

DETERMINANTS OF HUMAN MILK
COMPOSITION:
IMPACT OF METHODOLOGY, MATERNAL
OBESITY AND DIETARY INTAKE

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ABSTRACT

Human milk (HM) is uniquely designed for the infant, containing the nutrients and bioactive components that are required to support growth and development, and is known for its important role in infant health. However, it is becoming increasingly clear that the composition of HM varies considerably between mothers, and that these differences may affect the short and longer-term health of the child.

The composition of HM varies across lactation and even within a single feed, and as a result the method of HM sample collection can have a significant impact on the results of compositional analyses and complicate comparisons between studies. Therefore, the first part of this thesis details the design and outcomes of a systematic review describing the different collection methodologies applied in studies assessing HM composition and comparing the results obtained for HM macronutrient composition. The results of this systematic review were used to inform development of recommendations for a robust, consistent and practical sampling approach as an alternative to pooled 24-h collections – which has been referred to as the ‘gold standard method’, but which carries a relatively high participant burden and is impractical for large population studies. In addition to the method of collection (pre-feed, post-feed or pooled samples collection), it was also unclear to what extent HM macronutrient concentrations changed over periods of days to weeks and according to collection breast, and thus what frequency of sampling would be required to obtain representative results for HM composition of an individual. The next chapter aimed to complement the previous study by systematically evaluating the impact of day of collection during the week, time of day of collection and collection breast on HM macronutrient concentrations. The results of this study suggested that the pragmatic approach of collecting pre-feed samples at the same time point during the day,

and at one time point over a 3-week period, provided a reliable estimate of mature HM composition for an individual, irrespective of the breast of collection.

Previous studies had provided evidence that maternal overweight and obesity were potentially associated with alterations in HM composition, and that these may have a role in the intergenerational cycle of obesity. However, the extent of these differences across the full complement of nutritional factors and metabolic hormones in HM was unclear. The second part of this thesis reports the results of a systematic review and meta-analysis describing the effects of maternal obesity on the levels of macronutrients and metabolic hormones in HM. These studies suggest that maternal overweight and obesity are associated with altered concentrations of fat, lactose and key metabolic hormones in HM.

While these findings suggest that maternal obesity can impact HM composition, the mechanisms underlying these changes, in particular whether they were driven by metabolic or dietary factors was unclear. Further, the impact of changes to specific components of the maternal diet while breastfeeding on HM composition was unclear. To address this knowledge gap, we undertook a proof of concept clinical trial to assess the effect of improving dietary quality during breastfeeding on the concentrations of macronutrients and metabolic hormones in HM. The study indicated that significant reductions in maternal energy, carbohydrate, fat and sugar intake over a 2-week period were effective in reducing maternal weight and body fat mass and were associated with reduced concentrations of leptin, insulin and adiponectin in HM, but did not affect HM macronutrient composition, milk production or infant growth.

In conclusion, this thesis has made a significant contribution to the field of HM research. The research has provided evidence-based recommendations as to a HM sampling protocol for

improving replicability and consistency between studies, a contemporary synthesis of information on the impact of maternal obesity on HM composition and novel direct evidence of the impact of improved maternal diet quality on HM composition and production. These data are critical given the established importance of nutritional exposures in early infancy for an individual's life-long health outcomes, and therefore the potential impact of altered HM composition on long-term health of the child.

DECLARATION

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that 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 Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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Gabriela Esteves Leghi Voyer

LIST OF PUBLICATIONS

Leghi GE, Middleton PF, Muhlhausler BS. A methodological approach to identify the most reliable human milk collection method for compositional analysis: A systematic review protocol. *Systematic reviews*. 2018 Dec 1;7(1):122.

Leghi GE, Netting M, Muhlhausler BS. The short-term impact of dietary fat and sugar intake on breast milk composition: A clinical trial protocol. *Nutrition and Health*. 2020 Mar;26(1):65-72.

Leghi GE, Middleton PF, Netting MJ, Wlodek ME, Geddes DT, Muhlhausler BS. A systematic review of collection and analysis of human milk for macronutrient composition. *The Journal of Nutrition*. 2020 Apr 2;00:1-19.

Leghi GE, Netting MJ, Middleton PF, Wlodek ME, Geddes DT, Muhlhausler BS. The impact of maternal obesity on human milk macronutrient composition: A systematic review and meta-analysis. *Nutrients*. 2020 Apr;12(4):934.

CONFERENCE/SEMINAR PRESENTATIONS

ORAL PRESENTATIONS

“A methodological approach to identify the most reliable human milk collection method for compositional analyses: A systematic review” presented at Nutrition Society of Australia 43rd Annual Scientific Meeting, Newcastle, Australia (2019).

“What is the role of maternal obesity and diet in human milk composition?” presented at Australian Institute of Food Science and Technology Summer School, The University of Adelaide, Adelaide, Australia (2019).

“The short-term impact of dietary fat and sugar intake on breast milk composition” presented at Developmental Origins of Health and Disease Australia and New Zealand Conference, Sydney, Australia (2018).

“Breast milk hormones: why do we care?” presented at South Australian Health and Medical Research Institute Annual Scientific meeting, 3-minute thesis, Adelaide, Australia (2017).

“The effect of sampling methodology on the composition of breast milk: A systematic review” presented at the Postgraduate Symposium, School of Agriculture, Food and Wine, The University of Adelaide, Adelaide, Australia (2017).

“Improved understanding of human breast milk composition and role in nutritional programming” presented at Inaugural Trainee Forum, Women’s and Children’s Hospital, Adelaide, Australia (2017).

“Improved understanding of human milk composition and nutritional programming” presented at Children Nutrition Research Centre/FOODplus Early Career Research network event, South Australian Health and Medical Research Institute, Adelaide, Australia (2016).

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LIST OF ABBREVIATIONS

AA	Arachidonic acid
BIS	Bioelectrical impedance spectroscopy
BMI	Body mass index
BMIZ	BMI Z-score
CDI	Calculated daily intake
CI	Confidence interval
DHA	Docosahexaenoic acid
DPA	Docosapentaenoic acid
DXA	Dual-energy X-ray absorptiometry
EPA	Eicosapentaenoic acid
F	Female
GDM	Gestational diabetes
GLP-1	Glucagon-like peptide 1
HM	Human milk
HMO	Human milk oligosaccharides
IDQ	Index of diet quality
IgA	Immunoglobulin A
IUGR	Intrauterine growth restriction
LCPUFA	Long chain polyunsaturated fatty acids
LB	Left breast
M	Male
MD	Mean difference
MiRNAs	MicroRNAs

NOS	Newcastle-Ottawa scale
PUFA	Polyunsaturated fatty acids
PRISMA	Preferred reporting items for systematic reviews and meta-analysis
PRISMA-P	Preferred reporting items for systematic reviews and meta-analysis protocols
PYY	Peptide YY
RB	Right breast
WLZ	Weight-for-length Z-score
WHO	World Health Organization

CHAPTER 1. INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

The nutritional environment during the fetal and early postnatal period is an important determinant of an infant's future risk of obesity and metabolic diseases. Human milk (HM) is a critical factor in this relationship, since it contains essential nutrients and bioactive molecules known to promote infant health and survival and provides the sole source of nutrition for exclusively breastfed infants in the first few months after birth [1,2]. The World Health Organization (WHO) recommends that infants should be exclusively breastfed until 6 months' postpartum, with continued breastfeeding to 24 months of age and beyond [3]. There is compelling evidence that following the WHO breastfeeding recommendations reduces the incidence of gastrointestinal and respiratory infections and lowers infant mortality, particularly in low-income countries [4,5].

HM contains a massive array of components, all of which are thought to play a role in meeting the infants' energy and nutrient requirements. Macronutrients, including fat, protein and lactose, provide the major sources of energy and essential building blocks (e.g. amino acids) to support infant growth and development [6]. One of the more recently identified HM components are the metabolic hormones, including ghrelin, adiponectin, leptin and insulin [7-10]. These metabolic hormones form a major focus of this thesis, given their important role in regulating appetite and metabolism, and their potential role in contributing to growth and fat deposition in the infant [11-13]. There is also emerging evidence that the levels of key macronutrients (fat, protein and lactose) in HM may be related to infant growth and adiposity [11]. Consequently, variations in the levels of HM macronutrients and metabolic hormones

between women have the potential to influence the long-term health of the infant, including their risk of obesity and metabolic diseases. This has led to increasing interest in understanding the factors which determine HM composition.

The composition of HM varies considerably between individual women, and also changes markedly within women over the course of lactation and, in the case of some components, over the course of a single day [14-16]. A large body of literature has shown changes in fat and protein concentrations across the course of lactation, and there is emerging evidence that leptin and adiponectin concentrations in HM also decline as lactation progresses [17-19]. In the case of fat content, concentrations also appear to exhibit diurnal variation, and increase up to 3-fold between the beginning and end of a breastfeed or expression [20,21]. As a result, the method of HM collection can have a major influence on the concentrations of macronutrients, and potentially other bioactive factors, measured in the HM, and differences in HM collection methods can complicate comparisons between studies. Thus, there is a need for a standardised collection method for researchers undertaking compositional analyses of HM.

In addition to stage of lactation, gestational age at which a woman gives birth, the sex of the baby and whether the infant is exclusively or partially breastfed [11,22-24], there is growing evidence that maternal overweight or obesity is associated with alterations in HM composition [19,22,25]. The concentration of macronutrients, along with some bioactive factors in HM, including leptin, has been shown to be different in HM from obese mothers compared to those of normal weight [19,26]. The reason for the difference in HM composition between normal weight and overweight and/or obese women and to what extent these are related to maternal metabolic disturbances, maternal diet or even a combination of both, is currently unclear. While

maternal diet is generally considered to be an important determinant of HM composition, direct evidence of this is surprisingly sparse and while there are strong relationships between fatty acid intake in maternal diet and fatty acid composition in HM [27], the role of other dietary factors in determining HM composition is poorly understood.

The following chapter will provide a summary of the unique aspects of HM composition, with a specific focus on the macronutrients and metabolic hormones. This will include a discussion of the current knowledge of how HM composition changes across lactation, the role of different HM components in short-term growth and long-term health outcomes of the infant and determinants of HM composition, including maternal factors. Finally, the chapter will identify the key knowledge gaps to be addressed in this thesis and outline the specific thesis aims.

1.2 Human milk composition - key components

HM contains an extremely wide variety of different factors, all of which are thought to have a role in supporting infant growth and development. In addition to macronutrients (fat, protein and lactose), HM contains a multitude of bioactive and immunological factors, metabolites, appetite-regulating molecules, fatty acids, micronutrients and cells, human milk oligosaccharides (HMO) and bacteria (the HM microbiome), all of which have specific biological roles [28-30] (Figures 1.1 and 1.2). The following section will discuss the role of each of these HM components, and what is currently known about how their concentrations change across lactation. Emphasis will be placed on the macronutrients and metabolic hormones, which are the primary focus of this thesis.

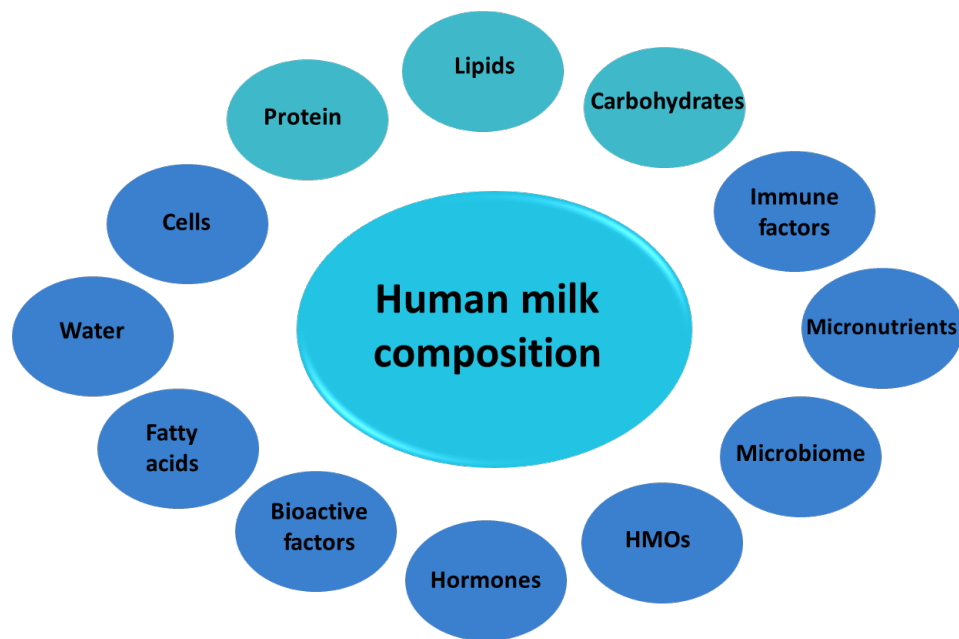


Figure 1.1. Summary of human milk components¹

¹HMO, human milk oligosaccharides

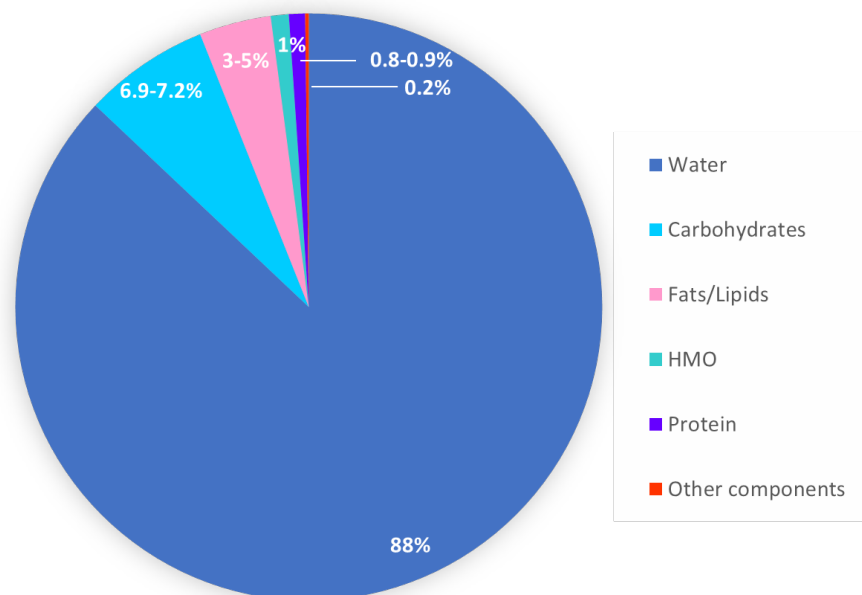


Figure 1.2. Distribution of human milk components by percentage volume^{1,2}

¹HMO, human milk oligosaccharides

²Sources [6,31,32]; other components include bioactive and immunological factors, metabolic hormones and micronutrients

1.2.1 Changes in HM composition across lactation

HM composition changes from the moment production begins and secretion of early milk occurs until breastfeeding ceases. Lactation, and HM type, is divided into 3 stages – colostrum, transitional milk and mature milk, each of which have different roles in supporting infant growth and development [6]. There is a substantial change in HM composition over the first 2 weeks postpartum, as HM shifts from colostrum to transitional milk. While changes in composition are less pronounced later in lactation than in the first 2 weeks postpartum, HM composition is still far from constant, and changes in both macronutrient concentrations and levels of key bioactive factors continue across the remainder of the lactation period in response to maternal and infant factors, including the frequency of feeding [6,33]. Table 1.1 summarises the different stages of HM including key compositional features at each lactation stage.

Table 1.1. Features of milk types across lactation¹

Type of milk	Characteristics	Timing
Colostrum	Main role: immunological High concentrations of sodium, chlorine, magnesium and total protein, including immunoprotective components secretory IgA, lactoferrin, leukocytes and epidermal growth factor. Low concentrations of lactose, potassium, citrate and calcium	From second half of pregnancy to 4 days postpartum
Transitional milk	Main role: to support the nutritional and developmental needs of the rapidly growing infant The concentration of protein and immunological factors, sodium, and chloride decreases, while the concentration of fat, lactose, citrate, and potassium increases.	From 5 days to 2 weeks postpartum
Mature milk	Main role: to support nutritional and developmental needs of the infant Relatively consistent in composition, but some changes occur across the lactation period.	From 2 weeks postpartum

¹Sources [6,33,34]; IgA, immunoglobulin A

1.2.2 HM components: macronutrients

The key macronutrients in HM are fat, protein and lactose, and together these comprise the major constituents of HM [25]. Fat, protein and lactose provide the main sources of energy and building blocks to support infant growth and development. In addition, the macronutrients in HM also contribute to developmental and immunoprotective processes [6].

Table 1.2 provides an overview of average values and ranges of typical macronutrient composition of mature full term HM, although it should be noted that there is considerable variation in levels between studies conducted in different countries and according to collection method [25,35].

Table 1.2. Variations in mature full term human milk macronutrient composition¹

Macronutrient	Expected average concentration (g/L)	Range (g/L)
Fat	36	32-40
Protein	9.5	7-12
Lactose	72.4	67-78

¹Sources [33,36]**1.2.2.1 Fat**

Fat makes up between 3 and 5% of HM by volume [32] and provides ~50% of HM energy [37]. It is also the macronutrient which is most variable between women and populations [38-41]. The fat content of HM varies across lactation [14,42] and over the course of the day within an individual woman, with higher concentrations reported at mid-morning [20,32,43] and lower levels overnight [20]. In addition, fat content in HM increases significantly (up to 3-fold) from the beginning to the end of a breastfeed or expression [20,44,45], and this is thought to play an important role in regulating feeding behaviour and development of the appetite control system in the breastfed infant [46]. Feeding-related factors, such as volume of milk removed from the breast and feeding frequency, can also influence the fat content of HM [20,47].

The fat component of HM is comprised of over 200 types of individual fatty acids (FA), which collectively make up ~85% of the triglycerides in HM [48]. The major fatty acids in HM include the omega-3 and omega-6 long chain polyunsaturated fatty acids (LCPUFA), docosahexaenoic acid (DHA), arachidonic acid (AA), eicosapentaenoic acid (EPA) and docosapentaenoic acid (DPA). Their main role in HM is to support development and growth in the infant. The omega-3 LCPUFA in HM have a particularly important role in supporting the development of infant's visual and central nervous system, and DHA is the predominant fatty acid in the cellular membranes of the infant brain and retina [49].

1.2.2.2 Protein

Protein levels in HM also vary significantly between mothers, although not to the same extent as fat content [39]. Protein makes up between 0.8 and 0.9% of HM by volume [32] and provides around 8% of the infant's energy requirement [50]. The average protein content in HM decreases over the course of lactation [16,44,51,52], especially from colostrum and transitional HM to mature milk [53,54]. This has been demonstrated in a number of studies; in a 2019 multicentre European study with 367 women it was reported that protein content decreased progressively over the first 3 months postpartum and between HM samples collected between early (16-100 days) and late (101-163 days) lactation [16], and similar findings were reported in other studies [19,53,54]. Unlike fat content, it appears that protein content in HM within individual women does not vary across the day, nor differ between breasts and is stable across a single feed, at least in HM from mothers of term infants [19,21,39].

The protein in HM is comprised of casein and whey protein fractions, each of which has distinct roles in supporting infant growth and development. Casein provides essential amino acids, minerals and peptides to the infant, whereas the whey protein, of which lysozyme is one of the major components, acts to eliminate foreign bacteria from HM and thereby protect the infant from infections [55]. The ratio of whey protein to casein protein is ~60:40 for most of the lactation period, although the concentration of both these proteins varies considerably in the first few days postpartum. Thus, whey protein content in HM is higher in early lactation, and decreases as lactation progresses and the infant immune system develops [56]. In addition to its role in supporting infant growth and providing protection against disease, there is evidence that the protein in HM may also have a role in regulating infant feeding, since the levels of both whey protein and casein protein, as well as total protein concentrations, have been reported to

be negatively correlated with the frequency of breastfeeds over a 24-h period. This has led to suggestions that the protein content of HM may participate in the development of satiety signals and programming of appetite in the infant, although there is currently limited direct evidence to support this speculation [21,57].

1.2.2.3 Lactose

Lactose is the major carbohydrate in HM, making up ~98% of the total carbohydrate content, between 6.9 and 7.2% of HM by volume and contributing ~40% of infant's energy requirement [32,58,59]. Lactose is responsible for maintaining adequate osmotic pressure in HM and improving calcium absorption in infants [60,61]. In addition, the disaccharide breaks down to glucose and galactose, which are both used as energy in the brain. Galactose is also required for the synthesis of galactolipids, which are critical for the development of central nervous system [62]. The levels of lactose in HM are considered the least variable among the macronutrients [33,39], with levels appearing to be stable both across lactation [25,53] and across a single feed [19], and exhibiting no diurnal variation. Lactose content does, however, vary between women and in relation to the volume of HM produced [63]. Thus, women who produce higher volumes of milk have higher HM lactose concentrations, at least in mature milk collected at 6 and 9 months postpartum [38].

1.2.3 HM components: metabolic hormones

In addition to the nutritional components, HM contains a wide range of other bioactive compounds. This thesis has a particular focus on the key metabolic hormones in HM given the critical role of these factors in regulating energy balance and appetite in child and adult life [64-67]. Importantly, studies have demonstrated that these compounds are transferred from HM to

the infant, which provides a plausible mechanism through which they could potentially regulate infant feeding behaviour and/or growth [68,69]. The most abundant metabolic hormones in HM are leptin, adiponectin, insulin and ghrelin, the roles of which will be discussed below.

1.2.3.1 Leptin

Leptin is a peptide hormone which plays a critical role in the regulation of appetite and energy balance by acting to suppress hunger signals, and thereby reduce appetite [70,71]. While this hormone is produced predominately by white adipose tissue, it is also synthesised by the placenta and by mammary epithelial cells [72]. Leptin levels in the circulation are directly related to body fat mass and leptin receptors are expressed in a number of tissues, including the placenta and adipose tissue [73]. The highest abundance of leptin receptor expression is in the brain, where leptin acts to stimulate energy expenditure, by activating sympathetic outputs to brown adipose tissue, and reduce appetite, by increasing the expression of anorexigenic and simultaneously decreasing the expression of orexigenic neuropeptides [74,75].

Leptin was first identified in HM in 1997 [7], and since then there has been considerable interest in understanding the role it may play in infant growth, development and body fat mass [12,76]. The levels of HM leptin vary considerably between studies, and between individual women in the same study, however the source of this variation remains unclear. Some studies have suggested that leptin concentrations in HM decrease progressively across lactation (from colostrum to 12 months postpartum) in healthy women [17-19], while another study has reported no change in HM leptin between 1 week and 3 months postpartum [26]. In addition, leptin concentrations in HM are positively associated with circulating leptin levels [23,77,78],

suggesting that variation could be, in part, due to changes in maternal leptin concentrations/adiposity.

