organs (Wells 2006; Ward et al. 2013).

The accuracy of approximately 6% (0.08 kg) was shown in in estimation of weight of subcutaneous and visceral adipose tissue as well as lean tissue in human adult cadavers (Despres et al. 1996). Although to date, there has been no validation of MRI in infants against a criterion method, recent calibration and validation study has shown good accuracy in both awake and anesthetized piglets (1% and 0.2% of weight for fat, respectively) (Kovner et al. 2010). MRI has also been compared with DXA and has shown high agreement with no bias in estimation of total FM and FFM ($R^2=0.88$ and $R^2=0.90$, respectively) of healthy infants ($n=14$) (Fields et al. 2015).

While MRI is a desirable method of the estimation of regional BC, particularly of intra-abdominal adipose tissue, it has relatively high cost and limited application due to infant movement and time required for scans acquisition. MRI has been used successfully in longitudinal imaging study of the infants brain without the need for sedation however, it required night scans to ensure acquisition of images during sleep (Dean III et al. 2013). Therefore, it is difficult to envisage MRI being used frequently for measurement of BC in the pediatric population.

### 1.6.2.5.2 Computed Tomography

CT is a tomographic technique where X-ray sources on a radial housing are transmitted through the body and emerging beam is detected on the opposite side of the subject. Unlike DXA, which is a 2D tomographic technique, CT not only measures bone mineral content and bone area, but also determines true 3D volumetric bone mineral density and muscle density (Ward et al. 2013). The rotation of source and detectors (or just the source) allows for 360° coverage for producing a cross-sectional image of the body region scanned. This is similar to MRI scan, but in addition each image contains information of tissue density at each pixel. This information together with the anatomical location of the pixel on the image allows better identification of the tissue type, resulting in excellent reconstruction of total body mass and masses of separate organs with high accuracy and precision (<1% for both) (Ellis 2000) and more accurate determination of visceral adipose tissue than MRI (Despres et al. 1996).

Despite its better accuracy and shorter scan times than MRI, the major disadvantage of CT for measuring infant BC is the radiation dose required for imaging, which is substantial (comparable to few years of background radiation depending on the
site of the scan), although can be reduced if lower resolution is acceptable (Starck et al. 1998).

1.6.2.6 Bioelectrical Impedance and Conductance Methods

Bioelectrical impedance, performed as two models, bioelectrical impedance analysis (BIA) and the more comprehensive bioelectrical impedance spectroscopy (BIS), are commonly used to estimate TBW. Total body electrical conductivity (TOBEC) is not longer available as an alternate bioelectrical method, although the results acquired with this technique are quite informative.

1.6.2.6.1 Bioelectrical Impedance Analysis

BIA became popular due to its ease of use, portability and relatively low cost compared to other methods of determining BC. It measures the electrical impedance, or resistance to a small and harmless electric current flow through the body tissues. The theoretical model treats the body as a single cylinder, with measurements taken between four electrodes that are usually placed on the wrist and ankle. For the single-frequency measurements (BIA, typically 50 kHz), a weak alternating current is passed through the outer pair of electrodes and measured with the inner pair. Current flows more easily through parts of the body that are composed mostly of water and electrolytes such as blood, urine and muscle, than it does through fat, bone or air-filled spaces. The measured impedance \( Z \), corrected for the reactive component, known as electrical resistance \( R \), is inversely and quantitatively related to the volume of conductive compartment and can be used to calculate TBW using validated prediction equations (Lukaski et al. 1985; Kyle et al. 2004a) and estimate FFM and FM. Changes in body fat and water balance are reflected as changes in tissue impedance and tissue electrical properties, and it is therefore hypothesized that these could be used for diagnosis and monitoring of various illnesses (Tuorkey 2012).

Most BIA equations based on the impedance index, height\(^2/R\), where \( R \) is resistance or impedance, as predictor of FFM (Kyle et al. 2004b). The inclusion of body height\(^2\) or weight in the regression model accounts to some extent for the variations in the geometric shape of the body and differences in trunk size (Kushner et al. 1992). Furthermore, many investigators have included some additional anthropometric terms, such as age, gender, race, BMI and others to the formula for the reduction of the standard error of the estimate (Ellis 2000).

In early studies of BIA it was not regarded as accurate, but in recent years technological improvements have made it more reliable for BC and tissue assessment
for both research and medical studies. Recent developments and improvements include the introduction of multifrequency BIS, which provides better estimates of TBW than single-frequency models, and an increased variety of electrode placements for estimates of regional and whole body composition (Buchholz et al. 2004).

The major disadvantage of BIA is that it requires the use of population specific equations, together with the appropriate hydration coefficient (proportion of the FFM that is water) for the studied population. While BIA provides reliable estimates of TBW, and predictive BIA equations have been developed for adolescents, children, toddlers and newborn infants (Ferreira et al. 2004; Mayfield et al. 1991; Lingwood et al. 2000; Ejlerskov et al. 2014), there are no validated prediction equations using BIA in neonates (Lingwood 2013).

The second major disadvantage of BIA is that its ability to determine BC is affected by the hydration status of the participant. FFM could therefore be overestimated in overhydrated or oedematous individuals, such as preterm infants, who have higher body water content compared to other pediatric populations (Ward et al. 2013). Poor predictive performance has been shown for BIS in infants younger than 4.5 months and could be due to better conductivity of the fat tissue due to greater vascularization and/or rapidly changing hydration rates due to feeding patterns (Lingwood et al. 2012; Sesmero et al. 2005).

Similar to other methods, BIA is presented with the challenge of keeping infants still for the measurement. Failure to keep still or being restrained have been shown to result in statistically higher resistance measurements (Sesmero et al. 2005), but the advantage of BIA and particularly BIS (see Section 1.6.2.6.2) is that several determinations can be taken in a few seconds.

No study has accounted for infant pre-/post-feed stomach or bladder volumes, yet rapid changes in these extracellular water pools may potentially affect measurements. For breastfed infants it is not possible to control the volume consumed or bladder volumes. Therefore it is necessary to know whether stomach and bladder volumes impact resistance measurements and whether they should be accounted for. The number of studies investigating the effect of hydration and liquid consumption on BIA measurements in infants are limited, with two studies finding no change in TBW using either impedance plethysmograph in low-birth-weight infants given enteral feeds (1–7 days; intake 34±16 mL; \( n=10 \)) (Mayfield et al. 1991) or BIS in term healthy infants given milk (1–24 weeks; intake 30–135mL; \( n=29 \)) (Sesmero et al. 2005). BIS is able to detect change in volume of extracellular water of approximately 40 mL in the adult arm.
(Czerniec et al. 2010), which is similar to the volumes in both studies. This lack of effect may be due to neither study reaching the BIS minimal detectable change limit, estimated at approximately 200 mL in infant TBW (Ellis 2002). Furthermore, the effect of MI of healthy term infants which may range from 0 to 350 g between 1 and 6 months of age (Kent et al. 2006), was not measured and needs to be investigated.

In addition, standardization of the method is required, to ensure that the same approach is used in terms of instrumentation, timing of the measurements, positioning of electrodes and data analysis, which will allow for better comparison between the studies.

The addition of BIA improves prediction of TBW compared to anthropometry only in 3 and 4.5 months old infants and correlation between impedance and FFM strengthens with infant age. Further, small bias means that this method is suitable for comparison between groups and monitoring changes over time within individuals (Lingwood et al. 2012; Lingwood 2013).

**1.6.2.6.2 Bioelectrical Impedance Spectroscopy**

BIS, or multiple frequency BIA (MFBIA), is a more complicated recent model of BIA, and is based on the modification of a mixture theory model (Hanai 1960) and positioning the whole body into a series of cylinders that represent body segments (Matthie et al. 1998; Van Loan et al. 1993). A wide range of frequencies is used for resistance measurements and multiple impedance parameters are derived from a single measurement. Single-frequency BIA operates at 50 kHz, where it primarily reflects the ECW compartment, although some current also passes through the cells. In case of BIS, a more accurate measurement of ECW can be achieved at a frequency of 0 kHz, when current travels between the cells only, and ICW compartment can also be calculated, and changes in these compartments can be monitored.

A complication of this method is that many data points are available for analysis, making it difficult to comprehend and analyze BC in pediatric population. The best model for dealing with BIS data is the Cole model, a parallel model, which uses resistance values at 0 and infinite frequencies (Cornish et al. 1996). While the method of fitting of Cole-Cole plot is described sufficiently (Lingwood et al. 2012), it requires further investigation and standardization, as fitting a semicircular model to neonatal data is more difficult than in adult due to higher variability in the curve with deviations at the higher and lower frequencies (Lingwood et al. 2012; Sesmero et al. 2005). BIS allows the whole curve to be evaluated and may overcome some of the difficulties of
measuring TBW in neonates using impedance technology, such as small size and
different proportions, hydration levels, skin characteristics and adipose tissue hydration
and composition, however, there is very limited use of BIS in neonates to date
(Lingwood 2013; Tint et al. 2016; Mialich et al. 2014).

There is a need for further evaluation of existing BIA prediction equations to
establish their value and limitations, development of new equations that are tailored
specifically to the studied populations and also for improved methods for monitoring
hydration status in neonates.

1.6.2.6.3 Total Body Electrical Conductivity
Unlike BIA or BIS, total body electrical conductivity (TOBEC) is based on the
introduction of electrical currents to the body by a large solenoid that generated a time-
varying electromagnetic field, and is relatively insensitive to the shifts of fluid between
the extracellular and intracellular compartments, so only the TBW can be monitored
successfully (Ellis 2000).

While TOBEC technology is not longer available, with exception of few research
settings, in the 1990s TOBEC measurements were used to monitor changes in TBW of
pregnant and lactating women (Butte et al. 1997; Motil et al. 1998), in infants (Butte et
al. 1995; Silliman et al. 1995; Bellu et al. 1997) and in childhood obesity (Ellis 1996),
providing results which are useful for understanding of specifics of conductivity
methodology in assessment of BC.

1.6.2.7 Simple measurements or indices

1.6.2.7.1 Body Mass Index
BMI ($BMI = \text{weight} / \text{height}^2$) is commonly used measure of adiposity (Cole et al.
1995). BMI is predictive of clinical outcomes, such as type II diabetes in adults, but in
children and infants its predictive value is not clear. Although highly correlated with
%FM, (Chan et al. 1998), BMI does not distinguish between FM and FFM, health status
and populations (Wells et al. 2016). It has a 2-fold range of variation in fatness for a
given BMI value in individual children (Wells 2000) and therefore in a number of
participants is misleading. This error is amplified in the clinical setting, where
nutritional management according to BC is required. On the contrary, used together
with bioelectrical impedance, BMI improved estimation of FM (Chan et al. 1998)
Given the issues with interpretation of BMI, few viable alternatives exist for population
or clinical measures, thus BMI is still being utilized as a measure of BC particularly in
large studies. Recent study reports that infant BMI peak characteristics are associated with duration of breastfeeding and BC at 3 years, thus may be of interest in understanding of early obesity (Jensen et al. 2015).

1.6.2.7.2 Circumferences
Circumferences of body parts and skeletal diameters have been investigated as alternative indicators of BC. These measures are affected by FM, muscle mass and skeletal size, and are therefore related to FM and lean body mass. These parameters are used in some BC prediction equations including those used for infants (Sen et al. 2010) and considered to be more precise than skinfold measures (Heyward et al. 2004a), since they can be measured with less error.

Visceral fat, located in the trunk, is considered to be more metabolically active, than subcutaneous fat (Despres et al. 2008). Waist circumference is a simple measure of central fatness, which is being used more frequently as a measure of regional adiposity in the field and clinical environments, as it may be better in predicting some outcomes, such as insulin resistance, than total body fat (Wells 2006). In adults, waist-to-hip ratio is associated with morbidity (Rimm et al. 1988) and in children waist circumference and waist-to-hip ratio were found to be better predictors of cardiovascular disease risk factors than BMI (Savva et al. 2000).

1.6.2.7.3 Skinfold Thickness
Traditionally, ST measurements have been used to assess the size of subcutaneous fat deposits or general adiposity. Skinfolds are measures of the thickness of subcutaneous adipose tissue. ST measurements are quick and relatively easy to obtain in many populations including the pediatric population. ST correlate with %FM ($r=0.7–0.9$), do not differ markedly among the common sites (Frerichs et al. 1979; Lohman et al. 1975) and can be used to predict body density and composition. Many equations have been developed for adults, children and infants (Heyward et al. 2004c; Brook 1971; Johnston et al. 1988; Deurenberg et al. 1990; Slaughter et al. 1988) with the sites most often included in prediction equations being the triceps, biceps, subscapular and suprailliac, since no single site is an accurate predictor (Lohman 1981).

There are multiple sources of error with ST, which include operator skill, type of caliper and participant factors such as hydration levels and tissue compressibility, side of the body, age, sex and health of individual. Thus ST measurements are considered unsuitable for longitudinal comparisons as their accuracy within individuals is poor (limits of agreement, ±9% fat) and varies depending on level of adiposity (Reilly et al. 1988).
1995; Wells et al. 1999) and are best used as raw values, indicating regional fatness (Wells 2006). In infants, particularly preterm, calipers also carry the risk of trauma (Petersen et al. 1995) and are disturbing for the infant and mother, while common issues, such as movement and infant skin compressibility, affect reliability (Ulbricht et al. 2012).

1.6.2.8 Ultrasound
US presents an alternative non-invasive, physiological, portable and inexpensive technique to study infant BC. US is routinely used in pregnancy for assessment of fetal growth. The US image is created by high frequency sound waves, which are attenuated during travel through tissue and reflected back to transducer. Each tissue has an intrinsic attenuation coefficient, thereby producing reflections of a different intensity allowing them to be differentiated within the US image. Further, stronger reflections tend to occur at different tissue boundaries (Chan et al. 2011; Wagner 2013). Transducers of higher frequencies (8–12 MHz) produce images of higher resolution however; the beam is attenuated to a greater degree resulting in a decrease in tissue penetration.

Skin thickness, subcutaneous fat thickness and %FM are valuable parameters for assessing and monitoring the nutritional state of the infant. US has already been introduced for this purpose (Petersen et al. 1995) but currently not widely used. US is able to obviate some of the limitations of the caliper technique in the pediatric population, such as age-related inter- and intra-subject variation in skinfold compressibility, inability to palpate the fat-muscle interface or to differentiate the layers of skin, differences in the types of calipers used and the pain/trauma factor (Kuczmarski et al. 1987).

Various measurements with US including ST have been performed to evaluate the growth and development of fetus and term and preterm infant and to assess changes in BC in these groups, which indicated that specific changes in body compartments could be detected with US. A fair correlation ($r=0.84$) was found between ST measured with caliper and US measurements of subcutaneous fat in a small group ($n=7$) of term and preterm infants (Petersen et al. 1995). Unlike caliper skinfold, US has also been used to measure depth and area of adipose and muscle tissue and is useful in assessing regional fat depots at defined anatomical sites in fetuses (Larciprete et al. 2003) and infants. ST measured at various sites as well as mid-arm and mid-thigh fat and lean mass and abdominal fat mass were significantly greater in fetuses with normal development compared to those with intrauterine growth restriction ($n=29$) (Larciprete et al. 2005).
A method to assess abdominal preperitoneal and subcutaneous adipose tissue has been adapted for the pediatric population (Holzhauer et al. 2009), which correlated well with estimates from CT scans, showing high reproducibility (0.89–0.97) and inter-observer agreement (0.90–0.96), making it an attractive tool for epidemiological studies in the early years of life.

Ahmad et al. have measured total leg cross-sectional area and muscle cross-sectional area and calculated subcutaneous fat cross-sectional area of right lower calf in preterm and term newborns (Ahmad et al. 2010). The significant correlations were found between limb US measurements of muscle ($r=0.71$, $r=0.82$) and fat ($r=0.81$, $r=0.82$) and regional and whole body DXA respectively, supporting the idea that relatively simple bedside measurements could be used to determine BC in neonates. US is sensitive enough to detect HM macronutrient-related changes in adipose and muscle tissue accrued at the measurement sites in the abdomen, scapula, mid-thigh and mid-arm in preterm infants (McLeod et al. 2013).

A combination of these methods could be utilized to measure ST in term infants in place of traditional calipers and assess regional adiposity. US offers a pain-free, more consistent solution and has been utilized in assessment of BC of adults, adolescents and infants (Petersen et al. 1995) and validated in adults against DXA, where %FM estimated with US skinfolds correlated better with this of DXA ($r=0.98$, standard error of the estimate (SEE)=2.0) than with BIA ($r=0.92$, SEE=4.4) and ADP ($r=0.94$, SEE=3.7) (Pineau et al. 2007). The technical error (TE) was also smaller for US skinfolds (TE=1%) compared with BIA (TE=2.57%) and ADP (TE=2.99%). Additionally, no bias was seen for both genders combined or separately with 95% limits of agreement ranging from ±2%, unlike BIA (±5.1%) and ADP (±5.8%). Recently Pineau et al. compared US skinfold measurements with DEXA in male and female athletes and confirmed good accuracy in producing FM and %FM estimates ($r=0.98$, SEE=0.96; $r=0.97$, SEE=1.79, respectively) (Pineau et al. 2009).

One can argue that high-resolution scanners are not cheap and technical skills are required, but with the recent introduction of new cheaper portable devices for the athletes to monitor training regimen such as BodyMetrix BX-200 US device (IntelaMetrix Inc., Livermore, CA, USA) this technology became accessible even to the general public. With limited validation studies in adults this device appears to be promising and has the advantage of being portable, non-invasive and inexpensive (Johnson 2013; Johnson et al. 2016; Wagner 2013), although it can not be used in
infants for other than for collecting raw skinfold data due to the equations used for the prediction of %FM.

Both, US and BIS present few advantages in research setting, such as relative ease of use, lower cost and risk and good tolerance by participants, which is important when working with infants and their mothers. While all BC measuring techniques have inherent limitations when used in infants and children, the combination of anthropometric measurements with BIS and US may offer relatively accurate, safe and non-invasive assessment of BC making them an attractive research tool for this population.

1.6.3 Infant body composition

Although limited cadaver data exists, several reference models have been developed to describe human BC over the lifespan. These are: Reference Fetus \((n=22)\) (Ziegler et al. 1976), Reference Infant \((n=44)\) (Fomon et al. 2002), Reference Children (Fomon et al. 1982), Reference Adolescents \((n=108)\) (Haschke 1983a; Haschke 1983b), and Reference Man and Woman (Ellis 1991; Snyder et al. 1984). Together with the work of other researchers, which described BC during human growth (Cheek 1968; Forbes 1987; Moore et al. 1963) they provide a cornerstone of human BC research at the tissue, cellular and elemental level. Since this pioneering research improvements in technology and new approaches have added more details to study of the BC of the neonate (Ellis 1998; Butte et al. 2000a). Koo et al. determined DXA reference values (Koo et al. 2000), de Bruin et al. has provided TOBEC reference values (de Bruin et al. 1995), and Olhager et al. presented MRI data during first 4 months of life (Olhager et al. 2003), with all different approaches showing a good visual agreement in curves for evolution of FM and FFM during first 12 months of life (Rigo 2006) (Figure 1.15). More recently ADP data on term infants has been collected (Ellis et al. 2007; Ma et al. 2004; Roggero et al. 2012; Fields et al. 2011) along with the creation or reference BC curves the preterm infant reference from data accumulated on 223 ethnically diverse infants born between 30 + 0 and 36 + 6 weeks of gestation (Demerath et al. 2016).

Unlike adults, BC in infants is changing substantially due to rapid growth (Fomon et al. 2002). Not only do water, lipid, protein and mineral content increase with age, but they do so at different rates (Ellis 1998). During this time a significant redistribution of body water is also occurs, with a decrease in extracellular and an increase in intracellular fluid volumes (Fomon et al. 1982).
A limited number of controversial BC studies exist of infants during the first year of life, particularly of breastfed infants whose FFM is significantly lower and %FM is significantly higher compared to formula-fed infants (Gale et al. 2012). Despite this, most of the BC studies in infants have been performed on mixed rather than fully...
breastfed cohorts (Fomon et al. 1982; Fomon et al. 2002) and in some instances studies make claims regarding breastfed infants despite some formula feeding being considered acceptable (Bartok 2011).

Some studies observed %FM to be consistently higher due to lower FFM in breastfed infants compared with formula-fed infants (Carberry et al. 2010; Fields et al. 2011; Butte et al. 2000b; Butte et al. 1995; Gale et al. 2012) while others have reported lower %FM (Bellu et al. 1997; Ay et al. 2009). This results could be explained by some breastfeeding (<2 months) being acceptable in formula-fed group and higher fat and protein intakes in the breastfed group in Bellu et al. study; while Ay et al. analyzed the effect of the length of breastfeeding on %FM rather than compared the groups. Following the introduction of other milks and supplementary foods results are contradictory as well, with marked differences between formula-fed and breastfed infants at 9 months of age are either diminishing and no longer apparent at 12 months of age (Butte et al. 2000b) or alternatively persisting beyond transitional period (Bellu et al. 1997; Dewey et al. 1993; Robinson et al. 2009), which could be contributed to significant differences in nutrient intakes between the groups in the latter studies all of which studied infants breastfed at least to 12 months and reported reduced adiposity. These results reason for further investigation of the effects of HM composition and MI on infant growth and BC beyond the exclusive breastfeeding period.

%FM changes in the first year of infant’s life with a gradual increase in first 6 months followed by gradual decrease to 12 months as reported in the reference models (Butte et al. 2000a; Fomon et al. 2002). Age differences in %FM of breastfed infants are mixed with some studies reporting an overall significant increase in %FM in the first 4 months of life (Fields et al. 2011; Roggero et al. 2010; Carberry et al. 2010) or no change between 2 and 3, 4 or 5 months (Gilchrist 2007) with detailed analysis between time points not always reported.

Some studies have also report sex differences in %FM of infants with females displaying higher %FM than males (Butte et al. 2000a; Carberry et al. 2010; Gilchrist et al. 2008), whilst one shows higher %FM in breastfed males only (Butte et al. 2000b). Other studies either show no difference (Gilchrist 2007; Roggero et al. 2010; Bellu et al. 1997) or have not analysed for sex differences (Fomon et al. 2002; Fields et al. 2011; Gale et al. 2012). These mixed results raise the importance of the differential sex approach to the reference BC data.

Since the results of many studies are contradictory due to the wide variability in techniques employed and cohorts analysed, the development of normative BC values
for breastfed males and females infants is necessary in order to gain better knowledge of the composition of infants’ growth, which will result in nutritional recommendations with the potential to reduce future risk of disease.

1.6.4 Effect of milk composition on infant body composition

There are many factors that affect infant BC, some of them modifiable and others not. Obvious factors programming BC are genetic and ethnic factors, which are difficult to modify although this maybe possible on an epigenetic level as shown in animal models investigating over- and under-nutrition during the prenatal period (Velazquez et al. 2015). In contrast, nutrition is highly modifiable and evidence suggests early nutrition may influence metabolic programming, thereby enhancing or compromising short and long-term health (Ratnasingham et al. 2017). Indeed, in utero maternal influences are apparent in obese women who generally deliver heavier infants with greater adiposity. Interestingly, this association ceases to exist by 6 weeks postpartum (Silliman et al. 1995) but seems to reappear later (between 12–24 months) (Dewey et al. 1993), confirming that early nutrition plays a role in regulation of infant BC.

Whilst volume of milk is known to associate with infant growth rate (Mitoulas et al. 2002; Dewey et al. 1991; Kent et al. 1999), information on effect of HM components on infant BC is limited. The macronutrient composition of HM feeds has been related to significant changes in the adipose and lean tissue of preterm infants (McLeod et al. 2013). Further, intakes of energy, protein, fat and carbohydrate are lower in breastfed infants at 3 and 6 months compared to formula-fed infants and are positively related to weight gain and FFM gain, but not FM (Butte et al. 2000b). Recent study of 614 dyads (Prentice et al. 2016) indicated that concentration of HM fat is implicated in development of infant BC showing negative association between fat concentration in HM samples collected at 4–8 weeks postpartum and infant delta weight (3–12 months), delta skinfolds (3–12 months), delta BMI (3–12 months), BMI and skinfolds at 12 months in a cohort with both exclusive and mixed (some formula supplementation allowed) breastfeeding. These relationships showed the same directions of associations in the exclusively breastfed subset, although with less significance, and should be interpreted with caution, since the fat concentration was analyzed in pooled samples collected once daily during two weeks rather than during 24-h milk production and timing of collection was not recorded (Prentice et al. 2016).

Another recent study suggested that early diet with higher fat intake may benefit the development of BC, reporting an inverse association between fat intake at 2 years
and body fat determined by BIA as well as serum leptin concentration at 20 years (Rolland-Cachera et al. 2013). Since fat concentration in HM is inversely related to the volume of MI (Nommsen et al. 1991), it is possible that infants consuming HM with lower fat concentrations may feel still hungry, ingest larger volumes of HM (and higher amounts of carbohydrate and protein) and increase the body weight (Prentice et al. 2016).

Breastfed infants consume not only less energy than formula-fed, but also less protein and fewer micronutrients. Amino acid and protein content may play a major role in infant weight gain providing rationale for slower growth trajectory and lower FFM of breastfed infants compared to formula-fed infants (Rigo et al. 2006; Roggero et al. 2008; Luque et al. 2015; Michaelsen et al. 2014), although it is impossible to identify the causal factors precisely. Protein intake is positively associated with weight gain (3–6 and 6–9 months) not only in formula-fed (n=46) but also in exclusively breastfed (n=73) infants (Heinig et al. 1993). Concentration of protein in HM collected at 4–8 weeks postpartum is positively associated with infant (n=614) BMI at 12 months, although no association is found with infant skinfolds, weight or 3–12 months weight gain, indicating that the relationship is not simple (Prentice et al. 2016).

Same study (Prentice et al. 2016) also showed a positive association between HM lactose and infant BMI, delta BMI (3–12 months), skinfolds and delta skinfolds (3–12 months) at 12 months. A higher estimated carbohydrate daily intake has also been reported to increase infant weight and lean mass gain (Butte et al. 2000b) and higher concentrations of glucose have been associated with higher relative weight and both fat and lean mass of breastfed infants (Fields et al. 2012). In addition individual HMO display differing associations with infant BC, although total concentration was not analyzed (Alderete et al. 2015). HMO are speculated to exert BC effects by enhancing the growth of beneficial gut bacteria (Bode 2012), as the gut microbiome has been implicated in the development of obesity (Turnbaugh et al. 2009; Chakraborti 2015).

Effect of maternal BC on infant BC via both composition and the quantity of HM becomes a new focus of research to understand the intergenerational nature of obesity allowing for the possibility of new interventions to improve the infant outcomes. Maternal influences on HM composition have been described in 1.2.5, 1.3.2.2 and 1.3.3.1. Smaller active HM peptides and bioactive components, believed to be involved in the development of appetite regulation and infant BC, include appetite hormones adiponectin, leptin, ghrelin, apelin, resistin, IGF-I, motilin, CCK and many others also play significant roles in the metabolic development of infants and may affect infant BC.
The effect of some HM appetite hormones on infant BC has been described in sections 1.3.2.4, 1.3.3.3 and 1.3.4.1.

1.7 CONCLUSIONS
Preconception and early life is a critical period when appetite control and BC are programmed and is the greatest window of opportunity for intervention to significantly improve infant outcome, since the trajectory of risk of NCD is set much earlier than the time of diagnosis (Figure 1.16). Further, these periods are influenced by the maternal factors and early nutrition, thus timely intervention may have a greater impact on disease risk later in life (Godfrey et al. 2016).

Figure 1.16 Conceptual framework illustrating a life course approach to NCD prevention and treatment. Reproduced from (Godfrey et al. 2016) by permission from Nestle Nutrition Institute.

A growing body of evidence suggests that breastfeeding has protective roles against NCD during adulthood and can have long-term beneficial health effects at both individual and population levels (Kelishadi et al. 2014). Furthermore, longer duration of breastfeeding is shown to attenuate the adverse effects of birth weight and early weight gain on infant fat mass (Ejlerskov et al. 2015). These findings suggest dose-dependent
effect of breastfeeding on development of infant BC, but the mechanisms of this effect are not fully understood.

The major focus of the currently limited research on infant appetite, growth and BC has been on the composition of HM, and to a lesser extent the effect of the volume of milk with only one study accounting for doses of the HM components that infant receives. Factors inhibiting the interpretation of these finding include: the conflicting results of studies that find either higher or lower %FM in breastfed infants; lack of knowledge of the volume and composition of milk fed to the infant, as well as the mode of delivery (bottle vs breast) (Bartok 2011), thus much more comprehensive study is required to determine relationships between HM and infant outcomes. Despite the increasing availability of the modern technology, rarely are HM composition analysis combined with simultaneous measurements of BC. No longitudinal study has combined the extensive analysis of both macronutrient and appetite control factors to gain a greater understanding of the complexity of growth and BC of the infant. However, the methodology of measuring of infant BC yet is still in the developmental phase. Whilst it is clear that there are many obstacles that currently exist for accurate measurement of infant BC, both BIS and US provide a novel non-invasive and relatively unexpansive tool to predict BC in this population.

Further insights emerging from the literature describing maternal hormones in HM and their effect on a developing infant highlight the need for a greater understanding of the synergy of the components of HM. Furthermore, utilization of ELISA for measuring multiple components in whole HM rather than skim would give an opportunity to finally assess the full effect of the components, rather than establish spurious associations with their reduced quantities in the skim milk. Future studies should focus on understanding of the changes that occur during infancy and lactation and potential factors that may underpin differences in infant BC, and thus differences in outcome such as infant growth and development and potentially health later in life. Findings from these studies will be vital to translation of the knowledge of appetite control and BC and their regulation during infancy into the nutritional recommendations for this period.

1.8 AIMS OF THE STUDY
The aim of this research is to investigate mechanisms involved in regulation of appetite control and development of BC of breastfed infants using cost-effective, non-invasive
techniques adapted for the use in infants in our laboratory to determine relationship between maternal BC, HM composition, MI, FFQ, GE and infant BC in a comprehensive longitudinal study.

**Major aims**

1. To assess the effect of the MI, feed duration and the volume of the infant’s stomach and bladder on the resistance values pre-/post-feed and impedance indices to establish the feasibility of using these values interchangeably during data collection.
2. To evaluate and compare the performance of BIS and US in determining %FM in term breastfed infants.
3. To optimize ELISA for leptin measurement in both whole and skim HM and compare leptin concentrations between both HM preparations.
4. To investigate associations between maternal adiposity and concentrations of HM adiponectin, leptin, fat, lactose, total carbohydrate, oligosaccharides, lysozyme, total, casein and whey protein, and whether these concentrations and the relationship between BMI and %FM in a breastfeeding population change over the first year of lactation.
5. To investigate the effects of concentrations and doses of HM appetite hormones and macronutrients on MI, FFQ, GR and GE, with further exploration of the effects of infant demographics, anthropometrics and BC on infant feeding and GE.
6. To simultaneously investigate relationships of concentrations and calculated daily intakes (CDI) of array of HM components such as appetite hormones, proteins and carbohydrates with the BC of healthy term breastfed infants during first year of life, with further exploration of relationships of infant 24-h MI and FFQ with HM components and maternal and infant BC.

**1.9 RESEARCH DESIGN**

In order to address the above aims and to optimise data collection our research adopted both longitudinal and cross-sectional study approach and used same participants for multiple analysis where inclusion/exclusion criteria allowed doing so. As a result, 114 data collection sessions, at which milk samples and/or maternal/infant BC data were
collected, were conducted; 73 sessions with longitudinal \((n=23)\) and 41 sessions with cross-sectional participants \((n=41)\). Given the complexity of the data collected, we were able to use some of the longitudinal participants data as cross-sectional for some of the analysis. Data was distributed for analysis as follows:

Forty-eight breastfeeding infants \((36\text{ cross-sectional and 12 longitudinal, of which 9 participated twice and 3 three times; } n=62\text{ sessions})\) were measured with BIS at 2, 5, 9 and/or 12 months for the study of the effect of milk intake on resistance measurements (Chapter 2).

Data from 58 breastfed infants \((35\text{ cross-sectional, 23 longitudinal})\) aged 2, 5, 9 or 12 months were used once in the methods comparison study (Chapter 3).

Data and milk samples from 59 mothers of breastfeeding infants \((38\text{ cross-sectional and 21 longitudinal, of which 2 participated twice, 11 three and 8 four times; } n=283\text{ samples})\) collected at 2, 5, 9 and/or 12 months was used for analysis in both study of the leptin concentration in skim and whole HM (Chapter 4) and in study of the associations between maternal BC and milk components (Chapter 5).

Data and milk samples from 27 breastfed infants \((13\text{ cross-sectional and 14 longitudinal; } n=41\text{ feeds})\) collected at 2 and 5 months after birth were used in the gastric emptying study (Chapter 6).

Data and milk samples from 20 breastfeeding dyads were used in the longitudinal study of associations between HM components and infant BC (Chapter 7).
CHAPTER 2

BIOIMPEDANCE SPECTROSCOPY IN THE INFANT: EFFECT OF MILK INTAKE AND EXTRACELLULAR FLUID RESERVOIRS ON RESISTANCE MEASUREMENTS IN TERM BREASTFED INFANTS*

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2.1 ABSTRACT

BIS is an accurate non-invasive method for measuring BC in adults, but in infants it requires further testing and validation. Of the few studies of bioimpedance conducted in infants, none have comprehensively investigated the effect of MI volume. This study assessed the effect of the MI, feed duration, and the volume of the infant’s stomach and bladder on the resistance values pre-/post-feed to establish the feasibility of using these values interchangeably during data collection. Forty-eight breastfeeding infants were measured at 2, 5, 9 and/or 12 months (n=62 sessions) within 1-2 minutes before the start and after the end of breastfeed. Median [IQR] time between measurements was 24 (20.0–30.0) min. Resistance measurements at 0 kHz and 50 kHz, and infinite frequency (R₀, R₅₀ and Rᵢₙfinity) and resistance of intracellular water (Rᵢcw) were analyzed with customized infant settings. MI was measured by test weights. Free-water volumes and free-water change were determined from stomach and bladder volumes calculated from US images. Small pre-to-post-feed changes (median [IQR]: R₀ -3.7 [-14.8, 14.3]; R₅₀ 0.3 [-10.4, 15.0]; Rᵢₙfinity 2.8 [-13.3, 35.5]; Rᵢcw 20.8 [-98.1, 290.9]) were not significantly different from zero (R₀: p=0.92; R₅₀: p=0.48; Rᵢₙfinity: p=0.32; Rᵢcw: p=0.097). No significant effect of MI or free-water change was detected. The lack of consistent change in resistance across a breastfeed provides flexibility in the timing of measurements of infants in the research setting, such that typically pre- and post-feed measures of resistance can be used interchangeably.
2.2 INTRODUCTION

Accurate assessment of infant fluid status and BC is essential for studies investigating effect of nutrition on the infant growth and development. BIS is non-invasive and an inexpensive method, commonly used in adults to calculate total TBW and estimate FFM and FM. Changes in body fat and water status are reflected as changes in tissue impedance and electrical properties (Tuorkey 2012). Although predictive BIS equations have been developed for adolescents, children, toddlers and newborn infants (Ferreira et al. 2004; Mayfield et al. 1991; Lingwood et al. 2000), there are no validated prediction equations using BIS in neonates (Lingwood 2013).

BIS has proven its ability to quantitatively assess hydration status in adult patients (Kraemer et al. 2006) and has been found to be accurate in children over two years of age, both healthy and those on haemodialysis (Wieskotten et al. 2008; Zaloszyc et al. 2012; Zaloszyc et al. 2013; Oh et al. 2014). In young infants, BIS is associated with poor predictive performance, possibly due to higher conductivity of adipose tissue with its increased vascularization and water content, and rapidly changing hydration rates caused by water loss and feeding (Sesmero et al. 2005; Lingwood et al. 2012). Before birth, the fetus has a constant supply of water through the placenta. After birth, the infant must adjust to life in a dry environment, a process that starts rapidly after birth and then gradually decreases through to adulthood (Lorenz 1997). Infants lose water through urine and skin (sweating and insensible water loss) and also from lungs and feces. Although infants have higher body water content and less ability to sweat, they also have a higher water turnover and a greater surface area compared with body mass, which causes them to lose more water from the skin compared to adults (Benelam et al. 2010). Water gains come from ingested fluids, food, and body metabolic water. Feeding patterns and volumes, which are highly variable both within and between breastfeeding dyads, combined with unpredictable bladder and bowel emptying, make it difficult to standardize hydration status and may contribute to BIS measurements variability. A technique to quantitatively assess infant body hydration status may be beneficial for method standardization.

To obtain maximal measurement reproducibility various requisites of standardisation have been established in adults, including timing of food intake before the test (Kushner et al. 1996). Fasting is not an option for improving measurement reproducibility in paediatric population, as hungry infants are often very unsettled,
making measurement difficult (or impossible) and influencing readings, whereas post-feed they are more content and relaxed.

Of the limited studies investigating BIS measurements in infants, two investigated the effects of liquid consumption. Mayfield et al. found no change in TBW using impedance plethysmograph in low-birth-weight infants given enteral feeds (1–7 days; intake 34±16 mL; n=10) (Mayfield et al. 1991). Sesmero et al. presented similar findings for term infants (1–24 weeks; intake 30–135mL; n=29) using BIS (Sesmero et al. 2005). This lack of effect, however, may be due to neither study reaching the BIS minimal detectable change limit, estimated at ~200 mL (10%; percentage change based on 3.5 kg infant with 15% fat) in infant TBW (Ellis 2002). Furthermore, the effect of MI, which may range from 0 to 350 g (1–6 months; 101.4±15.6 g) (Kent et al. 2006), was not measured.

No study has accounted for pre-/post-feed stomach or bladder volumes; yet they may increase the extracellular water pool affecting measurements. For breastfed infants, it is not possible to control the volume consumed or bladder volumes. Therefore, a method must be developed to account for these volumes to determine whether changes in stomach and/or bladder volume have an effect on resistance measurements.

This study aimed to determine the effect of MI and bladder volume on resistance measures pre-/post-feed and to investigate other factors that might influence resistance measures (feed duration, infant movement, length, weight and age) in order to establish the feasibility of using these measures interchangeably to improve BIS data collection in the first year of life.

2.3 SUBJECTS AND METHODS

2.3.1 Subjects

Predominantly Caucasian mothers of full-term healthy breastfed infants (n=48; 27 male, 21 female) were recruited primarily from the Australian Breastfeeding Association. Human Research Ethics Committee of The University of Western Australia approved the study, and participants provided written informed consent. Measurements were made when the infants were ~ two, five, nine or 12 months of age (Table 2.1). When an infant attended two or more sessions, the sessions were treated as independent observations.
At each study session, BIS, US and weight measurements were taken pre- and post-breastfeed (one infant received additional expressed HM). Infant crown-heel length was measured once to the nearest 0.1 cm, with perpendicular to the surface headpiece and footpiece and non-stretch tape on a hard surface. Clothing was removed, except for a dry diaper and a singlet.

2.3.2 Bioelectrical impedance measurements

Whole body bioimpedance (wrist to ankle) was measured using the Impedimed SFB7 bioelectrical impedance analyzer (ImpediMed, Brisbane, Queensland, Australia). This is a single channel, tetra-polar BIS device capable of measuring reactance and resistance at 256 logarithmically-spaced frequencies between 3 and 1000 kHz.

Multiple consecutive measurements (10–50) were performed with infants in supine position on a non-conductive surface within 1–2 min before the start and after the end of breastfeed. Median [IQR] time between measurements was 24 (20.0–30.0) min. After wiping skin with isopropyl alcohol, single Ag-AgCl gel electrodes (ImpediMed, Brisbane, Queensland, Australia) were applied on the right hand and foot. Two distal current drive electrodes were placed on the dorsal surface of the hand and the foot, at the metacarpal-phalangeal and metatarsal-phalangeal joints, and two voltage sense electrodes were placed 3cm proximal to the current electrodes. The electrodes were secured with 3M Micropore surgical tape (3M Health Care, Neuss, Germany). Electrodes remained on the limbs for the post-feed measurements. No direct contact was made with the infant’s skin during the measurements. Insulating material (cloth) was used to ensure no contact between the infant limbs or between infant and mother’s hands. Infant movement during the measurements was graded on a scale from 0 to 4 (0, complete absence of movements; 1, brief mild movements; 2, brief intense movements; 3, mild to intense constant movements; and 4, tense and crying) (Sesmero et al. 2005).

Collected data were transferred to a computer and analyzed by fitting the measured resistance and reactance at each frequency to a Cole-Cole plot of resistance against reactance, using Impedimed software (Bioimp version 5.2.2.0; ImpediMed, Brisbane, Queensland, Australia). All raw data were visually examined, and measurements were analyzed with settings customized for each infant to ensure goodness of fit as assessed by the standard error of the estimate according to Lingwood et al. (Lingwood et al. 2012). Values of resistance (ohm) at 0 kHz and 50 kHz, and infinite frequency (R₀, R₅₀ and R₉₀, respectively) were determined from the curve of
best fit. Resistance of intracellular water component (R_{icw}) was calculated using following formula (Lukaski 2013):

\[ R_{icw} = \frac{1}{\left( \frac{1}{R_{inf}} - \frac{1}{R_0} \right)} . \]

These frequencies were chosen as R_{50} is commonly used to predict TBW in empirically-derived prediction equations, R_0 and R_{icw} to predict extracellular and intracellular water volumes and R_{inf} to predict TBW using mixture theory modeling (Matthie 2008).

### 2.3.3 Stomach and bladder volumes

Scans of longitudinal and transverse planes of the stomach and bladder were performed in the semi-supine position according to the methods validated in infants (Perrella et al. 2013; Erasmie et al. 1989) using an Aplio 80 (Toshiba, Japan) US machine and Parker ultrasonic gel (Fairfield, NJ, USA). Longitudinal, transverse and sagittal diameters were measured, and the volume of both extracellular fluid reservoirs were calculated using the following formula:

\[ Volume (mL) = \text{longitudinal diameter (cm)} \times \text{anterioposterior diameter (cm)} \times \text{transverse diameter (cm)} \times 0.52 \]

Free-water change was calculated by subtracting combined bladder and stomach volumes pre-feed from combined volumes post-feed.

### 2.3.4 Infant milk intake

MI was determined by test-weighing the infant before and after feeding on an electronic balance (±2.0g; Medela Electronic Baby-Weigh Scales, Medela AG, Switzerland). MI was calculated by subtracting the initial weight from the final weight of the infant (Arthur et al. 1987).

### 2.3.5 Statistical analysis

Statistical analysis used R 2.9.0 for Mac OSX (R Core Team 2009) with packages nlme (Pinheiro et al. 2009), irr (Gamer et al. 2007) and multcomp (Hothorn et al. 2008). Descriptive statistics are reported as mean ± SD (normally distributed data) or range. \( p<0.05 \) was considered statistically significant. All tests were two tailed.

Linear mixed effect models grouping by infant were run and compared to linear regression models (analysis of variance). No significant individual patterns were found
(p>0.16); hence, measurements on the same infant at different time points were treated as independent.

Participants were recruited as part of a larger study; those with useable data were included here. Pilot data (n=5, two measurements under identical conditions) gave mean ± SD within participant differences of 7.4±7.7. Using α=0.05, 62 participants give the study power of 0.8 to detect a mean difference between pre- and post-feed resistance measures of 2.4.

BIS measurements were assessed for variability (CV (coefficient of variation)) and repeatability (intraclass correlation coefficient (ICC), 95% confidence interval (CI)) for a subset of infants (n=16, two per age/sex grouping).

Within time-point comparisons treated the first and second half of a recording as separate measures; summary BIS measures were calculated as per full recordings. Variability was assessed by calculating CV for the raw data for each infant by time-point by first/second half of recording (n=32), and compared using paired t-tests within frequencies. Repeatability was assessed by calculation of ICC and 95% CI and was considered to differ if the 95% CIs did not overlap. ICC>0.8 is considered acceptable for a research setting and >0.95 for the clinical setting (Landis et al. 1977). Between time-point comparisons compared pre- and post-feed measures using the same analyses as for the within time-point comparisons. Paired t-tests were used to compare variability at different frequencies, with pre- and post-feed data treated as independent measures.

Overall variation was determined from a repeated measures CV calculated from an intercept only linear mixed effects model.

Changes in resistance pre-/post-feed (∆R) were assessed by calculating bias/95% CI for pre- vs post-feed BIS as per Bland and Altman (Bland et al. 1986) (n=59). A 95% CI containing zero was interpreted as no consistent change.

Associations between ∆R and predictors/covariates of interest were assessed using linear regression. Model selection used forwards stepwise selection. Age was treated as a categorical variable and included in the models where an analysis of variance omnibus test indicated that one or more ages differed from the others.

Association between infant age (factor) and pre-feed resistance were assessed using Tukey’s all-pair comparisons based on OLS regression. Model appropriateness tested using standard residual diagnostics. Infant movements pre-/post-feed were compared using paired Student’s t-test.
2.4 RESULTS

Of the 62 infants (Table 2.1), 37 were measured once (33/37 complete data), 8 twice (14/16 complete data) and 3 three times (8/9 complete data). Missing data included: two bioimpedance measurements due to infant movement pre-feed and one post-feed (three feed duration times missing); two stomach volumes pre-/post-feed and four bladder volumes pre- and five post-feed (six free-water change calculations missing).

2.4.1 Bias, variability and repeatability

In a subset of infants \((n=16)\), no difference in variability pre- vs post-feed was seen for any of the frequencies \((R_0: p=0.36, R_{50}: p=0.47, R_{inf}: p=0.34, R_{icw}: p=0.47)\). There was no difference in variability between \(R_0\) and \(R_{50}\), and \(R_0\) and \(R_{icw}\) \((p=0.13\) for both), but all \(R_0\), \(R_{50}\) and \(R_{icw}\) were significantly less variable than \(R_{inf}\) \((p<0.001\) for all). Figure 2.1 presents Bland-Altman plots for pre- and post-feed resistance measures and impedance indices and illustrates that \(R_{inf}\) measures have greater variability which is also carried onto \(R_{inf}\) to the point that some of the individual data points on the plots for \(R_{inf}\) and \(R_{icw}\) and for corresponding indices present as outliers. Although these measurements are legitimate in that they are a result of machine output or calculated from the other measures, they are not presenting in an expected range and thus should be interpreted with caution.

Bias measurements \((n=59)\) were small, with the 95% CI containing zero for all frequencies except \(R_{icw}\). There was a higher level of agreement when comparing the first and second half of a set of measurements (within) than comparing pre- with post-feed (between) measurements. All but \(R_{icw}\) within measurements had ICCs>0.96 (CI 95%). Between pre- and post-feed measurements intraclass correlations also were strong (>0.8; Table 2.2; Figure 2.1).
**CHAPTER 2: BIOIMPEDANCE PRE- AND POST-FEED**

Table 2.1 *Subject demographic, anthropometric and impedance characteristics presented as total and grouped by infant age.*

<table>
<thead>
<tr>
<th>Age (months)</th>
<th>Time</th>
<th>Characteristic</th>
<th>Mean ± SD</th>
<th>Range</th>
<th>Mean ± SD</th>
<th>Range</th>
<th>Mean ± SD</th>
<th>Range</th>
<th>Mean ± SD</th>
<th>Range</th>
<th>Mean ± SD</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td></td>
<td>Sex (M:F)</td>
<td>8M/8F</td>
<td>(N=16)</td>
<td>9M/12F</td>
<td>(N=21)</td>
<td>12M/4F</td>
<td>(N=9)</td>
<td>5M/4F</td>
<td>(N=62)</td>
<td>34M/28F</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>Age (months)</td>
<td>2.02±0.25</td>
<td>1.33 - 2.40</td>
<td>4.9±0.31</td>
<td>4.30 - 5.47</td>
<td>9.14±0.34</td>
<td>8.13 - 9.47</td>
<td>12.2±0.22</td>
<td>11.90 - 12.57</td>
<td>6.3±3.58</td>
<td>1.33 - 12.57</td>
</tr>
<tr>
<td>9</td>
<td></td>
<td>Milk intake (mL)</td>
<td>93.9±26.3</td>
<td>39 - 136</td>
<td>88.6±47.1</td>
<td>15 - 232</td>
<td>89.1±51.6</td>
<td>19 - 189</td>
<td>51.7±40.7</td>
<td>10 - 129</td>
<td>84.7±44.3</td>
<td>10 - 232</td>
</tr>
<tr>
<td>12</td>
<td></td>
<td>Feed duration (min)</td>
<td>34.6±18.0</td>
<td>14 - 85</td>
<td>24.8±8.3</td>
<td>12 - 46</td>
<td>26.4±11.0</td>
<td>14 - 50</td>
<td>23.6±3.6</td>
<td>19 - 29</td>
<td>27.3±12.0</td>
<td>12 - 85</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Length (cm)</td>
<td>57.8±2.4</td>
<td>53.0 - 61.3</td>
<td>64.7±1.9</td>
<td>61.3 - 69.5</td>
<td>71.5±2.2</td>
<td>66.0 - 74.5</td>
<td>73.6±1.5</td>
<td>71.5 - 75.5</td>
<td>66.0±6.2</td>
<td>53.0 - 75.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Post-feed</td>
<td>5.416±0.726</td>
<td>4.296 - 6.425</td>
<td>7.377±0.930</td>
<td>5.756 - 8.835</td>
<td>9.177±0.780</td>
<td>7.783 - 10.740</td>
<td>9.361±0.975</td>
<td>7.210 - 10.320</td>
<td>7.623±1.750</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Stomach volume (mL)</td>
<td>Pre-feed</td>
<td>6.3±14.0</td>
<td>0 - 50</td>
<td>10.6±16.6</td>
<td>0 - 57</td>
<td>6.7±18.4</td>
<td>0 - 71</td>
<td>8.3±19.0</td>
<td>0 - 54</td>
<td>8.1±16.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Post-feed</td>
<td>103.1±37.1</td>
<td>52 - 191</td>
<td>99.4±49.7</td>
<td>13 - 237</td>
<td>101.4±82.3</td>
<td>19 - 329</td>
<td>60.9±40.7</td>
<td>1 - 108</td>
<td>95.8±57.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Bladder volume (mL)</td>
<td>Pre-feed</td>
<td>9.6±8.6</td>
<td>0 - 26</td>
<td>14.5±12.8</td>
<td>0 - 55</td>
<td>25.4±18.8</td>
<td>0 - 75</td>
<td>31.5±15.3</td>
<td>6 - 50</td>
<td>18.7±16.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Post-feed</td>
<td>16.1±13.6</td>
<td>0 - 48</td>
<td>13.6±10.7</td>
<td>0 - 37</td>
<td>21.5±15.8</td>
<td>0 - 59</td>
<td>29.8±13.4</td>
<td>8 - 51</td>
<td>18.7±14.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Free-water (mL)</td>
<td>Pre-feed</td>
<td>16.3±15.0</td>
<td>0 - 50</td>
<td>23.9±17.4</td>
<td>0 - 57</td>
<td>32.0±25.8</td>
<td>0 - 97</td>
<td>39.8±30.1</td>
<td>6 - 103</td>
<td>26.5±22.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Post-feed</td>
<td>118.0±36.2</td>
<td>61 - 202</td>
<td>114.4±48.2</td>
<td>23 - 237</td>
<td>122.9±81.3</td>
<td>52 - 359</td>
<td>90.6±47.4</td>
<td>21 - 139</td>
<td>114.3±56.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Free-water change (mL)</td>
<td>101.7±42.0</td>
<td>33 - 202</td>
<td>90.3±53.1</td>
<td>12 - 216</td>
<td>90.9±80.4</td>
<td>21 - 337</td>
<td>50.9±48.8</td>
<td>-29 - 109</td>
<td>87.7±60.0</td>
<td>-29 - 337</td>
</tr>
</tbody>
</table>

Abbreviations: F - female; icw - intracellular water; inf - infinity; L - length (cm); L²/R - impedance index; M - male; R - resistance (ohm). Subjects’ characteristics presented as mean ± SD and range for total group (n =62) and grouped by infant age.
### CHAPTER 2: BIOIMPEDANCE PRE- AND POST-FEED

#### Table 2.1 Continued.

<table>
<thead>
<tr>
<th>Age (months)</th>
<th>Time</th>
<th>Characteristic</th>
<th>Mean ± SD</th>
<th>Range</th>
<th>Mean ± SD</th>
<th>Range</th>
<th>Mean ± SD</th>
<th>Range</th>
<th>Mean ± SD</th>
<th>Range</th>
<th>Mean ± SD</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>R₀ (ohm)</td>
<td>Pre-feed</td>
<td>670.7±81.9</td>
<td>566.8 - 900.5</td>
<td>737.0±83.5</td>
<td>604.2 - 893.6</td>
<td>688.9±69.9</td>
<td>621.0 - 828.1</td>
<td>701.3±41.0</td>
<td>636.2 - 754.6</td>
<td>703.4±77.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Post-feed</td>
<td>675.9±90.3</td>
<td>560.6 - 913.6</td>
<td>729.8±78.1</td>
<td>661.3 - 919.8</td>
<td>691.1±80.1</td>
<td>601.5 - 844.4</td>
<td>691.7±42.9</td>
<td>621.7 - 742.5</td>
<td>700.5±79.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rₖₑ (ohm)</td>
<td>Pre-feed</td>
<td>627.7±82.8</td>
<td>532.7 - 862.7</td>
<td>691.6±82.5</td>
<td>559.3 - 855.6</td>
<td>651.9±69.5</td>
<td>577.3 - 786.8</td>
<td>659.4±43.8</td>
<td>602.2 - 727.0</td>
<td>661.3±77.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Post-feed</td>
<td>632.2±93.5</td>
<td>516.1 - 885.5</td>
<td>690.7±76.9</td>
<td>566.0 - 880.3</td>
<td>653.3±80.1</td>
<td>555.9 - 807.9</td>
<td>649.8±44.7</td>
<td>585.3 - 707.4</td>
<td>660.1±80.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rₑₑ (ohm)</td>
<td>Pre-feed</td>
<td>454.3±72.9</td>
<td>371.0 - 666.5</td>
<td>530.9±81.4</td>
<td>378.1 - 704.3</td>
<td>519.2±62.3</td>
<td>448.4 - 648.6</td>
<td>524.7±55.9</td>
<td>448.2 - 629.0</td>
<td>509.0±76.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Post-feed</td>
<td>458.6±90.6</td>
<td>324.2 - 689.7</td>
<td>541.6±77.0</td>
<td>433.0 - 712.4</td>
<td>513.5±96.6</td>
<td>305.8 - 693.6</td>
<td>520.3±58.6</td>
<td>448.5 - 630.0</td>
<td>509.8±88.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rₑₑ (ohm)</td>
<td>Pre-feed</td>
<td>1440.2±412.1</td>
<td>1074.2 - 2564.9</td>
<td>1942.9±536.6</td>
<td>1010.4 - 3324.7</td>
<td>2140.5±439.4</td>
<td>1529.2 - 3115.9</td>
<td>2177.3±692.1</td>
<td>1335.1 - 3779.0</td>
<td>1913.5±571.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Post-feed</td>
<td>1497.1±567.0</td>
<td>704.1 - 2814.2</td>
<td>2349.8±1321.6</td>
<td>1402.8 - 7402.8</td>
<td>2139.8±824.6</td>
<td>622.0 - 4107.9</td>
<td>2257.4±930.8</td>
<td>1462.4 - 4450.0</td>
<td>2069.0±1028.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>L¹/Rₑₑ</td>
<td>Pre-feed</td>
<td>4.98±0.66</td>
<td>3.89 - 5.76</td>
<td>5.77±0.80</td>
<td>4.41 - 7.38</td>
<td>7.50±0.92</td>
<td>5.95 - 8.82</td>
<td>7.75±0.63</td>
<td>6.86 - 8.63</td>
<td>6.35±1.34</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Post-feed</td>
<td>5.03±0.81</td>
<td>3.52 - 6.42</td>
<td>5.82±0.78</td>
<td>4.29 - 7.27</td>
<td>7.50±1.00</td>
<td>5.84 - 9.07</td>
<td>7.86±0.66</td>
<td>6.95 - 8.83</td>
<td>6.33±1.38</td>
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<tr>
<td></td>
<td></td>
<td>L¹/Rₑₑ</td>
<td>Pre-feed</td>
<td>5.34±0.74</td>
<td>4.06 - 6.35</td>
<td>6.16±0.90</td>
<td>4.61 - 7.90</td>
<td>7.94±1.00</td>
<td>6.50 - 9.49</td>
<td>8.25±0.72</td>
<td>7.29 - 9.12</td>
<td>6.75±1.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Post-feed</td>
<td>5.40±0.95</td>
<td>3.79 - 6.98</td>
<td>6.16±0.86</td>
<td>4.48 - 7.69</td>
<td>7.95±1.11</td>
<td>6.09 - 9.52</td>
<td>8.37±0.75</td>
<td>7.29 - 9.38</td>
<td>6.73±1.49</td>
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<td></td>
<td></td>
<td>L¹/Rₑₑ</td>
<td>Pre-feed</td>
<td>7.42±1.17</td>
<td>5.26 - 9.10</td>
<td>8.10±1.43</td>
<td>6.50 - 10.84</td>
<td>9.99±1.42</td>
<td>7.51 - 12.21</td>
<td>10.43±1.24</td>
<td>8.59 - 11.89</td>
<td>8.79±1.77</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Post-feed</td>
<td>7.57±1.70</td>
<td>5.03 - 11.10</td>
<td>7.90±1.23</td>
<td>5.41 - 9.89</td>
<td>10.36±2.39</td>
<td>7.02 - 16.72</td>
<td>10.53±1.32</td>
<td>8.18 - 11.81</td>
<td>8.81±2.13</td>
</tr>
<tr>
<td></td>
<td></td>
<td>L¹/Rₑₑ</td>
<td>Pre-feed</td>
<td>2.43±0.58</td>
<td>1.37 - 3.34</td>
<td>2.33±0.69</td>
<td>1.19 - 3.93</td>
<td>2.49±0.57</td>
<td>1.56 - 3.42</td>
<td>2.68±0.74</td>
<td>1.43 - 3.99</td>
<td>2.45±0.64</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Post-feed</td>
<td>2.54±0.99</td>
<td>1.25 - 5.11</td>
<td>2.08±0.63</td>
<td>0.56 - 3.15</td>
<td>2.86±1.62</td>
<td>1.19 - 8.22</td>
<td>2.67±0.78</td>
<td>1.16 - 3.59</td>
<td>2.48±1.08</td>
</tr>
</tbody>
</table>

**Abbreviations:** F - female; icw - intracellular water; inf - infinity; L - length (cm); L¹/R - impedance index; M - male; R - resistance (ohm). Subjects' characteristics presented as mean ± SD and range for total group (n=62) and grouped by infant age.
Table 2.2 Variability (bias and 95% CIs) and repeatability (CV, CI and ICC) of resistance measurements.

<table>
<thead>
<tr>
<th>R</th>
<th>CV within participant s</th>
<th>ICC agreement (95% CI)</th>
<th>CV within participants</th>
<th>ICC agreement (95% CI)</th>
<th>Bias (95% CI)</th>
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<tbody>
<tr>
<td>R₀</td>
<td>1.3</td>
<td>0.983 (0.966, 0.992)</td>
<td>2.4</td>
<td>0.942 (0.845, 0.979)</td>
<td>0.42 (-14.9, 15.8)</td>
</tr>
<tr>
<td>R₅₀</td>
<td>1.5</td>
<td>0.981 (0.961, 0.991)</td>
<td>2.5</td>
<td>0.945 (0.852, 0.980)</td>
<td>2.92 (-12.5, 18.3)</td>
</tr>
<tr>
<td>R₉₀</td>
<td>2.8</td>
<td>0.966 (0.931, 0.983)</td>
<td>3.9</td>
<td>0.931 (0.819, 0.975)</td>
<td>6.37 (-9.0, 21.7)</td>
</tr>
<tr>
<td>Rₒw</td>
<td>12.6</td>
<td>0.864 (0.739, 0.931)</td>
<td>14.9</td>
<td>0.805 (0.533, 0.927)</td>
<td>193.3 (178.0, 208.7)</td>
</tr>
</tbody>
</table>

Abbreviations: CI - confidence interval; CV - coefficient of variation; ICC - intraclass correlation coefficient; icw - intracellular water; inf - infinity; R - resistance (ohm). Limits of agreement are mean ± (1.96 x SD) for 95% CI.

2.4.2 Resistance pre-feed/post-feed

Changes in resistance post-feed – pre-feed (ΔR) were small (median [IQR]: R₀ 3.7 [-14.8, 14.3]; R₅₀ 0.3 [-10.4, 15.0]; R₉₀ 2.8 [-13.3, 35.5]; Rₒw 20.8 [-98.1, 290.9]) and not significantly different from zero (R₀: p=0.92; R₅₀: p=0.48; R₉₀: p=0.32; Rₒw: p=0.097) (Figures 2.1 and 2.2).
Figure 2.1 Bland-Altman plots examining agreement between pre- and post-feed resistance measures and impedance indices for four different frequencies ($R_0$, $R_{50}$, $R_{inf}$, and $R_{icw}$). The solid horizontal line is the mean difference, and the dotted lines are ±2SD. Data presented for four age groups as individual data points: ●, 2 months; ▲, 5 months; ●, 9 months; and ■, 12 months. Lines are local regression smoothers (LOESS) grouped as per legend: _ _ _, 2 months; _ _ _ , 5 months; _ _ _ , 9 months; and _ _ _ _ , 12 months.
Figure 2.2 Patterns of resistance measures ($R_0$, $R_{50}$, $R_{inf}$ and $R_{icw}$) and impedance indices ($L_2^2/R_0$, $L_2^2/R_{50}$, $L_2^2/R_{inf}$ and $L_2^2/R_{icw}$) pre- and post-feed by milk intake (mL). Lines are local regression smoothers (LOESS) and characters are individual data points grouped as per legend: ▲ and — denote pre-feed; ○ and ---- denote post-feed.
2.4.2.1 Infant age, length and weight

Significant negative univariate associations between ΔR and infant length were seen for R₀ and R₅₀ but not for R_inf or R_icw (R₀: p=0.019; R₅₀: p=0.007; R_inf: p=0.06; R_icw: p=0.44). A similar association was found between ΔR and infant weight for R₅₀ but not for R₀, R_inf or R_icw (R₀: p=0.08; R₅₀: p=0.036; R_inf: p=0.14; R_icw: p=0.39), with greater ΔR seen for shorter/smaller infants. After accounting for these covariates, no significant ΔR was seen for any frequency (Table 2.3).

Pre-feed resistance was highest at 5 months and lowest at 2 months (Table 2.1; Figure 2.3). Significant differences in pre-feed resistances were seen for R_inf between 2 and 5 months (p=0.015) and for R_icw between 2 and 5 (p=0.031), 2 and 9 (p=0.002), and 2 and 12 months (p=0.007). No other differences were significant (p>0.06).

Age was not significant in any model (R₀: p>0.06; R₅₀: p>0.09; R_inf: p>0.43; R_icw: p>0.56). Although resistance pre-/post-feed did not change consistently with infant age, a change in the trend from positive to negative was seen.

2.4.2.2 Impedance index

Changes in impedance indices post-feed – pre-feed (ΔL²/R) also were small (median [IQR]: L²/R₀ 0.04 [-1.11, 0.16]; L²/R₅₀ 0.00 [-0.13, 0.12]; L²/R_inf 0.06 [-0.51, 0.23]; L²/R_icw -0.03 [-0.38, 0.16]) and not significantly different from zero (L²/R₀: p=0.48; L²/R₅₀: p=0.79; L²/R_inf: p=0.86; L²/R_icw: p=0.78) (Figures 2.1 and 2.2).

Age was not significant in any model (L²/R₀: p>0.09; L²/R₅₀: p>0.14; L²/R_inf: p>0.33; L²/R_icw: p>0.31).

Pre-feed impedance indices were lowest at 2 and highest at 12 months, with the exception of L²/R_icw (Table 2.1; Figure 2.3). L²/R_icw was not significantly different at any time point (p>0.52). All four indices were not significantly different between 9 and 12 months (p>0.52). L²/R₀ (p=0.026) and L²/R₅₀ (p=0.041) were significantly different between 2 and 5 months, with the rest of the indices being significantly different between all other time points (p<0.001).

2.4.2.3 Milk intake

All MI were within the normal range (Kent et al. 2006). Compared to the overall sample mean, 12 month-old infants took smaller volumes (p=0.022, Table 2.1). There was no association between MI and both ΔR and ΔL²/R (Table 2.3). In the patterns of
resistance measures by MI, a split between pre- and post-feed was observed only for volumes higher than 150 mL (Figure 2.2).

![Line plots showing pattern by age and relationship between pre- and post-feed resistance measures and impedance indices for four different frequencies (R₀, R₅₀, R₉₀ and Rₑₓ) within an infant (individual data points).](Image)

**Figure 2.3** Line plots showing pattern by age and relationship between pre- and post-feed resistance measures and impedance indices for four different frequencies (R₀, R₅₀, R₉₀ and Rₑₓ) within an infant (individual data points).

### 2.4.2.4 Feed duration

Feed duration as a proxy measure of time interval between BIS measurements was significantly longer in 2-month-old infants ($p=0.017$, Table 2.1). There was no association between feed duration and both $\Delta R$ and $\Delta L²/R$ (Table 2.3).

### 2.4.2.5 Free-water change

There was no association between free-water change and both $\Delta R$ and $\Delta L²/R$ (Table 2.3).
2.4.2.6 Stomach and bladder volume

There was no association between both $\Delta R$ and $\Delta L^2/R$ and pre-feed stomach volume ($R_0$: $p=0.68$; $R_{50}$: $p=0.68$; $R_{\text{inf}}$: $p=0.91$; $R_{\text{icw}}$: $p=0.66$), (L$^2/R_0$: $p=0.85$; L$^2/R_{50}$: $p=0.90$; L$^2/R_{\text{inf}}$: $p=0.88$; L$^2/R_{\text{icw}}$: $p=0.82$) or post-feed stomach volume (Table 2.3).

Univariate associations were seen between pre-feed bladder volume and $\Delta R$ for $R_{50}$, $R_{\text{inf}}$ and $R_{\text{icw}}$ but not for $R_0$ ($R_0$: $p=0.08$; $R_{50}$: $p=0.04$; $R_{\text{inf}}$: $p=0.0006$; $R_{\text{icw}}$: $p=0.030$) and $\Delta L^2/R$ for all four impedance indices (L$^2/R_0$: $p=0.044$; L$^2/R_{50}$: $p=0.016$; L$^2/R_{\text{inf}}$: $p<0.001$; L$^2/R_{\text{icw}}$: $p<0.001$).

There were also significant associations between post-feed bladder volume and both $\Delta R$ and $\Delta L^2/R$ for $R_0$ and $R_{50}$, but not for $R_{\text{inf}}$ or $R_{\text{icw}}$ ($R_0$: $p=0.002$; $R_{50}$: $p=0.005$; $R_{\text{inf}}$: $p=0.15$; $R_{\text{icw}}$: $p=0.40$), (L$^2/R_0$: $p=0.0002$; L$^2/R_{50}$: $p=0.002$; L$^2/R_{\text{inf}}$: $p=0.53$; L$^2/R_{\text{icw}}$: $p=0.87$). After accounting for this, there was no significant $\Delta R$ or $\Delta L^2/R$ associated with change in bladder volume ($R_0$: $p=0.70$; $R_{50}$: $p=0.89$; $R_{\text{inf}}$: $p=0.99$; $R_{\text{icw}}$: $p=0.14$), (L$^2/R_0$: $p=0.48$; L$^2/R_{50}$: $p=0.94$; L$^2/R_{\text{inf}}$: $p=0.90$; L$^2/R_{\text{icw}}$: $p=0.64$).

2.4.2.7 Movement

Movements during measurements (pre-feed 2.16±1.1; post-feed 1.95±1.08) were not significantly different ($p=0.09$). The effect of movement post-feed on $\Delta R$ or $\Delta L^2/R$ was independent of pre-feed movement ($R_0$: $p=0.81$; $R_{50}$: $p=0.93$; $R_{\text{inf}}$: $p=0.34$; $R_{\text{icw}}$: $p=0.60$), (L$^2/R_0$: $p=0.57$; L$^2/R_{50}$: $p=0.58$; L$^2/R_{\text{inf}}$: $p=0.09$; L$^2/R_{\text{icw}}$: $p=0.09$) and of infant age ($R_0$: $p=0.28$; $R_{50}$: $p=0.35$; $R_{\text{inf}}$: $p=0.41$; $R_{\text{icw}}$: $p=0.43$), (L$^2/R_0$: $p=0.29$; L$^2/R_{50}$: $p=0.40$; L$^2/R_{\text{inf}}$: $p=0.22$; L$^2/R_{\text{icw}}$: $p=0.22$).
### CHAPTER 2: BIOIMPEDANCE PRE- AND POST-FEED

Table 2.3 Significance (p-values) of selected predictors for difference in resistance measures and impedance indices pre- and post-feed for four predictors accounting for at most one covariate

<table>
<thead>
<tr>
<th>Predictor</th>
<th>Added covariate</th>
<th>Effect of predictor</th>
<th>Effect of covariate</th>
<th>Effect of predictor</th>
<th>Effect of covariate</th>
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<td></td>
<td>R₀</td>
<td></td>
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<td>0.000</td>
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<table>
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<th>Predictor</th>
<th>Added covariate</th>
<th>Effect of predictor</th>
<th>Effect of covariate</th>
<th>Effect of predictor</th>
<th>Effect of covariate</th>
</tr>
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<td>R₅₀</td>
<td></td>
<td>L²/R₅₀</td>
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## CHAPTER 2: BIOIMPEDANCE PRE- AND POST-FEED

### Table 2.3, Continued

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<th>Effect of predictor</th>
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</tbody>
</table>
Abbreviations: icw - intracellular water; inf - infinity; L - length (cm); $L^2/R$ - impedance index; NA - not applicable; R - resistance (ohm). Each line in the table represents a separate model. Effects of predictor (added covariate - none) are the univariate regression results. Effects of covariate (length or weight) are results of multivariate models consisting of both predictor and covariate. Coefficients of predictors are changes in resistance difference per each unit more of the predictor after accounting for the added covariate (if any). Similarly, coefficients of covariates (length or weight) are changes in resistance difference associated with longer/heavier infants after accounting for the predictor. Covariates were included in the table if they were significant in at least one model. Length was not included as a covariate in models for impedance indices as it is a component of the formula. Bold text indicates p-values for the significant covariates retained in the final model (n=62.)
2.5 DISCUSSION

Our findings indicate that both resistance measurements and impedance indices pre- and immediately post breastfeeding are not significantly different. Contradictory studies in adults have shown either minimal change in resistance after eating/drinking (Kushner et al. 1996) or a significant increase in resistance after ingestion of liquid (1.2–1.8L) (Khaled et al. 1988). Any changes noted were not immediately after the fluid consumption but 15–20 min after. Similarly, in infant studies, a significant increase in R₀ values was recorded only in a group of 1-week-old infants 30 min after the feed (Sesmero et al. 2005).

The lack of significant effect of breastfeed volumes on resistance values or impedance indices suggests that either pre- or post-feed resistance measurements can be used in calculation of infant TBW, FM or %FM once the validity of these estimates has been established. Further, the lack of significant effects of MI, feed duration, free-water change, movement and post-stomach volume suggests no necessity to account for these during BIS measurements of infants, with the possible exception of infrequent feeders taking >200mL. These data question the sensitivity of BIS for measuring body water volumes and detecting change in these volumes. The maximum milk intake in this study (232 mL) was 2.9% of pre-feed body weight; yet, surprisingly, this magnitude of change was not detectable despite BIS being able to detect change in volume of extracellular water of ~ 40 mL in the adult arm (Czerniec et al. 2010). The explanation may lie in the fact that BIS measurements were performed using the adult wrist-to-ankle protocol. The measured impedance under such electrode arrangement is dominated by the impedance of the small cross-sectional area of arms and legs, whereas the larger cross-sectional area of trunk, where most of the free-water resides, contributes little (Matthie 2008; Kushner et al. 1996). Immediately after a breastfeed, the milk is still located in the trunk. Although trunk impedance, particularly R₀, may decrease post-feed, this is overshadowed by much larger arm and leg impedance. The important consequence of these observations is that breastfeeding would appear to have little effect on either resistance measurements or impedance indices in the breastfed infant.

A limitation of this study was the relatively narrow range of feed volumes with few feeds (1.6%) greater than 200 mL, which is the minimal detectable TBW change in infants estimated using mass balance or tracer dilution studies (Ellis 2002). Although, as noted above, the impedance technique is capable, under appropriate conditions, of detecting small changes in fluid volumes, it is not possible from the present data to
determine whether it can reliably detect changes with greater sensitivity than the 200 mL threshold for tracer or balance studies.