Differences in the volume of HM produced and the fat content of HM have also been suggested to contribute to variations in HM leptin. One study reported a positive association between feed volume and dose of leptin consumed by the infant [45]. However, other studies reported no associations between whole or skim leptin concentrations and either breastfeeding patterns or gastric emptying rate in the infant [79,80]. In relation to HM fat content, one study reported that HM leptin positively correlated with HM fat content in post-feed samples collected at 1 week postpartum [26], while other studies found no correlation between HM leptin or changes in HM leptin pre- to post-feed and HM fat concentrations in either colostrum or mature HM samples [45,81]. It has also been suggested that the substantial variation in leptin concentrations between studies could be related to differences in sample treatment, sample type (whole vs skim milk samples) and analytical method [82,83]. In support of this, leptin levels are consistently higher in whole milk when compared to skim milk samples, and this is thought to be due to potential artefacts created by interaction between the hormone and free cells and lipids within the HM sample [83].

1.2.3.2 Adiponectin

Adiponectin plays a major role in the regulation of energy metabolism [84], lipid and glucose metabolism and insulin sensitivity in both children and adults, particularly the regulation of hepatic and skeletal muscle glucose metabolism [85]. Adiponectin has also been shown to have the capacity to regulate food intake and energy expenditure [65], and circulating adiponectin

levels are inversely associated with weight and body mass index (BMI) in lactating women [86]. Interestingly, adiponectin concentrations in HM are significantly higher than concentrations of any other metabolic hormone, including leptin [9,76], which has led to suggestions that this hormone is likely to have an important role in supporting infant growth and development. To date, however, definitive evidence as to its role is lacking, although relationships between HM adiponectin concentrations and infant growth/adiposity indicate a potential involvement in the regulation of growth and fat deposition [13,87].

The source of adiponectin in HM is also unclear, but since concentrations in HM are positively correlated with those in the maternal blood [23,86,88], it appears that adiponectin is most likely synthesised outside the mammary gland and transferred into HM from the maternal circulation. As with leptin, concentrations of adiponectin also vary between women and have been reported to decrease across lactation [9,17,18] however, the source of this variation remains to be elucidated.

1.2.3.3 Insulin

Insulin plays a central role in regulating blood glucose homeostasis, liver metabolism and infant intestinal maturation [8,89]. Circulating insulin levels are positively correlated with maternal BMI and are lower than insulin concentrations in HM [17]. As a metabolic hormone, insulin has the potential to influence infant appetite, metabolism, growth and body fat deposition, however the role of HM insulin in these processes is not well understood [90].

Insulin concentrations in HM appear to decrease significantly from colostrum to mature HM [67,91] however, no differences are found between transitional to mature HM [26] or across the

remainder of lactation [22]. Insulin concentrations in HM have also been reported to be related to HM fat content, although the direction of this relationship appears to vary according to the stage of lactation. Thus, in one study insulin concentrations in colostrum were negatively correlated with HM fat content [91], while insulin concentrations in transitional HM were positively correlated with HM fat content in both pre-and post-feed samples [26]. Maternal metabolic disorders, in particular the presence of maternal insulin resistance, glucose intolerance or diabetes, have also been associated with alterations in HM insulin, with higher HM insulin levels identified in women with these metabolic conditions [18,91]. This suggests that insulin may be transferred from the maternal circulation into the HM of these women, with potential impacts on infant growth and development.

1.2.3.4 Ghrelin

Ghrelin, an orexigenic hormone produced mainly by the stomach [92], is believed to promote feeding behaviour and exert effects on long-term regulation of body weight and energy metabolism [93]. Besides the regulation of appetite, ghrelin is also responsible for gastric acid secretion, food intake and gut motility [93]. Ghrelin is present at lower concentrations in HM compared to the other metabolic hormones discussed in this section. Nevertheless, it has been suggested that changes in the levels of this hormone may still contribute to the regulation of appetite and feeding behaviour in the infant. In support of this, ghrelin concentrations in mature HM are higher before compared to after a feed, suggesting that this hormone may contribute to the initiation and termination of feeding [26]. However, direct evidence of the role of HM ghrelin in infant feeding behaviour and growth is lacking. The source of ghrelin in HM is also unclear. Some studies have suggested that ghrelin in HM is derived predominately from synthesis by the mammary epithelial cells [92]. However, another study reported a positive

correlation between ghrelin concentrations in colostrum and those in maternal serum [94], raising the potential that ghrelin is transferred into HM from the maternal circulation. As with other metabolic hormones, ghrelin concentrations change across the course of lactation, however, findings are not consistent. Thus, while some studies have reported that HM ghrelin concentrations increased from colostrum to mature milk [18,95], others have identified decreases in HM ghrelin concentrations from 1-2 weeks to 3-4 months postpartum [17,26].

1.2.4 Other HM components

In addition to the HM components discussed above, which form the major focus of this thesis, several other factors present in HM also have key roles in regulating infant growth and development, in particular the development of the infant gastrointestinal tract and immune system. HM contains a multitude of immune factors, including immunoglobulins, lactoferrin, cytokines and growth factors which have an essential role in conferring immunity in the first few days after birth and supporting the development of the infant immune system in the early infant period [96]. These factors are either produced by mammary epithelial cells or by cells in HM e.g. leukocytes [96-98]. Immune cell levels are highest in colostrum, and then decline as lactation progresses and the infant immune system matures [96,99]. The primary role of immune cells in protecting mammary glands from infection was first demonstrated around 30 years ago [100] and since then studies have shown that leukocytes can provide immune protection to infants [99]. Immunoglobulin A (IgA) is the most abundant antibody in HM comprising ~80-90% of the antibodies in colostrum and mature HM [96]. The primary role of IgA is to prevent attachment of microbes into mucosal membranes, e.g. the gastrointestinal tract, and to neutralise toxins from bacteria and viruses [101]. Similar to IgA, lactoferrin's role is to defend the infant from infection and disease, and it has the ability to destroy foreign bacteria, viruses

and fungi [96,102]. Cytokines and growth factors in HM are some of the components linked to anti-inflammatory roles and long-term protective effects against certain diseases, however more research is required to better understand their specific roles and mechanisms through which they act [96].

In addition to immune factors, HM contains other cellular and genetic components, including stem cells [103-105] and microRNAs (miRNAs), both of which have been suggested to contribute to communication between cells and the immune system [106,107]. The cellular component of HM is one of the least researched [108], however higher cellular content in colostrum and transitional milk compared to mature HM has been reported [109]. Previous research has also shown that total cell content of HM was strongly associated with HM fat concentrations. This appears to be because both fat and cell content seem to shift according to the degree of breast fullness, such that they are at their highest levels in the period up to 30 minutes following a feed [47]. Stem and progenitor cells were first identified in HM as recently as 2012, and these pluripotent cells are hypothesised to have a role in regulating cellular proliferation and maturation both locally in the mammary gland as well as in infant tissues [110]. The roles of non-immune HM cells in breastfed infants, particularly that of HM stem cells, is not yet fully understood but are likely to involve modulatory and developmental functions [111]. Further research to investigate the role of these components will be important, given their potential to support optimal infant development.

MiRNAs are small RNA molecules that regulate physiologic and pathologic processes, including immunological responses [112]. Over 386 different miRNAs have been identified in HM to date, and the majority have been shown to perform immune-regulatory roles which can,

ultimately, modulate the development of infant's immune system [113]. A 2013 study identified 21 novel miRNAs in HM and reported that both novel and known miRNAs were present in HM fat globules [114], which aligns with previous findings [115], suggesting that miRNAs in HM could contribute to the mechanisms leading to the positive health outcomes in breastfed compared to formula-fed infant. Interestingly, this same study identified that the levels of these novel miRNA species in HM were altered when women were asked to follow a high-fat diet [114], providing evidence to suggest that the concentrations of at least some miRNAs in HM may be influenced by maternal diet.

Historically, HM was considered to be a sterile biological fluid. More recently, however, it has become clear that the HM possesses its own distinct microbiome. The composition of this microbiome varies between individuals and across the course of lactation, and is thought to play an important role in supporting the development of infant microbiome and gastrointestinal tract [116,117]. While the source of the HM microbiome is still heavily debated, it is generally considered that a major source of these microbes is from transfer of bacteria from the maternal gut to mammary glands. In addition, once the HM is consumed by infant, the microbes colonise the infant gut [118,119]. As a result, it is hypothesised that the HM microbiome represents a link between maternal and infant gut microbiome and can contribute to early development of immune system in the infant [118,120]. The latest systematic review on the topic, published in 2017, suggested that *Streptococcus* and *Staphylococcus* were the most abundant bacterial genre in HM, irrespective of detection methods and geographic region [121].

Human milk oligosaccharides (HMO), a family of soluble glycans, are abundant in HM (third largest component in the milk, after lactose and lipids) and act as substrates for gut bacteria in

breastfed infants. More than 130 oligosaccharides have been identified in HM and HMOs are best known for their role in supporting the infant microbiome and immune system [122-124]. The HMOs also have a role in protecting the infant against pathogenic bacteria by preventing the adhesion of different microbes such as, *Salmonella*, *E.coli* and *Vibrio cholerae* strains to mucosal membranes, which blocks the initial step required to initiate the infection processes of these bacteria [125,126].

Micronutrients and antioxidant enzymes are also present in HM and are known to interact with each other as well as to support growth and development in the infant [127,128]. Vitamins A, K and B12 are present at higher levels in colostrum compared to mature HM samples [129]. The factors which determine the levels of these micronutrients in HM are still unclear, and for the majority of these, they are not directly related to maternal status, at least in women who are not deficient. In a systematic review that attempted to synthesise current evidence as to the role of different dietary components in determining HM composition, for example, vitamin C was the only micronutrient in HM reported to be associated with maternal dietary intake [27]. In the cases of some vitamins and minerals, including vitamin K, D, B12 and iron, supplementation of infants is recommended when there is inadequate supply from HM, which might occur in the context of sub-optimal maternal nutrition. The relationship between maternal status and levels of other vitamins in HM, including vitamins B1, B2 and B6, still requires further investigation [130].

1.2.5 Role of HM macronutrients and metabolic hormones in short and long-term infant outcomes

There is extensive literature that has compared the short- and long-term outcomes of infants who are breastfed compared with those fed with infant formula. The majority of these studies have reported differences in growth patterns and body composition between breastfed and formula-fed infants [131-135], such that formula-fed infants grow more rapidly in the first few months after birth compared with those that are breastfed. Previous research has speculated that differences in protein and energy intakes and promotion of appetite self-regulation from breastfeeding are the main mechanisms underlying the differences in growth patterns and fat accumulation [69,136,137]. The protein intake of breastfed infants is aligned with their nutritional requirements and is known to decrease across lactation however, the higher protein content of infant formula means that protein intakes of formula-fed infants are often in excess of requirements. As a result, formula-fed infants are exposed to higher protein intakes which promotes growth and fat deposition in early infancy and is associated with an increased fat mass and risk of obesity later in childhood [136-138]. It has also been suggested that the unique and dynamic composition of HM, in particular the changes in composition that occur during a feed and the presence of metabolic hormones that can regulate appetite, which are not present in infant formula, are also important for supporting the appropriate development of feeding and satiety cues [69].

There is also evidence, from both individual studies and systematic reviews, that being breastfed is associated with a lower risk of developing obesity and metabolic diseases later in life compared to formula feeding [5,139,140]. In one study, formula-feeding was associated not only with higher rates of weight gain in early infancy but also with increased risk of overweight

and obesity at 20 years of age, in comparison to infants who were breastfed [141]. However, the studies in this area to date have been predominately observational, and have suffered from inconsistencies in study design [142], inconsistent participant characteristics [143] and inability to control for confounding factors, in particular sociodemographic factors [144,145]. These deficiencies have led to questioning of a causal relationship between breastfeeding and future obesity risk. For instance, breastfeeding rates are higher in families with higher socioeconomic status in high-income countries [5], and this can occur in conjunction with a number of other health behaviours (e.g. higher food security, better health literacy and overall dietary quality) which are also associated with lower obesity risk. Further, more recent studies have suggested that breastfeeding is not always protective against the development of obesity and poor metabolic health [146,147].

One major criticism of the current literature is that it has failed to consider the variability of HM composition between women and how this impacts the long-term infant health outcomes. This was powerfully demonstrated by a landmark study which showed that the risk of overweight in infants of diabetic mothers at 2 years of age was significantly reduced if infants were provided with banked HM from non-diabetic women compared to infants who received HM from their own (diabetic) mother [148]. Although this study did not measure insulin or glucose in HM, it does raise the possibility that compositional changes as a result of maternal diabetes may contribute to the increased incidence of obesity and metabolic diseases in infants. [149]. It is also important to acknowledge the challenges inherent in distinguishing the effect of exposure to maternal diabetes before birth vs continuous exposure during breastfeeding [150].

1.2.5.1 Macronutrients

There is increasing evidence that variation in the concentrations of HM components, particularly macronutrients and metabolic hormones, may have relevance for both the short-term and longer-term growth and adiposity of the infant [11,90]. The strongest evidence of this to date comes from a 2016 study conducted in the UK, which involved 614 lactating women, and demonstrated that HM fat concentrations were negatively correlated with infant BMI and adiposity at 12 months, whereas HM carbohydrate (lactose) content was positively correlated with these measurements and HM protein was positively correlated only with infant BMI [11]. Other studies have suggested that the protein content of HM also has the potential to influence infant growth and body composition, and that these effects may vary according to protein type. A 2018 Australian study with 20 mothers/infants found no association between either the HM total protein concentration or calculated daily intake (CDI) of total protein and infant body composition (as assessed by ultrasound skinfold thickness) [151]. In the same study, however, a higher CDI of casein was associated with a higher fat mass and fat mass index, and lower fat-free mass in the infant at 5, 9 and 12 months [151]. While, to our knowledge, there have been no reports of associations between lactose concentrations in HM and infant growth and fat mass, a 2017 study conducted in the USA with 25 women reported that HM fructose content was positively correlated with infant body weight and body fat (as assessed by dual-energy X-ray absorptiometry) at 6 months [152]. While these studies raise the possibility that the levels of macronutrients in HM may have a key role in determining subsequent fat and lean mass in the infant, they are based on associations only, so it is not possible to attribute causality. Consequently, further studies are needed to confirm these relationships, and to explore additional roles of HM macronutrients in short and long-term infant health outcomes.

1.2.5.2 Metabolic hormones

In addition to macronutrients, the potential for bioactive factors in HM to contribute to short and long-term metabolic health outcomes of the infant continues to be investigated in the context of infant growth and fat deposition [90]. The metabolic hormones have received particular attention in this research, with a number of studies demonstrating relationships between the concentrations of these hormones in HM and infant growth and/or adiposity later in infancy. In the case of leptin, studies have shown that HM leptin concentrations measured at 1-4 months postpartum are inversely associated with infant weight-for-length Z-score (WLZ) and infant BMI Z-score (BMIZ) at 4 and 12 months postpartum [22], and infant length, body fat, total fat mass and trunk fat mass at 6 months postpartum [12]. The relationship of HM adiponectin to infant growth appears to be somewhat more complex. Thus, HM adiponectin measured from 6 weeks until 4 months postpartum has been shown to be inversely associated with weight and adiposity measurements in infants in the first 4 months after birth [22,153,154], but positively with body weight and adiposity of the infants beyond 12 months of age [88,154,155]. In addition, one of these studies controlled for a number of confounders, including maternal and infant serum adiponectin concentrations, duration of breastfeeding and time of introduction of solids and this did not alter the relationships between HM adiponectin and infant fat mass described above [88]. Therefore, these findings suggest that HM adiponectin may have different impacts on the short and longer-term growth and fat deposition in the infant [86].

Associations between HM insulin and ghrelin and infant measures of growth and adiposity have been reported in only a small number of studies to date, making it difficult to draw clear conclusions [18,90]. However, two studies have reported that HM insulin measured at 2 weeks,

1, 2, 3 and 4 months postpartum was negatively associated with infant weight, lean mass, WLZ and BMIZ at 1 month [156] and the change in WLZ between 2 weeks and 4 months [157], but only for infants with normal weight mothers. Similarly, a 2018 study conducted in Canada, which included 430 mothers and infants, showed that HM insulin concentrations at 4 months were negatively associated with infant WLZ and BMIZ at 4 and 12 months [22]. It is possible that these relationships may be related to insulin's role in metabolic control and appetite inhibition, which could contribute to slower growth in the early infant period. However, insulin concentrations in HM are also related to levels of several other hormones, making it difficult to separate these effects. For instance, insulin concentrations in mature HM has been previously reported to be positively associated with HM leptin and negatively associated with HM glucose [156]. In addition, circulating insulin and ghrelin concentrations are negatively related [158], raising the possibility that higher HM insulin is associated with lower HM ghrelin, which would also be expected to suppress appetite.

Ghrelin in HM has previously been associated with infant weight, growth and fat mass in 3 separate studies, the majority of which have reported a positive association between HM ghrelin and infant growth. A 2012 study with 25 mothers from Turkey reported that HM ghrelin at 4 months was positively associated with infant weight gain at 1- 4 months postpartum [159]. Similarly, a 2014 Russian study with 103 breastfeeding women separated infants into 3 groups according to their monthly weight gain (over the first 3 months), and showed that HM ghrelin at 1 month postpartum was significantly higher in mothers of those infants in the highest weight gain tertile, compared to those in lower tertiles [160]. While the mechanisms through which ghrelin in HM could mediate changes in infant growth/fat mass is unknown, one possibility is that the higher HM ghrelin stimulated infant appetite, and thereby increased infant milk intake,

resulting in more rapid weight gain and increased weight and fat mass. However, not all studies have demonstrated a growth-promoting effect of ghrelin. A 2018 study conducted in the USA with 41 mothers showed that a higher HM ghrelin measured at 2 weeks, 1, 2, 3 and 4 months was associated with a slower rate of fat-free mass accumulation, as calculated from the change in infant fat mass from 2 weeks to 4 months [157]. Therefore, further studies are required to confirm the effects of HM ghrelin on infant growth.

While further studies are needed, the evidence to date is consistent with the suggestion that the concentrations of macronutrients and other bioactive factors have the potential to influence growth and body composition in the infant, and thus contribute to their long-term metabolic outcomes. As a result, it is important to understand the factors, in addition to stage of lactation, that can influence the levels of these components in HM.

1.2.6 Other determinants of HM composition

As discussed above, HM composition varies substantially across the course of lactation and, in the case of fat in particular, also varies across the course of a day and even a single breastfeed. HM composition also varies significantly between women. While the reasons for this variability is not fully understood a number of factors, including the gestational age of the infant at birth, infant birth weight and infant sex, have been shown to affect HM composition and are therefore likely to contribute to compositional differences between women.

1.2.6.1 Gestational age

A systematic review and meta-analysis, published in 2014, indicated that HM macronutrient composition was significantly different between mothers of term and preterm infants,

particularly for protein content [24]. According to this meta-analysis, preterm HM was higher in protein from day 1 until 9 weeks postpartum, higher in fat content from day 1 until 2 weeks and had higher lactose content from day 1 until 4 weeks and again at 7-9 weeks postpartum [24]. However, the quality of the individual studies that were included in this meta-analysis was generally low, and the results from more recent studies have been mixed, with some producing results consistent with the meta-analysis, while others reported conflicting findings. In addition, the differences appear to vary according to the stage of lactation. In a Greek study involving 305 women, HM fat content was higher across lactation in mothers who had delivered preterm whilst HM protein was higher in mature milk only and HM lactose was higher in both transitional and mature HM [15]. In addition, other studies have reported that fat content was higher in transitional [161] and mature [162] preterm HM and higher at 3-8 weeks postpartum in term HM [161], while lactose concentrations were higher in preterm compared to term HM in mature samples [162]. Meanwhile, these same studies reported no difference in HM protein between preterm and term deliveries in early (colostrum and transitional) and mature HM [161,162].

The influence of preterm delivery on concentrations of metabolic hormones in HM has been assessed in only a very small number of studies, and these have produced inconsistent results. In one study, HM leptin concentrations in mature milk were up to 3-fold higher in HM from mothers of preterm compared to term infants, whereas insulin concentrations were not different between these groups [81]. In another study, however, HM leptin was lower in HM from mothers of preterm infants in colostrum, but not different in mature milk [163]. Interestingly, this appeared to be due to differences in the way that leptin concentrations changed over the course of lactation – decreasing in concentration between colostrum and mature HM in mothers

of term infants, but not exhibiting any change across this same period in mothers whose infants were born preterm [81]. Other studies, however, have found no relationship between gestational age and HM leptin over the first 6 months postpartum [77] and no differences in concentrations of leptin, adiponectin and insulin between term and preterm deliveries at 4 months postpartum [22].

1.2.6.2 Infant birth weight

A number of HM components have also been shown to be related to infant birth weight and/or fat mass. In some studies, infant birth weight and WLZ were negatively correlated with fat and protein content in mature milk [15,54], fat and lactose concentrations in transitional milk and fat content in colostrum [15]. The relationships identified between infant birth weight and HM composition are not, however, consistent between studies, with another study reporting a positive correlation between HM carbohydrate/lactose in mature milk and infant birth weight [15]. In addition, other studies have reported no association between infant birth weight and leptin [22,23,77], adiponectin [22,23] and insulin concentrations in HM transitional and mature samples [22]. Thus, the relationship between infant size at birth and HM composition and whether this is causal or coincidental, remains.

1.2.6.3 Infant sex

HM composition has also been shown to be influenced by infant sex, although this has not been a consistent finding across all studies and evidence is relatively limited. One study, by Powe and colleagues, reported that total energy levels were 25% higher in HM of women who breastfed male compared to female infants after adjustment for time since last feed [164]. This is potentially driven by a higher fat content in HM of mothers of male infants or higher HM

volume/infant intake. In support of this, a 2019 study reported that HM from mothers of both preterm and term male infants had higher fat concentrations compared to mothers of female infants [161]. It has also been demonstrated that breastfed male infants typically consume higher amounts of milk in comparison with females [165], which has the potential to contribute to the compositional differences between male and female HM. This is due to the fact that, since HM fat concentrations are inversely associated with the fullness of the breast [20], consuming a greater volume of milk at each feed would be expected to be associated with a greater degree of breast emptying, and thus higher fat content [20,164]. Interestingly, a study conducted in Northern Kenya suggested that the relationship between infant sex and HM composition may vary according to the mothers' socioeconomic background [166]. In this study, fat content was higher in HM of mothers with male infants who were of higher socioeconomic status, whereas fat content was higher in HM of mothers from female infants when they were of lower socioeconomic status. The reason for this difference, and whether it is related to maternal diet, lifestyle or other characteristics, is unclear.

The role of infant sex in determining hormone concentrations in HM has not been studied extensively to date. A study by Fields and colleagues, published in 2017, reported that the concentrations of leptin and insulin in HM were lower in obese mothers breastfeeding male vs female infants, which the authors hypothesised as due to greater suckling pressure in male infants suppressing leptin synthesis in the mammary glands [12]. In agreement, another study reported that leptin concentrations were lower in HM from mothers nursing male infants [23]. Other studies, however, have failed to identify any differences in HM adiponectin [22,23,167], leptin [22,77] or insulin [22] between male and female infants.

1.3 Human milk composition - the influence of sampling methodology

In addition to infant characteristics, variations in the timing of HM collection can also influence HM composition, as a result of variability in HM composition across the course of a day, during a feed or depending on the mode of collection (i.e. hand expression vs breast pump) [21,45,168].

1.3.1 Variations across the day and breastfeed

As indicated earlier, diurnal variations are observed for HM fat concentrations [21,45], with levels increasing over the course of the day, while protein and lactose remain stable across each 24-h period [21,169]. It is less clear to what extent fat, lactose and protein concentrations in mature HM change across shorter time periods, i.e. days or weeks, and no studies have evaluated this to date. There is also limited knowledge as to whether and to what extent the concentrations of metabolic hormones change over the course of the day or across a feed.

The levels of fat in HM varies to a greater extent than all other HM components assessed to date and increases up to 3-fold from the beginning to the end of a breastfeed or expression [45,170,171]. In contrast, both protein and lactose concentrations remain consistent across a feed [19,21]. In terms of the metabolic hormones, Andreas and colleagues showed that HM ghrelin levels in mature HM (collected at 3 months postpartum) decreased across a feed [26], and suggested that higher levels of this orexigenic hormone at the start of a feed could have a role in promoting infant feeding. However, only one study to date has measured ghrelin across the course of a feed, so more data are required. Similarly, information as to whether, and to what extent, concentrations of other metabolic hormones change across a breastfeed or expression is scarce, although the available data suggests that levels of both leptin [26,45,170,172] and adiponectin [19] are stable across a single feed. If this is the case, then it calls into question the suggested role of these metabolic hormones in regulating short-term feeding behaviour in the

infant [170]. Consequently, further studies that assess the biological roles of the metabolic hormones in HM are needed.

1.3.2 Other sources of variation in HM composition

Other factors that could potentially affect HM composition, particularly in relation to macronutrients, have only been reported by a limited number of studies. A 2013 Australian study assessed short-term changes in HM macronutrient composition between right and left breasts and found no significant differences in HM composition between breasts over a 3 week period [39] which aligns with findings from a later study [173]. The Australian study did, however, identify significant differences in the protein content of the HM collected from right and left breasts in 5 of the participating women [39]. This appeared to be explained by differences in breast volume, since there was also a negative association between breast volume and HM protein content in this same group.

The effect of mode of expression (i.e. hand expression or breast pump) in mature HM has also been evaluated in only a small number of studies. In one study, HM fat concentrations in pre-feed samples were lower in samples collected via hand expression compared to electric breast pump, but fat concentrations in post-feed samples and protein concentrations in either pre- or post-feed samples were not different between groups [47]. The authors of the study suggested that the differences in fat content in pre-feed samples could be explained by the greater homogenisation that would occur when samples were collected via breast pump compared to by hand [47]. Whilst a Cochrane review published in 2016 suggested potential differences in protein content between expression methods, these data appear to be confounded by lactation stage and are therefore not definitive [174]. Time since last breastfeed or expression has not

been directly assessed in regard to differences in HM composition, however it has been suggested that collecting samples at least 2-h after a previous feeding provides the best reflection of the HM composition of the subsequent feed [47,175,176].

While some studies have applied maternal fasting overnight before HM collection, this has generally been restricted to studies in which fasting blood samples are also collected [89,177]. In studies where maternal blood is not collected, no rationale for maternal fasting prior to milk collection are provided by authors [81,170]. The impact of maternal fasting on HM macronutrient content appears to be minimal, as demonstrated by studies that investigated the impact of Ramadan fasting on HM composition and reported no differences in fat, protein and lactose concentrations in HM during the fasting period [178,179]. While there were no effects of maternal fasting on HM macronutrient composition, however, one of the studies reported that micronutrient levels in HM, including levels of zinc, potassium and magnesium, were altered [178]. The effect of maternal fasting on concentrations of metabolic hormones in HM is particularly relevant since the levels of these hormones in the circulation can be impacted by fasting, and there are reports of relationship between maternal circulating leptin, insulin and adiponectin and their respective levels in HM [17,78,86]. However, the impacts of maternal fasting on metabolic hormone concentrations in HM along with mode of expression and time since last feed have not (to our knowledge) been studied.

1.3.3 Implications of sampling method on compositional analyses

Given the significant variation in HM composition based on time of day and time during a feed [36], the method of HM sampling can have a significant impact on the results of compositional analyses. While many different collection methods are used, the sub-sampling and pooling of

milk samples from a full breast expression (emptying of both breasts at every feed) across 24-h is considered to be the “gold standard” sampling method [33,38], in other words, the method which best represents the average of HM composition being consumed by the infant. However, a number of ethical and logistical issues have been raised with the use of this method. Thus, it is increasingly recognised that this approach represents a considerable burden for lactating women and their infants. In addition, this method is impractical for large scale epidemiological studies and also has the potential to interfere with infant feeding. Currently, there is no universally accepted standard collection method for HM studies and, as a result, many research groups have developed and applied alternative collection methods, which overcome some of the logical challenges of the “gold standard” approach. These methods include, but are not limited to, collection of samples at one or two time-points during the day (usually morning), rather than every feed across a 24-h period and/or limiting the collection to either pre-feed, mid-feed or post-feed samples. Despite the potentially considerable impact of collection method on HM composition, however, a comprehensive analysis of all existing collection methods applied in HM research has not yet been conducted, and thus the full extent of the variability and whether particular methods are preferred over others is unclear. In addition, whether these alternative methods provide equivalent compositional results as the “gold standard” approach and which, if any, are superior to others, also remains to be evaluated.

1.4 Maternal obesity and human milk composition

Global obesity rates have been increasing over the past decades, including among women of reproductive age [180,181]. A 2014 study applying a panel data model estimated that 24.3 million pregnant women were overweight and 14.6 million were obese globally at this time.

In the USA, a third of women were classified as obese, including 1.1 million pregnant women [182]. A large body of epidemiological, clinical and experimental animal evidence has shown that, in addition to increasing the risk of pregnancy and neonatal complications, maternal overweight and obesity are associated with a significant increase in the risk of obesity and metabolic diseases in the offspring [183-185]. One of the mechanisms through which this occurs may be due to changes in HM composition, since a number of studies have now reported differences in the levels of macronutrients and bioactive factors in HM from women who are overweight or obese, compared to those of normal weight [12,26,186]. This has also led to suggestions that the benefits of HM for infant growth and development could potentially be at least partially offset by the effects of maternal overweight and obesity and associated metabolic disorders on the composition of the milk [187], however direct evidence of this is extremely limited.

1.4.1 Macronutrients

The relationships between maternal overweight/obesity and HM macronutrient levels have been described in a number of studies, and a small sub-set of these studies have also reported associations between maternal BMI and/or fat mass and macronutrient concentrations [19,25,188]. In two of these studies, a positive correlation was identified between maternal fat mass and HM protein [19,25], and one also found a positive correlation between maternal BMI and both fat and protein concentrations in HM [25]. Other studies assessing associations between maternal pre-pregnancy BMI and fat, protein and lactose concentrations in HM, however, have failed to show any significant correlations [189-191]. Therefore, the results of studies investigating the impacts of maternal overweight/obesity on HM macronutrient composition have not been consistent, and not all have identified differences in macronutrient

composition between overweight/obese and normal weight women. In addition, no studies to date have systematically evaluated the current evidence regarding effects of maternal obesity on HM macronutrient concentrations.

1.4.2 Metabolic hormones

In comparison to effect on HM macronutrients, the impact of maternal overweight/obesity on the concentrations of metabolic hormones in HM has received considerably more attention in the published literature, presumably due to the known differences in the concentrations of these hormones in the circulation of overweight/obese individuals compared to those of normal weight [17,73]. The latest systematic review assessing the impact of maternal BMI on metabolic hormones was published in 2014 and concluded that the existing literature provided evidence of a positive relationship between HM leptin concentrations and maternal BMI, although the authors also acknowledged that there was considerable variability in findings between the studies [192]. Fewer studies have investigated the impact of maternal overweight/obesity on other metabolic hormones in HM, and results have not been consistent. In the case of HM adiponectin concentrations, some studies have found a positive correlation between maternal BMI and/or fat mass and HM adiponectin [18,193], while others found no association [22,94,194]. Differences in study design, time of HM collection and methodological details could explain the variability between studies [192]. Even fewer studies have examined relationships between maternal BMI and HM insulin and ghrelin concentrations, and again results are not consistent [22,26].

1.4.3 Clinical relevance

Overall, while there is variability in findings between studies, the available evidence suggests that the concentrations of at least some components in HM are altered in women who are overweight/obese and may therefore potentially contribute to the intergenerational cycle of obesity. However, the extent of these differences across the full complement of nutritional factors and metabolic hormones in HM and the reason for observed differences in HM of overweight/obese women have yet to be comprehensively studied. Specifically, it is not clear if changes in HM macronutrients and metabolic hormones in overweight and obese women are due to maternal metabolic dysregulation, differences in maternal dietary composition or a combination of both. This is particularly relevant since obesity and nutritionally poor diets often coincide in lactating women, making it challenging to identify their relative contribution to HM composition [195]. At present, it is unclear whether it would be sufficient for obese women to be advised to improve the quality of their diets during lactation in order to influence their HM composition, and further studies are needed to evaluate this.

1.5 Maternal dietary intake and human milk composition

Maternal dietary intake is often cited as important in the context of HM composition [33] however, direct evidence as to the relationship between diet, particularly dietary interventions, and HM components remains unclear. With the possible exception of fatty acid composition [129,196], the relationship between specific dietary components in HM factors is poorly defined. This was highlighted by the findings of a 2016 systematic review of studies that attempted to directly associate maternal intake of specific nutrients (excluding studies focused on supplements) and HM composition in healthy mothers of term infants [27]. The findings of this review suggested that, with the exception of direct correlations between maternal intake and

HM contents for vitamin C and the omega-3 fatty acid DHA, there were few studies that had shown a direct relationship between dietary intake and the concentrations of HM macro or micronutrients [27]. The authors of this review concluded that there is still limited evidence regarding the impact of maternal diet on HM composition, and that most, if not all, of the existing studies had limitations, including significant heterogeneity and inconsistent collection and analytical methodologies. A brief discussion of the key findings to date regarding dietary impacts on HM composition is presented below.

1.5.1 Macronutrients

There is limited information regarding the effect of maternal dietary intake on HM macronutrients, particularly from intervention studies. The DARLING study was one of the first to investigate relationships between maternal diet and HM composition and undertook a study in a sub-sample of 32 women from a larger cohort study in whom diet was assessed using a 3-day weighed food diary [38]. They found that maternal protein intake, both total quantity and intake as a percentage of energy, was positively associated with HM fat content, but only in those women for whom HM samples were collected after 16 weeks postpartum. Based on these data, one might speculate that maternal diet could influence HM composition to a greater extent later in lactation, compared to the earlier weeks. In addition, no relationships were identified between any other dietary factors and HM protein and lactose content in this study [38]. In a separate cross-sectional study of 102 women, in whom dietary intakes were assessed by a single dietary recall, no relationships were identified between maternal intakes of total energy, fat, protein or carbohydrate and HM macronutrient concentrations in the first 18 months postpartum [188]. It is important to note, however, that HM composition changes considerably during this period [197], so the relevance of these findings is unclear. A separate

study found no correlations between postpartum maternal dietary intake in the 24-h prior to sample collection and HM fat, protein and carbohydrate content at 4 days postpartum [198].

To date, the only study to our knowledge to assess the effect of dietary intervention on HM macronutrient content was conducted in healthy lactating women between 6 and 14 weeks postpartum. In this study, women were assigned to either a low-carbohydrate, high-fat diet (30% carbohydrate, 55% fat) or high-carbohydrate, low-fat (60% carbohydrate, 25% fat) diet, for an 8-day period, and HM samples collected before and after the intervention period. The results of this study indicated that HM fat content was increased following the high-fat diet, but not the high-carbohydrate, low-fat diet, while HM lactose and protein concentrations were not affected by either of the dietary interventions [199]. Overall, therefore, while there are some suggestions that HM composition could potentially be influenced by maternal intakes, evidence to date is limited.

1.5.2 Fatty acids

The fatty acid composition of HM is highly variable, and has been shown to shift rapidly in response to changes in the composition of fats in the maternal diet [200]. For instance, the content of trans-fatty acids in HM is directly correlated with maternal trans-fatty acid intakes [201,202]. Moreover, a large number of studies have consistently demonstrated that concentrations of most fatty acids in HM, including the omega-3 LCPUFA, vary substantially in accordance with the fatty acid composition of the maternal diet [203-205]. A review of the concentration of EPA and DHA in HM of women from several countries reported that EPA levels in HM are more conserved in comparison with DHA levels, which vary markedly in accordance with levels in habitual diets of different countries [205]. By way of example,

Australian women have low DHA contents in their HM, which is directly related to their low dietary DHA intakes [204]. In addition, fish oil or DHA supplementation during lactation is associated with a dose-dependent increase in omega-3 fatty acids and/or DHA concentrations in HM [206-208].

The close relationship between maternal dietary fatty acid intake and the fatty acid content of HM is further supported by the results from a cohort study in which maternal dietary intake was assessed using a food frequency questionnaire (covering 1-week period) in 100 lactating women at 3 months postpartum (mature HM) [208]. The study concluded that higher consumption of fatty fish was associated with increased concentrations of omega-3 fatty acids in HM. In addition, the intake of vegetable oils correlated with higher polyunsaturated fatty acids (PUFA) levels, particularly omega-6 PUFA, in HM, while saturated fatty acids in HM were higher in women consuming high-fat dairy products. The study also classified maternal dietary intake using an “index of diet quality” – IDQ (describing adherence to current Nordic nutrition recommendations) in the third trimester of pregnancy, and showed that a higher IDQ score was associated with increased PUFA levels in HM. In addition, IDQ scores were lower in women who were overweight or obese compared to those of normal weight, supporting the suggestion that dietary quality is typically lower in women with a higher BMI.

1.5.3 Metabolic Hormones

There are currently very few studies which have investigated the relationship between maternal dietary intake and metabolic hormone concentrations in HM. In one of the few published studies, conducted by Luoto and colleagues, [209] 256 pregnant women were randomly assigned to one of three groups: dietary intervention with probiotics; dietary intervention with

placebo and control group with placebo. The dietary intervention consisted of intensive dietary counselling administered by a nutritionist at each study visit, focused on limiting fat and increasing fibre content of the diet, and was provided from the first trimester of pregnancy until the end of exclusive breastfeeding. Colostrum samples were collected from 181 women. When both intervention groups were combined, the adiponectin levels in colostrum samples were higher in the dietary intervention groups compared to control group. Higher maternal protein intakes were also associated with higher adiponectin concentrations in the colostrum [209]. In another observational study which included 113 lactating women, however, no associations were identified between maternal energy or fat intakes, as assessed using a single-day dietary recall, and HM leptin concentrations between 10 days and 36 months postpartum [210].

Overall, information regarding the effects of maternal dietary intakes on HM macronutrient composition and metabolic hormone concentrations is particularly sparse, and further studies are required to improve our understanding in this much needed area.

1.5.4 Clinical significance

The investigation of the impact of early life nutrition on later life health outcomes of children has significant public health relevance. The assessment of the influence of maternal diet on HM is clearly complex; and separating the individual contributions of maternal dietary factors, from those of maternal metabolic health measures and other lifestyle factors is extremely challenging. Nevertheless, better understanding the role of maternal diet in influencing HM composition is important for studies to be translated into clinical practice. Whether it would be sufficient for overweight/obese women to be advised to improve the quality of their diets only during lactation in order to offset the consequences of exposure to maternal obesity *in utero*

remains unknown. Currently, there are no human studies addressing this question. While we have focussed on human studies in this review, animal studies, albeit limited, have reported that exposure to maternal high-fat diet only during the suckling period was more detrimental to metabolic consequences in the offspring e.g. leptin resistance, than exposure to maternal high-fat diet only before birth [211]. While this pre-clinical study raises the exciting possibility that the adverse effects of prenatal exposure to maternal obesity/high-fat diets could be mitigated by improved nutrition (and therefore improved milk quality) during the lactation period, further studies are required to confirm whether this translates to the human setting. There is a need for well-designed studies with the ability to control for external confounders to investigate and clarify the current gap in our understanding of the benefits of dietary interventions and improved nutrition in lactating women, particularly those who are overweight and obese.

1.6 Thesis outline and aims

The nutritional environment during the early postnatal period is an important determinant of an infant's future risk of obesity and metabolic diseases. HM composition is expected to be a critical factor in this relationship, with HM macronutrients and metabolic hormones playing a particularly important role. Therefore, the assessment of these HM nutritional and bioactive factors in the current context of growing rates of obesity in lactating women and consumption of energy dense and nutritionally poor diets is critical to better understand direct contributions and establish strategies to minimise the potential negative health outcomes for the infants.

The overarching aim of this thesis is to define an evidence-based standardised approach to HM collection and to advance understanding and knowledge as to the contribution of maternal

overweight/obesity and dietary factors to the concentrations of macronutrients and metabolic hormones in HM. Understanding HM composition as well as those factors that can influence this, is crucial for the management of infant feeding, especially in those with greater nutritional needs [33]. A better understanding of contemporary HM composition is critical given the established importance of nutritional exposures in early infancy for an individual's life-long health outcomes [13]. This is particularly important in light of the increasing number of pregnant/lactating women who are overweight or obese, nutritionally poor diets and the potential for HM to be indirectly contributing to an intergenerational cycle of obesity.

It is becoming increasingly clear that the composition of HM varies considerably between mothers and differs across the day and even within a single feed in the case of some components. Consequently, the method of HM sample collection can have a significant impact on the results of compositional analyses, particularly for HM fat content. In the absence of a universal standardised collection protocol, a wide range of different collection methods have been used in previous studies, but it is unclear which of these are most representative of infant intakes and which align most closely to the “gold standard” approach of sub-sampling from a full breast expression at each feed over a 24-hr period. **Therefore, the first aim of this thesis was to undertake a synthesis of the different collection methods used in studies of HM composition, and use this as a basis for determining the most reliable collection method.** Systematic reviews represent the highest level of evidence by reviewing and synthesising available data in a standardised and transparent way, and therefore the preferred approach to address this aim [212]. Chapter 2 of this thesis describes a comprehensive systematic review protocol aimed to identify the HM sampling method of collection which would provide the most reliable results for macronutrient compositional analyses. In Chapter 3, this protocol was

used to undertake a comprehensive systematic review to describe the different collection methodologies applied in studies assessing HM composition and compare the results obtained for HM macronutrient composition between collection methods.

In addition to differences in composition according to collection method, there was also limited evidence from existing studies regarding the extent to which macronutrient concentrations in mature HM change over shorter periods (days to weeks). **Therefore, the aim of Chapter 4 of this thesis was to assess the extent of changes in mature HM composition in the same woman according to time of day, collection breast and day of collection across a 3-week period.**

Maternal characteristics, in particular overweight and obesity, have the potential to impact HM composition [26,53], however, the strength of these relationships is currently unclear. There is considerable variability in the results obtained between individual studies, and either no extensive systematic synthesis of the existing literature, in the case of effects of maternal overweight/obesity on macronutrient composition and no contemporary synthesis of evidence in the case of effects on HM metabolic hormone concentrations. **Therefore, the aims of Chapters 5 and 6 of this thesis were to undertake a systematic review and meta-analyses to provide a contemporary synthesis of the evidence assessing the effects of maternal overweight and obesity, including maternal BMI and other adiposity measures, on the levels of HM macronutrients (Chapter 5) and metabolic hormones (Chapter 6).**

Maternal diet has been reported to be a major determinant of HM composition, however, direct evidence as to the effects of maternal dietary intake and HM components is sparse. The relative

contribution of metabolic disturbances associated with maternal obesity and maternal diet on HM composition is unclear, and no previous studies prior to this thesis had determined whether improving dietary quality during lactation would result in changes in HM composition. The relative contribution of different dietary components, particularly fat and sugar, to HM macronutrient and metabolic hormone concentrations was also unclear. **Therefore, the aim of Chapter 7 was to design a clinical trial protocol to assess the impact of test meals varying in fat and sugar content on post-prandial concentrations of HM macronutrients and metabolic hormones.**

Following the publication of this protocol however, the results of our systematic reviews suggested that investigating the impact of a longer-term dietary intervention (2 weeks) would provide more compelling proof-of-concept evidence regarding the potential for dietary interventions during the lactation period to favourably influence HM composition. This longer-term approach also enabled us to confirm that any changes in composition would occur in the absence of negative effects on milk production and/or infant growth. **Therefore, the aim of Chapter 8 was to assess the effect of improving dietary quality during breastfeeding on the concentrations of macronutrients and metabolic hormones in HM, maternal milk production and maternal body composition.**

CHAPTER 2. A METHODOLOGICAL APPROACH TO IDENTIFY THE MOST RELIABLE HUMAN MILK COLLECTION METHOD FOR COMPOSITIONAL ANALYSIS: A SYSTEMATIC REVIEW PROTOCOL

Gabriela E. Leghi, Philippa F. Middleton, and Beverly S. Muhlhausler

The manuscript entitled “A methodological approach to identify the most reliable human milk collection method for compositional analysis: a systematic review protocol” was published in the peer reviewed journal *Systematic reviews*, 2018;7.

Additional file 1 is listed as Appendix 1. PRISMA-P Checklist - Chapter 2

Additional file 2 is listed as Appendix 2. Search Strategy Example for PubMed – Chapter 2

Statement of Authorship

Title of Paper	A methodological approach to identify the most reliable human milk collection method for compositional analysis: a systematic review protocol.
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Principal Author

Name of Principal Author (Candidate)	Gabriela Esteves Leghi		
Contribution to the Paper	Contributed to the research idea, design and development. Drafted and constructed manuscript and had primary responsibility for final version.		
Overall percentage (%)	75		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	05.05.2020

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

- the candidate's stated contribution to the publication is accurate (as detailed above);
- permission is granted for the candidate to include the publication in the thesis; and
- the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Philippa Middleton		
Contribution to the Paper	Contributed to the research design, development and manuscript revision		
Signature		Date	05.05.2020

Name of Co-Author	Beverly Muhlhausler		
Contribution to the Paper	Contributed to the research idea, design, and manuscript revision. Supervised the work and had responsibility for final content. Acted as corresponding author.		
Signature		Date	05.05.2020

PROTOCOL

Open Access



A methodological approach to identify the most reliable human milk collection method for compositional analysis: a systematic review protocol

Gabriela E. Leghi^{1,2}, Philippa F. Middleton² and Beverly S. Muhlhausler^{1,2*} 

Abstract

Background: Breast milk composition has been reported to vary significantly between individual women and between different populations. However, the composition is also known to vary within the same woman between different days, within the same day, and even across the same feed. Therefore, it is unclear to what extent variations in composition are due to variations in sampling methodology between studies. The purpose of this systematic review is to compare the results obtained for breast milk macronutrient composition between studies utilizing different sampling methodologies and to use this as a basis to determine the most robust and consistent sampling approach as an alternative to full expression (gold standard).