Pragmatically, absence of systematic difference in both resistance and impedance indices pre- and immediately post-feed allows flexibility in the timing of BIS measurements. Post-feed measures could reliably be used rather than pre-feed measures, which is the standard recommendation for performing BIS in adults (Kushner et al. 1996). Such measurements should provide reliable estimates of TBW but not account for gut water volumes. If these are of interest, then focussed impedance measurements of the trunk could be undertaken. As the reproducible electrode location in the trunk is difficult in the small infant, measurements may be performed according to the principle of equipotentials (Cornish et al. 1999), using wrist and ankle electrode locations.

Variability (within participants CV) in all resistance measurements was observed (1.3%, 1.5%, 2.8% and 12.6% for $R_0$, $R_{50}$, $R_{inf}$ and $R_{icw}$, respectively). Although theoretically $R_{inf}$ should provide the best prediction of TBW and FFM, greater variability is seen in this parameter and in $R_{icw}$ (in the present study and those of others (Sesmero et al. 2005; Lingwood et al. 2012)). These errors will contribute to the precision of prediction of TBW. Consequently, prediction may be better performed in this group using resistance measured at 50 kHz as observed in neonates by Lingwood et al. (Lingwood et al. 2012), allowing for measurements to be obtained using simpler single frequency impedance devices. Prediction of TBW may be further improved when BIS data are available by the use of the impedance value at the characteristic frequency, $Z_c$, since at this frequency the ratio of the current flow through the extracellular and intracellular fluids is dependent only on the resistances of these water compartments and not cell membrane capacitance (Cornish et al. 1999; Cornish et al. 1996).

As was expected, infant length and weight were associated with $\Delta R$. One interesting feature of this is that, although greater $\Delta R$ were seen in smaller, shorter and younger infants, $\Delta R$ changed from positive to negative as the infants got longer/older. The observed difference in pre-feed $R_{inf}$ and $R_{icw}$ between 2 and 5 months may reflect a gradual decrease in extracellular fluid, as previously reported (Sesmero et al. 2005) and confirmed by electrolyte and fluid balance studies (Lorenz 1997). Intermediate values at 9 and 12 months could be a reflection of BC changes due to increased mobility. Longitudinal data will better describe changes in resistance measures in the first year of life.

A more accurate knowledge of changes in extracellular fluid volumes of the infant’s stomach and bladder was expected to explain $\Delta R$; however, this was not the
case with no significant effect of free-water change seen. The maximum free-water change of 337 mL (3.3% of pre-feed body weight) was observed but was not the result of the largest MI but rather the redistribution of the fluid in the trunk. Although accounting for bladder volume does not provide any benefit, measurement of MI may be useful. Pre-feed infant weight should be used in calculations to avoid overestimation of the FM while in infrequent feeders pre-feed BIS measurements might be more prudent.

2.6 CONCLUSIONS

In conclusion, measurement of resistance in the breastfeeding neonate is feasible, and reliable impedance data can be obtained. Feed volume did not significantly affect whole body resistance measurements or impedance indices, and consequently pre- or post-breastfeed measurements of resistance can be used interchangeably to improve resistance data collection in infants.
CHAPTER 3

DETERMINANTS OF BODY COMPOSITION IN BREASTFED INFANTS USING BIOIMPEDANCE SPECTROSCOPY AND ULTRASOUND SKINFOLDS - METHODS COMPARISON*

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CHAPTER 3: BODY COMPOSITION OF BREASTFED INFANTS

3.1 ABSTRACT

Accurate, noninvasive, and inexpensive methods are required to measure infant BC. US and BIS have been validated in adults and introduced in pediatric populations. The aim of this study was to evaluate the performance of both methods in determining %FM in breastfed infants. %FM of 2, 5, 9 and 12 month-old healthy, breastfed term infants \((n=58)\) was calculated using BIS-derived TBW equations and skinfold equations then compared to reference models. Skinfolds were measured with US at two and four sites (biceps, suprailiac and/or triceps, and subscapular). %FM differed widely within and between methods, with the degree of variation affected by infant age/sex. Not a single method/equation was consistent with the distributions of appropriate reference values for all age/sex groups. Moderate number of matches with references values (13–24 out of 36) was seen for both types of equations. High number of matches (25–36) was seen for US skinfold-based equations. %FM values calculated from US and BIS were not significantly different \((p = 0.35)\). Both BIS and US are practical for predicting %FM in infants. BIS calculations are highly dependent upon an appropriate set of validated age-matched equations.
3.2 INTRODUCTION

BC in early life plays an important role in the programming of long-term health outcomes including obesity, type II diabetes and cardiovascular disease (Wells et al. 2007). The majority of large studies reporting an association of infant development with disease later in life use anthropometric measurements or BMI to monitor growth that provide limited indices of adiposity as they fail to reflect body shape, fat distribution, and density.

The number of techniques available to assess infant BC is increasing. These measures have better accuracy than anthropometric measurements, but may be either time consuming, expensive, and invasive, expose infants to low levels of radiation (DXA and CT), require the infant to be restrained (MRI) or have weight restriction (ADP).

BIS is noninvasive and often used to estimate BC due to its ease of application and low cost. BIS measures the electrical impedance, or opposition to flow through the body tissues of a small harmless electric current. The measured impedance, corrected for the reactive component, known as electrical resistance is inversely and quantitatively related to the volume of conductive compartment which can be used to calculate TBW and estimate FFM and FM (Lukaski et al. 1985). BIS provides reliable estimates of TBW in adults and equations have been developed for children and infants (Ferreira et al. 2004; Lingwood et al. 2012). While in very young infants (<5 months) BIS is associated with poorer predictive performance that could be due to the higher conductivity of the infant’s adipose tissue with its increased vascularization and water content, and rapidly changing hydration status caused by feeding patterns (Sesmero et al. 2005; Lingwood et al. 2012; Collins et al. 2013), small bias indicates that BIS is suitable for comparison between groups and for longitudinal studies (Lingwood et al. 2012).

US presents an alternative noninvasive and inexpensive technique to study infant BC. The US image is created by high frequency waves, which are attenuated during travel through tissue and reflected back to transducer (Chan et al. 2011). US can be used to measure depth and area of adipose and muscle tissue. Serial measurements have been made to evaluate the growth and development of term and preterm infant and to assess changes in BC in both groups (Ahmad et al. 2010; McLeod et al. 2013). Tracking BC with US is sensitive enough to detect HM macronutrient-related changes in adipose and muscle tissue accrued at the measurement sites in preterm infants (McLeod et al. 2013).
A modification of this method was utilized to measure subcutaneous tissue thickness (SCTT) in term infants in place of traditional calipers. Calipers may disturb the infant (Petersen et al. 1995) and mother and common issues such as movement and infant skin compressibility affect reliability (Ulbricht et al. 2012). US offers a pain-free, more consistent solution and has been validated in adults against DXA (Pineau et al. 2007), and utilized in assessment of BC of adults, adolescents and infants (Petersen et al. 1995).

While all BC measuring techniques have inherent limitations when used in infants and children, the combination of anthropometric measurements with US and/or BIS may offer relatively accurate, safe and noninvasive assessment of BC, making them an attractive research tool for this population. The aim of this study was to compare these BIS and US assessments of BC of healthy term breastfed infants using a cross-sectional approach to determine the accuracy and feasibility of both methods over a wide range of infant body masses.

3.3 METHODS

3.3.1 Subjects

Full term healthy breastfed infants (n=58; 31 male and 27 female) of English speaking (predominantly Caucasian) mothers were recruited from the community, primarily from the Australian Breastfeeding Association. Inclusion criteria were: singletons; gestational age \( \geq 37 \) weeks; fully breastfed at 2 and 5 months and breastfed at the time of the study. Exclusion criteria were: infant health issues that could potentially influence growth and development and low maternal milk supply. Infants were measured once at either 2, 5, 9 or 12 months.

All mothers provided written informed consent to participate in the study, which was approved by The University of Western Australia, Human Research Ethics Committee.

3.3.2 Design

3.3.2.1 Study session

At each study session anthropometric (weight and length) and BIS measurements were taken pre-feed; US skinfold measurements were taken post-feed. Where pre-feed BIS were impractical, post-feed measurements were taken (Gridneva et al. 2016a). Clothing
was removed except for a dry diaper and a singlet.

3.3.2.2 Anthropometric measurements
A single weight and length measurement was performed on each infant. Infant’s weight was determined by weighing before breastfeeding using Medela Electronic Baby Weigh Scales (±2.0 g; Medela AG, Switzerland). Infant crown-heel length was measured once to the nearest 0.1 cm using nonstretch tape and headpiece and footpiece both applied perpendicular to the hard surface.

3.3.2.3 BIS measurements
Whole body bioimpedance (wrist to ankle) was measured using battery-operated Impedimed SFB7 bioelectrical impedance analyzer (ImpediMed, Brisbane, Queensland, Australia). This is a single channel, tetra-polar bioelectrical impedance spectrometer device capable of measuring reactance and resistance at 256 logarithmically spaced frequencies between 3 and 1,000 kHz. Before each session, the external calibration of the instrument was tested with a calibration RRC Test Cell (ImpediMed).

Infants were wearing a dry diaper and a singlet at the time of measurement. After wiping electrode sites with isopropyl alcohol, single Ag-AgCl gel electrodes (ImpediMed) were applied to the skin on the right hand and foot. Two distal current drive electrodes were placed on the dorsal surface of the hand and the foot, at the metacarpal-phalangeal and metatarsal-phalangeal joints; and two voltage sense electrodes were placed 3cm proximal to the current electrodes. 3M Micropore surgical tape (3M Health Care, Neuss, Germany) was used to secure the electrodes in position. Electrodes were left on the limbs for the second series of measurements after the breastfeed. No direct contact was made with the infant’s skin during the measurements and insulating material (cloth) was used to ensure no contact occurred between the infant’s limbs or between the infant and the mother’s hands. Series of measurements (pre- or post-feed) consisted of 10–50 consecutive measurements taken within 1–2 min with infants in supine position on a nonconductive surface.

Collected data were transferred to a computer and analyzed by fitting the measured resistance and reactance at each frequency to a Cole-Cole plot, using Bioimp version 5.2.2.0 software (ImpediMed). All raw data were visually examined and measurements were analyzed with settings customized for infants to ensure goodness of fit as assessed by the standard error of the estimate as per Lingwood *et al.* (Lingwood *et al.* 2012): Td correction off; identification of the curve that most closely resembles a semicircle and setting the frequency range to cover this portion of the curve
(upper/lower frequency limits of 1,000/12kHz); rejection limit of 1%; achieving standard error of the estimate <1.0 and then applying Td correction of 0). Values of resistance (ohm) at frequency of 50 kHz ($R_{50}$) were determined from the curve of best fit and averaged for analysis purposes. This frequency was chosen as $R_{50}$ is commonly used to predict TBW in empirically-derived prediction equations (Matthie 2008).

3.3.2.3 US skinfold measurements

Single measurements of SCTT were taken on the left side of the body with minimal compression. US scans of four anatomical sites (biceps, subscapular, suprailliac and triceps) were performed using the Aplio XG (Toshiba, Tokyo, Japan) machine and a high resolution PLT-1204BX 14-8 MHz transducer (McLeod et al. 2013). Sterile water-based Aquasonic 100 US transmission gel (Parker Laboratories, Fairfield, NJ) was placed between the probe and the infant’s skin to ensure penetration of the US beam, enhance imaging and to minimize tissue compression. The SCTT (skin thickness and the skin-fat interface to fat-muscle interface distance that were easily defined on the scan) was measured directly from images on the screen using electronic callipers (Pineau et al. 2007) (Figure 3.1). All of the measurements were performed by one experienced sonographer (DG) with previously reported high intraobserver reliability (McLeod et al. 2013).

US skinfold measurements were doubled for the use in skinfold equations developed for SCTT measurement with skinfold callipers (Ulbricht et al. 2012).

3.3.2.4 Equations and reference models

Equations and reference models were sourced from the literature. Equations for calculations of TBW, FFM and %FM using resistance ($R_{50}$, $\Omega$) and for calculations of BD and %FM using ST (mm) measurements are presented in Table 3.1. The %FM value calculated with six BIS-based and seven skinfold-based equations were compared with eight reference values data sets for %FM, measured by various reference techniques (Butte et al. 2000a; Gale et al. 2012; Carberry et al. 2010; Gilchrist 2007; Bellu et al. 1997; Fomon et al. 2002; Roggero et al. 2010; Fields et al. 2011).

All of the reference studies with the exception of Fomon (Fomon et al. 2002) state that either all or a proportion of infants were breastfed. In Butte’s study (Butte et al. 2000a; Gale et al. 2012) infants ($n=40$) were exclusively breastfed for 4 months with only 38% still breastfed at 12 months. Infants in Bellu’s study (Bellu et al. 1997) of 12 months old infants ($n=26$), were breastfed for at least 6 months and were not at the time of the study. Infants in Roggero (Roggero et al. 2010) ($n=59$), Fields (Fields et al. 2011)
(n=160) and Gilchrist (Gilchrist 2007) studies (n=80) were exclusively breastfed from birth to 6 months at which point all three studies had ended. While infants (n=30) in Carberry et al. study (Carberry et al. 2010) were predominantly or exclusively breastfed, they were followed up to 4.5 months only.

![Image of infant triceps (upper arm) with skin, subcutaneous fat, and muscle areas defined. The boundary between muscle and fat is marked by a bright line (fascia). A half skinfold measure is equal to the skin thickness plus the subcutaneous fat thickness and needs to be doubled for use in skinfold-based equation. Scale is presented on the left side of the scan with distance between 0 and 1 =10mm.]

**Figure 3.1** US image of infant triceps (upper arm) with skin, subcutaneous fat, and muscle areas defined. The boundary between muscle and fat is marked by a bright line (fascia). A half skinfold measure is equal to the skin thickness plus the subcutaneous fat thickness and needs to be doubled for use in skinfold-based equation. Scale is presented on the left side of the scan with distance between 0 and 1 =10mm.

The broad variation in time points in the reference models made it difficult to compare both the references’ and our time points. Combining the available references ensured that there is sufficient BC data to establish mean±SD values for several age/sex
groups up to 12 months.

Prediction equations were selected according to the following criteria: closest age match and predominantly Caucasian population. While it is recommended not to extrapolate from equations for older paediatric subjects to the early infant period, which is associated with chemical immaturity, it is not always possible to adapt even age-matched equations with the change in methodology as in case of using US to measure skinfolds. Therefore several equations were tested and few were chosen for further analysis. Those equations, not chosen produced erroneous results upon calculation or were discarded because of nonmatching variables.

Chosen BIA-based equations for TBW were established in infants and children of various ages (Lingwood et al. 2012; Fjeld et al. 1990; Bocage 1988; Kushner et al. 1992) then validated in healthy infants (Lingwood et al. 2012), as well as in well and malnourished children (Fjeld et al. 1990). Predicted TBW was converted to FFM using Butte et al. (Butte et al. 2000a) age-appropriate hydration factors and %FM was further calculated.

Chosen equations based on caliper skinfolds for %FM and BD were established for use in children and adolescents (Slaughter et al. 1988; Brook 1971; Durnin et al. 1967; Johnston et al. 1988); and evaluated in term and preterm infants (Schmelzle et al. 2002). Predicted BD was converted to %FM using both Siri (Siri 1961) and Lohman (Lohman 1984) equations. Details of the equations are given in Table 3.1.

3.3.2.5 Statistical analyses

Statistical analysis was carried out using R-software package (version 2.9.0 for Mac OS X; R Foundation for Statistical Computing, Vienna, Austria). The R packages nlme (version 3.1-96), multcomp (Hothorn et al. 2008) and ggplot2 (Wickham 2009) were used for linear mixed effect models, Tukey’s all pair comparisons, and data exploration and graphics respectively. Descriptive statistics are reported as mean ± SD (range), model parameters as estimate (95% CI). p-values <0.05 were considered statistically significant except where an adjustment for multiple comparisons false discovery rate was performed (Curran-Everett 2000). Analysis was carried out as overall, by age/sex groups and by sex within age.

Infants were measured once at either 2, 5, 9, and 12 months as they were recruited for a larger study. Forty participants, 10 in each age group (to achieve a wider body mass spread), allowed for detection of an effect size of 0.5. When 40 infants had been
## Table 3.1. Published equations used in the study.

<table>
<thead>
<tr>
<th>First author, year</th>
<th>Equation(^a)</th>
<th>Age at measurement</th>
<th>Study groups: no. of infants</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bioelectrical impedance</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lingwood, 2012</td>
<td>(FFM = 1.248 + 0.584W - 0.142S + \frac{0.002L^2}{R_{50}})</td>
<td>6 week old</td>
<td>54</td>
</tr>
<tr>
<td></td>
<td>(FFM = 1.458 + 0.498W - 0.197S + \frac{0.067L^2}{R_{50}})</td>
<td>3 month old</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>(FFM = 2.203 + 0.334W - 0.361S + \frac{0.188L^2}{R_{50}})</td>
<td>4.5 month old</td>
<td>53</td>
</tr>
<tr>
<td>Bocage, 1988</td>
<td>(TBW = \left(0.418W + \frac{1936}{R_{50}} + 0.8649\right) L / 100)</td>
<td>3 - 18 months old</td>
<td>NA</td>
</tr>
<tr>
<td>Fjeld, 1990</td>
<td>(TBW = 0.76 + \frac{0.18L^2}{R_{50}} + 0.39W)</td>
<td>3 - 30 months old</td>
<td>30</td>
</tr>
<tr>
<td>Kushner, 1992</td>
<td>(TBW = \frac{0.593L^2}{R_{50}} + 0.065W + 0.04)</td>
<td>Pre-pubertal; pre-school; premature infants</td>
<td>37; 44; 32</td>
</tr>
</tbody>
</table>

\(FFM = TBW/HF\)
\(\%FM = 100(W - FFM)/W\)
Table 3.1 Continued.

<table>
<thead>
<tr>
<th>First author, year</th>
<th>Equation*</th>
<th>Age at measurement</th>
<th>Study groups: no. of infants</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Slaughter, 1988</strong></td>
<td>Male %FM = 1.21Σ - 0.008Σ^2 - 1.7</td>
<td>8 - 17 years old</td>
<td>66</td>
</tr>
<tr>
<td></td>
<td>Female %FM = 1.33Σ - 0.013Σ^2 - 2.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Durnin, 1967</strong></td>
<td>Male d = 1.1533 - 0.0643 log(Σ SFT)</td>
<td>12 - 16 years old</td>
<td>86</td>
</tr>
<tr>
<td></td>
<td>Female d = 1.1359 - 0.0598 log(Σ SFT)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Johnston, 1988</strong></td>
<td>Male d = 1.166 - 0.07 log(Σ SFT)</td>
<td>8 - 14 years old</td>
<td>308</td>
</tr>
<tr>
<td></td>
<td>Female d = 1.144 - 0.06 log(Σ SFT)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Brook, 1971</strong></td>
<td>Male d = 1.1690 - 0.0788 log(Σ SFT)</td>
<td>1 - 11 years old</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>Female d = 1.2063 - 0.0999 log(Σ SFT)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Siri, 1961</strong></td>
<td>%FM = 100(4.55/d - 4.5)</td>
<td>Adult cadavers</td>
<td>NA</td>
</tr>
<tr>
<td><strong>Lohman, 1984</strong></td>
<td>%FM = 100(2.89/d - 4.89)</td>
<td>Pre-pubertal and pubertal children</td>
<td>NA</td>
</tr>
</tbody>
</table>
Published equations used in the study for predicting body density, fat-free mass, total body water or percentage fat mass using bioelectrical impedance and skinfolds in pediatric subjects. Abbreviations and symbols in formulas: d – body density (kg/L); FFM – fat-free mass (kg); FM – fat mass; %FM – percentage fat mass (%); HF – age-appropriate hydration factors calculated (Butte et al. 2000a) data; L – body length (cm); NA – not available in the reference; $R_{50}$ - impedance variable ($\Omega$); S – sex variable (male=1, female=2); $\sum$ - sum of two skinfolds, subscapular and triceps (mm); $\sum SFT$ – sum of four skinfolds, biceps, subscapular, suprailliac and triceps (mm); TBW – total body water (kg); W – weight, (kg).
recruited, an uneven gender split caused us to extend recruitment so as to achieve similar group sizes and gender balance. Using $\alpha=0.05$, final recruitment of 58 participants gave the study power of 0.94 to detect an effect size of 0.3 (Bausell et al. 2006).

One-sample Kolmogorov-Smirnov tests (Massey 1951; Kolmogorov-Smirnov 1951) were used to compare the %FM data for each of the predictors with each of the eight reference distributions. Each age/sex group was compared with the most appropriate reference distributions. If no sex specific reference was available, pooled data were used.

Where no SD was available in the reference model the SD was calculated as an average of SD of the given age/sex groups of other reference models. The Kolmogorov-Smirnov test requires that there be no ties in the data; 0.01 was added to one value when ties existed. $p$-values are not exact in those cases. Owing to the large number of comparisons, an adjustment for multiple comparisons false discovery rate was performed (Curran-Everett 2000). The calculated values were considered to be not significantly different from the reference values at $p>0.018$ for US skinfold measurements and $p>0.024$ for BIS. The number of matches between reference and sample distributions was classified as low (0–12 of possible 36 matches), moderate (13–24 matches) or high (25–36 matches).

Raw data ($n=58$) was used for calculating averages to compare overall equations performances in the whole group. General linear hypothesis test (Tukey’s all pair comparisons) was used to check for systematic differences between the equations. Some overall analyses were repeated after removing two equations with $<1/3$ of matches to the references.

CV for each measurement were calculated from infants with data for all 13 equations ($n=45$) omitting infants missing four skinfolds measurements ($n=13$).

Linear mixed effects models with random intercept per participant were used to determine whether %FM measurements differed systematically by equation and infant sex and/or age group. Where there were more than two levels of categorical variable, Tukey’s all pair comparisons was used to determine which levels differed. Possible sex differences between methods were tested with interaction between sex and equation.
3.4 RESULTS

3.4.1 Subjects

Infants were two (2.06±0.17, n=13), five (5.08±0.22, n=14), nine (9.17±0.35, n=17) or 12 (12.34±0.23, n=14) months of age at the time of measurement. Of the 58 infants, 54 were measured with BIS pre-feed and 4 post-feed (n=58). At the earlier stage of data collection only two skinfolds (triceps and subscapular) were measured with US, resulting in two skinfold measures for n=58 and four skinfolds measures for n=45. Subjects’ characteristics are presented in Table 3.2.

Two 2 m-old male infants were excluded from the study due to confirmed low maternal milk supply that could affect infant BC. One infant at the time of the visit was in second centile for weight and fifth for length according to WHO growth charts and had plateaued for 2 weeks, later dropping to first centile for weight. The second infant was in fifth centile for both weight and length.
### Table 3.2 Subject characteristics presented as total and grouped by the infant age.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>2</th>
<th>5</th>
<th>9</th>
<th>12</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SD (Range)</td>
<td>Mean ± SD (Range)</td>
<td>Mean ± SD (Range)</td>
<td>Mean ± SD (Range)</td>
<td>Mean ± SD (Range)</td>
</tr>
<tr>
<td>Sex (Male/Female)</td>
<td>7M/6F</td>
<td>7M/7F</td>
<td>10M/7F</td>
<td>7M/7F</td>
<td>31M/27F</td>
</tr>
<tr>
<td>Age (months)</td>
<td>2.06 ± 0.17 (^a) (1.80 - 2.37)</td>
<td>5.08 ± 0.22 (4.60 - 5.37)</td>
<td>9.17 ± 0.35 (8.13 - 9.77)</td>
<td>12.34 ± 0.23 (11.90 - 12.67)</td>
<td>7.35 ± 3.85 (1.80 - 12.67)</td>
</tr>
<tr>
<td>Length (cm)</td>
<td>58.4 ± 1.9 (54.5 - 61.3)</td>
<td>64.0 ± 2.2 (60.5 - 68.5)</td>
<td>71.2 ± 1.9 (68.0 - 74.5)</td>
<td>74.1 ± 2.6 (68.5 - 78.5)</td>
<td>67.28 ± 6.41 (54.5 - 78.5)</td>
</tr>
<tr>
<td>Body weight (kg)</td>
<td>5.458 ± 0.581 (4.420 - 6.335)</td>
<td>7.170 ± 1.088 (5.674 - 9.510)</td>
<td>8.997 ± 0.779 (7.683 - 10.605)</td>
<td>9.604 ± 0.968 (7.170 - 11.085)</td>
<td>7.909 ± 1.813 (4.420 - 11.085)</td>
</tr>
<tr>
<td>R50 (Ω)(^b)</td>
<td>646.8 ± 105.5 (516.1 - 862.7)</td>
<td>702.3 ± 93.7 (584.1 - 855.6)</td>
<td>685.5 ± 85.9 (582.8 - 811.5)</td>
<td>652.0 ± 45.2 (576.4 - 727.0)</td>
<td>672.8 ± 86.1 (516.1 - 862.7)</td>
</tr>
<tr>
<td>Biceps skinfold (mm)</td>
<td>16.0 ± 5.1 (8.8 - 23.8)</td>
<td>18.2 ± 6.4 (8.8 - 29.2)</td>
<td>16.3 ± 5.9 (7.4 - 26.8)</td>
<td>16.2 ± 4.7 (8.0 - 24.8)</td>
<td>16.7 ± 5.5 (7.4 - 29.2)</td>
</tr>
<tr>
<td>Triceps skinfold (mm)</td>
<td>15.6 ± 3.1 (10.6 - 20.2)</td>
<td>18.4 ± 5.2 (13.0 - 27.8)</td>
<td>18.7 ± 4.3 (11.2 - 25.8)</td>
<td>19.8 ± 6.4 (9.8 - 30.6)</td>
<td>18.2 ± 5.0 (9.8 - 30.6)</td>
</tr>
<tr>
<td>Subscapular skinfold (mm)</td>
<td>12.2 ± 2.2 (9.2 - 18.0)</td>
<td>11.5 ± 2.8 (6.6 - 16.4)</td>
<td>10.9 ± 3.8 (6.0 - 16.6)</td>
<td>11.4 ± 2.8 (5.4 - 14.8)</td>
<td>11.5 ± 3.0 (5.4 - 18.0)</td>
</tr>
<tr>
<td>Suprailiac skinfold (mm)</td>
<td>10.9 ± 3.5 (7.0 - 19.8)</td>
<td>14.6 ± 4.7 (10.2 - 24.6)</td>
<td>13.5 ± 4.0 (9.0 - 20.6)</td>
<td>11.3 ± 4.6 (4.8 - 19.6)</td>
<td>12.4 ± 4.3 (4.8 - 24.6)</td>
</tr>
</tbody>
</table>

\(^a\) Data expressed as mean ± SD and range. \(^b\) R50 (Ω)– whole body resistance (ohm) measured at 50 kHz. \(^c\) Skinfolds values presented in the table are double of the measurements that were acquired with the ultrasound.
### 3.4.2 Comparison of sample and reference distribution

Table 3.3 presents the probabilities that the sample distributions were drawn from each of the considered reference distributions. After an adjustment for false discovery rate values were considered to be not significantly different from the reference values at $p > 0.018$ for US skinfold measurements and $p > 0.024$ for BIS. Neither BIS nor US were consistent with distributions from appropriate reference values for all age/sex groups. A moderate number of matches (13–24 matches) with reference values were seen for both types of equations (three BIS-based and two US skinfold-based). High number of matches (25–36 matches) was predominantly seen for US skinfold-based equations (one BIS-based and four US skinfold-based).

### 3.4.3 % Fat Mass calculated with BIS and US

Calculated %FM values displayed wide variation within and between US and BIS, with the degree of variation affected by both infant age and sex (Table 3.4). Distributions of %FM calculated with two skinfolds matched with 27/36 reference values; use of four skinfolds gave between 10 and 30 matches. Figure 3.2 shows comparative performance of all of the equations for each infant. Table 3.5 presents the performance of all BIS and US skinfold equations in the whole group of infants (raw data, $n=58$). The Fjeld (BIS) and Johnston/Lohman (US) equations were excluded from further overall analysis as both were at the lower end of calculated %FM and had the lowest number of matches (<1/3) with the references.

### 3.4.4 Effect of sex on body composition

Overall, %FM was 2.0% (0.013, 3.9) lower in males ($p=0.036$). When the equations were considered separately, significant sex differences were only seen for those based on four US skinfolds ($p<0.001$ for all) with the endpoints of the 95% CIs between 2.00 and 7.08. Brook/Siri ($p=0.051$), Brook/Lohman ($p=0.050$), Slaughter ($p=0.16$) and all BIS equations ($p>0.075$) showed no significant difference in %FM between the sexes (Figure 4.2).
### Table 3.3 Associations between sample and reference distributions (p-values) established with one-sample Kolmogorov-Smirnov test.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Sex/ Age, mo*</th>
<th>Brook/ Lohman</th>
<th>Brook/ Siri</th>
<th>Durmin/ Lohman</th>
<th>Durmin/ Siri</th>
<th>Johnston/ Lohman</th>
<th>Johnston/ Siri</th>
<th>Slaughter</th>
<th>Bocage</th>
<th>Fjeld</th>
<th>Kushner</th>
<th>Lingwood, 6 weeks</th>
<th>Lingwood, 3 months</th>
<th>Lingwood, 4.5 months</th>
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</thead>
<tbody>
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<td>Butte, 2000 (Bf)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>31.5 ± 5.6</td>
<td>F/2</td>
<td>0.003</td>
<td>0.32&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&lt;0.001</td>
<td>0.103</td>
<td>&lt;0.001</td>
<td>0.004</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
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<tr>
<td>30.2 ± 4.0</td>
<td>M/2</td>
<td>&lt;0.001</td>
<td>0.16</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>0.005</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
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<tr>
<td>32.0 ± 4.4</td>
<td>F/5</td>
<td>0.66</td>
<td>0.12</td>
<td>0.050</td>
<td>0.45</td>
<td>0.002</td>
<td>0.17</td>
<td>0.002</td>
<td>0.001</td>
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<td>0.84</td>
<td>&lt;0.001</td>
<td>0.003</td>
<td>0.015</td>
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<tr>
<td>29.1 ± 4.7</td>
<td>M/5</td>
<td>0.28</td>
<td>0.23</td>
<td>&lt;0.001</td>
<td>0.14</td>
<td>&lt;0.001</td>
<td>0.052</td>
<td>0.19</td>
<td>0.065</td>
<td>&lt;0.001</td>
<td>0.75</td>
<td>0.094</td>
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<td>0.51</td>
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<tr>
<td>28.8 ± 5.0</td>
<td>F/9</td>
<td>0.96</td>
<td>0.32</td>
<td>0.15</td>
<td>0.27</td>
<td>0.002</td>
<td>0.49</td>
<td>0.034</td>
<td>0.55</td>
<td>&lt;0.001</td>
<td>0.017</td>
<td>0.022</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
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<tr>
<td>25.7 ± 5.2</td>
<td>M/9</td>
<td>0.52</td>
<td>0.033</td>
<td>0.028</td>
<td>0.66</td>
<td>0.014</td>
<td>0.41</td>
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<td>27.6 ± 4.3</td>
<td>F/12</td>
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<td>0.37</td>
<td>0.36</td>
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<td>0.57</td>
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<td>0.21</td>
<td>0.007</td>
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<td>&lt;0.001</td>
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<tr>
<td>25.6 ± 4.0</td>
<td>M/12</td>
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<td>0.15</td>
<td>&lt;0.001</td>
<td>0.42</td>
<td>&lt;0.001</td>
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<tr>
<td>32.5 ± 6.2</td>
<td>F/2</td>
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<td>&lt;0.001</td>
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<tr>
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<td>0.014</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
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<td>0.008</td>
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<td>&lt;0.001</td>
<td>0.23</td>
<td>0.003</td>
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<td>1.00</td>
<td>0.14</td>
<td>0.37</td>
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<td>0.003</td>
<td>0.004</td>
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<td>25.4 ± 3.8</td>
<td>M/12</td>
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<td>&lt;0.001</td>
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<td>&lt;0.001</td>
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<td>&lt;0.001</td>
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<td>0.002</td>
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<td>&lt;0.001</td>
<td>0.27</td>
<td>0.038</td>
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<td>0.20</td>
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<td>0.084</td>
<td>0.49</td>
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<td>25.0 ± 5.0</td>
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<td>0.013</td>
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<td>0.003</td>
<td>0.23</td>
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<td>0.22</td>
<td>0.005</td>
<td>0.56</td>
<td>0.022</td>
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<td>0.004</td>
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<td>0.61</td>
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<td>&lt;0.001</td>
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<td>0.45</td>
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</tr>
<tr>
<td>M/2</td>
<td>24.7 ± 4.0</td>
<td>0.27</td>
<td>0.006</td>
<td>&lt;0.001</td>
<td>0.37</td>
<td>&lt;0.001</td>
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<td>0.94</td>
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<td>&lt;0.001</td>
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<td>27.5 ± 3.1</td>
<td>0.10</td>
<td>0.021</td>
<td>&lt;0.001</td>
<td>0.19</td>
<td>&lt;0.001</td>
<td>0.086</td>
<td>0.25</td>
<td>0.13</td>
<td>&lt;0.001</td>
<td>0.42</td>
<td>0.85</td>
<td>0.59</td>
</tr>
<tr>
<td>Both/2</td>
<td>23.0 ± 4.3</td>
<td>0.078</td>
<td>&lt;0.001</td>
<td>0.40</td>
<td>0.007</td>
<td>0.003</td>
<td>0.067</td>
<td>0.13</td>
<td>0.001</td>
<td>&lt;0.001</td>
<td>0.043</td>
<td>0.076</td>
<td>0.11</td>
</tr>
<tr>
<td>Both/5</td>
<td>28.3 ± 4.1</td>
<td>0.76</td>
<td>0.029</td>
<td>0.021</td>
<td>0.97</td>
<td>&lt;0.001</td>
<td>0.56</td>
<td>0.13</td>
<td>0.601</td>
<td>&lt;0.001</td>
<td>0.12</td>
<td>0.020</td>
<td>0.38</td>
</tr>
<tr>
<td>Both/5</td>
<td>27.8 ± 4.5</td>
<td>0.95</td>
<td>0.011</td>
<td>0.058</td>
<td>0.83</td>
<td>&lt;0.001</td>
<td>0.64</td>
<td>0.33</td>
<td>0.10</td>
<td>&lt;0.001</td>
<td>0.07</td>
<td>0.050</td>
<td>0.28</td>
</tr>
<tr>
<td>Both/12</td>
<td>23.8 ± 3.1</td>
<td>0.14</td>
<td>&lt;0.001</td>
<td>0.11</td>
<td>0.018</td>
<td>0.001</td>
<td>0.15</td>
<td>0.012</td>
<td>0.43</td>
<td>&lt;0.001</td>
<td>0.13</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Notes:
- Time points presented here are time of measurements performed in this study and correspond with time points of most of the references with the exception of Butte, where infants were measured not at 2 and 5, but at 3 and 6 months.
- Calculations values were considered not significantly different (bold font) from the references values at p>0.018 for ultrasound skinfold measurements and p>0.024 for bioelectrical spectroscopy after adjusting for false discovery rate (Kolmogorov-Smirnov 1951).
- B – breastfed; BF – breastfed and formula-fed; BIS – bioelectrical impedance spectroscopy; %FM – percentage fat mass (%); US/2SF – ultrasound with two skinfolds; US/4SF – ultrasound with four skinfolds.
Table 3.4 Percentage fat mass of breastfed infants aged 2 to 12 months calculated with ultrasound and bioimpedance spectroscopy equations.

<table>
<thead>
<tr>
<th>Equations</th>
<th>%FM&lt;sup&gt;a&lt;/sup&gt; at 2 months</th>
<th>%FM at 5 months</th>
<th>%FM at 9 months</th>
<th>%FM at 12 months</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Female (n=6)</td>
<td>Male (n=7)</td>
<td>Female (n=7)</td>
<td>Male (n=7)</td>
</tr>
<tr>
<td>Ultrasound (2 skinfolds)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Slaughter&lt;sup&gt;b&lt;/sup&gt;</td>
<td>23.4±2.5&lt;sup&gt;d&lt;/sup&gt;</td>
<td>25.4±4.0</td>
<td>25.4±4.6</td>
<td>26.4±3.9</td>
</tr>
<tr>
<td>Reference matches</td>
<td>C,D,E,F</td>
<td>D,E</td>
<td>D,E,F,G</td>
<td>All but B,H</td>
</tr>
<tr>
<td>Ultrasound (4 skinfolds)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reference matches</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Durnin/Siri&lt;sup&gt;c&lt;/sup&gt;</td>
<td>30.2±1.0</td>
<td>24.6±2.0</td>
<td>33.0±1.4</td>
<td>26.3±2.5</td>
</tr>
<tr>
<td>Reference matches</td>
<td>A,B</td>
<td>D,E</td>
<td>All but C,H</td>
<td>All but B,H</td>
</tr>
<tr>
<td>Durnin/Lohman</td>
<td>25.2±1.1</td>
<td>19.2±2.1</td>
<td>28.7±1.5</td>
<td>21.0±2.7</td>
</tr>
<tr>
<td>Johnston/Siri</td>
<td>27.1±1.0</td>
<td>23.3±2.1</td>
<td>29.8±1.4</td>
<td>25.1±2.8</td>
</tr>
<tr>
<td>Reference matches</td>
<td>F</td>
<td>D,E,F</td>
<td>All but H</td>
<td>All but B,H</td>
</tr>
<tr>
<td>Johnston/Lohman</td>
<td>21.8±1.1</td>
<td>17.7±2.3</td>
<td>24.8±1.5</td>
<td>19.7±2.9</td>
</tr>
<tr>
<td>Reference matches</td>
<td>C,D,E</td>
<td>C</td>
<td>C,D</td>
<td>C</td>
</tr>
<tr>
<td>Brook/Siri</td>
<td>31.0±1.7</td>
<td>28.8±2.5</td>
<td>35.8±2.3</td>
<td>30.9±3.2</td>
</tr>
<tr>
<td>Reference matches</td>
<td>A,B</td>
<td>A</td>
<td>A,B,F</td>
<td>A,B,E,F</td>
</tr>
<tr>
<td>Brook/Lohman</td>
<td>26.0±1.8</td>
<td>23.7±2.6</td>
<td>31.2±2.5</td>
<td>25.9±3.4</td>
</tr>
<tr>
<td>Reference matches</td>
<td>D,E,F</td>
<td>D,E,F</td>
<td>All but H</td>
<td>All but H</td>
</tr>
<tr>
<td>Bioimpedance</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reference matches</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lingwood</td>
<td>23.4±2.0</td>
<td>22.0±1.6</td>
<td>26.4±1.4</td>
<td>27.2±1.8</td>
</tr>
<tr>
<td>6 weeks</td>
<td>C,D,E,F</td>
<td>D,F</td>
<td>C,D,G</td>
<td>A,C,D,E,G</td>
</tr>
<tr>
<td>Reference matches</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 months</td>
<td>22.9±2.2</td>
<td>21.3±2.4</td>
<td>28.3±1.6</td>
<td>28.2±2.4</td>
</tr>
<tr>
<td>Reference matches</td>
<td>C,D,E,F</td>
<td>C,D,F</td>
<td>C,D,E,F,G</td>
<td>All but H</td>
</tr>
<tr>
<td>4.5 months</td>
<td>19.8±3.6</td>
<td>15.5±3.6</td>
<td>28.7±2.4</td>
<td>26.9±4.0</td>
</tr>
<tr>
<td>Bocage</td>
<td>19.7±6.0</td>
<td>18.1±4.4</td>
<td>26.5±2.3</td>
<td>25.4±3.1</td>
</tr>
<tr>
<td>Reference matches</td>
<td>C,D,E</td>
<td>C</td>
<td>C,D,E,F,G</td>
<td>All but B,H</td>
</tr>
<tr>
<td>Kushner</td>
<td>22.6±11.1</td>
<td>17.8±7.4</td>
<td>32.8±3.6</td>
<td>28.2±5.1</td>
</tr>
<tr>
<td>Field</td>
<td>13.4±3.4</td>
<td>13.7±2.9</td>
<td>19.3±1.8</td>
<td>20.2±2.8</td>
</tr>
<tr>
<td>Reference matches</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>%FM - percentage fat mass (%). <sup>b</sup>Reference matches (distribution is not significantly different according to one-sample Kolmogorov-Smirnov test) are indicated for eight infant reference models: A – Butte mixed (Butte et al. 2000a); B – Butte breastfed only (Gale et al. 2012); C – (Fomon et al. 2002); D – (Fields et al. 2011); E – (Roggero et al. 2010); F – (Gilchrist 2007); G – (Carberry et al. 2010); H – (Bella et al. 1997). <sup>c</sup>Data expressed as mean ± SD. <sup>d</sup>Percentage fat mass of breastfed boys and girls aged 2, 5, 9 and 12 months calculated with various ultrasound and bioimpedance spectroscopy equations, presented together with the matches to the references.
**Figure 3.2** Calculated percentage fat mass of individual infants (n=58) using (a) ultrasound skinfolds or (b) bioimpedance-based total body water equations ordered from lowest to highest within a method. Each line represents an individual infant and connects all available percentage fat measurements for that infant to illustrate the differences between values calculated with the various equations. Ultrasound four skinfolds: Johnston/Lohman; Durnin/Lohman; Johnston/Siri; Brook/Lohman; Durnin/Siri; Brook/Siri. Ultrasound two skinfolds: Slaughter. Bioelectrical impedance spectroscopy: Fjeld; Bocage; Kushner; Lingwood, 6 weeks; Lingwood, 3 months; Lingwood, 4.5 months. Lines are grouped as: females - black; males - grey; — 2 months-old infants, – – 5 months-old, • • • 9 months-old, ⋅ – ⋅ 12 months-old.
### Table 3.5 Comparative performance of all ultrasound and bioimpedance spectroscopy equations.

<table>
<thead>
<tr>
<th>Equation</th>
<th>Method</th>
<th>%FM&lt;sup&gt;d&lt;/sup&gt; (Mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fjeld, 1990</td>
<td>BIS&lt;sup&gt;a&lt;/sup&gt;</td>
<td>19.0 ± 4.0&lt;sup&gt;b,c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Johnston, 1988/Lohman, 1984</td>
<td>US/4SF&lt;sup&gt;a&lt;/sup&gt;</td>
<td>20.6 ± 3.5&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Durnin, 1967/Lohman, 1984</td>
<td>US/4SF</td>
<td>22.9 ± 4.1&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Bocage, 1988</td>
<td>BIS</td>
<td>23.5 ± 4.7&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Slaughter, 1988</td>
<td>US/2SF&lt;sup&gt;a&lt;/sup&gt;</td>
<td>25.9 ± 4.1&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Johnston, 1988/Siri, 1961</td>
<td>US/4SF</td>
<td>26.0 ± 3.2&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Brook, 1971/Lohman, 1984</td>
<td>US/4SF</td>
<td>26.0 ± 4.3&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Kushner, 1992</td>
<td>BIS</td>
<td>26.5 ± 8.1&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Lingwood, 2012 (6wks)</td>
<td>BIS</td>
<td>27.7 ± 3.7&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Lingwood, 2012 (4.5mo)</td>
<td>BIS</td>
<td>28.1 ± 7.0&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Durnin, 1967/Siri, 1961</td>
<td>US/4SF</td>
<td>28.1 ± 3.8&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Lingwood, 2012 (3mo)</td>
<td>BIS</td>
<td>28.8 ± 4.5&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Brook, 1971/Siri, 1961</td>
<td>US/4SF</td>
<td>31.0 ± 4.0&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>(%); BIS - bioimpedance spectroscopy; %FM - percentage fat mass (%); US/2SF - ultrasound with two skinfolds; US/4SF - ultrasound with four skinfolds.<sup>b</sup>Mean ± SD of infant %FM calculated on the raw data for the whole group using BIS and US equations and presented from the lowest to the highest value.<sup>c</sup>(n=58);<sup>d</sup>(n=45).

### 3.4.5 Effect of age on body composition

Overall 2 months-old infants had significantly lower %FM than the 5, 9 and 12 months-olds (<i>p</i>&lt;0.001) with the differences between this group and each of the other three nearly of the same magnitude (4.6–5.5%) (Figure 3.2). In the detailed analysis, all BIS equations showed overall significant differences for age (<i>p</i>≤0.001) while all US skinfold equations showed no significant differences (<i>p</i>&gt;0.34). Looking at differences between the age groups all three Lingwood equations showed progressive increase in %FM between 2 and 5; 2 and 9; 2 and 12; 5 and 9; 5 and 12 months (<i>p</i>&lt;0.001 for all) but not...
between 9 and 12 months (p>0.88). The Fjeld equation showed a progressive increase in %FM between 2 and 5; 2 and 9; 2 and 12 months (p<0.001), but %FM was not significantly different between 5 and 9; 5 and 12 months; 9 and 12 months (p>0.28). The Bocage equation showed progressive increase in %FM between 2 and 5; 2 and 9 months (p<0.001) and 2 and 12 months (p=0.034), but %FM was not significantly different between 5 and 9; 5 and 12 months; 9 and 12 months (p>0.33). The Kushner equation showed a progressive increase in %FM only between 2 and 5 (p=0.002), and 2 and 9 months (p=0.005), but %FM was not significant between 2 and 12; 5 and 9; 5 and 12; and 9 and 12 months (p≥0.28).

3.4.6 BIS and US comparison

BIS-based equations displayed greater variability than US skinfold-based with calculated CVs (n=45) ranging from 15% to 34% and from 13% to 18% respectively. Overall %FM was 0.34% (-0.37, 1.04) lower when calculated with US skinfold equations (p=0.35) than with BIS. Figure 3.3 illustrates performance of all 13 equations in different age groups in comparison to few references.

3.4.7 Comparisons between the equations

Table 3.6 presents a cluster map of statistical differences between the %FM values calculated with all BIS and US equations and demonstrates that certain groupings exist between the equations independent of the method.
CHAPTER 3: BODY COMPOSITION OF BREASTFED INFANTS

Figure 3.3 Distributions of infant percentage fat mass calculated using each of the (a) ultrasound skinfolds or (b) bioimpedance-based total body water equations at four time points: 2 months (n=13), 5 months (n=14), 9 months (n=17) and 12 months (n=14), pooled by sex. Ultrasound skinfolds equations represented by grey scale in the following order from left to right: Brook/Lohman; Brook/Siri; Durnin/Lohman; Durnin/Siri; Johnston/Lohman; Johnston/Siri; Slaughter (two skinfolds). Bioelectrical impedance spectroscopy equations represented by grey scale in the following order from left to right: Bocage; Fjeld; Kushner; Lingwood, 6 weeks; Lingwood, 3 months; Lingwood, 4.5 months. Rectangles are selected reference ranges (mean±SD): --- Butte reported in Gale et al. (Gale et al. 2012) at all ages; • • • (Gilchrist 2007) at 2 and 5 months, (Bellu et al. 1997) at 12 months.
### Table 3.6. Cluster map of statistical differences between the percentage fat mass values calculated with all ultrasound and bioimpedance spectroscopy equations.

<table>
<thead>
<tr>
<th>Equation, Method (Reference)</th>
<th>Lohman/ Durnin</th>
<th>Lohman/ Johnston</th>
<th>Kushner</th>
<th>Slaughter</th>
<th>Lohman/ Brook</th>
<th>Siri/ Johnston</th>
<th>Lingwood 4.5 months</th>
<th>Lingwood 6 weeks</th>
<th>Lingwood 3 months</th>
<th>Siri/ Durnin</th>
<th>Siri/ Brook</th>
<th>Fjeld</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bocage 1988, BIS&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.00&lt;sup&gt;c&lt;/sup&gt;</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>0.011</td>
<td>0.015</td>
<td>0.017</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Durnin 1967/ Lohman 1984, US/4SF&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.061</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Johnston 1988/ Lohman 1984, US/4SF</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>0.49</td>
</tr>
<tr>
<td>Kushner 1992, BIS</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>0.41</td>
<td>0.85</td>
<td>0.017</td>
<td>0.47</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Slaughter 1988, US/2SF&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.00</td>
<td>1.00</td>
<td>0.029</td>
<td>0.19</td>
<td>&lt;0.01</td>
<td>0.049</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brook 1971/ Lohman 1984, US/4SF</td>
<td>1.00</td>
<td>0.10</td>
<td>0.40</td>
<td>&lt;0.01</td>
<td>0.13</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Johnston 1988/ Siri 1961, US/4SF</td>
<td>0.10</td>
<td>0.38</td>
<td>&lt;0.01</td>
<td>0.11</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lingwood 2012 4.5 months, BIS</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lingwood 2012 6 weeks, BIS</td>
<td>0.84</td>
<td>1.00</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lingwood 2012 3 months, BIS</td>
<td>1.00</td>
<td>0.049</td>
<td>&lt;0.01</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Durnin 1967/ Siri 1961, US/4SF</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brook 1971/ Siri 1961, US/4SF</td>
<td>&lt;0.01</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Equations are ordered so as to highlight the cluster pattern which demonstrates that certain groupings exist between the equations independent of the US or BIS method. <sup>b</sup>BIS - bioelectrical spectroscopy; US/2SF – ultrasound with two skinfolds; US/4SF – ultrasound with four skinfolds. <sup>c</sup>Statistical difference between all of the US and BIS equations results were calculated using a general linear hypothesis test (Tukey’s all pair comparisons). <sup>d</sup>Bold text (p>0.05) indicates no statistical differences between percentage fat mass values calculated with the equations.
3.5 DISCUSSION

With increasing pediatric obesity, the focus of the research is on associations between early nutrition, BC and risk of future disease. BC assessment during early infancy, when the window of opportunity for intervention still exists, is crucial and reference data, particularly for breastfed infants, is desperately needed. Our findings indicate that both BIS and US are practical for calculating %FM in breastfed infants, but accuracy of prediction is highly dependent upon the use of validated age-matched prediction equations.

A limited number of BC reference data exist for infants during the first year of life, particularly for breastfed infants whose FFM is significantly lower and %FM is significantly higher compared with formula-fed infants (Gale et al. 2012). The major strength of our study was that all infants were breastfed at the time of measurements (to 12 months); therefore this study describes the BC for infants fed according to WHO recommendations in the first year of life. We found that no single equation for calculation of %FM by BIS and US allowed a match to the references across the first 12 months therefore should these methods be used, it is important to make an informed selection of equations that will match preferred references for both sampling time points and sex (Tables 3.3, 3.4).

Overall, we found no difference between US and BIS calculations of %FM indicating that both methods are acceptable for measurements in the field. Further, there were more matches with reference values for US skinfold based equations (Table 3.3). Slaughter, a two skinfold method, had high level of matches with reference values without requiring additional calculations to determine %FM (Siri or Lohman), as required with four skinfold equations, making it a very attractive tool for large studies. If the four skinfold method is chosen, a greater number of matches with reference values were achieved using the Siri %FM equation vs Lohman %FM equation (Tables 3.1, 3.3) with the exception of Brook/Lohman. The Lohman %FM equation is based on pre-pubertal and pubertal children BC (Lohman 1984), taking into account the lower bone-mineral and higher water content of this population, and was expected to provide more matches. The Siri %FM equation, while it is based on adult cadaver BC (Siri 1961), is the most widely used in both adult and pediatric populations and in this study provided more matches overall. The Lohman equation often underestimated %FM, despite the fact that dermis and fat layers are not compressed with US, possibly making skinfold measurements larger than the caliper technique and resulting in lower BD and...
higher %FM (Kuczmar ski et al. 1987).

Examination of BIS equations showed Kushner (Kushner et al. 1992) and Bocage (Bocage 1988) calculations to have the highest number of matches with the reference values which is similar to that of US skinfolds with Durnin/Siri and Durnin/Lohman (Table 4.3). There is also a trend that the equations that showed the most matches also covered a larger number of reference studies. It is possible that greater variations of estimated values of Kushner and Bocage increase the chance of overlapping with multiple distributions. Equations should be chosen to be age matched to the study population otherwise erroneous results are obtained as demonstrated by the performance of the Lingwood 6 weeks, 3 months and 4.5 months equations at 9 and 12 months where no matches were seen with any of the reference studies (Tables 3.3, 3.4).

When we compare all tested equations for calculation of %FM (irrespective of method) the majority gave significantly different values (Table 3.6). However, Lingwood 6 weeks equation was most likely to give comparable results to the other equations. No clustering was noted for the type of the method indicating that most of the variation in calculated values lies in the equation.

Infant sex is believed to influence BC with males having lower %FM than females at some time points (Carberry et al. 2010; Butte et al. 2000a), although a number of studies have failed to show this (Roggero et al. 2010; Gilchrist 2007; Bellu et al. 1997) or have not analysed for it (Fomon et al. 2002; Fields et al. 2011; Gale et al. 2012). We showed that US (four skinfolds) was able to detect sex differences with the exception of Brook/Siri and Brook/Lohman yet BIS did not. The Bellu study (Bellu et al. 1997) using total body electrical conductivity, which is based on similar principles as BIS did not show sex differences and therefore, the similarity in technique might explain our BIS results. Results from ADP are mixed but Butte (Butte et al. 2000a) used a multicomponent model, which is considered to be more accurate.

%FM changes in the first year of life with gradual increase in first 6 months followed by gradual decrease to 12 months (Butte et al. 2000a; Fomon et al. 2002). Age differences in %FM are mixed with some studies reporting an overall significant increase in %FM in the first 4 months of life (Fields et al. 2011; Roggero et al. 2010; Carberry et al. 2010) or no change between 2 and 3, 4 or 5 months (Gilchrist 2007); and detailed analysis between time points is not always available. In our study, only BIS was able to detect age differences and match them to literature. The Lingwood equations were the most sensitive to age producing statistically different values between most of the groups, with the other three BIS equations finding differences only between
2 months and the remaining time points. The Bocage equation was established in 3–18 months old infants but only detected age differences at 2 months, as did Fjeld (3–30 months old infants) and Kushner (premature infants to adults). The lack of age specific equations for skinfolds for the first 12 months of life is an important part of why we were unable to detect age differences with US and further emphasises the need for development of new multiple age specific equations covering the first year of life.

We excluded two infants based on their growth and low maternal milk production. In the first infant BIS and US agreed, with a best age/sex fitted equation for %FM for BIS (Lingwood, 3 months) producing 11.1% and for US (Brook/Lohman) 17.5%. In the second infant the measurements were disparate with 14.9%FM for BIS and 28.2%FM for US. Interestingly, %FM values based on US skinfolds were low in the first infant that plateaued for 2 weeks before the visit, but were normally distributed for the second infant. The large discrepancy for second infant might be related to the reduction in FFM. This is similar to preterm infants that at the time of estimated arrival often have greater %FM and less FFM than newborn term infants, which is probably partially due to restricted nutrition in utero or in hospital environment (Johnson et al. 2012). It is possible that combination of BIS and US is detecting this BC abnormality whereas US alone is not able to do so due to the very nature of the measurement technique.

Our results support the claim that BIS and US are useful methods of measuring of BC in infants. Skin thickness, subcutaneous fat thickness and %FM are valuable parameters for assessing and monitoring the nutritional state of the infant. US was introduced for this purpose (Petersen et al. 1995) but currently not widely used. US is able to obviate some of the limitations of the caliper technique in the pediatric population, such as age-related inter and intrasubject variation in skinfold compressibility, inability to palpate the fat-muscle interface or to differentiate the layers of skin, differences in the types of calipers used, and pain/trauma factor (Kuczmarski et al. 1987). Petersen et al. reported some degree of difficulty with identification of the dermis-subcutaneous fat interface in preterm infants skin compared to adult skin using US (Petersen et al. 1995). We did not experience this difficulty, probably due to the fact that Petersen measured distance between peaks on skin echogram rather than on an actual scan, and that infants in our study were term thus the interface could be more defined. Recently Pineau et al. has validated both US skinfold and BIS measurements in adults against DXA. In that study US has shown very good accuracy, while BIS has shown less accuracy than US but better than ADP (Pineau et al. 2007). BIS predictions of %FM in infants improve with age compared with simple anthropometric
measurements (Lingwood et al. 2012) and could benefit pediatric populations if more equations developed for infants after 5 months of age.

The limitations of this study include the missing data of four skinfolds for 13 infants and, as a result, small numbers in particular sex/age groups; lack of available age-matched equations, necessitating running a full analysis of many equations; and limitation with resources for the study, resulting in absence of reference data for our subjects (notwithstanding the issues with existing reference methods in pediatric population).

### 3.6 CONCLUSIONS

In conclusion, high-frequency US measurement of SCTT is a precise and reliable method for assessment of %FM in breastfed infants. Accurate measurements of SCTT in a wide range of body masses are easily recorded, and the non-traumatic technique allows application of this method even in the smallest infants. BIS, on other hand presents some issues particularly in the earlier months of life but its performance improves with infant age. While both methods will further benefit from the development of more precise age appropriate equations, a number of the current equations are practical for assessing %FM in breastfed infants, particularly in longitudinal studies. This study further emphasises the critical need for development of sex/age specific normative BC values for assessment of the growth and nutritional status of breastfed infants during the first year of life.
CHAPTER 4

LEPTIN LEVELS ARE HIGHER IN WHOLE COMPARED TO SKIM HUMAN MILK, SUPPORTING A CELLULAR CONTRIBUTION*

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4.1 ABSTRACT
HM contains a plethora of metabolic hormones, including leptin, which is thought to participate in the regulation of the appetite of the developing infant. Leptin in HM is derived from a combination of de novo mammary synthesis and transfer from the maternal serum. Moreover, leptin is partially lipophilic and is also present in HM cells. However, leptin has predominately been measured in skim HM, which contains neither fat nor cells. We optimized an enzyme-linked immunosorbent assay for leptin measurement in both whole and skim HM and compared leptin levels between both HM preparations collected from 61 lactating mothers. Whole HM leptin ranged from 0.2 to 1.47 ng/mL, whilst skim HM leptin ranged from 0.19 to 0.9 ng/mL. Whole HM contained, on average, 0.24±0.01 ng/mL more leptin than skim HM (p<0.0001, n =287). No association was found between whole HM leptin and fat content (p=0.17, n=287), supporting a cellular contribution to HM leptin. No difference was found between pre- and post-feed samples (whole HM: p=0.29, skim HM: p=0.89). These findings highlight the importance of optimizing HM leptin measurement and assaying it in whole HM to accurately examine the amount of leptin received by the infant during breastfeeding.
4.2 INTRODUCTION

HM is a heterogeneous fluid composed of a combination of macro- and micro-nutrients, cells, and a plethora of biomolecules that provide the necessary elements to sustain infant growth, protection and development (Hassiotou et al. 2013a; Ballard et al. 2013; Hamosh 2001). The developmental effects of breastfeeding extend to programming of various organs and systems of the newborn, including that of appetite regulation (Hassiotou et al. 2014a). This early developmental programming results in a reduction in obesity and other metabolic diseases not only in the short-term, but also in adulthood (von Kries et al. 1999; Gillman et al. 2001; Armstrong et al. 2002; Bouret et al. 2012; Pico et al. 2007). The complex system of breastfeeding-mediated appetite regulation is attributable to various factors associated with the practice of breastfeeding, such as feeding on demand, but also potentially to a host of appetite regulatory molecules present in HM (Hassiotou et al. 2014a). These include whey and casein proteins, HMO, and recently discovered in HM appetite regulatory hormones, including the well-documented adipokines leptin, adiponectin, and many others (Savino et al. 2010; Savino et al. 2012b; Savino et al. 2012c; Alderete et al. 2015; Lopez Alvarez 2007).

Amongst these appetite molecules, leptin is the most widely studied, being primarily known for promoting satiety and energy expenditure in adults through binding to the full length leptin receptor (ObRb) expressed on the arcuate nucleus of the hypothalamus (Tartaglia et al. 1995). In addition, leptin stimulates cell proliferation, regulates blood pressure, and is also involved in the T-cell immune response, thus displaying pleiotropic roles (Bassi et al. 2015; Magarinos et al. 2007). White adipose tissue is one of the main sources of serum leptin, secreting leptin proportionally to the number of white adipocytes present in the body (Klein et al. 1996). Further, gastric chief cells, the placenta and the mammary epithelium also synthesise and secrete leptin in adults (Myers 2004; Mix et al. 2000; Smith-Kirwin et al. 1998; Hassiotou et al. 2014c).

In infants, HM is believed to be a major source of leptin early in life, with the endogenous leptin-synthesising mechanisms being still immature (Oliver et al. 2002). Leptin in HM has been hypothesised to be involved both in the short-term control of appetite and in developmental programming of appetite and energy signaling pathways, promoting efficient energy control and storage throughout life (Pico et al. 2007; Bouret et al. 2004a). Leptin administered during the first 14 days of life has been shown to act as a neurotrophic agent, promoting neural growth from the arcuate nucleus of the hypothalamus to additional appetite control centres located in the central nervous
system (Proulx et al. 2002). HM leptin may provide short-term appetite control in the infant also by up-regulating circulating melanocortins, potent anorexigenic agents that promote satiety (Miralles et al. 2006). Leptin in HM is sourced both endogenously from the mammary gland and from the maternal serum, following secretion from white adipocytes and gastric chief cells into the bloodstream (Pico et al. 2007). In the lactating mammary gland, serum-derived leptin combines with locally synthesised leptin by the mammary epithelium to yield the total leptin content of HM (Hassiotou et al. 2014c).

Leptin in HM has been predominately measured in skim HM, which does not contain the cellular and fat components of HM (Miralles et al. 2006; Weyermann et al. 2006; Schuster et al. 2011). Considering that the leptin peptide is capable of lipophilic interactions (Kline et al. 1997; Xie et al. 1991), it is plausible it may associate with the fat globule in whole HM. Moreover, HM cells, which are predominantly of epithelial origin in mature HM of healthy mother/infant dyads (Hassiotou et al. 2013a; Hassiotou et al. 2014b), are also thought to contribute to the leptin concentration of whole HM (Hassiotou et al. 2014c). Few previous studies have measured leptin in whole HM using RIA (Houseknecht et al. 1997; Smith-Kirwin et al. 1998). However, RIA is not considered appropriate for measuring leptin in a lipid-rich medium, such as whole HM due to interference of triglycerides with the binding of radioactive-labelled antigens to antibodies, which compromises the sensitivity of the assay (Resto et al. 2001; S.M. Grundy et al. 1979). Given the lack of an optimised assays to detect leptin in whole HM and the absence of reliable comparisons of leptin levels between whole and skim HM, we developed an ELISA as a more appropriate means of measuring leptin in HM, with two antibodies assisting in immobilising the leptin antigen, and compared the leptin concentration between pre- and post-feed samples, as well as whole and skim HM.

4.3 MATERIALS AND METHODS

4.3.1 Study participants
All procedures involving the recruitment of lactating mothers and HM sample collection and analyses were approved by, and conducted in accordance with, the guidelines of the Human Research Ethics Committee of The University of Western Australia (ethics approval number RA/4/1/4253). All mothers provided informed written consent in the form of a secure online questionnaire that was administered and securely stored by The University of Western Australia. A single sample of whole HM expressed by a mother in her first month of lactation was used for optimisation of the leptin assay. Following
assay optimisation, 61 lactating mothers (38 Caucasian, 23 non-Caucasian) with a mean maternal age of 33.6±4.39 years, of full-term healthy infants were recruited to assess leptin differences between whole and skim HM (Table 4.1). HM samples (~5 mL) were obtained at approximately 11:00 h aseptically, as previously described by Hassiotou et al. (Hassiotou et al. 2012a), from each breast before and after the infant fed from a single breast session either by using a Medela Symphony (Medela AG, Baar, Zug, Switzerland) breast pump or by hand expression. Samples were stored at -20 °C prior to analysis. Samples were collected at the second, fifth, ninth and 12th months of lactation (Table 4.1).

Table 4.1 Maternal and infant anthropometric and demographic characteristics (n=61). Table includes mothers who provided samples for multiple months.

<table>
<thead>
<tr>
<th>Stage of lactation (month)</th>
<th>2</th>
<th>5</th>
<th>9</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal age (years)</td>
<td>32.9 ± 4.21 a (28-40)</td>
<td>33.4 ± 4.27 (24-40)</td>
<td>34.0 ± 4.57 (25-43)</td>
<td>34.1 ± 4.35 (26-44)</td>
</tr>
<tr>
<td>Maternal BMI b</td>
<td>27.1 ± 7.15 (20.1-38.5)</td>
<td>23.5 ± 4.46 (18.0-35.2)</td>
<td>24.0 ± 5.15 (18.7-37.2)</td>
<td>24.8 ± 5.6 (18.2-34.6)</td>
</tr>
<tr>
<td>Parity</td>
<td>2.10 ± 0.75 (1-4)</td>
<td>2.13 ± 0.85 (1-4)</td>
<td>1.96 ± 0.94 (1-4)</td>
<td>2.05 ± 0.98 (1-4)</td>
</tr>
<tr>
<td>Infant sex (Male/Female)</td>
<td>12/9</td>
<td>16/16</td>
<td>17/13</td>
<td>12/12</td>
</tr>
<tr>
<td>Infant birth weight (kg)</td>
<td>3.58 ± 0.64 (2.66-4.23)</td>
<td>3.49 ± 0.45 (2.66-4.46)</td>
<td>3.49 ± 0.46 (2.82-4.46)</td>
<td>3.59 ± 0.46 (2.80-4.46)</td>
</tr>
<tr>
<td>Infant body length (cm)</td>
<td>57.6 ± 2.17 (54.2-61.3)</td>
<td>64.5 ± 2.09 (61.5-69.5)</td>
<td>70.9 ± 2.11 (68.0-74.5)</td>
<td>73.9 ± 2.38 (71.5-78.5)</td>
</tr>
</tbody>
</table>

a Values are mean ± SD (range). b BMI – body mass index.

4.3.2 Measurement of leptin in whole and skim human milk by ELISA

Whole HM samples were thawed at room temperature, vortexcised for 10 s and aliquoted (2×750 μL) into 1.5 mL microfuge tubes (Sarstedt, Numbrecht, Germany). One whole HM aliquot was centrifuged (05PR-22 Refrigerated Centrifuge, Hitachi, Tokyo, Japan) at 1500 × g for 10 min at 4°C and the resultant skim HM portion was aspirated. Both skim and whole HM aliquots were sonicated on ice at 100 Hz for 3 cycles of 5 s pulses, with a 20 s rest intervals using an ultrasonic processor VCX130 (Sonics & Material, Newton, CT, USA). Eleven dilutions ranging 1 to 50-fold were prepared from both milk preparations using 1% bovine serum albumin (BSA; Sigma-Aldrich, Castle Hill, NSW, Australia) in phosphate buffer saline (PBS; Gibco Life Technologies, Paisley, Scotland).
Leptin concentration for each dilution was measured using the Human Leptin ELISA DuoSet (R&D Systems, Minneapolis, MN, USA). Capture antibody (4 ng/mL, diluted with PBS, pH 7.4) was pipetted (100 µL per well) to coat the bottom of the wells of flat bottom 96-well microtitre plates (Flow Laboratories, McLean, VA, USA). Plates were sealed and incubated overnight at room temperature (RT). Wells were washed three times with PBS/Tween wash buffer (0.05% Tween 20; (Bio-Rad Laboratories, Gladesville, NSW, Australia) in PBS, pH 7.4), dispensed at 400 µL per well, using a plate washer (Innunowash 1575, Bio-Rad Laboratories, Hercules, CA, USA). Washed plates were inverted and blotted against absorbent paper to ensure no remaining solution was present inside the wells. Blocking buffer (1% w/v BSA in PBS, pH 7.4) was added (300 µL per well) to block non-specific binding sites. Plates were sealed and incubated for one hour at RT. Blocking buffer was washed according to the wash procedure described earlier. Diluted samples and standards (0–0.9 ng/mL) were added (100 µL per well) in duplicates and plates were sealed and incubated for 2 h at RT. Unbound components from samples and standards were washed, and biotinylated detection antibody (4 ng/mL, diluted in 1% w/v BSA in PBS, pH 7.4) was added (100 µL per well). Plates were sealed and incubated for 2 h at RT. Unbound detection antibody was washed, and streptavidin-horseradish peroxidase (HRP; R&D Systems, Minneapolis, MN, USA) (50 ng/mL in PBS, pH 7.4) was added (100 µL per well), and plates were sealed, wrapped in aluminium foil to avoid exposure to direct light, and incubated for 20 min at RT. Streptavidin-HRP was washed and substrate colour reagent (1:1 mixture of 12 mL/vial hydrogen peroxide and 4 mL/vial enhanced luminol, R&D Systems, Minneapolis, MN, USA) was added (100 µL per well). Plates were sealed and wrapped in aluminium foil and were incubated for 20 min at room RT. Sulphuric acid (1M; R&D Systems, Minneapolis, MN, USA) stop solution was added (50 µL per well) and absorbance was read at 450 nm by a plate spectrophotometer (Enspire Multimode Plate Reader, Waltham, MA, USA). Standard curves and leptin concentrations were calculated using linear regression (Figures 4.1 and 4.2).
CHAPTER 4: LEPTIN LEVELS IN SKIM AND WHOLE MILK

Figure 4.1 Standard curve for the leptin enzyme-linked immunoassay (ELISA) for whole and skim human milk. Standards were selected according to previous literature investigating levels of leptin in skim human milk, as well as recommendations provided by the leptin kit manufacturer (Cannon et al. 2015).

Figure 4.2 Leptin concentration for whole and skim human milk for each dilution tested. Values are mean ± SEM (n=100 diluted human milk preparations). Leptin levels in whole and skim human milk are shown by black and white bars, respectively.

Recovery assays to discern the optimal dilution factor for leptin detection were conducted on dilutions reporting leptin concentrations within the range of the protein standards used (Table 4.2). Following optimisation of the dilution factor, leptin concentration in matched whole and skim HM samples from the study population was
measured. All whole and skim HM samples were prepared according to the same centrifugation and sonication protocol used in the assay optimisation. Recovery of a known amount of the leptin protein when added to samples was 97.7±9.7% (n=10) (Table 4.2), with the leptin kit reporting an intra-assay variability of <5% and an inter-assay variability of <7.2%.

**Table 4.2 Recovery percentages for each dilution factor for skim and whole human milk leptin measurement.**

<table>
<thead>
<tr>
<th>Dilution Factor</th>
<th>Skim Human Milk Leptin (%)</th>
<th>Whole Human Milk Leptin (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>61.5 ± 2.09&lt;sup&gt;a&lt;/sup&gt;</td>
<td>17.1 ± 2.9</td>
</tr>
<tr>
<td>2</td>
<td>179.0 ± 0.82</td>
<td>14.0 ± 2.7</td>
</tr>
<tr>
<td>5</td>
<td>96.3 ± 1.2</td>
<td>14.0 ± 1.4</td>
</tr>
<tr>
<td>10</td>
<td>71.3 ± 1.6</td>
<td>97.1 ± 9.1</td>
</tr>
</tbody>
</table>

<sup>a</sup> Values are mean ± SD.

4.3.3 Measurement of fat content in human milk

The total fat content of HM samples was measured using the creamatocrit method (Khan et al. 2013a; Mitoulas et al. 2002). Samples were placed in micro-haematocrit tubes, plugged with sealant and centrifuged at 12,000 x g for ten min in a micro-haematocrit centrifuge (Hermle Z230H Labortechnik, Wehingen, Germany). The resultant milk column was placed on the creamatocrit analyser (Creamatocrit Plus, Medela Inc, McHenry, IL, USA) and the length of the fat layer and the total milk column was measured, from which the total fat content was calculated. It has been shown that Creamatocrit measurements strongly correlate with the biochemical spectroscopic esterified fatty acid assay (Mitoulas et al. 2002; Czank et al. 2009; Hassiotou et al. 2013b).

4.3.4 Statistical analyses

Statistical analyses were performed using Microsoft Excel 2013 (Microsoft Corporation, Redmond, WA, USA) and R 2.9.10 (R Core Team 2014) for Windows 10, with the additional R package “nlme” (R Core Team, Vienna, Austria) used for linear mixed effects modeling (Pinheiro et al. 2009). Student’s paired t-tests were conducted to assess leptin differences between matched skim and whole HM samples in the entire study population. Differences between whole and skim HM leptin concentrations were
subsequently analysed within each month of breastfeeding, also using matched Student’s t-test.

Linear mixed effects modeling was used to examine any associations between HM fat content and whole HM leptin concentration. Responses were modelled with and without controlling for the volume of milk that had been removed from the breast during the collection of the HM sample. To discern the significant random effects to use for each statistical model analysing the association between HM fat content and whole HM leptin concentration, three separate models were devised; one linear model with no random effects and two linear mixed effects models with the following random effects: the effect of general inter-individual variation present in the study population, and the effect of the stage of lactation in addition to inter-individual variation. Analysis of variance (ANOVA) tests were then used to compare each model with the same fixed effects, namely fat content and fat content when controlled for volume of milk removed from the breast. The final model for the association between whole HM leptin concentration and fat content accounted for inter-individual variation and the stage of lactation, as random effects when volume removed was not controlled for in the model. Similarly, when volume of milk removed from the breast was controlled for in the linear mixed effects models, inter-individual variation and stage of lactation were also considered as significant random variables to include in the analysis of associations between fat content and whole HM leptin concentration.