Methods: The EMBASE, MEDLINE/PubMed, Cochrane Library, Scopus, Web of Science, and ProQuest Dissertations and Theses Global databases will be searched for relevant articles. Observational studies, including cross-sectional, comparative cohort, and longitudinal cohort studies which involve lactating women who are breastfeeding (exclusively or not) or expressing (manually or using a breast pump) at any lactation stage will be included. This review will compare different methods of breast milk collection used in research studies which report macronutrient levels (protein, fat, lactose). Two review authors will independently screen titles and abstracts of studies identified by the literature search to determine articles for the full text screening. Quality assessment of included articles will be conducted independently by two review authors using the Newcastle-Ottawa scale.

Discussion: It is important to identify the most reliable and practical method of human milk collection which best represents the average composition of the milk that is being consumed by the infant. This systematic review will be critical for ensuring that we determine a robust and consistent sampling approach to use in future studies of evaluating breast milk composition in a larger population. Identifying a recommended standard collection protocol will also provide more opportunities for sharing and combining data from different research groups, thus enhancing replicability and knowledge in the field.

Systematic review registration: PROSPERO [CRD42017072563](https://www.crd42017072563)

Keywords: Systematic review, Human milk composition, Breast milk collection, Infant health

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Background

Breast milk is uniquely designed for the human infant; it is thought to contain adequate nutrients and bioactive components and is known for its important role in infant survival and health [1]. The World Health Organization (WHO) recommends that infants should be exclusively breastfed until around 6 months' postpartum, with continued breastfeeding to 12 months of age and beyond [2]. It is critical to understand contemporary breast milk composition given the established importance of nutritional exposures in early infancy for an individual's life-long health outcomes; a concept that has been reinforced by reports of significant relationships between the concentration of specific components in breast milk and infant growth/body composition and future risk of obesity [3].

Despite recognition of the importance of breastfeeding and human milk, however, research in this area is complicated by the fact that human milk composition is highly variable and can change according to time since last feed, period of the day, and stage of lactation, as well as between women and populations [4]. The method and time of sample collection has been shown to influence breast milk composition, including the levels of macronutrients (fat, protein, and lactose), micronutrients, as well as levels of other bioactive factors in breast milk [4]. For example, the milk fat content is lower at the beginning of the feed compared with the end of the feed and can increase up to three times from its initial concentration across a single feeding period [5].

Several studies have suggested that the "gold standard" method of sampling human milk for compositional analysis is to collect small samples from multiple complete breast expressions breastfed across a 24-h period [4, 6]. However, this approach is impractical for many women, particularly those who have a limited milk supply and/or whose infants refuse to consume expressed breast milk from a bottle, and is therefore less than ideal for studies in which the aim is to assess breast milk composition in a large population.

There are very few studies which have systematically evaluated the full range of breast milk components collected at different times during a feed and across a 24-h period and compared these to those obtained using this "gold standard" methodology.

Aim

The purpose of this systematic review is to compare the results obtained for breast milk macronutrient composition between studies utilizing different sampling methodologies and to use this as a basis to determine the most robust and consistent sampling approach as an alternative to full expression (gold standard).

Methods

Protocol

The present systematic review protocol has been developed based on Preferred Reporting Items for Systematic Reviews and Meta-Analysis Protocols (PRISMA-P) guidelines [7, 8] and Cochrane Handbook for Systematic Reviews of Interventions [9]. A PRISMA-P checklist file is attached (Additional file 1).

Eligibility criteria

Studies will be selected according to the criteria outlined below. Studies only reported as abstracts will not be included.

Study design

All observational studies, including cross-sectional, comparative cohort, and longitudinal cohort studies will be included.

Participants

Included studies will involve women who are breastfeeding (exclusively or partially) or expressing (manually or using a breast pump) at any lactation stage.

Method of collection

To be eligible for inclusion, studies must report the time of day of collection, state the method of collection, and whether collection was undertaken by manual expression, a breast milk pump (or either), or any other method.

Comparators

Comparison of average macronutrient composition obtained in studies using the gold standard collection methods (full expression of a breast or both breasts, or subsamples from all full expressions, across a 24-h period) vs other breast milk collection methods

Outcomes

Breast milk macronutrient composition (total protein, total fat, and lactose)

Timing

Any time during lactation

Setting

There will be no restrictions on the type of setting.

Language

Only articles reported in the English language will be included due to lack of resources for translation.

Information sources

Literature searches will be undertaken using the following electronic bibliographic databases: EMBASE, MEDLINE/PubMed, Cochrane Library, Scopus, Web of Science, and ProQuest Dissertations and Theses Global. The literature search will be limited to studies in humans, but no date range restrictions will be applied. For each selected article, abstracts and full articles will be obtained. In order to increase literature coverage, we will also scan the reference lists of included studies and systematic reviews identified during the screening process.

Search strategy

The specific search strategies will be developed by the research team with the input from an experienced research librarian. The literature search will be updated towards the end of the review, just prior to final analysis, to ensure maximum coverage of eligible studies. A draft search strategy for MEDLINE, including search terms, is included in Additional file 2. After the MEDLINE search is complete, we will adapt the search strategy to the subject headings and syntax of the other electronic databases.

Study records

Data management

The literature search will be initially performed using the selected databases and then uploaded into EndNote software [10], a reference management software that allows organization of references identified from different electronic databases. All search results will be entered into a single EndNote library, and duplicate studies will be identified and removed. After removal of duplicates, the search results will then be uploaded to Covidence [11], an Internet-based software program that facilitates article screening, data extraction, and collaboration among multiple reviewers. The team will develop and test screening questions and forms based on the eligibility criteria. Citation titles and abstracts will be uploaded to the software.

Selection process

The selection of articles for inclusion in the review will be undertaken in two stages. The first stage will involve screening the title and abstracts of the search results against the eligibility criteria. In the second stage, the full articles of these articles will be screened to confirm that they meet the eligibility criteria. At both stages, each article will be screened independently by two authors. Any articles where there is disagreement in eligibility status between the first two authors will be reviewed by the third author and any disagreements resolved by mutual discussion. The reviewers will not be blinded to journal of publication or authors and institutions at any stage of

the screening process. Reasons for excluding studies will be documented and reported, and the number of articles and reasons for exclusion at each stage of screening will be presented in a Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) flow diagram.

Data collection process

Two authors will independently extract data from each included study (which may have one or more publications) based on a standardized extraction form from the Cochrane Pregnancy and Childbirth group [12]. All authors will review and discuss the data extraction form prior to commencing data extraction, and all authors will complete and compare data extraction from a single selected study to ensure that there is consistent interpretation of the questions. To minimize inconsistency between reviewers, we will conduct training and calibration exercises using the data extraction form prior to the commencement of the systematic review.

Data extracted will include study design, sample size, number of milk samples analyzed, and methodological details about breast milk collection including:

- (i) Whether or not the women were exclusively breastfeeding
- (ii) Lactation day/stage when breast milk was collected (time postpartum)
- (iii) Time of day milk was expressed/collected
- (iv) Time since last feed/expression that milk samples were collected
- (v) Whether separate pre- or post-feed/expression samples were collected or milk samples were mixed
- (vi) Any details provided as to which breast was used for collection (e.g., some authors discuss the letdown response in the absence of suckling, so they ask women to breastfeed baby at one breast while collecting milk from the other)
- (vii) Whether milk was expressed manually or using a breast pump—duration of pumping and milk volume expressed will also be reported
- (viii) If a pump was used, then the type of pump
- (ix) Volume of milk consumed by infant (if samples collected at time of a feed)
- (x) Gestational age of infant at birth (e.g., preterm or term)

The data extraction form will be completed by at least two authors for each study. One author will be responsible for cross-checking each data extraction form for completeness. Any differences in the data extracted from a particular study will be resolved by discussion between the two reviewing authors, and in the case of a disagreement between these authors, reviewed by a third author. If missing data are identified, then reasonable efforts will be made to contact the corresponding authors of the

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relevant study by email (maximum three attempts) to obtain this missing information.

Risk of bias in individual studies

Quality assessment of each included articles will be conducted independently by two authors based on the Newcastle-Ottawa scale (NOS) which was designed specifically for assessment of non-randomized studies, including cohort studies [13], and adapted for cross-sectional studies. The information captured in the NOS will be used to guide the allocation of studies as being at either low or high risk of bias (or unclear), based on criteria such as attrition rate, sample size, and risk of confounding. In addition to the overall analysis of findings from all selected studies, we will also consider the findings from studies with low and high/unclear risk of bias separately to determine the potential influence of study quality on the overall conclusions.

Data synthesis

The extracted data will be presented as structured summary tables which include a description of study characteristics, including number of participants, timing (stage of lactation), type of breast milk collection, and results of macronutrient analyses (total protein, total fat, and/or lactose). Risk of bias for each study will also be included. The findings from different studies in relation to macronutrient concentrations (total fat, total protein, and/or lactose) will be summarized and compared between studies utilizing different methods of breast milk collection.

Discussion

What is the issue?

There is currently a lack of consensus on the most reliable alternative method of human milk collection to the subsampling and pooling of milk samples from a full breast expression at each feed across a 24-h period. While this method is considered the “gold standard,” it is invasive and impractical for population studies in which the goal is to obtain compositional information on a representative sample of women. Currently, an array of alternative collection methods is utilized by different research groups, and there is no universal/standardized sampling approach.

Why is this important?

It is important to identify the most reliable and practical method of human milk collection which will best represent the average composition of the milk that is being consumed by the infant. Breast milk is known to vary in composition across the day and during an individual feed, but to date, it is unclear whether one particular collection method is more closely aligned to the “gold standard” approach of sampling from all full breast milk

breastfeeds/expressions collected across a 24-h period. This systematic review will also compare the results obtained for macronutrient composition using different collection methods. Ultimately, the outcomes of this systematic review will identify a recommended standard collection protocol for use by all researchers working in human milk research, with the potential to significantly expand the opportunities for sharing and combining data from different research groups. This will enhance replicability and knowledge in the field.

Additional files

Additional file 1: PRISMA-P checklist. (DOCX 31 kb)

Additional file 2: Example Search Strategy - MEDLINE/PubMed. (DOCX 19 kb)

Abbreviations

NOS: Newcastle-Ottawa scale; PRISMA: Preferred Reporting Items for Systematic Reviews and Meta-Analysis; PRISMA-P: Preferred Reporting Items for Systematic Reviews and Meta-Analysis Protocols; WHO: World Health Organization

Acknowledgements

The data extraction form used for this protocol is modified from a standard template used by Cochrane Pregnancy and Childbirth group.

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Availability of data and materials

The datasets generated and/or analyzed during the current study will be available from the corresponding author on reasonable request.

Authors' contributions

GEL and BSM worked on the conception and design of the review in consultation with co-author PFM. GEL wrote the first draft of this protocol with substantial contribution from all authors. All authors read and approved final manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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CHAPTER 3. A SYSTEMATIC REVIEW OF COLLECTION AND ANALYSIS OF HUMAN MILK FOR MACRONUTRIENT COMPOSITION

Gabriela E. Leghi, Philippa F. Middleton, Merryn J. Netting, Mary E. Wlodek, Donna T. Geddes, and Beverly S. Muhlhausler

The manuscript entitled “A Systematic Review of Collection and Analysis of Human Milk for Macronutrient Composition” was published in the peer reviewed journal *The Journal of Nutrition*, 2020;00.

Supplemental table 1 is listed as Appendix 3. Search Strategy Example for MEDLINE/PubMed – Chapter 3

Supplemental table 2 is listed as Appendix 4. Reasons for exclusion of studies at time of data extraction – Chapter 3

Supplemental table 3 is listed as Appendix 5. Description of study population and methodological features of studies measuring fat concentrations in human milk – Chapter 3

Supplemental table 4 is listed as Appendix 6. Description of study population and methodological features of studies measuring protein concentrations in human milk – Chapter 3

Supplemental table 5 is listed as Appendix 7. Description of study population and methodological features of studies measuring lactose concentrations in human milk – Chapter 3

Statement of Authorship

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Principal Author

Name of Principal Author (Candidate)	Gabriela Esteves Leghi		
Contribution to the Paper	Contributed to the research idea, experimental design, data analysis and interpretation. Drafted and constructed manuscript and had primary responsibility for final version.		
Overall percentage (%)	70		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	05.05.2020

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

- the candidate's stated contribution to the publication is accurate (as detailed above);
- permission is granted for the candidate to include the publication in the thesis; and
- the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Philippa Middleton		
Contribution to the Paper	Contributed to the research design, development, data analysis and manuscript revision.		
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Name of Co-Author	Beverly Muhlhausler		
Contribution to the Paper	Contributed to the research idea, experimental design, data analysis and interpretation, and manuscript revision. Supervised the work and had responsibility for final content. Acted as corresponding author.		
Signature		Date	05.05.2020

Please cut and paste additional co-author panels here as required.

A Systematic Review of Collection and Analysis of Human Milk for Macronutrient Composition

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ABSTRACT

Background: As human milk (HM) composition varies by time and across even a single feed, methods of sample collection can significantly affect the results of compositional analyses and complicate comparisons between studies.

Objective: The aim was to compare the results obtained for HM macronutrient composition between studies utilizing different sampling methodologies. The results will be used as a basis to identify the most reliable HM sampling approach.

Methods: EMBASE, MEDLINE/PubMed, Cochrane Library, Scopus, Web of Science, and ProQuest databases were searched for relevant articles. Observational and interventional studies were included, and at least 2 authors screened studies and undertook data extraction. Quality assessment was conducted using the Newcastle-Ottawa scale and previously published pragmatic score.

Results: A total of 5301 publications were identified from our search, of which 101 studies were included ($n = 5049$ breastfeeding women). Methods used for HM collection were divided into 3 categories: collection of milk from all feeds over 24 h (32 studies, $n = 1309$ participants), collection at one time point (62 studies, $n = 3432$ participants), and “other methods” (7 studies, $n = 308$ participants). Fat and protein concentrations varied between collection methods within lactation stage, but there were no obvious differences in lactose concentrations. There was substantial variability between studies in other factors potentially impacting HM composition, including stage of lactation, gestational age, and analytical method, which complicated direct comparison of methods.

Conclusions: This review describes the first systematic evaluation of sampling methodologies used in studies reporting HM composition and highlights the wide range of collection methods applied in the field. This information provides an important basis for developing recommendations for best practices for HM collection for compositional analysis, which will ultimately allow combination of information from different studies and thus strengthen the body of evidence relating to contemporary HM composition. This trial was registered at PROSPERO as CRD42017072563, https://www.crd.york.ac.uk/prospero/display_record.php?ID=CRD42017072563 *J Nutr* 2020;00:1–19.

Keywords: systematic review, human milk composition, macronutrients, breast milk collection, infant health

Introduction

Human milk (HM) is uniquely designed for the human infant, containing the nutrients and bioactive components that are required to support optimal growth and development, and thus has an important role in infant survival and health (1). Consequently, the WHO recommends that infants should be exclusively breastfed until 6 mo postpartum, with continued breastfeeding to 2 y of age or beyond (2). It is critical to understand contemporary HM composition given the established importance of nutritional exposures in early infancy for an individual's lifelong health outcomes, reinforced by significant relations between the concentration of specific

components in HM and both short-term infant growth/body composition and future risk of obesity, metabolic disorders, and other noncommunicable diseases (3, 4).

Despite recognition of the importance of breastfeeding and HM, research in this area is complicated by the fact that HM composition is highly variable and can change according to the time of the day and stage of lactation, as well as between women and populations (5–8). The method and time of sample collection also have the potential to significantly influence HM composition, including the concentrations of macronutrients (fat, protein, and lactose, which ultimately impact energy content), as well as concentrations of other bioactive factors in

HM (5, 6, 9). For example, the fat concentration in HM can increase up to 3 times from its initial concentration across a single feed from 1 breast, and also increases progressively across the course of a day (10).

The sub-sampling and pooling of milk samples from a full breast expression at each feed across a 24 h period are considered the “gold standard” for HM collection (11, 12). However, this approach places a significant burden on the mother, is not possible in certain groups, and is impractical for population studies in which the goal is to obtain compositional information in a representative sample of women. Currently, several collection methods are utilized by different research groups, and there is no universal, standardized sampling approach. Thus, it is important to establish a reliable and practical method of HM collection that best represents the average composition of the milk that is being consumed by the infant, while ensuring that the method is practical, feasible, and has minimal interference with normal breastfeeding. To date, it is unclear which of the existing collection methods provide a measure of HM composition that is most closely aligned to the “gold standard” approach of 24 h pooled collection. The aim of this systematic review was to compare the results obtained for HM macronutrient composition between studies utilizing different sampling methodologies. The results will be used as a basis to identify the most reliable HM sampling approach.

Methods

Protocol

The systematic review protocol was developed based on Preferred Reporting Items for Systematic Reviews and Meta-Analysis Protocols (PRISMA-P) guidelines (13, 14) and the Cochrane Handbook for Systematic Reviews of Interventions (15). The full version of the protocol and further details, including PRISMA-P checklist file, has been previously published (16). The search strategy for MEDLINE/PubMed is presented in **Supplemental Table 1**. This trial was registered at PROSPERO as CRD42017072563.

Eligibility criteria

To be eligible for inclusion, studies must have included women who were currently breastfeeding (exclusively or partially) or routinely expressing HM (manually or using a breast pump). The studies could be conducted at any lactation stage and had to have reported the time of day of milk collection, the method of collection (i.e., whether samples were collected pre-feed, post-feed, or a full expression) and ≥ 1 measure of HM macronutrient composition (total protein, total fat and/or lactose). Studies not reported in the English language or only reported as abstracts were not included.

Information sources and search strategy

Literature searches were undertaken using the following electronic bibliographic databases: EMBASE, MEDLINE/PubMed, Cochrane Library, Scopus, Web of Science, ProQuest Dissertations, and Thesis

Global. The literature search was limited to studies in humans, but no date range restrictions were applied, and the last search was conducted in January 2018.

Selection process

The selection of articles for inclusion in the review was undertaken in 2 stages. The first stage involved screening the title and abstracts of the search results against the eligibility criteria. In the second stage, the full articles of papers selected in the title/abstract screening stage were screened to confirm that they met the eligibility criteria. At both stages, each article was screened independently by 2 authors. In the case of disagreement as to the eligibility status between the first 2 authors, the study was reviewed by a third author and any disagreements were resolved by mutual discussion.

Data extraction

Two authors independently extracted data from each included study based on a standardized extraction form modified from the Cochrane Pregnancy and Childbirth Group (17). Data extracted included methodological details [e.g., stage of lactation and time of day when milk was collected, method of collection, and analytical method for the reported macronutrient(s)], gestational age, mode of feeding (exclusively breastfeeding or mixed feeding), mode of collection (hand expression, manual/electric breast pump, or both), time since last feeding, volume collected per sample, number of samples collected per participant, and total number of samples analyzed in the study. In addition, information was extracted as to whether measures of maternal milk production or infant intake were undertaken in the study, including the use of test weighing (weighing infant before and after each feeding for 24 h), weighing of mothers before and after each feed, estimates from 24 h volume output, or use of deuterium oxide dilution technique. When missing data were identified, efforts were made to contact the corresponding authors and co-authors of the relevant study (maximum of 3 attempts).

Risk of bias in individual studies

Quality assessment of each included article was conducted independently by 2 authors based on the Newcastle-Ottawa scale and on a pragmatic score adapted from Andreas et al. (18). The risk of bias assessed the representativeness of the cohort (“truly represents” and “somewhat represents” the average lactating women in the community) and whether the study controlled for confounding factors (including, but not limited to, maternal and infant age, infant sex, and stage of lactation when the sample was collected). Based on the previously published pragmatic score, we also included additional categories: sample size (small = studies with <50 participants, medium = studies with between 50 and 100 participants, and large = studies with >100 participants); whether the study 1) stated feeding mode (exclusively or partially breastfeeding); 2) standardized stage of lactation when milk sample/s were collected; 3) stated mode of HM collection (manual expression and/or breast pump); 4) stated gestational age of infants; and 5) reported the analytical method used for macronutrient analyses. In the scoring matrix, studies were awarded a maximum of 1 point per category for binary questions (yes = 1, no = 0). For the sample size question, small, medium, and large studies were assigned 0.5, 1, and 1.5, points respectively. As a result, studies received a score out of a possible 8.5. The quality-assessment scores for each collection method are presented as means $\pm$ SDs. Other categories, including time since last feed and use of HM as the standard for protein analysis, were also included in the risk of bias assessment when these questions were applicable, but these data are presented separately since they were not relevant for all included studies.

Data synthesis

As the included data were highly diverse, it was not possible to conduct a meta-analysis and therefore findings are presented in the form of structured tables for each macronutrient (total protein, total fat, and lactose). Macronutrient concentrations are reported in grams per liter,

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Supplemental Tables 1–5 are available from the “Supplementary data” link in the online posting of the article and from the same link in the online table of contents at <https://academic.oup.com/jn/>.

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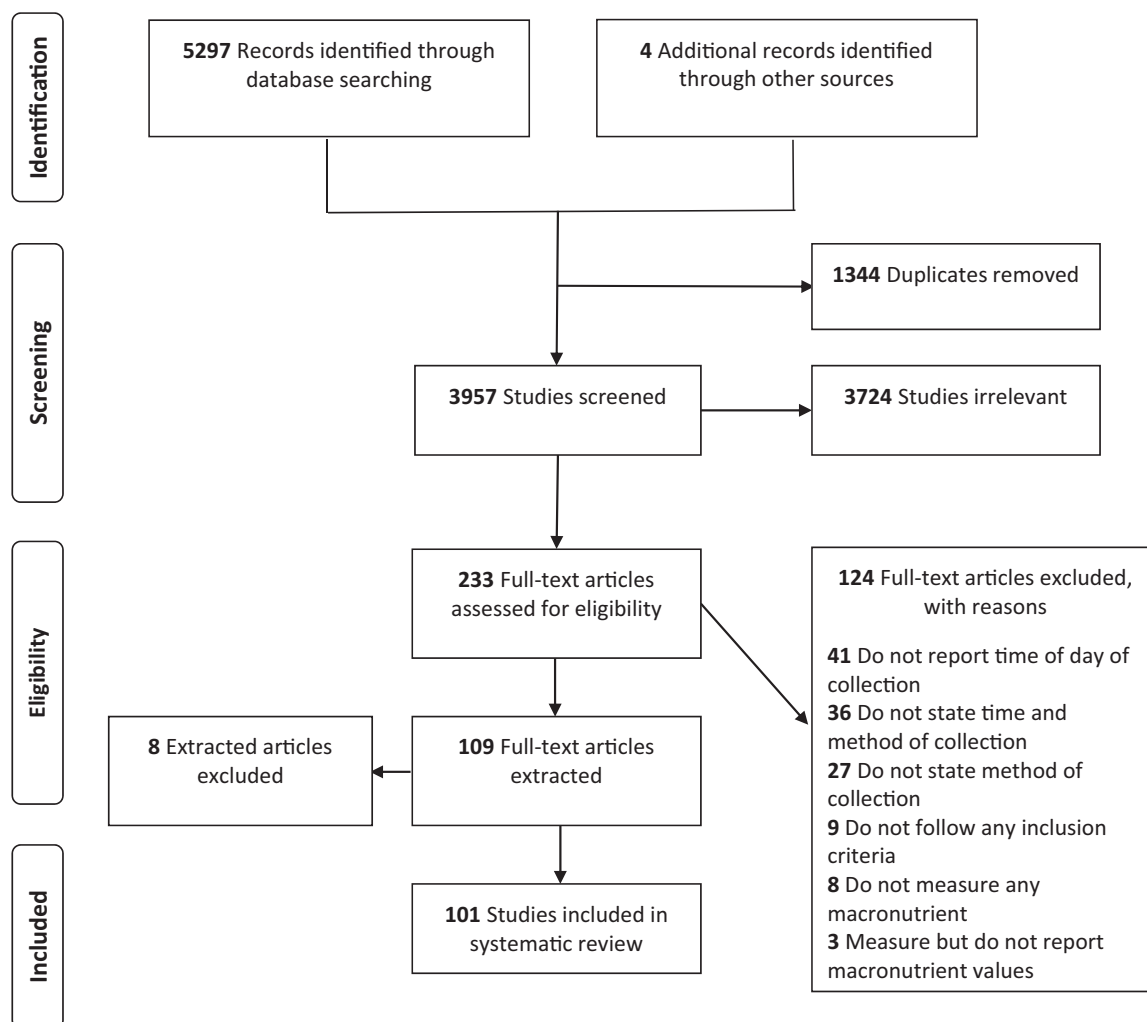


FIGURE 1 PRISMA flow diagram summarizing the process of article screening and reasons for exclusion. PRISMA, Preferred Reporting Items for Systematic Reviews and Meta-Analysis.

and any results reported in other units were first converted to grams per liter to allow direct comparison between studies. Concentrations that were reported as either percentage of creatinocrit (for fat) or total nitrogen (for protein) were converted to fat and total protein concentrations using standard conversion conventions (19) and formulas (20–22). Fat concentrations calculated from the cream percentage were based on the equation: $5.917 \times \text{cream percentage} + 3.968$ (19). For studies that only reported total nitrogen concentration, we considered the nonprotein nitrogen to represent 20% of this value (23, 24). Total protein was calculated based on the equation: $\text{protein nitrogen} = \text{total nitrogen} - \text{non protein nitrogen}$; $\text{protein nitrogen} \times 6.28 = \text{total protein}$ (20, 21). For the purpose of this systematic review, triglyceride concentrations were considered to be equivalent to fat concentration, and carbohydrate concentration was considered to be equivalent to lactose, since triglyceride makes up ~98% of the total lipids and lactose makes up ~98% of the total carbohydrates in HM (25, 26).

To provide a synthesis of macronutrient composition of HM at different lactation stages, mean macronutrient concentrations in colostrum, transitional, and mature milk were calculated for studies meeting specific inclusion criteria. These studies were conducted in term infants, clearly defined the stage of lactation when samples were collected, and reported analysis of HM samples collected at that stage (not pooled samples from different lactation stages). In the case of studies where samples were collected at one time point in the day, sample collection was conducted in the morning, as this was the most common approach.

Results

Summary of studies

Our search strategy identified a total of 5301 publications, of which 101 were included in this systematic review ($n = 5049$ breastfeeding women). A total of 8 studies were excluded after data extraction for reasons shown in **Supplemental Table 2**. **Figure 1** provides the Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) diagram for the search.

A summary of the included studies is presented in **Table 1**. The included studies were conducted in 38 different countries and regions, with sample sizes ranging from 1 to 156. Most of the studies were conducted in the United States ($n = 31$, $n = 1055$ women), followed by Australia ($n = 11$, $n = 267$ women). The majority of the studies ($n = 89$) were observational cohort studies, with 56 being longitudinal and 33 being cross-sectional in design. The remaining studies were interventional ($n = 9$) or case studies ($n = 3$). The studies included in this review had a wide range of publication dates, between 1959 and 2018, however, all but 3 studies (27–29) were published after 1980.

Samples were collected across a broad range of postpartum ages, from the day of birth to 26 mo postpartum, with most

TABLE 1 Summary of studies of human milk composition included in the systematic review¹

Reference	Site	Sample size	Stage of lactation	Outcomes measured		
				Milk fat or TGs	Milk protein or nitrogen	Milk lactose or carbohydrates
Milk samples collected across 24 h						
Full expression across 24 h						
Anderson et al., 1983 (30)	USA	23	3, 7, and 14 d	✓	✓	✓
Beijers et al., 1992 (31)	Netherlands	45	1 wk and weekly/biweekly (consecutive samples)		✓	
Bishara et al., 2008 (32)	Canada	24	21–30 d		✓	
Boutte et al., 1985 (33)	USA	9	— (average 3.2 mo)	✓		
Brown et al., 1982 (34)	Bangladesh	7	— (infants aged 6–29 mo)	✓	✓	✓
Brown et al., 1986 (35)	Bangladesh	58	0, 1, 2, 3, 4, 5, 6, 7, 8, and 9 mo	✓	✓	✓
Butte et al., 1984 (36)	USA	45	1, 2, 3, and 4 mo	✓	✓	✓
Cregan et al., 2002 (37)	Australia	38	5 d		✓	✓
Heinig et al., 1993 (38)	USA	73	3, 6, 9, and 12 mo	✓	✓	✓
Lovelady et al., 1990 (39)	USA	16	9–24 wk	✓	✓	✓
McCorry et al., 1999 (40)	USA	67	8–16 wk	✓	✓	✓
Motil et al., 1997 (41)	USA	22	6, 12, 18, and 24 wk	✓	✓	✓
Perez-Escamilla et al., 1995 (42)	Honduras	141	4, 5, and 6 mo	✓	✓	✓
Stafford et al., 1994 (43)	Mexico	10	—	✓	✓	✓
Stellwagen et al., 2013 (44)	USA	19	—	✓	✓	✓
Stuff and Nichols, 1989 (45)	USA	45	4–9 mo (consecutive samples)	✓	✓	✓
Pre- and post-feed across 24 h						
Canon et al., 2015 (9)	Australia	19	3–21 wk	✓	✓	✓
Daly et al., 1993 (46)	Australia	5	4–9 mo	✓		
Jackson et al., 1988 (47)	Thailand	25	3 wk–9 mo	✓		
Kent et al., 2006 (10)	Australia	71	1–6 mo	✓		
Khan et al., 2013 (6)	Australia	15	1–6 mo	✓	✓	✓
Mitoulas et al., 2002 (48)	Australia	17	1, 2, 4, 6, 9, and 12 mo	✓	✓	✓
Perrella and Geddes, 2016 (49)	Australia	1	4 mo	✓	✓	
Prentice et al., 1981 (50)	Gambia	60	1–18 mo	✓	✓	
Saint et al., 1986 (51)	Australia	9	2–12 mo	✓	✓	✓
Valentine et al., 1994 (52)	USA	15	1–4 wk	✓	✓	
Post-feed across 24 h						
Agostoni et al., 2001 (53)	Italy	95	1 d; 1, 3, 6, 9, and 12 mo	✓		
Agostoni et al., 2003 (54)	Italy	92	1 d; 1, 3, and 6 mo	✓		
Bauer and Gerss, 2011 (55)	Germany	112	1, 2, 3, 4, 5, 6, 7, and 8 wk		✓	✓
Marangoni et al., 2000 (56)	Italy	10	1, 3, 6, 9, and 12 mo	✓		
Marangoni et al., 2002 (57)	Italy	54	1 d and 3 mo	✓		
Morton et al., 2012 (58)	USA	67	Weekly from 1–8 wk	✓	✓	✓
Milk samples collected at one time point						
Full expression						
Aksit et al., 2002 (59)	Turkey	80	2 mo	✓		
Barbosa et al., 1997 (60)	Mexico	40	3 and 6 mo	✓	✓	✓
(Continued)						

(Continued)

TABLE 1 (Continued)

Reference	Site	Sample size	Stage of lactation	Outcomes measured		
				Milk fat or TGs	Milk protein or nitrogen	Milk lactose or carbohydrates
Butte et al., 1984 (61)	USA	21	2, 4, 6, 8, 10, and 12 wk	✓	✓	✓
Butte et al., 1992 (62)	Mexico	30	4 or 6 mo	✓	✓	✓
Chen et al., 1998 (63)	USA	40	5 d			
De Pee et al., 1997 (64)	Indonesia	155	3–6, 7–9, 10–12, and 13–18 mo	✓		
Dewey and Lönnerdal, 1983 (65)	USA	20	1, 2, 3, 4, 5, and 6 mo	✓	✓	✓
Foda et al., 2004 (66)	Japan	39	0–12 and 13–46 wk	✓	✓	✓
Goran et al., 2017 (67)	USA	25	1 and 6 mo	✓		✓
Heon et al., 2016 (68)	Canada	40	1, 3, and 6 wk	✓		
Jackson et al., 1994 (69)	USA	77	2, 3, 7, 14, 42, and 84 d	✓	✓	✓
Lönnerdal et al., 1976 (27)	Ethiopia	104	0–0.5, 0.5–1.5, 1.5–3.5, 3.5–6.5, 6.5 mo		✓	✓
	Sweden	–	4 and 5 d		✓	✓
Marquis et al., 2003 (70)	Peru	137	2 and 30 d	✓	✓	✓
Masters et al., 2002 (71)	USA	10	1–12 mo	✓		
Moran-Lew et al., 2015 (72)	Israel	32	1, 2, 3, 4, 5, 6, and 7 wk	✓	✓	✓
Mosley et al., 2007 (73)	USA	12	6–10 mo	✓	✓	✓
Neubauer et al., 1993 (74)	USA	46	2, 3, 7, 14, 42, and 84 d	✓	✓	✓
Park et al., 1999 (75)	USA	16	1–26 mo	✓		
Perrin, 2015 (76)	USA	19	11, 12, 13, 14, 15, 16, and 17 mo	✓	✓	✓
Ritzenthaler et al., 2005 (77)	USA	44	1–10 mo	✓	✓	✓
Sauer et al., 2017 (78)	USA	24	1, 7, 14, and 21 d	✓	✓	✓
Szlagatys-Sidorkiewicz et al., 2013 (79)	Poland	156	17–30 d	✓		
Thurl et al., 1993 (80)	Germany	5	8 and 57 d			✓
Wack et al., 1997 (81)	USA	133	0–60, 61–120, 121–180, 181–240, 241–300, 301–360, >360 d			✓
Williams et al., 2017 (82)	USA	16	– (average 5 mo)	✓	✓	✓
Young et al., 2017 (83)	USA	56	2 wk and 4 mo	✓	✓	✓
Pre- and post-feed						
Da Cunha et al., 2005 (84)	Brazil	77	15 d	✓		
Fornes and Dorea, 1995 (85)	Brazil	39	15, 30, 45, 60, 75, and 90 d	✓		
Gridneva et al., 2017 (86)	Australia	27	2 and 5 mo	✓	✓	✓
Grote et al., 2016 (8)	Europe	30	1, 2, 3, and 6 mo	✓	✓	✓
Hahn et al., 2018 (87)	Korea	80	4 wk	✓	✓	✓
Hassiotou et al., 2013 (88)	Australia	6	6–39 wk (consecutive samples)	✓	✓	
Karatas et al., 2011 (89)	Turkey	46	1–3 and 4–6 mo	✓		
Kuganathan et al., 2017 (7)	Australia	59	2, 5, 9, and 12 mo	✓	✓	✓
McDaniel et al., 1989 (90)	Not clear (USA or Canada)	24	2–20 mo	✓	✓	✓
Michaelsen et al., 1994 (91)	Denmark	91	4, 14, 28, 42, 56, 70, 84 d; 4, 5, 6, 7, and 8 mo	✓	✓	✓
Prentice et al., 1981 (92)	Gambia	120	1–18 mo (consecutive samples)	✓		
Schueler, 2011 (93)	USA	13	29–38 d	✓		
Pre-feed						
Al-Awadi and Sri Kumar, 2001 (94)	Kuwait ²	34	0–6, 6–12, and 12–18 mo		✓	
Antonakou et al., 2013 (95)	Greece	64	20–30 d; 3 and 6 mo	✓		

(Continued)

TABLE 1 (Continued)

Reference	Site	Sample size	Stage of lactation	Outcomes measured		
				Milk fat or TGs	Milk protein or nitrogen	Milk lactose or carbohydrates
Bachour et al., 2012 (96)	Lebanon	66	10–365 d	✓	✓	
Chavalitramong et al., 1981 (97)	Thailand	135	0–7, 8–14, 15–21, 22–28, 29–90, 91–180, 180–270, >270 d		✓	
Donangelo et al., 1989 (98)	Brazil	83	1–5, 6–30, and 31–280d		✓	
Dudzík et al., 2008 (99)	Poland	51	3, 21 and 100 d		✓	✓
Fujita et al., 2012 (100)	Kenya	83	0–20 mo		✓	
Khin-Maung et al., 1980 (101)	Burma	23	1–4, 4–7, and 7–12 mo	✓	✓	✓
Picciano and Guthrie, 1976 (28)	USA	50	6–12 wk	✓	✓	
Trugo et al., 1988 (102)	Brazil	59	1–5 and 6–36 d	✓	✓	
Tyson et al., 1976 (29)	Chile	9 ³	13–71 d (consecutive samples)	✓	✓	
Mid-feed						
Allen et al., 1991 (103)	USA	13	21, 45, 90, and 180 d	✓	✓	✓
Dutta et al., 2014 (104)	India	33	7, 28, 90, and 180 d	✓	✓	✓
Kon et al., 2014 (105)	Russia	103	1, 2, and 3 mo	✓	✓	
Lubetzky et al., 2015 (106)	Israel	72	3, 7, and 14 d	✓	✓	✓
Mandel et al., 2005 (107)	Israel	61	2–6 and >12 mo	✓	✓	✓
Pines et al., 2016 (108)	Israel	57	1–7 mo	✓	✓	✓
Quinn, 2013 (109)	The Philippines	103	<18 mo	✓	✓	✓
Quinn et al., 2016 (110)	Nepal	82	—	✓	✓	✓
Post-feed						
Bener et al., 2001 (111)	United Arab Emirates	26	—	✓	✓	✓
Cant et al., 1991 (112)	Canada	36	2–6 and 10–14 mo	✓		
Molto-Puigmartí et al., 2011 (113)	Spain	43	2–4, 8–12, and 28–32 d	✓		
Rakicioglu et al., 2006 (114)	Turkey	21	2–5 mo	✓	✓	✓
Vitolo et al., 1993 (115)	Brazil	136	1–2 d	✓	✓	
Milk samples collected by other methods						
Bassir, 1959 (116)	Nigeria	16	1, 2, and 3 mo; mixed mid-feed and post-feed	✓	✓	
Britton, 1986 (117)	USA	1	15 d; mixed pre-feed and mid-feed		✓	
De Luca et al., 2016 (118)	France	100	1 mo; expression lasted for as long as infant suckled	✓	✓	✓
Eilers et al., 2011 (119)	Germany	77	3 and 28 d; pumping for 10 min	✓	✓	
Neville et al., 1984 (120)	USA	18 ⁴	1–7 mo; mixed mid-feed and post-feed	✓	✓	✓
Shehadeh et al., 2006 (121)	Israel	84	3 mo or >12 mo; from 3 min until emptying the breast	✓	✓	✓
Woolridge et al., 1990 (122)	UK	12	4, 5, and 6 wk; pre-feed and post-feed for 12 h	✓	✓	

¹Every effort was made to follow a standardized approach when capturing information from studies into the summary tables. Any variation to the approach was due to the way in which the variable was reported in the respective paper. Methods of collection: studies were organized according to the method of collection described in the paper and the following definitions of HM methods of collection. Full expression: defined as milk collected when a single breast or both breasts were completely emptied. Pre-feed: defined as milk collected prior to infant feeding or milk collected for 3 min after milk flow began. Mid-feed: defined as milk collected at middle of the feeding or expression (after pre-feed and before post-feed). Post-feed: defined as milk collected at the end of the feeding or expression. Site: country/region where study participants were recruited. Sample size: number of participants who completed the study and provided milk samples considered for milk analyses according to our eligibility criteria. When this information was unavailable or unclear, we reported the number of individuals who participated in the study. Stage of lactation: period of lactation during which the milk samples were collected. If multiple samples were collected, e.g., on a weekly or daily basis, each time point or time window in lactation when samples were collected was reported (e.g., “3, 7 and 14 d postpartum” indicates that milk samples were collected at each of these time points, while “21–30 d postpartum” indicates that milk sample was collected between these time points and the study did not provide exact time point of collection). HM, human milk; TG(s), triglyceride(s); —, information not stated or unclear. The checkmark symbols indicate which macronutrient was assessed by each study.

²Nationalities of non-Kuwaitis group included American, Egyptian, Indian, Czech, and Taiwanese.

³Results only reported for study 3.

⁴Results only reported for protocol 2 and 3.

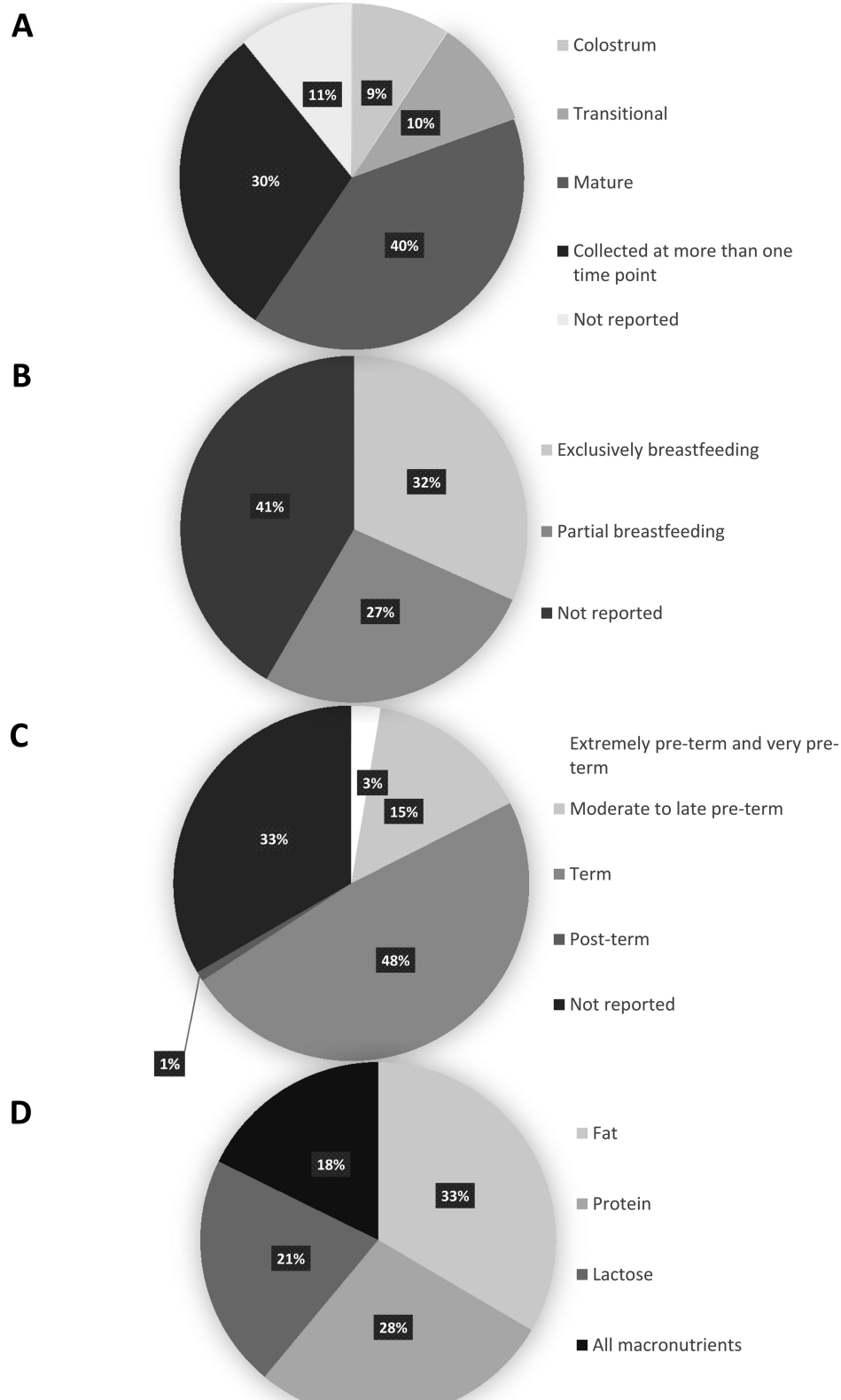


FIGURE 2 Distribution of included studies of human milk composition according to the type of milk collected (A), mode of feeding (B), gestational age of the infant (C), and milk macronutrient concentrations measured (D).

(78 studies) collecting mature milk and 58 studies collecting samples at more than one stage of lactation (Figure 2A). Of those studies that reported mode of feeding ($n = 59$), the number in which women were exclusively breastfeeding compared with partially breastfeeding was similar (Figure 2B). HM samples

were collected from mothers of infants born at different gestational ages, ranging from extremely preterm (<28 weeks' gestation) to post-term (>42 weeks' gestation); however, the majority of studies were conducted in mothers of term infants (Figure 2C).