Similarly, the association between leptin concentration in whole HM samples and the corresponding fat content was also analysed within each month of lactation. Given that the volume of HM removed during feeding was only collected for 74 samples out of the entire study population, the liner mixed effects models used for intra-month analysis did not control for volume of HM removed from the breast. As with the analysis between the association of leptin levels in whole HM samples and fat content for the entire population, for each month, three statistical models were devised; one linear model with no random effects, and two linear mixed effects models with the following random variables included: the effect of inter-individual variation present in the study population and the effect of the stage of lactation in addition to inter-individual variation. Analysis of variance (ANOVA) was then used to compare each model within each month. For each month of breastfeeding, the only significant random effect found was general inter-individual variation. \( p<0.05 \) was considered statistically significant. All values presented are mean ± SD, unless stated otherwise. All \( R^2 \) values were generated from linear regression line of best fit equations.
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4.4 RESULTS

4.4.1 Participants

The demographic characteristics of mothers and infants in the study population are shown in Table 4.2. All infants (n=61) were born at term, healthy, and were growing appropriately for their age according to WHO growth charts for exclusively breastfed infants (World Health Organization 2009a; World Health Organization 2009b). Mean maternal BMI was highest at 27.1±7.15 kg/m² during the second month of lactation and lowest during the fifth month of lactation at 23.5±4.46 kg/m² (Table 5.1). Compared to the second month of lactation, maternal BMI decreased by 2.30±1.55 kg/m² over the first 12 months of breastfeeding (p<0.01).

4.4.2 Leptin optimisation

Measurement of leptin in whole and skim HM was optimised using an ELISA-based assay. One- to 20-fold dilutions for both skim and whole HM yielded leptin concentrations within the standard range of the assay (0–0.9 ng/mL), whilst dilutions above 20-fold were outside of the upper protein standard used (Figure 4.2). Mean leptin concentrations for 15-fold (whole HM: 0.8±0.07 ng/mL, skim HM: 0.75±0.09 ng/mL) and 20-fold (whole HM: 0.9±0.11 ng/mL, skim HM: 0.9±0.09 ng/mL) dilutions were close to the highest protein standard, thus further consideration was not given to these dilution factors (Figure 4.2). Ten-fold dilution of whole HM yielded the best recovery rates (97.1±9.1%) (Table 4.2). Five-fold diluted skim HM recovered 96.3±1.2% of leptin (Table 4.2). Therefore, subsequent samples were diluted by 10-fold and five-fold with the diluent reagent for whole and skim HM samples, respectively, given these dilution factors recovered the highest percentage of leptin protein when the assay was performed for whole and skim HM.

4.4.3 Whole and skim human milk leptin

Leptin levels measured using the optimised assay were compared between whole and skim HM obtained during different stages of lactation from 61 lactating mothers. Whole HM leptin levels ranged 0.2–1.47 ng/mL, whilst a 0.19–0.9 ng/mL range was obtained for skim HM leptin (Figure 4.3). Whole HM leptin was 0.24±0.01 ng/mL higher than skim HM leptin across all samples (p<0.0001, n=287) (Figure 4.3). Leptin levels were also higher in whole HM compared to skim HM within each month of lactation (Table 4.3). Matched pre-feed whole HM samples contained 0.24±0.07 ng/mL more leptin than the paired pre-feed skim preparations (p<0.01, n=157), with 0.25 ng/mL±0.05 ng/mL
more leptin measured in post-feed whole HM samples compared to matched skim post-feed aliquots \( (p<0.01, n=137) \).

![Figure 4.3](image)

**Figure 4.3** Comparison between whole and skim human milk (HM) leptin concentration \( (n=287) \). *** Indicates significant difference between matched whole and skim human milk leptin values \( (p < 0.001) \).

**Table 4.3** Leptin concentrations for whole and skim human milk at each month of lactation. Values are mean±SD.

<table>
<thead>
<tr>
<th>Month of Lactation</th>
<th>Whole Human Milk Leptin (ng/mL)</th>
<th>Skim Human Milk Leptin (ng/mL)</th>
<th>p-Value (^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>0.50 ± 0.16 (^a)</td>
<td>0.32 ± 0.16</td>
<td>( p &lt; 0.0001 )</td>
</tr>
<tr>
<td>5</td>
<td>0.48 ± 0.16</td>
<td>0.26 ± 0.07</td>
<td>( p &lt; 0.0001 )</td>
</tr>
<tr>
<td>9</td>
<td>0.56 ± 0.11</td>
<td>0.22 ± 0.03</td>
<td>( p &lt; 0.0001 )</td>
</tr>
<tr>
<td>12</td>
<td>0.54 ± 0.14</td>
<td>0.21 ± 0.02</td>
<td>( p &lt; 0.0001 )</td>
</tr>
</tbody>
</table>

\(^a\) Values are mean ± SD. \(^b\) p-values indicate significant differences between whole and skim milk leptin concentrations at given time points.

No association was observed between whole and skim HM leptin \( (p=0.55, n=287) \) (Figure 4.4a). HM fat content was not related to leptin concentration in whole HM when the volume of milk removed from the breast during sample collection was not accounted for \( (p=0.52, n=283) \) (Figure 4.4b) or accounted for \( (p=0.24, n=74) \) in the
analysis. Further, no association between leptin levels in whole HM samples and fat content were found within each stage of lactation (Table 4.4).

Figure 4.4 (a) No association was detected between skim and whole HM leptin levels in matched samples ($R^2=0.001, p=0.552, n=287$); (b) No association was detected between fat content and leptin concentration in whole HM ($R^2=0.0004, p=0.17, n=284$). The solid black line is the line of best fit.

Table 4.4 Association between leptin levels in whole human milk and fat content at each stage of lactation.

<table>
<thead>
<tr>
<th>Month of Lactation</th>
<th>N (samples)</th>
<th>$R^2$</th>
<th>p-Value $^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>66</td>
<td>0.0013</td>
<td>0.782</td>
</tr>
<tr>
<td>5</td>
<td>72</td>
<td>0.018</td>
<td>0.686</td>
</tr>
<tr>
<td>9</td>
<td>83</td>
<td>0.069</td>
<td>0.577</td>
</tr>
<tr>
<td>12</td>
<td>66</td>
<td>0.153</td>
<td>0.889</td>
</tr>
</tbody>
</table>

$^a$ p-values indicate absence of associations between whole milk leptin concentrations and fat concentrations at given time points.

No differences between pre-feed and post-feed whole ($p=0.29, n=77$) and skim ($p=0.89, n=76$) leptin levels were detected after accounting for the volume of milk consumed by the infant during the session (Figure 4.5). Post-feed samples contained 36.2±2.82 g/L more fat compared to matched pre-feed samples ($p<0.01, n=37$).
CHAPTER 4: LEPTIN LEVELS IN SKIM AND WHOLE MILK

Figure 4.5 After accounting for the volume of milk consumed by the infant during the session, no differences in pre- and post-feed (a) whole and (b) skim human milk (HM) leptin values were detected in the study population.

4.5 DISCUSSION

This study has shown that whole HM contains significantly higher levels of leptin compared to skim HM and that sampling either before or after a breastfeed does not influence this level.

Leptin has been previously shown to be present in HM and has been hypothesised to participate in the short- and long-term regulation of appetite in the breastfed infant (Pico et al. 2007; Bouret et al. 2006). In addition, HM leptin may be involved in other functions in the breastfed infant given its known pleiotropic properties, and in mammary development (Lord et al. 1998; Lord et al. 2002). Although many studies have previously measured leptin in HM, optimisation of the methodology has not been well documented and most studies have focused on the levels of leptin in skim HM. However, leptin has been proposed to have lipophilic properties (Kline et al. 1997) and is also synthesised by mammary epithelial cells (Hassiotou et al. 2014c), which comprise the majority of cells in mature HM from healthy mother/infant dyads (Hassiotou et al. 2013a; Ho et al. 1979; Boutinaud et al. 2002). It is therefore conceivable that leptin in HM is associated with the fat and/or cells, which are not present in its skim fraction, suggesting that previous measurements of skim HM leptin have underestimated the concentration of leptin in HM. In this study, we performed a comparison of leptin levels between whole and skim HM and optimised an ELISA assay to accurately measure it in both HM preparations.
Leptin levels were found to be, on average, two-fold higher in whole HM compared to skim HM (Figure 4.3, Table 4.3). There are limited studies that have compared whole and skim HM leptin levels and they utilized RIA-based methodologies. While results of our study are in agreement with two previous studies that found higher levels of leptin in whole than skim HM, the actual values are different. Houseknecht et al. (Houseknecht et al. 1997) reported whole HM leptin levels (10.1±2.6 ng/mL, n=23) that were approximately seven times higher than in skim HM (1.5±0.87 ng/mL, n=23) and, on average, 20 times higher than levels found in this study (Figure 4.3). Moreover, Smith-Kirwin et al. (Smith-Kirwin et al. 1998) reported 56-fold higher leptin levels in whole HM (73.2±39.0 ng/mL, n=8) compared to skim HM (~1.3 ng/mL), and a 130-fold higher mean whole HM leptin concentration compared to the present study. For skim HM both studies observed leptin levels 1.5–2.5 times greater than those found here (Smith-Kirwin et al. 1998). The higher absolute leptin levels measured in these studies may be attributable to the analysis. Both studies used the RIA technique, which is not as reliable as ELISA in measuring leptin levels in whole HM due to its known inaccuracies of immune-reactive antibodies binding to the epitopes of antigens suspended in a lipid-rich medium, such as whole HM, or of interference of iron and emulsifiers with the assay (Resto et al. 2001; S.M. Grundy et al. 1979). In addition, differences in the technique optimisation between the two previous studies are apparent, as the whole milk leptin level in one study is seven times greater than the other. Both commercially available RIA and ELISA kits are originally designed to measure leptin in serum, not in HM; therefore, optimization is critical. On the other hand, our results are comparable to leptin levels in many other studies that measured it in skim HM using ELISA, or whole and skim milk using RIA. ELISA in skim HM detected similar leptin levels (from 0.30±0.04 ng/mL at 1 month to 0.10±0.02 ng/mL at 12 months, n=72) in a study by Bronsky et al. (Bronsky et al. 2011), (0.28±0.38 ng/mL, n=651) in a study by Weyermann et al. (Weyermann et al. 2006), and (0.16±0.04 ng/mL, n=28) in a study by Miralles et al. (Miralles et al. 2006). RIA in skim HM also detected close leptin levels: (0.18±0.15 ng/mL, n=23) in a study by Schuster et al. (Schuster et al. 2011) and (1.00±0.80 ng/mL, n=13) in a study by Schueler et al. (Schueler et al. 2013). RIA results in whole HM (1.34±0.14, n=24) in study by Bielicki et al. (Bielicki et al. 2004) were also comparable to our whole HM leptin levels.

Whilst it has been hypothesised that leptin may be associated with fat globules present in whole HM due to its lipophilic nature (Smith-Kirwin et al. 1998; Kline et al. 1997) this was not borne out in this study (Figure 4.4b, Table 4.4) despite analyzing
CHAPTER 4: LEPTIN LEVELS IN SKIM AND WHOLE MILK

pre- and post-feeding milk samples with a wide range of fat content of 11.0–128.8 g/L. The lack of an association between HM fat content and whole HM leptin levels suggests that fat may not have a strong contribution to HM leptin levels compared to the cellular fraction of HM. Lactocytes, myoepithelial cells, and stem cells present in HM have been previously shown to express the leptin gene (Hassiotou et al. 2014c). Additionally, flow cytometric analysis of HM cells has revealed that the majority of lactocytes and stem cells contain the leptin protein (Hassiotou et al. 2014c). Given that lactocytes are the dominant cell type in mature whole HM when both the mother and infant are healthy (Hassiotou et al. 2013a; Ho et al. 1979; Boutinaud et al. 2002; King et al. 2002; Hassiotou et al. 2015), it is likely that lactocytes contribute significantly to HM leptin levels. Indeed, the cellular fraction of HM can constitute a significant portion of milk, comparable to its skim and fat fractions (Hassiotou et al. 2013a; Hassiotou et al. 2014b). This warrants investigation to further discern the cellular contribution to leptin levels in whole HM, and emphasises the need to assay whole HM for leptin, and potentially for other appetite hormones, to obtain accurate measurements of the levels of these hormones in HM. Importantly, the procedures of whole HM preparation for such measurements must enable complete lysis of the milk cells for accurate results. This is also very important during sample preparation for whole HM ELISA, achieved in the present study by sonication.

Higher leptin levels in whole HM compared to the skim fraction indicate that infants ingest a larger dose of leptin than that calculated from skim HM. Whilst HM cells likely contribute to the increased leptin level in whole HM the bioavailability of this source is unknown. However, we speculate that the process of digestion would release leptin protein from HM cells. It is also possible that these cells are absorbed through the stomach mucosa after ingestion and enter the circulation, as has been confirmed with HM leukocytes and stem cells (Hassiotou et al. 2012a; Weiler et al. 1983).

The lack of an association between whole HM leptin and milk fat content may be also attributable to the biochemical properties of the leptin peptide. Although paradoxical to the notion that leptin is synthesised by white adipocytes which exhibit a lipophilic nature, leptin may also consist of hydrophilic regions, enabling it to primarily interact with aqueous fluids. The specific hydrophilic regions exhibited on the leptin peptide are hypothesised to be conserved cysteine residues tethered to disulphide bridges (Kline et al. 1997; Imagawa et al. 1998), which may form polar bonds with water molecules, given sulphide’s strong electronegativity properties (Bergstrom 1926).
Leptin crystallization studies could further confirm its hydrophilic properties (Imagawa et al. 1998), providing insight into the lack of an association between the leptin protein and the fat component of whole HM.

Upon analysis we also found no difference between the concentration of whole HM leptin in pre- and post-feed samples, indicating the small samples taken either pre- or post-feed provide the same levels of leptin despite differences in fat content. To measure the cellular contribution of leptin in HM, it may be possible to acquire larger pre- and post-feed sample volumes and examine the number of cells in the sample. Hassiotou et al. (Hassiotou et al. 2013a) has shown that cell content increases post-feed, as does fat, when larger sample volumes were attained or the breast was well drained of milk.

4.6 CONCLUSIONS

We describe the first standardised and optimised ELISA assay for measurement of leptin in both skim and whole HM, demonstrating higher concentrations of leptin in whole HM preparations compared to skim HM samples. Further, we provide evidence supporting the lack of an association between the fat component of HM and its leptin content, suggesting a contribution of HM cells, which merits further investigation. Accurate analysis of whole HM leptin will assist in clarifying the biological role of this milk component for the breastfed infant, improving our understanding of early developmental programming of appetite and its implications for obesity prevention later in life.
ASSOCIATIONS BETWEEN MATERNAL BODY COMPOSITION AND APPETITE HORMONES AND MACRONUTRIENTS IN HUMAN MILK*

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** - Equal first authors
5.1 ABSTRACT

HM appetite hormones and macronutrients may mediate satiety in breastfed infants. This study investigated associations between maternal adiposity and concentrations of HM leptin, adiponectin, protein and lactose, and whether these concentrations and the relationship between BMI and %FM in a breastfeeding population change over the first year of lactation. Lactating women (n=59) provided milk samples (n=283) at the 2nd, 5th, 9th and/or 12th month of lactation. Concentrations of leptin, adiponectin, total protein and lactose were measured. Maternal %FM was measured using BIS. Higher maternal %FM was associated with higher leptin concentrations in both whole (0.006±0.002 ng/mL, p=0.008) and skim HM (0.005±0.002 ng/mL, p=0.007), and protein (0.16±0.07 g/L, p=0.028) concentrations. Adiponectin and lactose concentrations were not associated with %FM (0.01±0.06 ng/mL, p=0.81; 0.08±0.11 g/L, p=0.48, respectively). Whole milk concentrations of adiponectin and leptin did not differ significantly over the first year of lactation. These findings suggest that the level of maternal adiposity during lactation may influence the early appetite programming of breastfed infants by modulating concentrations of HM components.
5.2 INTRODUCTION

HM is the optimal nutrition for term infants as it contains a uniquely balanced profile of macronutrients along with micronutrients, hormones, antibodies, bioactive molecules (Lonnerdal 2000) and cells (Hartmann et al. 2011; Hassiotou et al. 2012b), which adequately support the nutritional needs, appropriate growth, immunoprotection and physiological development of the infant (Fichter et al. 2011; Isaacs et al. 2010). It is well documented that prolonged breastfeeding is associated with decreased prevalence of overweight and obesity across the life course (Thompson 2012; Weng et al. 2012).

Enhanced appetite control in adults who have been breastfed as infants has been partly attributed to regulatory appetite hormones present in HM (Miralles et al. 2006; Hassiotou et al. 2014a; Savino et al. 2013), which include leptin and adiponectin (Miralles et al. 2006; Bronsky et al. 2011). In rat pups, acute or chronic administration of leptin by intraperitoneal injection has been shown not to reduce food consumption but to modulate the expression of neuropeptides and receptors involved in the regulation of feeding behaviour (NPY and proopiomelanocortin (POMC)) (Proulx et al. 2002), while in mice pups leptin deficiency caused profound disruptions in the development of the projections of the arcuate nucleus of the hypothalamus (Bouret et al. 2004b). These findings indicate that leptin plays a neurotrophic role and contributes to the developmental programming of the hypothalamic appetite circuitry during the neonatal period, preceding leptin’s acute regulation of food intake in adults. Adiponectin has anti-inflammatory properties and improves both fatty acid metabolism and sensitivity to insulin (Newburg et al. 2010). In mice, adiponectin inhibits tension-sensitive gastric vagal afferent mechanosensitivity, modulating satiety signals in both lean and obese animals, while simultaneously increasing the mechanosensitivity of mucosal gastric vagal afferent in an obesity-induced model (Kentish et al. 2015).

Additionally, the concentration of macronutrients in HM, namely protein, fat and lactose, may also be involved in regulation of the infant appetite control (Hassiotou et al. 2014a; Geddes et al. 2013). Differences in the concentrations of these factors may partly explain the variability in breastfeeding patterns observed in infants who feed on demand (Kent et al. 2006). As such, an understanding of the factors that affect concentrations of appetite hormones and macronutrients in HM is critical, as it presents a unique opportunity for the prevention of unfavourable early developmental programming and subsequent obesity.

Leptin is secreted into the maternal circulation by white adipocytes and is subsequently transferred into the mammary ductal system via diffusion or a receptor-
mediated transport mechanism (Weyermann et al. 2006). Leptin is also contributed by lactocytes (Hassiotou et al. 2014c; Smith-Kirwin et al. 1998). Previous studies have identified a positive correlation between maternal BMI and both maternal serum (Schuster et al. 2011; Savino et al. 2016) and skim HM leptin (Schuster et al. 2011; Savino et al. 2016; Houseknecht et al. 1997; Schueler et al. 2013), despite differing methodologies in leptin measurement (Andreas et al. 2014). However, no consistent relationship between maternal adiposity and HM leptin concentrations has been shown across lactation, with Miralles et al. (2006) reporting only moderate correlations between maternal BMI and HM leptin during the first 6 months of lactation ($R=0.387; p<0.01$) (Miralles et al. 2006), while Bronsky et al. (2011) saw no consistent relationship over the first 12 months of lactation (Bronsky et al. 2011), despite utilizing a similar leptin measurement methodology.

There are a number of factors that may contribute to these conflicting findings. BMI is a poor measure of adiposity, as it fails to adequately differentiate between adipose tissue and lean body mass (Prentice et al. 2001; Shah et al. 2012). Investigations between the relationship of maternal BMI and leptin have been conducted predominantly in skim HM, which excludes the fat and cellular components of HM (Schuster et al. 2011; Schueler et al. 2013). Further, leptin concentrations have been shown to be higher in whole HM compared to skim HM (Houseknecht et al. 1997; Smith-Kirwin et al. 1998; Kugananthan et al. 2016).

In contrast, maternal serum concentrations of adiponectin are lower if weight and BMI are higher (Arita et al. 1999; Savino et al. 2012b). Results associating maternal BMI and adiponectin concentrations in HM are conflicting (Andreas et al. 2014), with several studies showing no associations (Weyermann et al. 2007; Brunner et al. 2014; Bronsky et al. 2011) and two studies counter-intuitively reporting a positive association (Martin et al. 2006; Woo et al. 2009). As with leptin, use of BMI as a measure of maternal adiposity may contribute to these conflicting findings. Also, both increasing (Ozarda et al. 2012; Bronsky et al. 2011) and decreasing (Martin et al. 2006; Wang et al. 2011; Savino et al. 2012b) trends in adiponectin concentrations across the lactation period have been reported.

Similar to its effects on appetite hormones, it is postulated that maternal adiposity influences macronutrient concentrations in HM. Again, results are conflicting. Excessive adipose tissue storage has been shown to impair amino acid and monosaccharide metabolism and transport (Mihalik et al. 2012; Kelley et al. 1996), yet increased serum amino acid levels were found in mothers with more adipose tissue (Xie
et al. 2014). In lactating women, higher concentrations of HM protein were associated with lower BMI in one study (Bachour et al. 2012), yet with higher BMI (Grote et al. 2016) and higher adiposity (Nommsen et al. 1991) in others. BMI also was found to associate positively with concentration of HM galactose (Grote et al. 2016). There is a possibility that the effect of maternal BC only becomes evident in late but not early lactation, when the fat accumulated during pregnancy is depleted (Nommsen et al. 1991). More precise measurements of maternal adiposity across the lactation period are needed to elucidate effects on HM composition.

This study investigated relationships between maternal adiposity and HM leptin, adiponectin, total protein and lactose. Further, it investigated the relationship between %FM and BMI, and the change in maternal adiposity and component concentrations over the first year of lactation.

5.3 MATERIALS AND METHODS

5.3.1 Study participants
Fifty-nine predominantly Caucasian, English-speaking, breastfeeding mothers were recruited via the Australian Breastfeeding Association and through external networking. Inclusion criteria were: healthy singletons, gestational age ≥37 weeks, fully breastfed at 2 and/or 5 months (World Health Organization 2007) and maternal intention to breastfeed until 12 months. The exclusion criterion was: maternal smoking. Participants were recruited during their 2nd, 5th, 9th and 12th month of lactation and invited to come back at any subsequent time points. Twenty-one participants contributed samples at two or more time points. All participants provided informed written consent and answered a secure online questionnaire that was administered and securely stored at the university. This study was approved by the Human Research Ethics Committee of The University of Western Australia (RA/4/1/4253) and registered with the Australian New Zealand Clinical Trials Registry (ACTRN12616000368437).

5.3.2 Human milk sample collection
HM samples were collected on site at our research laboratory at King Edward Memorial Hospital for Women (Subiaco, Perth, Australia). Pre-feed and post-feed milk samples (~5 mL each) were obtained from the breast(s) the infant fed from by hand expression or with a breast pump and were analysed separately. Samples were collected between 9:30 and 11:30 a.m. to minimise possible circadian influences on the milk composition.
CHAPTER 5: MATERNAL BODY COMPOSITION

Samples were stored at -20 °C for later biochemical analysis.

### 5.3.3 Anthropometry and body composition

Maternal weight was measured using an electronic scale (±0.1 kg; Seca, Chino, CA, USA). Height was self-reported by participants or measured against a calibrated marked wall (accuracy ±0.1 cm). BMI was calculated as kg/m².

%FM was measured with whole body bioimpedance (wrist to ankle) using an ImpediMed SFB7 tetra-polar bioelectrical impedance analyser (Impedimed, Brisbane, Australia) with the participant in a supine position on a non-conductive surface according to the manufacturer’s instructions. Before each session, the external calibration of the bioelectrical impedance analyser was tested with a calibration Test Cell (ImpediMed, Brisbane, Australia). Ten consecutive measurements of %FM were taken within 1–2 min and averaged. Within-participant coefficient of variation for maternal %FM was 0.21%. All measurements were made after the breastfeeding session.

### 5.3.4 Leptin and adiponectin measurements

Total Leptin concentrations in whole and skim HM were measured using the DuoSet Human Leptin ELISA (R&D Systems, Minneapolis, MN, USA) as described previously (Kugananthan et al. 2016). The detection limit was 0.05 ng/mL with a recovery of 96.3%±1.2% (n=10) for skim milk and 97.1%±9.1% (n=10) for whole milk leptin and an inter-assay CV of <7.2%.

Adiponectin was measured in whole HM using the Biovendor Human Adiponectin Sandwich ELISA kit (Life Technologies, Asheville, NC, USA). The detection limit was 1 ng/mL, with a recovery of 96.2%±3.2% (n=10) and an inter-assay CV of <2.5%.

### 5.3.5 Protein and lactose measurements

Protein content was measured using the Bradford assay according to the methods of Mitoulas et al. (Mitoulas et al. 2002). The detection limit was 1.03 g/L, with a recovery of 97.2%±1.4% (n=10) and an inter-assay CV of <1.9%.

Lactose concentration was measured using the enzymatic–spectrophotometric method outlined by Kuhn et al. (Kuhn et al. 1967) according to the methods of Mitoulas et al. (Mitoulas et al. 2002). The detection limit was 30 mM, with a recovery of 98.2%±4.1% (n=10) and an inter-assay CV of <3.5%.
5.3.6 Statistical analyses

Statistical analyses were performed using R 2.15.1 for Windows (R Core Team 2014). The packages nlme (Pinheiro et al. 2011), lattice (Sarkar 2008), and RColorBrewer (Neuwirth 2014) were used for linear mixed effects modelling and data representation respectively. Descriptive statistics are reported as mean ± SD and range unless otherwise stated; model parameters are presented as estimate ± standard error (SE).

In order to collect systematic information over time and at fixed moments in time and to make better use of the collected data, a combined data approach that considers individual-level random effects to account for participants measured at two or more study sessions was adopted. We further contrasted the results from the combined data and from the longitudinal subset to confirm our findings.

During this study, infants were measured at least at one of the four time points (2, 5, 9 and 12 months postpartum). An approximate sample size was calculated using the ‘Linear multiple regression: fixed model: r² increase’ option in G*Power (Faul et al. 2009) as if this was a cross-sectional study with equal numbers at each time. Allowing four predictors (one main effect, three group contrasts), α=0.05 and 22 participants at each time point (88 sample points = 22 participants × 4 time points) gave the study power of 0.80 to detect an effect size of 0.15. This approach was selected as there is no closed form expression suitable for the calculation of sample sizes for this research design (Diggle et al. 2002), with the consideration that longitudinal study design is more powerful. To maintain predicted power and to address issues relating to missed visits, such as inability to attend due to illness, and unwillingness of mothers approached at 2 months (n=8) to commit to a study that requires breastfeeding to 12 months, the recruitment of participants continued past 22, resulting in 111 sessions for 59 (21 longitudinal, 38 cross-sectional) participants.

BC data at 9 months of lactation is missing for two longitudinal participants. Missing data also occurred for all milk components due to insufficient milk sample volumes. Missing data was dealt with using complete case (regression models) or available case (descriptive statistics) approaches. Milk samples were not pooled for biochemical analysis; thus, measures were not averaged. Sample sizes are presented in Table 5.1.
### Table 5.1 Sample sizes used in statistical analyses.

<table>
<thead>
<tr>
<th>Month of Lactation</th>
<th>2</th>
<th>5</th>
<th>9</th>
<th>12</th>
<th>Total</th>
</tr>
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<tbody>
<tr>
<td>Participants a</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>38</td>
</tr>
<tr>
<td>Cross-sectional</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>38</td>
</tr>
<tr>
<td>Longitudinal</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>21</td>
</tr>
<tr>
<td>Total</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>59</td>
</tr>
<tr>
<td>Sessions</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cross-sectional</td>
<td>8</td>
<td>8</td>
<td>13</td>
<td>9</td>
<td>38</td>
</tr>
<tr>
<td>Longitudinal</td>
<td>15</td>
<td>21</td>
<td>19</td>
<td>18</td>
<td>73</td>
</tr>
<tr>
<td>Total</td>
<td>23</td>
<td>29</td>
<td>32</td>
<td>27</td>
<td>111</td>
</tr>
<tr>
<td>Samples (complete cases)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cross-sectional</td>
<td>19</td>
<td>21</td>
<td>33</td>
<td>27</td>
<td>100</td>
</tr>
<tr>
<td>Longitudinal</td>
<td>41</td>
<td>55</td>
<td>47</td>
<td>40</td>
<td>183</td>
</tr>
<tr>
<td>Total</td>
<td>60</td>
<td>76</td>
<td>80</td>
<td>67</td>
<td>283</td>
</tr>
<tr>
<td>Samples (available cases) b</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Whole milk adiponectin</td>
<td>66</td>
<td>79</td>
<td>86</td>
<td>72</td>
<td>303</td>
</tr>
<tr>
<td>Whole milk leptin</td>
<td>66</td>
<td>79</td>
<td>86</td>
<td>72</td>
<td>303</td>
</tr>
<tr>
<td>Skim milk leptin</td>
<td>62</td>
<td>77</td>
<td>85</td>
<td>71</td>
<td>295</td>
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<tr>
<td>Total protein</td>
<td>64</td>
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<td>69</td>
<td>298</td>
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<tr>
<td>Lactose</td>
<td>65</td>
<td>78</td>
<td>86</td>
<td>67</td>
<td>296</td>
</tr>
</tbody>
</table>

* The number of participants at each time point is the same as the number of sessions, thus not specified in the table; *b* The number of samples (including pre-feed and post-feed) analysed; this differs by component.

Linear models were used to investigate associations between maternal BMI or %FM (predictors) and each of the composition variables (responses), with and without controlling for month of lactation (four-level factor or linear predictor). Associations with month of lactation were assessed using omnibus F-tests and specific post-hoc tests comparing each of the subsequent time points with 2 months. Appropriate random effects were selected by comparing four models for each analysis using a likelihood ratio test. Models were (a) linear regression, and linear mixed effects models with random effects of one of: (b) effect of general inter-individual variation present in the study population; (c) effect of the month of lactation samples were collected at, in addition to inter-individual variation; and (d) the effect of pre- and post-feed samples along with inter-individual variation. Whether the overall effect of maternal adiposity on HM component concentrations differs by month of lactation was also investigated by including interactions between BMI/%FM and the month of lactation (factor only). To allow for realistic interpretation of the intercept values in the model outputs, maternal BMI and %FM have been centred at the upper bounds of the ‘healthy’ range (25 kg/m² for BMI, and 33% for %FM) (World Health Organization 2000; Gallagher *et al.* 2000). Where significant outlier values were identified from a kernel density plot, models were
run with and without these values to determine how they might be influencing the findings.

An intercept-only linear mixed effects model was used to calculate the coefficient of variation for maternal %FM measurements (\(n=10\), 10 measurements each).

## 5.4 RESULTS

### 5.4.1 Participants

Participant adiposity measures and HM components’ concentrations are shown in Table 2. Mean maternal age was 33.4 ± 4.2 years and parity was 1.8 ± 0.8 at the start of the study. At the first session attended at either 2, 5, 9 or 12 months postpartum participants were classified as being underweight (BMI <18.5, 5%, \(n=3\); %FM <21, 7%, \(n=4\)), of normal weight (BMI 18.5–24.9, 54%, \(n=32\); %FM 21–32.9, 50%, \(n=29\)), overweight (BMI 25–29.9, 24%, \(n=14\); %FM 33–38.9, 28%, \(n=16\)) or obese (BMI >30, 17%, \(n=10\); %FM >39, 15%, \(n=9\)) (Gallagher et al. 2000). Infant male/female ratio was 33/26.

### Table 5.2 Maternal adiposity and human milk components concentrations presented at the months after birth for combined subset (\(n=59\)) expressed as mean ± standard deviation (SD, range). Some participants (\(n=21\)) contributed milk samples at multiple time points.

<table>
<thead>
<tr>
<th>Month of Lactation</th>
<th>2 ((n=23))</th>
<th>5 ((n=29))</th>
<th>9 ((n=32))</th>
<th>12 ((n=27))</th>
<th>Total ((n=111))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal BMI  (^c)</td>
<td>27.0 ± 7.3  (^{ab})</td>
<td>23.5 ± 4.5</td>
<td>23.9 ± 5.2</td>
<td>24.4 ± 5.5</td>
<td>24.6 ± 5.7</td>
</tr>
<tr>
<td>(20.1–51.3)</td>
<td>(17.0–35.2)</td>
<td>(16.9–37.2)</td>
<td>(18.2–37.2)</td>
<td>(16.9–51.3)</td>
<td></td>
</tr>
<tr>
<td>Maternal fat mass (%)</td>
<td>34.9 ± 6.4</td>
<td>32.5 ± 6.0</td>
<td>30.9 ± 7.9</td>
<td>31.3 ± 7.2</td>
<td>32.3 ± 7.0</td>
</tr>
<tr>
<td>(19.6–49.3)</td>
<td>(23.2–47.2)</td>
<td>(16.7–47.9)</td>
<td>(19.4–45.3)</td>
<td>(16.7–49.3)</td>
<td></td>
</tr>
<tr>
<td>Whole milk leptin (ng/mL)</td>
<td>0.55 ± 0.29</td>
<td>0.50 ± 0.17</td>
<td>0.53 ± 0.15</td>
<td>0.54 ± 0.13</td>
<td>0.53 ± 0.19</td>
</tr>
<tr>
<td>(0.21–2.24)</td>
<td>(0.20–0.85)</td>
<td>(0.21–0.99)</td>
<td>(0.24–0.89)</td>
<td>(0.20–2.24)</td>
<td></td>
</tr>
<tr>
<td>Skim milk leptin (ng/mL)</td>
<td>0.34 ± 0.21</td>
<td>0.27 ± 0.07</td>
<td>0.26 ± 0.09</td>
<td>0.26 ± 0.08</td>
<td>0.28 ± 0.12</td>
</tr>
<tr>
<td>(0.19–1.46)</td>
<td>(0.20–0.48)</td>
<td>(0.19–0.76)</td>
<td>(0.19–0.54)</td>
<td>(0.19–1.46)</td>
<td></td>
</tr>
<tr>
<td>Adiponectin (ng/mL)</td>
<td>11.12 ± 4.39</td>
<td>9.30 ± 3.94</td>
<td>8.46 ± 2.26</td>
<td>11.07 ± 7.88</td>
<td>9.88 ± 5.05</td>
</tr>
<tr>
<td>Total protein (g/L)</td>
<td>12.94 ± 6.15</td>
<td>11.7 ± 5.70</td>
<td>10.83 ± 4.63</td>
<td>12.83 ± 6.74</td>
<td>11.96 ± 5.82</td>
</tr>
<tr>
<td>(6.54–31.51)</td>
<td>(7.00–34.76)</td>
<td>(3.32–29.69)</td>
<td>(6.60–36.89)</td>
<td>(3.32–36.89)</td>
<td></td>
</tr>
<tr>
<td>Lactose (g/L)</td>
<td>67.54 ± 9.05</td>
<td>68.07 ± 8.10</td>
<td>68.37 ± 8.83</td>
<td>69.70 ± 9.11</td>
<td>68.41 ± 8.75</td>
</tr>
<tr>
<td>(50.35–89.06)</td>
<td>(50.92–110.07)</td>
<td>(51.81–100.05)</td>
<td>(51.00–98.36)</td>
<td>(50.35–110.07)</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Data are mean ± SD and ranges. \(^b\) Concentrations of components are measured in both pre- and post-feed milk samples. \(^c\) BMI - body mass index.
5.4.2 Changes in components’ concentrations with feeding (pre- and post-feed)

HM component concentrations did not differ between pre-feed and post-feed samples in univariate models or after accounting for the month of lactation as a linear effect model or as a factor (Table 5.3).

Table 5.3 Relative concentrations of pre-feed human milk samples compared to post-feed samples with and without accounting for possible month of lactation effects.

<table>
<thead>
<tr>
<th>Predictor</th>
<th>Univariate a PE ± SE</th>
<th>p</th>
<th>Accounting for Month of Lactation (Linear) b PE ± SE</th>
<th>p</th>
<th>Accounting for Month of Lactation (Factor) b PE ± SE</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adiponectin (ng/mL)</td>
<td>0.45 ± 0.42</td>
<td>0.29</td>
<td>0.45 ± 0.42</td>
<td>0.30</td>
<td>0.45 ± 0.42</td>
<td>0.29</td>
</tr>
<tr>
<td>Leptin (ng/mL)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Whole milk</td>
<td>−0.008 ± 0.016</td>
<td>0.65</td>
<td>−0.008 ± 0.017</td>
<td>0.65</td>
<td>0.007 ± 0.016</td>
<td>0.66</td>
</tr>
<tr>
<td>Skim milk</td>
<td>−0.002 ± 0.009</td>
<td>0.84</td>
<td>−0.002 ± 0.009</td>
<td>0.86</td>
<td>−0.001 ± 0.009</td>
<td>0.87</td>
</tr>
<tr>
<td>Protein (g/L)</td>
<td>−0.14 ± 0.32</td>
<td>0.68</td>
<td>−0.14 ± 0.32</td>
<td>0.68</td>
<td>−0.14 ± 0.32</td>
<td>0.68</td>
</tr>
<tr>
<td>Lactose (g/L)</td>
<td>−0.36 ± 0.91</td>
<td>0.69</td>
<td>−0.38 ± 0.88</td>
<td>0.66</td>
<td>−0.39 ± 0.88</td>
<td>0.66</td>
</tr>
</tbody>
</table>

Data are parameter estimate ± SE. Analyses were run on pre- and post-feed samples using complete case approach. a Effects of predictors taken from univariate linear mixed effects models; b Effects of predictors taken from linear mixed effects models that accounted for the month of lactation as linear main effect or as a factor; c Month of lactation is significant (p<0.036). PE—parameter estimate; SE—standard error.

5.4.3 Associations in combined subset

5.4.3.1 Differences in concentrations of HM components at different months of lactation

Table 5.4 presents the changes in HM components’ concentrations in the combined subset (n=57) at four time points during first 12 months of lactation.

While component concentrations differed by the month of lactation within participants for all components (lactose: p=0.031; adiponectin, whole and skim milk leptin, protein: p<0.001), no consistent month of lactation-related patterns were seen for whole milk leptin (p>0.47), protein (p>0.37) and lactose (p>0.26).

Skim milk leptin decreased non-linearly over the months of lactation (univariate: p=0.024). Post-hoc tests showed that adiponectin concentration at 9 months was −2.27±0.88 ng/mL lower (p=0.013) than that at 2 months of lactation (univariate: p=0.042) (Table 5.4).
Table 5.4 Associations between human milk appetite hormones and maternal adiposity. Values are parameter estimates ± standard error (n=57). Some participants (n=21) contributed milk samples at multiple time points.

<table>
<thead>
<tr>
<th>Predictor</th>
<th>Adiponectin (ng/mL)</th>
<th>Whole Milk Leptin (ng/mL)</th>
<th>Skim Milk Leptin (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PE ± SE</td>
<td>p</td>
<td>PE ± SE</td>
</tr>
<tr>
<td>BMI</td>
<td>0.10 ± 0.07</td>
<td>0.17</td>
<td>0.01 ± 0.003</td>
</tr>
<tr>
<td>%FM</td>
<td>0.01 ± 0.06</td>
<td>0.81</td>
<td>0.006 ± 0.002</td>
</tr>
<tr>
<td>Month d</td>
<td>-</td>
<td>0.042</td>
<td>-</td>
</tr>
<tr>
<td>Intercept</td>
<td>10.58 ± 0.71</td>
<td>-</td>
<td>0.55 ± 0.03</td>
</tr>
<tr>
<td>5 e</td>
<td>-1.39 ± 0.87</td>
<td>0.12</td>
<td>-0.06 ± 0.05</td>
</tr>
<tr>
<td>9 e</td>
<td>-2.27 ± 0.88</td>
<td>0.013</td>
<td>-0.01 ± 0.05</td>
</tr>
<tr>
<td>12 e</td>
<td>-0.26 ± 0.93</td>
<td>0.78</td>
<td>-0.02 ± 0.05</td>
</tr>
<tr>
<td>Adjusted model for %FM (month of lactation as linear main effect) c</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intercept</td>
<td>9.76 ± 0.61</td>
<td>-</td>
<td>0.52 ± 0.03</td>
</tr>
<tr>
<td>%FM</td>
<td>0.008 ± 0.06</td>
<td>0.90</td>
<td>0.006 ± 0.002</td>
</tr>
<tr>
<td>Month d</td>
<td>-0.05 ± 0.09</td>
<td>0.57</td>
<td>0.003 ± 0.004</td>
</tr>
<tr>
<td>Adjusted model for %FM (month of lactation as a factor) c</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intercept</td>
<td>10.59 ± 0.72</td>
<td>-</td>
<td>0.54 ± 0.03</td>
</tr>
<tr>
<td>%FM</td>
<td>-0.01 ± 0.06</td>
<td>0.86</td>
<td>0.006 ± 0.002</td>
</tr>
<tr>
<td>Month d</td>
<td>-</td>
<td>0.044</td>
<td>-</td>
</tr>
<tr>
<td>5 e</td>
<td>-1.40 ± 0.88</td>
<td>0.12</td>
<td>-0.04 ± 0.04</td>
</tr>
<tr>
<td>9 e</td>
<td>-2.30 ± 0.90</td>
<td>0.014</td>
<td>0.01 ± 0.05</td>
</tr>
<tr>
<td>12 e</td>
<td>-0.29 ± 0.95</td>
<td>0.77</td>
<td>0.007 ± 0.05</td>
</tr>
<tr>
<td>Adjusted model for BMI (month of lactation as linear main effect) c</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intercept</td>
<td>9.76 ± 0.60</td>
<td>-</td>
<td>0.52 ± 0.03</td>
</tr>
<tr>
<td>BMI</td>
<td>0.09 ± 0.07</td>
<td>0.19</td>
<td>0.01 ± 0.003</td>
</tr>
<tr>
<td>Month d</td>
<td>-0.04 ± 0.09</td>
<td>0.62</td>
<td>0.002 ± 0.004</td>
</tr>
<tr>
<td>Adjusted model for BMI (month of lactation as a factor) c</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intercept</td>
<td>10.51 ± 0.71</td>
<td>-</td>
<td>0.54 ± 0.003</td>
</tr>
<tr>
<td>BMI</td>
<td>0.07 ± 0.07</td>
<td>0.33</td>
<td>0.01 ± 0.003</td>
</tr>
<tr>
<td>Month d</td>
<td>-</td>
<td>0.063</td>
<td>-</td>
</tr>
<tr>
<td>5 e</td>
<td>-1.23 ± 0.89</td>
<td>0.17</td>
<td>-0.03 ± 0.04</td>
</tr>
<tr>
<td>9 e</td>
<td>-2.13 ± 0.89</td>
<td>0.021</td>
<td>0.01 ± 0.04</td>
</tr>
<tr>
<td>12 e</td>
<td>-0.16 ± 0.94</td>
<td>0.87</td>
<td>0.004 ± 0.05</td>
</tr>
</tbody>
</table>

Data are parameter estimate ± SE. Analyses were run on pre- and post-feed samples using complete case approach. a Significant p-values are in bold font; b Effects of predictors taken from univariate linear mixed effects models; c Effects of predictors taken from linear mixed effects models that accounted for the month of lactation as linear main effect or as a factor; d Omnibus F-test; e Post-hoc test with reference 2 months. Abbreviations: BMI - body mass index; %FM - percentage fat mass; PE - parameter estimate; SE - standard error.

5.4.3.2 Associations between maternal adiposity and HM leptin

Table 5.4 presents associations between adiposity and HM components’ concentrations seen in the combined subset (n=57) at four time points during first 12 months of lactation.
Higher %FM and BMI were associated with higher concentrations of both whole (Figures 5.1a and 5.1b) and skim milk (Figures 5.2a and 5.2b) leptin (Table 5.4). Accounting for the month of lactation as a main linear effect or as a factor did not change the associations with %FM and BMI for both whole and skim milk leptin. Significant negative interactions were seen between %FM and the month of lactation for whole milk leptin, (2 m: reference; 5 m: \(-0.02\pm0.01, p=0.023\); 9 m: \(-0.02\pm0.01, p=0.003\); 12 m: \(-0.03\pm0.01, p<0.001\); month of lactation as a factor: \(p=0.008\)), and for skim milk leptin (2 m: reference; 5 m: \(-0.02\pm0.01, p<0.001\); 9 m: \(-0.02\pm0.01, p<0.001\); 12 m: \(-0.02\pm0.01, p=0.002\); month of lactation as a factor: \(p<0.001\)); and also between BMI and the month of lactation for whole milk leptin, (2 m: reference; 5 m: \(-0.02\pm0.01, p=0.026; 9 m: -0.03\pm0.01, p<0.001; 12 m: -0.03\pm0.01, p<0.001; month of lactation as a factor: \(p<0.001\)), and for skim milk leptin (2 m: reference; 5 m: \(-0.02\pm0.01, p<0.001; 9 m: -0.02\pm0.01, p<0.001; 12 m: -0.01\pm0.01, p=0.005; month of lactation as a factor: \(p=0.001\)), indicating that the association between adiposity and leptin weakens over the first 12 months of lactation.

Figure 5.1 Associations between whole human milk (HM) leptin and (a) maternal percentage fat mass, and (b) maternal BMI. Combined subset data points (measured in pre- and post-feed samples) are shown as cross-sectional (pale blue) and longitudinal (dark blue). Lines are fixed effects from univariate linear mixed effect models: pale blue dotted line - combined cohort (Table 5.4); medium blue dashed line - combined cohort with outliers removed (Section 5.4.3.2); solid dark blue line - longitudinal cohort (Table S5.1).

Removing statistically significant outliers resulted in either weakening or an absence of the association between either %FM or BMI and whole and skim milk leptin in the univariate models (%FM: 0.004±0.002 ng/mL, \(p=0.066\); 0.003±0.001 ng/mL, \(p=0.043\), respectively; BMI: 0.004±0.002 ng/mL, \(p=0.065\); 0.004±0.002 ng/mL, \(p=0.043\), respectively.
and after accounting for the month of lactation as a linear effect (%FM: 0.004±0.002 ng/mL, \( p=0.066 \), age: \( p=0.17 \); 0.002±0.001 ng/mL, \( p=0.12 \), age: \( p=0.30 \), respectively; BMI: 0.005±0.002 ng/mL, \( p=0.053 \), age: \( p=0.25 \); 0.004±0.002 ng/mL, \( p=0.039 \), age: \( p=0.17 \), respectively) or as a factor (%FM: 0.004±0.002 ng/mL, \( p=0.044 \), age: \( p=0.37 \); 0.002±0.001 ng/mL, \( p=0.14 \), age: \( p=0.15 \), respectively; BMI: 0.004±0.002 ng/mL, \( p=0.072 \), age: \( p=0.53 \); 0.004±0.002 ng/mL, \( p=0.044 \), age: \( p=0.37 \), respectively). No interaction between either %FM or BMI and the month of lactation as a factor was seen (%FM: whole milk leptin: \( p=0.37 \); skim milk leptin: \( p=0.13 \); BMI: whole milk leptin: \( p=0.24 \); skim milk leptin: \( p=0.18 \)).

**Figure 5.2** Associations between skim human milk (HM) leptin and (a) maternal percentage fat mass, and (b) maternal BMI. Combined subset data points are shown as cross-sectional (pale purple) and longitudinal (dark purple). Lines are fixed effects from univariate linear mixed effect models: pale purple dotted line - combined cohort (Table 5.4); medium purple dashed line - combined cohort with outliers removed (Section 5.4.3.2); solid dark purple line - longitudinal cohort (Table S5.1).

### 5.4.3.3 Associations between maternal adiposity and HM adiponectin

HM adiponectin was not significantly associated with either %FM or BMI in the univariate models (Table 5.4) or after accounting for the month of lactation. No interactions were seen between the month of lactation as a factor and either %FM (\( p=0.51 \)) or BMI (\( p=0.62 \)).

Removing a statistically significant outlier did not change the conclusion (%FM: \( p \geq 0.50 \); BMI: \( p \geq 0.083 \)) and no interaction between the month of lactation and either %FM (\( p=0.54 \)) or BMI (\( p=0.081 \)) was seen.
**5.4.3.4 Associations between maternal adiposity and HM protein**

Higher %FM was associated with higher concentrations of protein in HM in univariate model (Table 5.5; Figure 5.3a). Accounting for the month of lactation made the association between %FM and protein concentrations weaker but still significant. BMI was not associated with concentrations of protein in HM in the univariate model (Figure 5.3b) or after accounting for the month of lactation. No interaction with the month of lactation as a factor was seen for both %FM ($p=0.21$) and BMI ($p=0.16$).

**Table 5.5** Associations between human milk macronutrients and maternal adiposity. Values are parameter estimates ± standard error ($n=57$). Some participants ($n=21$) contributed milk samples at multiple time points.

<table>
<thead>
<tr>
<th>Predictor</th>
<th>Lactose (g/L)</th>
<th>Protein (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PE ± SE</td>
<td>$p$</td>
</tr>
<tr>
<td><strong>Univariate models</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMI</td>
<td>0.06 ± 0.14</td>
<td>0.66</td>
</tr>
<tr>
<td>%FM</td>
<td>0.08 ± 0.11</td>
<td>0.48</td>
</tr>
<tr>
<td>Month</td>
<td>–</td>
<td>0.65</td>
</tr>
<tr>
<td>Intercept</td>
<td>67.32 ± 1.43</td>
<td>–</td>
</tr>
<tr>
<td>5 $^e$</td>
<td>0.56 ± 1.89</td>
<td>0.77</td>
</tr>
<tr>
<td>9 $^e$</td>
<td>1.31 ± 1.88</td>
<td>0.49</td>
</tr>
<tr>
<td>12 $^e$</td>
<td>2.35 ± 1.97</td>
<td>0.24</td>
</tr>
<tr>
<td><strong>Adjusted model for %FM (month of lactation as linear main effect)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intercept</td>
<td>68.89 ± 1.13</td>
<td>–</td>
</tr>
<tr>
<td>%FM</td>
<td>0.09 ± 0.11</td>
<td>0.43</td>
</tr>
<tr>
<td>Month</td>
<td>0.08 ± 0.15</td>
<td>0.59</td>
</tr>
<tr>
<td><strong>Adjusted model for %FM (month of lactation as a factor)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intercept</td>
<td>67.58 ± 1.44</td>
<td>–</td>
</tr>
<tr>
<td>%FM</td>
<td>0.06 ± 0.11</td>
<td>0.55</td>
</tr>
<tr>
<td>Month</td>
<td>–</td>
<td>0.67</td>
</tr>
<tr>
<td>5 $^e$</td>
<td>0.60 ± 1.85</td>
<td>0.75</td>
</tr>
<tr>
<td>9 $^e$</td>
<td>1.37 ± 1.87</td>
<td>0.50</td>
</tr>
<tr>
<td>12 $^e$</td>
<td>2.27 ± 1.96</td>
<td>0.25</td>
</tr>
<tr>
<td><strong>Adjusted model for BMI (month of lactation as linear main effect)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intercept</td>
<td>67.71 ± 2.92</td>
<td>–</td>
</tr>
<tr>
<td>BMI</td>
<td>0.06 ± 0.13</td>
<td>0.63</td>
</tr>
<tr>
<td>Month</td>
<td>0.20 ± 0.17</td>
<td>0.26</td>
</tr>
<tr>
<td><strong>Adjusted model for BMI (month of lactation as a factor)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intercept</td>
<td>67.55 ± 1.44</td>
<td>–</td>
</tr>
<tr>
<td>BMI</td>
<td>0.06 ± 0.13</td>
<td>0.66</td>
</tr>
<tr>
<td>Month</td>
<td>–</td>
<td>0.69</td>
</tr>
<tr>
<td>5 $^e$</td>
<td>0.63 ± 1.88</td>
<td>0.74</td>
</tr>
<tr>
<td>9 $^e$</td>
<td>1.29 ± 1.87</td>
<td>0.49</td>
</tr>
<tr>
<td>12 $^e$</td>
<td>2.22 ± 1.95</td>
<td>0.26</td>
</tr>
</tbody>
</table>

Data are parameter estimate ± SE. Analyses were run on pre- and post-feed samples using complete case approach. $^a$ Significant $p$-values are in bold font; $^b$ Effects of predictors taken from univariate linear mixed effects models; $^c$ Effects of predictors taken from linear mixed effects models that accounted for the month of lactation as linear main effect or as a factor; $^d$ Omnibus F-test; $^e$ Post-hoc test with reference 2 months. Abbreviations: BMI - body mass index; %FM - percentage fat mass; PE - parameter estimate; SE - standard error.


### Figure 5.3 Associations between protein concentration and (a) maternal percentage fat mass, and (b) maternal BMI. Combined subset data points are shown as cross-sectional (pale orange) and longitudinal (dark orange). Lines are fixed effects from univariate linear mixed effect models: pale orange dotted line - combined cohort (Table 5.5); solid dark red line - longitudinal cohort (Table S5.1).

#### 5.4.3.5 Associations between maternal adiposity and HM lactose

Neither %FM or BMI were associated with concentrations of lactose in HM in univariate model (Table 5.5) or after accounting for the month of lactation. No interaction with the month of lactation as a factor was seen for BMI ($p=0.19$), but a significant positive interaction was seen between %FM and the month of lactation (2 m: reference; 5 m: $0.12\pm0.33$, $p=0.71$; 9 m: $-0.14\pm0.30$, $p=0.63$; 12 m: $0.58\pm0.31$, $p=0.068$; month of lactation as a factor: $p=0.029$), indicating that association between %FM and lactose strengthens over the first 12 months of lactation.

#### 5.4.4 Associations in the longitudinal subset

##### 5.4.4.1 Participants

Longitudinal ($n=21$) participants’ characteristics and HM components’ concentrations are shown in Table S5.2. Participants in longitudinal subset were generally leaner than in the combined subset, but none were underweight. At the first session they were classified as: normal weight (BMI 18.5–24.9, 67%, $n=14$; %FM 21–32.9, 57%, $n=12$), overweight (BMI 25–29.9, 19%, $n=4$; %FM 33–38.9, 29%, $n=6$) or obese (BMI >30, 14%, $n=3$; %FM >39,14%, $n=3$) (Gallagher et al. 2000). Infant male:female ratio was 10:11.
5.4.4.2 Longitudinal changes in concentrations of HM components

Table S5.1 presents the changes in HM component concentrations in the longitudinal subset (n=21 participants, 73 sessions). While component concentrations differed by the month of lactation within participants for all components (lactose: \( p=0.020 \); adiponectin, whole and skim milk leptin, protein: \( p<0.001 \)), no consistent month of lactation-related patterns were seen for adiponectin \((p>0.32)\), whole milk leptin \((p>0.11)\) or lactose \((p>0.46)\). Although the overall pattern for protein was not significant \((p>0.10)\), post-hoc tests showed that protein concentration at 9 months was \(3.80\pm1.65\ \text{g/L} \) lower than that at 2 months of lactation (univariate, \( p=0.027 \)). Skim milk leptin decreased non-linearly over the months of lactation (univariate: \( p=0.007 \)).

5.4.4.3 Associations between maternal BMI and %FM

A strong relationship \((p<0.001)\) was observed between maternal BMI and %FM in the longitudinal subset, with a one-unit increase in BMI associated with a \(1.07\%\pm0.17\% \) increase in %FM. After accounting for the month of lactation there was a significant overall difference in %FM with the month of lactation \((p=0.008)\) and the association between maternal BMI and %FM remained significant \((1.01\%\pm0.17,\ p<0.001)\).

Over the first year of lactation, maternal %FM decreased in non-linear fashion (largest drop between 9 and 12 months) by more than 2% after accounting for the month of lactation as a factor \((-2.26\%\pm0.67\%,\ p=0.002;\ \text{age}:\ p<0.001)\) or by 0.23% per month after accounting for the month of lactation as a linear effect \((-0.23\%\pm0.06\ %,\ p<0.001)\) (Figure 5.4a).

Over the first year of lactation, maternal BMI decreased in an almost linear fashion; decreasing by \(-1.05\pm0.24\ \text{kg/m}^2\ (p<0.001;\ \text{month of lactation}:\ p<0.001)\) over the ten months of the study when accounting for the month of lactation as a factor, or by \(-0.10\pm0.02\ \text{kg/m}^2\ (p<0.001)\) per month when accounting for the month of lactation as a linear effect (Figure 5.4b).
Figure 5.4 Longitudinal changes in (a) maternal percentage fat mass and (b) maternal BMI from 2 to 12 months of lactation. Lines are colour-coordinated for the individual participants (e.g. dark orange in panel (a) is a same dark orange in panel (b)) for illustrative purposes only (n=21).

### 5.4.4.4 Associations between maternal adiposity and HM components in longitudinal subset

No associations were seen between measures of maternal adiposity and adiponectin (%FM: $p>0.36$; BMI: $p>0.39$), whole (%FM: $p>0.051$; BMI: $p>0.082$) and skim milk leptin (%FM: $p>0.51$; BMI: $p>0.78$) and lactose concentrations (%FM: $p>0.56$; BMI: $p>0.68$) in either univariate models or after accounting for the month of lactation (Table S5.1).

Higher %FM was associated with higher protein concentration in the univariate model ($0.19\pm0.09 \text{ g/L, } p=0.035$); when the month of lactation was accounted for, this association was no longer seen ($0.14\pm0.09 \text{ g/L, } p=0.12$).

No interaction with the month of lactation as a factor was seen for adiponectin (%FM: $p=0.87$; BMI: $p=0.52$), whole milk leptin (%FM: $p=0.62$; BMI: $p=0.36$), skim milk leptin (%FM: $p=0.37$), protein (%FM: $p=0.78$; BMI: $p=0.25$) and lactose (%FM: $p=0.22$; BMI: $p=0.30$).

Significant negative interactions were seen between BMI and the month of lactation for skim milk leptin, (2 m: reference; 5 m: $-0.02\pm0.01$, $p=0.005$; 9 m: $-0.01\pm0.01$, $p=0.059$; 12 m: $-0.01\pm0.01$, $p=0.059$; month of lactation as a factor: $p=0.043$), indicating that association between BMI and skim milk leptin weakens over the first 12 months of lactation.
5.5 DISCUSSION
The hormonal regulation of appetite plays a central role in infant developmental programming facilitating a lifelong healthy balance between energy intake and expenditure (Hassiotou et al. 2014a). Concentrations of appetite hormones and macronutrients present in HM influence regulation of appetite, energy expenditure pathways and growth trajectory in the developing infant (Woo et al. 2012; Miralles et al. 2006; Luque et al. 2015). Maternal adiposity may play a critical role in regulation of concentrations of HM leptin, adiponectin, protein and lactose, and thus of the ingested dose of these components by the infant. This study found some associations of higher maternal adiposity with higher concentrations of HM leptin and protein, but no associations with adiponectin or lactose. Concentrations of whole milk leptin, adiponectin, protein and lactose did not systematically change with milk removal during a breastfeed, or over the first year of lactation - a period which includes both exclusive breastfeeding and the introduction of complementary foods.

This study has shown that the greater the maternal %FM or BMI, the higher the concentrations of HM leptin. This is logical in that a greater amount of adipose tissue results in more leptin secreted into the circulation and thus increased amounts are transferred into the milk. In young infants, HM is believed to be a major source of leptin, due to immature endogenous leptin-synthesising mechanisms (Oliver et al. 2002). Leptin may provide both short and long term appetite control acting as a neurotrophic molecule targeting the hypothalamus to stimulate neural connections with other key appetite nuclei (Bouret et al. 2004a). Higher HM leptin concentrations are associated with lower infant weight, weight gain and adiposity (Fields et al. 2012; Miralles et al. 2006) while higher concentrations in infant serum are associated with greater lean body mass (TBW) (Savino et al. 2002; Savino et al. 2008), suggesting a pivotal role in regulating infant growth and BC.

One might speculate that increased amounts of HM leptin supplied to an infant may be detrimental to the development of infant appetite control and growth. However, concentrations of leptin do not reflect the 24-h dose received by the infant and indeed 24-h MI are variable between dyads (Kent et al. 2013). In particular, obese mothers are more likely to have reduced milk production due to incomplete differentiation of mammary epithelial cells (Twigger et al. 2015). Conversely, lean mothers with very low plasma leptin concentrations may produce milk with low leptin levels, thus signalling marginal environment and promoting rapid infant growth while supported by maternal metabolism (Quinn et al. 2015). Lower infant serum leptin levels would thus reduce the
neurotrophic effects on the hypothalamic appetite circuitry and lower satiety stimulation (Miralles et al. 2006; Bouret et al. 2004b). Accordingly, maintenance of healthy maternal adiposity during pregnancy and lactation may ensure appropriate levels of leptin supply to the infant, supporting the optimal programming of appetite control in infancy.

Whilst the lack of relationship between either maternal %FM or BMI and HM leptin in the longitudinal subset (Figures 5.1 and 5.2) may seem counter-intuitive, there is a marked reduction in the variability of both the maternal BC and HM leptin levels, restricting applicability of the results (Tables S5.1, S5.2). However, HM leptin concentrations have not been shown to differ between obese and non-obese mothers (Uysal et al. 2002). Furthermore, the contribution by the lactocytes to leptin levels in HM (Hassiotou et al. 2014c; Smith-Kirwin et al. 1998) is not known. These limitations may explain why some studies (n=11) find an association and others do not (n=4) (Andreas et al. 2014). Moreover, the majority of previous studies analysed leptin in skim HM in cross-sectional cohorts and are restricted to the first and third months postpartum (Andreas et al. 2014), limiting their ability to determine leptin profiles during lactation in women of varying BC. Only three studies have analysed %FM using DXA (Schueler et al. 2013), skinfold measures (Quinn et al. 2015) and BIA (Khodabakhshi et al. 2015) and they found a strong association between %FM and skim milk leptin, consistent with the results from our study. Interestingly, Khodabakhshi et al. (2015) (Khodabakhshi et al. 2015) found association between HM leptin and both %FM and BMI only in the subset of mothers of obese infants but not in the subset of mothers of normal infants, although these two groups did not differ by BMI or leptin concentration.

Concentrations of adiponectin in HM were not related to maternal %FM and BMI in either the combined or longitudinal subsets in this study. This is not consistent with the physiological inverse relationship between the number of white adipocytes and serum adiponectin levels in humans (Arita et al. 1999), however it is in agreement with a recent meta-analysis by Andreas et al. (Andreas et al. 2014) who reported the absence of a consistent relationship, either positive or negative, between maternal BMI and colostrum or skim HM adiponectin. We have now expanded the absence of a relationship to maternal %FM and whole HM adiponectin. Thus, it is unlikely that maternal adiposity plays a major role in influencing HM adiponectin levels and it suggests the majority of HM adiponectin may be synthesized and controlled by the mammary gland (Anderson et al. 2016), highlighting the importance of this HM
hormone for the infant. Indeed, it appears that HM adiponectin levels regulate infant growth with higher levels of HM adiponectin being associated with lower WAZ and WLZ z-scores at 6 months of age (Woo et al. 2009) and higher WAZ and WLZ scores over the first 2 years of life (Woo et al. 2012). These results were also supported by Brunner et al. (Brunner et al. 2014), who found that higher concentrations of HM adiponectin at 6 weeks were associated with lower infant FFM and weight at 4 months as well as greater weight and FM at 1 and 2 years of age. The follow up at 3, 4 and 5 years of age has not shown any relationship with the exception of the positive association between HM adiponectin levels at 4 months postpartum and FM at 4 years (Meyer et al. 2016). This reversal of the initial trend in early life is speculated to be related to the timing of cessation of breastfeeding (Woo et al. 2012). High HM adiponectin levels may initially down-regulate infant growth, and later promote adipogenesis and adipocyte hypertrophy (Bieswal et al. 2006). Conversely lean populations with lower concentrations of HM adiponectin demonstrate a positive association with the infant WAZ scores. This suggests that the association between HM adiponectin and infant growth may in fact be parabolic, further highlighting the pleiotropic effects of adiponectin during development and the adaptive mechanisms that humans display in the marginal environments (Anderson et al. 2016). Our recent study of GE and breastfeeding patterns in fully breastfed term infants has established that higher concentrations and doses of HM adiponectin are associated with longer times between breastfeeds (GE time) (Gridneva et al. 2017), which may partially explain the growth-regulating effect of adiponectin in some populations. As such, investigations into other factors that may affect adiponectin concentrations in HM and its effect on infant growth and BC development are warranted.

The few studies investigating relationships between HM protein composition and maternal adiposity are contradictory, with some reporting a positive association between protein and maternal adiposity (BMI or percentage ideal weight) (Grote et al. 2016; Nommsen et al. 1991) and one a negative association between protein and BMI (Bachour et al. 2012). Increased serum amino acid concentrations are present in mothers with more adipose tissue (Xie et al. 2014), leading to more amino acids transferred to the breast and HM (A. De Luca et al. 2016), explaining the positive relationship between maternal adiposity and HM protein concentrations (Nommsen et al. 1991). This study has found that higher %FM but not BMI was associated with higher protein concentrations, which is similar to Quinn et al. (Quinn et al. 2015) who reported %FM to be more precise measure of adiposity reporting stronger correlation.
with HM leptin, thus the more precise measure of maternal BC is desirable in mechanistic research.

The measured concentrations of lactose in our cohort were consistent with the normal range in HM (Ballard et al. 2013) and were not related to maternal adiposity profiles. HM provides a constant source of carbohydrates to the infant during early life, ensuring adequate nourishment, maturation and development of their relatively immature physiological systems (Michaelsen et al. 1994; Makinde et al. 1998; Grote et al. 2016). Given the fact that lactose is important for maintaining a constant osmotic pressure in HM (Martin et al. 2016), maternal adiposity is not expected to have a significant impact on lactose concentration.

Despite significant changes in maternal %FM and BMI over the 12 months of lactation, and the introduction of complimentary foods, the measured HM components have remained relatively stable (Tables 5.4, 5.5 and S5.2). However, adiponectin concentration decreased significantly in the combined subset as well as in the concentration of protein in the longitudinal subset, both at 9 months only. The temporary drop in protein concentration was consistent with differences described by Nommsen et al. (Nommsen et al. 1991). Whereas some studies have reported a decrease in concentrations of leptin (Karatas et al. 2011) and adiponectin (Martin et al. 2006; Wang et al. 2011; Savino et al. 2012b; Anderson et al. 2016) (measured predominantly in skim HM), others found the opposite trend for adiponectin (Ozarda et al. 2012; Bronsky et al. 2011), no change (Andreas et al. 2016) or significant fluctuations (Schuster et al. 2011; Bronsky et al. 2011) for leptin. More research is required to clarify these relationships.

In this study we have measured leptin in both skim and whole milk. Interestingly, longitudinal changes observed in concentrations of skim milk leptin in this study were not confirmed in whole HM. Caution in the interpretation of leptin concentrations measured in skim HM should be exercised, as whole milk measures are more indicative of the level of hormone consumed by the infant (Kugananthan et al. 2016). The consistent concentrations of adiponectin and leptin in whole HM over the first 12 months of life may be indicative of a continuing roles of appetite programming, priming neural connections involved in the appetite circuitry, thereby contributing to long-lasting enhanced appetite control and BC of breastfed infants throughout life.

Sampling protocols are of prime importance when investigating relationships between HM components and BC. In this study we confirmed no systematic change between pre- and post-feed samples for concentrations of protein, lactose and whole and
skim HM leptin throughout the first 12 months of lactation (Table 5.3), and report for the first time that whole milk adiponectin concentrations also do not differ significantly pre- to post-feed. Care however should still be taken as fat content (Kent 2007), ghrelin (Karatas et al. 2011) and glucagon-like peptide 1 (GLP-1) (Schueler et al. 2013) change between pre- and post-feed, further highlighting the importance of prudent sampling.

The limitations of this study are the modest number of longitudinal participants, resulting from time constraints associated with multiple measurement time points. Further, our population was predominantly Caucasian and of high social-economic status; therefore, the results may not be applicable to participants from other backgrounds.

5.6 CONCLUSIONS
This study found that elevated maternal adiposity was associated with higher concentrations of leptin and protein of HM in a cross-sectional cohort however; these findings were not confirmed in a smaller longitudinal cohort. Clarification of the relationships between maternal body composition and human milk appetite regulators will identify periods of lactation where interventions may influence programming of early appetite control and body composition of breastfed infants.

5.7 SUPPLEMENTARY MATERIAL
The following are available in the Appendices:
Table S5.1 Longitudinal changes and associations between human milk components and maternal adiposity. Values are parameter estimates ± standard error (n=21).
Table S5.2 Maternal adiposity and human milk components concentrations presented at the months after birth for longitudinal subset (n=21 participants, 73 sessions). Values are mean ± standard deviation (range).
CHAPTER 6

EFFECT OF HUMAN MILK APPETITE HORMONES, MACRONUTRIENTS AND INFANT CHARACTERISTICS ON GASTRIC EMPTYING AND BREASTFEEDING PATTERNS OF TERM FULLY BREASTFED INFANTS*

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6.1 ABSTRACT
HM components influence infant feeding patterns and nutrient intake, yet it is unclear how they influence GE, a key component of appetite regulation. This study analysed GE of a single breastfeed, HM appetite hormones, macronutrients and demographics, anthropometrics, BC of term fully breastfed infants (n=41, 2 and/or 5 months). Stomach volumes (SV) were calculated from pre-/post-feed US scans, then repeatedly until the next feed. Feed volume (FV) was measured by the test-weigh method. HM samples were analysed for adiponectin, leptin, fat, lactose, total carbohydrate, lysozyme, and total, whey and casein protein. Linear regression/mixed effect models were used to determine associations between GE, feed variables and HM components, infant anthropometrics and adiposity. Higher FV were associated with faster (−0.07 [−0.10, −0.03], p<0.001) GE rate, higher post-feed SV (0.82 [0.53, 1.12], p<0.001), and longer GE times (0.24 [0.03, 0.46], p=0.033). Higher whey protein concentration was associated with higher post-feed SV (4.99 [0.84, 9.13], p=0.023). Longer GE time was associated with higher adiponectin concentration (2.29 [0.92, 3.66], p=0.002) and dose (0.02 [0.01, 0.03], p=0.005), and lower casein:whey ratio (−65.89 [−107.13, −2.66], p=0.003). FV and HM composition influence GE and breastfeeding patterns in term breastfed infants.
6.2 INTRODUCTION

HM Breastfeeding and its longer duration are associated with reduced risks of developing obesity and other chronic non-communicable diseases later in life (Geddes et al. 2013; Marseglia et al. 2015). This unique protection could be the result of many mechanisms associated with both nutritive and non-nutritive components of HM (Savino et al. 2009) as well as breastfeeding patterns and behaviour (Sievers et al. 2002; Bartok 2011). It has been shown that HM has the pleiotropic role, providing immune and anti-inflammatory protection (Le Huërou-Luron et al. 2016; Manti et al. 2016) and endocrine, developmental, neural, and psychological benefits (Marseglia et al. 2015).

Non-nutritive HM components such as hormones, growth factors, neuropeptides, and anti-inflammatory and immune-modulating agents influence the growth, development, and function of the GI tract during early infancy (Goldman 2000), while some micronutrients act as nutritional antioxidants, improving GI functions (Hanson et al. 2016); however, there is much to be learned about the spectrum of HM programming agents, how their patterns change throughout lactation period, and their short-term effect on the GE rate of the breastfed infants.

GE is a process by which ingested food is mechanically and chemically partially broken down and delivered to the duodenum at a controlled rate for further digestion and absorption (Hellstrom et al. 2006; Hunt 1980). While well studied in the preterm population (Gomez et al. 2003; Carlos et al. 1997; S. Perrella et al. 2015), in healthy term fully breastfed infants the GE rate and its relationship with breastfeeding patterns are not fully understood.

GE rate and patterns are known to depend on the nature and macronutrient composition of the ingested meal. HM or formula in the infant stomach separates into two phases, a liquid phase consisting of water, whey proteins, lactose, etc., and a semi-solid phase consisting of curd formed by casein and lipids. The semi-solid phase typically empties more slowly than the liquid phase. Different proportions of these phases in part explain the difference between GE patterns of formula-fed and breastfed infants - linear and curvilinear, respectively (Gomez et al. 2003; Cavell 1979).

HM has a unique composition, including nutrients, growth factors, immune factors, and hormones. Despite numerous investigations into the different effects of HM and formula, few components, including major macronutrients, have been studied in connection with the GE of breastfed term infants.
Fatty acids profiles are not associated with GE rate in preterm infants (Armand et al. 1996), while in term infants more rapid GE has been attributed to the fat and protein components of feeds with similar lactose concentration and osmolality (Cavell 1981).

Both osmolality and carbohydrate content are known to influence the rate of GE in adults (Vist et al. 1995), but in infants results are dependent on the type of carbohydrate (Hunt et al. 1982; Husband et al. 1970).

Proteins from different HM fractions such as whey and casein are resistant to proteolysis in the infant stomach (Lonnerdal 2010) and the protein content of a food has also been shown to influence appetite and its regulation (Michaelsen et al. 2012). Infant formula generally empties more slowly than HM in term infants; further, formulas with different casein:whey protein ratio exhibit different GE rates, with casein-predominant formulas emptying slower than whey-predominant formulas (Billeaud et al. 1990). Thus the casein:whey ratio of HM could play an important role in controlling GE in the breastfed infant.

HM lysozyme, also present in whey in a relatively high concentration, catalyzes the hydrolysis of specific bonds in Gram-negative bacteria cell walls and plays multiple roles in digestive strategy, such as controlling the microbiome in the stomach and speeding up the digestion of microbial protein, which may affect gastric motility and GE rate (Artym et al. 2013; Wang et al. 2012).

The satiety hormone leptin and the appetite-stimulating hormone adiponectin are also present in HM. Although not transferred to the infant circulation in direct manner, levels of HM leptin and adiponectin from HM have been found to correlate with levels of these hormones in infant serum (Savino et al. 2016; Wang et al. 2011) and are known to affect both appetite control and infant BC (Woo et al. 2009; Miralles et al. 2006), but are yet to be investigated in relation to GE in the term infant. In animal models (rat, mouse), injection of leptin into the fourth ventricle has been shown to delay GE (Smedh et al. 1998) and oral administration reduced food intake (Sanchez et al. 2005). Leptin in HM is by far the most studied appetite hormone, but predominantly in skim milk (Kugananthan et al. 2016). Leptin measured in skim HM was not associated with time between feeds (Cannon et al. 2015; Cannon et al. 2017) or GE (Cannon et al. 2017) in term breastfed infants, emphasizing the need for studies including whole milk leptin, where the levels of leptin are shown to be higher (Kugananthan et al. 2016).

Adiponectin has the highest concentration of any appetite hormone in HM. It is present in a biologically active form that is resistant to digestion (Newburg et al. 2010). In the animal model, adiponectin inhibits tension-sensitive gastric vagal afferent
mechanosensitivity, modulating satiety signals in both lean and obese animals, while simultaneously increasing the mechanosensitivity of mucosal gastric vagal afferent in the obesity-induced model (Kentish et al. 2015). In humans, elevated serum levels of adiponectin are associated with more rapid GE in diabetic patients (Iwase et al. 2009). It is not known whether adiponectin levels impact GE in the infant and this warrants further investigation.

The volume of milk taken at a single feed varies greatly both within and between infants (Kent et al. 2006). This may be affected by HM composition, with greater breastfeeding frequency associated with lower total 24-h protein intakes and higher lactose concentrations (Khan et al. 2013a). This suggests that the variations in HM components between mothers may potentially influence GE rate and time, and therefore feeding patterns.

This study investigated the effects of HM appetite hormones (whole milk adiponectin and leptin, skim milk leptin) and macronutrients (fat, total carbohydrates, lactose, oligosaccharides, total protein, casein and whey protein, lysozyme) on feeding frequency and GE. Further exploration of infant demographics, anthropometrics, and BC was carried out to determine relationships with infant feeding and GE.

6.3 MATERIALS AND METHODS

6.3.1 Participants
Lactating mothers and their infants (n=27) were recruited predominantly through the Australian Breastfeeding Association. Inclusion criteria were: healthy singletons, gestational age ≥37 weeks, fully breastfed on demand at the point of measurement. Exclusion criteria were: infant health issues requiring medication that could potentially influence GE rate (e.g., reflux), indications of low maternal milk production or infant growth issues. All mothers provided written informed consent to participate in the study, which was approved by the University of Western Australia, Human Research Ethics Committee (RA/1/4253) and registered with the Australian New Zealand Clinical Trials Registry (ACTRN12616000368437).

6.3.2 Study design
Participants arrived at our laboratory at King Edward Memorial Hospital for Women (Subiaco, Perth, WA, Australia) in the morning (09:30–11:30 a.m.) to avoid circadian influence on the outcomes, and stayed for two consecutive breastfeeding sessions.
Before the first feed (F1) infants were weighed and had US stomach volumes (SV) recorded (pre-feed residual, R1). Mothers expressed a pre-feed sample (fore-milk) of milk from the feeding breast/breasts and then breastfed their infants as usual. Immediately after F1, infant SV images and infant weights were taken, and mothers expressed a post-feed (hind-milk) milk sample. Subsequent scans of the stomach were scheduled at 15–20 min intervals (although attending infants’ needs caused some variation) until the infant cued for the next feed (F2), when a final SV immediately before F2 was measured (pre-feed residual, R2).

To assess infant BC, BIS measurements were taken pre-feed, unless impractical – then they were taken post-feed (Gridneva et al. 2016a). US skinfold, length, and head circumference measurements were taken post-feed. This combination of two methods for measuring infant BC was used to ensure safe, non-invasive and accurate assessment and to avoid the inherent limitations of a singular technique (Gridneva et al. 2016b). Clothing was removed for the measurements except for a dry diaper and a singlet.

6.3.3 Feeding frequency
Mothers were asked how frequently their infant feeds, and the self-reported typical time between the feeds (e.g., every three hours) during the week prior to the study session was taken as a proxy measure of FFQ.

6.3.4 Feed volume measurement
The volume of milk transferred from a breast/breasts by the infant was determined by weighing the infant immediately before and after the breastfeed using electronic scales (±2.0 g, Medela Electronic Baby Weigh Scales, Medela Inc., McHenry, IL, USA). MI (g) was calculated by deducting the initial weight from the final weight of the infant (Arthur et al. 1987) and was converted to mL (feed volume; FV) using HM density of 1.03 g/mL (Neville et al. 1988).

6.3.5 Stomach measurements with ultrasound
The infant’s stomach was scanned using the Aplio XG (Toshiba, Tokyo, Japan) machine, with a high-resolution PVT-674BT (6MHz) transducer and Parker ultrasonic gel (Fairfield, NJ, USA). Three to nine (median [IQR]: 5 [5; 6]) serial measurements of infant stomachs were taken 3 to 62 min apart (16±10). Scans were performed with the infant in the semi-supine position according to the method validated in preterm infants (Perrella et al. 2013). Briefly, the sagittal and transverse planes of the stomach were used to measure the longitudinal (L), anterior-posterior (AP) and transverse (T)
diameters directly from images on the US screen using electronic calipers (Figure 6.1). One experienced sonographer with good intra- and interrater reliability (Perrella et al. 2013) performed all of the measurements. Gastric volume (mL) was calculated from the above measured diameters using following equation for an ellipsoidal body:

\[
\text{Stomach volume (mL)} = L (\text{mm}) \times \text{AP (mm)} \times T (\text{mm}) \times 0.52.
\]

![Figure 6.1 Measurements of infant’s stomach with ultrasound. Ultrasound images of infant’s stomach: (a) transverse view with anterior-posterior (AP) and transverse (T) diameter measurements; (b) longitudinal view with longitudinal (L) diameter (maximum length) measurement. Stomach volume (mL) = longitudinal diameter (mm) \times \text{antior-posterior diameter (mm)} \times \text{transverse diameter (mm)} \times 0.52.](image)

**6.3.6 Milk sample collection**

Mothers hand-expressed or pumped small (1–2 mL) pre- and post-feed milk samples into separate 5-mL polypropylene plastic vials (Disposable Products, Adelaide, SA, Australia). Fat concentration was measured (below) and samples were frozen at −20 °C for further biochemical analysis.

**6.3.7 Biochemical analysis**

**6.3.7.1 Fat content**

Percentage fat was measured in pre- and post-feed samples immediately after sample collection with the creamatocrit method (Fleet et al. 1964) using the Creamatocrit Plus device (Medela Inc., McHenry, IL, USA). Fat concentration of the pre- and post-feed milk samples (g/L) was calculated from the cream content of the milk samples, based on the equation (Meier et al. 2006):

\[
\text{Fat (g/L)} = 3.56 + (5.917 \times \text{cream percentage}).
\]
Fat concentration in the volume consumed by the infant was further calculated (Mitoulas 2000):

\[
\text{Fat (g/L)} = 0.53 \times \text{Fat pre-feed} + 0.47 \times \text{Fat post-feed}.
\]

### 6.3.7.2 Sample preparation

Prior to further analysis, all samples were thawed for two hours at RT and aliquotted into 1.5-mL tubes (Sarstedt, Numbrecht, Germany). Components’ concentrations were determined in both pre- and post-feed samples in case of adiponectin, skim and whole milk leptin, fat, and lactose, and in pooled samples in case of total protein, casein, whey protein, total carbohydrates, and lysozyme. Concentrations of pre- and post-feed samples were averaged to arrive at the concentration used for statistical analyses. Whole milk was used for measuring whole milk adiponectin and leptin concentration. Milk samples were defatted (by centrifugation at RT in a Beckman Microfuge 11 (Aberdon Enterprise Inc., Elk Grove Village, IL, USA) at 10,000 \(\times\) g for 10 min and removing the fat layer by clipping it off with the top of the tube (Keller et al. 1986)) for analysis of skim milk leptin, total protein, lysozyme, lactose, and total carbohydrates concentrations. The standard assays were adapted for and carried out using a JANUS workstation (PerkinElmer, Inc., Waltham, MA, USA) and measured on EnSpire (PerkinElmer, Inc., Waltham, MA, USA).

### 6.3.7.3 Leptin

Leptin concentration in HM was measured using the R & D Systems Human Leptin ELISA DuoSet kit (Minneapolis, MN, USA) optimized to measure leptin in sonicated skim HM, as previously described by Cannon et al. (Cannon et al. 2015) and further modified to measure leptin in skim and whole HM milk as described by Kugananthan et al. (Kugananthan et al. 2016). Recovery of leptin was 97.7\%±9.7\% \((n=10)\) with a detection limit of 0.05 ng/mL and an inter-assay CV of <7.2%.

### 6.3.7.4 Adiponectin

Adiponectin concentration in whole milk was measured using the Biovendor Human Adiponectin Sandwich ELISA kit (Life Technologies, Asheville, NC, USA). Adiponectin recovery was 96.2\%±3.2\% \((n=10)\) with a detection limit of 1 ng/mL and an inter-assay CV of <2.5%.
6.3.7.5 Protein
Casein and whey proteins were separated by the method fully described by Kunz and Lonnerdal (Kunz et al. 1989), and Khan et al. (Khan et al. 2012a). Protein concentrations (total protein of skim HM, casein and whey proteins) were measured using the Bradford Protein Assay adapted from Mitoulas et al. (Mitoulas et al. 2002). Recovery of protein was 100.6±5.2% (n=5) with a detection limit of 0.031 g/L and an inter-assay CV of 7.8% (n=18). Casein:whey ratio was calculated as follows:

\[ \text{Casein:whey ratio} = \frac{\text{casein concentration}}{\text{whey protein concentration}}. \]

6.3.7.6 Lysozyme
Lysozyme concentration was determined using a modified turbidimetric assay (Selsted et al. 1980). Hen egg white lysozyme (EC 3.2.1.17, Sigma, St. Louis, MA, USA) standards (range 0.00075–0.0125 g/L) and skim milk samples were diluted 10-fold with 0.1 M of Na₂HPO₄/1.1 mM of citric acid (pH 5.8) buffer. Twenty-five microliters of standards or diluted skim milk samples were placed into the wells of a plate (Greiner Bio-One, Frickenhausen, Germany), 175 μL of Micrococcus lysodeiktikus suspension (0.075% w/v, ATCC No. 4698, Sigma, St. Louis, MA, USA) was added into each well and plate was incubated at RT for 1 h. The absorbance was measured at 450 nm. Recovery of lysozyme was 97.0±5.0% (n=8) with a detection limit of 0.007 g/L and an inter-assay CV of 13.0% (n=8).

6.3.7.7 Carbohydrates
Defatted milk was deproteinized with trichloroacetic acid (Euber et al. 1979) before dehydration by sulphuric acid (Albalasmeh et al. 2013). This technique reliably estimates concentrations and carbon content for monosaccharides, disaccharides, and polysaccharides. Total carbohydrates were analyzed by UV-spectrophotometry. Recovery of total carbohydrates was 101.4±2.1% (n=7) with a detection limit of 0.007 g/L and an inter-assay CV of 3.3% (n=7).

Lactose concentration was measured using the enzymatic spectrophotometric method of Kuhn and Lowenstein (Kuhn et al. 1967), adapted from Mitoulas et al. (Mitoulas et al. 2002), with recovery of 98.2±4.1% (n=10), detection limit of 30 mM and inter-assay CV of 3.5%.

The HMO concentration (g) was calculated by deducting concentration of lactose (g) from concentration of total carbohydrates (g). The glucose and galactose were not
measured or accounted for, as their concentrations in HM are small and comparable or less than the assays errors (Newburg et al. 1995).

6.3.8 Hormone and macronutrient dose

Doses were defined as the amount of hormone/macronutrient ingested during a breastfeed and calculated as average of the pre- and post-feed HM component concentration, multiplied by the corresponding FV. When an infant fed from both breasts at the breastfeeding session, hormone/macronutrient doses from these individual breastfeeds were calculated separately and added together.

6.3.9 Infants’ anthropometrics and body composition

6.3.9.1 Anthropometric measurements

Infants’ weight was determined by weighing before breastfeeding using Medela Electronic Baby Weigh Scales (±2.0 g; Medela Inc., McHenry, IL, USA). Clothing was removed except for a dry diaper and a singlet. Infant crown-heel length was measured once to the nearest 0.1 cm using non-stretch tape and headpiece and footpiece, both applied perpendicular to the hard surface. Infant head circumference was measured with non-stretch tape. Infant BMI was calculated according to the following formula:

\[
BMI = \frac{\text{Body weight (kg)}}{\text{(Height (m))}^2}
\]

6.3.9.2 Body composition with bioelectrical impedance spectroscopy

Infants’ whole body bioimpedance were measured using the Impedimed SFB7 bioelectrical impedance analyzer (ImpediMed, Brisbane, Queensland, Australia) applying an adult protocol (wrist to ankle) according to the manufacturer’s instructions and analysed with settings customized for each infant according to Lingwood et al. (Lingwood et al. 2012) and Gridneva et al. (Gridneva et al. 2016b). Values of resistance (ohm) at frequency of 50 kHz \(R_{50}\) were determined from the curve of best fit, averaged for analysis purposes and used in the Lingwood et al. age matched (3 and 4.5 mo infants) equations for fat-free mass (FFM) of 2 and 5 mo infants respectively (Lingwood et al. 2012):

\[
FFM 3 \text{ mo} = 1.458 + 0.498 \times W - 0.197 \times S + 0.067 \times L^2/R_{50}
\]

\[
FFM 4.5 \text{ mo} = 2.203 + 0.334 \times W - 0.361 \times S + 0.185 \times L^2/R_{50},
\]
where L is body length (cm), R50 is resistance (Ω), S is sex (male = 1, female = 2) and W is infant weight (kg).

%FM was calculated as follows:

\[ \%FM = 100(\text{Weight (kg)} - \text{FFM (kg)})/\text{Weight (kg)}. \]

### 6.3.9.3 Body composition with ultrasound skinfold measurements

Infant US skinfold measurements were carried out using the Aplio XG (Toshiba, Tokyo, Japan) US machine, PLT-1204BX 14-8 MHz transducer and sterile water-based Parker ultrasonic gel (Fairfield, NJ, USA). Single US scans of four anatomical sites (biceps, subscapular, suprailiac, and triceps) were performed on the left side of the body with minimal compression. ST (skin thickness and the skin–fat interface to fat–muscle interface distance) was measured directly from images on the screen using electronic calipers. One experienced sonographer (DG) with good intra- and interrater reliability (Perrella et al. 2013) performed all of the measurements.

The doubled US ST was used in Brook BD age-matched (3–18 months) equations (Brook 1971) developed for skinfolds measured with calipers:

\[
\begin{align*}
\text{Male BD} &= 1.1690 - 0.0788 \times \log(\sum SFT) \\
\text{Female BD} &= 1.2063 - 0.0999 \times \log(\sum SFT),
\end{align*}
\]

where BD is infant body density (kg/L) and \(\sum SFT\) is a sum of four skinfolds (mm).

Predicted BD (kg/L) was converted to %FM using the Lohman equation (Lohman 1984):

\[ \%FM = 100 \times (5.28/BD - 4.89). \]

### 6.3.10 Statistical analysis

Statistical analysis was performed in R 2.9.0 (R Core Team 2009) for Mac OSX using additional packages nlme (Pinheiro et al. 2016); lattice (Sarkar 2008), lattice extra (Sarkar et al. 2013), and car (Fox et al. 2011); MASS (Venables et al. 2002), sfsmisc (Maechler et al. 2015) and multcomp (Hothorn et al. 2008) for mixed effects modelling, data representation, robust regression, and multiple comparisons of means, respectively. Descriptive statistics are reported as mean ± SD (range) or median (IQR) unless otherwise stated; model parameters are presented as estimate ± SE, and, where appropriate, an approximate 95% CI.
Measurements missing due to insufficient sample volume: skim milk leptin, whole milk leptin, adiponectin, total protein, whey and casein protein, lactose and total carbohydrate \((n=3)\); lysozyme \((n=5)\). Measurements of fat \((n=14)\) were missing as a result of either insufficient sample volumes or absence of separate FV from breasts where both breast were offered during one feed. Also missing were FFQ as reported by mothers \((n=6)\), measurements of length, head circumference, infant BMI, %FM measured with BIS \((n=4)\) and %FM measured with US skinfolds \((n=5)\).

GE time was determined as the time from the start of F1 to the start of F2 and included the time between two feeds and feed duration. Feed duration was included as up to 80% of HM consumed by term healthy breastfed infants in the first 4–5 min (Cannon et al. 2016). GE during breastfeeding was defined as the volume of milk to have left the stomach, calculated as the difference between the immediate post-feed SV and the sum of R1 and FV.

Due to the lack of term infant GE studies focusing on SV, no power calculation/sample size determination could be performed for this study. A goal of 20 infants at each two and five months was selected with the expectation that this would be sufficient to show overall patterns. When available, infants were included in both subsets to allow for investigation of longitudinal patterns. Linear mixed effects models allow us to treat the individual feeds as separate, without having to assume independence, when there may be correlations between feeds within infants.

Influences on GE rate were analysed by first fitting a time curve to the sequential post-feed SV using linear mixed effects models; as curves differed significantly within and between infants \((p<0.001)\), random time curves were fitted to feeds within infants. Time terms (linear, square root) were selected as per the fractional polynomial method of (Royston et al. 1994); this model also considered possible confounding effects of FV (median-centred) and feed duration (median-centred). Interaction terms involving the time curve indicated changes in the GE rate; main effects indicated overall effects on post-feed SV but not the GE rate. The addition of one term to this base model was used to investigate associations with (a) concentrations/doses of hormones/macronutrients; (b) infant characteristics/anthropometrics/BC; (c) R1. Whether the overall effect of HM component concentrations differs by FV was investigated by including interactions between FV and concentration measures. Models using the selected technique did not converge for fat concentration, lysozyme concentration, or lysozyme dose. Omitting the random effect of FV within infant provided converging models, but no evidence of an association with fat or lysozyme was seen. Given the complexity of linear mixed effects
models used to analyse GE rate, no further adjustments were performed and $p<0.05$ was considered to be statistically significant.

Associations between pre-feed residual SV, FV, immediate post-feed SV, feed duration, FFQ and both hormone and macronutrient concentrations and doses, and infant anthropometrics/BC parameters were tested using robust linear regression. Mixed effects models were considered, but were not significantly better ($p>0.1$) Robust linear regression (rlm) was chosen so as to address heteroscedasticity in the data and points with high leverage in the majority of the predictors; MM-estimation (M-estimation with Tukey’s biweight, initialized by a specific S-estimator) accounting for appropriate covariates was used (Venables et al. 2002). Approximate p-values were determined using the Wald test. Multivariate models accounting for FV were used for testing the relationship with FV-dependent predictor (fat dose and concentration).

Possible age differences in HM components, infant characteristics, and GE/breastfeeding parameters were analyzed with either linear mixed effects models or robust linear regression models; model type was determined using likelihood ratio tests. Linear mixed effects models were used to analyze relationships of GE during feed time with HM components and infant characteristics. R1, FV and feed duration were not associated with SV reduction during the feed time therefore, univariate models were run. Multivariate linear mixed effects models accounting for R1, FV and feed duration were used in analysis of relationships of immediate post-feed SV with HM components and infant characteristics.

Owing to the large number of comparisons, a false discovery rate adjustment (Curran-Everett 2000) was performed on associated subgroupings of results with one or more $p$-values $<0.05$. $p$-values were considered to be significant at $<0.011$ for GE time, $<0.031$ for FFQ, $<0.038$ for R2, and $<0.008$ for associations between HM components’ concentrations.

### 6.4 RESULTS

#### 6.4.1 Participants

Characteristics of the 27 participants (2 months ($n=20$; longitudinal: 7 females, 7 males; cross-sectional: 2 females, 4 males); 5 months ($n=21$; longitudinal: 7 females, 7 males; cross-sectional: 1 male, 6 females); overall $n=41$ feeds) are described in Table 6.1. At the study session, infants fed from one ($n=3$) or both ($n=18$) breasts.
### Table 6.1 Participant characteristics expressed as mean ± SD and range.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>2 mo</th>
<th>5 mo</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SD</td>
<td>Range</td>
<td>Mean ± SD</td>
</tr>
<tr>
<td><strong>Infant characteristics</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Infant age (weeks)</td>
<td>9 ± 1</td>
<td>6–10</td>
<td>22 ± 1</td>
</tr>
<tr>
<td>Infant length (cm)</td>
<td>57 ± 2</td>
<td>53–61</td>
<td>65 ± 2 ***</td>
</tr>
<tr>
<td>Infant weight (kg)</td>
<td>5.3 ± 0.8</td>
<td>4.2–6.3</td>
<td>7.2 ± 1.0 ***</td>
</tr>
<tr>
<td>Infant BMI</td>
<td>15.9 ± 1.3</td>
<td>13.9–18.1</td>
<td>17.6 ± 1.7 ***</td>
</tr>
<tr>
<td>HC (cm)</td>
<td>39 ± 1</td>
<td>37–42</td>
<td>43 ± 2 ***</td>
</tr>
<tr>
<td>Fat Mass with BIS (%)</td>
<td>21.4 ± 3.6</td>
<td>11.1–27.1</td>
<td>28.9 ± 3.2 ***</td>
</tr>
<tr>
<td>Fat Mass with US (%)</td>
<td>24.2 ± 3.6</td>
<td>17.5–30.5</td>
<td>26.6 ± 3.6</td>
</tr>
<tr>
<td><strong>BF/GE characteristics</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Feed volume (mL)</td>
<td>86 ± 34</td>
<td>35–140</td>
<td>85 ± 33</td>
</tr>
<tr>
<td>SV after feed 1 (mL)</td>
<td>87 ± 36</td>
<td>32–141</td>
<td>93 ± 41</td>
</tr>
<tr>
<td>Feed duration (min)</td>
<td>28 ± 14</td>
<td>11–72</td>
<td>20 ± 8</td>
</tr>
<tr>
<td>SV reduction (mL) d</td>
<td>5 ± 21</td>
<td>(-42)–33</td>
<td>4 ± 26</td>
</tr>
<tr>
<td>GE time (min) e</td>
<td>94 ± 29</td>
<td>44–153</td>
<td>88 ± 18</td>
</tr>
<tr>
<td>Residual 1 (mL)</td>
<td>6 ± 12</td>
<td>0–50</td>
<td>11 ± 19</td>
</tr>
<tr>
<td>Residual 2 (mL)</td>
<td>20 ± 20</td>
<td>0–81</td>
<td>15 ± 15</td>
</tr>
<tr>
<td>Feeding frequency (h) f</td>
<td>2.3 ± 0.7</td>
<td>1.0–4.0</td>
<td>2.7 ± 0.8</td>
</tr>
</tbody>
</table>

Data are mean ± SD and ranges. a n=20; b n=21. c n=41 feeds. d Stomach volume reduction during feed time is calculated as the difference between the sum of residual 1 and feed volume and the immediate stomach volume after Feed 1 to the start of Feed 2 (time between feeds plus feed duration). e GE time is the time from the start of Feed 1 to the start of Feed 2. f Feeding frequency self-reported by mothers as to how often infant feeds (e.g., every three hours). *** Indicates significant differences (p<0.001) between two- and five-month-old infants. Abbreviations: BF - breastfeeding; BIS - bioimpedance spectroscopy; GE - gastric emptying; HC - head circumference; SV - stomach volume; US - ultrasound.

#### 6.4.2 Influence of infant age

Infant anthropometrics and %FM measured with BIS significantly differed by infant age (p<0.001), while breastfeeding and GE parameters did not change significantly (p>0.067) (Table 6.1).

Lower whey protein concentration (5.51±0.96 g/L 5 mo vs 6.41±1.39 g/L 2 mo, p=0.034) and subsequently a higher casein:whey ratio (0.32±0.14 5 mo vs 0.22±0.07 2 mo, p=0.035) were observed at 5 months. All other measured appetite hormones and macronutrient concentrations did not differ significantly by infant age (p>0.053).
6.4.3 Analysed human milk components

Appetite hormones and macronutrient concentrations and doses per feed are presented in Table 6.2. Higher skim milk leptin concentrations were associated with lower whole milk leptin concentrations (−0.25 [−0.34, −0.16], p<0.001) and higher protein concentrations were associated with higher whey protein concentrations (0.68 [0.41, 0.95], p<0.001). Higher HMO concentrations were associated with higher total carbohydrates concentrations (p<0.001) and lower lactose concentrations (p<0.001).

Table 6.2 Concentrations and doses of measured HM hormones and macronutrients.

<table>
<thead>
<tr>
<th>Components</th>
<th>Concentration</th>
<th>Dose Per Feed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SD</td>
<td>Range</td>
</tr>
<tr>
<td>Adiponectin (ng/mL, ng)</td>
<td>10.02 ± 4.08</td>
<td>6.18–22.58</td>
</tr>
<tr>
<td>WM leptin (ng/mL, ng)</td>
<td>0.51 ± 0.18</td>
<td>0.23–1.10</td>
</tr>
<tr>
<td>SM leptin (ng/mL, ng)</td>
<td>0.28 ± 0.12</td>
<td>0.20–0.84</td>
</tr>
<tr>
<td>Total protein (g/L, g)</td>
<td>11.29 ± 2.56</td>
<td>7.60–24.16</td>
</tr>
<tr>
<td>Casein (g/L, g)</td>
<td>1.54 ± 0.53</td>
<td>0.69–3.45</td>
</tr>
<tr>
<td>Whey protein (g/L, g)</td>
<td>5.97 ± 1.26</td>
<td>3.82–9.08</td>
</tr>
<tr>
<td>Casein:whey ratio</td>
<td>0.27 ± 0.11</td>
<td>0.10–0.73</td>
</tr>
<tr>
<td>Lysozyme (g/L, g)</td>
<td>0.14 ± 0.12</td>
<td>0.05–0.48</td>
</tr>
<tr>
<td>TCH (g/L, g)</td>
<td>82.72 ± 7.89</td>
<td>67.08–97.49</td>
</tr>
<tr>
<td>Lactose (g/L, g)</td>
<td>65.84 ± 5.14</td>
<td>53.49–77.94</td>
</tr>
<tr>
<td>HMO (g/L, g)</td>
<td>16.88 ± 9.89</td>
<td>(−10.86)b–35.77</td>
</tr>
<tr>
<td>Fat (g/L, g)</td>
<td>42.74 ± 12.10</td>
<td>17.42–66.79</td>
</tr>
</tbody>
</table>

Data are mean ± SD and ranges, n=41 feeds. a Casein:whey ratios for doses are the same as for concentrations. b Negative values are seen for human milk oligosaccharides (HMO) when lactose measurements are higher than total carbohydrates. Abbreviations: SM - skim milk; TCH - total carbohydrates; WM - whole milk.

6.4.4 Gastric emptying rate

The overall decreasing curvilinear pattern of GE (linear: 0.04 [−0.17, 0.24], p=0.72; square root: −10.5 [−12.7, −8.2], p<0.001) is shown in Figure 6.2. Higher FV were associated with faster (−0.07 [−0.10, −0.03], p<0.001) GE rate (Figure 6.3) and higher overall post-feed SV (0.82 [0.53, 1.12], p<0.001). No association was seen between feed duration and post-feed SV (−0.25 [−0.68, 0.18], p=0.23).

Immediate post-feed stomach volumes were not associated with R1 (p = 0.91).
**Figure 6.2** Overall curvilinear pattern of gastric emptying (n=41 feeds). The lines represent the overall pattern of changes in stomach volume as measured by ultrasound imaging. Bold line represents local regression smoother (LOESS, span = 0.9). Dotted lines represent confidence interval.

**Figure 6.3** Overall gastric emptying of individual feeds in term breastfed infants (n=41 feeds). Feeds are grouped by milk intake (MI) to illustrate the effect of the feed volumes; approximately equal numbers are included in each panel. Data points represent stomach volumes calculated from ultrasound images; connecting lines link measurements from the same feed. Bold line represents local regression smoother (LOESS, span=0.9).
CHAPTER 6: GASTRIC EMPTYING IN BREASTFED INFANTS

After accounting for time post-feed, FV, and feed duration, as per the above model, larger R1 volumes (0.55 [0.24, 0.86], p=0.003) and higher whey protein concentrations (4.99 [0.84, 9.13], p=0.023) were associated with larger post-feed SV, while the casein:whey ratio (2.2 ± 0.88, p=0.030) and lactose concentration (−0.04 ± 0.02, p=0.037) modified the GE curve depending on FV. Higher casein:whey ratios at lower FV were associated with faster GE, and at higher FV with slower GE, while higher lactose concentrations at lower FV were associated with slower GE, and at higher FV with faster GE. No other associations with post-feed stomach volumes or changes to the GE curves were found (Table 6.3).

Table 6.3 HM components and infant characteristics and their associations with feed variables and gastric emptying.

<table>
<thead>
<tr>
<th>Predictors</th>
<th>Feed Volume a Estimate ± SE (95% CI)</th>
<th>p-Value</th>
<th>Gastric Emptying Time a Estimate ± SE (95% CI)</th>
<th>p-Value</th>
<th>Post-Feed Stomach Volumes b Estimate ± SE (95% CI)</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Concentrations</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adiponectin (ng/mL)</td>
<td>1 ± 1.3 (−36.6, 134.5)</td>
<td>0.44</td>
<td>2.3 ± 0.7 (0.9, 3.7)</td>
<td><strong>0.002</strong></td>
<td>1.3 ± 0.7 (−0.2, 2.7)</td>
<td>0.081</td>
</tr>
<tr>
<td>Whole milk leptin (ng/mL)</td>
<td>9.9 ± 29.9 (−36.6, 134.5)</td>
<td>0.74</td>
<td>6.8 ± 15.8 (−24.2, 37.8)</td>
<td>0.67</td>
<td>−9.5 ± 13.3 (−35.8, 16.9)</td>
<td>0.48</td>
</tr>
<tr>
<td>Skim milk leptin (ng/mL)</td>
<td>49 ± 43.6 (−36.6, 134.5)</td>
<td>0.26</td>
<td>6.7 ± 24.3 (−41, 54.4)</td>
<td>0.78</td>
<td>39.8 ± 18.4 (−0.8, 80.3)</td>
<td>0.054</td>
</tr>
<tr>
<td>Total protein (g/L)</td>
<td>−2.1 ± 2.1 (−6.2, 1.9)</td>
<td>0.30</td>
<td>−0.9 ± 1.1 (−3.1, 1.3)</td>
<td>0.41</td>
<td>1.1 ± 1 (−1.2, 3.4)</td>
<td>0.30</td>
</tr>
<tr>
<td>Whey protein (g/L)</td>
<td>−5.5 ± 4.2 (−13.8, 2.7)</td>
<td>0.19</td>
<td>5.8 ± 2.2 (1.6, 10.1)</td>
<td>0.011</td>
<td>5 ± 1.9 (0.8, 9.1)</td>
<td><strong>0.023</strong></td>
</tr>
<tr>
<td>Casein (g/L)</td>
<td>2.6 ± 10.2 (−17.4, 22.5)</td>
<td>0.80</td>
<td>−12.4 ± 4.7 (−21.5, −3.2)</td>
<td>0.013</td>
<td>−2 ± 4.4 (−11.6, 7.6)</td>
<td>0.66</td>
</tr>
<tr>
<td>Casein:whey ratio</td>
<td>24.5 ± 46.1 (−65.9, 114.9)</td>
<td>0.59</td>
<td>−65.9 ± 21 (−107.1, −24.7)</td>
<td><strong>0.003</strong></td>
<td>−17.3 ± 20.1 (−61.4, 26.9)</td>
<td>0.41</td>
</tr>
<tr>
<td>Lysozyme (g/L)</td>
<td>−81.4 ± 46.2 (−172, 9.1)</td>
<td>0.079</td>
<td>−19.5 ± 28.3 (−75, 36)</td>
<td>0.49</td>
<td>23.3 ± 15.4 (−7.2, 53.8)</td>
<td>0.13</td>
</tr>
<tr>
<td>Total carbohydrates (g/L)</td>
<td>−1.1 ± 0.7 (−2.3, 0.2)</td>
<td>0.12</td>
<td>−0.6 ± 0.4 (−1.3, 0.1)</td>
<td>0.10</td>
<td>−0.5 ± 0.3 (−1.2, 0.1)</td>
<td>0.089</td>
</tr>
<tr>
<td>Lactose (g/L)</td>
<td>0.7 ± 1.1 (−1.4, 2.7)</td>
<td>0.51</td>
<td>0.2 ± 0.6 (−0.9, 1.3)</td>
<td>0.76</td>
<td>0.03 ± 0.49 (−1, 1.1)</td>
<td>0.96</td>
</tr>
<tr>
<td>HMO (g/L)</td>
<td>−0.8 ± 0.5 (−1.9, 0.2)</td>
<td>0.13</td>
<td>−0.4 ± 0.3 (−1, 0.2)</td>
<td>0.16</td>
<td>−0.4 ± 0.2 (−0.9, 0.1)</td>
<td>0.13</td>
</tr>
<tr>
<td>Fat (g/L)</td>
<td>−0.69 ± 0.6 (−1.8, 0.5)</td>
<td>0.26</td>
<td>−0.1 ± 0.3 (−0.6, 0.5)</td>
<td>0.79</td>
<td>−0.1 ± 0.3 (−0.9, 0.6)</td>
<td>0.71</td>
</tr>
</tbody>
</table>

Data are parameter estimate ± SE and 95% CI, n = 41 feeds. a Effects of predictors taken from univariate regression models; b Effects of predictors taken from linear mixed effects models that accounted for postprandial time, feed volume and feed duration. c After the false discovery rate adjustment the p-values were considered to be significant at <0.011 for GE time (bold font); d n/a—dosage is dependent on feed volume. Abbreviations: BIS—bioimpedance spectroscopy; HMO—human milk oligosaccharides; US—ultrasound skinfolds.
### Table 6.3 Continued.

<table>
<thead>
<tr>
<th>Predictors</th>
<th>Feed Volume&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Gastric Emptying Time&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Post-Feed Stomach Volumes&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Estimate ± SE (95% CI)</td>
<td>p-Value</td>
<td>Estimate ± SE (95% CI)</td>
</tr>
<tr>
<td><strong>Doses</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adiponectin (ng)</td>
<td>n/a&lt;sup&gt;d&lt;/sup&gt;</td>
<td>n/a&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.02 ± 0.01 (0.01, 0.03)</td>
</tr>
<tr>
<td>Whole milk leptin (ng)</td>
<td>n/a</td>
<td>n/a</td>
<td>−0.1 ± 0.2 (-0.4, 0.2)</td>
</tr>
<tr>
<td>Skim milk leptin (ng)</td>
<td>n/a</td>
<td>n/a</td>
<td>−0.2 ± 0.2 (-0.7, 0.3)</td>
</tr>
<tr>
<td>Total protein (g)</td>
<td>n/a</td>
<td>n/a</td>
<td>−25.9 ± 12.4 (-50.2, -1.7)</td>
</tr>
<tr>
<td>Whey protein (g)</td>
<td>n/a</td>
<td>n/a</td>
<td>47.6 ± 18.7 (10.8, 84.3)</td>
</tr>
<tr>
<td>Casein (g)</td>
<td>n/a</td>
<td>n/a</td>
<td>−119 ± 53.3 (-223.4, -14.6)</td>
</tr>
<tr>
<td>Lysozyme (g)</td>
<td>n/a</td>
<td>n/a</td>
<td>−276.2 ± 370.7 (-1002.9, 450.4)</td>
</tr>
<tr>
<td>Total carbohydrates (g)</td>
<td>n/a</td>
<td>n/a</td>
<td>−4.1 ± 1.8 (-7.6, -0.5)</td>
</tr>
<tr>
<td>Lactose (g)</td>
<td>n/a</td>
<td>n/a</td>
<td>−5.8 ± 2.7 (-11.1, -0.6)</td>
</tr>
<tr>
<td>HMO (g)</td>
<td>n/a</td>
<td>n/a</td>
<td>−3.1 ± 3.1 (-9.2, 3)</td>
</tr>
<tr>
<td>Fat (g)</td>
<td>n/a</td>
<td>n/a</td>
<td>−2.8 ± 2.7 (-8.1, 2.4)</td>
</tr>
</tbody>
</table>

**Demographics**

| Infant sex (Male) | −2.2 ± 10.7 (-23.1, 18.8) | 0.84 | −1.5 ± 7.5 (-16.3, 13.2) | 0.84 | −8.4 ± 4.6 (-17.8, 1.1) | 0.081 |
| Infant age (months) | −0.9 ± 3.6 (-7.9, 6) | 0.80 | −1.8 ± 2.5 (-6.6, 3) | 0.47 | −1.5 ± 1.4 (-4.5, 1.6) | 0.32 |

**Anthropometrics**

| Infant length (cm) | −0.03 ± 1.3 (-2.6, 2.6) | 0.98 | −1.3 ± 0.9 (-3, 0.4) | 0.15 | −0.5 ± 0.6 (-1.8, 0.8) | 0.44 |
| Infant weight (kg) | 0.7 ± 4.1 (-7.4, 8.8) | 0.87 | −2.3 ± 2.9 (-7.9, 3.4) | 0.43 | −2.3 ± 1.8 (-6.3, 1.7) | 0.23 |
| Head circumference (cm) | −2.5 ± 2.6 (-7.5, 2.5) | 0.34 | −1.4 ± 1.8 (-4.9, 2.1) | 0.42 | −1.8 ± 1.2 (-4.5, 0.8) | 0.15 |
| Infant BMI | −0.2 ± 3.2 (-6.5, 6) | 0.94 | −1.5 ± 2.2 (-5.8, 2.8) | 0.48 | −3.2 ± 1.5 (-6.6, 0.2) | 0.062 |

**Body composition**

| Fat mass with US (%) | 0.6 ± 1.4 (-2.2, 3.4) | 0.67 | −0.3 ± 0.9 (-2.2, 1.5) | 0.71 | −0.6 ± 0.7 (-2.1, 1.0) | 0.42 |
| Fat mass with BIS (%) | 0.4 ± 1.1 (-1.8, 2.5) | 0.74 | −0.4 ± 0.7 (-1.9, 1) | 0.56 | −0.5 ± 0.5 (-1.5, 0.5) | 0.35 |

Data are parameter estimate ± SE and 95% CI, n = 41 feeds. <sup>a</sup> Effects of predictors taken from univariate regression models; <sup>b</sup> Effects of predictors taken from linear mixed effects models that accounted for postprandial time, feed volume and feed duration. <sup>c</sup> After the false discovery rate adjustment the p-values were considered to be significant at <0.011 for GE time (bold font); <sup>d</sup> n/a - dosage is dependent on feed volume. Abbreviations: BIS - bioimpedance spectroscopy; HMO - human milk oligosaccharides; US - ultrasound skinfolds.
6.4.5 Feed volume, feed duration, and gastric emptying during breastfeeding

Higher FVs were associated with higher SV measured immediately post-feed (0.79 [0.51, 1.07], \( p<0.001 \)) and longer GE times (0.24 [0.03, 0.46], \( p=0.033 \)). FV was not associated with either concentrations of measured HM components or infant’s characteristics/anthropometrics/BC (Table 6.3).

Feed duration was not associated with FV (0.06 [-0.03, 0.15], \( p=0.20 \)) or R1 volume (0.01 [-0.17, 0.19], \( p=0.91 \)).

After accounting for R1 (1.07 [0.47, 1.7], \( p=0.002 \)), FV (1.00 [0.71, 1.3], \( p<0.001 \)) and feed duration (-0.30 [-0.96, 0.36], \( p=0.34 \)), immediate post-feed SV were not associated with either measured HM components (\( p>0.068 \)) or infant’s demographics/anthropometrics/BC (\( p>0.46 \)). SV reduction during breastfeeding was not associated with either measured HM components (\( p>0.11 \)); infant’s demographics/anthropometrics/BC (\( p>0.48 \)); R1, FV or feed duration (\( p>0.34 \)).

6.4.6 Gastric emptying time

The GE time was not associated with feed duration (0.35 [-0.29, 0.98], \( p=0.28 \)), but was negatively associated with R2 (-0.63 [-1.05, -0.21], \( p=0.005 \)) after accounting for FV (\( p<0.001 \)). Longer GE times were associated with higher adiponectin concentration (2.3 [0.9, 3.7], \( p=0.002 \)) and dose (0.02 [0.01, 0.03], \( p=0.005 \)), and lower casein:whey ratio (-65.9 [-107.1, -24.7], \( p=0.003 \)). No associations with infant characteristics were seen (Table 6.3).

6.4.7 Pre-feed residuals

Infants cued for F1 and F2 with different residual volumes (R1 and R2) present in their stomachs (Table 6.1). Larger FV were associated with smaller R1 volumes (\( p=0.002 \)), with each -0.92 [-1.47, -0.37] mL of R1 volume resulting in extra mL of FV. Larger R2 volumes were associated with larger FV (\( p=0.006 \)), each additional mL of FV resulting in 0.21 [0.07, 0.35] mL greater R2.

There was no association between R2 and R1 in univariate model (0.11 [-0.19, 0.42], \( p=0.46 \)). After accounting for FV and GE time (\( p<0.001 \) for both) larger R2 volumes were associated with larger R1 volumes (0.36 [0.11, 0.60], \( p=0.005 \)).

After accounting for FV, R2 was not associated with any concentration of HM components (\( p>0.038 \) after adjusting for multiple comparisons).
6.4.8 Feeding frequency

There were no associations between FFQ and HM components and doses after adjusting for multiple comparisons (Table 6.4).

Table 6.4 Associations between infant feeding frequency and HM components.

<table>
<thead>
<tr>
<th>Predictors</th>
<th>Feeding Frequency (h) a Estimate ± SE (95% CI) b</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Concentrations</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adiponectin (ng/mL)</td>
<td>−0.001 ± 0.03 (−0.06, 0.06)</td>
<td>0.96</td>
</tr>
<tr>
<td>Whole milk leptin (ng/mL)</td>
<td>−1.1 ± 0.7 (−2.5, 0.3)</td>
<td>0.13</td>
</tr>
<tr>
<td>Skim milk leptin (ng/mL)</td>
<td>0.8 ± 1.6 (−2.3, 4)</td>
<td>0.60</td>
</tr>
<tr>
<td>Total protein (g/L)</td>
<td>−0.05 ± 0.05 (−0.15, 0.04)</td>
<td>0.28</td>
</tr>
<tr>
<td>Whey protein (g/L)</td>
<td>−0.1 ± 0.1 (−0.3, 0.1)</td>
<td>0.42</td>
</tr>
<tr>
<td>Casein (g/L)</td>
<td>0.04 ± 0.2 (−0.4, 0.5)</td>
<td>0.86</td>
</tr>
<tr>
<td>Casein:whey protein ratio</td>
<td>0.4 ± 1.1 (−1.7, 2.5)</td>
<td>0.68</td>
</tr>
<tr>
<td>Lysozyme (g/L)</td>
<td>−0.4 ± 1.1 (−2.5, 1.7)</td>
<td>0.71</td>
</tr>
<tr>
<td>Total carbohydrates (g/L)</td>
<td>0.01 ± 0.02 (−0.03, 0.04)</td>
<td>0.73</td>
</tr>
<tr>
<td>Lactose (g/L)</td>
<td>−0.05 ± 0.02 (−0.1, −0.01)</td>
<td>0.031</td>
</tr>
<tr>
<td>HMO (g/L)</td>
<td>0.01 ± 0.02 (−0.03, 0.04)</td>
<td>0.73</td>
</tr>
<tr>
<td>Fat (g/L)</td>
<td>−0.02 ± 0.01 (−0.04, 0.01)</td>
<td>0.19</td>
</tr>
<tr>
<td><strong>Doses</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adiponectin (ng/mL)</td>
<td>0.0002 ± 0.0003 (−0.0004, 0.0008)</td>
<td>0.50</td>
</tr>
<tr>
<td>Whole milk leptin (ng/mL)</td>
<td>−0.002 ± 0.01 (−0.01, 0.01)</td>
<td>0.80</td>
</tr>
<tr>
<td>Skim milk leptin (ng/mL)</td>
<td>0.01 ± 0.01 (−0.01, 0.03)</td>
<td>0.59</td>
</tr>
<tr>
<td>Total protein (g/L)</td>
<td>0.1 ± 0.3 (−0.5, 0.8)</td>
<td>0.67</td>
</tr>
<tr>
<td>Whey protein (g/L)</td>
<td>0.1 ± 0.7 (−1.3, 1.5)</td>
<td>0.89</td>
</tr>
<tr>
<td>Casein (g/L)</td>
<td>2.1 ± 2 (−1.7, 5.9)</td>
<td>0.27</td>
</tr>
<tr>
<td>Lysozyme (g/L)</td>
<td>−5.7 ± 17.2 (−39.4, 27.9)</td>
<td>0.73</td>
</tr>
<tr>
<td>Total carbohydrates (g/L)</td>
<td>0.1 ± 0.1 (0, 0.2)</td>
<td>0.22</td>
</tr>
<tr>
<td>Lactose (g/L)</td>
<td>0.04 ± 0.06 (−0.08, 0.17)</td>
<td>0.49</td>
</tr>
<tr>
<td>HMO (g/L)</td>
<td>0.3 ± 0.1 (0, 0.5)</td>
<td>0.051</td>
</tr>
<tr>
<td>Fat (g/L)</td>
<td>−0.2 ± 0.1 (−0.5, 0)</td>
<td>0.085</td>
</tr>
</tbody>
</table>

Data are parameter estimate ± SE and 95% CI, n=41 feeds. a Feeding frequency self-reported by mothers as to how often infant feeds (e.g., every three hours). b Effects of predictors are results of univariate regression model. c After the false discovery rate adjustment the p-values were considered to be significant at <0.031 (highlighted). Abbreviations: HMO - human milk oligosaccharides.

A longer time between the feeds was seen when infants were longer, heavier, and had higher %FM measured with BIS (Table 6.5) in univariate models. The associations for length and weight were not significant after accounting for the other (p>0.38); the association for %FM measured with BIS was not significant after accounting for infant length (p=0.095).
Table 6.5  Associations between infant feeding frequency and HM components and infant characteristics.

| Predictors                  | Feeding Frequency (h)  
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Estimate ± SE (95% CI)</td>
<td>p-Value</td>
<td></td>
</tr>
<tr>
<td><strong>Demographics</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Infant sex (Male)</td>
<td>-0.2 ± 0.3 (-0.7, 0.4)</td>
<td>0.53</td>
<td></td>
</tr>
<tr>
<td>Infant age (months)</td>
<td>0.2 ± 0.1 (0, 0.3)</td>
<td>0.078</td>
<td></td>
</tr>
<tr>
<td><strong>Anthropometrics</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Infant length (cm)</td>
<td>0.1 ± 0.03 (0.04, 0.15)</td>
<td><strong>0.004</strong></td>
<td></td>
</tr>
<tr>
<td>Infant weight (kg)</td>
<td>0.2 ± 0.1 (0.1, 0.4)</td>
<td><strong>0.010</strong></td>
<td></td>
</tr>
<tr>
<td>Head circumference (cm)</td>
<td>0.1 ± 0.1 (0, 0.2)</td>
<td>0.23</td>
<td></td>
</tr>
<tr>
<td>Infant BMI</td>
<td>0.13 ± 0.1 (0, 0.3)</td>
<td>0.10</td>
<td></td>
</tr>
<tr>
<td><strong>Body composition</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% fat mass with US</td>
<td>0.07 ± 0.03 (0, 0.13)</td>
<td>0.040</td>
<td></td>
</tr>
<tr>
<td>% fat mass with BIS</td>
<td>0.08 ± 0.02 (0.03, 0.12)</td>
<td><strong>0.002</strong></td>
<td></td>
</tr>
</tbody>
</table>

Data are parameter estimate ± SE and 95% CI, n=41 feeds.  
* Feeding frequency self-reported by mothers as to how often infant feeds (e.g., every three hours).  
* Effects of predictors are results of univariate regression model.  
* After the false discovery rate adjustment the p-values were considered to be significant at <0.031 (highlighted).  
Abbreviations: BIS - bioimpedance spectroscopy; US - ultrasound skinfolds.

6.5 DISCUSSION

Our research shows that HM components, such as adiponectin, whey protein, casein:whey ratios, lactose, total carbohydrates, and oligosaccharides are associated with GE and breastfeeding patterns of breastfed infants. GE is a mechanism involved in satiety; therefore milk components influencing GE have the potential to affect infant MI and therefore growth and development in early life and subsequently health later in life.

Given the assumption that HM composition potentially influences GE (S. Perrella et al. 2015; Meyer et al. 2015), in term infants we expected the appetite hormones to be associated with infant GE rate, such that high concentrations and/or doses of leptin would result in slower GE (Smedh et al. 1998), whereas adiponectin might induce faster GE (Iwase et al. 2009) consistent with both animal and human models. However, neither the concentrations nor doses of these hormones were related to GE rate. Previously skim milk leptin was not found to be associated with either GE rate or GE time (Cannon et al. 2015; Cannon et al. 2017), which we have confirmed with this larger study cohort. It was speculated that whole milk leptin, which is known to be of higher concentration, might be the reason for the negative finding (Kugananthan et al. 2016). While our measures of whole milk leptin were typically higher, there is an opinion that values of this magnitude are unlikely to contribute considerably to infant
serum levels (Lonnerdal et al. 2000), so only the local pathways would be engaged in GE regulation. As such we were unable to find a relationship between whole milk leptin and GE. This is in contrast to animal studies showing reduced GE (Smedh et al. 1998) or food intake (Sanchez et al. 2005) after injection or oral administration of leptin, respectively. However, it is possible that the long-term energy expenditure regulatory effect of leptin (Bouret et al. 2004b) may mask its short-term satiety effect on GE. Alternatively, if levels of leptin are contributing significantly to serum levels, there is a possibility that the number of receptors in the stomach of the young infant is low. Further, short-term satiety signaling through hypothalamic neurons is not fully mature, both of which would allow the infant to maintain a high physiological drive to feed to ensure adequate growth (Bouret et al. 2004b; Miralles et al. 2006). Gender differences in infant serum leptin levels associated with adiposity (Petridou et al. 2005) have also been speculated to play a role in gastric response to HM leptin, although we did not find any relationships between infant sex/adiposity and both GE rate and GE time.

In contrast to leptin, we found that increased levels and doses of adiponectin were associated with longer GE times. This finding may partially explain the growth-regulating effect of adiponectin in infants in the first six months of life (Woo et al. 2009), when high HM adiponectin concentration is associated with lower infant weight and adiposity. Further, adiponectin is 20-fold higher in concentration compared to leptin and is therefore, likely to have greater biological significance (Savino et al. 2013). The lack of association between adiponectin and GE rate is in agreement with studies of rats that showed that gastric epithelium and glands are populated with adiponectin receptors, which downregulate gastric motility (Kentish et al. 2015; Gonzalez et al. 2010). Conversely, the findings are in contrast to studies of type 2 diabetic adults, in which elevated levels of adiponectin were associated with faster GE (Iwase et al. 2009). Further, other HM hormones such as ghrelin, CCK, and insulin may counteract or interact synergistically with leptin (Chaudhri et al. 2006; Perry et al. 2012) and/or adiponectin.

Our study examined an extensive array of macronutrients beyond fat, total protein and lactose. Consistent with the findings of Cannon et al. (Cannon et al. 2017), there were no associations with fat and total protein and either GE rate or GE time. Studies in dogs indicate that all three major macronutrients activate the ileal brake, resulting in reduction of GE; limited human studies support the findings for fat and carbohydrates, while associations with protein are not so straightforward (Maljaars et al. 2008; Van Citters et al. 2006). We were unable to find associations between the HM fat content
and GE, consistent with findings of Khan et al. (Khan et al. 2013a) and Kent et al. (Kent et al. 2006) regarding the FFQ. This may be because lipids initiate the ileal brake when they reach the ileum via hydrolysis of triacylglycerol into fatty acids thereby, producing a delay in GE in humans (Maljaars et al. 2008). Further analysis of HM fatty acids may shed more light on GE in breastfed infants.

However, we have found that higher whey protein concentrations are associated with larger post-feed SV, although we did not see any interaction with time, so no effect on GE rate was detected. This contradicts the results of studies of GE conducted on breastfed and formula-fed infants or studies of formula with different casein:whey ratio (Cavell 1979; Khoshoo et al. 2002) in which a fast or slow GE rate was explained by concentrations of whey protein or casein, respectively. Previous studies, however, could not adequately analyze the effect of the whey protein concentration in conjunction with volume, as they only reported gastric half-emptying time, restricted monitoring time, and/or controlled infants’ volume intakes. The whey fraction of HM is highly soluble in the gastric juices and rapidly empties from the stomach compared to other proteins such as casein. Whey isolate, however, was associated with a lower gastric inhibitory polypeptide (GIP) response in adults, consistent with decreased rate of GE (Stanstrup et al. 2014). It may very well be that whey protein speeds up the initial stage of GE (probably during the breastfeeding time), but once it activates jejunal or ileal brakes the overall GE is reduced.

While lactose is related to GE rate, it is affected by FV; at the middle range FV (71–108 mL) lactose has no relationship with GE, whereas at lower FV higher lactose concentrations are associated with slower GE, and at higher FV with faster GE. These results are consistent with Khan et al. (Khan et al. 2013a), who reported an association of higher lactose concentration with increased FFQ. These findings could be an important addition to the evaluation of the digestive and metabolic effect of lower breastfeeding frequency and larger FV, common in Western countries, contrary to the lactation practices in traditional societies (Hale et al. 2007).

In terms of casein:whey ratios the effect is opposite to that of lactose where at lower FV higher casein:whey ratios are associated with faster GE, and at higher FV with slower GE, which may explain the contradictory findings for casein associations with GE rate in previous studies (S. Perrella et al. 2015; Billeaud et al. 1990). Bovine milk casein was found to activate the ileal brake in adults, resulting in reduced food intake, although its effect on GE was not significant (van Avesaat et al. 2015). The finding of smaller volumes resulting in more rapid GE rate might be explained by the
time casein spends in the acidic environment of the stomach. While soluble whey proteins rapidly enter the small intestine mostly intact, casein transit is delayed due to the curd formation. When it exits the stomach, it is mainly in the form of degraded peptides (Boirie et al. 1997). If the FV are small some casein may exit intact, thereby speeding up GE, while if the FV are large, casein curdles and degrades to the opioid peptides that slow down GE (Daniel et al. 1990). However, this mechanism does not fully explain why higher casein:whey ratios of HM were associated with shorter GE time, which could be due to the smaller amounts of whey protein reaching the small intestine and having less effect on jejunal or ileal brakes (Van Citters et al. 2006). Our finding that higher whey protein concentrations are associated with larger post-feed SV further supports this possible explanation.

Further, \(k\)-casein has been shown to inhibit the binding of *Helicobacter pylori* to human mucosa in vitro (Stromqvist et al. 1995). \(H.\ pylori\) are Gram-negative bacteria present in the stomach, and are known to downregulate levels of ghrelin and leptin in the stomach (Francois et al. 2011), which may significantly affect GE. The protective action of HM \(k\)-casein is reinforced by lysozyme, one of the major whey proteins. While we have not seen any significant associations between lysozyme and GE, lysozyme contributes to the control of the GI bacterial population (Montagne et al. 2001), and could be upregulated to control the bacterial population in the GI tract (Rubio 2014) and increase digestion of microbial protein (Wang et al. 2012), all of which could potentially influence GE. In a clinical study of preterm infants, lysozyme added to donor HM or formula was associated with increased body weight, normalization of the stool, and improved feed tolerance (Bol'shakova et al. 1984). While all of this suggests that lysozyme could potentially have an effect on GE in certain circumstances, given that we have studied a healthy population the magnitude of the effect could be insignificant.

GE during feed administration has been previously documented in preterm infants. In this study, an average of 20% of FV is emptied from the stomach during breastfeeding compared with 10% in preterm (Perrella et al. 2014). This is probably due to a more mature GI tract in term infants and the effect of both larger FV and present pre-feed residuals, which were associated with faster GE rate, but not to the longer feed duration time in term infants or milk composition as no associations were found.

While we speculated that milk composition might regulate the MI of the infant and/or the residual volume in the stomach prior to cueing for the next feed, we were unable to show this. Rather, FV is more strongly associated with GE rate than variations
in milk composition. Gastric mechanosensation is an important factor in the regulation of satiation during food intake. Indeed, gastric distention is an important determinant of GE (Hellstrom et al. 2001), and volume-related suppression of GE rate has been reported in animal models (Lorenz 1985). The observed volume-related acceleration of GE with larger FV emptying more quickly in term breastfed infants is consistent with our previous findings (Cannon et al. 2017). The biggest effect of volume was seen after the feed, and as the post-prandial period progressed, the magnitude of this effect decreased (Figure 6.2). This may also explain the variability in the time between each feed for an infant over a 24-h period (Cannon et al. 2015; Kent et al. 2006). FFQ decreases between one and three months of lactation, while MI during each breastfeeding session increases, with both parameters remaining constant up to six months (Kent et al. 2013). This is attributed to the fact that as infants mature they become able to consume larger FV (Kent et al. 2006), resulting in a longer time between feeds. Also, larger FV are generally consumed at night or in the early morning when the frequency of feeding declines (Kent et al. 2006; Cannon et al. 2015). This decline in FFQ also coincides with higher nocturnal concentrations of leptin and fat, and lower concentrations of lactose in HM (Cannon et al. 2015), although relationships between both FFQ and FV and these components’ concentrations are yet to be evaluated.

The recommendations for breastfeeding are to feed on demand. Interestingly, we found that the majority of infants cued for a feed when milk was still present in the stomach, albeit in variable volumes (Table 6.1). This suggests that the reduction of gastric distension, which regulates hunger sensations, plays a greater role in signaling time to feed (Sepple et al. 1989). Further, it may be beneficial to the developing infant to have the gastric mucosa exposed to HM anti-inflammatory components, such as lysozyme or immunomodulatory agents and growth factors, all of which contribute to the maturation of the GI tract (Goldman 2000). Thus it may be detrimental to prescribe decreasing the frequency of feeding in breastfed infants or expect the infant stomach to be empty in order to feed again (Lawrence et al. 2011).

Furthermore, interesting associations were observed between infant MI and volumes remaining in the stomach prior to the first and second feed. Smaller residual volumes prior to the first feed were associated with greater MI, and greater MI were associated with larger volumes in the stomach prior to feeding again. This suggests that breastfed infants may appear to be consuming HM volumes in a variable pattern, but due to varying residuals may actually be feeding to a predetermined stomach volume,
which is also supported by the positive relationship between both pre-feed residuals (R1, R2). In fasting adults, ghrelin was found to increase and spontaneously decrease at the time points of the customary meals (Natalucci et al. 2005), supporting the involvement of the brain in GI tract regulation. Further studies monitoring two or more consecutive feeds or even 24-h GE measurements and analyses of ghrelin in HM would clarify this finding.

In healthy adults, post-lag GE and colonic transfer is reported to be faster in men than in women (Degen et al. 1996). In this study infant sex, age, anthropometrics, and BC were not associated with GE and breastfeeding parameters, with the exception of FFQ. FFQ decreases in the first three months of lactation and then remains stable until six months (Butte et al. 1984; Kent et al. 2013). The absence of a significant association between FFQ and age, together with associations with anthropometric and BC parameters, illustrates that FFQ is dictated by the growth and development of an infant rather than the infant age. These findings further underline the need for breastfeeding on demand, with the frequency linked to individual infant growth rates rather than scheduled feeding, which could exert a detrimental effect on infant growth.

While the monitoring of a single feed limits the analysis possibilities, examination of multiple feeds requires the study to be carried out in the mother’s home for long periods of time. The sample size is not a limitation of the study, as although no associations between milk composition and GE rate were detected, we were able to clearly show a relationship between FV and GE rate as well as associations between milk composition and other GE parameters.

6.6 CONCLUSIONS
HM appetite hormones and macronutrients and FV affect GE and feeding patterns in term breastfed infants. Adiponectin, whey protein, and casein:whey ratio are associated with GE, while the effects of casein:whey ratios and lactose concentrations on GE vary with FV. Larger FV result in a faster GE rate. Thus, milk composition and FV play an important role in appetite regulation via gastric function.
CHAPTER 7: HUMAN MILK AND INFANT BODY COMPOSITION

7.1 ABSTRACT
HM components may influence infant feeding patterns, appetite regulation and BC. This study aimed to investigate associations between breastfed term infant anthropometrics, BC, feeding parameters, and the concentrations and calculated daily intakes (CDI) of HM adiponectin, leptin (ng) and macronutrients (g) in first 12 months of life. The BC of breastfeeding dyads (n=20) was measured at 2, 5, 9 and/or 12 months after birth with US skinfolds (infants) and BIS (infants and mothers). 24-h MI and FFQ were measured. HM was analysed for adiponectin, leptin; lactose, total carbohydrates; lysozyme; whey, casein and total protein concentrations, and CDI (g) were also calculated. Statistical analysis used linear regression and mixed effects models. FFQ had positive associations with CDI of whole milk leptin (0.001±0.002; p<0.001), whey protein (0.79±0.27, p=0.007), lysozyme (14.10±4.39 g, p=0.004), total carbohydrates (0.06±0.02, p=0.005) and lactose (0.10±0.02, p<0.001). CDI of casein was negatively associated with infant FFM (-0.50±0.15, p=0.010). Total carbohydrate concentration (g/L) was positively associated with infant length (cm) (0.05±0.01; p<0.001), weight (kg) (0.013±0.004; p=0.005) and FFM (0.02±0.004, p<0.001). Concentrations and doses of HM components may influence the development of infant BC in the first year of life. This time is a critical window of infant programming and HM potentially influences risk of later disease via modulation of BC.
7.2 INTRODUCTION

Breastfeeding is associated with reduced risk of developing obesity and a range of other chronic NCD later in life (Geddes et al. 2013). The development of BC in early life is known to play an important role in the programming of these health outcomes (Wells et al. 2007). This reduction in risk may be a result of multiple mechanisms associated not only with composition of HM but also infant breastfeeding patterns and behavior (Sievers et al. 2002; Savino et al. 2009; Bartok 2011), all of which may influence the growth and development of the breastfed infants.

Differences in the weight and BC between breastfed and formula-fed infants have been attributed to the stark compositional differences between HM and formula (Butte et al. 2000b; Luque et al. 2015). In term infants, the protein, fat, carbohydrate and energy intakes of HM were found to be lower than those of formula, and at 3 and 6 months were positively correlated with both weight and FFM gain (Butte et al. 2000b). Protein content appears to play a key role in infant growth, providing rationale for the breastfed infants’ slower growth trajectory and lower FFM (Rigo et al. 2006; Roggero et al. 2008). Despite MI being associated with infant weight gain there has been no investigation of the effect of either 24-h MI or FFQ on infant BC, yet these factors are highly variable between infants. These variations suggest a self-regulatory mechanism of MI, which may be in part driven by HM components (Kon et al. 2014) that are associated with FFQ (Khan et al. 2013a).

The protein lysozyme in HM is well known for its immune effects; degrading the cell walls of Gram-positive bacteria (Montagne et al. 2001). Lysozyme is also implicated in the homeostasis of bone and cartilage growth (Reynolds 1972) and oral administration of lysozyme in preterm infants increased weight gain (Bol'shakova et al. 1984), thus it is possible that HM lysozyme could contribute to the BC of breastfed infants.

More recently, new biologically active appetite hormones such as adiponectin and leptin have been discovered in HM. Higher concentrations of HM adiponectin and leptin have an age-related effect on infant weight suggesting an active role in energy homeostasis (Woo et al. 2009; Woo et al. 2012; Miralles et al. 2006; Fields et al. 2012). The limited research in this area has not established relationships between HM leptin and infant BC, however, concentrations were examined only in skim milk and anthropometric measures or BMI were used, rather than BC measurements (Uysal et al. 2002; Fields et al. 2012). BMI is a limited index of adiposity that fails to reflect body shape, fat distribution and density (Prentice et al. 2001) and may lead to misleading
conclusions (Uysal et al. 2002) therefore, a combination of accurate non-invasive methods to measure infant BC is needed (Gridneva et al. 2016b).

It is important to understand the mechanisms by which breastfeeding may influence infant BC, as this will allow for more targeted interventions that may impact the current increase in both infant and adult overweight and obesity. Thus, the aim of this longitudinal study was to simultaneously investigate relationships of array of HM components, such as appetite hormones, proteins and carbohydrates with the BC of healthy term breastfed infants during first year of life. Further, exploration of relationships of infant 24-h MI and feeding frequency with HM components and BC were carried out.

7.3 SUBJECTS AND METHODS

7.3.1 Subjects
Breastfed infants (n=20; 10 male, 10 female) of English-speaking, predominantly Caucasian, mothers of higher social-economic status from a developed country were recruited from the community, primarily from the Australian Breastfeeding Association. Inclusion criteria were: healthy singletons, gestational age ≥37 weeks, fully breastfed at 2 and 5 months (World Health Organization 2015) and maternal intention to breastfeed until 12 months. Exclusion criteria were: infant factors that could potentially influence growth and development of BC, maternal smoking and low milk supply. All mothers provided written informed consent to participate in the study, which was approved by The University of Western Australia Human Research Ethics Committee (RA/1/4253, RA/4/1/2639) and registered with the Australian New Zealand Clinical Trials Registry (ACTRN1261600368437).

7.3.2 Study session
Measurements were made when the infants were 2 and/or 5, 9 and 12 months of age. Participants visited our laboratory at King Edward Memorial Hospital for Women (Subiaco, Perth, WA) for up to 4 monitored breastfeeding sessions between March 2013 and September 2015. At each study session the infant was weighed pre-feed, and then the mother breastfed her infant. Infant BIS measurements were taken pre-feed, unless impractical, then they were taken post-feed (Gridneva et al. 2016a). US skinfold and anthropometric measurements were taken post-feed. This combination of two methods for measuring infant BC was used to ensure safe, non-invasive and accurate assessment
and to avoid the inherent limitations of a singular technique (Gridneva et al. 2016b). Clothing was removed for the measurements except for a dry diaper and a singlet.

Maternal weight, height and BIS measurements were recorded. Small (1–2 mL) milk samples pre-/post-feed were collected into polypropylene 5-mL polypropylene vials (Disposable Products, Adelaide, Australia) from the breast/s that infant was fed from and samples were frozen at -20°C for biochemical analysis. Current FFQ of the infants was self-reported by mothers.

7.3.3 Anthropometric Measurements
Infants weight was determined before breastfeeding using Medela Electronic Baby Weigh Scales (±2.0 g; Medela AG, Switzerland). Infant crown-heel length was measured once to the nearest 0.1 cm using non-stretch tape and a headpiece and a footpiece, both applied perpendicularly to the hard surface. Infant head circumference was measured with a non-stretch tape to the nearest 0.1 cm. Infant BMI was calculated according to the following formula:

\[
BMI = \frac{\text{Body weight (kg)}}{\text{Height (m)}^2}.
\]

Maternal weight was measured using Seca electronic scales (±0.1 kg; California, USA). Height was self-reported by participants or measured against a calibrated marked wall (accuracy ±0.1 cm). Maternal BMI was calculated as above.

7.3.4 Bioimpedance spectroscopy measurements
Whole body bioimpedance (wrist to ankle) of infants and mothers was measured using the Impedimed SFB7 bioelectrical impedance analyzer (ImpediMed, Brisbane, Queensland, Australia) according to the manufacturer’s instructions. Mothers were measured in supine position on a non-conductive surface. A series of ten consecutive measurements (%FM, FM and FFM) were taken within 1–2 minutes and averaged for data analysis. Within participant CV for maternal %FM was 0.21%.

Infants’ whole body bioimpedance was measured by applying an adult protocol as used previously in infants (Gridneva et al. 2016b; Lingwood et al. 2012), but with data analyzed with settings customized for infants (Gridneva et al. 2016b; Lingwood et al. 2012). Values of resistance (ohm) at a frequency of 50 kHz (R_{50}) were determined from the curve of best fit, averaged for analysis purposes and used in BIS age-matched equations for FFM (below). Within participant coefficient of variation for infant R_{50} was 1.5% (Gridneva et al. 2016a).
7.3.5 Ultrasound skinfold measurements

Infant skinfolds were measured using the Aplio XG (Toshiba, Tokyo, Japan) US machine with a 14-8 MHz transducer (PLT-1204BX) and sterile water-based Parker ultrasonic gel (Fairfield, NJ, USA) as described previously (Gridneva et al. 2016b). Single US scans of four anatomical sites (biceps, subscapular, suprailiac and triceps) were performed on the left side of the body with minimal compression. ST (skin thickness and the skin-fat interface to fat-muscle interface distance) was measured directly from images on the screen using electronic calipers. One experienced sonographer with good intra- and interrater reliability (McLeod et al. 2013) performed all of the measurements. US measurements were doubled (Ulbricht et al. 2012) for use in skinfold equations (2-skinfolds (US 2SF): triceps, subscapular; 4-skinfolds (US 4SF): biceps, subscapular, suprailiac and triceps) developed for SCTT measurement with skinfold callipers.

7.3.6 Body composition equations

BIS- and US-based prediction equations were sourced from the literature, evaluated (Gridneva et al. 2016b) and selected according to the following criteria: absence of significant difference from the reference distribution, closest age match, predominantly Caucasian population. Equations used in the calculation of infant BC (Lingwood et al. 2012; Bocage 1988; Slaughter et al. 1988; Brook 1971; Lohman 1984; Butte et al. 2000a) are presented in Table 7.1.

7.3.7 24-hour milk intake and feeding frequency

MI was measured by mothers using the 24-h milk production protocol, weighing infants at home with the Medela Electronic Baby Weigh Scales pre- and post each breastfeed during a 24-h period plus one breastfeeding, and recording amounts of HM consumed by the infant (including expressed HM if any) (Arthur et al. 1987). 24-h MI was determined as previously described with potential underestimation of 3-10% (Arthur et al. 1987) and FFQ (meals per day) was recorded (Kent et al. 2006). 24-h MI was measured at three time points: between 2 and 5 months, when MI is shown to be stable (Kent et al. 2006), and within two weeks of 9 and 12 months. Given that measuring 24-h MI is not always practical, particularly at the later stages of lactation, mothers were also asked how frequently the infant fed, and self-reported the typical time between the feeds (e.g., each 2 hours) during the week prior to the study session as a proxy measure of FFQ.
Table 7.1 Published equations for prediction of infant body composition used in the study.