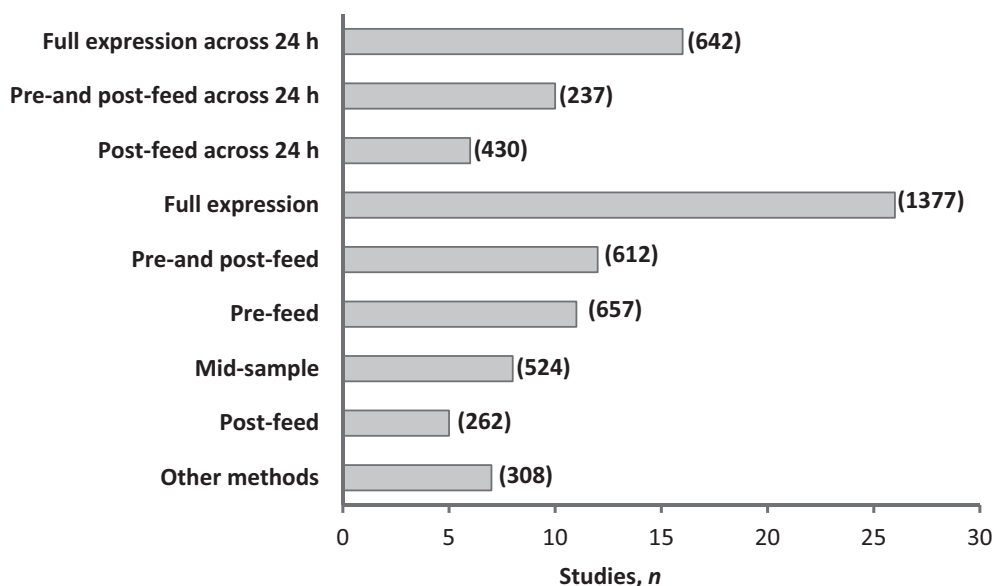


FIGURE 3 The number of studies applying each human milk collection method. Number of studies is depicted by the bar chart, with the corresponding number of participants shown in parentheses.

All studies included a measure of at least one macronutrient, and 45 studies included measures of all 3 macronutrients. Total fat was the most commonly measured macronutrient (85 studies), followed by total protein (70 studies) and lactose (54 studies) (Figure 2D).

Summary of methods used

The wide range of HM collection methods were divided into 3 broad categories, as follows:

1. Studies in which milk samples were collected at each feeding over a 24 h period ($n = 32$ studies, 1309 participants). This included studies in which either full expression, pre- and post-feed samples, or post-feed samples only collected at each feed over at least one 24 h period.
2. Studies in which milk samples were collected at one time point during the day ($n = 62$ studies, 3432 participants). This category was subdivided into studies in which a full expression, pre and post-feed samples, pre-feed samples only, mid-feed samples only, or post-feed samples only had been collected.
3. Studies in which milk samples were collected by other methods (7 studies, 308 participants), which included all studies whose method of collection did not fit into any of the other categories.

Additional details of the number of studies and participants in each of the sub-categories above are provided in Figure 3.

The most frequently used method was collection of a full expression at one time point, followed by pooling of full expressions of all feeds across 24 h and collection of pre- and post-feed samples at one time point. Of the studies collecting HM samples at one time point ($n = 62$), the majority ($n = 47$) collected samples only in the morning, followed by afternoon and evening samples ($n = 7$). Samples were collected at more than one time ($n = 6$) or the time of collection was unclear ($n = 2$) in 8 studies. Measures of maternal milk production and/or infant milk intake were measured by 46 studies. Of these, most collecting HM samples across a 24 h period measured milk production (29 out of 32 studies), whereas only 15 of the

62 studies collecting HM samples at one time point and 2 of the 7 studies that collected HM samples via other methods had assessed milk production as well as milk composition.

Determinants of methods of collection

A broad range of collection methods were used, independent of the macronutrient(s) being measured in the study (Figure 4). Most studies collected samples at one time point, regardless of whether they reported fat concentration (50 of 85 studies), protein concentration (42 of 70 studies), or lactose concentrations (35 of 54 studies). For each macronutrient, the method of collection used was highly variable (Figure 4).

Geographically, 31 studies were performed in United States, with 24 collecting full expression (15 at one time point and 9 across 24 h), whereas of the 11 studies conducted in Australia, 7 adopted the collection of 24 h pre- and post-feed samples. The 2 largest studies ($n = 155$ and 156 participants) both utilized full breast expression collected at one time point. No other clear patterns as to the choice of method in the individual studies could be identified.

Determinants of macronutrient concentrations

The mean fat, protein, and lactose concentrations in colostrum, transitional, and mature HM obtained in samples collected from mothers of term infants using different HM collection methods are presented in Tables 2–4. Relatively few studies meeting the inclusion criteria measured macronutrient concentrations in transitional HM or colostrum; however, the available data suggested that fat concentration was lower and protein concentration higher in colostrum and transitional HM compared with mature HM (Tables 2 and 3), while lactose concentration was similar across lactation stages (Table 4). In mature HM, fat and protein concentrations were similar between studies that collected full expressions across 24 h or pooled pre- and post-feed samples either at one time point (morning) or across 24 h, but were higher in studies that collected either post-feed samples or a full expression samples at one time point (morning) (Tables 2 and 3). In the case of fat, but not protein, the concentrations in HM were lower in studies that collected pre-feed samples (Table 2). Lactose concentration of HM was similar in studies

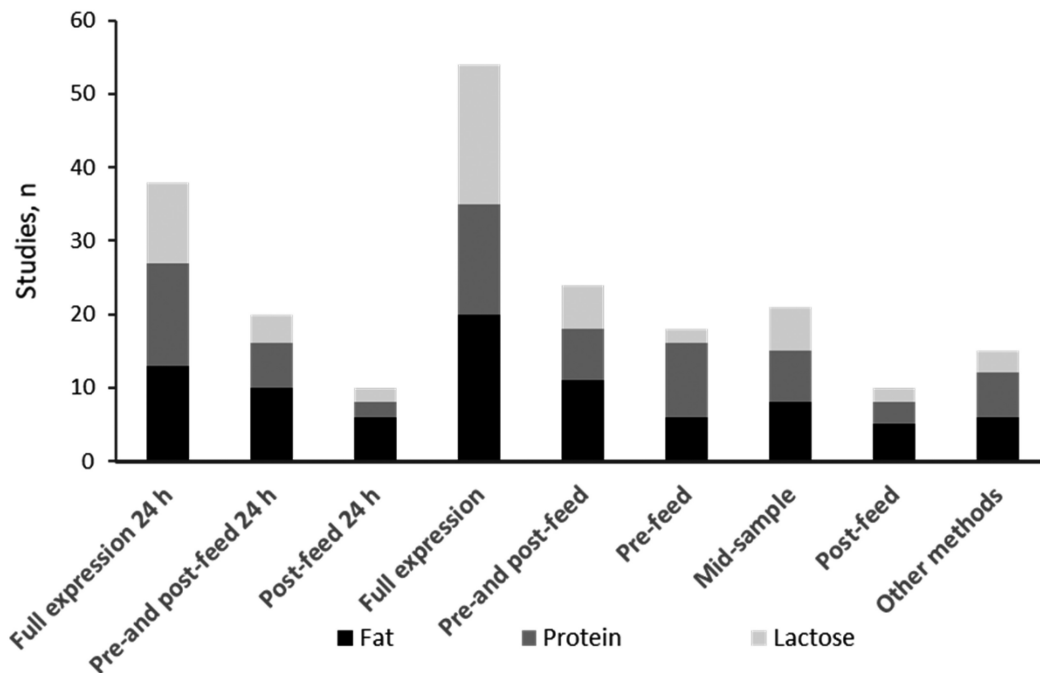


FIGURE 4 The number of studies applying each of the different human milk collection methods for the measurement of fat, protein, and lactose.

that had undertaken a full expression either at one time point or across 24 h and for pre- and post-feed or post-feed across a 24 h period, and there were insufficient studies to compare between other methods (Table 4). A full list of macronutrient concentrations obtained for all studies included in the review, including studies undertaken in preterm infants and studies where the stage of lactation was not stated or unclear and therefore not included in the major synthesis, is presented in Supplemental Tables 3–5.

Comparison of analytical methods

Sixteen different analytical methods were reported for fat analysis, with the most common being the creatatocrit method ($n = 28$) and Folch extraction ($n = 13$). While direct comparisons between studies are complicated by the significant variability in HM fat concentrations existing between individual women and between populations, there did not appear to be any significant difference in the average fat concentration of HM at any stage of lactation depending on the analytical method used. It is important to note, however, that not all studies applied the same formula for converting the creatatocrit value

to the percentage fat, making it difficult to undertake a direct comparison.

Protein was analyzed by 16 different analytical methods across the studies, with the most popular being the Kjeldahl method, including micro-Kjeldahl and modified versions ($n = 22$) and Lowry assay (and modifications thereof) ($n = 7$). Protein concentrations varied considerably between studies, independently of the analytical and collection methods, but no clear patterns could be identified. Of the studies that reported protein concentrations, 9 reported concentrations of protein nitrogen (36, 41, 45, 61, 116) and total nitrogen (34, 35, 52, 74).

A total of 15 different analytical methods were used for the measurement of lactose concentrations, with the most common being the enzymatic method, including enzymatic-spectrophotometer ($n = 14$), followed by automated analyzer ($n = 10$; Yellow Springs Instrument and Technicon). The choice of analytical method seemed to be a more important factor influencing lactose concentration than method and time of milk collection. For instance, some studies using the Mid-Infrared Milk (MIRIS) analyzer, Sweden ($n = 5$) tended to report lower

TABLE 2 Fat concentrations in colostrum, transitional, and mature milk according to human milk collection method¹

Method of collection	Time of collection	Mature		Transitional		Colostrum	
		Included studies, <i>n</i> (total participants, <i>n</i>)	Milk concentration, g/L	Included studies, <i>n</i> (total participants, <i>n</i>)	Milk concentration, g/L	Included studies, <i>n</i> (total participants, <i>n</i>)	Milk concentration, g/L
Full expression	24 h period	8 (424)	35.2 ± 3.1	1 (23)	31.0 ± 10.0	1 (23)	18.0 ± 6.0
Pre- and post-feed	24 h period	6 (148)	34.5 ± 10.1	0	—	0	—
Post-feed	24 h period	5 (332)	44.6 ± 2.5	0	—	4 (220)	17.6 ± 4.1
Full expression	Morning	6 (449)	42.4 ± 13.1	2 (77)	36.5 ± 1.9	1 (137)	24.2 ± 1.0
Pre- and post-feed	Morning	4 (180)	36.0 ± 11.5	0	—	0	—
Pre-feed	Morning	2 (114)	31.1 ± 5.3	0	—	0	—
Mid-feed	Morning	1 (103)	38.9 ± 6.5	0	—	0	—
Post-feed	Morning	1 (43)	30.6 ± 6.5	1 (43)	31.1 ± 15.3	2 (179)	28.7 ± 3.8

¹Data are presented as means ± SDs unless otherwise indicated.

TABLE 3 Protein concentrations in colostrum, transitional, and mature milk according to human milk collection method¹

Method of collection	Time of collection	Mature		Transitional		Colostrum	
		Included studies, <i>n</i> (total participants, <i>n</i>)	Milk concentration, g/L	Included studies, <i>n</i> (total participants, <i>n</i>)	Milk concentration, g/L	Included studies, <i>n</i> (total participants, <i>n</i>)	Milk concentration, g/L
Full expression	24 h period	8 (432)	9.4 ± 2.1	1 (23)	17.0 ± 2.0	1 (23)	23.0 ± 6.0
Pre- and post-feed	24 h period	4 (52)	9.9 ± 1.6	0	—	0	—
Post-feed	24 h period	1 (112)	16.0	0	—	0	—
Full expression	Morning	4 (233)	13.3 ± 4	2 (77)	11.3 ± 1.3	1 (137)	29.0 ± 2.2
Pre- and post-feed	Morning	4 (207)	10.4 ± 1.8	1 (91)	13.3 ± 3.4	1 (91)	18.0 ± 3.5
Pre-feed	Morning	2 (101)	12.6 ± 0.9	0	—	2 (110)	18.9 ± 0.1
Mid-feed	Morning	1 (103)	16.1 ± 1.6	0	—	0	—
Post-feed	Morning	0	—	0	—	0	—

¹Data are presented as means ± SDs unless otherwise indicated.

concentrations (72, 108) compared with studies using other methods.

Direct comparison of methods of collection within a study

A total of 21 studies included in this systematic review directly compared the same analyte (fat, *n* = 10; protein, *n* = 7; lactose, *n* = 4) for HM samples obtained using different collection methods. All studies reported that fat concentration differed according to the method of collection, with post-feed concentrations significantly higher compared with pre-feed concentrations. There were, however, no systematic differences reported for either protein or lactose concentrations according to the method of collection.

Quality assessment

The risk of bias assessment is summarized in Table 5. Overall quality of the studies was relatively low, with a median ± SD score of 5.6 ± 1.6 (out of a possible 8.5) across all studies, and 29 studies scoring <50% of the maximum score. The highest scores were similar for studies that utilized full expression across 24 h (5.9 ± 1.7), mid-feed (5.9 ± 1.1), post-feed across 24 h (5.8 ± 1.2), and pre- and post-feed (5.8 ± 1.4) across 24 h, while those studies in which HM samples were collected post-feed at one time point appeared to have lower scores (3.9 ± 2.2).

A large number of studies, particularly those that collected samples at only one time point, failed to report key pieces of information. Of relevant studies collecting samples at one time point (full expression, pre- and post-feed, pre-feed, and most of the other methods), less than half of the studies standardized the time since last feed or expression for which the sample was collected (*n* = 22 of 55 studies). The majority of samples

utilizing Bradford assays for protein measurement (*n* = 9 of 12 studies) used HM samples as standards.

Discussion

The current review describes the first systematic evaluation of the range of sampling methodologies used in studies reporting HM composition. The review has highlighted the wide range of collection methods applied in the field, as well as the substantial variability between studies in other factors that may impact HM composition, including stage of lactation, whether infants are term or preterm, and the analytical method applied to measure macronutrient concentrations. This information provides an important basis for developing recommendations for best practices for HM collection for compositional analysis, which, if it were to be adopted by researchers in the field, would considerably expand the opportunities for combining data from different research groups and thus advance knowledge in the field.

Selecting a HM sampling method requires researchers to identify the most representative method of collection that is feasible for a given study. Based on the evidence synthesized in this review, and the relative strengths and weaknesses of different collection methods (Table 6), collection of pre- and post-feed samples (and pooling prior to analysis) for all feeds across a 24 h period represents the most appropriate alternative to the “gold standard.” Pre- and post-feed sampling avoids issues associated with collection of full breast expressions, while still obtaining representative compositional data and accounting for diurnal variations in HM composition. Where collection of samples at all feeds across 24 h is not feasible,

TABLE 4 Lactose concentrations in colostrum, transitional, and mature milk according to human milk collection method¹

Method of collection	Time of collection	Mature		Transitional		Colostrum	
		Included studies, <i>n</i> (total participants, <i>n</i>)	Milk concentration, g/L	Included studies, <i>n</i> (total participants, <i>n</i>)	Milk concentration, g/L	Included studies, <i>n</i> (total participants, <i>n</i>)	Milk concentration, g/L
Full expression	24 h period	6 (224)	63.5 ± 6.3	1 (23)	67.0 ± 5.0	1 (23)	62.0 ± 9.0
Pre- and post-feed	24 h period	3 (51)	63.2 ± 2.8	0	—	0	—
Post-feed	24 h period	1 (112)	62.0 ± 9	0	—	0	—
Full expression	Morning	4 (237)	64.3 ± 8.6	1 (56)	65.0 ± 9.0	2 (177)	57.9 ± 5.9
Pre- and post-feed	Morning	3 (116)	71.8 ± 5.4	—	—	0	—
Pre-feed	Morning	1 (51)	69.5 ± 4.9	—	—	1 (51)	69.0
Mid-feed	Morning	0	—	—	—	0	—
Post-feed	Morning	0	—	—	—	0	—

¹Data are presented as means ± SDs unless otherwise indicated.

TABLE 5 Risk of bias assessment for studies measuring macronutrient concentrations in human milk¹

Reference	Representativeness of cohort	Controls for confounders	Sample size (small, medium, or large study)	State mode of feeding	Standard postpartum HM collection	State mode collection (manual/pump)	State gestational age	Report analytical method	Score
Full expression across 24 h									
Anderson et al., 1983 (30)	✓	✓	Small	✓	✓	✓	✓	✓	7.5
Beijers et al., 1992 (31)	✓	x	Small	x	x	✓	✓	✓	4.5
Bishara et al., 2008 (32)	✓	x	Small	x	✓	✓	✓	✓	5.5
Boutte et al., 1985 (33)	✓	x	Small	✓	x	✓	x	✓	4.5
Brown et al., 1982 (34)	x	x	Small	x	x	✓	x	✓	2.5
Brown et al., 1986 (35)	x	x	Medium	✓	✓	✓	x	✓	5
Butte et al., 1984 (36)	✓	x	Small	✓	✓	✓	✓	✓	6.5
Cregan et al., 2002 (37)	✓	✓	Small	✓	✓	x	✓	✓	6.5
Heinig et al., 1993 (38)	✓	✓	Medium	✓	✓	x	✓	✓	7
Lovelady et al., 1990 (39)	✓	✓	Small	✓	✓	✓	✓	✓	7.5
McCroy et al., 1999 (40)	x	✓	Medium	✓	✓	✓	✓	✓	7
Motil et al., 1997 (41)	✓	✓	Small	✓	✓	x	✓	✓	6.5
Perez-Escamilla et al., 1995 (42)	✓	✓	Large	✓	✓	✓	✓	✓	8.5
Stafford et al., 1994 (43)	✓	x	Small	x	x	✓	x	✓	3.5
Stelwagen et al., 2013 (44)	x	x	Small	✓	x	✓	✓	✓	4.5
Stuff et al., 1989 (45)	✓	✓	Small	✓	✓	✓	✓	✓	7.5
Overall									5.9 ± 1.7
Pre- and post-feed across 24 h									
Camon et al., 2015 (9)	✓	✓	Small	✓	✓	✓	✓	✓	7.5
Daly et al., 1993 (46)	x	x	Small	✓	✓	x	x	✓	3.5
Jackson et al., 1988 (47)	✓	✓	Small	✓	✓	✓	✓	✓	7.5
Kent et al., 2006 (10)	✓	✓	Small	✓	✓	✓	✓	✓	7.5
Khan et al., 2013 (6)	✓	✓	Small	✓	✓	✓	✓	✓	7.5
Mitoulas et al., 2002 (48)	✓	x	Small	✓	✓	✓	✓	✓	6.5
Perrella and Geddes, 2016 (49)	x	x	Small	✓	✓	✓	✓	✓	5.5
Prentice et al., 1981 (50)	✓	x	Medium	x	x	✓	x	✓	4
Saint et al., 1986 (51)	x	x	Small	✓	x	✓	✓	✓	4.5
Valentine et al., 1994 (52)	x	x	Small	x	✓	✓	x	✓	3.5
Overall									5.8 ± 1.8

(Continued)

TABLE 5 (Continued)

Reference	Representativeness of cohort	Controls for confounders	Sample size (small, medium, or large study)	State mode of feeding	Standard postpartum HM collection	State mode collection (manual/pump)	State gestational age	Report analytical method	Score
Post-feed across 24 h									
Agostoni et al., 2001 (53)	✓	x	Large	✓	✓	x	✓	✓	6.5
Agostoni et al., 2003 (54)	✓	x	Large	✓	✓	x	✓	✓	6.5
Bauer and Gerss, 2011 (55)	✓	✓	Large	x	✓	✓	✓	✓	7.5
Marangoni et al., 2000 (56)	✓	x	Small	x	✓	x	✓	✓	4.5
Marangoni et al., 2002 (57)	✓	x	Medium	x	✓	x	✓	✓	5
Morton et al., 2012 (58)	x	x	Medium	x	✓	✓	✓	✓	5
Overall									5.8 ± 1.2
Full expression									
Aksit et al., 2002 (59)	✓	x	Medium	✓	✓	✓	✓	✓	7
Barbosa et al., 1997 (60)	x	x	Small	x	✓	✓	x	✓	3.5
Butte et al., 1984 (61)	✓	✓	Small	x	✓	✓	✓	✓	6.5
Butte et al., 1992 (62)	✓	✓	Small	x	✓	✓	✓	✓	6.5
Chen et al., 1998 (63)	✓	✓	Small	✓	✓	✓	✓	✓	7.5
De Pee et al., 1997 (64)	✓	x	Large	x	✓	✓	x	✓	5.5
Dewey and Lönnerdal, 1983 (65)	✓	x	Small	✓	✓	✓	x	✓	5.5
Foda et al., 2004 (66)	x	x	Small	x	✓	✓	x	✓	3.5
Goran et al., 2017 (67)	✓	x	Small	✓	✓	✓	✓	✓	6.5
Heon et al., 2016 (68)	✓	x	Small	✓	✓	✓	✓	✓	6.5
Jackson et al., 1994 (69)	✓	x	Medium	x	✓	✓	✓	✓	6
Lönnerdal et al., 1976 (27)	✓	x	Large	x	✓	✓	x	✓	5.5
Marquis et al., 2003 (70)	x	✓	Large	x	✓	✓	✓	✓	6.5
Masters et al., 2002 (71)	✓	x	Small	x	x	✓	x	✓	3.5
Moran-Lev et al., 2015 (72)	x	✓	Small	✓	✓	✓	✓	✓	6.5
Mosley et al., 2007 (73)	✓	✓	Small	✓	✓	✓	x	✓	6.5
Neubauer et al., 1993 (74)	✓	✓	Small	x	✓	✓	✓	✓	6.5
Park et al., 1999 (75)	x	x	Small	x	x	✓	x	✓	2.5
Perrin, 2015 (76)	✓	✓	Small	✓	✓	✓	✓	✓	7.5
Ritzenthaler et al., 2005 (77)	✓	x	Small	x	x	✓	x	✓	3.5
Sauer et al., 2017 (78)	x	x	Small	x	✓	✓	✓	✓	4.5
Śliagatys-Sidorkiewicz et al., 2013 (79)	✓	x	Large	✓	✓	✓	✓	✓	7.5
Thurl et al., 1993 (80)	x	x	Small	✓	✓	✓	x	✓	4.5
Wack et al., 1997 (81)	✓	x	Large	x	✓	✓	x	✓	5.5
Williams et al., 2017 (82)	✓	x	Small	x	x	✓	x	✓	3.5
Young et al., 2017 (83)	✓	✓	Medium	✓	✓	✓	✓	✓	8
Overall									5.6 ± 1.5

(Continued)

TABLE 5 (Continued)