<table>
<thead>
<tr>
<th>First author, year (reference)</th>
<th>Months after birth used for</th>
<th>Equation</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bioimpedance equations</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lingwood, 2012 (Lingwood et al. 2012)</td>
<td>2mo</td>
<td>( FFM = 1.458 + 0.498W - 0.197S + \frac{0.067L^2}{R_{50}} )</td>
</tr>
<tr>
<td></td>
<td>5mo</td>
<td>( FFM = 2.203 + 0.334W - 0.361S + \frac{0.185L^2}{R_{50}} )</td>
</tr>
<tr>
<td>Bocage, 1988 (Bocage 1988)</td>
<td>9mo, 12mo</td>
<td>( TBW = \left( \frac{0.418W + 1936}{R_{50}} + 0.8649 \right) \frac{L}{100} )</td>
</tr>
<tr>
<td><strong>Fat-free mass</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>( FFM = \frac{TBW}{HF} )</td>
</tr>
<tr>
<td><strong>Fat mass</strong></td>
<td></td>
<td>( FM = W - FFM )</td>
</tr>
<tr>
<td><strong>Percentage fat mass</strong></td>
<td></td>
<td>( %FM = \frac{FM}{W} \times 100 )</td>
</tr>
<tr>
<td><strong>Skinfold equations</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Two skinfolds</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Slaughter, 1988 (Slaughter et al. 1988)</td>
<td>All</td>
<td>( Male %FM = 1.21\Sigma - 0.008\Sigma^2 - 1.7 )</td>
</tr>
<tr>
<td></td>
<td></td>
<td>( Female %FM = 1.33\Sigma - 0.013\Sigma^2 - 2.5 )</td>
</tr>
<tr>
<td>Four skinfolds</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brook, 1971 (Brook 1971)</td>
<td>All</td>
<td>( Male d = 1.1690 - 0.0788 \log(\Sigma SFT) )</td>
</tr>
<tr>
<td></td>
<td>All</td>
<td>( Female d = 1.2063 - 0.0999 \log(\Sigma SFT) )</td>
</tr>
<tr>
<td><strong>Percentage fat mass</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lohman, 1984 (Lohman 1984)</td>
<td></td>
<td>( %FM = 100\left( \frac{5.28}{d} - 4.89 \right) )</td>
</tr>
</tbody>
</table>

Published equations (presented as per source) used in the study for predicting body density, fat-free mass, total body water or percentage fat mass using bioelectrical impedance and skinfolds in pediatric subjects. Abbreviations: \( d \) – body density (kg/L); \( FFM \) – fat-free mass (kg); \( FM \) – fat mass; \( \%FM \) – percentage fat mass (%); \( HF \) – age-appropriate hydration factors calculated from (Butte et al. 2000a) data; \( L \) – body length (cm); \( R_{50} \) - impedance variable (\( \Omega \)); \( S \) – sex variable (\( male = 1, female =2 \)); \( \Sigma \) - sum of two skinfolds, subscapular and triceps (mm); \( \Sigma SFT \) - sum of four skinfolds, biceps, subscapular, suprailiac and triceps (mm); \( TBW \) - total body water (kg); \( W \) - weight, (kg).
7.3.8 Calculated daily intakes of hormones and macronutrients
MIs from the 24-h milk productions, and HM component concentrations (averaged) from samples taken at the study sessions were used for the calculation of daily intakes (CDI). These CDI were considered representative of a typical daily intake due to absence of significant short-term changes in macronutrient concentrations (Khan et al. 2013b).

7.3.9 Sample preparation
Prior to further analysis, HM samples were thawed for 2 h at RT, mixed on Intelli-Mixer RM-2M (ELMI, Riga, Latvia) at 50 revolutions per min in “UU” mode for 15 s, then, after gentle inversion (3 times), aliquoted into 1.5 mL tubes (Sarstedt, Numbrecht, Germany). Pre- and post-feed samples of whole HM were used for measuring whole milk leptin and adiponectin concentrations. Pre- and post-feed samples were pooled for measuring casein and whey protein. Milk samples were defatted by centrifugation at RT in a Beckman Microfuge 11 (Aberdon Enterprise Inc, Elk Grove Village, IL, USA) at 10,000 × g for 10 min and removing the fat layer by clipping it off together with the top of the tube (Keller et al. 1986). Skim HM was used for measuring skim milk leptin, total protein, lysozyme, lactose, and total carbohydrates concentrations. Standard assays were adapted for and carried out using a JANUS workstation (PerkinElmer, Inc., Waltham, USA) and measured on EnSpire (PerkinElmer, Inc., Waltham, USA).

7.3.10 Leptin
Leptin concentration in whole and skim HM was measured using the R&D Systems Human Leptin ELISA DuoSet kit (R & D system, Minneapolis, USA) with a protocol to measure leptin in skim HM optimised by Cannon et al. (Cannon et al. 2015), and further modified for measurement of leptin in skim and whole HM by Kugananthan et al. (Kugananthan et al. 2016). Recovery of leptin was 97.1±9.1% (n=10) with a detection limit of 0.05 ng/mL and an inter-assay CV of <7.2%.

7.3.11 Adiponectin
Adiponectin concentration was measured in whole HM using the Biovendor Human Adiponectin Sandwich ELISA kit (Life Technologies, Asheville, USA). Adiponectin recovery was 96.2±3.2% (n=10) with a detection limit of 1 ng/mL and an inter-assay CV of <2.5%.

7.3.11 Protein
Casein and whey proteins were separated by the method described by Kunz and
Lonnerdal (Kunz et al. 1989) and Khan et al. (Khan et al. 2012a). Protein concentrations (total protein, casein, and whey proteins) were measured using the Bradford Protein Assay adapted from Mitoulas et al. (Mitoulas et al. 2002). Recovery of protein was 100.6%±5.2% (n=5) with a detection limit of 0.031 g/L and an inter-assay CV of 7.8% (n=18). Casein:whey ratio was calculated as follows:

\[
\text{Casein:whey ratio} = \frac{\text{casein concentration}}{\text{whey protein concentration}}
\]

7.3.12 Lysozyme
Lysozyme concentration was determined using a modified turbidimetric assay (Selsted et al. 1980). Standards (range 0.00075 - 0.0125 g/l) of hen egg white lysozyme (EC 3.2.1.17, Sigma, St. Louis, Missouri, USA) and SHM samples were diluted 10-fold with 0.1M of Na\textsubscript{2}HPO\textsubscript{4}/1.1mM of citric acid (pH 5.8) buffer. Twenty-five micro litres of standards or diluted SHM samples were placed into the wells of a plate (Greiner bio-one, Frickenhausen, Germany), 175μl of Micrococcus lysodeiltikus suspension (0.075% w/v, ATCC No. 4698, Sigma, St. Louis, Missouri, USA) was added into each well and plate was incubated at RT for 1 h. The absorbance was measured at 450 nm. Recovery of lysozyme was 97.0%±5.0% (n=8) with a detection limit of 0.007 g/L and an inter-assay CV of 13.0% (n=8).

7.3.13 Carbohydrates
Skim HM was deproteinized with trichloroacetic acid (Euber et al. 1979) before dehydration by sulphuric acid (Albalasmeh et al. 2013). This technique reliably estimates concentrations and carbon content for monosaccharides, disaccharides, and polysaccharides. Total carbohydrates were analyzed by UV-spectrophotometry at 315nm. Recovery of added lactose was 101.4%±2.1% (n=7) with a detection limit of 0.007 g/L and an inter-assay CV of 3.3% (n=7).

Lactose concentration was measured using the enzymatic-spectrophotometric method of Kuhn and Lowenstein (Kuhn et al. 1967) adapted from Mitoulas, et al. (Mitoulas et al. 2002) with recovery of 98.2±4.1% (n=10), detection limit of 30 mM and inter-assay CV of 3.5%.

The total HMO concentration (g/L) was calculated by deducting concentration of lactose (g/L) from concentration of total carbohydrates (g/L). Glucose and galactose were not measured or accounted for, as their concentrations in HM are small and not detectable in this assay (Newburg et al. 1995).
7.3.14 Statistical analyses

Statistical analysis was performed in R 2.9.0 for Mac OSX (R Core Team 2009). Additional packages were used for linear mixed effect models (nlme) (Pinheiro et al. 2009), intra-class correlations (irr) (Gamer et al. 2012), and Tukey’s all pair comparisons (multcomp) (Hothorn et al. 2008). Descriptive statistics are reported as mean ± standard deviation (SD) (range); model parameters as estimate ± SE (approximate 95% CI).

During this longitudinal study infants were measured at four time points (2 and/or 5, 9 and 12 months). An approximate sample size was calculated using the ‘Linear multiple regression: fixed model: $r^2$ increase’ option in G*Power (Faul et al. 2009) as if this was a cross-sectional study with equal numbers at each time. Allowing four predictors (3 for age comparisons), $\alpha=0.05$ and 14 participants (56 sample points = 14 participants x 4 time points) gave the study power of 0.80 to detect an effect size of 0.15. This approach was selected, as there is no closed form expression suitable for the calculation of sample sizes for this research design (Diggle et al. 2002), with the consideration that longitudinal study design is more powerful. Recruitment of participants at the 5 month point was introduced, as many mothers would not commit to a study that required breastfeeding to 12 months, when approached at 2 months ($n=8$). As a result, required number of participants was increased to 20 in order to maintain predicted power; this also addressed issues relating to missed visits.

Session data at 2 months of age is missing for 5 infants, and 2 infants at 9 and 12 months. Missing data also occurred due to insufficient milk samples. The following measures from the 80 expected were missing as result: infant weight ($n=9$); infant head circumference and %FM, FM, FFM measured with US 2SF, and maternal age, weight, height, BMI, %FM, FM and FFM ($n=10$); infant length, measures of total protein, whey and casein protein, ($n=11$); infant BMI and %FM, FM, FFM measured with US 4SF, measures of skim and whole HM leptin, adiponectin, lactose and total carbohydrate ($n=12$); infant %FM, FM, FFM measured with BIS ($n=13$); measures of lysozyme ($n=15$); self-reported FFQ ($n = 20$). Missing data also occurred due to difficulties with conducting 24-h MI measurements at the later stages of lactation. The following measurements from the 60 expected were missing: FFQ from 24-hour milk productions ($n = 28$) and 24-h MI ($n = 29$). Missing data was dealt with using available case analysis.

Linear mixed effects models with random intercept per participant were used to determine whether BC measurements (%FM, FM and FFM) differed systematically by
age, measurement method (US2, US4 and BIS) and infant sex. As interactions between sex and methods were non-significant ($p>0.52$), reported associations are for pooled data. Months after birth were accounted for in all models except compositional comparisons; results reported account for this, regardless of significance. General linear hypothesis tests (Tukey’s all pair comparisons) were used to check for systematic differences in response variables at different months after birth/different measurement methods. An intercept only linear mixed effects model was used to calculate the coefficient of variation for maternal %FM measurements ($n=10, 10$ measurements each). The two measurements of FFQ were compared using an oneway intra-class correlation for agreement of single measures.

Fitting a sequence of models was used to test for associations between response and predictor variables. For each set of response and predictor variables, four models were fit: linear regressions and random intercept linear mixed effects models, with and without infant age as a covariate. Selection of appropriate models used likelihood ratio tests.

Due to the exploratory nature of this small proof-of-concept study any significant associations ($p<0.05$) were considered to be of interest and there has been no adjustment for multiple comparisons, thus all raw significant p-values are reported with the exception of the abstract where significance level is set at 0.01 and below. Given the number of models being run, it is not realistic to report the parameter estimates for all of them. Thus, majority of results are presented as statistically significant associations only and unreported results of any possible pairwise associations are considered to be not significant.

7.4 RESULTS

7.4.1 Subjects
Twenty-two infants were recruited; 2 infants (1 male, 1 female) were excluded from the study after the 2-month visit (starting supplementation with the formula; personal circumstances). Sixteen out of 18 infants (89%) measured at 12 months still continued to breastfeed. One male stopped breastfeeding 2 weeks before the 12-month appointment and one female stopped at 10 months after birth. Both infants and their mothers were measured at 12 months.

Demographic, anthropometric and breastfeeding characteristics at the four study sessions are presented in Table 7.2. Males were heavier (0.85 [0.12, 1.57], $p=0.025$) and
had larger head circumferences (1.89 [0.81, 2.96], \( p = 0.002 \)) than females, while no significant difference between sexes was seen for either length (1.68 [-0.24, 3.59], \( p = 0.083 \)) or BMI (1.09 [-0.15, 2.32], \( p = 0.081 \)).

Table 7.2 Subject demographic, anthropometric and feeding characteristic \( ^a \).

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Months after birth</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2 (( n = 15 )) Mean ± SD (Range)</td>
</tr>
<tr>
<td><strong>Mothers</strong></td>
<td></td>
</tr>
<tr>
<td>Age (yrs)</td>
<td>33.3 ± 4.2 ( ^b ) (24 - 40)</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>78.8 ± 19.3 (57.5 - 116.2)</td>
</tr>
<tr>
<td>BMI</td>
<td>27.2 ± 5.5 (20.4 - 35.5)</td>
</tr>
<tr>
<td>Fat-free Mass (kg)</td>
<td>49.5 ± 8.2 (38.2 - 66.2)</td>
</tr>
<tr>
<td>Fat Mass (kg)</td>
<td>29.3 ± 11.8 (15.2 - 50.0)</td>
</tr>
<tr>
<td>Fat Mass (%)</td>
<td>36.0 ± 6.4 (25.7 - 44.7)</td>
</tr>
<tr>
<td><strong>Infants</strong></td>
<td></td>
</tr>
<tr>
<td>Sex (M/F)</td>
<td>9M/6F</td>
</tr>
<tr>
<td>Age (months)</td>
<td>2.04 ± 0.14 (1.87 - 2.33)</td>
</tr>
<tr>
<td>Length (cm)</td>
<td>58.1 ± 1.9 (54.2 - 60.0)</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>5.630 ± 0.660 (4.420 - 7.400)</td>
</tr>
<tr>
<td>BMI</td>
<td>16.6 ± 1.2 (14.5 - 18.1)</td>
</tr>
<tr>
<td>Head circumference (cm)</td>
<td>39.7 ± 1.6 (37.0 - 42.0)</td>
</tr>
<tr>
<td><strong>Breastfeeding</strong></td>
<td></td>
</tr>
<tr>
<td>24-h milk intake (mL)</td>
<td>n/a ( ^c ) (490 - 1255)</td>
</tr>
<tr>
<td>24-h feeding frequency (MP)</td>
<td>n/a ( ^c ) (5 - 12)</td>
</tr>
<tr>
<td>Feeding frequency (SR)</td>
<td>2.3 ± 0.4 ( ^d ) (1.5 – 3.0)</td>
</tr>
</tbody>
</table>

\( ^a \) Subject characteristics presented grouped by the months after birth. \( ^b \) Data are mean ± SD and ranges. \( ^c \) Milk intake and feeding frequency as meals per 24-hours was determined from 24-h milk.
production (MP) measured between 2 and 5 months (presented at 5 months here, n=17) and within 2 weeks of 9 (n=6) and 12 months (n=8). Maternal self-report (SR) of feeding frequency at the time of the visit as an average time between feeds (e.g., each 2 h) (n=11, n=19, n=17, n=13 at 2, 5, 9 and 12 months respectively).

7.4.2 Infant body composition
Infant BC is presented in Table 7.3. FFM was significantly higher in males overall (0.66 [0.19, 1.14] kg, p=0.009) and, when the equations were considered separately, measured with US 2SF, US 4SF or BIS (0.55 [0.07, 1.03] kg, p=0.027; 0.70 [0.20, 1.20] kg, p=0.009; and 0.74 [0.25, 1.22] kg, p=0.005, respectively). Differences were not seen for %FM and FM overall (males %FM: -0.38 [-3.02, 2.26] %, p=0.77; FM: 0.19 [-0.17, 0.55] kg, p=0.27) or when the equations were considered separately (p>0.30 and p>0.95, respectively). Measurement method was not associated with differences in overall %FM (p>0.074), FM (p>0.11) or FFM (p>0.15).

7.4.3 Milk components
Milk components concentrations and CDI at 4 time points are detailed in Table 7.4. Higher total protein concentrations (g/L) were associated with higher whey protein (g/L) (0.25±0.06; p<0.001) and casein (g/L) (0.08±0.02; p<0.001) concentrations. Higher whey protein concentrations (g/L) were associated with higher adiponectin concentrations (ng/mL) (0.68±0.030; p=0.030). Higher whole milk leptin concentrations (ng/mL) were associated with lower casein concentrations (-0.07±0.03; p=0.026) and lower casein:whey ratio (-0.31±0.15; p=0.038). Higher total carbohydrates were associated with higher HMO concentrations (g/L) (0.98±0.06; p<0.001), while higher lactose (g/L) was associated with lower HMO concentrations (g/L) (-0.89±0.28; p=0.002).

7.4.4 Effect of month after birth
Maternal weight, BMI, FM and %FM decreased significantly after 5 months postpartum (Table 7.5), while FFM did not differ (p=0.10).

Infant anthropometrics, FM and FFM increased significantly as age increased, while %FM initially increased and then decreased (Table 7.5). Significant changes in concentrations over time occurred in skim milk leptin (decreased), lysozyme (increased), whey protein (lowest at 5 months), casein (highest at 5 months) and casein:whey ratio (highest at 5 months). FFQ and 24-h MI decreased across the lactation, as did the CDI of adiponectin, skim milk leptin, total carbohydrates, casein, whey and total protein (Table 7.5).
Table 7.3 Body composition of breastfed infants aged 2 to 12 months calculated with ultrasound and bioimpedance equations.

<table>
<thead>
<tr>
<th>Equations</th>
<th>2 months</th>
<th>5 months</th>
<th>9 months</th>
<th>12 months</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Female</td>
<td>Male</td>
<td>Pooled</td>
<td>Female</td>
</tr>
<tr>
<td>Ultrasound 2-skinfolds</td>
<td>n = 6</td>
<td>n = 8</td>
<td>n = 14</td>
<td>n = 10</td>
</tr>
<tr>
<td>FFM (kg)</td>
<td>3.74±0.27</td>
<td>4.42±0.36</td>
<td>4.23±0.44</td>
<td>5.08±0.49</td>
</tr>
<tr>
<td>FM (kg)</td>
<td>1.21±0.25</td>
<td>1.63±0.53</td>
<td>1.40±0.25</td>
<td>1.78±0.29</td>
</tr>
<tr>
<td>FM (%)</td>
<td>24.2±3.1</td>
<td>26.5±5.1</td>
<td>24.7±2.3</td>
<td>25.8±2.6</td>
</tr>
<tr>
<td>Ultrasound 4-skinfolds</td>
<td>n = 6</td>
<td>n = 7</td>
<td>n = 13</td>
<td>n = 10</td>
</tr>
<tr>
<td>FFM (kg)</td>
<td>3.69±0.30</td>
<td>4.53±0.44</td>
<td>4.20±0.47</td>
<td>5.05±0.49</td>
</tr>
<tr>
<td>FM (kg)</td>
<td>1.25±0.26</td>
<td>1.54±0.40</td>
<td>1.43±0.24</td>
<td>1.81±0.37</td>
</tr>
<tr>
<td>FM (%)</td>
<td>25.2±3.5</td>
<td>25.1±3.6</td>
<td>25.3±2.5</td>
<td>26.2±3.6</td>
</tr>
<tr>
<td>Bioimpedance spectroscopy</td>
<td>n = 6</td>
<td>n = 7</td>
<td>n = 13</td>
<td>n = 10</td>
</tr>
<tr>
<td>FFM (kg)</td>
<td>3.84±0.27</td>
<td>4.56±0.33</td>
<td>4.36±0.43</td>
<td>4.79±0.37</td>
</tr>
<tr>
<td>FM (kg)</td>
<td>1.10±0.22</td>
<td>1.30±0.26</td>
<td>1.27±0.26</td>
<td>2.07±0.40</td>
</tr>
<tr>
<td>FM (%)</td>
<td>22.1±2.3</td>
<td>22.0±2.5</td>
<td>22.4±2.6</td>
<td>30.0±3.0</td>
</tr>
</tbody>
</table>

* Body composition (%FM - percentage fat mass, FM - fat mass, FFM - fat-free mass) of breastfed infants aged 2, 5, 9 and 12 months calculated with various equations: ultrasound 2-skinfolds - (Slaughter et al. 1988) at all ages; ultrasound 4-skinfolds - (Brook 1971) at all ages; bioimpedance spectroscopy - (Lingwood et al. 2012) 3mo equation at 2 months and 4.5mo equation at 5 months; and (Bocage 1988) at 9 and 12 months. * Mean ± SD (all such values).
<table>
<thead>
<tr>
<th>Components</th>
<th>2 Mean ± SD (Range)</th>
<th>5 Months after birth Mean ± SD (Range)</th>
<th>9 Months after birth Mean ± SD (Range)</th>
<th>12 Months after birth Mean ± SD (Range)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Concentrations</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adiponectin (ng/mL)</td>
<td>11.14 ± 5.79 b</td>
<td>8.42 ± 1.69</td>
<td>8.44 ± 1.33</td>
<td>11.22 ± 4.22</td>
</tr>
<tr>
<td></td>
<td>(6.61 - 21.56) b</td>
<td>(6.18 - 22.58)</td>
<td>(6.41 - 12.86)</td>
<td>(5.66 - 19.38)</td>
</tr>
<tr>
<td>Whole milk leptin (ng/mL)</td>
<td>0.50 ± 0.18</td>
<td>0.49 ± 0.17</td>
<td>0.56 ± 0.11</td>
<td>0.50 ± 0.11</td>
</tr>
<tr>
<td></td>
<td>(0.24 - 0.77)</td>
<td>(0.23 - 0.71)</td>
<td>(0.42 - 0.67)</td>
<td>(0.34 - 0.74)</td>
</tr>
<tr>
<td>Skim milk leptin (ng/mL)</td>
<td>0.34 ± 0.20</td>
<td>0.26 ± 0.08</td>
<td>0.21 ± 0.02</td>
<td>0.21 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>(0.20 - 0.84)</td>
<td>(0.20 - 0.40)</td>
<td>(0.19 - 0.27)</td>
<td>(0.19 - 0.40)</td>
</tr>
<tr>
<td>Total protein (g/L)</td>
<td>11.03 ± 1.40</td>
<td>11.90 ± 4.31</td>
<td>9.69 ± 1.12</td>
<td>10.72 ± 2.84</td>
</tr>
<tr>
<td></td>
<td>(7.60 - 12.32)</td>
<td>(7.93 - 24.16)</td>
<td>(7.25 - 14.96)</td>
<td>(5.89 - 16.80)</td>
</tr>
<tr>
<td>Casein (g/L)</td>
<td>1.24 ± 0.24</td>
<td>1.51 ± 0.44</td>
<td>1.11 ± 0.38</td>
<td>1.07 ± 0.35</td>
</tr>
<tr>
<td></td>
<td>(0.69 - 1.57)</td>
<td>(0.78 - 3.45)</td>
<td>(0.49 - 2.00)</td>
<td>(0.65 - 1.87)</td>
</tr>
<tr>
<td>Whey protein (g/L)</td>
<td>6.44 ± 1.62</td>
<td>5.43 ± 0.90</td>
<td>5.43 ± 0.93</td>
<td>7.61 ± 1.85</td>
</tr>
<tr>
<td></td>
<td>(4.12 - 9.08)</td>
<td>(3.82 - 7.38)</td>
<td>(3.94 - 9.40)</td>
<td>(4.49 - 9.76)</td>
</tr>
<tr>
<td>Casein:whey ratio</td>
<td>0.21 ± 0.07</td>
<td>0.28 ± 0.05</td>
<td>0.21 ± 0.08</td>
<td>0.16 ± 0.09</td>
</tr>
<tr>
<td></td>
<td>(0.10 - 0.31)</td>
<td>(0.13 - 0.73)</td>
<td>(0.07 - 0.38)</td>
<td>(0.09 - 0.36)</td>
</tr>
<tr>
<td>Lysozyme (g/L)</td>
<td>0.11 ± 0.13</td>
<td>0.12 ± 0.06</td>
<td>0.14 ± 0.02</td>
<td>0.14 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>(0.01 - 0.46)</td>
<td>(0.08 - 0.29)</td>
<td>(0.10 - 0.16)</td>
<td>(0.12 - 0.65)</td>
</tr>
<tr>
<td>Total carbohydrates (g/L)</td>
<td>86.74 ± 9.23</td>
<td>80.68 ± 7.90</td>
<td>87.81 ± 11.10</td>
<td>88.35 ± 21.16</td>
</tr>
<tr>
<td></td>
<td>(67.08 - 97.94)</td>
<td>(69.27 - 94.13)</td>
<td>(60.90 - 105.62)</td>
<td>(56.94 - 126.94)</td>
</tr>
<tr>
<td>Lactose (g/L)</td>
<td>64.45 ± 4.08</td>
<td>64.29 ± 5.89</td>
<td>65.34 ± 5.30</td>
<td>66.93 ± 3.98</td>
</tr>
<tr>
<td></td>
<td>(59.10 - 77.94)</td>
<td>(53.49 - 70.55)</td>
<td>(57.55 - 79.02)</td>
<td>(60.07 - 79.33)</td>
</tr>
<tr>
<td>HMO d (g/L)</td>
<td>22.29 ± 10.71</td>
<td>16.38 ± 9.89</td>
<td>22.47 ± 9.15</td>
<td>21.41 ± 22.27</td>
</tr>
<tr>
<td><strong>CDI e</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adiponectin (ng)</td>
<td>n/a c</td>
<td>7958 ± 4585 c</td>
<td>4094 ± 1469</td>
<td>4040 ± 1520</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(3584 - 22439)</td>
<td>(2142 - 5600)</td>
<td>(2532 - 6352)</td>
</tr>
<tr>
<td>Whole milk leptin (ng)</td>
<td>n/a</td>
<td>364 ± 186</td>
<td>271 ± 74</td>
<td>217 ± 96</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(123 - 892)</td>
<td>(132 - 349)</td>
<td>(122 - 350)</td>
</tr>
<tr>
<td>Skim milk leptin (ng)</td>
<td>n/a</td>
<td>196 ± 77</td>
<td>100 ± 23</td>
<td>97 ± 39</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(106 - 370)</td>
<td>(62 - 130)</td>
<td>(51 - 159)</td>
</tr>
<tr>
<td>Total protein (g/L)</td>
<td>n/a</td>
<td>9.14 ± 4.05</td>
<td>4.84 ± 1.76</td>
<td>4.30 ± 2.34</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(4.51 - 21.07)</td>
<td>(2.18 - 7.48)</td>
<td>(1.93 - 7.23)</td>
</tr>
<tr>
<td>Casein (g)</td>
<td>n/a</td>
<td>1.44 ± 0.83</td>
<td>0.55 ± 0.20</td>
<td>0.54 ± 0.38</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(0.56 - 3.63)</td>
<td>(0.32 - 0.80)</td>
<td>(0.24 - 1.19)</td>
</tr>
<tr>
<td>Whey protein (g)</td>
<td>n/a</td>
<td>4.22 ± 1.28</td>
<td>2.85 ± 1.12</td>
<td>2.98 ± 1.40</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(2.34 - 6.43)</td>
<td>(1.70 - 4.64)</td>
<td>(1.15 - 4.40)</td>
</tr>
<tr>
<td>Lysozyme (g)</td>
<td>n/a</td>
<td>0.090 ± 0.076</td>
<td>0.062 ± 0.014</td>
<td>0.068 ± 0.024</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(0.049 - 0.359)</td>
<td>(0.042 - 0.081)</td>
<td>(0.052 - 0.174)</td>
</tr>
<tr>
<td>Total carbohydrates (g)</td>
<td>n/a</td>
<td>62.50 ± 16.00</td>
<td>45.00 ± 9.98</td>
<td>43.13 ± 32.56</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(42.85 - 98.41)</td>
<td>(28.15 - 58.33)</td>
<td>(22.18 - 100.92)</td>
</tr>
<tr>
<td>Lactose (g)</td>
<td>n/a</td>
<td>50.75 ± 15.52</td>
<td>32.96 ± 8.33</td>
<td>29.56 ± 13.22</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(32.49 - 88.54)</td>
<td>(19.60 - 43.03)</td>
<td>(18.02 - 51.44)</td>
</tr>
<tr>
<td>HMO d (g)</td>
<td>n/a</td>
<td>11.76 ± 6.0</td>
<td>12.04 ± 2.99</td>
<td>13.56 ± 20.26</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(1.97 - 21.61)</td>
<td>(8.55 - 15.67)</td>
<td>(1.49 - 49.48)</td>
</tr>
</tbody>
</table>

* Milk components’ concentrations and 24-hour component intakes are presented grouped by the months after birth. b Data are mean ± SD and ranges. c CDIs of milk components were calculated between 2 and 5 months (presented at 5 months here, n=17) and within 2 weeks of 9 (n=6) and 12 months (n=8). d HMO – human milk oligosaccharides. HMO concentration was calculated by deducting concentration of lactose (g/L) from concentration of total carbohydrates (g/L).
### Table 7.5 Significant differences by infant age/lactation duration within measured parameters a.

<table>
<thead>
<tr>
<th>Measure</th>
<th>5 and 2</th>
<th>9 and 2</th>
<th>12 and 2</th>
<th>9 and 5</th>
<th>12 and 5</th>
<th>12 and 9</th>
<th>p overall</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mother characteristics</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>0.37</td>
<td>0.027 b</td>
<td>0.009 b</td>
<td>0.50</td>
<td>0.27</td>
<td>0.98</td>
<td>0.014</td>
</tr>
<tr>
<td></td>
<td>-1.10 ± 0.68</td>
<td>-1.98 ± 0.71</td>
<td>-2.23 ± 0.71</td>
<td>-0.88 ± 0.62</td>
<td>-1.13 ± 0.62</td>
<td>-0.25 ± 0.64</td>
<td></td>
</tr>
<tr>
<td>BMI</td>
<td>0.35</td>
<td>0.016</td>
<td>0.007</td>
<td>0.40</td>
<td>0.25</td>
<td>0.99</td>
<td>0.009</td>
</tr>
<tr>
<td></td>
<td>-0.38 ± 0.23</td>
<td>-0.71 ± 0.24</td>
<td>-0.78 ± 0.24</td>
<td>-0.33 ± 0.21</td>
<td>-0.39 ± 0.21</td>
<td>-0.06 ± 0.22</td>
<td></td>
</tr>
<tr>
<td>Fat mass (kg)</td>
<td>0.99</td>
<td>0.25</td>
<td>0.003</td>
<td>0.28</td>
<td>0.002</td>
<td>0.27</td>
<td>0.002</td>
</tr>
<tr>
<td></td>
<td>-0.15 ± 0.50</td>
<td>-0.97 ± 0.52</td>
<td>-1.81 ± 0.52</td>
<td>-0.82 ± 0.46</td>
<td>-1.67 ± 0.46</td>
<td>-0.85 ± 0.47</td>
<td></td>
</tr>
<tr>
<td>Fat mass (%)</td>
<td>0.98</td>
<td>0.62</td>
<td>0.004</td>
<td>0.28</td>
<td>&lt;0.001</td>
<td>0.066</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.22 ± 0.57</td>
<td>-0.72 ± 0.59</td>
<td>-2.03 ± 0.59</td>
<td>-0.94 ± 0.52</td>
<td>-2.25 ± 0.52</td>
<td>-1.31 ± 0.54</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Infant characteristics</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Length (cm)</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>6.81 ± 0.41</td>
<td>12.36 ± 0.42</td>
<td>16.49 ± 0.42</td>
<td>5.55 ± 0.36</td>
<td>9.68 ± 0.37</td>
<td>4.13 ± 0.37</td>
<td></td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>1.78 ± 0.13</td>
<td>3.09 ± 0.13</td>
<td>3.85 ± 0.13</td>
<td>1.31 ± 0.12</td>
<td>2.08 ± 0.12</td>
<td>0.77 ± 0.12</td>
<td></td>
</tr>
<tr>
<td>Head circumference (cm)</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>3.11 ± 0.20</td>
<td>5.71 ± 0.21</td>
<td>6.72 ± 0.21</td>
<td>2.60 ± 0.18</td>
<td>3.61 ± 0.19</td>
<td>1.01 ± 0.19</td>
<td></td>
</tr>
<tr>
<td>BMI</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>1.11 ± 0.29</td>
<td>1.14 ± 0.30</td>
<td>0.61 ± 0.30</td>
<td>0.03 ± 0.26</td>
<td>-0.50 ± 0.26</td>
<td>-0.52 ± 0.27</td>
<td></td>
</tr>
<tr>
<td>Fat-free mass US2 (kg)</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>1.21 ± 0.12</td>
<td>2.23 ± 0.12</td>
<td>2.86 ± 0.12</td>
<td>1.02 ± 0.11</td>
<td>1.65 ± 0.11</td>
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<td>&lt;0.001</td>
<td>&lt;0.001</td>
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<td>1.26 ± 0.12</td>
<td>2.28 ± 0.12</td>
<td>3.02 ± 0.12</td>
<td>1.02 ± 0.11</td>
<td>1.76 ± 0.11</td>
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<tr>
<td>Fat-free mass BIS (kg)</td>
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<td>&lt;0.001</td>
<td>&lt;0.001</td>
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<tr>
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<td>0.96 ± 0.13</td>
<td>2.15 ± 0.13</td>
<td>2.76 ± 0.13</td>
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<td>Fat mass US4 (kg)</td>
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<td>&lt;0.001</td>
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<tr>
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<td>0.47 ± 0.10</td>
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<td>0.81 ± 0.10</td>
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<td>Fat mass BIS (kg)</td>
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<td>&lt;0.001</td>
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<tr>
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<td>0.86 ± 0.12</td>
<td>0.97 ± 0.12</td>
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<tr>
<td>Fat mass BIS (%)</td>
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<td>0.073</td>
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<td>0.006</td>
<td>0.96</td>
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<td>6.27 ± 1.12</td>
<td>3.39 ± 1.15</td>
<td>2.85 ± 1.18</td>
<td>-2.88 ± 1.01</td>
<td>-3.42 ± 1.05</td>
<td>-0.54 ± 1.06</td>
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Table 7.5 Continued.

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<th>Measure</th>
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<th>12 and 2</th>
<th>9 and 5</th>
<th>12 and 5</th>
<th>12 and 9</th>
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<td>Skim milk leptin (ng/mL)</td>
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<td>0.009</td>
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<td>-0.10 ± 0.03</td>
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<tr>
<td>Whey protein (g/L)</td>
<td>0.016</td>
<td>0.75</td>
<td>0.99</td>
<td>0.15</td>
<td>0.005</td>
<td>0.52</td>
<td>0.006</td>
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<td>-1.06 ± 0.36</td>
<td>-0.36 ± 0.36</td>
<td>-0.13 ± 0.39</td>
<td>0.69 ± 0.33</td>
<td>1.19 ± 0.36</td>
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<tr>
<td>Casein (g/L)</td>
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<td>0.87</td>
<td>1.00</td>
<td>0.002</td>
<td>0.043</td>
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<td>-0.01 ± 0.16</td>
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<td>1.00</td>
<td>1.00</td>
<td>0.001</td>
<td>0.006</td>
<td>0.98</td>
<td>0.002</td>
</tr>
<tr>
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<td>0.11 ± 0.03</td>
<td>-0.01 ± 0.03</td>
<td>0.01 ± 0.03</td>
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<td>0.85</td>
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<td>0.03 ± 0.04</td>
<td>0.15 ± 0.04</td>
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<td>0.12 ± 0.04</td>
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<td><strong>Feeding characteristics</strong></td>
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<tr>
<td>Feeding frequency (SR) e</td>
<td>0.82</td>
<td>0.045</td>
<td>&lt;0.001</td>
<td>0.17</td>
<td>&lt;0.001</td>
<td>0.003</td>
<td>&lt;0.001</td>
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<tr>
<td></td>
<td>0.46 ± 0.53</td>
<td>1.40 ± 0.54</td>
<td>3.14 ± 0.58</td>
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<td>Feeding frequency (MP) f</td>
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<td>n/a</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
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<td>n/a</td>
<td>-2.91 ± 0.81</td>
<td>-3.24 ± 0.77</td>
<td>-0.34 ± 0.90</td>
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<tr>
<td>24-h milk intake (mL) f</td>
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<td>n/a</td>
<td>n/a</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>0.99</td>
<td>&lt;0.001</td>
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<td>n/a</td>
<td>n/a</td>
<td>-340 ± 77</td>
<td>-349 ± 77</td>
<td>-9 ± 87</td>
<td></td>
</tr>
<tr>
<td>C DI of milk components f</td>
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<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
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</tr>
<tr>
<td>Adiponectin (ng)</td>
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<td>n/a</td>
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<td>0.035</td>
<td>0.99</td>
<td>0.016</td>
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<td>-3824 ± 1635</td>
<td>-4074 ± 1546</td>
<td>-249 ± 1915</td>
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</tr>
<tr>
<td>Skim milk leptin (ng)</td>
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<td>n/a</td>
<td>n/a</td>
<td>0.005</td>
<td>0.002</td>
<td>1.00</td>
<td>&lt;0.001</td>
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<tr>
<td></td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>-103 ± 30</td>
<td>-106 ± 28</td>
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<tr>
<td>Total protein (g)</td>
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<td>n/a</td>
<td>n/a</td>
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<td>-4.32 ± 1.52</td>
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<td>Whey protein (g)</td>
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<td>n/a</td>
<td>n/a</td>
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<td>0.045</td>
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<td>0.016</td>
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<td>n/a</td>
<td>-1.48 ± 0.60</td>
<td>-1.44 ± 0.57</td>
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</tr>
<tr>
<td>Casein (g)</td>
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<td>n/a</td>
<td>n/a</td>
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<td>0.016</td>
<td>1.00</td>
<td>0.005</td>
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<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>-0.84 ± 0.30</td>
<td>-0.85 ± 0.29</td>
<td>-0.01 ± 0.35</td>
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<tr>
<td>Total carbohydrates (g)</td>
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<td>n/a</td>
<td>n/a</td>
<td>0.11</td>
<td>0.086</td>
<td>1.00</td>
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<td>n/a</td>
<td>-17.97 ± 8.57</td>
<td>-19.00 ± 8.57</td>
<td>-1.03 ± 10.42</td>
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</tr>
<tr>
<td>Lactose (g)</td>
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<td>n/a</td>
<td>n/a</td>
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<td>0.006</td>
<td>0.95</td>
<td>0.002</td>
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<tr>
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<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>-18.44 ± 6.41</td>
<td>-20.78 ± 6.07</td>
<td>-2.33 ± 7.51</td>
<td></td>
</tr>
</tbody>
</table>
Systematic differences in the measured variables between different months after birth were calculated using general linear hypothesis test (Tukey’s all pair comparisons). Bold text indicates significant difference (p<0.05) between two time points. Only variables with at least one significant difference between two time points are presented; all other variables tested that not presented here did not differ significantly. Parameter estimate and standard error of estimate. US2 – ultrasound 2-skinfolds; US4 – ultrasound 4-skinfolds; BIS – bioimpedance spectroscopy. Feeding frequency was self-reported by mothers (SR) at the time of the visit as an average time between feeds (e.g., each 2 h) (n=11, n=19, n=17, n=13 at 2, 5, 9 and 12 mo respectively). 24-h milk intake and feeding frequency as meals per 24-h was measured at 24-h milk production (MP) and CDIs calculated between 2 and 5 months (presented at 5 months here, n=17) and within 2 weeks of 9 (n=6) and 12 months (n=8).
7.4.5 Maternal body composition and milk components

Greater maternal weight, FM and FFM (kg) were associated with higher whole HM leptin concentrations (ng/mL) (0.002±0.001, \( p=0.026 \); 0.003±0.002, \( p=0.027 \); 0.005±0.002, \( p=0.044 \), respectively) (Figure 7.1), while higher skim HM leptin concentrations (ng/mL) were associated only with greater maternal height (0.004±0.002, \( p=0.011 \)).

Higher maternal BMI, weight, and FFM were associated with higher whey protein concentrations (g/L) (0.10±0.04, \( p=0.022 \); 0.03±0.01, \( p=0.036 \); 0.06±0.03, \( p=0.040 \), respectively) (Figure 7.1). No other associations between HM components and maternal characteristics were seen.

![Graphs showing significant positive associations between maternal fat mass and whole milk leptin concentration; maternal fat-free mass and whole milk leptin concentration; maternal weight and whey protein concentration; maternal fat-free mass and whey protein concentration.](image)

**Figure 7.1** Significant positive associations between: (a) maternal fat mass and whole milk leptin concentration; (b) maternal fat-free mass and whole milk leptin concentration; (c) maternal weight and whey protein concentration; (d) maternal fat-free mass and whey protein concentration.
concentration. Lines represent linear regression and grouped by the month of lactation (n=14, n=20, n=18 (n=17 for whole milk leptin), n=15 at 2, 5, 9 and 12 mo respectively).

7.4.6 Infant body composition and milk components

Significant associations between infant anthropometrics and BC and milk composition (components concentrations and CDI) were seen after accounting for the month after birth.

Higher adiponectin concentrations (ng/mL) were associated with lower infant FFM 4SF (kg) (-0.03±0.02; p=0.040) (Figure 7.2), while higher adiponectin CDI (ng) were associated with lower FFM 4SF (-0.0001±0.00002; p=0.016), and higher FM (kg) (FM 2SF: 0.00004±0.00002; p=0.049; FM 4SF: 0.0001±0.00002, p=0.021) and larger skinfolds (mm) (biceps: 0.001±0.0002, p=0.020; suprailiac: 0.0004±0.0002; p=0.042).

Higher whole milk leptin concentrations (ng/mL) were associated with lower biceps skinfolds (-8.63±3.59; p=0.021) (Figure 7.2). Higher skim milk leptin concentrations (ng/mL) were associated with smaller infant head circumference (cm) (-1.85±0.84 ng/mL; p=0.034), while higher skim milk leptin CDI (ng) were associated with higher %FM 2SF (0.05±0.01; p<0.001), and higher FM (FM 2SF: 0.005±0.001, p<0.001; FM 4SF: 0.004±0.001, p=0.013).

Higher total carbohydrate concentrations (g/L) were associated with longer (cm) (0.05±0.01; p=0.001), heavier (kg) (0.013±0.004; p=0.005) infants with higher FFM.
(FFM 2SF: 0.01±0.04, \( p=0.034 \); FFM 4SF: 0.01±0.04, \( p=0.021 \); FFM BIS: 0.02±0.004, \( p<0.001 \)) and lower %FM BIS (-0.10±0.04; \( p=0.014 \)) (Figure 7.3), while higher CDI (g) were associated with greater triceps thickness (0.16±0.04; \( p=0.002 \)), higher %FM 4SF (0.11±0.04; \( p=0.011 \)) and FM 4SF (0.01±0.004; \( p=0.030 \)).

![Figure 7.3](image)

**Figure 7.3** Significant positive associations between: (a) total carbohydrates concentration and infant length; (b) total carbohydrates concentration and infant weight; and (c) total carbohydrates concentration and infant fat-free mass measured with bioelectrical spectroscopy (BIS). (d) Significant negative association between total carbohydrates concentration and infant fat mass measured with BIS. Lines represent linear regression and grouped by the month of lactation (n=13 (n=14 for weight), n=20, n=18 (n=19 for weight), n=13 (n=12 for fat and fat-free mass) at 2, 5, 9 and 12 mo respectively).

Higher HMO concentrations (g/L) were associated with longer (0.03±0.01; \( p=0.039 \)) infants with higher FFM BIS (0.02±0.004, \( p<0.001 \)), while higher CDI (g) were associated with higher triceps thickness (0.19±0.09; \( p=0.044 \)).
Higher CDI of lactose (g) were associated with higher FM 2SF (0.01±0.01; p=0.024) and %FM 4SF (0.11±0.05; p=0.046).

Higher casein concentrations (g/L) were associated with larger skinfolds (biceps: 2.87±1.15, p=0.016; triceps: 2.50±1.18, p=0.039) while higher CDI (g) were associated with lower length (cm) (-1.32±0.64; p=0.049) and FFM (FM 2SF: -0.37±0.15, p=0.038; FFM 4SF: -0.50±0.15, p=0.010), and greater skinfolds thickness (biceps: 3.45±1.44, p=0.024; triceps: 3.11±1.17, p=0.026), FM 4SF (0.32±0.11, p=0.024) and %FM 4SF (2.42±1.14, p=0.044).

Higher CDI of lysozyme (g) were associated with higher infant BMI (11.03±4.57; p=0.025), biceps thickness (33.27±14.61; p=0.032), %FM 4SF (23.12±10.93; p=0.046) and FM 4SF (3.0±1.23; p=0.023).

### 7.4.7 Relationship between infant and maternal body composition

Significant negative associations between infant and mother anthropometrics/BC were seen after accounting for months after birth.

Greater maternal %FM and FM (kg) were associated with lower infant FFM measured with US 2SF (%FM: -0.04±0.02, p=0.006; FM: -0.03±0.01, p=0.023) (Figure 7.4) and US 4SF (%FM: -0.04±0.02, p=0.028; FM: -0.03±0.01, p=0.038).

![Figure 7.4](image_url) **Figure 7.4** Significant negative associations between: (a) maternal fat mass and infant fat-free mass measured with ultrasound (2 skinfolds; US 2 SF); (b) maternal percentage fat mass (%FM) and infant fat-free mass measured with US 2 SF. Lines represent linear regression and grouped by the month of lactation (n=14, n=20, n=18, n=18 at 2, 5, 9 and 12 mo respectively).
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Greater maternal weight (kg), FFM (kg) and height (cm) were associated with lower infant subscapular thickness (mm) (-0.05±0.03, \( p=0.042 \); -0.13±0.06, \( p=0.028 \); -0.14±0.06, \( p=0.020 \), respectively).

Higher maternal BMI was associated with lower infant FFM measured with all methods, (US 2SF: -0.06±0.02, \( p=0.010 \); US 4SF: -0.06±0.02, \( p=0.013 \); BIS -0.05±0.03, \( p=0.035 \)).

7.4.8 24-hour milk intake, feeding frequency and milk components

A moderate level of agreement (ICC=0.574 [0.29, 0.77], \( p<0.001 \)) was seen between FFQ measured with 24-h MP as meals per 24-h and FFQ self-reported by mothers as hours between meals. Short intervals between feeds were associated with higher self-reported values than 24-h MP values; this effect was not seen with longer intervals between feeds. No associations between FFQ and maternal characteristics were seen with the exception of maternal height (self-reported FFQ (h): -0.07±0.03, \( p<0.017 \)).

Higher 24-h MI was associated with higher FFQ (24-h MP: 0.007±0.002, \( p<0.001 \); self-reported: -0.004±0.002; \( p=0.021 \)). Higher FFQ was associated with larger 24-h MI (24-h MP: 67.4±12.6 mL, \( p<0.001 \); self-reported: -46.9±19.2 mL, \( p=0.021 \)); higher total protein concentration (g/L) (self-reported: -0.19±0.08 g/L, \( p=0.020 \)); and higher CDI of whole milk leptin (ng) (24-h MP: 0.001±0.002; \( p<0.001 \)), total protein (g) (24-h MP: 0.28±0.11; \( p=0.020 \)), whey protein (g) (24-h MP: 0.79±0.27, \( p=0.007 \); self-reported: -0.61±0.24, \( p=0.018 \)), lysozyme (g) (24-h MP: 14.10±4.39 g, \( p=0.004 \)), total carbohydrates (g) (24-h MP: 0.06±0.02, \( p=0.005 \); self-reported: -0.04 ± 0.02, \( p=0.025 \)) and lactose (g) (24-h MP: 0.10±0.02, \( p<0.001 \); self-reported: -0.05±0.02, \( p=0.040 \)).

7.4.9 Infant body composition and feeding parameters

Significant associations between infant BC and feeding parameters (FFQ, 24-h MI) were seen after accounting for the month after birth. FFQ and 24-h MI did not differ by infant sex (\( p>0.098 \)). Higher 24-h MI was associated with higher infant FM 2SF (kg) (0.001±0.0003; \( p=0.014 \)) and %FM 4SF (0.01±0.004; \( p=0.033 \)) (Figure 7.5). Higher 24-h MP FFQ (meals/24-h) was associated with higher %FM BIS (0.80±0.38; \( p=0.045 \), while lower self-reported FFQ (more hours between feeds) was associated with higher infant weight (0.10±0.04; \( p=0.016 \)) and FFM (FFM 2SF: 0.11±0.03, \( p=0.002 \); FFM 4SF: 0.14±0.03, \( p<0.001 \); FFM BIS: 0.09±0.04, \( p=0.025 \), and lower %FM 4SF (-0.61±0.30; \( p=0.049 \)).
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Figure 7.5 Significant positive associations between: (a) 24-h milk intake and infant fat mass measured with ultrasound (2 skinfolds; US 2SF); (b) 24-h milk intake and infant percentage fat mass (%FM) measured with ultrasound (4 skinfolds; US 4SF). Lines represent linear regression and grouped by the month of lactation ($n=16$ ($n=15$ for percentage fat mass), $n=6$, $n=8$, between 2 and 5, and at 9 and 12 mo respectively).

7.5 DISCUSSION

With pediatric obesity increasing, research is focusing on associations between early nutrition, BC and risk of future disease. This study sheds new light on the mechanisms by which breastfeeding may influence infant BC. For the first time, concentrations of casein, HM total carbohydrates and HMO have been associated with infant BC. More importantly, CDI of adiponectin, leptin, casein, lysozyme, total carbohydrates, lactose and HMO are also differentially related to infant anthropometry and BC. Further, FFQ and 24-h MI are also implicated in development of infant BC emphasizing the critical role of breastfeeding in programming growth in the first year of life.

The total protein content of HM is low yet highly bioavailable and this has been implicated in the decreased risk of later obesity for breastfed infants (Luque et al. 2015). Currently, excessive protein is considered to be responsible for the accelerated growth trajectory of formula-fed infants compared to breastfed infants, leading to greater weight gain and increased lean (Roggero et al. 2008; Koletzko et al. 2009a) or fat mass (Luque et al. 2015) in early life. Within the normal developmental context of breastfeeding we found that neither greater concentrations nor CDI of total protein were associated with infant anthropometrics or adiposity.
On the other hand, both concentrations and CDI of casein, but not whey, were implicated in infant BC, with greater levels associated with increased adiposity and limited accretion of lean body mass (Section 7.4.6). Maternal BC however, was associated with higher concentrations of whey protein, but not casein in the HM, reflecting the intrinsic synthesis of casein in the breast as opposed to movement of whey components from the maternal blood into the milk.

These results confirm that the range of total protein in HM is not problematic (neither concentration or dose). However, casein appeared to be associated with infant BC and is not associated with maternal BC, suggesting it is not modifiable, although large studies would be required to determine if casein is predictive of infant BC. This also draws attention to the fact the different casein levels in formula may have effects on infant BC.

The most abundant proteins in the HM whey fraction include α-lactalbumin and immune factors lactoferrin, lysozyme and secretory IgA (Ballard et al. 2013). Lysozyme accounts for 6% of the whey fraction and has both immunological and nutritional properties (Montagne et al. 2001). We found that a greater CDI of lysozyme was associated with an increase in infant adiposity (Section 7.4.6), indicating the importance of HM lysozyme in the growth of the term infant. Previous studies have found dose dependent effects of lysozyme added to formula on the weight gain of preterm infants (Bol'shakova et al. 1984). Furthermore, lysozyme concentrations continued to increase over the first 12 months of lactation (Tables 7.4 and 7.5), suggesting this component is important for both the protection and development of infant BC.

Smaller active HM peptides, believed to be involved in the development of appetite regulation and infant BC, include adiponectin and leptin. Adiponectin regulates lipid and glucose metabolism, stimulates food intake, participates in energy balance, and has anti-inflammatory effects (Catli et al. 2014). We found higher adiponectin concentrations to be associated with lower lean body mass. A recent study showed that higher HM adiponectin concentration (measured at 6 weeks and 4 months of age) was associated with lower weight and lean tissue accretion which changed to higher weight, lean and adipose tissue accretion after 4 months of age (Brunner et al. 2014). Our results show that adiponectin concentration did not change over time, nor did the relationship with BC. The contrast in results may be explained by differences in our statistical (accounting for individual curves), biochemical (adiponectin measured in whole HM with ELISA) and BC (BIS and US) methods. Shorter breastfeeding duration
in previous studies could also have had an impact on BC, since the infants in our study continued to breastfeed at the time of the measurements. Further, increased concentration of adiponectin was associated with increased concentrations of whey protein (Section 7.4.3), thus the absence of a relationship between whey and total protein concentration and infant BC suggests adiponectin, whilst dependent on protein content, is independently associated with BC.

The most studied adipokine in HM is leptin, which is implicated in short and long term satiety and regulation of energy intake (Miralles et al. 2006). Unfortunately, three out of the four studies that have measured leptin in whole HM in relation to infant BC used RIA and were limited to the first 3 months postpartum (Dundar et al. 2005; Bielicki et al. 2004; A. De Luca et al. 2016) providing mixed results. One study measured the concentration of leptin in whole HM using ELISA, but leptin was measured only at 1 and 3 months postpartum and BMI and weight and weight gain were the measures of BC and these showed negative associations (Miralles et al. 2006). In our study higher concentration of whole HM leptin was associated with lower biceps skinfold. Additionally we found a negative association between maternal and infant BC and high leptin concentrations are linked to higher maternal adiposity raising the speculation of a link between HM leptin and infant BC (Section 7.4.7). Since FM assessed by skinfolds correlated closely with FM values determined by dual X-ray absorptiometry in infants under 4 months (Schmelzle et al. 2002), investigation of larger numbers of infants might expand this finding further.

We also measured skim HM leptin and found higher concentrations were associated with reduced head circumference, while higher CDIs were associated with higher adiposity. A recent study that measured milk intake and skim HM leptin found higher CDI were associated with increased weight gain (Kon et al. 2014). Caution in interpretation of skim HM leptin concentration results should be exercised as, although consistent with previous literature (Miralles et al. 2006; Fields et al. 2012), the statistical associations were different to that of whole HM leptin (Section 7.4.3). Further, whole HM leptin concentration did not differ by infant age, yet skim HM leptin concentration differed (Table 7.5), contributing to the possibility of misinterpretation. Given infants receive whole milk, and whole HM leptin analysis requires less sample manipulation, further study with more infants should be carried out to confirm these relationships.

Maternal associations with leptin also differed according to skim or whole HM was analysis. Higher whole HM leptin concentrations were associated with higher
maternal weight, fat and lean body mass, whereas skim HM leptin concentrations were associated with lower height. Our whole HM leptin results with respect to maternal BC are consistent with published literature using skim HM leptin analysis and BMI (Andreas et al. 2014).

HM carbohydrates are a major macronutrient and contribute approximately 44% of the energy of the HM (Casadio et al. 2010). Higher estimated carbohydrate daily intake has been related to increased infant weight and lean mass gain (Butte et al. 2000b). However, we found that while higher concentrations of total HM carbohydrates and HMO were associated with greater infant weight and lean body mass, greater CDI of HM total carbohydrates, lactose and HMO were related to increased adiposity. We were unable to account for carbohydrates provided by solid foods however, our HMO results were consistent with effects seen in breastfeed infants up to the age of 6 months, where individual HMO displayed differing associations with infant BC (Alderete et al. 2015). HMO may exert BC effects by enhancing the growth of beneficial bacteria of the infant gut microbiome (Bode 2012), and the microbiome has been implicated in the development of obesity (Turnbaugh et al. 2009; Laursen et al. 2017). Further work including HMO and infant gut microbiome analysis will serve to clarify the role of HMO in the development of infant BC.

Breastfeeding frequency is highly variable and likely a reflection of appetite regulation. For the first time we have shown increased FFQ measured by 24-h test weighing was associated with increased infant adiposity. This was confirmed by maternal self-reported FFQ however, this parameter was also associated with decreased infant lean body mass and weight. Self-reports of frequency may be misleading and therefore results should be interpreted cautiously, as we found a moderate intraclass correlation between the 2 measures with a bias to greater frequency being self-reported for frequent feeders. Increased FFQ measured by both methods was associated with higher 24-h MI (and as result higher CDI for the majority of measured HM components), and 24-h MI in turn was associated with increased infant adiposity but not infant weight. Few studies have investigated the effect of 24-h MI and particularly the effect of higher CDI of various HM components on infant growth velocity (Kon et al. 2014). These results emphasise the importance of including these measures in order to elucidate the mechanisms by which HM components affect infant BC and the degree by which the mother influences HM composition.

The limitations of this proof-of-concept study are the small number of 24-h MP at the later stages of lactation, the modest number of participants as a result of time
constraints associated with multiple measurement time points, and the absence of reference data for our subjects. Our population was predominantly Caucasian term healthy fully breastfed singletons from mothers of higher social-economical status therefore, the results may not be applicable to infants from other backgrounds.

7.6 CONCLUSIONS
In conclusion, this study found maternal BC was associated with HM composition, which in turn was associated with breastfeeding patterns and development of infant BC over the first 12 months of life. These results confirm that the first year of life is a critical window of infant developmental programming that has the potential for intervention to improve outcomes for the infant.
CHAPTER 8

GENERAL DISCUSSION
Health inequalities are common in modern societies, with obesity identified as a major risk factor for metabolic disease and therefore a leading cause of death. As such the obesity pandemic has become a massive economic global burden (J.C.K. Wells 2016; Via 2012). The importance of lactocrine programming has been highlighted recently, with breastfeeding identified as one of the preventative measures for NCD (Prescott 2016; Socha et al. 2016; Godfrey et al. 2016). Indeed breastfeeding is associated with lower rates of childhood obesity, which is itself a risk factor for later obesity. Further research into the mechanisms of the lactocrine programming (Figure 8.1) will explain variations in breastfeeding patterns and infant growth, explore possibilities of linking them to maternal factors and identify possible points of intervention. The exact mechanism by which breastfeeding confers a lowered risk of obesity is not known nor how HM composition impacts infant outcomes. More extensive knowledge in these areas could serve to construct early intervention and inform nutritional guidelines providing a greater impetus to breastfeed.

**Figure 8.1** Framework for possible interconnecting pathways of lactocrine programming of the infant as researched, and points of intervention for potential improvement of infant growth, development and health.
Unravelling the multiple ways in which breastfeeding modulates infant growth and BC requires consideration of HM composition and appetite control mechanisms along with robust measures of infant BC.

HM research has been hampered by the lack of rigorous development and optimisation of compositional methods. Leptin is a classic example, where a variety of methods, including ELISA, RIA and mixed immunoassay, have been employed to measure concentrations in HM. This is further exacerbated by the fact that most analyses of HM leptin preformed in skim HM, yet the leptin is higher in whole milk. (Houseknecht et al. 1997; Smith-Kirwin et al. 1998). After extensive optimisation of the ELISA assay for whole milk leptin (Kugananthan et al. 2016), which is more relevant to investigation of infant BC, we also failed to find a relationship of leptin with HM fat content (Chapter 5) jeopardising the theory of leptin affiliation with the HM fat (Smith-Kirwin et al. 1998; Resto et al. 2001).

Prudent sampling is imperative for robust human lactation research. We found no significant change between pre- and post-feed concentrations of whole HM leptin and adiponectin, as well as total protein and lactose (Chapter 5). These findings indicate that concentrations of these particular hormones and macronutrients may have no effect on infant satiation and likely do not signal to the infant to cease a breastfeed. This finding is further supported by the lack of associations between concentrations of these components and infant MI (Chapter 6). On the other hand, some of the HM components have shown differences in concentrations across a breastfeed and these may be more likely to have an effect on satiety or GE. For example, fat (Kent 2007) and GLP-1 (Schueler et al. 2013) are higher in post-feed samples, while ghrelin is reduced in post-feed samples (Karatas et al. 2011). Whilst it is assumed that they may have an effect, the differences have not been interpreted in the context of MI, which would be much more informative.

Past research has focused mainly on relationships between HM concentrations and infant BC, without taking into account breastfeeding patterns and other appetite regulators such as GE. The question then arises as to whether dose (24-h MI) is a more relevant factor rather than concentration as it represents the total amount of a component ingested by the infant.

Breastfeeding patterns are highly variable in breastfed infants and likely a reflection of appetite regulation. During the exclusive BF period FFQ is associated with infant size and adiposity (Chapter 6), with the smallest, shortest and leanest infants feeding more frequently. Thus intervening in rapid weight gain infants by decreasing
FFQ should be considered with caution. Intervention could change total milk volume by down regulating milk synthesis or the infant may increase FV by emptying the breast more completely. Therefore monitoring MI would be prudent if offering this advice.

As with methods of measurement of BC, there is an absence of suitable validated techniques for GE restricting the evaluation and understanding of the breastfed infant GE as a mechanism of appetite control. We have further validated the direct US technique (Perrella et al. 2013) to apply this method to the term population (Cannon et al. 2017). We have shown that HM components are associated with GE therefore, potentially impact appetite regulation and BC, as evidenced by relationships between FFQ and infant BC (Chapters 7).

In order to examine relationship of HM components and infant BC it is important to choose a robust method of measurement. We chose the US and BIS method as they are the most likely methods that could be scaled up for larger studies. Our investigations have refined BIS protocols and highlighted the need for the development of more accurate calculations for BC in infants. Using two methods we have attempted to exploit the accuracies and somewhat account for limitations of both methods.

Similar to infant BC, maternal BC should be accurately assessed when determining relationships between maternal factors and HM composition. Very few studies measured maternal %FM in relationship to HM composition. To determine whether there was a relationship between maternal and infant BC and HM composition we conducted analyses of an array of HM components in a cross-sectional and longitudinal study, together with measurements of anthropometry and adiposity. For the first time we found that maternal BC (higher %FM, FM, FFM, BMI and weight) was associated with increased concentration of whole HM leptin (Figure 8.2). This is consistent with the only other study measuring whole HM leptin with ELISA using pre-pregnancy BMI as a measure of adiposity (Miralles et al. 2006) confirming maternal BC may influence HM leptin.

Clearly, the variation in the reported literature indicates that the relationships between maternal adiposity and HM leptin are not straightforward. Other studies have reported both presence and absence of such associations (Andreas et al. 2014). Even when different groups are compared no consistent results have been reported, for example HM leptin concentrations in obese mothers (n=50) was double of that of normal-weight mothers (n=50) (A. De Luca et al. 2016). However, these results were not replicated in a smaller cohort (obese: n=17; non-obese: n=33) (Uysal et al. 2002). Future longitudinal studies with comprehensive design involving more participants
including those considered overweight and obese may address these irregularities and elucidate the relationships of HM leptin with maternal BC.

Figure 8.2 Possible pathways of lactocrine programming of the infant by the appetite hormones. BMI – body mass index; CDI – calculated daily intake; Conc. – concentration; doses – amounts of human milk component ingested during a single breastfeed; FFM – fat-free mass; FM – fat mass; %FM – percentage fat mass; GE – gastric emptying; MI – milk intake; - negative association; + positive association

Purple arrows indicate the direct associations between components and infant body composition. In the case where direct relationships between infant body composition and human milk components are supported by the relationships of human milk components and infant gastric emptying factors and breastfeeding parameters, these relationships have been included and could be integrated into possible pathway. Orange arrows indicate further possible pathways, although no association of component unit with infant body composition has been established (or not analysed in case of doses). Red cross indicates no associations detected with maternal adiposity.

The absence of any associations of HM leptin with any GE factors (Chapter 6) combined with the varying associations of leptin with infant anthropometry and adiposity (Figure 8.2; Chapter 7) indicates that long-term effects of leptin in human infants are possibly stronger than the potential short-term satiety effects observed in rodent models. This highlights the difficulty of extrapolating results in animal models to humans (Wang et al. 2014). Also, the lower rate of leptin clearance in humans indicates that it may stay active for relatively longer time (Margetic et al. 2002; Klein et al. 1996), modifying the short-term effects.
While adiponectin was not associated with maternal BC or anthropometry, there were interesting relationships with GE. For the first time it was shown that higher concentrations and doses of adiponectin were associated with longer GE time (time between feeds) in term breastfed infants (Chapter 6), which may be implicated in the growth-regulating effect of adiponectin in the first 4-7 months of life (Woo et al. 2012; Brunner et al. 2014; Wang et al. 2011), evidenced by high HM adiponectin concentrations being associated with lower infant weight and FFM. Extended GE times may culminate in fewer feeds per day and potentially lower 24-h MI, restricting both volume and energy to the infant and slowing the growth. This hypothesis was further supported by our longitudinal study of infant BC that showed that higher HM adiponectin concentrations being associated with lower infant FFM over the first year of life (Chapter 7; Figure 8.2).

Whilst several studies have investigated longitudinal changes in both HM leptin and adiponectin (Tables 1.1, 1.2, 2.1 and 2.2), only two studies have measured these hormones in HM at several time points up to 12 months of lactation, reporting higher leptin at 12 months (Bronsky et al. 2011), and one reporting lower and another higher concentrations of adiponectin at 12 months (Bronsky et al. 2011; Woo et al. 2012). We found that the concentrations of both HM leptin and adiponectin did not change over the 12 months (Chapters 5 and 7), emphasising the importance of these appetite hormones for infant development and warranting further optimization of methodologies to allow whole HM to be tested for the variety of other bioactive components.

Similar to HM leptin, higher concentrations of HM protein (Figure 8.3) are associated with increased maternal %FM, FM, FFM, BMI and weight, and are not identical in all tested cohorts (Chapters 5 and 7). In the combined cross-sectional and longitudinal cohort we observed positive associations between maternal %FM and total protein (Chapter 5). These associations disappeared after the removal of cross-sectional participants. In the longitudinal study of infant BC, we have found for the first time positive associations between maternal weight, FFM, BMI and whey protein, with %FM showing no associations (Chapter 7). As with leptin, there are studies reporting the both presence and absence of such associations (A. De Luca et al. 2016; Nommsen et al. 1991). Increasingly it appears that the mother may modulate a number of components in the milk, suggesting that milk composition may be modifiable. Indeed larger longitudinal studies including measurement of maternal BC are required to confirm this.
Figure 8.3 Possible pathways of lactocrine programming of the infant by the human milk proteins. BMI – body mass index; CDI – calculated daily intake; Conc. – concentrations; doses – amounts of human milk component ingested during a single breastfeed; FFM – fat-free mass; FM – fat mass; %FM – percentage fat mass; GE – gastric emptying; MI – milk intake; PFSV – post-feed stomach volume; - negative association; + positive association. Purple arrows indicate the direct associations between components and infant BC. In the case where direct relationships between infant body composition and human milk components are supported by the relationships of human milk components and infant gastric emptying factors and breastfeeding parameters, these relationships have been included and could be integrated into possible pathway. Orange arrows indicate further possible pathways, although no association of component unit with infant body composition has been established (or not analysed in case of doses). Red crosses indicate no associations detected with maternal adiposity.

Protein composition rather than total protein appears to influence GE either indirectly or directly. For example, the casein:whey ratio either increased or decreased GE rate depending on the volume of milk consumed by the infant, such that higher casein:whey ratios were associated with faster GE of small FV and slower GE rate of larger FV (Chapter 6; Figure 8.3). This explains the contrasting results in the literature for breastfeeding and formula fed infants. However, this agrees with recent data from enteral fed preterm infants, where faster GE during feed delivery (S.L. Perrella et al.
2015; S. Perrella et al. 2015) and faster postprandial GE rate (Perrella 2015b) were observed with considerably smaller volumes than the term infant (19±3.3 and 35±4.5 mL for 2 and 3-hourly feeding respectively).

Furthermore, higher casein concentrations and doses were associated with shorter GE time, which may result in higher FFQ, and in turn, higher infant adiposity; and higher whey protein concentrations and doses were associated with longer GE time, which may have an opposite effect on adiposity (Chapter 7; Figure 8.3). For the first time HM casein and whey protein concentrations were investigated with respect to infant anthropometry and BC. Higher casein concentrations were associated with greater skinfold measures and similarly, CDI had positive associations with measures of adiposity (FM and %FM). CDI however, also had negative associations with length and FFM (Chapter 7). This suggests that higher daily doses of casein may down-regulate the accrual of lean body mass in infants, probably via decreased time between feeds and increase in volume of milk consumed. These results further clarify the role of the protein as a potent appetite regulator and draw attention to the importance of the protein composition of the HM rather than the total concentration.

In this study lysozyme has been highlighted as important not only for the protection of the infant but for infant growth and now BC, in particular adiposity (Chapter 7). This confirms the results of a study of preterm infants administered lysozyme (Bol'shakova et al. 1984), where the lysozyme group demonstrated better growth and improved GI function. Although this was not explained by any of our measures of GE during exclusive breastfeeding period (Chapter 6), increased CDI of lysozyme was also associated with increased FFQ, which was further associated with 24-h MI (Chapter 7). The effects of higher amounts of HM lysozyme may be mediated through improvement of infant gut health and optimized digestion, resulting in increased absorption of nutrients and subsequently, increased fat accretion. Or alternatively, lysozyme, not unlike leptin, also enhances innate and adaptive immune responses (Prescott 2016). As such lysozyme may be implicated in the two-way relationship between the obesity and immune status. These findings warrant further investigation of other immunological factors of HM in relation to infant growth and BC.

New insights were gained regarding relationships of HM carbohydrates with infant GE and BC (Figure 8.4). In this study, we established that concentrations of total HMO were positively associated with lean mass, and CDI with adiposity (triceps). Whilst the numbers of participants are low, this makes biological sense in that HMO are resistant to digestion and have been shown to modulate the gut microbiome therefore,
having an indirect affect on BC. This would be interesting to pursue within a larger cohort to gain greater understanding of HMO role in infant BC. In contrast, both total carbohydrates and lactose are associated with GE, FFQ and 24-h milk volume ingested by the infant culminating in relationships to adiposity (Figure 8.4).

**Figure 8.4** Possible pathways of lactocrine programming of the infant by the human milk carbohydrates. BMI – body mass index; CDI – calculated daily intake; Conc. – concentrations; doses – amounts of human milk component ingested during a single breastfeed; FFM – fat-free mass; FM – fat mass; %FM – percentage fat mass; GE – gastric emptying; MI – milk intake; - negative association; + positive association. Purple arrows indicate the direct associations between components and infant BC. In the case where direct relationships between infant body composition and human milk components are supported by the relationships of human milk components and infant gastric emptying factors and breastfeeding parameters, these relationships have been included and could be integrated into possible pathway. Orange arrows indicate further possible pathways, although no association of component unit with infant body composition has been established (or not analysed in case of doses). Red crosses indicate no associations detected with maternal adiposity.

This highlights the complexity of pathways affected by HM components and the dose that the infant receives, as evidenced by our finding that higher lactose concentrations associated with slower GE rate of small FV and faster GE rate of large FV (Chapter 6). The suggestion that the effects of lactose are modulated via milk volume can be translated to the clinical case of colic in breastfed infants. One
explanation of colic is lactose overload. It is believed that high volumes of HM result in the rapid delivery of lactose to small intestine and with malabsorption, leading to dysbiosis and colic (Woolridge et al. 1988). These findings lend support to this theory and indicate that if lactose overload is suspected/diagnosed it may be possible to relieve the symptoms by offering smaller more frequent feeds.

These results suggest a differential effect of the concentrations and doses of the HM components on infant anthropometry and adiposity, but translation is cautionary given the consistency of dose relationships with infant BC. CDI may be a more relevant factor than concentrations to examine the nutritional physiology of the breastfed infant. These findings indicate possibility of intervention via modulation of both MI and infant gut microbiome, which is implicated in adiposity and development of obesity (Koleva et al. 2015) by the decrease in bacterial diversity and shifts of gut bacteria at the phylum level resulting in increased energy intake and fat storage, and low-grade systemic inflammation (Prescott 2016). Future studies should take more comprehensive approach to measurement of HM components (concentration and dose) when investigating relationships between the gut microbiome and infant BC.

Whilst the numbers of participants in some of these studies may be considered relatively low, these small proof of concept studies have provided the methods and design to identify relationships between the breastfeeding mother, HM components, MI, feeding patterns, GE and infant BC. These results also unravel some of the complex mechanisms by which breastfeeding confers some degree of protection from obesity and reduces risk factor for later NCD. Thus these studies have formed the basis of a larger more extensive observational cohort study to be initiated in 2017.

In summary, these comprehensive studies have made significant contributions to the fields of BC measurement, GE assessment and analyses of HM composition, improving understanding of the mechanisms of appetite control and development of infant BC during the first year of life. We have evaluated the use of non-invasive BIS and US in both cross-sectional and longitudinal studies, standardised BIS for measurements of resistance in breastfed infants and optimized ELISA for measurement of leptin in whole HM. We have shown that maternal BC is a likely modifiable factor that influences HM composition, which was further, for the first time, linked to the infant appetite control via the GE and breastfeeding parameters, such as FFQ and MI, and further to infant anthropometrics and BC. We also accounted for the dose of the HM components (CDI, and dose ingested during the single breastfeed), highlighting the importance of the component dose in investigations of infant appetite control and BC,
where the dose-response of the effects is completely unknown. We have successfully designed a new non-invasive, holistic approach to data collection in the field of BC and infant nutrition, which we can build upon in the future research into lactocrine programming of the human infant.


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APPENDICES
### Table S5.1. Longitudinal changes and associations between human milk components and maternal adiposity. Values are parameter estimates ± standard error (n=21).

<table>
<thead>
<tr>
<th>Predictor</th>
<th>Adiponectin (ng/mL)</th>
<th>Whole Milk Leptin (ng/mL)</th>
<th>Skim Milk Leptin (ng/mL)</th>
<th>Lactose (g/L)</th>
<th>Protein (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PE ± SE</td>
<td>p</td>
<td>PE ± SE</td>
<td>p</td>
<td>PE ± SE</td>
</tr>
<tr>
<td>BMI</td>
<td>0.11 ± 0.13</td>
<td>0.41</td>
<td>0.005 ± 0.003</td>
<td>0.11</td>
<td>0.001 ± 0.002</td>
</tr>
<tr>
<td>%FM</td>
<td>-0.08 ± 0.10</td>
<td>0.40</td>
<td>0.004 ± 0.003</td>
<td>0.12</td>
<td>0.001 ± 0.002</td>
</tr>
<tr>
<td>Month d</td>
<td>-</td>
<td>0.32</td>
<td>-</td>
<td>0.39</td>
<td>-</td>
</tr>
<tr>
<td>Intercept</td>
<td>10.25 ± 0.99</td>
<td>-</td>
<td>0.51 ± 0.04</td>
<td>-</td>
<td>0.32 ± 0.03</td>
</tr>
<tr>
<td>5 e</td>
<td>-0.69 ± 1.12</td>
<td>0.54</td>
<td>-0.02 ± 0.05</td>
<td>0.67</td>
<td>-0.05 ± 0.03</td>
</tr>
<tr>
<td>9 e</td>
<td>-1.69 ± 1.17</td>
<td>0.15</td>
<td>0.06 ± 0.05</td>
<td>0.26</td>
<td>-0.10 ± 0.03</td>
</tr>
<tr>
<td>12 e</td>
<td>0.32 ± 1.25</td>
<td>0.80</td>
<td>0.02 ± 0.05</td>
<td>0.68</td>
<td>-0.11 ± 0.03</td>
</tr>
</tbody>
</table>

**Adjusted model for %FM (month of lactation as linear main effect) c**

| Intercept  | 9.84 ± 0.86 | - | 0.49 ± 0.03 | - | 0.31 ± 0.02 | - | 67.23 ± 1.04 | - | 13.08 ± 0.95 | - |
| %FM        | -0.09 ± 0.10 | 0.36 | 0.005 ± 0.003 | 0.051 | -0.001 ± 0.002 | 0.67 | -0.05 ± 0.10 | 0.58 | 0.14 ± 0.09 | 0.12 |
| Month d    | -0.05 ± 0.12 | 0.70 | 0.008 ± 0.005 | 0.11 | -0.01 ± 0.003 | 0.001 | -0.17 ± 0.15 | 0.46 | -0.30 ± 0.16 | 0.070 |

**Adjusted model for %FM (month of lactation as a factor) c**

| Intercept  | 10.29 ± 1.00 | - | 0.50 ± 0.04 | - | 0.32 ± 0.02 | - | 68.30 ± 1.16 | - | 13.41 ± 1.21 | - |
| %FM        | -0.07 ± 0.10 | 0.47 | 0.005 ± 0.003 | 0.061 | -0.001 ± 0.002 | 0.71 | -0.05 ± 0.09 | 0.56 | 0.14 ± 0.09 | 0.12 |
| Month d    | -         | 0.35 | -       | 0.24 | -       | 0.008 | -       | 0.50 | -       | 0.23 |
| 5 e        | -0.71 ± 1.11 | 0.53 | -0.01 ± 0.05 | 0.81 | -0.05 ± 0.03 | 0.097 | -2.05 ± 1.53 | 0.18 | -1.26 ± 1.57 | 0.43 |
| 9 e        | -1.79 ± 1.17 | 0.13 | 0.07 ± 0.05 | 0.15 | -0.10 ± 0.03 | 0.003 | -1.94 ± 1.59 | 0.23 | -3.33 ± 1.66 | 0.051 |
| 12 e       | 0.07 ± 1.29 | 0.96 | 0.05 ± 0.05 | 0.34 | -0.11 ± 0.04 | 0.003 | -2.16 ± 1.69 | 0.20 | -2.54 ± 1.79 | 0.16 |
Table S5.1. Continued.

<table>
<thead>
<tr>
<th>Predictor</th>
<th>Adiponectin (ng/mL)</th>
<th>Whole milk Leptin (ng/mL)</th>
<th>Skim Milk Leptin (ng/mL)</th>
<th>Lactose (g/L)</th>
<th>Protein (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PE ± SE</td>
<td>p</td>
<td>PE ± SE</td>
<td>p</td>
<td>PE ± SE</td>
</tr>
<tr>
<td>Adjusted model for BMI (month of lactation as linear main effect) c</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intercept</td>
<td>9.78 ± 0.85</td>
<td>−</td>
<td>0.50 ± 0.03</td>
<td>−</td>
<td>0.31 ± 0.02</td>
</tr>
<tr>
<td>BMI</td>
<td>0.11 ± 0.13</td>
<td>0.42</td>
<td>0.006 ± 0.003</td>
<td>0.082</td>
<td>-0.001 ± 0.002</td>
</tr>
<tr>
<td>Month d</td>
<td>-0.002 ± 0.12</td>
<td>0.99</td>
<td>0.006 ± 0.005</td>
<td>0.19</td>
<td>-0.01 ± 0.003</td>
</tr>
<tr>
<td>Adjusted model for BMI (month of lactation as a factor) c</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intercept</td>
<td>10.27 ± 0.99</td>
<td>−</td>
<td>0.51 ± 0.03</td>
<td>−</td>
<td>0.32 ± 0.02</td>
</tr>
<tr>
<td>BMI</td>
<td>0.11 ± 0.13</td>
<td>0.39</td>
<td>0.005 ± 0.003</td>
<td>0.10</td>
<td>-0.001 ± 0.002</td>
</tr>
<tr>
<td>Month d</td>
<td>-0.002 ± 0.12</td>
<td>0.99</td>
<td>0.006 ± 0.005</td>
<td>0.19</td>
<td>-0.01 ± 0.003</td>
</tr>
</tbody>
</table>

Data are parameter estimate ± SE. Analyses were run on pre- and post-feed samples using complete case approach. a Significant p – values are in bold font. b Effects of predictors taken from univariate linear mixed effects models. c Effects of predictors taken from linear mixed effects models that accounted for the month of lactation as linear main effect or as a factor. d Omnibus F-test. e Post-hoc test with reference 2 months. Abbreviations: BMI – body mass index; %FM – percentage fat mass; PE – parameter estimate; SE – standard error.
Table S5.2. Maternal adiposity and human milk components concentrations presented at the months after birth for longitudinal subset (n=21 participants, 73 sessions). Values are mean ± standard deviation (range).

<table>
<thead>
<tr>
<th>Month of Lactation</th>
<th>2 (n = 15)</th>
<th>5 (n = 21)</th>
<th>9 (n = 19)</th>
<th>12 (n = 18)</th>
<th>Total (n = 73)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal BMI a</td>
<td>26.0 ± 5.4</td>
<td>23.7 ± 5.0</td>
<td>25.9 ± 5.8</td>
<td>23.1 ± 6.1</td>
<td>24.5 ± 5.5</td>
</tr>
<tr>
<td>(20.1–35.5)</td>
<td>(19.0–35.2)</td>
<td>(19.2–37.2)</td>
<td>(18.5–37.2)</td>
<td>(18.5–37.2)</td>
<td></td>
</tr>
<tr>
<td>Maternal fat mass (%)</td>
<td>35.0 ± 5.3</td>
<td>33.0 ± 6.2</td>
<td>33.3 ± 7.4</td>
<td>29.7 ± 7.6</td>
<td>32.7 ± 6.8</td>
</tr>
<tr>
<td>(25.7–43.0)</td>
<td>(23.2–47.2)</td>
<td>(23.0–44.3)</td>
<td>(19.4–44.5)</td>
<td>(19.4–47.2)</td>
<td></td>
</tr>
<tr>
<td>Whole milk leptin (ng/mL)</td>
<td>0.50 ± 0.16</td>
<td>0.49 ± 0.17</td>
<td>0.57 ± 0.12</td>
<td>0.54 ± 0.13</td>
<td>0.52 ± 0.15</td>
</tr>
<tr>
<td>(0.21–0.92)</td>
<td>(0.20–0.82)</td>
<td>(0.21–0.79)</td>
<td>(0.24–0.86)</td>
<td>(0.20–0.92)</td>
<td></td>
</tr>
<tr>
<td>Skim milk leptin (ng/mL)</td>
<td>0.32 ± 0.17</td>
<td>0.27 ± 0.08</td>
<td>0.22 ± 0.03</td>
<td>0.23 ± 0.05</td>
<td>0.26 ± 0.10</td>
</tr>
<tr>
<td>(0.19–0.90)</td>
<td>(0.20–0.48)</td>
<td>(0.19–0.35)</td>
<td>(0.19–0.41)</td>
<td>(0.19–0.90)</td>
<td></td>
</tr>
<tr>
<td>Adiponectin (ng/mL)</td>
<td>10.75 ± 4.99</td>
<td>9.80 ± 4.27</td>
<td>8.91 ± 2.60</td>
<td>11.02 ± 7.25</td>
<td>10.07 ± 4.99</td>
</tr>
<tr>
<td>(5.62–25.62)</td>
<td>(6.02–29.67)</td>
<td>(6.07–20.29)</td>
<td>(4.74–52.00)</td>
<td>(4.74–52.00)</td>
<td></td>
</tr>
<tr>
<td>Total protein (g/L)</td>
<td>13.63 ± 6.80</td>
<td>12.42 ± 6.39</td>
<td>10.12 ± 2.42</td>
<td>10.67 ± 2.44</td>
<td>11.71 ± 5.18</td>
</tr>
<tr>
<td>(6.54–31.51)</td>
<td>(7.09–34.76)</td>
<td>(3.32–15.73)</td>
<td>(6.95–16.80)</td>
<td>(3.32–34.76)</td>
<td></td>
</tr>
<tr>
<td>Lactose (g/L)</td>
<td>68.24 ± 10.47</td>
<td>66.28 ± 5.84</td>
<td>66.40 ± 6.62</td>
<td>66.31 ± 6.10</td>
<td>66.75 ± 7.33</td>
</tr>
<tr>
<td>(50.35–89.06)</td>
<td>(50.92–79.48)</td>
<td>(54.74–90.61)</td>
<td>(51.00–79.33)</td>
<td>(50.35–90.61)</td>
<td></td>
</tr>
</tbody>
</table>

Data are mean ± SD and ranges. Concentrations of components are measured in both pre- and post-feed milk samples. a BMI - body mass index.
COPIES OF THE PUBLISHED VERSIONS OF CHAPTERS 2 - 6
Bioimpedance spectroscopy in the infant: effect of milk intake and extracellular fluid reservoirs on resistance measurements in term breastfed infants

INTRODUCTION

Accurate assessment of infant fluid status and body composition is essential for studies investigating the effect of nutrition on the infant growth and development. Bioimpedance spectroscopy (BIS) is non-invasive and an inexpensive method, commonly used in adults to calculate total body water (TBW) and estimate fat-free mass. Changes in body fat and water status are reflected as changes in tissue impedance and electrical properties. Although predictive BIS equations have been developed for adolescents, children, toddlers and newborn infants, there are no validated prediction equations using BIS in neonates.

BACKGROUND/OBJECTIVES: Bioimpedance spectroscopy is an accurate non-invasive method for measuring body composition in adults, but in infants it requires further testing and validation. Of the few studies of bioimpedance conducted in infants, none have comprehensively investigated the effect of milk intake and volume. This study assessed the effect of the milk intake, feed duration and the volume of the infant’s stomach and bladder on the resistance values pre-/post-feed to establish the feasibility of using these values interchangeably during data collection.

SUBJECTS/METHODS: Forty-eight breastfeeding infants were measured at 2, 5, 9 and/or 12 months (n = 62 sessions) within 1–2 min before the start and after the end of breastfeed. Median (IQR) time between measurements was 24 (20.0–30.0) min. Resistance measurements at 0 and 50 kHz, and infinite frequency (R0, R50 and Rinfi) and resistance of intracellular water (Ricw) were analysed with customised infant settings. Milk intake was measured by test weights. Free-water volumes and free-water change were determined from stomach and bladder volumes calculated from ultrasound images.

RESULTS: Small pre-to-post-feed changes (median (IQR): R0 = 3.7 (–14.8, 14.3); R50 0.3 (–10.4, 15.0); Rinfi 2.8 (–13.3, 35.5); Ricw 20.8 (–98.1, 290.9)) were not significantly different from zero (R0: P = 0.92; R50: P = 0.48; Rinfi: P = 0.32; Ricw: P = 0.097). No significant effect of milk intake or free-water change was detected.

CONCLUSIONS: The lack of consistent change in resistance across a breastfeed provides flexibility in the timing of measurements of infants in the research setting, such that typically pre- and post-feed measures of resistance can be used interchangeably.

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Values of resistance (ohm) at 0 and 50 kHz, and infinite frequency ($R_{in}$ and $R_{out}$, respectively) were determined from the curve of best fit. Resistance of intracellular water component ($R_{icw}$) was calculated using the following formula: $R_{icw} = 1/(1/R_{in} - 1/(1/R_{out}))$. These frequencies were chosen as $R_{in}$ is commonly used to predict TBW in empirically derived prediction equations, $R_{out}$ and $R_{icw}$ to predict extracellular and intracellular water volumes and $R_{out}$ to predict TBW using mixture theory modelling.

Stomach and bladder volumes

Scans of longitudinal and transverse planes of the stomach and bladder were performed in the semi-supine position according to the methods validated in infants, using an Aplio 80 (Toshiba, Japan) ultrasound machine and Parker ultrasonic gel (Fairfield, NJ, USA). Longitudinal, transverse and sagittal diameters were measured, and the volume of both extracellular fluid reservoirs was calculated using the following formula:

$$\text{Volume (mL)} = \text{longitudinal diameter (cm)} \times \text{transverse diameter (cm)} \times 0.52$$

Free-water change was calculated by subtracting combined bladder and stomach volumes pre-feed from combined volumes post-feed.

Infant milk intake

Milk intake was determined by test weighing the infant pre- and post-feed on an electronic balance (±0.2 g; Medela Electronic Baby-Weigh Scales, Medela AG, Switzerland). Milk intake was calculated by subtracting the initial weight from the final weight of the infant.

Statistical analyses

Statistical analyses were performed using R 2.9.0 for Mac OS X with packages nlme, irr and multcomp. Descriptive statistics are reported as mean ± s.d. (normally distributed data) or range. $P < 0.05$ was considered statistically significant. All tests were two tailed.

Linear mixed effect models grouping by infant were run and compared with linear regression models (analysis of variance). No significant individual patterns were found ($P > 0.16$); hence, measurements on the same infant at different time points were treated as independent.

Participants were recruited as part of a larger study; those with usable data were included here. Pilot data ($n = 5$, two measurements under identical conditions) gave mean ± s.d. within participant differences of 7.4 ± 7.7. Using $\alpha = 0.05$, 62 participants give the study power of 0.8 to detect a mean difference between pre- and post-feed resistance measures of 2.4.

BIS measurements were assessed for variability (CV (coefficient of variation)) and repeatability (intraclass correlation coefficient (ICC), 95% confidence interval (CI)) for a subset of infants ($n = 16$, two per age/sex grouping).

Within time-point comparisons treated the first and second half of a recording as separate measures; summary BIS measures were calculated as per full recordings. Variability was assessed by calculating CV for the raw data for each infant by time point by first/second half of recording ($n = 32$) and compared using paired t-tests within frequencies. Repeatability was assessed by calculation of ICC and 95% CI and was considered to differ if the 95% CIs did not overlap. ICC > 0.8 is considered acceptable for a research setting and > 0.95 for the clinical setting. Between time-point comparisons compared pre- and post-feed measures using the same analyses as for the within time-point comparisons. Paired t-tests were used to compare variability at different frequencies, with pre- and post-feed data treated as independent measures.

Overall variation was determined from a repeated measures CV calculated from an intercept only linear mixed effects model.

Changes in resistance pre-/post feed ($\Delta R$) were assessed by calculating bias/95% CI for pre- vs post-feed BIS as per Bland and Altman ($n = 59$). A 95% CI containing zero was interpreted as no consistent change.

Associations between $\Delta R$ and predictors/covariates of interest were assessed using linear regression. Model selection used backwards stepwise selection. Age was treated as a categorical variable and included in the models where an analysis of variance omnibus test indicated that one or more age differed from the others.

Associations between infant age (factor) and pre-feed resistance were assessed using Tukey's all-pair comparisons based on OLS regression. Model appropriateness tested using standard residual diagnostics. Infant movements pre-/post feed were compared using paired Student's t-test.
<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Mean ± s.d.</th>
<th>Range</th>
<th>Mean ± s.d.</th>
<th>Range</th>
<th>Mean ± s.d.</th>
<th>Range</th>
<th>Mean ± s.d.</th>
<th>Range</th>
<th>Mean ± s.d.</th>
<th>Range</th>
<th>Mean ± s.d.</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Milk intake (ml)</td>
<td>93.9 ± 26.3</td>
<td>39-136</td>
<td>106.6 ± 16.6</td>
<td>50-200</td>
<td>50-200</td>
<td>50-200</td>
<td>6.7 ± 18.4</td>
<td>0-71</td>
<td>8.3 ± 19.0</td>
<td>0-54</td>
<td>8.3 ± 19.0</td>
<td>0-54</td>
</tr>
<tr>
<td>Length (cm)</td>
<td>57.8 ± 2.4</td>
<td>53.0-61.3</td>
<td>64.7 ± 1.9</td>
<td>61.3-69.5</td>
<td>71.5 ± 2.2</td>
<td>66.0-74.5</td>
<td>73.6 ± 1.5</td>
<td>71.5-77.5</td>
<td>66.0 ± 6.2</td>
<td>53.0-75.5</td>
<td>66.0 ± 6.2</td>
<td>53.0-75.5</td>
</tr>
<tr>
<td>Body weight (kg)</td>
<td>Pre-feed 5.319 ± 0.727</td>
<td>4.20-6.335</td>
<td>7.289 ± 0.923</td>
<td>5.67-8.715</td>
<td>9.084 ± 0.773</td>
<td>7.68-10.605</td>
<td>9.307 ± 0.985</td>
<td>7.17-10.290</td>
<td>7.537 ± 1.756</td>
<td>4.20-10.605</td>
<td>7.537 ± 1.756</td>
<td>4.20-10.605</td>
</tr>
<tr>
<td>Stomach volume (ml)</td>
<td>Pre-feed 63 ± 14.0</td>
<td>0-50</td>
<td>106.6 ± 16.6</td>
<td>50-200</td>
<td>50-200</td>
<td>50-200</td>
<td>6.7 ± 18.4</td>
<td>0-71</td>
<td>8.3 ± 19.0</td>
<td>0-54</td>
<td>8.3 ± 19.0</td>
<td>0-54</td>
</tr>
<tr>
<td>Bladder volume (ml)</td>
<td>Pre-feed 9.6 ± 0.8</td>
<td>0-26</td>
<td>14.5 ± 12.8</td>
<td>0-55</td>
<td>50-200</td>
<td>50-200</td>
<td>25.4 ± 18.8</td>
<td>0-75</td>
<td>31.5 ± 15.3</td>
<td>6-50</td>
<td>31.5 ± 15.3</td>
<td>6-50</td>
</tr>
<tr>
<td>Free water (ml)</td>
<td>Pre-feed 163 ± 15.0</td>
<td>0-50</td>
<td>23.9 ± 17.4</td>
<td>0-55</td>
<td>50-200</td>
<td>50-200</td>
<td>32.0 ± 25.8</td>
<td>0-97</td>
<td>39.8 ± 30.1</td>
<td>6-103</td>
<td>39.8 ± 30.1</td>
<td>6-103</td>
</tr>
<tr>
<td>Free-water change (ml)</td>
<td>Pre-feed 101.7 ± 42.0</td>
<td>33-202</td>
<td>90.3 ± 53.1</td>
<td>12-216</td>
<td>50-200</td>
<td>50-200</td>
<td>12.9 ± 81.3</td>
<td>52-359</td>
<td>90.9 ± 80.4</td>
<td>21-337</td>
<td>90.9 ± 80.4</td>
<td>21-337</td>
</tr>
</tbody>
</table>

Abbreviations: F, female; icw, intracellular water; inf, infinity; L, length (cm); \( L^2/R \), impedance index; M, male; R, resistance (ohm). Subjects’ characteristics presented as mean ± s.d. and range for total group (N = 62) and grouped by infant age.
RESULTS

Of the 62 infants (Table 1), 37 were measured once (33/37 complete data), 8 twice (14/16 complete data) and 3 three times (8/9 complete data). Missing data included the following: two bioimpedance measurements due to infant movement pre-feed and one post feed (three feed duration times missing); two stomach volumes pre-/post feed and four bladder volumes pre- and five post feed (six free-water change calculations missing).

Bias, variability and repeatability

In a subset of infants \((n=16)\), no difference in variability pre- vs post feed was seen for any of the frequencies \((R_0, P=0.36; R_{50}, P=0.47; R_{inf}, P=0.34; R_{icw}, P=0.47)\). There was no difference in variability between \(R_0\) and \(R_{50}\), and \(R_0\) and \(R_{icw}\) \((P=0.13\) for both), but all \(R_0\), \(R_{50}\) and \(R_{icw}\) were significantly less variable than \(R_{inf}\) \((P<0.001\) for all). Figure 1 presents Bland–Altman plots for pre- and post-feed resistance measures and impedance indices and illustrates that \(R_{inf}\) measures have greater variability, which is also carried onto \(R_{ref}\) to the point that some of the individual data points on the plots for \(R_{ref}\) and \(R_{inf}\) and for corresponding indices present as outliers. Although these measurements are legitimate in that they are a result of machine output or calculated from the other measures, they are not presenting in an expected range and thus should be interpreted with caution.

Bias measurements \((n=59)\) were small, with the 95% CI containing zero for all frequencies, except \(R_{icw}\). There was a higher level of agreement when comparing the first and second half of a set of measurements (within) than comparing pre- with post-feed (between) measurements. All but \(R_{icw}\) within measurements had ICCs > 0.96 (95% CI). Between pre- and post-feed measurements intraclass correlations also were strong (> 0.8; Table 2; Figure 1).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Within measures (paired) ((n=16))</th>
<th>Pre- vs post-measures ((n=16))</th>
<th>((n=59))</th>
</tr>
</thead>
<tbody>
<tr>
<td>(R_0)</td>
<td>CV (within participants)</td>
<td>1.3</td>
<td>2.4</td>
</tr>
<tr>
<td></td>
<td>ICC agreement (95% CI)</td>
<td>0.983 (0.966, 0.992)</td>
<td>0.981 (0.961, 0.991)</td>
</tr>
<tr>
<td>(R_{50})</td>
<td></td>
<td>1.5</td>
<td>2.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.981 (0.961, 0.991)</td>
<td>0.966 (0.931, 0.983)</td>
</tr>
<tr>
<td>(R_{inf})</td>
<td></td>
<td>2.8</td>
<td>3.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.966 (0.961, 0.991)</td>
<td>1.966 (0.931, 0.983)</td>
</tr>
<tr>
<td>(R_{icw})</td>
<td></td>
<td>12.6</td>
<td>14.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.864 (0.739, 0.931)</td>
<td>0.864 (0.739, 0.931)</td>
</tr>
</tbody>
</table>

Abbreviations: CI, confidence interval; CV, coefficient of variation; ICC, intraclass correlation coefficient; icw, intracellular water; inf, infinity; \(R\), resistance (ohm). Limits of agreement are mean ± (1.96 × s.d.) for 95% CI.
Resistance pre-feed/post-feed
Changes in resistance post-feed–pre-feed (ΔR) were small (median (IQR): Rc = 3.7 (−4.8, 14.3); Rs = 0.3 (−10.4, 15.0); Rif = 2.8 (−13.3, 35.5); Rcw = 20.8 (−98.1, 290.9)) and not significantly different from zero (Rc: P = 0.92; Rs: P = 0.48; Rif: P = 0.32; Rcw: P = 0.097; Figures 1 and 2).

Infant age, length and weight. Significant negative univariate associations between ΔR and infant length were seen for Rc and Rs but not for Rif or Rcw (Rc: P = 0.019; Rs: P = 0.007; Rif: P = 0.06; Rcw: P = 0.44). A similar association was found between ΔR and infant weight for Rs but not for Rc, Rif or Rcw (Rc: P = 0.08; Rs: P = 0.036; Rif: P = 0.14; Rcw: P = 0.39), with greater ΔR seen for shorter/smaller infants. After accounting for these covariates, no significant ΔR was seen for any frequency (Table 3).

Pre-feed resistance was highest at 5 months and lowest at 2 months (Table 1; Figure 3). Significant differences in pre-feed resistances were seen for Rif between 2 and 5 months (P = 0.015) and for Rcw between 2 and 5 (P = 0.031), 2 and 9 (P = 0.002), and 2 and 12 months (P = 0.007). No other differences were significant (P > 0.06).

Age was not significant in any model (Rc: P > 0.06; Rs: P > 0.09; Rif: P > 0.43; Rcw: P > 0.56). Although resistance pre/post feed did not change consistently with infant age, a change in the trend from positive to negative was seen.

Impedance index. Changes in impedance indices post-feed–pre-feed (ΔL'/R) also were small (median (IQR): L'/Rc = 0.04 (−1.11, 0.16); L'/Rs = 0.00 (−0.13, 0.12); L'/Rif = 0.06 (−0.51, 0.23); L'/Rcw = 0.03 (−0.38, 0.16)) and not significantly different from zero (L'/Rc: P = 0.48; L'/Rs: P = 0.79; L'/Rif: P = 0.86; L'/Rcw: P = 0.78; Figures 1 and 2).

Age was not significant in any model (L'/Rc: P > 0.09; L'/Rs: P > 0.14; L'/Rif: P > 0.33; L'/Rcw: P > 0.31).

Pre-feed impedance indices were lowest at 2 and highest at 12 months, with the exception of L'/Rcw (Table 1; Figure 3). All L'/Rcw was not significantly different at any time point (P > 0.52). All four indices were not significantly different between 9 and 12 months (P > 0.52). L'/Rc (P = 0.026) and L'/Rcg (P = 0.041) were significantly different between 2 and 5 months, with the rest of the indices being significantly different between all other time points (P < 0.001).

Milk intake. All milk intakes were within the normal range. Compared with the overall sample mean, 12-month-old infants took smaller volumes (P = 0.022, Table 1). There was no association between milk intake and both ΔR and ΔL'/R (Table 3). In the patterns of resistance measures by milk intake, a split between pre- and post-feed was observed only for volumes higher than 150 ml (Figure 2).

Feed duration. Feed duration as a proxy measure of time interval between BIS measurements was significantly longer in 2-month-old infants (P = 0.017, Table 1). There was no association between feed duration and both ΔR and ΔL'/R (Table 3).

Free-water change. There was no association between free-water change and both ΔR and ΔL'/R (Table 3).

Stomach and bladder volume. There was no association between both ΔR and ΔL'/R and pre-feed stomach volume (Rc: P = 0.68; Rs: P = 0.68; Rif: P = 0.91; Rcw = 0.66), (L'/Rc: P = 0.85; L'/Rs: P = 0.90; L'/Rif: P = 0.88; L'/Rcw: P = 0.82) or post-feed stomach volume (Table 3).

Univariate associations were seen between pre-feed bladder volume and ΔR for Rcg, Rif and Rcw but not for Rc (Rc: P = 0.08; Rs: P = 0.04; Rif: P = 0.0006; Rcw = 0.030) and ΔL'/R for all four impedance indexes (L'/Rc: P = 0.044; L'/Rcg: P = 0.016; L'/Rif: P < 0.001; L'/Rcw: P < 0.001).

There were also significant associations between post-feed bladder volume and both ΔR and ΔL'/R for Rc and Rs but not for Rif or Rcw (Rc: P = 0.002; Rs: P = 0.005; Rif: P = 0.15; Rcw = 0.40), (L'/Rc: P = 0.0002; L'/Rs: P = 0.002; L'/Rif: P = 0.53; L'/Rcw: P = 0.87). After accounting for this, there was no significant ΔR or ΔL'/R associated with a change in bladder volume (Rc: P = 0.70; Rs: P = 0.89; Rif: P = 0.99; Rcw = 0.14), (L'/Rc: P = 0.48; L'/Rs: P = 0.94; L'/Rif: P = 0.90; L'/Rcw: P = 0.64).

Figure 2. Patterns of resistance measures (Rc, Rs, Rif and Rcw) and impedance indices (L'/Rc, L'/Rs, L'/Rif and L'/Rcw) pre- and post-feed by milk intake (ml). Lines are local regression smoothers (LOESS), and character are individual data points grouped as per legend: ▲ and — denote pre-feed; ● and --- denote post-feed.
Table 3. Significance (P-values) of selected predictors for difference in resistance measures and impedance indices pre- and post feed for four predictors accounting for at most one covariate

<table>
<thead>
<tr>
<th>Predictor</th>
<th>Added covariate</th>
<th>Effect of predictor</th>
<th>Effect of covariate</th>
<th>Effect of predictor</th>
<th>Effect of covariate</th>
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<tr>
<td></td>
<td></td>
<td>Coefficient</td>
<td>P-value</td>
<td>Coefficient</td>
<td>P-value</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>Milk Intake (ml)</td>
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<td>0.929</td>
<td>−0.002</td>
<td>0.929</td>
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<tr>
<td></td>
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<td>0.683</td>
<td>−1.552</td>
<td>0.019</td>
</tr>
<tr>
<td></td>
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<td>0.753</td>
<td>−4.191</td>
<td>0.077</td>
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<tr>
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<td>None</td>
<td>0.222</td>
<td>0.52</td>
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<td>NA</td>
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<tr>
<td></td>
<td>Length (cm)</td>
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<td>0.891</td>
<td>−1.497</td>
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<td>0.849</td>
<td>−3.978</td>
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<tr>
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<td>Length (cm)</td>
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<td>0.648</td>
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<tr>
<td>Post-feed stomach volume (ml)</td>
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<td>NA</td>
<td>NA</td>
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<tr>
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<td>0.485</td>
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<td>0.061</td>
<td>0.396</td>
<td>−4.418</td>
<td>0.065</td>
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<tr>
<td>Milk intake (ml)</td>
<td>None</td>
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<td>0.843</td>
<td>−0.001</td>
<td>0.406</td>
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<td>0.88</td>
<td>−1.746</td>
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<td>0.951</td>
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<tr>
<td>Feed duration (min)</td>
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<td>0.547</td>
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<td>Length (cm)</td>
<td>0.007</td>
<td>0.984</td>
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<tr>
<td>Free-water change (ml)</td>
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<td>0.032</td>
<td>0.622</td>
<td>−1.774</td>
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<td>Weight (kg)</td>
<td>0.044</td>
<td>0.408</td>
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<td>0.014</td>
</tr>
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<td>Post-feed stomach volume (ml)</td>
<td>None</td>
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<td>0.299</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
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<td>−1.742</td>
<td>0.009</td>
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<td></td>
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<td>0.066</td>
<td>0.355</td>
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<td>0.033</td>
</tr>
<tr>
<td>Milk intake (ml)</td>
<td>None</td>
<td>0.072</td>
<td>0.614</td>
<td>−0.001</td>
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</tr>
<tr>
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<td>Length (cm)</td>
<td>0.038</td>
<td>0.786</td>
<td>−1.869</td>
<td>0.073</td>
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<tr>
<td></td>
<td>Weight (kg)</td>
<td>0.047</td>
<td>0.743</td>
<td>−5.224</td>
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</tr>
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<td>Feed duration (min)</td>
<td>None</td>
<td>0.231</td>
<td>0.669</td>
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<td>0.987</td>
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<td>0.073</td>
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<td></td>
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<td>0.164</td>
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<tr>
<td>Free-water change (ml)</td>
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<td>0.143</td>
<td>−0.004</td>
<td>0.072</td>
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<td>0.139</td>
<td>0.193</td>
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<td>0.092</td>
</tr>
<tr>
<td>Post-feed stomach volume (ml)</td>
<td>None</td>
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<td>0.417</td>
<td>−0.002</td>
<td>0.412</td>
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<td>0.067</td>
<td>0.553</td>
<td>−1.974</td>
<td>0.06</td>
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<tr>
<td></td>
<td>Weight (kg)</td>
<td>0.081</td>
<td>0.476</td>
<td>−5.94</td>
<td>0.115</td>
</tr>
</tbody>
</table>

Abbreviations: icw, intracellular water; inf, infinity; L, length (cm); $L^2/R$, impedance index; NA, not applicable; R, resistance (ohm). Each line in the table represents a separate model. Effects of predictor (added covariate—none) are the univariate regression results. Effects of covariate (length or weight) are results of multivariate models consisting of both predictor and covariate. Coefficients of predictors are changes in resistance difference per each unit more of the predictor after accounting for the added covariate (if any). Similarly, coefficients of covariates (length or weight) are changes in resistance difference associated with longer/heavier infants after accounting for the predictor. Covariates were included in the table if they were significant in at least one model. Length was not included as a covariate in models for impedance indices, as it is a component of the formula. Bold text indicates P-values for the significant covariates retained in the final model.
shown either minimal change in resistance after eating/drinking 

are not significant different (P = 0.09). The effect of movement post-feed on \( \Delta R \) or \( \Delta L^2/R \) was independent of pre-feed movement (\( R_c; P = 0.81 \); \( R_{50}; P = 0.93 \); \( R_{inf}; P = 0.34 \); \( R_{icw}; P = 0.60 \); (\( L^2/R_c; P = 0.57 \); \( L^2/R_{50}; P = 0.58 \); \( L^2/R_{inf}; P = 0.09 \); \( L^2/R_{icw}; P = 0.09 \)), and of infant age (\( R_c; P = 0.28 \); \( R_{50}; P = 0.35 \); \( R_{inf}; P = 0.41 \); \( R_{icw}; P = 0.43 \); (\( L^2/R_c; P = 0.29 \); \( L^2/R_{50}; P = 0.40 \); \( L^2/R_{inf}; P = 0.22 \); \( L^2/R_{icw}; P = 0.22 \)).

**DISCUSSION**

Our findings indicate that both resistance measurements and impedance indices pre- and immediately post breastfeeding are not significantly different. Contradictory studies in adults have shown either minimal change in resistance after eating/drinking or a significant increase in resistance after ingestion of liquid (1.2–1.8 l). Any changes noted were not immediately after the fluid consumption but 15–20 min after. Similarly, in infant studies, a significant increase in \( R_0 \) values was recorded only in a group of 1-week-old infants 30 min after the feed. The lack of significant effect of breastfeed volumes on resistance values or impedance indices suggests that either pre- or post-feed resistance measurements can be used in calculation of infant TBW, fat mass or %fat mass once the validity of these estimates has been established. Further, the lack of significant effects of milk intake, feed duration, free-water change, movement and post-stomach volume suggests no necessity to account for these during BIS measurements of infants, with the possible exception of infrequent feeders taking >200 ml. These data question the sensitivity of BIS for measuring body water volumes and detecting change in these volumes. The maximum milk intake in this study (232 ml) was 2.9% of pre-feed body weight; yet, surprisingly, this magnitude of change was not detectable despite BIS being able to detect a change in volume of extracellular water of ~40 ml in the adult arm. The explanation may lie in the fact that BIS measurements were performed using the adult wrist-to-ankle protocol. The measured impedance under such electrode arrangement is dominated by the impedance of the small cross-sectional area of arms and legs, whereas the larger cross-sectional area of trunk, where most of the free-water resides, contributes.

![Figure 3. Line plots showing pattern by age and relationship between pre- and post-feed resistance measures and impedance indices for four different frequencies (\( R_{icw}, R_{50}, R_{inf} \) and \( R_{c} \)) within an infant (individual data points).](image)
little.\textsuperscript{19,15} Immediately after a breastfeed, the milk is still located in the trunk. Although trunk impedance, particularly $R_b$ may decrease post feed, this is overshadowed by much larger arm and leg impedance. The important consequence of these observations is that breastfeeding would appear to have little effect on either resistance measurements or impedance indices in the breastfeeding infant.

A limitation of this study was the relatively narrow range of feed volumes with few feeds (1.6%) greater than 200 ml, which is the minimal detectable TBW change in infants estimated using mass balance or tracer dilution studies.\textsuperscript{16} Although, as noted above, the impedance technique is capable, under appropriate conditions, of detecting small changes in fluid volumes, it is not possible from the present data to determine whether it can reliably detect changes with greater sensitivity than the 200 ml threshold for tracer or balance studies.

Pragmatically, the absence of systematic difference in both resistance and impedance indices pre- and immediately post feed allows flexibility in the timing of BIS measurements. Post-feed measures could reliably be used rather than pre-feed measures, which is the standard recommendation for performing BIS in adults.\textsuperscript{17} Such measurements should provide reliable estimates of TBW but not account for gut water volumes. If these are of interest, then focussed impedance measurements of the trunk could be undertaken. As the reproducible electrode location in the trunk is difficult in the small infant, measurements may be performed according to the principle of equipotentials,\textsuperscript{17} using wrist and ankle electrode locations.

Variability (within participants CV) in all resistance measurements was observed (1.3%, 1.5%, 2.8% and 12.6% for $R_a$, $R_{sp}$, $R_c$ and $R_{cv}$, respectively). Although theoretically $R_a$ should provide the best prediction of TBW and fat-free mass, greater variability is seen in this parameter and in $R_{cv}$ (in the present study and those of others, for example, Sesmero et al.\textsuperscript{12} and Lingwood et al.\textsuperscript{15}). These errors will contribute to the precision of prediction of TBW. Consequently, prediction may be better performed in this group using resistance measured at 50 kHz as observed in neonates by Lingwood et al.,\textsuperscript{12} allowing for measurements to be obtained using simpler single frequency impedance devices. Prediction of TBW may be further improved when BIS data are available by the use of the impedance value at the characteristic frequency, $Z_c$, as at this frequency the ratio of the current flow through the extra- and intracellular is dependent only on the resistances of these water compartments and not cell membrane capacitance.\textsuperscript{11,12}

As expected, infant length and weight were associated with $\Delta R$. One interesting feature of this is that, although greater $\Delta R$ were seen in smaller, shorter and younger infants, $\Delta R$ changed from positive to negative as the infants got longer/older. The observed difference in pre-feed $R_{inf}$ and $R_{cv}$ between 2 and 5 months may reflect a gradual decrease in extracellular fluid, as previously reported\textsuperscript{11} and confirmed by electrolyte and fluid balance studies.\textsuperscript{13} Intermediate values at 9 and 12 months could be a reflection of body composition changes due to increased mobility. Longitudinal data will better describe changes in resistance measures in the first year of life.

A more accurate knowledge of the changes in extracellular fluid volumes of the infant's stomach and bladder was expected to explain $\Delta R$; however, this was not the case with no significant effect of free-water change seen. The maximum free-water change of 337 ml (3.3% of pre-feed body weight) was observed but was not the result of the largest milk intake but rather the redistribution of the fluid in the trunk. Although accounting for bladder volume does not provide any benefit, measurement of milk intake may be useful. Pre-feed infant weight should be used in calculations to avoid overestimation of the fat mass, whereas in infrequent feeders pre-feed BIS measurements might be more prudent.

In conclusion, measurement of resistance in the breastfeeding neonate is feasible, and reliable impedance data can be obtained. Feed volume did not significantly affect whole body resistance measurements or impedance indices, and consequently pre- or post-breastfeed measurements of resistance can be used interchangeably to improve best body data collection in infants.

**CONFLICT OF INTEREST**

LCW provides consultancy services to Medela, Ltd, which had no involvement in the inception, conduct of this research or in writing of the manuscript. The remaining authors declare no conflict of interest.

**ACKNOWLEDGEMENTS**

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Determinants of body composition in breastfed infants using bioimpedance spectroscopy and ultrasound skinfolds—methods comparison

Zoya Gridneva¹, Anna R. Hepworth¹, Leigh C. Ward², Ching T. Lai¹, Peter E. Hartmann¹ and Donna T. Geddes¹

BACKGROUND: Accurate, noninvasive, and inexpensive methods are required to measure infant body composition. Ultrasound (US) and bioimpedance spectroscopy (BIS) have been validated in adults and introduced in pediatric populations. The aim of this study was to evaluate the performance of both methods in determining percentage fat mass (%FM) in breastfed infants.

METHODS: %FM of 2, 5, 9, and 12 mo-old healthy, breastfed term infants (n = 58) was calculated using BIS-derived total body water equations and skinfold equations then compared with reference models. Skinfolds were measured with US at two and four sites (biceps, suprailiac and/or triceps, and subcapsular).

RESULTS: %FM differed widely within and between methods, with the degree of variation affected by infant age/sex. Not a single method/equation was consistent with the distributions of appropriate reference values for all age/sex groups. Moderate number of matches with reference values (13–24 out of 36) was seen for both types of equations. High number of matches (25–36) was seen for US skinfold-based equations. %FM values calculated from US and BIS were not significantly different (P = 0.35).

CONCLUSION: Both BIS and US are practical for predicting %FM in infants. BIS calculations are highly dependent upon an appropriate set of validated age-matched equations.

Body composition (BC) in early life plays an important role in the programming of long-term health outcomes including obesity, type II diabetes, and cardiovascular disease (1). The majority of large studies reporting an association of infant development with disease later in life use anthropometric measurements or BMI to monitor growth that provide limited indices of adiposity as they fail to reflect body shape, fat distribution, and density.

The number of techniques available to assess infant BC is increasing. These measures have better accuracy than anthropometric measurements, but may be either time consuming, expensive, and invasive, expose infants to low levels of radiation (dual-energy X-ray absorptiometry and computed tomography), require the infant to be restrained (magnetic resonance imaging) or have weight restriction (air displacement plethysmography).

Bioelectrical impedance spectroscopy (BIS) is noninvasive and often used to estimate BC due to its ease of application and low cost. BIS measures the electrical impedance, or opposition to flow through the body tissues of a small harmless electric current. The measured impedance, corrected for the reactive component, known as electrical resistance is inversely and quantitatively related to the volume of conductive compartment which can be used to calculate total body water (TBW) and estimate fat-free mass (FFM) and fat mass (FM) (2). BIS provides reliable estimates of TBW in adults and equations have been developed for children and infants (3,4). While in very young infants (<5 mo) BIS is associated with poorer predictive performance that could be due to the higher conductivity of the infant’s adipose tissue with its increased vascularization and water content, and rapidly changing hydration status caused by feeding patterns (4–6), small bias indicates that BIS is suitable for comparison between groups and for longitudinal studies (4).

Ultrasound (US) presents an alternative noninvasive and inexpensive technique to study infant BC. The US image is created by high frequency waves, which are attenuated during travel through tissue and reflected back to transducer (7). US can be used to measure depth and area of adipose and muscle tissue. Serial measurements have been made to evaluate the growth and development of term and preterm infant and to assess changes in BC in both groups (8,9). Tracking BC with US is sensitive enough to detect breastmilk macronutrient-related changes in adipose and muscle tissue accrued at the measurement sites in preterm infants (9). A modification of this method was utilized to measure subcutaneous tissue thickness (SCTT) in term infants, in place of traditional calipers. Calipers may disturb the infant (10) and mother while common issues such as movement and infant skin compressibility affect reliability (11). US offers a pain-free, more consistent solution and has been validated in adults against dual-energy

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X-ray absorptiometry (12), utilized in assessment of BC of adults, adolescents, and infants (10).

While all BC measuring techniques have inherent limitations when used in infants and children, the combination of anthropometric measurements with US and/or BIS may offer relatively accurate, safe, and noninvasive assessment of BC, making them an attractive research tool for this population. The aim of this study was to compare these BIS and US assessments of BC of healthy term breastfed infants using a cross-sectional approach to determine the accuracy and feasibility of both methods over a wide range of infant body masses.

METHODS

Subjects

Full term healthy breastfed infants (n = 58; 31 male and 27 female) of English speaking (predominantly Caucasian) mothers were recruited from the community, primarily from the Australian Breastfeeding Association. Inclusion criteria were: singletons; gestational age ≥ 37 wk; fully breastfed at 2 and 5 mo and breastfed at the time of the study. Exclusion criteria were: infant health issues that could potentially influence growth and development and low maternal milk supply. Infants were measured once at either 2, 5, 9, or 12 mo.

All mothers provided written informed consent to participate in the study, which was approved by The University of Western Australia, Human Research Ethics Committee.

Design

Study session. At each study session anthropometric (weight and length) and BIS measurements were taken prefeed; US skinfold measurements were taken postfeed. Where prefeed BIS were impractical, postfeed US measurements were performed. Clothing was removed except for a dry diaper and a singlet.

Anthropometric measurements. A single weight and length measurement was performed on each infant. Infant’s weight was determined by weighing before breastfeeding using Medela Electronic Baby Weigh Scales (± 2.0 g; Medela AG, Switzerland). Infant crown-heel length was measured once to the nearest 0.1 cm using nonstretch tape and headpiece and footpiece both applied perpendicular to the hard surface.

BIS measurements. Whole body bioimpedance (wrist to ankle) was measured using battery-operated ImpediMed SFB7 bioelectrical impedance analyzer (ImpediMed, Brisbane, Queensland, Australia). This is a single channel, tetra-polar bioelectrical impedance spectrometer device capable of measuring reactance and resistance at 256 logarithmically spaced frequencies between 3 and 1,000 kHz. Before each session, the external calibration of the instrument was tested with a calibration RRC Test Cell (ImpediMed).

Infants were wearing a dry diaper and a singlet at the time of measurement. After wiping electrode sites with isopropyl alcohol, single

Figure 1. US image of infant triceps (upper arm) with skin, subcutaneous fat, and muscle areas defined. The boundary between muscle and fat is marked by a bright line (fascia). A half skinfold measure is equal to the skin thickness plus the subcutaneous fat thickness and needs to be doubled for use in skinfold-based equation. Scale is presented on the left side of the scan with distance between 0 and 1 = 10 mm.

Table 1. Subject characteristics presented as total and grouped by the infant age

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Mean ± SD (Range)</th>
<th>Mean ± SD (Range)</th>
<th>Mean ± SD (Range)</th>
<th>Mean ± SD (Range)</th>
<th>Mean ± SD (Range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SEX (male/female)</td>
<td>7M/6F</td>
<td>7M/7F</td>
<td>10M/7F</td>
<td>7M/7F</td>
<td>31M/27F</td>
</tr>
<tr>
<td>Age (months)</td>
<td>2.06 ± 0.17*</td>
<td>5.08 ± 0.22</td>
<td>9.17 ± 0.35</td>
<td>12.34 ± 0.23</td>
<td>7.35 ± 3.85</td>
</tr>
<tr>
<td>Length (cm)</td>
<td>58.4 ± 1.9</td>
<td>64.0 ± 2.2</td>
<td>71.2 ± 1.9</td>
<td>74.1 ± 2.6</td>
<td>67.28 ± 6.41</td>
</tr>
<tr>
<td>(54.5–61.3)</td>
<td>(60.5–68.5)</td>
<td>(68.0–74.5)</td>
<td>(68.5–78.5)</td>
<td>(54.5–78.5)</td>
<td></td>
</tr>
<tr>
<td>Body weight (kg)</td>
<td>5.45±0.581</td>
<td>7.170 ± 1.088</td>
<td>8.997 ± 0.779</td>
<td>9.604 ± 0.968</td>
<td>7.909 ± 1.813</td>
</tr>
<tr>
<td>R_{so} (Ω)*</td>
<td>646.8 ± 105.5</td>
<td>702.3 ± 93.7</td>
<td>685.5 ± 85.9</td>
<td>652.0 ± 45.2</td>
<td>672.8 ± 86.1</td>
</tr>
<tr>
<td>(516.1–862.7)</td>
<td>(584.1–855.6)</td>
<td>(582.8–811.5)</td>
<td>(576.4–727.0)</td>
<td>(516.1–862.7)</td>
<td></td>
</tr>
<tr>
<td>Biceps skinfold (mm)*</td>
<td>16.0 ± 5.1</td>
<td>18.2 ± 6.4</td>
<td>16.3 ± 5.9</td>
<td>16.2 ± 4.7</td>
<td>16.7 ± 5.5</td>
</tr>
<tr>
<td>(8.8–23.8)</td>
<td>(8.8–29.2)</td>
<td>(7.4–26.8)</td>
<td>(8.0–24.8)</td>
<td>(7.4–29.2)</td>
<td></td>
</tr>
<tr>
<td>Triceps skinfold (mm)*</td>
<td>15.6 ± 3.1</td>
<td>18.4 ± 5.2</td>
<td>18.7 ± 4.3</td>
<td>19.8 ± 6.4</td>
<td>18.2 ± 5.0</td>
</tr>
<tr>
<td>(10.6–20.2)</td>
<td>(13.0–27.8)</td>
<td>(11.2–25.8)</td>
<td>(9.8–30.6)</td>
<td>(9.8–30.6)</td>
<td></td>
</tr>
<tr>
<td>Subscapular skinfold (mm)*</td>
<td>12.2 ± 2.2</td>
<td>11.5 ± 2.8</td>
<td>10.9 ± 3.8</td>
<td>11.4 ± 2.8</td>
<td>11.5 ± 3.0</td>
</tr>
<tr>
<td>(9.2–18.0)</td>
<td>(6.6–16.4)</td>
<td>(6.0–16.6)</td>
<td>(5.4–14.8)</td>
<td>(5.4–18.0)</td>
<td></td>
</tr>
<tr>
<td>Suprailiac skinfold (mm)*</td>
<td>10.9 ± 3.5</td>
<td>14.6 ± 4.7</td>
<td>13.5 ± 4.0</td>
<td>11.3 ± 4.6</td>
<td>12.4 ± 4.3</td>
</tr>
<tr>
<td>(7.0–19.8)</td>
<td>(10.2–24.6)</td>
<td>(9.0–20.6)</td>
<td>(4.8–19.6)</td>
<td>(4.8–24.6)</td>
<td></td>
</tr>
</tbody>
</table>

*Data expressed as mean ± SD and range. *R_{so} (Ω) – whole body resistance (ohm) measured at 50 kHz. *Skinfolds values presented in the table are double of the measurements that were acquired with the ultrasound.
Articles

Body composition of breastfed infants

Table 2. Associations between sample and reference distributions (P-values) established with one-sample Kolmogorov–Smirnov test
Reference
%FMa

Sex/age, Brook/
mob Lohman

Method

Brook/
Siri

Durnin/
Lohman

Durnin/
Siri

Johnston/ Johnston/
Lohman
Siri
Slaughter

US/4SFa US/4SF

US/4SF

US/4SF

US/4SF

US/4SF

Lingwood, Lingwood, Lingwood,
6 wk
3 mo
4.5 mo

Bocage

Fjeld

Kushner

US/2SFa

BISa

BIS

BIS

BIS

BIS

BIS

Butte, BFa (17)
31.5 ± 5.6

F/2

0.003

0.32c

<0.001

0.103

<0.001

0.004

<0.001

<0.001

<0.001

0.012

<0.001

<0.001

<0.001

30.2 ± 4.0

M/2

<0.001

0.16

<0.001

<0.001

<0.001

<0.001

0.005

<0.001

<0.001

<0.001

<0.001

<0.001

<0.001

32.0 ± 4.4

F/5

0.66

0.12

0.050

0.45

0.002

0.17

0.002

0.001

<0.001

0.84

<0.001

0.003

0.015

29.1 ± 4.7

M/5

0.28

0.23

<0.001

0.14

<0.001

0.052

0.19

0.065

<0.001

0.75

0.094

0.39

0.51

28.8 ± 5.0

F/9

0.96

0.32

0.15

0.27

0.002

0.49

0.034

0.55

<0.001

0.017

0.022

<0.001

<0.001

25.7 ± 5.2

M/9

0.52

0.033

0.028

0.66

0.014

0.41

0.90

0.29

<0.001

0.44

<0.001

<0.001

<0.001

27.6 ± 4.3

F/12

0.57

0.16

0.37

0.36

0.004

0.91

0.57

0.004

<0.001

0.021

0.007

<0.001

<0.001

25.6 ± 4.0

M/12

0.39

0.15

<0.001

0.42

<0.001

0.16

0.24

0.27

0.006

0.81

<0.001

<0.001

<0.001

Butte, Ba (15)
32.5 ± 6.2

F/2

0.002

0.16

<0.001

0.045

<0.001

0.002

<0.001

<0.001

<0.001

0.011

<0.001

<0.001

<0.001

31.7 ± 3.5

M/2

<0.001

0.014

<0.001

<0.001

<0.001

<0.001

<0.001

<0.001

<0.001

<0.001

<0.001

<0.001

<0.001

32.3 ± 4.4

F/5

0.59

0.16

0.039

0.52

0.001

0.13

<0.001

<0.001

<0.001

0.92

<0.001

0.001

0.009

31.1 ± 3.9

M/5

0.031

0.87

<0.001

0.008

<0.001

0.001

0.012

0.002

<0.001

0.23

0.003

0.053

0.096

27.8 ± 4.6

F/9

1.00

0.14

0.37

0.12

0.008

0.85

0.12

0.66

<0.001

0.003

0.004

<0.001

<0.001

27.8 ± 4.8

M/9

0.11

0.28

0.002

0.21

<0.001

0.078

0.37

0.023

<0.001

0.29

0.012

0.002

0.009

26.6 ± 4.7

F/12

0.66

0.11

0.67

0.17

0.025

0.97

0.90

0.023

<0.001

0.040

0.003

<0.001

<0.001

25.4 ± 3.8

M/12

0.43

0.11

<0.001

0.52

<0.001

0.20

0.20

0.32

0.007

0.79

<0.001

<0.001

<0.001

21.1 ± 4.4

F/2

0.004

<0.001

0.004

<0.001

0.24

<0.001

0.084

0.66

<0.001

0.45

0.32

0.23

0.49

19.9 ± 4.1

M/2

0.015

<0.001

0.21

0.002

0.038

0.015

0.004

0.24

<0.001

0.26

0.024

0.11

0.031

26.0 ± 4.3

F/5

0.048

<0.001

0.22

0.002

0.38

0.048

0.94

0.55

<0.001

<0.001

0.27

0.038

0.078

25.3 ± 3.9

M/5

0.76

<0.001

0.026

0.34

0.005

0.82

0.37

0.98

<0.001

0.20

0.13

0.084

0.49

Fomon (21)

25.0 ± 5.0

F/9

0.49

0.013

0.67

0.003

0.23

0.15

0.73

0.050

0.044

<0.001

<0.001

<0.001

<0.001

24.0 ± 5.2

M/9

0.66

0.004

0.082

0.24

0.033

0.61

0.42

0.70

0.005

0.21

<0.001

<0.001

<0.001

23.7 ± 3.7

F/12

0.25

0.004

0.61

0.002

0.56

0.12

0.39

0.19

0.017

0.12

<0.001

<0.001

<0.001

22.5 ± 3.6

M/12

0.77

0.004

0.036

0.31

0.005

0.86

0.034

0.47

0.46

0.20

<0.001

<0.001

<0.001

Fields (22)
24.0 ± 3.7

F/2

0.18

<0.001

0.16

<0.001

0.027

0.009

0.86

0.054

<0.001

0.48

0.22

0.33

0.013

22.7 ± 4.3

M/2

0.50

<0.001

0.003

0.12

<0.001

0.50

0.21

0.005

<0.001

0.010

0.18

0.20

0.002

27.7 ± 5.0

F/5

0.22

0.005

0.56

0.022

0.12

0.22

0.43

0.38

<0.001

0.013

0.11

0.24

0.35

26.2 ± 4.1

M/5

0.82

0.004

0.006

0.61

0.001

0.70

0.73

0.83

<0.001

0.47

0.32

0.24

0.88

22.4 ± 4.0

F/2

0.023

<0.001

0.020

<0.001

0.21

<0.001

0.35

0.29

<0.001

0.45

0.70

0.67

0.14

24.7 ± 4.0

M/2

0.27

0.006

<0.001

0.37

<0.001

0.094

0.94

<0.001

<0.001

<0.001

0.009

0.011

<0.001

29.5 ± 3.3

F/5

0.64

0.004

0.36

0.040

0.008

0.68

0.045

0.022

<0.001

0.060

0.005

0.18

0.54

27.5 ± 2.1

M/5

0.10

0.021

<0.001

0.19

<0.001

0.086

0.25

0.13

<0.001

0.42

0.85

0.59

0.55

23.0 ± 4.3

Both/2 0.078

<0.001

0.40

0.007

0.003

0.067

0.13

0.001

<0.001

0.043

0.076

0.11

<0.001

28.3 ± 4.1

Both/5

0.76

0.029

0.021

0.97

<0.001

0.56

0.13

0.601

<0.001

0.12

0.020

0.38

0.54

Both/5

0.95

0.011

0.058

0.83

<0.001

0.64

0.33

0.10

<0.001

0.07

0.050

0.28

0.61

Both/12

0.14

<0.001

0.11

0.018

0.001

0.15

0.012

0.43

<0.001

0.13

<0.001

<0.001

<0.001

30

17

22

24

10

27

27

22

2

24

12

16

13

Roggero (18)

Gilchrist (19)

Carberry (16)
27.8 ± 4.5
Bellu (20)
23.8 ± 3.1
Matches/36

B, breastfed; BF, breastfed and formula-fed; BIS, bioimpedance spectroscopy; %FM, percentage fat mass (%); US/2SF, ultrasound with two skinfolds; US/4SF, ultrasound with four skinfolds.
b
Time points presented here are time of measurements performed in this study and correspond with time points of most of the references with the exception of Butte, where infants
were measured not at 2 and 5, but at 3 and 6 mo.
c
Calculations values were considered not significantly different (bold font) from the references values at P > 0.018 for US skinfold measurements and P > 0.024 for bioelectrical
spectroscopy after adjusting for false discovery rate 40.
a

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Pediatric RESEARCH 3


Ag–AgCl gel electrodes (ImpediMed) were applied to the skin on the right hand and foot. Two distal current drive electrodes were placed on the dorsal surface of the hand and the foot, at the metacarpal-phalangeal and metatarsal-phalangeal joints; and two voltage sense electrodes were placed 3 cm proximal to the current electrodes. 3M Micropore surgical tape (3M Health Care, Neuss, Germany) was used to secure the electrodes in position. Electrodes were left on the limbs for the second series of measurements after the breastfeed. No direct contact was made with the infant’s skin during the measurements and insulating material (cloth) was used to ensure no contact occurred between the infant’s limbs or between the infant and the mother’s hands. Series of measurements (pre or postfeed) consisted of 10–50 consecutive measurements taken within 1–2 min with infants in supine position on a nonconductive surface.

Collected data were transferred to a computer and analyzed by fitting the measured resistance and reactance at each frequency to a Cole–Cole plot using Bioimp version 5.2.2.0 software (ImpediMed). All raw data were visually examined and measurements were analyzed with settings customized for infants to ensure goodness of fit as assessed by the standard error of the estimate as per Lingwood et al. (4): Td correction off; identification of the curve that most closely resembles a semicircle and setting the frequency range to cover this portion of the curve (upper/lower frequency limits of 1,000/12 kHz); rejection limit of 1%; achieving standard error of the estimate <1.0 and then applying Td correction of 0). Values of resistance (ohm) at frequency of 50 kHz (R50) were determined from the curve of best fit and averaged for analysis purposes. This frequency was chosen as R50 is commonly used to predict TBW in empirically derived prediction equations (14).

**US skinfold measurements.** Single measurements of SCCT were taken on the left side of the body with minimal compression. US scans of four anatomical sites (biceps, subscapular, suprailliac, and triceps)
were performed using the Aplio XG (Toshiba, Tokyo, Japan) machine and a high-resolution PLT-1204BX 14–8 MHz transducer (9). Sterile water-based Aquasonic 100 US transmission gel (Parker Laboratories, Fairfield, NJ) was placed between the probe and the infant’s skin to ensure penetration of the US beam, enhance imaging and to minimize tissue compression. The SCTT (skin thickness and the skin-fat interface to fat-muscle interface distance that were easily defined on the scan) was measured directly from images on the screen using electronic calipers (12) (Figure 1). All of the measurements were performed by one experienced sonographer (DG) with previously reported high intraobserver reliability (9).

US skinfold measurements were doubled for the use in skinfold equations developed for SCTT measurement with skinfold calipers (11).

### Equations and reference models

Equations and reference models were sourced from the literature. Equations for calculations of TBW, FFM, and %FM using resistance (R<sub>∞</sub>, Ω) and for calculations of body density and %FM using skinfold thickness (mm) measurements are presented in Table 6. The %FM value calculated with six BIS-based and seven skinfold-based equations were compared with eight reference values data sets for %FM, measured by various reference techniques (15–22).

All of the reference studies with the exception of Fomon (21) state that either all or a proportion of infants were breastfed. In Butter’s study (15) infants (n = 40) were exclusively breastfed for 4 mo with only 38% still breastfed at 12 mo. Infants in Bellu’s study (20) of 12 mo old infants (n = 26), were breastfed for at least 6 mo and were not at the time of the study. Infants in Roggero et al. (18) (n = 59), Fields et al. (22) (n = 160), and Gilchrist et al. (19) study (n = 80) were exclusively breastfed from birth to 6 mo at which point all three studies had ended. While infants (n = 30) in Carberry et al. study (16) were predominantly or exclusively breastfed, they were followed up to 4.5 mo only.

The broad variation in time points in the reference models made it difficult to compare both the references’ and our time points. Combining the available references ensured that there is sufficient BC data to establish mean ± SD values for several age/sex groups up to 12 mo.
Prediction equations were selected according to the following criteria: closest age match and predominantly Caucasian population. While it is recommended not to extrapolate from equations for older pediatric subjects to the early infant period, which is associated with chemical immaturity, it is not always possible to adapt even age-matched equations with the change in methodology as in case of using US to measure skinfolds. Therefore, several equations were tested and only few were chosen for further analysis. Those equations, not chosen produced erroneous results upon calculation or were discarded because of nonmatching variables.

Chosen BIA-based equations for TBW were established in infants and children of various ages (4, 23–25) then validated in healthy infants (4), as well as in well and malmournished children (25). Predicted TBW was converted to FFM using Butte et al. (17) age-appropriate hydration factors and %FM was further calculated.

Chosen equations based on caliper skinfolds for %FM and body density were established for use in children and adolescents (26–29); and evaluated in term and preterm infants (30). Predicted body density was converted to %FM using both Siri (31) and Lohman (32) equations.

Details of the equations are given in Table 6.

Statistical Analyses

Statistical analysis was carried out using R-software package (version 2.9.0 for Mac OSX; R Foundation for Statistical Computing, Vienna, Austria). The R-packages nlme (version 3.1–96), multcomp (33), ggplot2 (34) were used for linear mixed effect models, Tukey’s all pair comparisons, data exploration, and graphics respectively. Descriptive statistics are reported as mean ± SD (range), model parameters as estimate (95% CI). P-values < 0.05 were considered statistically significant except where an adjustment for multiple comparisons false discovery rate was performed (35). Analysis was carried out as overall, by age/sex groups and by sex within age.

Infants were measured once at either 2, 5, 9, and 12 mo as they were recruited for a larger study. Forty participants, 10 in each age group (to achieve a wider body mass spread), allowed for detection of an effect size of 0.5. When 40 infants had been recruited, an uneven gender split caused us to extend recruitment to achieve similar group sizes and gender balance. Using α = 0.05, final recruitment of 58 participants gave the study power of 0.94 to detect an effect size of 0.3 (36).

One-sample Kolmogorov–Smirnov (KS) tests (37) were used to compare the %FM data for each of the predictors with each of the eight reference distributions. Each age/sex group was compared with the most appropriate reference distributions. If no sex specific reference was available, pooled data were used. Where no SD was available in the reference model the SD was calculated as an average of SDs of the given age/sex groups of other reference models. The KS test requires that there be no ties in the data; 0.01 was added to one value when ties existed. P-values are not exact in those cases. Owing to the large number of comparisons, an adjustment for multiple comparisons false discovery rate was performed (35). The calculated values were considered not significantly different from the reference values at P > 0.018 for US skinfold measurements and P > 0.024 for BIS.

The number of matches between reference and sample distributions was classified as low (0–12 of possible 36 matches), moderate (13–24 matches) or high (25–36 matches).

Raw data (n = 58) was used for calculating averages to compare overall equations performances in the whole group. General linear hypothesis test (Tukey’s all pair comparisons) was used to check for systematic differences between the equations. Some overall analyses were repeated after removing two equations with < 1/3 of matches to the references.

CVs for each measurement were calculated from infants with data for all 13 equations (n = 45) omitting infants missing four skinfolds measurements (n = 13).

Linear mixed effects models with random intercept per participant were used to determine whether %FM measurements differed systematically by equation and infant sex and/or age group. Where there were more than two levels of categorical variable, Tukey’s all
pair comparisons were used to determine which levels differed. Possible sex differences between methods were tested with interaction between sex and equation.

RESULTS

Subjects

Infants were two (2.06±0.17, n = 13), five (5.08±0.22, n = 14), nine (9.17±0.35, n = 17) or 12 (12.34±0.23, n = 14) mo of age at the time of measurement. Of the 58 infants, 54 were measured with BIS prefeed and four postfeed (n = 58). At the earlier stage of data collection only two skinfolds (triceps and subscapular) were measured with US, resulting in two skinfold measures for n = 58 and four skinfolds measures for n = 45. Subjects’ characteristics are presented in Table 1.

Two 2 mo-old male infants were excluded from the study due to confirmed low maternal milk supply that could affect infant BC. One infant at the time of the visit was in second centile for weight and fifth for length according to WHO growth charts and had plateaued for 2 wk, later dropping to first centile for weight. The second infant was in fifth centile for both weight and length.

Comparison of Sample and Reference Distribution

Table 2 presents the probabilities that the sample distributions were drawn from each of the considered reference distributions. After an adjustment for false discovery rate values were considered not significantly different from the reference values at P > 0.018 for US skinfold measurements and P > 0.024 for BIS. Neither BIS nor US were consistent with distributions from appropriate reference values for all age/sex groups. A moderate number of matches (13–24 matches) with reference values were seen for both types of equations (three BIS-based and two US skinfold-based). High number of matches (25–36 matches) was predominantly seen for US skinfold-based equations (one BIS-based and four US skinfold-based).

% FM Calculated with BIS and US

Calculated %FM values displayed wide variation within and between US and BIS, with the degree of variation affected by both infant age and sex (Table 3). Distributions of %FM calculated with two skinfolds matched with 27/36 reference values; use of four skinfolds gave between 10 and 30 matches. Figure 2 shows comparative performance of all of the equations for each infant. Table 4 presents the performance of all BIS and US skinfold equations in the whole group of infants (raw data, n = 58). The Fjeld (BIS) and Johnston/Lohman (US) equations were excluded from further overall analysis as both were at the lower end of calculated %FM and had the lowest number of matches (<1/3) with the references.

Effect of Sex on BC

Overall, %FM was 2.0% (0.013, 3.9) lower in males (P = 0.036). When the equations were considered separately, significant sex differences were only seen for those based on four US skinfolds (P < 0.001 for all) with the endpoints of the 95% CIs between 2.00 and 7.08. Brook/Siri (P = 0.051), Brook/Lohman (P = 0.050), Slaughter (P = 0.16) and all BIS equations (P > 0.075)
Table 6. Published equations used in the study

<table>
<thead>
<tr>
<th>First author, y (reference)</th>
<th>Equation*</th>
<th>Age at measurement</th>
<th>Study groups: no. of infants</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bioelectrical impedance</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lingwood, 2012 (4)</td>
<td>$FFM^b = 1.248 + 0.584W^b - 0.1425^b + \frac{0.002L^b}{R_{50}^2}$</td>
<td>6wk old</td>
<td>54</td>
</tr>
<tr>
<td></td>
<td>$FFM = 1.458 + 0.498W - 0.1975 + \frac{0.067L^2}{R_{50}^2}$</td>
<td>3 mo old</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>$FFM = 2.203 + 0.334W - 0.361S + \frac{0.185L^2}{R_{50}^2}$</td>
<td>4.5 mo old</td>
<td>53</td>
</tr>
<tr>
<td>Bocage, 1988 (24)</td>
<td>$TBW^b = \left(0.418W + \frac{1936}{R_{50}^2} + 0.8649\right)\frac{L}{100}$</td>
<td>3–18 mo old</td>
<td>NA*</td>
</tr>
<tr>
<td>Fjeld, 1990 (25)</td>
<td>$TBW = 0.76 + \frac{0.18L^2}{R_{50}} + 0.39W$</td>
<td>3–30 mo old</td>
<td>30</td>
</tr>
<tr>
<td>Kushner, 1992 (23)</td>
<td>$TBW = \frac{0.593L^2}{R_{50}} + 0.065W + 0.04$</td>
<td>Prepubertal; preschool; and premature infants</td>
<td>37; 44; and 32</td>
</tr>
<tr>
<td></td>
<td>$FFM = TBW / HF^b$</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>%FM$^b = 100(W - FFM)/W$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Skinfolds</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Slaughter, 1988 (26)</td>
<td>Male %FM = 1.21\sum S^2 - 0.008\sum S^2 - 1.7</td>
<td>8–17 y old</td>
<td>66</td>
</tr>
<tr>
<td></td>
<td>Female %FM = 1.33\sum S - 0.013\sum S^2 - 2.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Durmin, 1967 (28)</td>
<td>Male $d = 1.1533 - 0.0643 \log(\sum SFT^b)$</td>
<td>12–16 y old</td>
<td>86</td>
</tr>
<tr>
<td></td>
<td>Female $d = 1.1369 - 0.0598 \log(\sum SFT)$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Johnston, 1988 (29)</td>
<td>Male $d = 1.166 - 0.07 \log(\sum SFT)$</td>
<td>8–14 y old</td>
<td>308</td>
</tr>
<tr>
<td></td>
<td>Female $d = 1.144 - 0.06 \log(\sum SFT)$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brook, 1971 (27)</td>
<td>Male $d = 1.1690 - 0.0788 \log(\sum SFT)$</td>
<td>1–11 y old</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>Female $d = 1.2063 - 0.0999 \log(\sum SFT)$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Siri, 1961 (31)</td>
<td>%FM = 100\left(\frac{4.95}{d} - 4.5\right)</td>
<td>Adult cadavers</td>
<td>NA</td>
</tr>
<tr>
<td>Lohman, 1984 (32)</td>
<td>%FM = 100\left(\frac{5.28}{d} - 4.89\right)</td>
<td>Prepubertal and pubertal children</td>
<td>NA</td>
</tr>
</tbody>
</table>

*Published equations used in the study for predicting body density, fat-free mass, total body water or percentage fat mass using bioelectrical impedance and skinfolds in pediatric subjects. Abbreviations and symbols in formulas: $d$, body density (kg/l); $FFM$, fat-free mass (kg); %FM, percentage fat mass (%); $HF$, age-appropriate hydration factors calculated from Butte et al. (17) data; $L$, body length (cm); NA, not available in the reference; $R_{50}$, impedance variable (Ω); $S$, skinfold variable (mm); $\sum SFT$, sum of four skinfolds, biceps, subscapular, suprailiac, and triceps (mm); TBW, total body water (kg); $W$, weight (kg).

**Effect of Age on BC**

Overall 2 mo-old infants had significantly lower %FM than the 5, 9, and 12 mo-olds ($P < 0.001$) with the differences between this group and each of the other three nearly of the same magnitude (4.6–5.5%) (Figure 2). In the detailed analysis, all BIS equations showed overall significant differences for age ($P \leq 0.001$) while all US skinfold equations showed no significant differences ($P > 0.34$). Looking at differences between the age groups all three Lingwood equations showed progressive increase in %FM between 2 and 5; 2 and 9; 2 and 12; 5 and 9; 5 and 12 mo ($P < 0.001$ for all) but not between 9 and 12 mo ($P > 0.88$). The Fjeld equation showed a progressive increase in %FM between 2 and 5; 2 and 9; 2 and 12 mo ($P < 0.001$), but %FM was not significantly different between 5 and 9; 5 and 12 mo; 9 and 12 mo ($P > 0.28$). The Bocage equation showed progressive increase in %FM between 2 and 5; 2 and 9 mo ($P < 0.001$); and 2 and 12 mo ($P = 0.034$), but %FM was not significantly different between 5 and 9; 5 and 12 mo; 9 and 12 mo ($P > 0.33$). The Kushner equation showed a progressive increase in %FM only between 2 and 5 ($P = 0.002$); and 2 and 9 mo ($P = 0.005$), but %FM was not significant between 2 and 12; 5 and 9; 5 and 12; and 9 and 12 mo ($P \geq 0.28$).
Body composition of breastfed infants

Comparisons Between the Equations

Table 5 presents a cluster map of statistical differences between the %FM values calculated with all BIS and US equations and demonstrates that certain groupings exist between the equations independent of the method.

DISCUSSION

With increasing pediatric obesity, the focus of the research is on associations between early nutrition, BC and risk of future disease. BC assessment during early infancy, when the window of opportunity for intervention still exists, is crucial and reference data, particularly for breastfed infants is desperately needed. Our findings indicate that both BIS and US are practical for calculating %FM in breastfed infants, but accuracy of prediction is highly dependent upon the use of validated age-matched prediction equations.