Reference	Representativeness of cohort	Controls for confounders	Sample size (small, medium, or large study)	State mode of feeding	Standard postpartum HM collection	State mode collection (manual/pump)	State gestational age	Report analytical method	Score
Pre- and post-feed									
Da Cunha et al., 2005 (84)	✓	✓	Medium	✓	✓	✓	✓	✓	8
Fornes and Dorea, 1995 (85)	✓	x	Small	✓	✓	✓	x	✓	5.5
Gridneva et al., 2017 (86)	✓	✓	Small	x	✓	✓	✓	✓	6.5
Grote et al., 2016 (8)	✓	✓	Small	✓	✓	✓	✓	✓	7.5
Hahn et al., 2018 (87)	x	x	Medium	✓	✓	x	x	✓	4
Hassiotou et al., 2013 (88)	✓	✓	Small	x	✓	✓	x	✓	5.5
Karatas et al., 2011 (89)	✓	x	Small	✓	✓	x	✓	✓	5.5
Kuganathan et al., 2017 (7)	✓	✓	Medium	✓	✓	✓	✓	✓	8
McDaniel et al., 1989 (90)	x	✓	Small	x	x	✓	x	✓	3.5
Michaelsen et al., 1994 (91)	✓	x	Medium	✓	x	x	✓	✓	5
Prentice et al., 1981 (92)	✓	✓	Large	x	x	✓	x	✓	5.5
Schueler, 2011 (93)	✓	x	Small	✓	✓	✓	x	✓	5.5
Overall									5.8 ± 1.4
Pre-feed									
Al-Awadi and Srikumar, 2001 (94)	✓	x	Small	✓	✓	x	x	✓	4.5
Antonakou et al., 2013 (95)	✓	x	Medium	✓	✓	✓	✓	✓	7
Bachour et al., 2012 (96)	x	x	Medium	x	x	✓	x	✓	3
Chavalittamrong et al., 1981 (97)	x	x	Large	x	✓	✓	x	✓	4.5
Donangelo et al., 1989 (98)	✓	x	Medium	✓	✓	✓	x	✓	6
Dudzic et al., 2008 (99)	✓	x	Medium	✓	✓	✓	✓	x	6
Fujita et al., 2012 (100)	✓	✓	Medium	✓	x	✓	x	✓	6
Khin-Maung et al., 1980 (101)	x	x	Small	✓	✓	✓	x	✓	4.5
Picciano and Guthrie, 1976 (28)	✓	x	Small	✓	✓	✓	✓	✓	6.5
Trugo et al., 1988 (102)	x	x	Medium	✓	✓	✓	✓	✓	6
Tyson et al., 1976 (29)	✓	x	Small	✓	✓	✓	✓	✓	6.5
Overall									5.5 ± 1.2

(Continued)

TABLE 5 (Continued)

Reference	Representativeness of cohort	Controls for confounders	Sample size (small, medium, or large study)	State mode of feeding	Standard postpartum HM collection	State mode collection (manual/pump)	State gestational age	Report analytical method	Score
Mid-feed									
Allen et al., 1991 (103)	x	x	Small	✓	✓	✓	x	✓	4.5
Dutta et al., 2014 (104)	✓	x	Small	✓	✓	✓	✓	✓	6.5
Kon et al., 2014 (105)	✓	x	Large	x	✓	✓	✓	x	5.5
Lubetzky et al., 2015 (106)	✓	x	Medium	x	✓	✓	✓	✓	6
Mandel et al., 2005 (107)	✓	✓	Medium	✓	✓	✓	✓	✓	8
Pines et al., 2016 (108)	✓	x	Medium	✓	✓	✓	x	✓	6
Quinn, 2013 (109)	✓	✓	Large	x	x	✓	x	✓	5.5
Quinn et al., 2016 (110)	✓	✓	Medium	x	x	✓	x	✓	5
Overall									5.9 ± 1.1
Post-feed									
Bener et al., 2001 (111)	x	x	Small	x	x	x	x	✓	1.5
Cant et al., 1991 (112)	x	x	Small	x	x	x	x	✓	1.5
Molto-Puigmartí et al., 2011 (113)	✓	x	Small	x	✓	✓	✓	✓	5.5
Rakicioglu et al., 2006 (114)	✓	x	Small	✓	✓	✓	x	✓	5.5
Vitolo et al., 1993 (115)	x	x	Large	x	✓	✓	✓	✓	5.5
Overall									3.9 ± 2.2
Other methods									
Bassir, 1959 (116)	x	x	Small	x	✓	✓	x	x	2.5
Britton, 1986 (117)	x	x	Small	✓	✓	✓	✓	✓	5.5
De Luca et al., 2016 (118)	✓	✓	Large	✓	✓	✓	✓	✓	8.5
Eilers et al., 2011 (119)	✓	x	Medium	x	✓	✓	✓	x	5
Neville et al., 1984 (120)	✓	x	Small	✓	x	✓	✓	✓	5.5
Shehadeh et al., 2006 (121)	✓	x	Medium	✓	✓	✓	✓	✓	7
Woolridge et al., 1990 (122)	✓	x	Small	x	✓	x	✓	✓	4.5
Overall									5.5 ± 1.9

¹The quality-assessment scores for each collection method are presented as means ± SDs. Sample size is defined as small = studies with <50 participants, medium = studies with between 50 and 100 participants, and large = studies with > 100 participants. The checkmark symbol indicates yes and cross symbol indicates no.

TABLE 6 Methodological and practical considerations of different human milk collection methods¹

Time of collection	Methods of collection	Best represents feed	Accounts for diurnal changes	Minimal interference with infant feeding	Suitable for large studies	Minimal issues HM limited supply	Minimal issues transport/storage of samples	Less burden mothers/infants
24 h sampling	Full expression	✓	✓	x	x	x	x	x
24 h sampling	Pre- and post-feed	x	✓	✓	x	x	x	x
24 h sampling	Post-feed	x	✓	✓	x	✓	x	x
One sample	Full expression	✓	x	x	x	x	✓	x
One sample	Pre- and post-feed	x	x	✓	✓	✓	✓	✓
One sample	Pre-feed	x	x	✓	✓	✓	✓	✓
One sample	Mid-feed	x	x	✓	✓	✓	✓	✓
One sample	Post-feed	x	x	✓	✓	✓	✓	✓

¹ HM, human milk. The checkmark symbol indicates yes and cross symbol indicates no.

collection of pre- and post-feed samples 3 times/d may be more practical and would still account for diurnal variations in composition. If sample collection at one time point is the only pragmatic option, for example in large population-based studies, the recommended approach would be collection of pre- and post-feed samples (pooled for analysis) at the same time of day for all participants, preferably the morning, and at a consistent time after the previous feeding (at least 2 h when the breast has synthesized a reasonable amount of milk). These recommendations are particularly relevant for the analysis of fat concentration, given that the amount of milk in the breast is related to fat concentration and this varies over the day (10). In addition, other factors, such as the breast from which samples are collected, how milk samples are obtained (hand expression vs. manual/electric pump), as well as stage of lactation should also be standardized across all participants. Further to this reporting, and ideally standardizing, other factors known to influence HM composition [including mode of feeding (exclusively breastfeeding, partial breastfeeding, and formula feeding), term vs. preterm, stage of lactation] are also critical (7, 8, 55, 123–125). The key points to consider when planning a study measuring HM composition are presented in Table 7.

This systematic review has identified a number of different HM collection methods that were applied across the studies. While, perhaps unsurprisingly, the method of collection used tended to be consistent across research groups, there was no clear rationale for selection of the collection method. It was notable, however, that the “gold standard” method of 24 h collection of full expression of all feeds tended to be applied to a greater extent in older studies, and 2 of these studies stated that this method was selected as it was believed to be the most representative of milk consumed by the infant (30, 39). More recent studies, however, tended to collect one expression or pre-/post-feed samples at one time point across the day, usually the

morning. This may reflect the tightening of ethical requirements around human research over time, particularly the need to justify the burden to participants in the context of the research question. In addition, full expressions require the infant to be fed via other means (bottle or cup), which then limits the population for recruitment. Collection of full breast expressions from all feedings over a 24 h period represents a substantial burden for the mother, is not practical for large population-based studies, and may have the potential to interfere with normal breastfeeding (126, 127). Indeed, 2 of the studies in this review elected to collect samples at only one time point to minimize interference with infant feeding (103, 110). Consequently, it can be argued that applying the “gold standard” approach may restrict the women who are willing and able to participate, and that the results obtained from these studies may therefore not be representative of the general population of breastfeeding women.

In the move away from collection of full expressions at all feeds across a 24 h period, towards collection at one time point, there also appeared to be parallel tendency to collect smaller sample volumes either pre-, post-, or during a feed. While assessing the composition of a full expression is generally thought to be most representative of the milk that the infant consumes (36, 127), 1 study included in this review directly compared a full expression with pooled pre- and post-feed samples from the same woman and found little difference in protein concentrations between these approaches (32). This is important, since full breast expressions have a higher participant burden than spot collections and may not be possible in certain women/population groups. If equivalent results can be obtained by pooling pre- and post-feed samples, this increases the opportunity to access broader population groups and undertake larger studies, both of which are important for the generalizability of the findings.

We had expected that the macronutrient measured would be an important determinant of the choice of method of HM

TABLE 7 Key recommendations to consider when planning a study measuring human milk macronutrient composition¹

Overall	Report and standardize			
	Collection procedure	Other factors	Analytical method	Milk production
Same sampling procedure for all participating women	Time of day of collection	Stage of lactation	Validated for HM	Maternal milk production and/or
	Collection mode (hand expression and/or breast pump)	Infant gestational age	Use of HM standards, where applicable	Infant milk intake across 24 h whenever possible
	Collection breast	Mode of feeding (exclusively or partial breastfeeding)		
	Time since last feed or expression			

¹ HM, human milk.

collection, particularly for fat concentration (10). However, this speculation was not supported by the findings. Nevertheless, the collection method did influence macronutrient composition. Higher fat concentrations in post-feed samples compared with pre-feed samples were expected, given emptying of the breast has been consistently associated with increases in fat concentration across a feed or breast expression (9, 10). However, the higher mean HM protein concentration reported by studies that had collected samples post-feed or full expression at one time point was not anticipated (6). This may imply that protein concentrations in HM have the potential to be impacted by collection method, although other factors, including whether mothers were partially or full breastfeeding and the analytical method applied, may also contribute to these differences. Indeed, synthesizing the information related to average macronutrient composition by lactation stage and collection method was complicated by the considerable heterogeneity between studies in relation to other factors known to impact HM composition (7, 8, 55, 123–125), which again emphasizes the importance of reporting, and ideally standardizing, these factors.

An unexpected finding was the substantial variability in the analytical methods that were applied to assess all macronutrients in the HM samples, which also complicated comparisons between studies and needs to be considered when interpreting the results. There was an overall lack of detail in reporting of analytical methods, as well as handling of the samples post-collection. As sample treatment and management, including transport conditions, homogenization, freeze-thaw cycles, and duration and conditions of storage, all have the potential to impact HM composition (128–130), it is critical that these factors are reported with sufficient detail to enable results of compositional analyses to be interpreted appropriately.

Our review highlights the general low quality of many of the included studies in the HM area, particularly regarding lack of detailed information on HM collection procedures. Many studies were excluded at the full-text screening stage due to not reporting key factors, including time of day of collection or method of sample collection. Most studies did not provide detailed information on other factors that could impact HM composition, including stage of lactation, parity, and gestational age of the infant at birth. Time since the last breastfeeding that HM samples were collected was also reported in less than half of the studies collecting samples at one time point, despite evidence that HM samples collected at least 2 h after a previous feeding (131), and when the breast is full or near full, provide the most reliable representation of the HM composition of the subsequent feed (132). Most included studies did not assess measures of maternal HM production or infant intake, limiting the ability to account for volume, which could impact sampling for fat concentration. Inclusion of objective measures of milk production/infant intake, such as test weighing or deuterium-labeled water, in addition to compositional analysis, would improve estimates of variability due to maternal and infant factors.

A major strength of this systematic review is the comprehensive analysis across broad range of studies, geographical regions, study populations, and study designs, including observational cohort (longitudinal and cross-sectional), interventional, and case studies. Further, lactating women across different regions, populations, settings (urban and rural), ethnic and sociodemographic backgrounds, and stages of lactation were included. A clear limitation of our review was the restricted number of studies in which we could undertake comparison of results

from different studies using the “gold standard” sampling method versus alternative methods of collection and to calculate the average concentrations at different lactation stages, which limited the generalizability of these findings.

In conclusion, this systematic evaluation highlights the wide range of sampling methods applied in studies assessing HM composition around the world. Our findings, particularly the quality assessment, reinforce the need for establishing a standardized method for HM collection to ensure accurate analysis of milk components with respect to infant outcomes. In addition, the reporting and standardization of collection procedures, validation of analytical methodology, and inclusion of HM production measures would contribute to a more representative compositional analysis. Our work provides research groups in this field with valuable guidance on the details required when designing and reporting studies, particularly in relation to the methodology used for HM collection.

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CHAPTER 4. DAILY VARIATION OF MACRONUTRIENT CONCENTRATIONS IN MATURE MILK OVER 3 WEEKS

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4.1 Abstract

Human milk (HM) composition is known to be highly variable, both between individuals and across the duration of lactation. It is less clear, however, to what extent fat, lactose and protein concentrations in HM change daily over shorter time periods in mature HM, and no studies have evaluated this to date. The aim of this study was to systematically assess and compare HM macronutrient concentrations in samples collected at different times of day, from left and right breasts and daily across a 3-week period in the same woman. Fifteen lactating women (1.6 to 4.9 months postpartum) collected daily pre-feed HM samples from both breasts each morning for 21 consecutive days and completed intensive sampling once a week (morning, afternoon and evening samples) during this period. Concentrations of fat, protein and lactose in HM did not differ according to time of day, day of week or breast used for collection. The results of this study suggest that pre-feed samples collected at any point across a 3-week period and from either the left or right breast provide comparable measures of fat, protein and lactose concentrations in mature HM, in pragmatic studies where women are collecting their own HM samples.

Clinical trial registration: Australian New Zealand Clinical Trials Registry (ACTRN12619000606189).

Keywords: human milk, lactation, sampling, macronutrients, fat, protein, lactose

4.2 Introduction

Human milk (HM) composition is highly variable, both between individuals and across lactation [16,53,169]. Fat concentration is generally considered to be the most dynamic, and varies across a single breastfeed or expression, as well as across the day, in the majority of studies [20,21,169]. Protein and lactose concentrations are generally reported to be more stable, both across a feed and over the course of the day, however not all studies have produced consistent results [19,21,169]. Considerable shifts in macronutrient (protein, fat, lactose) composition have also been reported to occur across lactation, particularly in the first few weeks postpartum [53,161]. It is less clear, however, to what extent fat, lactose and protein concentrations in HM change daily over shorter time periods in mature HM, and no studies have evaluated this to date.

There have been suggestions that there are differences in the composition of HM sampled from different breasts in the same woman [39]. This is potentially due to differences in breast fullness and time since the infant last fed from that specific breast, since both of these factors have been associated with differences in fat content [20]. However, evaluation of between-breast differences in HM macronutrient composition is limited and data in the existing literature are not consistent. Three studies have found no significant differences in HM concentrations of fat, protein or lactose [173] or in casein and whey proteins [213] between breasts over a 24-h period or over 3 week period (sampling HM once a week) [39]. One of the studies did, however, identify significant differences between breasts in HM protein content in 5 of the 20 participants [39].

The dynamic variations in HM macronutrient composition, as well as potential differences in composition between breasts, suggests that the method used to collect the sample has the potential to have a significant impact on the results obtained from analyses of macronutrient composition. Our recent systematic review of collection methods used in studies of HM macronutrient composition revealed the use of a broad range of different sampling methodologies [214]. However, very few studies have directly compared the impact of the different collection methods on fat, protein and lactose concentrations. As a result, the extent to which factors such as the time of day, day of the week and/or breast of collection can impact HM composition, and therefore need to be factored in when designing studies investigating HM composition, remains unclear. This information is particularly important for studies undertaken in the community, in which women collect their own HM samples, since there is less capacity to precisely control the time of day and time relative to feeds that samples are collected. In addition, since collection of repeated samples increases participant burden, it is important to establish whether collection of multiple samples is necessary, or whether it is possible to obtain representative information on HM composition in an individual woman using a less frequent sampling regimen.

Therefore, the primary aim of this study was to use the opportunity presented by a clinical study involving regular collection of a large number of HM samples from breastfeeding women over a 3-week period to systematically evaluate the impact of day of collection during the week, time of day of collection and collection breast on HM macronutrient concentrations.

4.3 Methods

4.3.1 Study design

This study utilised samples collected from women who were participating in an open label dietary intervention trial which aimed to determine the effect of improving dietary quality in lactating women for 2 weeks on the composition of their HM. Women completed a 1 week baseline period, during which they consumed their habitual diet. Following the baseline week, women completed a 2-week dietary intervention phase targeted towards reducing fat and sugar intake, during which they were provided with all meals and snacks. This study was approved by the Human Research Ethics Committee of The University of Western Australia (RA/4/20/4953) and registered with the Australian New Zealand Clinical Trials Registry (ACTRN12619000606189).

4.3.2 Study participants

Women were approached to participate in the study through community centres and existing networks. Women were eligible to participate in the study if they were exclusively breastfeeding a term singleton infant who was between 6 and 20 weeks postpartum at the time of the first study visit and growing normally (according to WHO standards) [215]. Women and infant pairs were excluded from participation if there were any known major infant congenital abnormalities or health issues that could affect feeding behaviour, pregnancy complications (e.g. gestational diabetes, preeclampsia, fetal growth restriction), maternal diabetes, maternal diseases known to affect gastric absorption, restrictive diets (e.g. gluten free, dairy free, milk free, vegan) or maternal smoking.

4.3.3 Human milk sample collection

Women were instructed to collect daily HM samples (~5 mL) each morning before the first morning feed and at least 2 hours after the previous feed every day throughout the 3-week study. In addition to this, they were instructed to undertake intensive milk sampling once a week throughout the study. This involved collecting 3 HM samples: before the first feed in the morning, one in the afternoon and one in the evening for one 24-h period. As per the study protocol, the total number of HM samples collected across the study period for each woman was 54 samples (27 from each breast). At each time point, women were asked to obtain pre-feed samples from each breast after first wiping their hands and breasts with an anti-bacterial wipe (to ensure aseptic collection) either by hand expression or by breast pump. Participants stored the samples in their home freezer until weekly collection by study staff. Samples were transported in cooler bags with ice to maintain low temperature until reaching the biochemical lab where samples were stored at -80°C for later analysis.

4.3.4 Sample preparation

All samples were thawed for 2 h at room temperature, mixed on Intelli-Mixer (RM-2, ELMI, Ltd, Riga, Latvia) using UU mode for 15s at 50rpm and after 3 gentle top-to-bottom inversions, aliquoted into 1.5 mL microtubes (Sarstedt, Numbrecht, Germany) prior to analysis. Whole milk (500µl) samples were thawed and transferred into a homogeniser tube containing zirconium beads (3.0mm diameter, D1032-30, Benchmark Scientific, USA). The milk samples were homogenised 3 cycles at 2500rpm, 5 second per cycles with 3 second pause using a microtube homogeniser (BeadBug 6, Benchmark Scientific, USA). Whole milk was used to measure fat concentrations and HM samples were defatted for measurement of protein and lactose concentrations. The standard assays were carried out using a JANUS workstation

(PerkinElmer, Inc., Waltham, MA, USA) and measured on EnSpire (PerkinElmer, Inc., Waltham, MA, USA).

4.3.5 Macronutrient measurements

Fat concentration of milk samples was measured by a modified creatocrit method [216]. The thawed samples were mixed with Intelli-mixer (RM-2, ELMI Ltd, Riga, Latvia) using UU mode for 15s at 50rpm and followed by 3 gentle top-to-bottom inversions. The mixed milk was sampled in duplicate using glass capillary-tubes (41A2502, Kimble-Chase, USA), sealed one end with tube sealing compound (43510, Kimble-Chase, USA) and spun in a flat-bed centrifuge (CEN 96221, Phoenix Scientific Industries Ltd, USA) designed for capillary-tubes for 10min. Spun capillary-tubes were read on Creamatocrit Plus™ device (Medela, AG) to determine the fat content in percentage. The read capillary-tubes were then cut and obtained the skim milk portion for the protein and lactose assay.

Protein concentration in the milk sample was measured by a modified Bradford method using a commercial protein reagent (Bio-Rad Laboratories, USA) [217]. Protein standards were prepared from an aliquot of HM and the protein concentration determined by the Kjeldahl method, as described by Atwood and Hartmann (1992) [218] and the protein assay as described by Mitoulas et al (2002) [169]. The recovery of a known amount of protein added to the milk samples was $101 \pm 2.1\%$ (n=6). The detection limit of this assay was 0.03g/L and the inter-assay CV was 18% (n=22).

4.3.6 Statistical analysis

The data was analysed by fitting a linear mixed effects model to each of the response variables: fat concentration, protein concentration and lactose concentration. Day (indicator), breast (indicator) and time of day (indicator) were input as explanatory variables for each of the models. In all models, the mothers were fitted as random effects as to account for the within mother variation. Model selection was carried out using backwards selection. All statistical analysis, including descriptive statistics, was computed using the statistical programming language R [220].

4.4 Results

4.4.1 Study participants

Of the 66 women currently breastfeeding or expressing HM who initially expressed interest in participating and requested further information, 38 confirmed their interest and completed the eligibility assessment. Of the 18 who were eligible and enrolled in the study, 15 women completed the study. Clinical and sociodemographic characteristics of the study participants (mothers and infants) are shown in Table 4.1. Study participants were predominantly of Caucasian background (n=13), with one woman identifying as Hispanic and one not providing information on ethnicity. The majority of participants had completed a University degree (n=12). The age of the infants at the time of study entry ranged from 1.6 to 4.9 months, and there were approximately equal numbers of males and females.

Table 4.1. Demographic characteristics of participating mothers and infants^{1,2}

Characteristics	Mean $\pm$ SD	Range
Mothers		
Age (years)	32 $\pm$ 3	27-37
Pre-pregnancy weight ² (kg)	69.4 $\pm$ 10.8	55-89
Pre-pregnancy BMI ² (kg/m ²)	25.1 $\pm$ 4.1	17.2-32.7
Current weight (kg)	71.4 $\pm$ 10.3	54.4-80.7
Current BMI (kg/m ²)	25.8 $\pm$ 4.1	17.1-33.0
Infants		
Age (months)	3.1 $\pm$ 0.8	1.6-4.9
Sex ² (M/F)	8/6	-
Birth weight ² (kg)	3.6 $\pm$ 0.4	2.9-4.3
Birth length ² (cm)	51.1 $\pm$ 1.7	48-53
Current weight (kg)	6.1 $\pm$ 1.0	4.6-7.6
Current length (cm)	60.3 $\pm$ 3.3	55.2-65.5

¹BMI, body mass index; F, female; M, male; SD, standard deviation²Missing information from one participant

A total of 796 HM samples were provided by the 15 participants and analysed in duplicate for each of the macronutrients (Table 4.2). Eleven women provided all 54 HM samples and the remaining 4 women provided between 47 and 53 HM samples.