A limited number of BC reference data exist for infants during the first year of life, particularly for breastfed infants whose FFM is significantly lower and %FM is significantly higher compared with formula-fed infants (15). The major strength of our study was that all infants were breastfed at the time of measurements (to 12 mo); therefore this study describes the BC for infants fed according to WHO recommendations in the first year of life. We found that no single equation for calculation of %FM by BIS and US allowed a match to the references across the first 12 mo therefore should these methods be used, it is important to make an informed selection of equations that will match preferred references for both sampling time points and sex (Tables 2 and 3).

Overall, we found no difference between US and BIS calculations of %FM indicating that both methods are acceptable for measurements in the field. Further, there were more matches with reference values for US skinfold based equations (Table 2). Slaughter, a two skinfold method, had high level of matches with reference values without requiring additional calculations to determine %FM (Siri or Lohman), as required with four skinfold equations, making it a very attractive tool for large studies. If the four skinfold method is chosen, a greater number of matches with reference values were achieved using the Siri %FM equation vs Lohman %FM equation (Tables 2 and 6) with the exception of Brook/Lohman. The Lohman %FM equation is based on prepubertal and pubertal children BC (32), taking into account the lower bone-mineral and higher water content of this population, and was expected to provide more matches. The Siri %FM equation, while it is based on adult cadaver BC (31), is the most widely used in both adult and pediatric populations and in this study provided more matches overall. The Lohman equation often underestimated %FM, despite the fact that dermis and fat layers are not compressed with US, possibly making skinfold measurements larger than the caliper technique and resulting in lower body density and higher %FM (38).

Examination of BIS equations showed Kushner (23) and Bocage (24) calculations to have the highest number of matches with the reference values, which is similar to that of US skinfolds with Durnin/Siri and Durnin/Lohman ((Table 2). There is also a trend that the equations that showed the most matches also covered a larger number of reference studies. It is possible that greater variations of estimated values of Kushner and Bocage increase the chance of overlapping with multiple distributions. Equations should be chosen to be age matched to the study population otherwise erroneous results are obtained as demonstrated by the performance of the Lingwood 6 wk, 3 mo and 4.5 mo equations at 9 and 12 mo where no matches were seen with any of the reference studies (Tables 2 and 3).

When we compare all tested equations for calculation of %FM (irrespective of method) the majority gave significantly different values (Table 5). However, Lingwood 6 wk equation was most likely to give comparable results to the other equations. No clustering was noted for the type of the method indicating that most of the variation in calculated values lies in the equation.

Infant sex is believed to influence BC with males having lower %FM than females at some time points (16,17), although a number of studies have failed to show this (18–20) or have not analyzed for it (15,21,22). We showed that US (four skinfolds) was able to detect sex differences with the exception of Brook/Siri and Brook/Lohman yet BIS did not. The Bellu’s study (20) using total body electrical conductivity, which is based on similar principles as BIS did not show sex differences and therefore the similarity in technique might explain our BIS results. Results from air displacement plethysmography are mixed but Butte (17) used a multicomponent model, which is considered more accurate.

%FM changes in the first year of life with gradual increase in first 6 mo followed by gradual decrease to 12 mo (17,21). Age differences in %FM are mixed with some studies reporting an overall significant increase in %FM in the first 4 mo of life (16,18,22) or no change between 2 and 3; 4 or 5 mo (19); and detailed analysis between time points is not always available. In our study, only BIS were able to detect age differences and match them to literature. The Lingwood equations were the most sensitive to age producing statistically different values between most of the groups, with the other three BIS equations finding differences only between 2 mo and the remaining time points. The Bocage equation was established in 3–18 mo old infants but only detected age differences at 2 mo, as did Fjeld (3–30 mo old infants) and Kushner (premature infants to adults). The lack of age specific equations for skinfolds for the first 12 mo of life is an important part of why we were unable to detect age differences with US and further emphasizes the need for development of new multiple age specific equations covering the first year of life.
We excluded two infants based on their growth and low maternal milk production. In the first infant BIS and US agreed, with a best age/sex fitted equation for %FM for BIS (Lingwood, 3 mo) producing 11.1% and for US (Brook/Lohman) 17.5%. In the second infant, the measurements were disparate with 14.9%FM for BIS and 28.2%FM for US. Interestingly, %FM values based on US skinfolds were low in the first infant that plateaued for 2 wk before the visit, but were normally distributed for the second infant. The large discrepancy for second infant might be related to the reduction in FFM. This is similar to preterm infants that at the time of estimated arrival often have greater %FM and less FFM than newborn term infants, which is probably partially due to restricted nutrition in utero or in hospital environment (39). It is possible that combination of BIS and US is detecting this BC abnormality whereas US alone is not able to do so due to the very nature of the measurement technique.

Our results support the claim that BIS and US are useful methods of measuring of BC in infants. Skin thickness, subcutaneous fat thickness and %FM are valuable parameters for assessing and monitoring the nutritional state of the infant. US was introduced for this purpose (10) but currently not widely used. US is able to obviate some of the limitations of the caliper technique in the pediatric population such as age-related inter and intrasubject variation in skinfold compressibility, inability to palpate the fat-muscle interface or to differentiate the layers of skin, differences in the types of calipers used, and pain/trauma factor (38). Petersen et al. reported some degree of difficulty with identification of the dermis-subcutaneous fat interface in preterm infants’ skin compared with adult skin using US (10). We did not experience this difficulty, probably because Petersen measured distance between peaks on skin echogram rather than on an actual scan, and that infants in our study were term thus the interface could be more defined. Recently Pineau et al. has validated both US skinfold and BIS measurements in adults against dual-energy X-ray absorptiometry. In that study US has shown very good accuracy, while BIS has shown less accuracy than US but better than air displacement plethysmography (12). BIS predictions of %FM in infants improve with age compared with simple anthropometric measurements (4) and could benefit pediatric populations if more equations developed for infants after 5 mo of age.

The limitations of this study include the missing data of four skinfolds for 13 infants and, as a result, small numbers in particular sex/age groups; lack of available age-matched equations, necessitating running a full analysis of many equations; and limitation with resources for the study, resulting in absence of reference data for our subjects (notwithstanding the issues with existing reference methods in pediatric population).

In conclusion, high-frequency US measurement of SCTT is a precise and reliable method for assessment of %FM in breastfed infants. Accurate measurements of SCTT in a wide range of body masses are easily recorded, and the nontraumatic technique allows application of this method even in the smallest infants. BIS, on other hand presents some issues particularly in the earlier mo of life but its performance improves with infant age. While both methods will further benefit from the development of more precise age appropriate equations, a number of the current equations are practical for assessing %FM in breastfed infants, particularly in longitudinal studies. This study further emphasizes the critical need for development of sex/age specific normative BC values for assessment of the growth and nutritional status of breastfed infants during the first year of life.

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Leptin Levels Are Higher in Whole Compared to Skim Human Milk, Supporting a Cellular Contribution

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Abstract: Human milk (HM) contains a plethora of metabolic hormones, including leptin, which is thought to participate in the regulation of the appetite of the developing infant. Leptin in HM is derived from a combination of de novo mammary synthesis and transfer from the maternal serum. Moreover, leptin is partially lipophilic and is also present in HM cells. However, leptin has predominately been measured in skim HM, which contains neither fat nor cells. We optimised an enzyme-linked immunosorbent assay for leptin measurement in both whole and skim HM and compared leptin levels between both HM preparations collected from 61 lactating mothers. Whole HM leptin ranged from 0.2 to 1.47 ng/mL, whilst skim HM leptin ranged from 0.19 to 0.9 ng/mL. Whole HM contained, on average, 0.24 ± 0.01 ng/mL more leptin than skim HM (\( p < 0.0001, n = 287 \)). No association was found between whole HM leptin and fat content (\( p = 0.17, n = 287 \)), supporting a cellular contribution to HM leptin. No difference was found between pre- and post-feed samples (whole HM: \( p = 0.29 \), skim HM: \( p = 0.89 \)). These findings highlight the importance of optimising HM leptin measurement and assaying it in whole HM to accurately examine the amount of leptin received by the infant during breastfeeding.

Keywords: leptin; human milk; whole human milk; skim human milk; appetite; obesity

1. Introduction

Human milk (HM) is a heterogeneous fluid composed of a combination of macro- and micro-nutrients, cells, and a plethora of biomolecules that provide the necessary elements to sustain infant growth, protection and development [1–3]. The developmental effects of breastfeeding extend to the programming of various organs and systems of the newborn, including that of appetite regulation [4]. This early developmental programming results in a reduction in obesity and other metabolic diseases not only in the short-term, but also in adulthood [5–9]. The complex system of breastfeeding-mediated appetite regulation is attributable to various factors associated with the practice of breastfeeding, such as feeding on demand, but also potentially to a host of appetite regulatory molecules present in HM [4]. These include whey and casein proteins, HM oligosaccharides, and recently discovered in HM appetite regulatory hormones, including the well-documented adipokine leptin, adiponectin, and many others [10–14].

Amongst these appetite molecules, leptin is the most widely studied, being primarily known for promoting satiety and energy expenditure in adults through binding to the full length leptin receptor (ObRb) expressed on the arcuate nucleus of the hypothalamus [15]. In addition, leptin stimulates cell proliferation, regulates blood pressure, and is also involved in the T-cell immune response, thus displaying pleiotropic roles [16,17]. White adipose tissue is one of the main sources of serum...
leptin, secreting leptin proportionally to the number of white adipocytes present in the body [18]. Further, gastric chief cells, the placenta, and the mammary epithelium also synthesise and secrete leptin in adults [19–22].

In infants, HM is believed to be a major source of leptin early in life, with the endogenous leptin-synthesising mechanisms being still immature [23]. Leptin in HM has been hypothesised to be involved both in the short-term control of appetite and in developmental programming of appetite and energy-signalling pathways, promoting efficient energy control and storage throughout life [9,24]. Leptin administered during the first 14 days of life has been shown to act as a neurotrophic agent, promoting neural growth from the arcuate nucleus of the hypothalamus to additional appetite control centres located in the central nervous system [25]. HM leptin may provide short-term appetite control in the infant also by up-regulating circulating melanocortins, potent anorexigenic agents that promote satiety [26]. Leptin in HM is sourced both endogenously from the mammary gland and from the maternal serum, following secretion from white adipocytes and gastric chief cells into the bloodstream [9]. In the lactating mammary gland, serum-derived leptin combines with locally-synthesised leptin by the mammary epithelium to yield the total leptin content of HM [22].

Leptin in HM has been predominately measured in skim HM, which does not contain the cellular and fat components of HM [26–28]. Considering that the leptin peptide is capable of lipophilic interactions [29,30], it is plausible it may associate with the fat globule in whole HM. Moreover, HM cells, which are predominantly of epithelial origin in mature HM of healthy mother/infant dyads [1,31], are also thought to contribute to the leptin concentration of whole HM [22]. Few previous studies have measured leptin in whole HM using a radioimmuno-assay (RIA) [21,32]. However, RIA is not considered appropriate for measuring leptin in a lipid-rich medium such as whole HM due to interference of triglycerides with the binding of radioactive-labelled antigens to antibodies, which compromises the sensitivity of the assay [33,34]. Given the lack of an optimised assay to detect leptin in whole HM and the absence of reliable comparisons of leptin levels between whole and skim HM, we developed an enzyme-linked immunosorbent assay (ELISA) as a more appropriate means of measuring leptin in HM, with two antibodies assisting in immobilising the leptin antigen, and compared the leptin concentration between pre- and post-feed samples, as well as whole and skim HM.

2. Materials and Methods

2.1. Study Participants

All procedures involving the recruitment of lactating mothers and HM sample collection and analyses were approved by, and conducted in accordance with, the guidelines of the Human Research Ethics Committee of The University of Western Australia (ethics approval number RA/4/1/4253). All mothers provided informed written consent in the form of a secure online questionnaire that was administered and securely stored by The University of Western Australia. A single sample of whole HM expressed by a mother in her first month of lactation was used for optimisation of the leptin assay. Following assay optimisation, 61 lactating mothers (38 Caucasian, 23 non-Caucasian) with a mean maternal age of 33.6 ± 4.39 years, of full-term healthy infants were recruited to assess leptin differences between whole and skim HM (Table 1). HM samples (~5 mL) were obtained at approximately 1100 h aseptically, as previously described by Hassiotou et al. [35], from each breast before and after the infant fed from a single breast session either by using a Medela Symphony (Medela AG, Baar, Zug, Switzerland) breast pump or by hand expression. Samples were stored at −20 °C prior to analysis. Samples were collected at the second, fifth, ninth, and 12th months of lactation (Table 1).
<table>
<thead>
<tr>
<th>Stage of Lactation (Month)</th>
<th>2</th>
<th>5</th>
<th>9</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal age (years)</td>
<td>32.9±4.21 (28–40)</td>
<td>33.4±4.27 (24–40)</td>
<td>34.0±4.57 (25–43)</td>
<td>34.1±4.35 (26–44)</td>
</tr>
<tr>
<td>Maternal BMI</td>
<td>27.1±7.15 (20.1–38.5)</td>
<td>23.5±4.46 (18.0–35.2)</td>
<td>24.0±5.15 (18.7–37.2)</td>
<td>24.8±5.6 (18.2–34.6)</td>
</tr>
<tr>
<td>Parity</td>
<td>2.10±0.75 (1–4)</td>
<td>2.13±0.85 (1–4)</td>
<td>1.96±0.94 (1–4)</td>
<td>2.05±0.98 (1–4)</td>
</tr>
<tr>
<td>Infant sex (Male/Female)</td>
<td>12/9</td>
<td>16/16</td>
<td>17/13</td>
<td>12/12</td>
</tr>
<tr>
<td>Infant birth weight (kg)</td>
<td>3.58±0.64 (2.66–4.23)</td>
<td>3.49±0.45 (2.66–4.46)</td>
<td>3.49±0.46 (2.82–4.46)</td>
<td>3.59±0.46 (2.80–4.46)</td>
</tr>
<tr>
<td>Infant body length (cm)</td>
<td>57.6±2.17 (54.2–61.3)</td>
<td>64.5±2.09 (61.5–69.5)</td>
<td>70.9±2.11 (68.0–74.5)</td>
<td>73.9±2.38 (71.5–78.5)</td>
</tr>
</tbody>
</table>

BMI: body mass index.

2.2. Measurement of Leptin in Whole and Skim Human Milk by an Enzyme-Linked Immunosorbent Assay (ELISA)

Whole HM samples were thawed at room temperature, vortexed for 10 s and aliquoted (2 × 750 µL) into 1.5 mL microfuge tubes (Sarstedt, Numbrecht, Germany). One whole HM aliquot was centrifuged (05PR-22 Refrigerated Centrifuge, Hitachi, Tokyo, Japan) at 1500 × g for 10 min at 4°C and the resultant skim HM portion was aspirated. Both skim and whole HM aliquots were sonicated on ice at 100 Hz for three cycles of 5 s pulses, with a 20 s rest interval using an ultrasonic processor VCX130 (Sonics and Material, Newton, CT, USA). Eleven dilutions ranging from 1 to 50-fold were prepared from both milk preparations using 1% bovine serum albumin (BSA; Sigma-Aldrich, Castle Hill, NSW, Australia) in phosphate-buffered saline (PBS; Gibco Life Technologies, Paisley, Scotland).

Leptin concentration for each dilution was measured using the Human Leptin ELISA DuoSet (R&D Systems, Minneapolis, MN, USA). Capture antibody (4 ng/mL, diluted with PBS, pH 7.4) was pipetted (100 µL per well) to coat the bottom of the wells of flat bottom 96-well microtiter plates (Flow Laboratories, McLean, VA, USA). Plates were sealed and incubated overnight at room temperature. Wells were washed three times with PBS/Tween wash buffer (0.05% Tween 20; Bio-Rad Laboratories, Gladesville, NSW, Australia) in PBS, pH 7.4), dispensed at 400 µL per well, using a plate washer (Immunowash 1575, Bio-Rad Laboratories, Hercules, CA, USA). Washed plates were inverted and blotted against absorbent paper to ensure no remaining solution was present inside the wells. Blocking buffer (1% w/v BSA in PBS, pH 7.4) was added (300 µL per well) to block non-specific binding sites. Plates were sealed and incubated for one hour at room temperature. Blocking buffer was washed according to the wash procedure described earlier. Diluted samples and standards (0–0.9 ng/mL) were added (100 µL per well) in duplicates and plates were sealed and incubated for 2 h at room temperature. Unbound components from samples and standards were washed, and biotinylated detection antibody (4 ng/mL, diluted in 1% w/v BSA in PBS, pH 7.4) was added (100 µL per well). Plates were sealed and incubated for 2 h at room temperature. Unbound detection antibody was washed, and streptavidin-horseradish peroxidase (HRP; R&D Systems, Minneapolis, MN, USA) (50 ng/mL in PBS, pH 7.4) was added (100 µL per well), and plates were sealed, wrapped in aluminium foil to avoid exposure to direct light, and incubated for 20 min at room temperature. Streptavidin-HRP was washed and substrate colour reagent (1:1 mixture of 12 mL/vial hydrogen peroxide and 4 mL/vial enhanced luminol, R&D Systems, Minneapolis, MN, USA) was added (100 µL per well). Plates were sealed and wrapped in aluminium foil and were incubated for 20 min at room temperature. Sulphuric acid (1 M, R&D Systems, Minneapolis, MN, USA) stop solution was added (50 µL per well) and absorbance was read at 450 nm by a plate spectrophotometer (Enspire Multimode Plate Reader, Waltham, MA, USA). Standard curves and leptin concentrations were calculated using linear regression (Figures 1 and 2).
were placed in micro-haematocrit tubes, plugged with sealant and centrifuged at 12,000 × g for 10 min. Standards were selected according to previous literature investigating levels of leptin in skim human milk, as well as recommendations provided by the leptin kit manufacturer [36].

Recovery assays to discern the optimal dilution factor for leptin detection were conducted on dilutions reporting leptin concentrations within the range of the protein standards used (Table 2). Following optimisation of the dilution factor, leptin concentration in matched whole and skim HM samples from the study population was measured. All whole and skim HM samples were prepared according to the same centrifugation and sonication protocol used in the assay optimisation. Recovery of a known amount of the leptin protein when added to samples was 97.7% ± 9.7% (n = 10) (Table 2), with the leptin kit reporting an intra-assay variability of <5% and an inter-assay variability of <7.2%.

**Table 2.** Recovery percentages for each dilution factor for skim and whole human milk leptin measurement.

<table>
<thead>
<tr>
<th>Dilution Factor</th>
<th>Skim Human Milk Leptin (%)</th>
<th>Whole Human Milk Leptin (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>61.5 ± 2.09</td>
<td>17.1 ± 2.9</td>
</tr>
<tr>
<td>2</td>
<td>179.0 ± 0.82</td>
<td>14.0 ± 2.7</td>
</tr>
<tr>
<td>5</td>
<td>96.3 ± 1.2</td>
<td>14.0 ± 1.4</td>
</tr>
<tr>
<td>10</td>
<td>71.3 ± 1.6</td>
<td>97.1 ± 9.1</td>
</tr>
</tbody>
</table>

**2.3. Measurement of Fat Content in Human Milk**

The total fat content of HM samples was measured using the creamatocrit method [37,38]. Samples were placed in micro-haematocrit tubes, plugged with sealant and centrifuged at 12,000 × g for ten min.
in a micro-haematocrit centrifuge (Hermle Z230H Labortechnik, Wehingen, Germany). The resultant milk column was placed on the creamatocrit analyser (Creamatocrit Plus, Medela Inc., McHenry, IL, USA) and the length of the fat layer and the total milk column was measured, from which the total fat content was calculated. It has been shown that Creamatocrit measurements strongly correlate with the biochemical spectroscopic esterified fatty acid assay [38–40].

2.4. Statistical Analyses

Statistical analyses were performed using Microsoft Excel 2013 (Microsoft Corporation, Redmond, WA, USA) and R 2.9.10 (R Core Team, Vienna, Austria) [41] for Windows 10, with the additional R package “nlme” (R Core Team, Vienna, Austria) used for linear mixed effects modelling [42]. Student’s paired t-tests were conducted to assess leptin differences between matched skim and whole HM samples in the entire study population. Differences between whole and skim HM leptin concentrations were subsequently analysed within each month of breastfeeding, also using matched Student’s t-test.

Linear mixed effects modelling was used to examine any associations between HM fat content and whole HM leptin concentration. Responses were modelled with and without controlling for the volume of milk that had been removed from the breast during the collection of the HM sample. To discern the significant random effects to use for each statistical model analysing the association between HM fat content and whole HM leptin concentration, three separate models were devised; one linear model with no random effects and two linear mixed effects models with the following random effects: the effect of general inter-individual variation present in the study population, and the effect of the stage of lactation in addition to inter-individual variation. Analysis of variance (ANOVA) tests were then used to compare each model with the same fixed effects, namely fat content and fat content when controlled for volume of milk removed from the breast. The final model for the association between whole HM leptin concentration and fat content accounted for inter-individual variation and the stage of lactation as random effects when volume removed was not controlled for in the model. Similarly, when volume of milk removed from the breast was controlled for in the linear mixed effects models, inter-individual variation and stage of lactation were also considered as significant random variables to include in the analysis of associations between fat content and whole HM leptin concentration.

Similarly, the association between leptin concentration in whole HM samples and the corresponding fat content was also analysed within each month of lactation. Given that the volume of HM removed during feeding was only collected for 74 samples out of the entire study population, the liner mixed effects models used for intra-month analysis did not control for volume of milk removed from the breast. As with the analysis between the association of leptin levels in whole HM samples and fat content for the entire population, for each month, three statistical models were devised; one linear model with no random effects, and two linear mixed effects models with the following random variables included: the effect of inter-individual variation present in the study population and the effect of the stage of lactation in addition to inter-individual variation. Analysis of variance (ANOVA) was then used to compare each model within each month. For each month of breastfeeding, the only significant random effect found was general inter-individual variation. $p < 0.05$ was considered statistically significant. All values presented are mean ± standard deviation (SD), unless stated otherwise. All $R^2$ values were generated from the linear regression line of best fit equations.

3. Results

3.1. Participants

The demographic characteristics of mothers and infants in the study population are shown in Table 2. All infants (n = 61) were born at term, healthy, and were growing appropriately for their age according to the World Health Organisation’s (WHO) growth charts for exclusively-breastfed infants [43,44]. Mean maternal body mass index (BMI) was highest at 27.1 ± 7.15 kg/m$^2$ during the second month of lactation and lowest during the fifth month of lactation at 23.5 ± 4.46 kg/m$^2$ (Table 1). Compared to the second month of lactation, maternal BMI decreased by 2.30 ± 1.55 kg/m$^2$ over the first 12 months of breastfeeding ($p < 0.01$).
3.2. Leptin Optimisation

Measurement of leptin in whole and skim HM was optimised using an ELISA-based assay. One- to 20-fold dilutions for both skim and whole HM yielded leptin concentrations within the standard range of the assay (0–0.9 ng/mL), whilst dilutions above 20-fold reported values Redmond outside of the upper protein standard used (Figure 2). Mean leptin concentrations for 15-fold (whole HM: 0.8 ± 0.07 ng/mL, skim HM: 0.75 ± 0.09 ng/mL) and 20-fold (whole HM: 0.9 ± 0.11 ng/mL, skim HM: 0.9 ± 0.09 ng/mL) dilutions were close to the highest protein standard, thus further consideration was not given to these dilution factors (Figure 2). Ten-fold dilution of whole HM yielded the best recovery rates (97.7% ± 9.7%) (Table 2). Five-fold-diluted skim HM recovered 96.3% ± 1.2% of leptin (Table 2). Therefore, subsequent samples were diluted by 10-fold and five-fold with the diluent reagent for whole and skim HM samples, respectively, given these dilution factors recovered the highest percentage of leptin protein when the assay was performed for whole and skim HM.

3.3. Whole and Skim Human Milk Leptin

Leptin levels measured using the optimised assay were compared between whole and skim HM obtained during different stages of lactation from 61 lactating mothers. Whole HM leptin levels ranged from 0.2–1.47 ng/mL, whilst a 0.19–0.9 ng/mL range was obtained for skim HM leptin (Figure 3). Whole HM leptin was 0.24 ± 0.01 ng/mL higher than skim HM leptin across all samples (p < 0.0001, n = 287) (Figure 3). Leptin levels were also higher in whole HM compared to skim HM within each month of lactation (Table 3). Matched pre-feed whole HM samples contained 0.24 ± 0.07 ng/mL more leptin than pre-feed skim preparations (p < 0.01, n = 157), with 0.25 ng/mL ± 0.05 ng/mL more leptin measured in post-feed whole HM samples compared to paired skim post-feed aliquots (p < 0.01, n = 137).

![Figure 3](image)

**Figure 3.** Comparison between whole and skim human milk (HM) leptin concentration (n = 287). *** Indicates significant difference between matched whole and skim human milk leptin values (p < 0.001).

No association was observed between whole and skim HM leptin (p = 0.55, n = 287) (Figure 4a). HM fat content was not related to leptin concentration in whole HM when the volume of milk removed from the breast during sample collection was not accounted for (p = 0.52, n = 283) (Figure 4b) or accounted for (p = 0.24, n = 74) in the analysis. Further, no association between leptin levels in whole HM samples and fat content were found within each stage of lactation (Table 4).
Table 3. Leptin concentrations for whole and skim human milk at each month of lactation. Values are mean ± SD.

<table>
<thead>
<tr>
<th>Month of Lactation</th>
<th>Whole Human Milk Leptin (ng/mL)</th>
<th>Skim Human Milk Leptin (ng/mL)</th>
<th>p-Value *</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>0.50 ± 0.16</td>
<td>0.32 ± 0.16</td>
<td>p &lt; 0.0001</td>
</tr>
<tr>
<td>5</td>
<td>0.48 ± 0.16</td>
<td>0.26 ± 0.07</td>
<td>p &lt; 0.0001</td>
</tr>
<tr>
<td>9</td>
<td>0.56 ± 0.11</td>
<td>0.22 ± 0.03</td>
<td>p &lt; 0.0001</td>
</tr>
<tr>
<td>12</td>
<td>0.54 ± 0.14</td>
<td>0.21 ± 0.02</td>
<td>p &lt; 0.0001</td>
</tr>
</tbody>
</table>

* p-values indicate significant differences between whole and skim human milk leptin concentrations at given time points.

Figure 4. (a) Association between skim and whole human milk (HM) leptin (n = 287). No association was detected between skim and whole HM leptin levels in matched samples ($R^2 = 0.001, p = 0.552$); (b) no association was detected between fat content and leptin concentration in whole HM ($R^2 = 0.0004, p = 0.17, n = 284$). The solid black line is the line of best fit.

Table 4. Association between leptin levels in whole human milk and fat content at each stage of lactation.

<table>
<thead>
<tr>
<th>Month of Lactation</th>
<th>N (Samples)</th>
<th>$R^2$</th>
<th>p-Value *</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>66</td>
<td>0.0013</td>
<td>0.782</td>
</tr>
<tr>
<td>5</td>
<td>72</td>
<td>0.018</td>
<td>0.686</td>
</tr>
<tr>
<td>9</td>
<td>83</td>
<td>0.069</td>
<td>0.577</td>
</tr>
<tr>
<td>12</td>
<td>66</td>
<td>0.153</td>
<td>0.889</td>
</tr>
</tbody>
</table>

* p-values indicate absence of associations between whole human milk leptin concentrations and fat concentrations at given time points.

No differences between pre- and post-feed whole ($p = 0.29, n = 74$) and skim ($p = 0.89, n = 74$) leptin levels were detected after accounting for the volume of milk consumed by the infant during the session (Figure 5). Post-feed samples contained $36.2 ± 2.82$ g/L more fat compared to matched pre-feed samples ($p < 0.01, n = 74$).
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4. Discussion

This study has shown that whole HM contains significantly higher levels of leptin compared to skim HM and that sampling either before or after a breastfeed does not influence this level.

Leptin has been previously shown to be present in HM and has been hypothesised to participate in the short- and long-term regulation of appetite in the breastfed infant [9,45]. In addition, HM leptin may be involved in other functions in the breastfed infant given its known pleiotropic properties, and in mammary development [46,47]. Although many studies have previously measured leptin in HM, optimisation of the methodology has not been well documented and most studies have focused on the levels of leptin in skim HM. However, leptin has been proposed to have lipophilic properties [29] and is also synthesised by mammary epithelial cells [22], which comprise the majority of cells in mature HM from healthy mother/infant dyads [1,48,49]. It is, therefore, conceivable that leptin in HM is associated with the fat and/or cells, which are not present in its skim fraction, suggesting that previous measurements of skim HM leptin have underestimated the concentration of leptin in HM. In this study, we performed a comparison of leptin levels between whole and skim HM and optimised an ELISA assay to accurately measure it in both HM preparations.

Leptin levels were found to be, on average, two-fold higher in whole HM compared to skim HM (Figure 3, Table 3). There are limited studies that have compared whole and skim HM leptin levels and they utilized RIA based methodologies. While results of our study are in agreement with two previous studies that found higher levels of leptin in whole than skim HM, the actual values are different. Houseknecht et al. [32] reported whole HM leptin levels (10.1 ± 2.6 ng/mL, n = 23) that were approximately seven times higher than in skim HM (1.5 ± 0.87 ng/mL, n = 23) and, on average, 20 times higher than levels found in this study (Figure 3). Moreover, Smith-Kirwin et al. [21] reported 56-fold higher leptin levels in whole HM (73.2 ± 39.0 ng/mL, n = 8) compared to skim HM (approximately 1.3 ng/mL), and a 130-fold higher mean whole HM leptin concentration compared to the present study. For skim HM both studies observed leptin levels 1.5–2.5 times greater than those found here [21]. The higher absolute leptin levels measured in these studies may be attributable to the analysis. Both studies used the RIA technique, which is not as reliable as ELISA in measuring leptin levels in whole HM due to its known inaccuracies of immune-reactive antibodies binding to the epitopes of antigens suspended in a lipid-rich medium, such as whole HM, or of interference of iron and emulsifiers with the assay [33,34]. In addition, differences in the technique optimisation between the two previous studies are apparent, as the whole HM leptin level in one study is seven times greater than the other. Both commercially available RIA and ELISA kits are originally designed to measure leptin in serum, not in HM; therefore, optimisation is critical. On the other hand, our results are comparable to leptin levels in many other studies that measured it in skim HM using ELISA,
or whole and skim HM using RIA. ELISA in skim HM detected similar leptin levels (from 0.30 ± 0.04 ng/mL at 1 month to 0.10 ± 0.02 ng/mL at 12 months, n = 72) in a study by Bronsky et al. [50], (0.28 ± 0.38 ng/mL, n = 651) in a study by Weyermann et al. [27], and (0.16 ± 0.04 ng/mL, n = 28) in a study by Miralles et al. [26]. RIA in skim HM also detected close leptin levels: (0.18 ± 0.15 ng/mL, n = 23) in a study by Schuster et al. [28] and (1.00 ± 0.80 ng/mL, n = 13) in a study by Schueller et al. [31]. RIA results in whole HM (1.34 ± 0.14, n = 24) in a study by Bielicki et al. [52] were also comparable to our whole HM leptin levels.

Whilst it has been hypothesised that leptin may be associated with fat globules present in whole HM due to its lipophilic nature [21,29] this was not borne out in this study (Figure 4b, Table 4) despite analysing pre- and post-feeding milk samples with a wide range of fat content of 11.0–128.8 g/L. The lack of an association between HM fat content and whole HM leptin levels suggests that fat may not have a strong contribution to HM leptin levels compared to the cellular fraction of HM. Lactocytes, myoepithelial cells, and stem cells present in HM have been previously shown to express the leptin gene [22]. Additionally, flow cytometric analysis of HM cells has revealed that the majority of lactocytes and stem cells contain the leptin protein [22]. Given that lactocytes are the dominant cell type in mature whole HM when both the mother and infant are healthy [1,48,49,53,54], it is likely that lactocytes contribute significantly to HM leptin levels. Indeed, the cellular fraction of HM can constitute a significant portion of milk, comparable to its skim and fat fractions [1,31]. This warrants investigation to further discern the cellular contribution to leptin levels in whole HM, and emphasises the need to assay whole HM for leptin and, potentially, for other appetite hormones, to obtain accurate measurements of the levels of these hormones in HM. Importantly, the procedures of whole HM preparation for such measurements must enable complete lysis of the milk cells for accurate results. This is also very important during sample preparation for whole HM ELISA, achieved in the present study by sonication.

Higher leptin levels in whole HM compared to the skim fraction indicate that infants ingest a larger dose of leptin than that calculated from skim HM. Whilst HM cells likely contribute to the increased leptin level in whole HM the bioavailability of this source is unknown. However, we speculate that the process of digestion would release leptin proteins from HM cells. It is also possible that these cells are absorbed through the stomach mucosa after ingestion and enter the circulation, as has been confirmed with HM leukocytes and stem cells [35,55].

The lack of an association between whole HM leptin and milk fat content may be also attributable to the biochemical properties of the leptin peptide. Although paradoxical to the notion that leptin is synthesised by white adipocytes which exhibit a lipophilic nature, leptin may also consist of hydrophilic regions, enabling it to primarily interact with aqueous fluids. The specific hydrophilic regions exhibited on the leptin peptide are hypothesised to be conserved cysteine residues tethered to disulphide bridges [29,56], which may form polar bonds with water molecules, given sulphide’s strong electronegativity properties [57]. Leptin crystallization studies could further confirm its hydrophilic properties [56], providing insight into the lack of an association between the leptin protein and the fat component of whole HM.

Upon analysis, we also found no difference between the concentration of whole HM leptin in pre- and post-feed samples, indicating the small samples taken either pre- or post-feed provide the same levels of leptin despite differences in fat content. To measure the cellular contribution of leptin in HM, it may be possible to acquire larger pre- and post-feed sample volumes and examine the number of cells in the sample. Hassiotou et al. [1] has shown that cell content increases post-feed, as does fat, when larger sample volumes were attained or the breast was well drained of milk.

5. Conclusions

We describe the first standardised and optimised ELISA assay for the measurement of leptin in both skim and whole HM, demonstrating higher concentrations of leptin in whole HM preparations compared to skim HM samples. Further, we provide evidence supporting the lack of an association between the fat component of HM and its leptin content, suggesting a contribution of HM cells, which merits further investigation. Accurate analysis of whole HM leptin will assist in clarifying the
biological role of this milk component for the breastfed infant, improving our understanding of early developmental programming of appetite and its implications for obesity prevention later in life.

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**Author Contributions:** Sambavi Kugananthan designed the study, conducted experiments and data analyses, interpreted results, and wrote the manuscript; Ching Tat Lai conducted experiments and data analyses, and critically reviewed the manuscript; Zoya Gridneva conducted experiments, collected the data, and critically reviewed the manuscript; Peter J. Mark designed the study, interpreted results, and critically reviewed the manuscript; Donna T. Geddes designed the study, interpreted results, and critically reviewed the manuscript; Foteini Kakulas designed the study, interpreted results and critically reviewed the manuscript.

**Conflicts of Interest:** The authors declare that Medela AG provides an unrestricted research grant to Donna T. Geddes from which salaries to Foteini Kakulas, Donna T. Geddes and Ching Tat Lai are paid. Medela AG provided a Top-up Scholarship for Zoya Gridneva, and has provided speaker’s fees to Donna T. Geddes for educational lectures. The funding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

**Abbreviations**
The following abbreviations are used in this manuscript:

- HM: Human milk
- ELISA: Enzyme linked immunosorbent assay
- RIA: Radioimmunoassay
- PBS: Phosphate-buffered solution
- BMI: Body mass index
- SD: Standard deviation
- SEM: Standard error of the mean

**References**


Associations between Maternal Body Composition and Appetite Hormones and Macronutrients in Human Milk

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Abstract: Human milk (HM) appetite hormones and macronutrients may mediate satiety in breastfed infants. This study investigated associations between maternal adiposity and concentrations of HM leptin, adiponectin, protein and lactose, and whether these concentrations and the relationship between body mass index and percentage fat mass (%FM) in a breastfeeding population change over the first year of lactation. Lactating women (n = 59) provided milk samples (n = 283) at the 2nd, 5th, 9th and/or 12th month of lactation. Concentrations of leptin, adiponectin, total protein and lactose were measured. Maternal %FM was measured using bioimpedance spectroscopy. Higher maternal %FM was associated with higher leptin concentrations in both whole (0.006 ± 0.002 ng/mL, p = 0.008) and skim HM (0.005 ± 0.002 ng/mL, p = 0.007), and protein (0.16 ± 0.07 g/L, p = 0.028) concentrations. Adiponectin and lactose concentrations were not associated with %FM (0.01 ± 0.06 ng/mL, p = 0.81; 0.08 ± 0.11 g/L, p = 0.48, respectively). Whole milk concentrations of adiponectin and leptin did not differ significantly over the first year of lactation. These findings suggest that the level of maternal adiposity during lactation may influence the early appetite programming of breastfed infants by modulating concentrations of HM components.

Keywords: leptin; adiponectin; maternal body composition; percentage fat mass; lactation; human milk; breastfeeding; appetite hormones; macronutrients; protein; lactose

1. Introduction

Human milk (HM) is the optimal nutrition for term infants as it contains a uniquely balanced profile of macronutrients along with micronutrients, hormones, antibodies, bioactive molecules [1] and cells [2,3], which adequately support the nutritional needs, appropriate growth, immunoprotection and physiological development of the infant [4,5]. It is well documented that prolonged breastfeeding is associated with decreased prevalence of overweight and obesity across the life course [6,7]. Enhanced appetite control in adults who have been breastfed as infants has been partly attributed to regulatory appetite hormones present in HM [8–10], which include leptin and adiponectin [8,11]. In rat pups, acute or chronic administration of leptin by intraperitoneal injection has been shown not to reduce food consumption but to modulate the expression of neuropeptides and receptors involved in the regulation of feeding behaviour (neuropeptide Y (NPY) and proopiomelanocortin (POMC)) [12], while in mice pups leptin deficiency caused profound disruptions in the development of the projections of
the arcuate nucleus of the hypothalamus [13]. These findings indicate that leptin plays a neurotrophic role and contributes to the developmental programming of the hypothalamic appetite circuitry during the neonatal period, preceding leptin’s acute regulation of food intake in adults. Adiponectin has anti-inflammatory properties and improves both fatty acid metabolism and sensitivity to insulin [14]. In mice, adiponectin inhibits tension-sensitive gastric vagal afferent mechanosensitivity, modulating satiety signals in both lean and obese animals, while simultaneously increasing the mechanosensitivity of mucosal gastric vagal afferent in an obesity-induced model [15].

Additionally, the concentration of macronutrients in HM, namely protein, fat and lactose, may also be involved in regulation of the infant appetite control [9,16]. Differences in the concentrations of these factors may partly explain the variability in breastfeeding patterns observed in infants who feed on demand [17]. As such, an understanding of the factors that affect concentrations of appetite hormones and macronutrients in HM is critical, as it presents a unique opportunity for the prevention of unfavourable early developmental programming and subsequent obesity.

Leptin is secreted into the maternal circulation by white adipocytes and is subsequently transferred into the mammary ductal system via diffusion or a receptor-mediated transport mechanism [18]. Leptin is also contributed by lactocytes [19,20]. Previous studies have identified a positive correlation between maternal body mass index (BMI) and both maternal serum [21,22] and skim HM leptin [21–24], despite differing methodologies in leptin measurement [25]. However, no consistent relationship between maternal adiposity and HM leptin concentrations has been shown across lactation, with Miralles et al. (2006) reporting only moderate correlations between maternal BMI and HM leptin during the first 6 months of lactation ($R = 0.387; p < 0.01$) [8], while Bronsky et al. (2011) saw no consistent relationship over the first 12 months of lactation [11], despite utilizing a similar leptin measurement methodology.

There are a number of factors that may contribute to these conflicting findings. BMI is a poor measure of adiposity, as it fails to adequately differentiate between adipose tissue and lean body mass [26,27]. Investigations between the relationship of maternal BMI and leptin have been conducted predominantly in skim HM, which excludes the fat and cellular components of HM [21,24]. Further, leptin concentrations have been shown to be higher in whole HM compared to skim HM [20,23,28].

In contrast, maternal serum concentrations of adiponectin are lower if weight and BMI are higher [29,30]. Results associating maternal BMI and adiponectin concentrations in HM are conflicting [25], with several studies showing no associations [11,31,32] and two studies counter-intuitively reporting a positive association [33,34]. As with leptin, use of BMI as a measure of maternal adiposity may contribute to these conflicting findings. Also, both increasing [11,35] and decreasing [30,33,36] trends in adiponectin concentrations across the lactation period have been reported.

Similar to its effects on appetite hormones, it is postulated that maternal adiposity influences macronutrient concentrations in HM. Again, results are conflicting. Excessive adipose tissue storage has been shown to impair amino acid and monosaccharide metabolism and transport [37,38], yet increased serum amino acid levels were found in mothers with more adipose tissue [39]. In lactating women, higher concentrations of HM protein were associated with lower BMI in one study [40], yet with higher BMI [41] and higher adiposity [42] in others. BMI also was found to associate positively with concentration of HM galactose [41]. There is a possibility that the effect of maternal body composition (BC) only becomes evident in late but not early lactation, when the fat accumulated during pregnancy is depleted [42]. More precise measurements of maternal adiposity across the lactation period are needed to elucidate effects on HM composition.

This study investigated relationships between maternal adiposity and HM leptin, adiponectin, total protein and lactose. Further, it investigated the relationship between percentage fat mass (%FM) and BMI, and the change in maternal adiposity and component concentrations over the first year of lactation.
2. Materials and Methods

2.1. Study Participants

Fifty-nine predominantly Caucasian, English-speaking, breastfeeding mothers were recruited via the Australian Breastfeeding Association (ABA) and through external networking. Inclusion criteria were: healthy singletons, gestational age ≥ 37 weeks, fully breastfed at 2 and/or 5 months [43] and maternal intention to breastfeed until 12 months. The exclusion criterion was: maternal smoking. Participants were recruited during their 2nd, 5th, 9th and 12th month of lactation and invited to come back at any subsequent time points. Twenty-one participants contributed samples at two or more time points. All participants provided informed written consent and answered a secure online questionnaire that was administered and securely stored at the university. This study was approved by the Human Research Ethics Committee of The University of Western Australia (RA/4/1/4253) and registered with the Australian New Zealand Clinical Trials Registry (ACTRN12616000368437).

2.2. Human Milk Sample Collection

HM samples were collected on site at our research laboratory at King Edward Memorial Hospital for Women (Subiaco, Perth, Australia). Pre-feed and post-feed milk samples (~5 mL each) were obtained from the breast(s) the infant fed from by hand expression or with a breast pump and were analysed separately. Samples were collected between 9:30 and 11:30 a.m. to minimise possible circadian influences on the milk composition. Samples were stored at 20°C for later biochemical analysis.

2.3. Anthropometry and Body Composition

Maternal weight was measured using an electronic scale (±0.1 kg; Seca, Chino, CA, USA). Height was self-reported by participants or measured against a calibrated marked wall (accuracy ± 0.1 cm). BMI was calculated as kg/m².

Percentage fat mass (%FM) was measured with whole body bioimpedance (wrist to ankle) using an ImpediMed SFB7 tetra-polar bioelectrical impedance analyser (Impedimed, Brisbane, Australia) with the participant in a supine position on a non-conductive surface according to the manufacturer’s instructions. Before each session, the external calibration of the bioelectrical impedance analyser was tested with a calibration Test Cell (ImpediMed, Brisbane, Australia). Ten consecutive measurements of %FM were taken within 1–2 min and averaged. Within-participant coefficient of variation for maternal %FM was 0.21%. All measurements were made after the breastfeeding session.

2.4. Leptin and Adiponectin Measurements

Leptin concentrations in whole and skim HM were measured using the DuoSet Human Leptin enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Minneapolis, MN, USA) as described previously [28]. The detection limit was 0.05 ng/mL with a recovery of 96.3% ± 1.2% (n = 10) for skim milk and 97.1% ± 9.1% (n = 10) for whole milk leptin and an inter-assay coefficient of variation (CV) of <7.2%.

Adiponectin was measured in whole HM using the Biovendor Human Adiponectin Sandwich ELISA kit, (Life Technologies, Asheville, NC, USA). The detection limit was 1 ng/mL, with a recovery of 96.2% ± 3.2% (n = 10) and an inter-assay CV of <2.5%.

2.5. Protein and Lactose Measurements

Protein content was measured using the Bradford assay according to the methods of Mitoulas et al. [44]. The detection limit was 1.03 g/L, with a recovery of 97.2% ± 1.4% (n = 10) and an inter-assay CV of <1.9%.

Lactose concentration was measured using the enzymatic–spectrophotometric method outlined by Kuhn et al. [45] according to the methods of Mitoulas et al. [44]. The detection limit was 30 mM, with a recovery of 98.2% ± 4.1% (n = 10) and an inter-assay CV of <3.5%.
2.6. Statistical Analyses

Statistical analyses were performed using R 2.15.1 for Windows [46]. The packages nlme [47] and lattice [48], and RColorBrewer [49] were used for linear mixed effects modeling and data representation respectively. Descriptive statistics are reported as mean ± standard deviation (SD) and range unless otherwise stated; model parameters are presented as estimate ± standard error (SE).

In order to collect systematic information over time and to make better use of the collected data, a combined data approach that considers individual-level random effects to account for participants measured at two or more study sessions was adopted. We further contrasted the results from the combined data and from the longitudinal subset to confirm our findings.

During this study, infants were measured at least at one of the four time points (2, 5, 9 and 12 months postpartum). An approximate sample size was calculated using the 'Linear multiple regression: fixed model: $r^2$ increase' option in G*Power [50] as if this was a cross-sectional study with equal numbers at each time. Allowing four predictors (one main effect, three group contrasts), $\alpha = 0.05$ and 22 participants at each time point (88 sample points $= 22$ participants $\times 4$ time points) gave the study power of 0.80 to detect an effect size of 0.15. This approach was selected as there is no closed form expression suitable for the calculation of sample sizes for this research design [51], with the consideration that longitudinal study design is more powerful. To maintain predicted power and to address issues relating to missed visits, such as inability to attend due to illness and unwillingness of mothers approached at 2 months ($n = 8$) to commit to a study that requires breastfeeding to 12 months, the recruitment of participants continued past 22, resulting in 111 sessions for 59 (21 longitudinal, 38 cross-sectional) participants.

BC data at 9 months of lactation is missing for two longitudinal participants. Missing data also occurred for all milk components due to insufficient milk sample volumes. Missing data was dealt with using complete case (regression models) or available case (descriptive statistics) approaches. Milk samples were not pooled for biochemical analysis; thus, measures were not averaged. Sample sizes are presented in Table 1.

### Table 1. Sample sizes used in statistical analyses.

<table>
<thead>
<tr>
<th>Month of Lactation</th>
<th>2</th>
<th>5</th>
<th>9</th>
<th>12</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Participants *</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cross-sectional</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>38</td>
</tr>
<tr>
<td>Longitudinal</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>21</td>
</tr>
<tr>
<td>Total</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>59</td>
</tr>
<tr>
<td>Sessions</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cross-sectional</td>
<td>8</td>
<td>8</td>
<td>13</td>
<td>9</td>
<td>38</td>
</tr>
<tr>
<td>Longitudinal</td>
<td>15</td>
<td>21</td>
<td>19</td>
<td>18</td>
<td>73</td>
</tr>
<tr>
<td>Total</td>
<td>23</td>
<td>29</td>
<td>32</td>
<td>27</td>
<td>111</td>
</tr>
<tr>
<td>Samples (complete cases)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cross-sectional</td>
<td>19</td>
<td>21</td>
<td>33</td>
<td>27</td>
<td>100</td>
</tr>
<tr>
<td>Longitudinal</td>
<td>41</td>
<td>55</td>
<td>47</td>
<td>40</td>
<td>183</td>
</tr>
<tr>
<td>Total</td>
<td>60</td>
<td>76</td>
<td>80</td>
<td>67</td>
<td>283</td>
</tr>
<tr>
<td>Samples (available cases) **</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Whole milk adiponectin</td>
<td>66</td>
<td>79</td>
<td>86</td>
<td>72</td>
<td>303</td>
</tr>
<tr>
<td>Whole milk leptin</td>
<td>66</td>
<td>79</td>
<td>86</td>
<td>72</td>
<td>303</td>
</tr>
<tr>
<td>Skim milk leptin</td>
<td>62</td>
<td>77</td>
<td>85</td>
<td>71</td>
<td>295</td>
</tr>
<tr>
<td>Total protein</td>
<td>64</td>
<td>78</td>
<td>87</td>
<td>69</td>
<td>298</td>
</tr>
<tr>
<td>Lactose</td>
<td>65</td>
<td>78</td>
<td>86</td>
<td>67</td>
<td>296</td>
</tr>
</tbody>
</table>

* The number of participants at each time point is the same as the number of sessions, thus not specified in the table; ** The number of samples (including pre-feed and post-feed) analysed; this differs by component.

Linear models were used to investigate associations between maternal BMI or %FM (predictors) and each of the composition variables (responses), with and without controlling for month of lactation.
(four-level factor or linear predictor). Associations with month of lactation were assessed using omnibus F-tests and specific post-hoc tests comparing each of the subsequent time points with 2 months. Appropriate random effects were selected by comparing four models for each analysis using a likelihood ratio test. Models were (a) linear regression, and linear mixed effects models with random effects of one of: (b) effect of general inter-individual variation present in the study population; (c) effect of the month of lactation samples were collected at, in addition to inter-individual variation; and (d) the effect of pre- and post-feed samples along with inter-individual variation. Whether the overall effect of maternal adiposity on HM component concentrations differs by month of lactation was also investigated by including interactions between BMI/%FM and the month of lactation (factor only). To allow for realistic interpretation of the intercept values in the model outputs, maternal BMI and %FM have been centred at the upper bounds of the ‘healthy’ range (25 kg/m\(^2\) for BMI, and 33% for %FM) [52,53]. Where significant outlier values were identified from a kernel density plot, models were run with and without these values to determine how they might be influencing the findings.

An intercept-only linear mixed effects model was used to calculate the coefficient of variation for maternal %FM measurements (\(n = 10\), 10 measurements each).

3. Results

3.1. Participants

Participant adiposity measures and HM components’ concentrations are shown in Table 2. Mean maternal age was 33.4 ± 4.2 years and parity was 1.8 ± 0.8 at the start of the study. At the first session attended at either 2, 5, 9 or 12 months postpartum participants were classified as being underweight (BMI < 18.5, 5%, n = 3; %FM < 21, 7%, n = 4), of normal weight (BMI 18.5–24.9, 54%, n = 32; %FM 21–32.9, 50%, n = 29), overweight (BMI 25–29.9, 24%, n = 14; %FM 33–38.9, 28%, n = 16) or obese (BMI > 30, 17%, n = 10; %FM > 39, 15%, n = 9) [53]. Infant male/female ratio was 33/26.

Table 2. Maternal adiposity and human milk components concentrations presented at the months after birth for combined subset (\(n = 59\)) expressed as mean ± standard deviation (SD, range).

<table>
<thead>
<tr>
<th>Month of Lactation</th>
<th>2 (n = 23)</th>
<th>5 (n = 29)</th>
<th>9 (n = 32)</th>
<th>12 (n = 27)</th>
<th>Total (n = 111)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal BMI a</td>
<td>27.0 ± 7.3 (20.1–51.3)</td>
<td>23.5 ± 4.5 (17.0–35.2)</td>
<td>23.9 ± 5.2 (16.9–37.2)</td>
<td>24.4 ± 5.5 (18.2–37.2)</td>
<td>24.6 ± 5.7 (16.9–51.3)</td>
</tr>
<tr>
<td>Maternal fat mass (%)</td>
<td>34.9 ± 6.4 (19.6–49.3)</td>
<td>32.5 ± 6.0 (23.2–47.2)</td>
<td>30.9 ± 7.9 (16.7–47.9)</td>
<td>31.3 ± 7.2 (19.4–45.3)</td>
<td>32.3 ± 7.0 (16.7–49.3)</td>
</tr>
<tr>
<td>Whole milk leptin (ng/mL)</td>
<td>0.55 ± 0.29 (0.21–2.24)</td>
<td>0.50 ± 0.17 (0.20–0.85)</td>
<td>0.53 ± 0.15 (0.21–0.99)</td>
<td>0.54 ± 0.13 (0.24–0.89)</td>
<td>0.53 ± 0.19 (0.20–2.24)</td>
</tr>
<tr>
<td>Skim milk leptin (ng/mL)</td>
<td>0.34 ± 0.21 (0.19–1.46)</td>
<td>0.27 ± 0.07 (0.20–0.48)</td>
<td>0.26 ± 0.09 (0.19–0.76)</td>
<td>0.26 ± 0.08 (0.19–0.54)</td>
<td>0.28 ± 0.12 (0.19–1.46)</td>
</tr>
<tr>
<td>Total protein (g/L)</td>
<td>12.94 ± 6.15 (6.54–31.51)</td>
<td>11.7 ± 5.70 (7.00–34.76)</td>
<td>10.83 ± 4.63 (3.32–29.69)</td>
<td>12.83 ± 6.74 (6.60–36.89)</td>
<td>11.96 ± 5.82 (3.32–36.89)</td>
</tr>
<tr>
<td>Lactose (g/L)</td>
<td>67.54 ± 9.05 (50.35–89.06)</td>
<td>68.07 ± 8.10 (50.92–110.07)</td>
<td>68.37 ± 8.83 (51.81–100.05)</td>
<td>69.70 ± 9.11 (51.00–98.36)</td>
<td>68.41 ± 8.75 (50.35–110.07)</td>
</tr>
</tbody>
</table>

Data are mean ± SD and ranges. Concentrations of components are measured in both pre- and post-feed milk samples. a BMI—body mass index.
3.2. Changes in Components’ Concentration with Feeding (Pre- and Post-Feed)

HM component concentrations did not differ between pre-feed and post-feed samples in univariate models or after accounting for the month of lactation as a linear effect model or as a factor (Table 3).

Table 3. Relative concentrations of pre-feed human milk samples compared to post-feed samples with and without accounting for possible month of lactation effects.

<table>
<thead>
<tr>
<th>Predictor</th>
<th>Univariate a</th>
<th>Accounting for Month of Lactation (Linear) b</th>
<th>Accounting for Month of Lactation (Factor) b</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PE ± SE</td>
<td>p</td>
<td>PE ± SE</td>
</tr>
<tr>
<td>Adiponectin (ng/mL)</td>
<td>0.45 ± 0.42</td>
<td>0.29</td>
<td>0.45 ± 0.42</td>
</tr>
<tr>
<td>Leptin (ng/mL)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Whole milk</td>
<td>-0.008 ± 0.016</td>
<td>0.65</td>
<td>-0.008 ± 0.017</td>
</tr>
<tr>
<td>Skim milk</td>
<td>-0.002 ± 0.009</td>
<td>0.84</td>
<td>-0.002 ± 0.009</td>
</tr>
<tr>
<td>Protein (g/L)</td>
<td>-0.14 ± 0.32</td>
<td>0.68</td>
<td>-0.14 ± 0.32</td>
</tr>
<tr>
<td>Lactose (g/L)</td>
<td>-0.36 ± 0.91</td>
<td>0.69</td>
<td>-0.38 ± 0.88</td>
</tr>
</tbody>
</table>

Data are parameter estimate ± SE. Analyses were run on pre- and post-feed samples using complete case approach. a Effects of predictors taken from univariate linear mixed effects models; b Effects of predictors taken from linear mixed effects models that accounted for the month of lactation as linear main effect or as a factor; c Month of lactation is significant (p < 0.036). PE—parameter estimate; SE—standard error.

3.3. Associations in Combined Subset

3.3.1. Differences in Concentrations of Human Milk Components at Different Months of Lactation

Table 4 presents the changes in HM components’ concentrations in the combined subset (n = 57) at four time points during first 12 months of lactation.

While component concentrations differed by the month of lactation within participants for all components (lactose: p = 0.031; adiponectin, whole and skim milk leptin, protein: p < 0.001), no consistent month of lactation-related patterns were seen for whole milk leptin (p > 0.47), protein (p > 0.37) and lactose (p > 0.26).

Skim milk leptin decreased non-linearly over the months of lactation (univariate: p = 0.024). Post-hoc tests showed that adiponectin concentration at 9 months was -2.27 ± 0.88 ng/mL lower (p = 0.013) than that at 2 months of lactation (univariate: p = 0.042) (Table 4).

Table 4. Associations between human milk appetite hormones and maternal adiposity. Values are parameter estimates ± standard error (n = 57). Some participants (n = 21) contributed milk samples at multiple time points.

<table>
<thead>
<tr>
<th>Predictor</th>
<th>Adiponectin (ng/mL)</th>
<th>Whole Milk Leptin (ng/mL)</th>
<th>Skim Milk Leptin (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PE ± SE</td>
<td>p</td>
<td>PE ± SE</td>
</tr>
<tr>
<td>BMI</td>
<td>0.10 ± 0.07</td>
<td>0.17</td>
<td>0.01 ± 0.003</td>
</tr>
<tr>
<td>%FM</td>
<td>0.01 ± 0.06</td>
<td>0.81</td>
<td>0.006 ± 0.002</td>
</tr>
<tr>
<td>Month d</td>
<td>-</td>
<td>0.042</td>
<td>-</td>
</tr>
<tr>
<td>Intercept</td>
<td>10.58 ± 0.71</td>
<td>0.05 ± 0.03</td>
<td>-</td>
</tr>
<tr>
<td>5 e</td>
<td>-1.39 ± 0.87</td>
<td>0.12</td>
<td>-0.06 ± 0.05</td>
</tr>
<tr>
<td>9 e</td>
<td>-2.27 ± 0.88</td>
<td>0.013</td>
<td>-0.01 ± 0.05</td>
</tr>
<tr>
<td>12 e</td>
<td>-0.26 ± 0.93</td>
<td>0.78</td>
<td>-0.02 ± 0.05</td>
</tr>
</tbody>
</table>

Adjusted model for %FM (month of lactation as linear main effect) c

<table>
<thead>
<tr>
<th>Predictor</th>
<th>PE ± SE</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMI</td>
<td>9.76 ± 0.61</td>
<td>0.52 ± 0.03</td>
</tr>
<tr>
<td>%FM</td>
<td>0.008 ± 0.06</td>
<td>0.006 ± 0.002</td>
</tr>
<tr>
<td>Month d</td>
<td>-0.05 ± 0.09</td>
<td>0.32 ± 0.02</td>
</tr>
</tbody>
</table>

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### Table 4. Cont.

<table>
<thead>
<tr>
<th>Predictor</th>
<th>Adiponectin (ng/mL)</th>
<th>Whole Milk Leptin (ng/mL)</th>
<th>Skim Milk Leptin (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PE ± SE</td>
<td>p</td>
<td>PE ± SE</td>
</tr>
<tr>
<td>Intercept</td>
<td>10.59 ± 0.72</td>
<td>-</td>
<td>0.54 ± 0.03</td>
</tr>
<tr>
<td>%FM</td>
<td>-0.01 ± 0.06</td>
<td>0.86</td>
<td>0.006 ± 0.002</td>
</tr>
<tr>
<td>Month d</td>
<td>0.044</td>
<td>-</td>
<td>0.51</td>
</tr>
<tr>
<td>5 e</td>
<td>-1.40 ± 0.88</td>
<td>0.12</td>
<td>-0.04 ± 0.04</td>
</tr>
<tr>
<td>9 e</td>
<td>-2.30 ± 0.90</td>
<td>0.014</td>
<td>0.01 ± 0.05</td>
</tr>
<tr>
<td>12 e</td>
<td>-0.29 ± 0.95</td>
<td>0.77</td>
<td>0.007 ± 0.05</td>
</tr>
</tbody>
</table>

**Adjusted model for %FM (month of lactation as a factor)**

<table>
<thead>
<tr>
<th>Predictor</th>
<th>Adiponectin (ng/mL)</th>
<th>Whole Milk Leptin (ng/mL)</th>
<th>Skim Milk Leptin (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>9.76 ± 0.60</td>
<td>-</td>
<td>0.52 ± 0.03</td>
</tr>
<tr>
<td>BMI</td>
<td>0.09 ± 0.07</td>
<td>0.19</td>
<td>0.01 ± 0.003</td>
</tr>
<tr>
<td>Month d</td>
<td>-0.04 ± 0.09</td>
<td>0.62</td>
<td>0.002 ± 0.004</td>
</tr>
</tbody>
</table>

**Adjusted model for BMI (month of lactation as linear main effect)**

<table>
<thead>
<tr>
<th>Predictor</th>
<th>Adiponectin (ng/mL)</th>
<th>Whole Milk Leptin (ng/mL)</th>
<th>Skim Milk Leptin (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>10.51 ± 0.71</td>
<td>-</td>
<td>0.54 ± 0.03</td>
</tr>
<tr>
<td>BMI</td>
<td>0.07 ± 0.07</td>
<td>0.33</td>
<td>0.01 ± 0.003</td>
</tr>
<tr>
<td>Month d</td>
<td>-0.063</td>
<td>-</td>
<td>0.74</td>
</tr>
<tr>
<td>5 e</td>
<td>-1.23 ± 0.89</td>
<td>0.17</td>
<td>-0.03 ± 0.04</td>
</tr>
<tr>
<td>9 e</td>
<td>-2.13 ± 0.89</td>
<td>0.021</td>
<td>0.01 ± 0.04</td>
</tr>
<tr>
<td>12 e</td>
<td>-0.16 ± 0.94</td>
<td>0.87</td>
<td>0.004 ± 0.05</td>
</tr>
</tbody>
</table>

**Adjusted model for %FM (month of lactation as a factor)**

Data are parameter estimate ± SE. Analyses were run on pre- and post-feed samples using complete case approach.

- Significant p-values are in bold font
- Effects of predictors taken from univariate linear mixed effects models
- Effects of predictors taken from linear mixed effects models that accounted for the month of lactation as linear main effect or as a factor
- Omnibus F-test
- Post-hoc test with reference 2 months

3.3.2. Associations between Maternal Adiposity and HM Leptin

Table 4 presents associations between adiposity and HM components’ concentrations seen in the combined subset (n = 57) at four time points during first 12 months of lactation.

Associations between Maternal Adiposity and HM Leptin

Higher %FM and BMI were associated with higher concentrations of both whole (Figure 1a,b) and skim milk (Figure 2a,b) leptin (Table 4). Accounting for the month of lactation as a main linear effect or as a factor did not change the associations with %FM and BMI for whole and skim milk leptin. Significant negative interactions were seen between %FM and the month of lactation for whole milk leptin, (2 m: reference; 5 m: −0.02 ± 0.01, p = 0.023; 9 m: −0.02 ± 0.01, p = 0.003; 12 m: −0.03 ± 0.01, p < 0.001; month of lactation as a factor: p = 0.008), and for skim milk leptin (2 m: reference; 5 m: −0.02 ± 0.01, p < 0.001; 9 m: −0.02 ± 0.01, p < 0.001; 12 m: −0.02 ± 0.01, p = 0.002; month of lactation as a factor: p < 0.001); and also between BMI and the month of lactation for whole milk leptin, (2 m: reference; 5 m: −0.02 ± 0.01, p = 0.026; 9 m: −0.03 ± 0.01, p < 0.001; 12 m: −0.03 ± 0.01, p < 0.001; month of lactation as a factor: p < 0.001), and for skim milk leptin (2 m: reference; 5 m: −0.02 ± 0.01, p < 0.001; 9 m: −0.02 ± 0.01, p < 0.001; 12 m: −0.01 ± 0.01, p = 0.005; month of lactation as a factor: p = 0.001), indicating that the association between adiposity and leptin weakens over the first 12 months of lactation.

Removing statistically significant outliers resulted in either weakening or an absence of the association between either %FM or BMI and whole and skim milk leptin in the univariate models (%FM: 0.004 ± 0.002 ng/mL, p = 0.066; 0.003 ± 0.001 ng/mL, p = 0.043, respectively; BMI: 0.004 ± 0.002 ng/mL, p = 0.065; 0.004 ± 0.002 ng/mL, p = 0.066, respectively) and after accounting for the month of lactation as a linear effect (%FM: 0.004 ± 0.002 ng/mL, p = 0.039, age: p = 0.17; 0.002 ± 0.001 ng/mL, p = 0.12, age: p = 0.030, respectively; BMI: 0.005 ± 0.002 ng/mL, p = 0.053, age: p = 0.25; 0.004 ± 0.002 ng/mL, p = 0.039, age: p = 0.17, respectively) or as a factor (%FM:
0.004 ± 0.002 ng/mL, p = 0.044, age: p = 0.37; 0.002 ± 0.001 ng/mL, p = 0.14, age: p = 0.15, respectively; BMI: 0.004 ± 0.002 ng/mL, p = 0.072, age: p = 0.53; 0.004 ± 0.002 ng/mL, p = 0.044, age: p = 0.37, respectively). No interaction between either %FM or BMI and the month of lactation as a factor was seen (%FM: whole milk leptin: p = 0.37; skim milk leptin: p = 0.13; BMI: whole milk leptin: p = 0.24; skim milk leptin: p = 0.18).

Figure 1. Associations between whole human milk (HM) leptin and (a) maternal percentage fat mass; and (b) maternal BMI. Combined subset data points (measured in pre- and post-feed samples) are shown as cross-sectional (pale blue) and longitudinal (dark blue). Lines are fixed effects from univariate linear mixed effect models: pale blue dotted line—combined cohort (Table 4); medium blue dashed line—combined cohort with outliers removed (Section 3.3.2); solid dark blue line—longitudinal cohort (Table S1).

Figure 2. Associations between skim human milk (HM) leptin and (a) maternal percentage fat mass; and (b) maternal BMI. Combined subset data points are shown as cross-sectional (pale purple) and longitudinal (dark purple). Lines are fixed effects from univariate linear mixed effect models: pale purple dotted line—combined cohort (Table 4); medium purple dashed line—combined cohort with outliers removed (Section 3.3.2); solid dark purple line—longitudinal cohort (Table S1).
3.3.3. Associations between Maternal Adiposity and HM Adiponectin

HM adiponectin was not significantly associated with either %FM or BMI in the univariate models (Table 4) or after accounting for the month of lactation. No interactions were seen between the month of lactation as a factor and either %FM ($p = 0.51$) or BMI ($p = 0.62$).

Removing a statistically significant outlier did not change the conclusion (%FM: $p = 0.50$; BMI: $p = 0.083$) and no interaction between the month of lactation and either %FM ($p = 0.54$) or BMI ($p = 0.081$) was seen.

3.3.4. Associations between Maternal Adiposity and HM Protein

Higher %FM was associated with higher concentrations of protein in HM in univariate model (Table 5; Figure 3a). Accounting for the month of lactation made the association between %FM and protein concentrations weaker but still significant. BMI was not associated with concentrations of protein in HM in the univariate model (Figure 3b) or after accounting for the month of lactation. No interaction with the month of lactation as a factor was seen for both %FM ($p = 0.21$) and BMI ($p = 0.16$).

Table 5. Associations between human milk macronutrients and maternal adiposity. Values are parameter estimates ± standard error ($n = 57$). Some participants ($n = 21$) contributed milk samples at multiple time points.

<table>
<thead>
<tr>
<th>Predictor</th>
<th>Lactose (g/L)</th>
<th>Protein (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PE ± SE</td>
<td>$p$</td>
</tr>
<tr>
<td><strong>Univariate models</strong> $^b$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMI</td>
<td>0.06 ± 0.14</td>
<td>0.66</td>
</tr>
<tr>
<td>%FM</td>
<td>0.08 ± 0.11</td>
<td>0.48</td>
</tr>
<tr>
<td>Month $^d$</td>
<td>-</td>
<td>0.65</td>
</tr>
<tr>
<td>Intercept</td>
<td>67.32 ± 1.43</td>
<td>-</td>
</tr>
<tr>
<td>5 $^e$</td>
<td>0.56 ± 1.89</td>
<td>0.77</td>
</tr>
<tr>
<td>9 $^e$</td>
<td>1.31 ± 1.88</td>
<td>0.49</td>
</tr>
<tr>
<td>12 $^e$</td>
<td>2.35 ± 1.97</td>
<td>0.24</td>
</tr>
<tr>
<td><strong>Adjusted model for %FM (month of lactation as linear main effect)</strong> $^c$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intercept</td>
<td>68.89 ± 1.13</td>
<td>-</td>
</tr>
<tr>
<td>%FM</td>
<td>0.09 ± 0.11</td>
<td>0.43</td>
</tr>
<tr>
<td>Month $^d$</td>
<td>0.08 ± 0.15</td>
<td>0.59</td>
</tr>
<tr>
<td><strong>Adjusted model for %FM (month of lactation as a factor)</strong> $^c$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intercept</td>
<td>67.58 ± 1.44</td>
<td>-</td>
</tr>
<tr>
<td>%FM</td>
<td>0.06 ± 0.11</td>
<td>0.55</td>
</tr>
<tr>
<td>Month $^d$</td>
<td>-</td>
<td>0.67</td>
</tr>
<tr>
<td>5 $^e$</td>
<td>0.60 ± 1.85</td>
<td>0.75</td>
</tr>
<tr>
<td>9 $^e$</td>
<td>1.37 ± 1.87</td>
<td>0.50</td>
</tr>
<tr>
<td>12 $^e$</td>
<td>2.27 ± 1.96</td>
<td>0.25</td>
</tr>
<tr>
<td><strong>Adjusted model for BMI (month of lactation as linear main effect)</strong> $^c$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intercept</td>
<td>67.71 ± 2.92</td>
<td>-</td>
</tr>
<tr>
<td>BMI</td>
<td>0.06 ± 0.13</td>
<td>0.63</td>
</tr>
<tr>
<td>Month $^d$</td>
<td>0.20 ± 0.17</td>
<td>0.26</td>
</tr>
<tr>
<td><strong>Adjusted model for BMI (month of lactation as a factor)</strong> $^c$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intercept</td>
<td>67.55 ± 1.44</td>
<td>-</td>
</tr>
<tr>
<td>BMI</td>
<td>0.06 ± 0.13</td>
<td>0.66</td>
</tr>
<tr>
<td>Month $^d$</td>
<td>-</td>
<td>0.69</td>
</tr>
<tr>
<td>5 $^e$</td>
<td>0.63 ± 1.88</td>
<td>0.74</td>
</tr>
<tr>
<td>9 $^e$</td>
<td>1.29 ± 1.87</td>
<td>0.49</td>
</tr>
<tr>
<td>12 $^e$</td>
<td>2.22 ± 1.95</td>
<td>0.26</td>
</tr>
</tbody>
</table>

Data are parameter estimate ± SE. Analyses were run on pre- and post-feed samples using complete case approach. $^a$ Significant $p$-values are in bold font; $^b$ Effects of predictors taken from univariate linear mixed effects models; $^c$ Effects of predictors taken from linear mixed effects models that accounted for the month of lactation as linear main effect or as a factor; $^d$ Omnibus F-test; $^e$ Post-hoc test with reference 2 months. Abbreviations: BMI—body mass index; %FM—percentage fat mass; PE—parameter estimate; SE—standard error.
FM and lactose strengthens over the first 12 months of lactation.

Table longitudinal subset, with a one-unit increase in BMI associated with a 1.07% increase in leptin, 0.32% increase in adiponectin, and 0.30% increase in whole milk leptin. Male BMI difference was 0.58 ± 0.31, p = 0.068; month of lactation as a factor: p = 0.029), indicating that association between %FM and lactose strengthens over the first 12 months of lactation.

3.4. Associations in the Longitudinal Subset

3.4.1. Participants

Longitudinal (n = 21) participants’ characteristics and HM components’ concentrations are shown in Table S2. Participants in longitudinal subset were generally leaner than in the combined subset, but none were underweight. At the first session they were classified as: normal weight (BMI 18.5–24.9, 67%, n = 14; %FM 21–32.9, 57%, n = 12), overweight (BMI 25–29.9, 19%, n = 4; %FM 33–38.9, 29%, n = 6) or obese (BMI > 30, 14%, n = 3; %FM > 39.14%, n = 3) [53]. Infant male:female ratio was 10:11.

3.4.2. Longitudinal Changes in Concentrations of Human Milk Components

Table S1 presents the changes in HM component concentrations in the longitudinal subset (n = 21 participants, 73 sessions). While component concentrations differed by the month of lactation within participants for all components (lactose: p = 0.020; adiponectin, whole and skim milk leptin, protein: p < 0.001), no consistent month of lactation-related patterns were seen for adiponectin (p > 0.32), whole milk leptin (p > 0.11) or lactose (p > 0.46). Although the overall pattern for protein was not significant (p > 0.10), post-hoc tests showed that protein concentration at 9 months was 3.80 ± 1.65 g/L lower than that at 2 months of lactation (univariate, p = 0.027). Skim milk leptin decreased non-linearly over the months of lactation (univariate: p = 0.007).

3.4.3. Associations between Maternal Body Mass Index and Percentage Fat Mass

A strong relationship (p < 0.001) was observed between maternal BMI and %FM in the longitudinal subset, with a one-unit increase in BMI associated with a 1.07% ± 0.17% increase in

Figure 3. Associations between protein concentration and (a) maternal percentage fat mass; and (b) maternal BMI. Combined subset data points are shown as cross-sectional (pale orange) and longitudinal (dark orange). Lines are fixed effects from univariate linear mixed effect models: pale orange dotted line—combined cohort (Table 5); solid dark red line—longitudinal cohort (Table S1).

3.3.5. Associations between Maternal Adiposity and HM Lactose

Neither %FM or BMI were associated with concentrations of lactose in HM in univariate model (Table 5) or after accounting for the month of lactation. No interaction with the month of lactation as a factor was seen for BMI (p = 0.19), but a significant positive interaction was seen between %FM and the month of lactation (2 m: reference; 5 m: 0.12 ± 0.33, p = 0.71; 9 m: −0.14 ± 0.30, p = 0.63; 12 m: 0.58 ± 0.31, p = 0.068; month of lactation as a factor: p = 0.029), indicating that association between %FM and lactose strengthens over the first 12 months of lactation.
%FM. After accounting for the month of lactation there was a significant overall difference in %FM with the month of lactation ($p = 0.008$) and the association between maternal BMI and %FM remained significant ($1.01 \pm 0.17, p < 0.001$).

Over the first year of lactation, maternal %FM decreased in non-linear fashion (largest drop between 9 and 12 months) by more than 2% after accounting for the month of lactation as a factor ($-2.26\% \pm 0.67\%, p = 0.002$; age: $p < 0.001$) or by 0.23% per month after accounting for the month of lactation as a linear effect ($-0.23\% \pm 0.06\%, p < 0.001$) (Figure 4a).

Over the first year of lactation, maternal BMI decreased in an almost linear fashion; decreasing by $-1.05 \pm 0.24$ kg/m$^2$ ($p < 0.001$; month of lactation: $p < 0.001$) over the ten months of the study when accounting for the month of lactation as a factor, or by $-0.10 \pm 0.02$ kg/m$^2$ ($p < 0.001$) per month when accounting for the month of lactation as a linear effect (Figure 4b).

**Figure 4.** Longitudinal changes in (a) maternal percentage fat mass and (b) maternal BMI from 2 to 12 months of lactation. Lines are colour-coordinated for the individual participants (e.g., dark orange in panel (a) is a same dark orange in panel (b)) for illustrative purposes only ($n = 21$).

### 3.4.4. Associations between Maternal Adiposity and Human Milk Components in Longitudinal Subset

No associations were seen between measures of maternal adiposity and adiponectin (%FM: $p > 0.36$; BMI: $p > 0.39$), whole (%FM: $p > 0.082$) and skim milk leptin (%FM: $p > 0.51$; BMI: $p > 0.78$) and lactose concentrations (%FM: $p > 0.56$; BMI: $p > 0.68$) in either univariate models or after accounting for the month of lactation (Table S1).

Higher %FM was associated with higher protein concentration in the univariate model ($0.19 \pm 0.09$ g/L, $p = 0.035$); when the month of lactation was accounted for, this association was no longer seen ($0.14 \pm 0.09$ g/L, $p = 0.12$).

No interaction with the month of lactation as a factor was seen for adiponectin (%FM: $p = 0.87$; BMI: $p = 0.52$), whole milk leptin (%FM: $p = 0.62$; BMI: $p = 0.36$), skim milk leptin (%FM: $p = 0.37$), and lactose (%FM: $p = 0.22$; BMI: $p = 0.30$).

Significant negative interactions were seen between BMI and the month of lactation for skim milk leptin, (2 m: reference; 5 m: $-0.02 \pm 0.01$, $p = 0.005$; 9 m: $-0.01 \pm 0.01$, $p = 0.059$; 12 m: $-0.01 \pm 0.01$, $p = 0.059$; month of lactation as a factor: $p = 0.043$), indicating that association between BMI and skim milk leptin weakens over the first 12 months of lactation.

### 4. Discussion

The hormonal regulation of appetite plays a central role in infant developmental programming facilitating a lifelong healthy balance between energy intake and expenditure [9]. Concentrations
of appetite hormones and macronutrients present in HM influence regulation of appetite, energy expenditure pathways and growth trajectory in the developing infant [8,54,55]. Maternal adiposity may play a critical role in regulation of concentrations of HM leptin, adiponectin, protein and lactose, and thus of the ingested dose of these components by the infant. This study found some associations of higher maternal adiposity with higher concentrations of HM leptin and protein, but no associations with adiponectin or lactose. Concentrations of whole milk leptin, adiponectin, protein and lactose did not systematically change with milk removal during a breastfeed, or over the first year of lactation—a period which includes both exclusive breastfeeding and the introduction of complementary foods.

This study has shown that the greater the maternal %FM or BMI, the higher the concentrations of HM leptin. This is logical in that a greater amount of adipose tissue results in more leptin secreted into the circulation and thus increased amounts are transferred into the milk. In young infants, HM is believed to be a major source of leptin, due to immature endogenous leptin-synthesising mechanisms [56]. Leptin may provide both short and long term appetite control acting as a neurotrophic molecule targeting the hypothalamus to stimulate neural connections with other key appetite nuclei [57]. Higher HM leptin concentrations are associated with lower infant weight, weight gain and adiposity [8,58] while higher concentrations in infant serum are associated with greater lean body mass (total body water) [59,60], suggesting a pivotal role in regulating infant growth and BC.

One might speculate that increased amounts of HM leptin supplied to an infant may be detrimental to the development of infant appetite control and growth. However, concentrations of leptin do not reflect the 24-h dose received by the infant and indeed 24-h milk intakes are variable between dyads [61]. In particular, obese mothers are more likely to have reduced milk production due to incomplete differentiation of mammary epithelial cells [62]. Conversely, lean mothers with very low plasma leptin concentrations may produce milk with low leptin levels, thus signalling marginal environment and promoting rapid infant growth while supported by maternal metabolism [63]. Lower infant serum leptin levels would thus reduce the neurotrophic effects on the hypothalamic appetite circuitry and lower satiety stimulation [8,13]. Accordingly, maintenance of healthy maternal adiposity during pregnancy and lactation may ensure appropriate levels of leptin supply to the infant, supporting the optimal programming of appetite control in infancy.

Whilst the lack of relationship between maternal %FM/BMI and HM leptin in the longitudinal subset (Figures 1 and 2) may seem counter-intuitive, there is a marked reduction in the variability of both the maternal BC and HM leptin levels, restricting applicability of the results (Tables S1 and S2). However, HM leptin concentrations have not been shown to differ between obese and non-obese mothers [64]. Furthermore, the contribution by the lactocytes to leptin levels in HM [19,20] is not known. These limitations may explain why some studies (n = 11) find an association and others do not (n = 4) [25]. Moreover, the majority of previous studies analysed leptin in skim HM in cross-sectional cohorts and are restricted to the first and third months postpartum [25], limiting their ability to determine leptin profiles during lactation in women of varying BC. Only three studies have analysed %FM using dual-energy X-ray absorptiometry (DXA) [24], skinfold measures [63] and bioelectrical impedance analysis [65] and they found a strong association between %FM and skim milk leptin, consistent with the results from our study. Interestingly, Khodabakhshi et al. (2015) [65] found association between HM leptin and both %FM and BMI only in the subset of mothers of obese infants but not in the subset of mothers of normal infants, although these two groups did not differ by BMI or leptin concentration.

Concentrations of adiponectin in HM were not related to maternal %FM and BMI in either the combined or longitudinal subsets in this study. This is not consistent with the physiological inverse relationship between the number of white adipocytes and serum adiponectin levels in humans [29], however it is in agreement with a recent meta-analysis by Andreas et al. [25] who reported the absence of a consistent relationship, either positive or negative, between maternal BMI and colostrum or skim
HM adiponectin. We have now expanded the absence of a relationship to maternal %FM and whole HM adiponectin. Thus, it is unlikely that maternal adiposity plays a major role in influencing HM adiponectin levels and it suggests the majority of HM adiponectin may be synthesized and controlled by the mammary gland [66] highlighting the importance of this HM hormone for the infant. Indeed, it appears that HM adiponectin levels regulate infant growth with higher levels of HM adiponectin being associated with lower weight for age (WAZ) and weight for length (WLZ) z-scores at 6 months of age [34] and higher WAZ and WLZ scores over the first 2 years of life [54]. These results were also supported by Brunner et al. [32], who found that higher concentrations of HM adiponectin at 6 weeks were associated with lower infant fat-free mass and weight at 4 months as well as greater weight and fat mass at 1 and 2 years of age. The follow up at 3, 4 and 5 years of age has not shown any relationship with the exception of the positive association between HM adiponectin levels at 4 months postpartum and fat mass at 4 years [67]. This reversal of the initial trend in early life is speculated to be related to the timing of cessation of breastfeeding [54]. High HM adiponectin levels may initially down-regulate infant growth, and later promote adipogenesis and adipocyte hypertrophy [68]. Conversely lean populations with lower concentrations of HM adiponectin demonstrate a positive association with the infant WAZ scores. This suggests that the association between HM adiponectin and infant growth may in fact be parabolic, further highlighting the pleiotropic effects of adiponectin during development and the adaptive mechanisms that humans display in the marginal environments [66]. Our recent study of gastric emptying and breastfeeding patterns in fully breastfed term infants has established that higher concentrations and doses of HM adiponectin are associated with longer times between breastfeeds (gastric emptying time) [69], which may partially explain the growth-regulating effect of adiponectin in some populations. As such, investigations into other factors that may affect adiponectin concentrations in HM and its effect on infant growth and BC development are warranted.

The few studies investigating relationships between HM protein composition and maternal adiposity are contradictory, with some reporting a positive association between protein and maternal adiposity (BMI or percentage ideal weight) [41,42] and one a negative association between protein and BMI [40]. Increased serum amino acid concentrations are present in mothers with more adipose tissue [39], leading to more amino acids transferred to the breast and HM [70], explaining the positive relationship between maternal adiposity and HM protein concentrations [42]. This study has found that higher %FM but not BMI was associated with higher protein concentrations, which is similar to Quinn et al. [63] who reported %FM to be more precise measure of adiposity reporting stronger correlation with HM leptin, thus the more precise measure of maternal BC is desirable in mechanistic research.

The measured concentrations of lactose in our cohort were consistent with the normal range in HM [71] and were not related to maternal adiposity profiles. HM provides a constant source of carbohydrates to the infant during early life, ensuring adequate nourishment, maturation and development of their relatively immature physiological systems [41,72,73]. Given the fact that lactose is important for maintaining a constant osmotic pressure in HM [74], maternal adiposity is not expected to have a significant impact on lactose concentration.

Despite significant changes in maternal %FM and BMI over the 12 months of lactation, and the introduction of complimentary foods, the measured HM components have remained relatively stable (Tables 4 and 5 and Table S2). However, adiponectin concentration decreased significantly in the combined subset as well as in the concentration of protein in the longitudinal subset, both at 9 months only. The temporary drop in protein concentration was consistent with differences described by Nommsen et al. [42]. Whereas some studies have reported a decrease in concentrations of leptin [75] and adiponectin [30,33,36,66] (measured predominantly in skim HM), others found the opposite trend for adiponectin [11,35], no change [76] or significant fluctuations [11,21] for leptin. More research is required to clarify these relationships.

In this study we have measured leptin in both skim and whole milk. Interestingly, longitudinal changes observed in concentrations of skim milk leptin in this study were not confirmed in whole
HM. Caution in the interpretation of leptin concentrations measured in skim HM should be exercised, as whole milk measures are more indicative of the level of hormone consumed by the infant [28]. The consistent concentrations of adiponectin and leptin in whole HM over the first 12 months of life may be indicative of a continuing roles of appetite programming, priming neural connections involved in the appetite circuitry, thereby contributing to long-lasting enhanced appetite control and BC of breastfed infants throughout life.

Sampling protocols are of prime importance when investigating relationships between HM components and BC. In this study we confirmed no systematic change between pre- and post-feed samples for concentrations of protein, lactose and whole and skim HM leptin throughout the first 12 months of lactation (Table 3), and report for the first time that whole milk adiponectin concentrations also do not differ significantly pre- to post-feed. Care however should still be taken as fat content [77], ghrelin [75] and glucagon-like peptide 1 (GLP-1) [24] change between pre- and post-feed, further highlighting the importance of prudent sampling.

The limitations of this study are the modest number of longitudinal participants, resulting from time constraints associated with multiple measurement time points. Further, our population was predominantly Caucasian and of high social-economic status; therefore, the results may not be applicable to participants from other backgrounds.

5. Conclusions

This study found that elevated maternal adiposity was associated with higher concentrations of leptin and protein of HM in a cross-sectional cohort however; these findings were not confirmed in a smaller longitudinal cohort. Clarification of the relationships between maternal body composition and human milk appetite regulators will identify periods of lactation where interventions may influence programming of early appetite control and body composition of breastfed infants.

Supplementary Materials: The following are available online at http://www.mdpi.com/2072-6643/9/3/252/s1, Table S1: Longitudinal changes and associations between human milk components and maternal adiposity. Values are parameter estimates ± standard error (n = 21), Table S2: Maternal adiposity and human milk components concentrations presented at the months after birth for longitudinal subset (n = 21 participants, 73 sessions). Values are mean ± standard deviation (range).

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Author Contributions: Sambavi Kugananthan designed the study, conducted experiments and data analysis and interpretation, and wrote the manuscript; Zoya Gridneva designed the study, collected the data, conducted data analysis and interpretation, and wrote the manuscript; Ching T. Lai conducted experiments and critically reviewed the manuscript; Anna R. Hepworth provided consultation for research design, conducted data analysis and interpretation, and critically reviewed the manuscript; Peter J. Mark designed the study, interpreted results, and critically reviewed the manuscript; Donna T. Geddes designed the study, collected the data, interpreted results, and critically reviewed the manuscript; Foteini Kakulas designed the study, interpreted results and critically reviewed the manuscript.

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References


Article

Effect of Human Milk Appetite Hormones, Macronutrients, and Infant Characteristics on Gastric Emptying and Breastfeeding Patterns of Term Fully Breastfed Infants

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Abstract: Human milk (HM) components influence infant feeding patterns and nutrient intake, yet it is unclear how they influence gastric emptying (GE), a key component of appetite regulation. This study analyzed GE of a single breastfeed, HM appetite hormones/macronutrients and demographics/anthropometrics/body composition of term fully breastfed infants (n = 41, 2 and/or 5 mo). Stomach volumes (SV) were calculated from pre-/post-feed ultrasound scans, then repeatedly until the next feed. Feed volume (FV) was measured by the test-weigh method. HM samples were analyzed for adiponectin, leptin, fat, lactose, total carbohydrate, lysozyme, and total/whey/casein protein. Linear regression/mixed effect models were used to determine associations between GE/feed variables and HM components/infant anthropometrics/adiposity. Higher FVs were associated with faster \((0.07 \ [0.10, \ -0.03], \ p < 0.001)\) GE rate, higher post-feed SVs \((0.82 \ [0.53, \ 1.12], \ p < 0.001)\), and longer GE times \((0.24 \ [0.03, \ 0.46], \ p = 0.033)\). Higher whey protein concentration was associated with higher post-feed SVs \((4.99 \ [0.84, \ 9.13], \ p = 0.023)\). Longer GE time was associated with higher adiponectin concentration \((2.29 \ [0.92, \ 3.66], \ p = 0.002)\) and dose \((0.02 \ [0.01, \ 0.03], \ p = 0.005)\), and lower casein:whey ratio \((-65.89 \ [-107.13, \ -2.66], \ p = 0.003)\). FV and HM composition influence GE and breastfeeding patterns in term breastfed infants.

Keywords: human milk; term breastfed infants; gastric emptying; feeding frequency; ultrasound; stomach volumes; appetite hormones; macronutrients; feed volume; anthropometrics; body composition

1. Introduction

Breastfeeding and its longer duration are associated with reduced risks of developing obesity and other chronic non-communicable diseases later in life [1,2]. This unique protection could be the result of many mechanisms associated with both nutritive and non-nutritive components of human milk (HM) [3] as well as breastfeeding patterns and behaviour [4,5]. It has been shown that HM has the pleiotropic role, providing immune and anti-inflammatory protection [6,7] and endocrine, developmental, neural, and psychological benefits [2]. Non-nutritive HM components such as hormones, growth factors, neuropeptides, and anti-inflammatory and immune-modulating
agents influence the growth, development, and function of the gastrointestinal (GI) tract during early infancy [8], while some micronutrients act as nutritional antioxidants, improving GI functions [9]; however, there is much to be learned about the spectrum of HM programming agents, how their patterns change throughout lactation period, and their short-term effect on the gastric emptying (GE) rate of the breastfed infants.

GE is a process by which ingested food is mechanically and chemically partially broken down and delivered to the duodenum at a controlled rate for further digestion and absorption [10,11]. While well studied in the preterm population [12–14], in healthy term fully breastfed infants the GE rate and its relationship with breastfeeding patterns are not fully understood.

GE rate and patterns are known to depend on the nature and macronutrient composition of the ingested meal. HM or formula in the infant stomach separates into two phases, a liquid phase consisting of water, whey proteins, lactose, etc., and a semi-solid phase consisting of curd formed by casein and lipids. The semi-solid phase typically empties more slowly than the liquid phase. Different proportions of these phases in part explain the difference between GE patterns of formula-fed and breastfed infants—linear and curvilinear, respectively [12,15].

HM has a unique composition, including nutrients, growth factors, immune factors, and hormones. Despite numerous investigations into the different effects of HM and formula, few components, including major macronutrients, have been studied in connection with the GE of breastfed term infants.

Fatty acids profiles are not associated with GE rate in preterm infants [16], while in term infants more rapid GE has been attributed to the fat and protein components of feeds with similar lactose concentration and osmolality [17].

Both osmolality and carbohydrate content are known to influence the rate of GE in adults [18], but in infants results are dependent on the type of carbohydrate [19,20].

Proteins from different HM fractions such as whey and casein are resistant to proteolysis in the infant stomach [21] and the protein content of a food has also been shown to influence appetite and its regulation [22]. Infant formula generally empties more slowly than HM in term infants; further, formulas with different casein:whey protein ratio exhibit different GE rates, with casein-predominant formulas emptying slower than whey-predominant formulas [23]. Thus the casein:whey ratio of HM could play an important role in controlling GE in the breastfed infant.

HM lysozyme, also present in whey in a relatively high concentration, catalyzes the hydrolysis of specific bonds in Gram-negative bacteria cell walls and plays multiple roles in digestive strategy, such as controlling the microbiome in the stomach and speeding up the digestion of microbial protein, which may affect gastric motility and GE rate [24,25].

The satiety hormone leptin and the appetite-stimulating hormone adiponectin are also present in HM. Although not transferred to the infant circulation in direct manner, levels of HM leptin and adiponectin from HM have been found to correlate with levels of these hormones in infant serum [26,27] and are known to affect both appetite control and infant body composition (BC) [28,29], but are yet to be investigated in relation to GE in the term infant. In animal models (rat, mouse), injection of leptin into the fourth ventricle has been shown to delay GE [30] and oral administration reduced food intake [31]. Leptin in HM is by far the most studied appetite hormone, but predominantly in skim milk [32]. Leptin measured in skim HM was not associated with time between feeds [33,34] or GE [34] in term breastfed infants, emphasizing the need for studies including whole milk leptin, where the levels of leptin are shown to be higher [32]. Adiponectin has the highest concentration of any appetite hormone in HM. It is present in a biologically active form that is resistant to digestion [35]. In the animal model adiponectin inhibits tension-sensitive gastric vagal afferent mechanosensitivity, modulating satiety signals in both lean and obese animals, while simultaneously increasing the mechanosensitivity of mucosal gastric vagal afferent in the obesity-induced model [36]. In humans, elevated serum levels of adiponectin are associated with more rapid GE in diabetic patients [37]. It is not known whether adiponectin levels impact GE in the infant and this warrants further investigation.
The volume of milk taken at a single feed varies greatly both within and between infants [38]. This may be affected by HM composition, with greater breastfeeding frequency associated with lower total 24-h protein intakes and higher lactose concentrations [39]. This suggests that the variations in HM components between mothers may potentially influence GE rate and time, and therefore feeding patterns.

This study investigated the effects of HM appetite hormones (whole milk adiponectin and leptin, skim milk leptin) and macronutrients (fat, total carbohydrates, lactose, oligosaccharides, total protein, casein and whey protein, lysozyme) on feeding frequency and GE. Further exploration of infant demographics, anthropometrics, and BC was carried out to determine relationships with infant feeding and GE.

2. Materials and Methods

2.1. Participants

Lactating mothers and their infants \((n = 27)\) were recruited predominantly through the Australian Breastfeeding Association. Inclusion criteria were: healthy singletons, gestational age \(\geq 37\) weeks, fully breastfed on demand at the point of measurement. Exclusion criteria were: infant health issues requiring medication that could potentially influence GE rate (e.g., reflux), indications of low maternal milk production or infant growth issues. All mothers provided written informed consent to participate in the study, which was approved by the University of Western Australia, Human Research Ethics Committee (RA/1/4253) and registered with the Australian New Zealand Clinical Trials Registry (ACTRN12616000368437).

2.2. Study Design

Participants arrived at our laboratory at King Edward Memorial Hospital for Women (Subiaco, Perth, WA, Australia) in the morning (09:30–11:30 a.m.) to avoid circadian influence on the outcomes, and stayed for two consecutive breastfeeding sessions. Before the first feed (F1) infants were weighed and had ultrasound stomach volumes recorded (pre-feed residual, R1). Mothers expressed a pre-feed sample (fore-milk) of milk from the feeding breast/breasts and then breastfed their infants as usual. Immediately after F1, infant stomach volumes images and infant weights were taken, and mothers expressed a post-feed (hind-milk) milk sample. Subsequent scans of the stomach were scheduled at 15–20 min intervals (although attending infants’ needs caused some variation) until the infant cued for the next feed (F2), when a final stomach volume immediately before F2 was measured (pre-feed residual, R2).

To assess infant BC bioimpedance spectroscopy measurements were taken pre-feed, unless impractical—then they were taken post-feed [40]. Ultrasound skinfold, length, and head circumference measurements were taken post-feed. This combination of two methods for measuring infant BC was used to ensure safe, non-invasive and accurate assessment and to avoid the inherent limitations of a singular technique [41]. Clothing was removed for the measurements except for a dry diaper and a singlet.

2.3. Feeding Frequency

Mothers were asked how frequently their infant feeds, and the self-reported typical time between the feeds (e.g., every three hours) during the week prior to the study session was taken as a proxy measure of feeding frequency.

2.4. Feed Volume Measurement

The volume of milk transferred from a breast/breasts by the infant was determined by weighing the infant immediately before and after the breastfeed using electronic scales (±2.0 g, Medela Electronic Baby Weigh Scales, Medela Inc., McHenry, IL, USA). Milk intake (g) was calculated by deducting the
initial weight from the final weight of the infant [42] and was converted to mL (feed volume; FV) using HM density of 1.03 g/mL [43].

2.5. Stomach Measurements with Ultrasound

The infant’s stomach was scanned using the Aplio XG (Toshiba, Tokyo, Japan) machine, with a high-resolution PVT-674BT (6MHz) transducer and Parker ultrasonic gel (Fairfield, NJ, USA). Three to nine (median [IQR]: 5 [5; 6]) serial measurements of infant stomachs were taken 3 to 62 min apart (16 ± 10). Scans were performed with the infant in the semi-supine position according to the method validated in preterm infants [44]. Briefly, the sagittal and transverse planes of the stomach were used to measure the longitudinal (L), anterior-posterior (AP) and transverse (T) diameters directly from images on the ultrasound screen using electronic calipers (Figure 1). One experienced sonographer with good intra- and interrater reliability [44] performed all of the measurements. Gastric volume (mL) was calculated from the above measured diameters using following equation for an ellipsoidal body:

\[
\text{Stomach volume (mL)} = L \text{(mm)} \times AP \text{(mm)} \times T \text{(mm)} \times 0.52. \tag{1}
\]

![Figure 1](image.png)

**Figure 1.** Measurements of infant’s stomach with ultrasound. Ultrasound images of infant’s stomach: (a) transverse view with anterior-posterior (AP) and transverse (T) diameter measurements; (b) longitudinal view with longitudinal (L) diameter (maximum length) measurement. Stomach volume (mL) = longitudinal diameter (mm) × anterior-posterior diameter (mm) × transverse diameter (mm) × 0.52.

2.6. Milk Sample Collection

Mothers hand-expressed or pumped small (1–2 mL) pre- and post-feed milk samples into separate 5-mL polypropylene plastic vials (Disposable Products, Adelaide, SA, Australia). Fat concentration was measured (below) and samples were frozen at −20 °C for further biochemical analysis.

2.7. Biochemical Analysis

2.7.1. Fat Content

Percentage fat was measured in pre- and post-feed samples immediately after sample collection with the creamatocrit method [45] using the Creamatocrit Plus device (Medela Inc., McHenry, IL, USA). Fat concentration of the pre- and post-feed milk samples (g/L) was calculated from the cream content of the milk samples, based on the equation [46]:

\[
\text{Fat (g/L)} = 3.56 + (5.917 \times \text{cream percentage}). \tag{2}
\]
Fat concentration in the volume consumed by the infant was further calculated [47]:

\[
\text{Fat (g/L)} = 0.53 \times \text{Fat}_{\text{pre-feed}} + 0.47 \times \text{Fat}_{\text{post-feed}}.
\]  

(3)

2.7.2. Sample Preparation

Prior to further analysis, all samples were thawed for two hours at room temperature (RT) and aliquoted into 1.5-mL tubes (Sarstedt, Numbrecht, Germany). Components’ concentrations were determined in both pre- and post-feed samples in case of adiponectin, skim and whole milk leptin, fat, and lactose, and in pooled samples in case of total protein, casein, whey protein, total carbohydrates, and lysozyme. Concentrations of pre- and post-feed samples were averaged to arrive at the concentration used for statistical analyses. Whole milk was used for measuring whole milk adiponectin and leptin concentration. Milk samples were defatted (by centrifugation at RT in a Beckman Microfuge 11 (Aberdon Enterprise Inc., Elk Grove Village, IL, USA) at 10,000 \( \times \) g for 10 min and removing the fat layer by clipping it off with the top of the tube [48]) for analysis of skim milk leptin, total protein, lysozyme, lactose, and total carbohydrates concentrations. The standard assays were adapted for and carried out using a JANUS workstation (PerkinElmer, Inc., Waltham, MA, USA) and measured on EnSpire (PerkinElmer, Inc., Waltham, MA, USA).

2.7.3. Leptin

Leptin concentration in HM was measured using the R & D Systems Human Leptin enzyme linked immunosorbent assay (ELISA) DuoSet kit (Minneapolis, MN, USA) optimized to measure leptin in sonicated skim HM, as previously described by Cannon et al. [33] and further modified to measure leptin in skim and whole HM milk as described by Kugananthan et al. [32]. Recovery of leptin was 97.7\% \pm 9.7\% (n = 10) with a detection limit of 0.05 ng/mL and an inter-assay CV of <7.2\%.

2.7.4. Adiponectin

Adiponectin concentration in whole milk was measured using the Biovendor Human Adiponectin Sandwich ELISA kit (Life Technologies, Asheville, NC, USA). Adiponectin recovery was 96.2\% \pm 3.2\% (n = 10) with a detection limit of 1 ng/mL and an inter-assay CV of <2.5\%.

2.7.5. Protein

Casein and whey proteins were separated by the method fully described by Kunz and Lonnerdal [49], and Khan et al. [50]. Protein concentrations (total protein of skim HM, casein and whey proteins) were measured using the Bradford Protein Assay adapted from Mitoulas et al. [51]. Recovery of protein was 100.6\% \pm 5.2\% (n = 5) with a detection limit of 0.031 g/L and an inter-assay CV of 7.8\% (n = 18). Casein:whey ratio was calculated as follows:

\[
\text{Casein:whey ratio} = \frac{\text{casein concentration}}{\text{whey protein concentration}}.
\]  

(4)

2.7.6. Lysozyme

Lysozyme concentration was determined using a modified turbidimetric assay [52]. Hen egg white lysozyme (EC 3.2.1.17, Sigma, St. Louis, MA, USA) standards (range 0.00075–0.0125 g/L) and skim milk samples were diluted 10-fold with 0.1 M of Na\(_2\)HPO\(_4\)/1.1 mM of citric acid (pH 5.8) buffer. Twenty-five microliters of standards or diluted skim milk samples were placed into the wells of a plate (Greiner Bio-One, Frickenhausen, Germany), 175 \( \mu \)L of Micrococcus lysodeiktikus suspension (0.075% w/v, ATCC No. 4698, Sigma, St. Louis, MA, USA) was added into each well and plate was incubated at RT for 1 h. The absorbance was measured at 450 nm. Recovery of lysozyme was 97.0\% \pm 5.0\% (n = 8) with a detection limit of 0.007 g/L and an inter-assay CV of 13.0\% (n = 8).
2.7.7. Carbohydrates

Defatted milk was deproteinized with trichloroacetic acid [53] before dehydration by sulphuric acid [54]. This technique reliably estimates concentrations and carbon content for monosaccharides, disaccharides, and polysaccharides. Total carbohydrates were analyzed by UV-spectrophotometry. Recovery of total carbohydrates was 101.4% ± 2.1% (n = 7) with a detection limit of 0.007 g/L and an inter-assay CV of 3.3% (n = 7).

Lactose concentration was measured using the enzymatic spectrophotometric method of Kuhn and Lowenstein [55], adapted from Mitoulas et al. [51], with recovery of 98.2% ± 4.1% (n = 10), detection limit of 30 mM and inter-assay CV of 3.5%.

The human milk oligosaccharides (HMO) concentration (g) was calculated by deducting concentration of lactose (g) from concentration of total carbohydrates (g). The glucose and galactose were not measured or accounted for as their concentrations in HM are small and comparable or less than the assays errors [56].

2.8. Hormone and Macronutrient Dose

Doses were defined as the amount of hormone/macronutrient ingested during a breastfeed and calculated as average of the pre- and post-feed HM component concentration, multiplied by the corresponding FV. When an infant fed from both breasts at the breastfeeding session, hormone/macronutrient doses from these individual breastfeeds were calculated separately and added together.

2.9. Infants’ Anthropometrics and Body Composition

2.9.1. Anthropometric Measurements

Infants’ weight was determined by weighing before breastfeeding using Medela Electronic Baby Weigh Scales (±2.0 g; Medela Inc., McHenry, IL, USA). Clothing was removed except for a dry diaper and a singlet. Infant crown-heel length was measured once to the nearest 0.1 cm using non-stretch tape and headpiece and footpiece, both applied perpendicular to the hard surface. Infant head circumference was measured with non-stretch tape. Infant BMI was calculated according to the following formula:

\[ BMI = \frac{\text{Body weight (kg)}}{\text{Height (m)}^2}. \]  

2.9.2. Body Composition with Bioelectrical Impedance Spectroscopy

Infants’ whole body bioimpedance were measured using the Impedimed SFB7 bioelectrical impedance analyzer (ImpediMed, Brisbane, Queensland, Australia) applying an adult protocol (wrist to ankle) according to the manufacturer’s instructions and analyzed with settings customized for each infant according to Lingwood et al. [57] and Gridneva et al. [41]. Values of resistance (ohm) at frequency of 50 kHz (RS0) were determined from the curve of best fit, averaged for analysis purposes and used in the Lingwood et al. age matched (3 and 4.5 mo infants) equations for fat-free mass (FFM) of 2 and 5 mo infants respectively [57]:

\[ FFM \ 3 \ mo = 1.458 + 0.498 \times W - 0.197 \times S + 0.067 \times L^2 / R_{S0} \]  
\[ FFM \ 4.5 \ mo = 2.203 + 0.334 \times W - 0.361 \times S + 0.185 \times L^2 / R_{S0}, \]  

where \( L \) is body length (cm), \( R_{S0} \) is resistance (Ω), \( S \) is sex (male = 1, female = 2) and \( W \) is infant weight (kg).

%FM was calculated as follows:

\[ \%FM = \frac{100(\text{Weight (kg)} - \text{FFM (kg)})}{\text{Weight (kg)}}. \]
2.9.3. Body Composition with Ultrasound Skinfold Measurements

Infant ultrasound skinfold measurements were carried out using the Aplio XG (Toshiba, Tokyo, Japan) ultrasound machine, PLT-1204BX 14-8 MHz transducer and sterile water-based Parker ultrasonic gel (Fairfield, NJ, USA). Single ultrasound scans of four anatomical sites (biceps, subscapular, suprailiac, and triceps) were performed on the left side of the body with minimal compression. Skinfold thickness (skin thickness and the skin–fat interface to fat–muscle interface distance) was measured directly from images on the screen using electronic calipers. One experienced sonographer (DG) with good intra- and interrater reliability [44] performed all of the measurements.

The doubled ultrasound skinfold thickness was used in Brook body density ($d$) age-matched (3–18 mo) equations [58] developed for skinfolds measured with calipers:

\[
\text{Male } d = 1.1690 - 0.0788 \times \log (\sum \text{SFT})
\]

\[
\text{Female } d = 1.2063 - 0.0999 \times \log (\sum \text{SFT}),
\]

where $d$ is infant body density (kg/L) and $\sum \text{SFT}$ is a sum of four skinfolds (mm).

Predicted body density was converted to %FM using the Lohman equation [59]:

\[
\%\text{FM} = 100 \times \left( \frac{5.28}{d} - 4.89 \right),
\]

where $d$ is the infant body density (kg/L).

2.10. Statistical Analysis

Statistical analysis was performed in R 2.9.0 [60] for Mac OSX using additional packages nlme [61]; lattice [62], lattice extra [63], and car [64]; MASS [65], sfsmisc [66] and multcomp [67] for mixed effects modeling, data representation, robust regression, and multiple comparisons of means, respectively. Descriptive statistics are reported as mean ± standard deviation (SD) (range) or median (IQR) unless otherwise stated; model parameters are presented as estimate ± standard error (SE), and, where appropriate, an approximate 95% confidence interval (95% CI).

Measurements missing due to insufficient sample volume: skim milk leptin, whole milk leptin, adiponectin, total protein, whey and casein protein, lactose and total carbohydrate ($n = 3$); lysozyme ($n = 5$). Measurements of fat ($n = 14$) were missing as a result of either insufficient sample volumes or absence of separate feed volumes from breasts where both breast were offered during one feed. Also missing were feeding frequency as reported by mothers ($n = 6$), measurements of length, head circumference, infant BMI, %FM measured with bioelectrical spectroscopy ($n = 4$) and %FM measured with ultrasound skinfolds ($n = 5$).

GE time was determined as the time from the start of F1 to the start of F2 and included the time between two feeds and feed duration. Feed duration was included as up to 80% of HM consumed by term healthy breastfed infants in the first 4–5 min [68]. GE during breastfeeding was defined as the volume of milk to have left the stomach, calculated as the difference between the immediate post-feed stomach volumes and the sum of R1 and FV.

Due to the lack of term infant gastric-emptying studies focusing on stomach volume, no power calculation/sample size determination could be performed for this study. A goal of 20 infants at each two and five months was selected with the expectation that this would be sufficient to show overall patterns. When available, infants were included in both subsets to allow for investigation of longitudinal patterns. Linear mixed effects models allow us to treat the individual feeds as separate, without having to assume independence, when there may be correlations between feeds within infants.

Influences on GE rate were analyzed by first fitting a time curve to the sequential post-feed stomach volumes using linear mixed effects models; as curves differed significantly within and between infants ($p < 0.001$), random time curves were fitted to feeds within infants. Time terms (linear, square root) were selected as per the fractional polynomial method of [69]; this model also considered
possible confounding effects of FV (median-centred) and feed duration (median-centred). Interaction terms involving the time curve indicated changes in the GE rate; main effects indicated overall effects on post-feed stomach volumes but not the GE rate. The addition of one term to this base model was used to investigate associations with (a) concentrations/doses of hormones/macronutrients; (b) infant characteristics/anthropometrics/BC; (c) R1. Whether the overall effect of HM component concentrations differs by feed volume was investigated by including interactions between FV and concentration measures. Models using the selected technique did not converge for fat concentration, lysozyme concentration, or lysozyme dose. Omitting the random effect of feed within infant provided converging models, but no evidence of an association with fat or lysozyme was seen. Given the complexity of linear mixed effects models used to analyze GE rate, no further adjustments were performed and \( p < 0.05 \) was considered to be statistically significant.

Associations between pre-feed residual stomach volumes, FV, immediate post-feed stomach volumes, feed duration, feeding frequency and both hormone and macronutrient concentrations and doses, and infant anthropometrics/BC parameters were tested using robust linear regression. Mixed effects models were considered, but were not significantly better (\( p > 0.1 \)) Robust linear regression (rlm) was chosen so as to address heteroscedasticity in the data and points with high leverage in the majority of the predictors; MM-estimation (M-estimation with Tukey’s biweight, initialized by a specific S-estimator) accounting for appropriate covariates was used [65]. Approximate \( p \)-values were determined using the Wald test. Multivariate models accounting for FV were used for testing the relationship with FV-dependent predictor (fat dose and concentration).

Possible age differences in HM components, infant characteristics, and GE/breastfeeding parameters were analyzed with either linear mixed effects models or robust linear regression models; model type was determined using likelihood ratio tests. Linear mixed effects models were used to analyze relationships of GE during feed time with HM components and infant characteristics. R1, FV and feed duration were not associated with stomach volume reduction during the feed time, therefore univariate models were run. Multivariate linear mixed effects models accounting for R1, FV and feed duration were used in analysis of relationships of immediate post-feed stomach volumes with HM components and infant characteristics.

Owing to the large number of comparisons, a false discovery rate adjustment [70] was performed on associated subgroupings of results with one or more \( p \)-values < 0.05. \( p \)-values were considered to be significant at <0.011 for GE time, <0.031 for feeding frequency, <0.038 for R2, and <0.008 for associations between HM components’ concentrations.

3. Results

3.1. Participants

Characteristics of the 27 participants (2 months (\( n = 20 \)); longitudinal: 7 females, 7 males; cross-sectional: 2 females, 4 males); 5 months (\( n = 21 \)); longitudinal: 7 females, 7 males; cross-sectional: 6 females); overall \( n = 41 \) feeds) are described in Table 1. At the study session, infants fed from one (\( n = 23 \)) or both (\( n = 18 \)) breasts.

3.2. Influence of Infant Age

Infant anthropometrics and %FM measured with bioimpedance spectroscopy significantly differed by infant age (\( p < 0.001 \)), while breastfeeding and GE parameters did not change significantly (\( p > 0.067 \)) (Table 1).

Lower whey protein concentration (5.51 ± 0.96 g/L 5 mo vs. 6.41 ± 1.39 g/L 2 mo, \( p = 0.034 \)) and subsequently a higher casein:whey ratio (0.32 ± 0.14 5 mo vs. 0.22 ± 0.07 2 mo, \( p = 0.035 \)) were observed at 5 months. All other measured appetite hormones and macronutrient concentrations did not differ significantly by infant age (\( p > 0.053 \)).
3.3. Analyzed Human Milk Components

Appetite hormones and macronutrient concentrations and doses per feed are presented in Table 2. Higher skim milk leptin concentrations were associated with lower whole milk leptin concentrations ($-0.25 [-0.34, -0.16], p < 0.001$) and higher protein concentrations were associated with higher whey protein concentrations (0.68 [0.41, 0.95], $p < 0.001$). Higher HMO concentrations were associated with higher total carbohydrates concentrations ($p < 0.001$) and lower lactose concentrations ($p < 0.001$).

### Table 1. Participant characteristics expressed as mean ± SD and range.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>2 mo $^a$</th>
<th>Range</th>
<th>5 mo $^b$</th>
<th>Range</th>
<th>Total $^c$</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infant age (weeks)</td>
<td>$9 \pm 1$</td>
<td>6–10</td>
<td>$22 \pm 1$</td>
<td>18–23</td>
<td>$16 \pm 7$</td>
<td>6–23</td>
</tr>
<tr>
<td>Infant length (cm)</td>
<td>$57 \pm 2$</td>
<td>53–61</td>
<td>$65 \pm 2 ***$</td>
<td>62–69</td>
<td>$61 \pm 4$</td>
<td>53–69</td>
</tr>
<tr>
<td>Infant weight (kg)</td>
<td>$5.3 \pm 0.8$</td>
<td>4.2–6.3</td>
<td>$7.2 \pm 1.0 ***$</td>
<td>5.8–9.5</td>
<td>$6.3 \pm 1.3$</td>
<td>4.2–9.5</td>
</tr>
<tr>
<td>Infant BMI</td>
<td>$15.9 \pm 1.3$</td>
<td>13.9–18.1</td>
<td>$17.6 \pm 1.7 ***$</td>
<td>14.9–20.4</td>
<td>$16.7 \pm 1.7$</td>
<td>13.9–20.4</td>
</tr>
<tr>
<td>HC (cm)</td>
<td>$39 \pm 1$</td>
<td>37–42</td>
<td>$43 \pm 2 ***$</td>
<td>40–46</td>
<td>$41 \pm 2$</td>
<td>37–46</td>
</tr>
<tr>
<td>Fat Mass with BIS (%)</td>
<td>$21.4 \pm 1$</td>
<td>11.1–27.1</td>
<td>$28.9 \pm 3.2 ***$</td>
<td>21.7–35.8</td>
<td>$25.3 \pm 5.0$</td>
<td>11.1–35.8</td>
</tr>
<tr>
<td>Fat Mass with US (%)</td>
<td>$24.2 \pm 3.6$</td>
<td>17.5–30.3</td>
<td>$26.6 \pm 3.6$</td>
<td>20.8–35.9</td>
<td>$25.5 \pm 3.8$</td>
<td>17.5–35.9</td>
</tr>
</tbody>
</table>

Data are mean ± SD and ranges. $^a$ $n = 20$; $^b$ $n = 21$. $^c$ $n = 41$ feeds. $^d$ Stomach volume reduction during feed time is calculated as the difference between the sum of residual 1 and feed volume and the immediate stomach volume after Feed 1.

### Table 2. Concentrations and doses of measured HM hormones and macronutrients.

<table>
<thead>
<tr>
<th>Components</th>
<th>Concentration</th>
<th>Dose Per Feed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SD</td>
<td>Range</td>
</tr>
<tr>
<td>Adiponectin (ng/mL, ng)</td>
<td>10.02 ± 4.08</td>
<td>6.18–22.58</td>
</tr>
<tr>
<td>WM leptin (ng/mL, ng)</td>
<td>0.51 ± 0.18</td>
<td>0.23–1.10</td>
</tr>
<tr>
<td>SM leptin (ng/mL, ng)</td>
<td>0.28 ± 0.12</td>
<td>0.20–0.84</td>
</tr>
<tr>
<td>Total protein (g/L, g)</td>
<td>11.29 ± 2.56</td>
<td>7.60–24.16</td>
</tr>
<tr>
<td>Casein (g/L, g)</td>
<td>1.54 ± 0.53</td>
<td>0.69–3.45</td>
</tr>
<tr>
<td>Whey protein (g/L, g)</td>
<td>5.97 ± 1.26</td>
<td>3.82–9.08</td>
</tr>
<tr>
<td>Casein:whey ratio</td>
<td>0.27 ± 0.11</td>
<td>0.10–0.73</td>
</tr>
<tr>
<td>Lysozyme (g/L, g)</td>
<td>0.14 ± 0.12</td>
<td>0.05–0.48</td>
</tr>
<tr>
<td>TCH (g/L, g)</td>
<td>82.72 ± 7.89</td>
<td>67.08–97.49</td>
</tr>
<tr>
<td>Lactose (g/L, g)</td>
<td>65.84 ± 5.14</td>
<td>53.49–77.94</td>
</tr>
<tr>
<td>HMO (g/L, g)</td>
<td>16.88 ± 9.89</td>
<td>(−10.86) $^b$</td>
</tr>
<tr>
<td>Fat (g/L, g)</td>
<td>42.74 ± 12.10</td>
<td>17.42–66.79</td>
</tr>
</tbody>
</table>

Data are mean ± SD and ranges, $n = 41$ feeds. $^a$ Casein:whey ratios for doses are the same as for concentrations.

Negative values are seen for human milk oligosaccharides (HMO) when lactose measurements are higher than total carbohydrates. Abbreviations: SM—skim milk; TCH—total carbohydrates; WM—whole milk.

3.4. Gastric Emptying Rate

The overall decreasing curvilinear pattern of GE (linear: 0.04 $[-0.17, 0.24]$, $p = 0.72$; square root: $-10.5 [-12.7, -8.2]$, $p < 0.001$) is shown in Figure 2. Higher FVs were associated with faster
were associated with larger post-feed stomach volumes, while the casein:whey ratio (2.2 ± 0.82 [0.53, 1.12], \( p < 0.001 \)) and lactose concentration (4.99 ± 0.07 [0.84, 9.13], \( p = 0.003 \)) modified the GE curve depending on milk intake (MI) to illustrate the effect of the feed volumes; approximately equal numbers are included in each panel. Data points represent stomach volumes calculated from ultrasound images; connecting lines link measurements from the same feed. Bold line represents local regression smoother (LOESS, span = 0.9). Dotted lines represent confidence interval.

**Figure 2.** Overall curvilinear pattern of gastric emptying (\( n = 41 \) feeds). The lines represent the overall pattern of changes in stomach volume as measured by ultrasound imaging. Bold line represents local regression smoother (LOESS, span = 0.9). Dotted lines represent confidence interval.

**Figure 3.** Gastric emptying of individual feeds in term breastfed infants (\( n = 41 \) feeds). Feeds are grouped by milk intake (MI) to illustrate the effect of the feed volumes; approximately equal numbers are included in each panel. Data points represent stomach volumes calculated from ultrasound images; connecting lines link measurements from the same feed. Bold line represents local regression smoother (LOESS, span = 0.9).

Immediate post-feed stomach volumes were not associated with R1 (\( p = 0.91 \)). After accounting for time post-feed, FV, and feed duration, as per the above model, larger R1 volumes (0.55 [0.24, 0.86], \( p = 0.003 \)) and higher whey protein concentrations (4.99 [0.84, 9.13], \( p = 0.023 \)) were associated with larger post-feed stomach volumes, while the casein:whey ratio (2.2 ± 0.88, \( p = 0.030 \)) and lactose concentration (−0.04 ± 0.02, \( p = 0.037 \)) modified the GE curve depending on
FV. Higher casein:whey ratios at lower FVs were associated with faster GE, and at higher FVs with slower GE, while higher lactose concentrations at lower FVs were associated with slower GE, and at higher FVs with faster GE. No other associations with post-feed stomach volumes or changes to the GE curves were found (Table 3).

<table>
<thead>
<tr>
<th>Table 3. HM components and infant characteristics and their associations with feed variables and gastric emptying.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Predictors</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Concentrations</td>
</tr>
<tr>
<td>Adiponectin (ng/mL)</td>
</tr>
<tr>
<td>Whole milk leptin (ng/mL)</td>
</tr>
<tr>
<td>Skin milk leptin (ng/mL)</td>
</tr>
<tr>
<td>Total protein (g/L)</td>
</tr>
<tr>
<td>Whey protein (g/L)</td>
</tr>
<tr>
<td>Casein (g/L)</td>
</tr>
<tr>
<td>Casein/whey ratio</td>
</tr>
<tr>
<td>Lysozyme (g/L)</td>
</tr>
<tr>
<td>Total carbohydrates (g/L)</td>
</tr>
<tr>
<td>Lactose (g/L)</td>
</tr>
<tr>
<td>HMO (g/L)</td>
</tr>
<tr>
<td>Fat (g/L)</td>
</tr>
<tr>
<td>Doses</td>
</tr>
<tr>
<td>Adiponectin (ng)</td>
</tr>
<tr>
<td>Whole milk leptin (ng)</td>
</tr>
<tr>
<td>Skin milk leptin (ng)</td>
</tr>
<tr>
<td>Total protein (g)</td>
</tr>
<tr>
<td>Whey protein (g)</td>
</tr>
<tr>
<td>Casein (g)</td>
</tr>
<tr>
<td>Lysozyme (g)</td>
</tr>
<tr>
<td>Total carbohydrates (g)</td>
</tr>
<tr>
<td>Lactose (g)</td>
</tr>
<tr>
<td>HMO (g)</td>
</tr>
<tr>
<td>Fat (g)</td>
</tr>
</tbody>
</table>
3.5. Feed Volume, Feed Duration, and Gastric Emptying during Breastfeeding

Higher FVs were associated with higher stomach volumes measured immediately post-feed (0.79 [0.51, 1.07], p < 0.001) and longer GE times (0.24 [0.03, 0.46], p = 0.033). FV was not associated with either concentrations of measured HM components or infant’s characteristics/anthropometrics/BC (Table 3).

Feed duration was not associated with FV (0.06 [−0.03, 0.15], p = 0.20) or R1 volume (0.01 [−0.17, 0.19], p = 0.91).

After accounting for R1 (1.07 [0.47, 1.7], p = 0.002), FV (1.00 [0.71, 1.3], p < 0.001) and feed duration (−0.30 [−0.96, 0.36], p = 0.34), immediate post-feed stomach volumes were not associated with either measured HM components (p > 0.068) or infant’s demographics/anthropometrics/BC (p > 0.46). Stomach volume reduction during breastfeeding was not associated with either measured HM components (p > 0.11); infant’s demographics/anthropometrics/BC (p > 0.48); R1, FV or feed duration (p > 0.34).

3.6. Gastric Emptying Time

The GE time was not associated with feed duration (0.35 [−0.29, 0.98], p = 0.28), but was negatively associated with R2 (−0.63 [−1.05, −0.21], p = 0.005) after accounting for FV (p < 0.001). Longer GE times were associated with higher adiponectin concentration (2.3 [0.9, 3.7], p = 0.002) and dose (0.02 [0.01, 0.03], p = 0.005), and lower casein:whey ratio (−65.9 [−107.1, −24.7], p = 0.003). No associations with infant characteristics were seen (Table 3).

3.7. Pre-Feed Residuals

Infants cued for F1 and F2 with different residual volumes (R1 and R2) present in their stomachs (Table 1). Larger FVs were associated with smaller R1 volumes (p = 0.002), with each −0.92 [−1.47, −0.37] mL of R1 volume resulting in extra mL of FV. Larger R2 volumes were associated with larger FVs (p = 0.006), each additional mL of FV resulting in 0.21 [0.07, 0.35] mL greater R2.

Table 3. Cont.

<table>
<thead>
<tr>
<th>Predictors</th>
<th>Feed Volume a</th>
<th>Gastric Emptying Time b</th>
<th>Post-Feed Stomach Volumes b</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Estimate ± SE (95% CI)</td>
<td>p-Value</td>
<td>Estimate ± SE (95% CI)</td>
</tr>
<tr>
<td><strong>Demographics</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Infant sex (Male)</td>
<td>−2.2 ± 10.7</td>
<td>0.84</td>
<td>−1.5 ± 7.5</td>
</tr>
<tr>
<td></td>
<td>(−23.1, 18.8)</td>
<td></td>
<td>(−16.3, 13.2)</td>
</tr>
<tr>
<td>Infant age (months)</td>
<td>−0.9 ± 3.6</td>
<td>0.80</td>
<td>−1.8 ± 2.5</td>
</tr>
<tr>
<td></td>
<td>(−7.9, 6)</td>
<td></td>
<td>(−6.6, 3)</td>
</tr>
<tr>
<td><strong>Anthropometrics</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Infant length (cm)</td>
<td>−0.03 ± 1.3</td>
<td>0.98</td>
<td>−1.3 ± 0.9</td>
</tr>
<tr>
<td></td>
<td>(−2.6, 2.6)</td>
<td></td>
<td>(−3.0, 0.4)</td>
</tr>
<tr>
<td>Infant weight (kg)</td>
<td>0.7 ± 4.1</td>
<td>0.87</td>
<td>−2.3 ± 2.9</td>
</tr>
<tr>
<td></td>
<td>(−7.4, 8.8)</td>
<td></td>
<td>(−7.9, 3.4)</td>
</tr>
<tr>
<td>Head circumference (cm)</td>
<td>−2.5 ± 2.6</td>
<td>0.34</td>
<td>−1.4 ± 1.8</td>
</tr>
<tr>
<td></td>
<td>(−7.5, 2.5)</td>
<td></td>
<td>(−4.9, 2.1)</td>
</tr>
<tr>
<td>Infant BMI</td>
<td>−0.2 ± 3.2</td>
<td>0.94</td>
<td>−1.5 ± 2.2</td>
</tr>
<tr>
<td></td>
<td>(−6.5, 6)</td>
<td></td>
<td>(−5.8, 2.8)</td>
</tr>
<tr>
<td><strong>Body composition</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fat mass with US (%)</td>
<td>0.6 ± 1.4</td>
<td>0.67</td>
<td>−0.3 ± 0.9</td>
</tr>
<tr>
<td></td>
<td>(−2.2, 3.4)</td>
<td></td>
<td>(−2.2, 1.5)</td>
</tr>
<tr>
<td>Fat mass with BIS (%)</td>
<td>0.4 ± 1.1</td>
<td>0.74</td>
<td>−0.4 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>(−1.8, 2.5)</td>
<td></td>
<td>(−1.9, 1.1)</td>
</tr>
</tbody>
</table>

Data are parameter estimate ± SE and 95% CI, n = 41 feeds. a Effects of predictors taken from univariate regression models; b Effects of predictors taken from linear mixed effects models that accounted for postprandial time, feed volume and feed duration. c After the false discovery rate adjustment the p-values were considered to be significant at <0.011 for GE time (bold font); d n/a—dosage is dependent on feed volume. Abbreviations: BIS—bioimpedance spectroscopy; HMO—human milk oligosaccharides; US—ultrasound skinfolds.
There was no association between R2 and R1 in univariate model (0.11 [−0.19, 0.42], \(p = 0.46\)). After accounting for FV and GE time (\(p < 0.001\) for both) larger R2 volumes were associated with larger R1 volumes (0.36 [0.11, 0.60], \(p = 0.005\)). After accounting for FV, R2 was not associated with any concentration of HM components (\(p ≥ 0.038\) after adjusting for multiple comparisons).

### 3.8. Feeding Frequency

A longer time between the feeds was seen when infants were longer, heavier, and had higher %FM measured with BIS (Table 4) in univariate models. The associations for length and weight were not significant after accounting for the other (\(p > 0.38\)); the association for %FM measured with BIS was not significant after accounting for infant length (\(p = 0.095\)).

**Table 4.** Associations between infant feeding frequency and HM components and infant characteristics.

<table>
<thead>
<tr>
<th>Predictors</th>
<th>Feeding Frequency (h) (a)</th>
<th>(\text{Estimate} ± \text{SE} \ (95% \text{CI}) \ b)</th>
<th>(p)-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Concentrations</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adiponectin (ng/mL)</td>
<td>(-0.001 ± 0.03 \ (−0.06, 0.06))</td>
<td>0.96</td>
<td></td>
</tr>
<tr>
<td>Whole milk leptin (ng/mL)</td>
<td>(-1.1 ± 0.7 \ (−2.5, 0.3))</td>
<td>0.13</td>
<td></td>
</tr>
<tr>
<td>Skim milk leptin (ng/mL)</td>
<td>0.8 ± 1.6 \ (−2.3, 4)</td>
<td>0.60</td>
<td></td>
</tr>
<tr>
<td>Total protein (g/L)</td>
<td>(-0.05 ± 0.05 \ (−0.15, 0.04))</td>
<td>0.28</td>
<td></td>
</tr>
<tr>
<td>Whey protein (g/L)</td>
<td>(-0.1 ± 0.1 \ (−0.3, 0.1))</td>
<td>0.42</td>
<td></td>
</tr>
<tr>
<td>Casein (g/L)</td>
<td>0.04 ± 0.2 \ (−0.4, 0.5)</td>
<td>0.86</td>
<td></td>
</tr>
<tr>
<td>Casein:whey protein ratio</td>
<td>0.4 ± 1.1 \ (−1.7, 2.5)</td>
<td>0.68</td>
<td></td>
</tr>
<tr>
<td>Lysozyme (g/L)</td>
<td>(-0.4 ± 1.1 \ (−2.5, 1.7))</td>
<td>0.71</td>
<td></td>
</tr>
<tr>
<td>Total carbohydrates (g/L)</td>
<td>0.01 ± 0.02 \ (−0.03, 0.04)</td>
<td>0.73</td>
<td></td>
</tr>
<tr>
<td>Lactose (g/L)</td>
<td>(-0.05 ± 0.02 \ (−0.1, −0.01))</td>
<td>0.031</td>
<td></td>
</tr>
<tr>
<td>HMO (g/L)</td>
<td>0.01 ± 0.02 \ (−0.03, 0.04)</td>
<td>0.73</td>
<td></td>
</tr>
<tr>
<td>Fat (g/L)</td>
<td>(-0.02 ± 0.01 \ (−0.04, 0.01))</td>
<td>0.19</td>
<td></td>
</tr>
<tr>
<td><strong>Doses</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adiponectin (ng/mL)</td>
<td>0.0002 ± 0.0003 \ (−0.0004, 0.0008)</td>
<td>0.50</td>
<td></td>
</tr>
<tr>
<td>Whole milk leptin (ng/mL)</td>
<td>(-0.002 ± 0.01 \ (−0.01, 0.01))</td>
<td>0.80</td>
<td></td>
</tr>
<tr>
<td>Skim milk leptin (ng/mL)</td>
<td>0.01 ± 0.01 \ (−0.01, 0.03)</td>
<td>0.59</td>
<td></td>
</tr>
<tr>
<td>Total protein (g/L)</td>
<td>0.1 ± 0.3 \ (−0.5, 0.8)</td>
<td>0.67</td>
<td></td>
</tr>
<tr>
<td>Whey protein (g/L)</td>
<td>0.1 ± 0.7 \ (−1.3, 1.5)</td>
<td>0.89</td>
<td></td>
</tr>
<tr>
<td>Casein (g/L)</td>
<td>2.1 ± 2 \ (−1.7, 5.9)</td>
<td>0.27</td>
<td></td>
</tr>
<tr>
<td>Lysozyme (g/L)</td>
<td>(-5.7 ± 17.2 \ (−39.4, 27.9))</td>
<td>0.73</td>
<td></td>
</tr>
<tr>
<td>Total carbohydrates (g/L)</td>
<td>0.1 ± 0.1 \ (0.02)</td>
<td>0.22</td>
<td></td>
</tr>
<tr>
<td>Lactose (g/L)</td>
<td>0.04 ± 0.06 \ (−0.08, 0.17)</td>
<td>0.49</td>
<td></td>
</tr>
<tr>
<td>HMO (g/L)</td>
<td>0.3 ± 0.1 \ (0.5)</td>
<td>0.051</td>
<td></td>
</tr>
<tr>
<td>Fat (g/L)</td>
<td>(-0.2 ± 0.1 \ (−0.5, 0))</td>
<td>0.085</td>
<td></td>
</tr>
<tr>
<td><strong>Demographics</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Infant sex (Male)</td>
<td>(-0.2 ± 0.3 \ (−0.7, 0.4))</td>
<td>0.53</td>
<td></td>
</tr>
<tr>
<td>Infant age (months)</td>
<td>0.2 ± 0.1 \ (0.3)</td>
<td>0.078</td>
<td></td>
</tr>
<tr>
<td><strong>Anthropometrics</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Infant length (cm)</td>
<td>0.1 ± 0.03 \ (0.04, 0.15)</td>
<td>(0.004^c)</td>
<td></td>
</tr>
<tr>
<td>Infant weight (kg)</td>
<td>0.2 ± 0.1 \ (0.1, 0.4)</td>
<td>(0.010)</td>
<td></td>
</tr>
<tr>
<td>Head circumference (cm)</td>
<td>0.1 ± 0.1 \ (0, 0.2)</td>
<td>0.23</td>
<td></td>
</tr>
<tr>
<td>Infant BMI</td>
<td>0.13 ± 0.1 \ (0, 0.3)</td>
<td>0.10</td>
<td></td>
</tr>
<tr>
<td><strong>Body composition</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% fat mass with US</td>
<td>0.07 ± 0.03 \ (0.13)</td>
<td>0.040</td>
<td></td>
</tr>
<tr>
<td>% fat mass with BIS</td>
<td>0.08 ± 0.02 \ (0.03, 0.12)</td>
<td>(0.002)</td>
<td></td>
</tr>
</tbody>
</table>

Data are parameter estimate ± SE and 95% CI, \(n = 41\) feeds. \(a\) Feeding frequency self-reported by mothers as to how often infant feeds (e.g., every three hours). \(b\) Effects of predictors are results of univariate regression model. \(c\) After the false discovery rate adjustment the \(p\)-values were considered to be significant at \(<0.031\) (highlighted). Abbreviations: BIS—bioimpedance spectroscopy; HMO—human milk oligosaccharides; US—ultrasound skinfolds.
4. Discussion

Our research shows that HM components, such as adiponectin, whey protein, casein:whey ratios, lactose, total carbohydrates, and oligosaccharides are associated with gastric emptying and breastfeeding patterns of breastfed infants. GE is a mechanism involved in satiety, therefore milk components influencing GE have the potential to affect infant milk intake and therefore growth and development in early life and subsequently health later in life.

Given the assumption that HM composition potentially influences GE [14,71], in term infants we expected the appetite hormones to be associated with infant GE rate such that high concentrations and/or doses of leptin would result in slower GE [30], whereas adiponectin might induce faster GE [37] consistent with both animal and human models. However, neither the concentrations nor doses of these hormones were related to GE rate. Previously skim milk leptin was not found to be associated with either GE rate or GE time [33,34], which we have confirmed with this larger study cohort. It was speculated that whole milk leptin, which is known to be of higher concentration, might be the reason for the negative finding [32]. While our measures of whole milk leptin were typically higher, there is an opinion that values of this magnitude are unlikely to contribute considerably to infant serum levels [72] so only the local pathways would be engaged in GE regulation. As such we were unable to find a relationship between whole milk leptin and GE. This is in contrast to animal studies showing reduced GE [30] or food intake [31] after injection or oral administration of leptin, respectively. However, it is possible that the long-term energy expenditure regulatory effect of leptin [73] may mask its short-term satiety effect on GE. Alternatively, if levels of leptin are contributing significantly to serum levels, there is a possibility that the number of receptors in the stomach of the young infant is low. Further, short-term satiety signaling through hypothalamic neurons is not fully mature, both of which would allow the infant to maintain a high physiological drive to feed to ensure adequate growth [28,73]. Gender differences in infant serum leptin levels associated with adiposity [74] have also been speculated to play a role in gastric response to HM leptin, although we did not find any relationships between infant sex/adiposity and both GE rate and GE time.

In contrast to leptin, we found that increased levels and doses of adiponectin were associated with longer GE times. This finding may partially explain the growth-regulating effect of adiponectin in infants in the first six months of life [29], when high HM adiponectin concentration is associated with lower infant weight and adiposity. Further adiponectin is 20-fold higher in concentration compared to leptin and is therefore likely to have greater biological significance [75]. The lack of association between adiponectin and GE rate is in agreement with studies of rats that showed that gastric epithelium and glands are populated with adiponectin receptors, which downregulate gastric motility [36,76]. Conversely, the findings are in contrast to studies of type 2 diabetic adults, in which elevated levels of adiponectin were associated with faster GE [37]. Further, other HM hormones such as ghrelin, cholecystokinin, and insulin may counteract or interact synergistically with leptin [77,78] and/or adiponectin.

Our study examined an extensive array of macronutrients beyond fat, total protein and lactose. Consistent with the findings of Cannon et al. [34] there were no associations with fat and total protein and either GE rate or GE time. Studies in dogs indicate that all three major macronutrients activate the ileal brake, resulting in reduction of GE; limited human studies support the findings for fat and carbohydrates while associations with protein are not so straightforward [79,80]. We were unable to find associations between the HM fat content and GE consistent with findings of Khan et al. [39] and Kent et al. [38] regarding the feeding frequency. This may be because lipids initiate the ileal brake when they reach the ileum via hydrolysis of triacylglycerol into fatty acids, thereby producing a delay in GE in humans [79]. Further analysis of HM fatty acids may shed more light on GE in breastfed infants.

However, we have found that higher whey protein concentrations are associated with larger post-feed stomach volumes, although we did not see any interaction with time, so no effect on GE rate was detected. This contradicts the results of studies of GE conducted on breastfed and formula-fed infants or studies of formula with different casein:whey ratio [15,81] in which a fast or slow GE rate
was explained by concentrations of whey protein or casein, respectively. Previous studies, however, could not adequately analyze the effect of the whey protein concentration in conjunction with volume, as they only reported gastric half-emptying time, restricted monitoring time, and/or controlled infants’ volume intakes. The whey fraction of HM is highly soluble in the gastric juices and rapidly empties from the stomach compared to other proteins such as casein. Whey isolate, however, was associated with a lower gastric inhibitory polypeptide (GIP) response in adults, consistent with decreased rate of GE [82]. It may very well be that whey protein speeds up the initial stage of GE (probably during the breastfeeding time), but once it activates jejunal or ileal brakes the overall GE is reduced.

While lactose is related to GE rate, it is affected by FV; at the middle range FVs (71–108 mL) lactose has no relationship with GE, whereas at lower FVs higher lactose concentrations are associated with slower GE, and at higher FVs with faster GE. These results are consistent with Khan et al. [39], who reported an association of higher lactose concentration with increased feeding frequency. These findings could be an important addition to the evaluation of the digestive and metabolic effect of lower breastfeeding frequency and larger FVs, common in Western countries, contrary to the lactation practices in traditional societies [83].

In terms of casein:whey ratios the effect is opposite to that of lactose where at lower FVs higher casein:whey ratios are associated with faster GE, and at higher FVs with slower GE, which may explain the contradictory findings for casein associations with GE rate in previous studies [14,23]. Cows’ milk casein was found to activate the ileal brake in adults, resulting in reduced food intake, although its effect on GE was not significant [84]. The finding of smaller volumes resulting in more rapid GE rate might be explained by the time casein spends in the acidic environment of the stomach. While soluble whey proteins rapidly enter the small intestine mostly intact, casein transit is delayed due to the curd formation. When it exits the stomach it is mainly in the form of degraded peptides [85]. If the FVs are small some casein may exit intact, thereby speeding up GE, while if the FVs are large, casein curdles and degrades to the opioid peptides that slow down GE [86]. However, this mechanism does not explain why higher casein:whey ratios of HM were associated with shorter GE time, which could be due to the smaller amounts of whey protein reaching the small intestine and having less effect on jejunal or ileal brakes [80]. Our finding that higher whey protein concentrations are associated with larger post-feed stomach volumes further supports this possible explanation.

Further, k-casein has been shown to inhibit the binding of Helicobacter pylori to human mucosa in vitro [87]. Helicobacter pylori are Gram-negative bacteria present in the stomach, and are known to downregulate levels of ghrelin and leptin in the stomach [88], which may significantly affect GE. The protective action of HM k-casein is reinforced by lysozyme, one of the major whey proteins. While we have not seen any significant associations between lysozyme and GE, lysozyme contributes to the control of the GI bacterial population [89], and could be upregulated to control the bacterial population in the GI tract [90] and increase digestion of microbial protein [24], all of which could potentially influence GE. In a clinical study of preterm infants, lysozyme added to donor HM or formula was associated with increased body weight, normalization of the stool, and improved feed tolerance [91]. While all of this suggests that lysozyme could potentially have an effect on GE in certain circumstances, given that we have studied a healthy population the magnitude of the effect could be insignificant.

GE during feed administration has been previously documented in preterm infants. In this study, an average of 20% of feed volume is emptied from the stomach during breastfeeding compared with 10% in preterm [92]. This is probably due to a more mature GI tract in term infants and the effect of both larger FVs and present pre-feed residuals, which were associated with faster GE rate, but not to the longer feed duration time in term infants or milk composition as no associations were found.

While we speculated that milk composition might regulate the milk intake of the infant and/or the residual volume in the stomach prior to cueing for the next feed, we were unable to show this. Rather, FV is more strongly associated with GE rate than variations in milk composition. Gastric mechanosensation is an important factor in the regulation of satiation during food intake. Indeed, gastric distention is an important determinant of GE [93], and volume-related suppression of GE rate
has been reported in animal models [94]. The observed volume-related acceleration of GE with larger FVs emptying more quickly in term breastfed infants is consistent with our previous findings [34]. The biggest effect of volume was seen after the feed and as the post-prandial period progressed the magnitude of this effect decreased (Figure 2). This may also explain the variability in the time between each feed for an infant over a 24-h period [33,38]. Feeding frequency decreases between one and three months of lactation, while milk intake during each breastfeeding session increases, with both parameters remaining constant up to six months [95]. This is attributed to the fact that as infants mature they become able to consume larger FVs [38], resulting in a longer time between feeds. Also, larger FVs are generally consumed at night or in the early morning when the frequency of feeding declines [33,38]. This decline in feeding frequency also coincides with higher nocturnal concentrations of leptin and fat, and lower concentrations of lactose in HM [33], although relationships between both feeding frequency and FV and these components’ concentrations are yet to be evaluated.

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The recommendations for breastfeeding are to feed on demand. Interestingly, we found that the majority of infants cued for a feed when milk was still present in the stomach, albeit in variable volumes (Table 1). This suggests that the reduction of gastric distension, which regulates hunger sensations, plays a greater role in signaling time to feed [96]. Further, it may be beneficial to the developing infant to have the gastric mucosa exposed to HM anti-inflammatory components such as lysozyme or immunomodulatory agents and growth factors, all of which contribute to the maturation of the GI tract [8]. Thus it may be detrimental to prescribe decreasing the frequency of feeding in breastfed infants or expect the infant stomach to be empty in order to feed again [97].

Furthermore, interesting associations were observed between infant milk intake and volumes remaining in the stomach prior to the first and second feed. Smaller residual volumes prior to the first feed were associated with greater milk intakes, and greater milk intakes were associated with larger volumes in the stomach prior to feeding again. This suggests that breastfed infants may appear to be consuming HM volumes in a variable pattern, but due to varying residuals may actually be feeding to a predetermined stomach volume, which is also supported by the positive relationship between both pre-feed residuals (R1, R2). In fasting adults ghrelin was found to increase and spontaneously decrease at the time points of the customary meals [98], supporting the involvement of the brain in GI tract regulation. Further studies monitoring two or more consecutive feeds or even 24-h GE measurements and analyses of ghrelin in HM would clarify this finding.

In healthy adults post-lag GE and colonic transfer is reported to be faster in men than in women [99]. In this study infant sex, age, anthropometrics, and BC were not associated with GE and breastfeeding parameters, with the exception of feeding frequency. Feeding frequency decreases in the first three months of lactation and then remains stable until six months [95,100]. The absence of a significant association between feeding frequency and age, together with associations with anthropometric and body composition parameters, illustrates that feeding frequency is dictated by the growth and development of an infant rather than the infant age. These findings further underline the need for breastfeeding on demand, with the frequency linked to individual infant growth rates rather than scheduled feeding, which could exert a detrimental effect on infant growth.

While the monitoring of a single feed limits the analysis possibilities, examination of multiple feeds requires the study to be carried out in the mother’s home for long periods of time. The sample size is not a limitation of the study, as although no associations between milk composition and GE rate were detected, we were able to clearly show a relationship between FV and GE rate as well as associations between milk composition and other GE parameters.

5. Conclusions

Human milk appetite hormones and macronutrients and feed volume affect gastric emptying and feeding patterns in term breastfed infants. Adiponectin, whey protein, and casein:whey ratio are associated with GE, while the effects of casein:whey ratios and lactose concentrations on GE vary with
feed volume. Larger feed volumes result in a faster GE rate. Thus, milk composition and feed volume play an important role in appetite regulation via gastric function.

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Author Contributions: Zoya Gridneva conceived and designed of the study, collected the data, performed biochemical analysis, performed the statistical analyses, and wrote the manuscript; Sambavi Kugananthan adapted the methods for measuring adiponectin and leptin in whole milk, performed the hormone analysis, and critically reviewed the manuscript; Anna R. Hepworth was involved in research design, data analysis, and interpretation, and critically reviewed the manuscript; Wan J. Tie performed biochemical analysis and critically reviewed the manuscript; Ching T. Lai performed biochemical analysis, provided technical support, and critically reviewed the manuscript; Leigh C. Ward provided technical and educational support, contributed to the design of the study, and critically reviewed the manuscript; Peter E. Hartmann substantially contributed to the conception and design of the study, contributed reagents/materials/analysis tools for research, and critically reviewed the manuscript; Donna T. Geddes conceived and designed of the study, carried out the ultrasonography component of the study, provided the ultrasound images, and critically reviewed the manuscript. All authors have read and approved the final manuscript as submitted and agree to be accountable for all aspects of the work.

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