Table 4.2. Samples analysed for macronutrient concentrations in human milk¹

Weeks	Samples per woman	Total samples, <i>n</i>
1	17-19	270
2	16-18	263
3	12-20	263
Total		796
Breast	Samples, <i>n</i>	
Left	24-28	400
Right	23-27	396

¹N, number of samples

4.4.2 HM macronutrient concentrations across days of the week and between breasts

There were no differences in HM content of either fat (week 1, $p = 0.34$; week 2, $p = 0.27$; week 3, $p = 0.15$, Figure 4.1), protein (week 1, $p = 0.37$; week 2, $p = 0.36$; week 3, $p = 0.97$, Figure 4.2) or lactose (week 1, $p = 0.51$; week 2, $p = 0.19$; week 3, $p = 0.11$, Figure 4.3) either between different study days in any of the 3 study weeks or across the 3-week study period. There were also no differences in concentrations of either fat (week 1, $p = 0.51$; week 2, $p = 0.35$ week 3, $p = 0.95$), protein (week 1, $p = 0.62$; week 2, $p = 0.96$; week 3, $p = 0.23$) or lactose (week 1, $p = 0.40$; week 2, $p = 0.25$; week 3, $p = 0.15$) between right and left breasts at any point in the study.

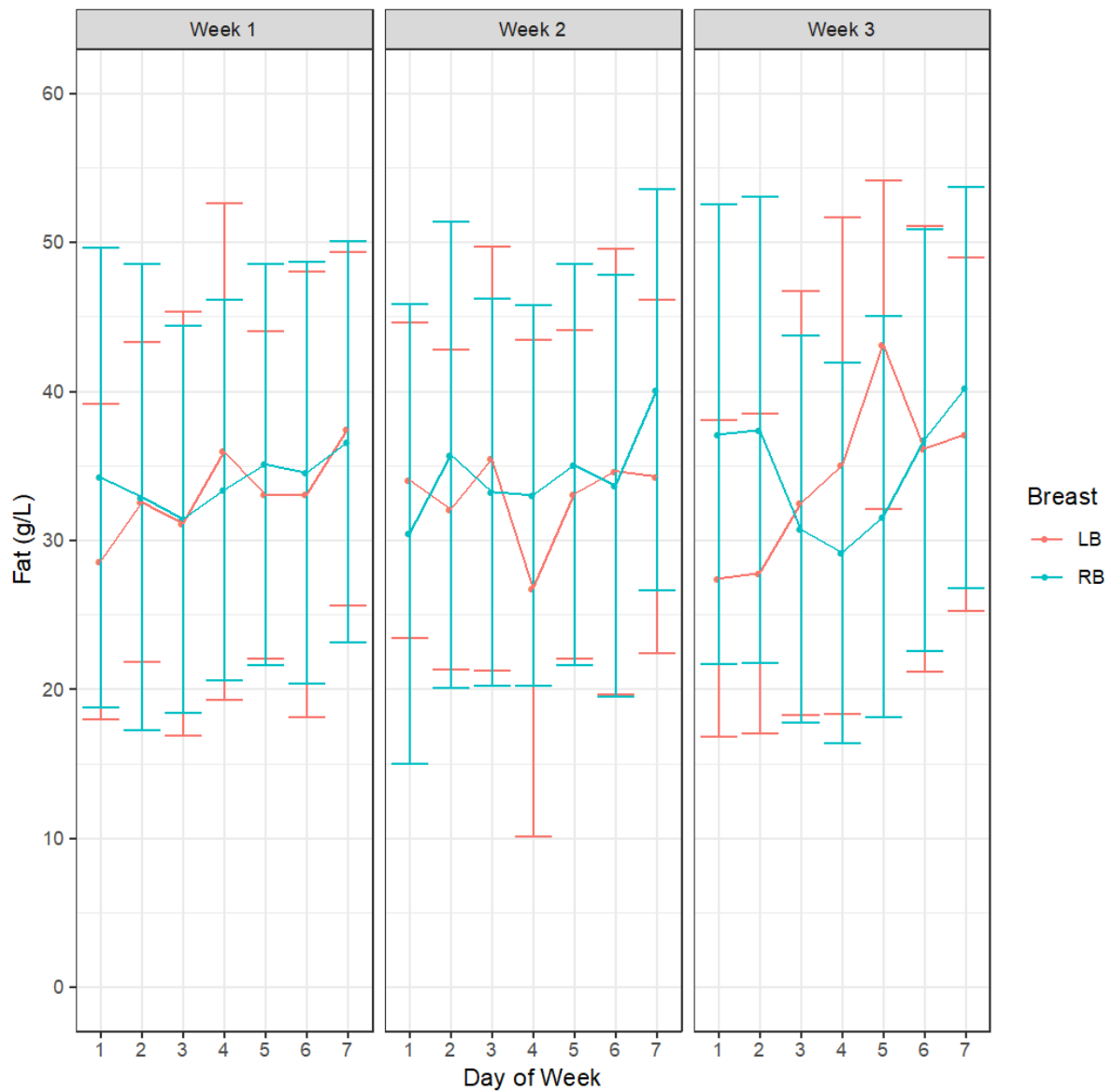


Figure 4.1. HM fat concentrations within participants across the days and between breasts for week 1, week 2 and week 3. Left breast (LB, red); Right breast (RB blue). Values are shown as mean and standard deviation.

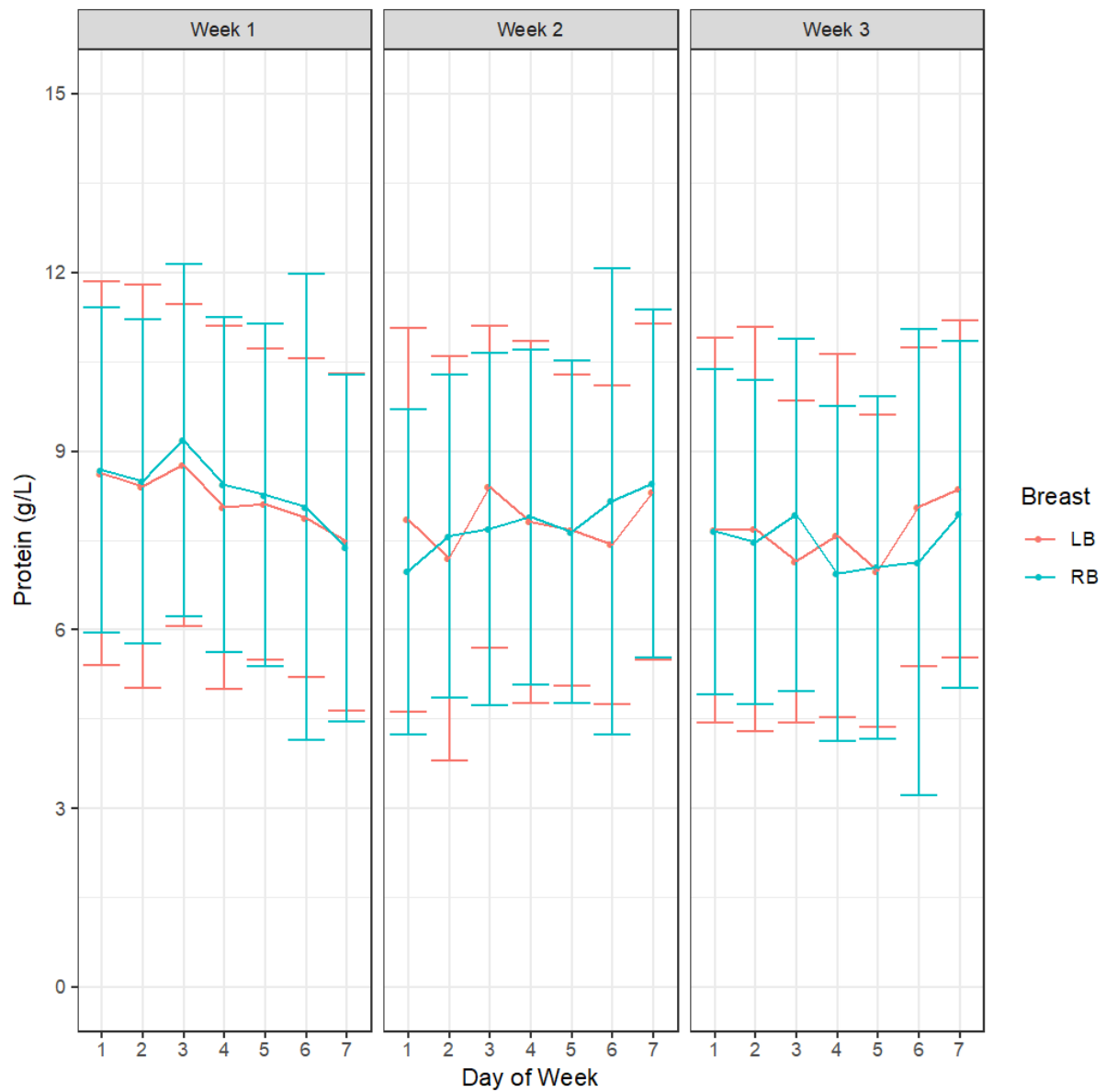


Figure 4.2. HM protein concentrations within participants across the days and between breasts for week 1, week 2 and week 3. Left breast (LB, red); Right breast (RB blue). Values are shown as mean and standard deviation.

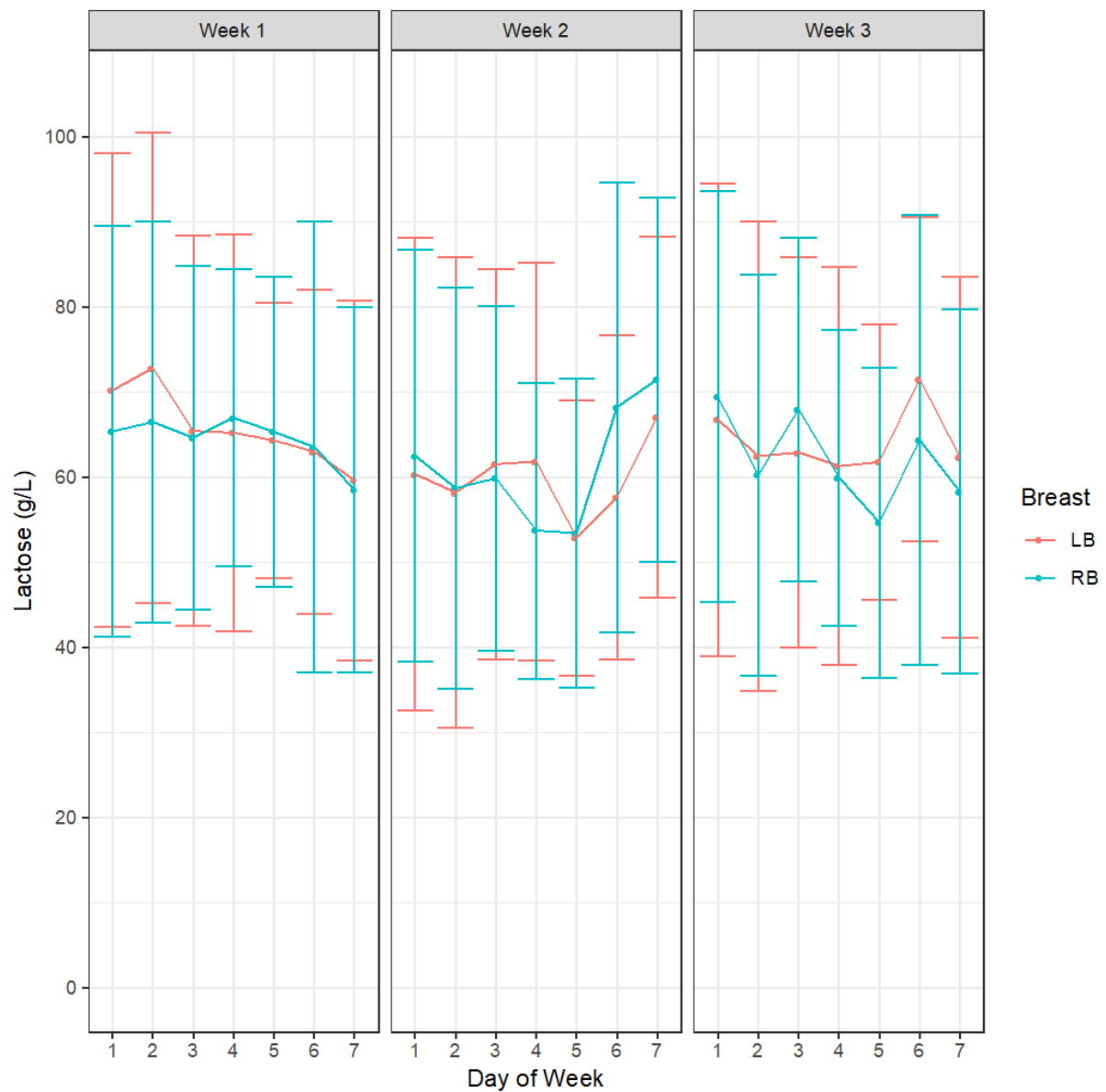


Figure 4.3. HM lactose concentrations in HM within participants across the days and between breasts for week 1, week 2 and week 3. Left breast (LB, red); Right breast (RB blue). Values are shown as mean and standard deviation.

4.4.3 Impact of time of day of collection on HM macronutrient concentrations

There were no significant differences in the concentrations of protein and lactose in HM between samples collected in the morning, afternoon and evening in any of the study weeks (Table 4.3). The fat content was not different throughout the day with the exception of week 2 of the study, where fat content was significantly higher in HM samples collected in the afternoon compared to those collected in the morning or evening ($p = 0.001$) (Table 4.3).

Table 4.3. Mean fat, protein and lactose concentrations in HM collected at different times of day across the 3 study weeks¹

	Morning	Afternoon	Evening	<i>p</i> values
Fat (g/L)				
Week 1	33.26±14.10	36.85±11.35	33.24±7.70	0.350
Week 2	32.55±14.93	43.43±20.24	34.39±13.18	0.001
Week 3	34.02±13.70	39.00±15.06	35.83±13.50	0.280
Protein (g/L)				
Week 1	8.24±3.08	8.19±2.92	8.32±1.63	0.964
Week 2	7.82±2.62	7.74±1.90	7.83±2.19	0.942
Week 3	7.56±2.52	7.38±3.19	7.42±2.49	0.811
Lactose (g/L)				
Week 1	64.89±21.82	68.88±26.04	62.77±22.03	0.408
Week 2	60.69±20.53	61.13±19.23	61.85±20.36	0.927
Week 3	64.06±23.00	61.32±26.43	58.47±19.86	0.051

¹Values are shown as mean ± standard deviation

4.5 Discussion

Given the dynamic nature of HM composition, concerns exist with HM sampling regimes. In the current study, in which women collected samples daily for 3 weeks and three times daily for one day each week, we did not identify any significant differences in concentrations of any macronutrients in HM according to either the day or time of collection or between breasts. These results suggest that it is unnecessary to sample mature milk repeatedly in established lactation where mothers are exclusively HM feeding.

As previously reported, there is considerable variation between women in HM macronutrient composition [19,38,161]. However, what is less understood are the acute temporal changes in HM composition within an individual and, therefore, how frequently it is necessary to collect HM samples in order to obtain a reliable estimate of the composition of an individual's milk. We found the macronutrients to be remarkably consistent with no differences in the levels of fat, protein or lactose in pre-feed HM samples that were self-collected from the same woman daily across a 3-week period. While no previous studies to our knowledge have sampled this intensively, the results are consistent with a prior study which found no differences in HM fat, protein and lactose concentrations between HM samples collected from the same individual once a week for 3 weeks [39]. These findings have relevance for longitudinal studies, since they provide confidence that less frequent sampling (weekly or less) is appropriate for obtaining reliable estimates of mature HM composition in an individual woman. In the case of population studies, these findings support the suggestion that a HM sample collected from a woman at a single time point in lactation can provide a reasonably reliable estimate of her milk composition. Therefore, this approach would be appropriate as a basis for estimating average composition in a population. It is important to note, however, that all women in this sample

were between ~2 and 5 months postpartum, and thus producing mature HM, and were exclusively HM feeding, and more substantial changes across time are likely to be observed earlier postpartum, when women are producing colostrum or transitional milk [6,161] and at weaning, when composition changes again [11,165,221].

Time of day of sample collection has also been suggested to have an influence on HM composition, due to variations in fat concentrations across the day as a result of different timings between feeds [21,222]. We found no difference in mean protein or lactose content between samples collected at different times of day, which is consistent with previous reports [21,213] and would support the absence of significant daily variability in the levels of these macronutrients in human milk. In contrast to a number of previous studies that have reported lower fat concentrations in HM samples collected at night, compared to earlier in the day [20,32], we found no consistent difference in fat content of HM across the day over the 3 study weeks, with the exception of the afternoon sample at week 2. Since the fat content of HM is strongly related to the fullness of the breast and time since last feed [20], this difference may well account for this result. Overall, our findings suggest that the time of HM collection relative to infant feeding, infant feeding pattern and/or breast fullness at the time of collection are likely to be more important determinants of HM fat content than the time of day of collection per se.

There is limited evidence from reports of differences in HM collected from left and right breasts in the same woman at the same time point, which would suggest that it is important to consistently sample from the same breast over time. Consistent with the majority of previous studies, we found no difference in concentrations of fat, protein or lactose in HM collected daily from right and left breasts at the same time point [39,169,173]. This is also in line with data

suggesting that breast dominance, which typically does vary between breasts, does not appear to affect HM macronutrient concentrations [173], although it has been associated with differences in milk production [20,169,223]. It therefore appears that other factors, including breast fullness and time since last feed, which are consistently reported to affect HM fat content, are more important factors to consider in a sampling protocol, rather than specifically needing to distinguish between right and left breasts.

A major strength of this study is the availability of both daily HM samples as well as samples collected at different times of day across a 3-week period. This enabled us to assess daily, within-day and between-breasts changes over time, and the collection of a large number of samples (~50 samples per participant and > 750 samples in total) for the assessment of macronutrient composition. Further, no women in the study sample had conditions, such as diabetes and gestational diabetes or were smokers, all of which have been shown to impact milk composition and could therefore have confounded the results [149,224]. The fact that all samples were collected prior to feeding means that we are not able to use these data to directly compare the composition of pre-feed, post-feed or pooled samples. As indicated above, the primary objective was to assess changes in HM composition in the same woman over time and required women to collect a large number of samples over a short time period, thus pre-feed collection was a pragmatic and appropriate choice. When the aim of the study is to obtain an estimate of what the infant is actually consuming, however, a different sampling approach, including collection of pre- and post-feed samples over a 24-hour period and measures of milk production may be required [214].

4.6 Conclusion

In conclusion, our findings indicate that the pragmatic approach of collecting pre-feed samples at the same time point during the day across individual days provides a reliable estimate of HM composition for an individual, irrespective of the breast of collection. These findings are important for the design of future studies to investigate HM composition, and would tend to suggest that there is limited need for repeated sampling of women across a week in order to obtain a reliable estimate of the macronutrient content of her mature breast milk. The results also imply that other factors, such as infant feeding pattern, time since last feed and breast fullness, are likely to be more important determinants of HM fat content than time of day of collection, and challenge the current dogma that fat content increases progressively across the course of the day. Further studies which directly investigate the key drivers of the variation in fat content of HM are warranted.

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Conflicts of interest: The authors declare that they have no competing interests.

Abbreviations:

Human milk (HM)

Left breast (LB)

Right breast (RB)

CHAPTER 5. THE IMPACT OF MATERNAL OBESITY ON HUMAN MILK MACRONUTRIENT COMPOSITION: A SYSTEMATIC REVIEW AND META-ANALYSIS

Gabriela E. Leghi, Merryn J. Netting, Philippa F. Middleton, Mary E. Wlodek, Donna T. Geddes, and Beverly S. Muhlhausler

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Name of Principal Author (Candidate)	Gabriela Esteves Leghi		
Contribution to the Paper	Contributed to the research idea, experimental design, data analysis and interpretation. Drafted and constructed manuscript and had primary responsibility for final version.		
Overall percentage (%)	70		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	05.05.2020

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

- the candidate's stated contribution to the publication is accurate (as detailed above);
- permission is granted for the candidate to include the publication in the thesis; and
- the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Contribution to the Paper	Contributed to the research design, development, data analysis and manuscript revision.		
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Contribution to the Paper	Contributed to the research idea, experimental design, data analysis and interpretation, and manuscript revision. Supervised the work and had responsibility for final content. Acted as corresponding author.		
Signature		Date	05.05.2020

Please cut and paste additional co-author panels here as required.

Review

The Impact of Maternal Obesity on Human Milk Macronutrient Composition: A Systematic Review and Meta-Analysis

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Abstract: Maternal obesity has been associated with changes in the macronutrient concentration of human milk (HM), which have the potential to promote weight gain and increase the long-term risk of obesity in the infant. This article aimed to provide a synthesis of studies evaluating the effects of maternal overweight and obesity on the concentrations of macronutrients in HM. EMBASE, MEDLINE/PubMed, Cochrane Library, Scopus, Web of Science, and ProQuest databases were searched for relevant articles. Two authors conducted screening, data extraction, and quality assessment independently. A total of 31 studies (5078 lactating women) were included in the qualitative synthesis and nine studies (872 lactating women) in the quantitative synthesis. Overall, maternal body mass index (BMI) and adiposity measurements were associated with higher HM fat and lactose concentrations at different stages of lactation, whereas protein concentration in HM did not appear to differ between overweight and/or obese and normal weight women. However, given the considerable variability in the results between studies and low quality of many of the included studies, further research is needed to establish the impact of maternal overweight and obesity on HM composition. This is particularly relevant considering potential implications of higher HM fat concentration on both growth and fat deposition during the first few months of infancy and long-term risk of obesity.

Keywords: Systematic review; maternal obesity; body mass index (BMI); adiposity; human milk composition; macronutrient; infant health

1. Introduction

The World Health Organization (WHO) defines overweight as a body mass index (BMI) greater than or equal to 25 kg/m² and obesity as a BMI equal or greater than 30 kg/m² [1]. The number of women who are overweight or obese while pregnant and breastfeeding has increased markedly in the past few decades, in line with the global increase in obesity rates [2]. In Australia, around 50% of pregnant women are overweight or obese when attending their first antenatal appointment [3,4], and similar statistics have been reported in the USA and UK [5]. The increasing body weights of pregnant

and breastfeeding women has led to a growing interest in the consequences of maternal overweight and obesity for both pregnancy outcomes and infant and child health [6,7].

There are increasing suggestions that, in addition to increasing the risk of pregnancy and neonatal complications, maternal overweight and obesity may be associated with changes in the macronutrient composition of human milk (HM) [8–10]. This is of clinical relevance, given the increasing number of studies that have reported associations between the levels of specific HM components, including fat, protein, and lactose in HM, as well as growth and fat deposition in the infant in the first 12 months of age [11,12]. Thus, changes in HM composition have the potential to promote weight gain and fat deposition in the infant [11], and thereby increase their risk of obesity and metabolic disease later in life. The global increase in the incidence of overweight and obesity has raised concerns about the impact this may be having on contemporary HM composition at a population level, and the potential for these compositional changes to be contributing to the current intergenerational cycle of obesity.

While a previous systematic review examined the effect of maternal overweight and obesity on levels of metabolic hormones in HM [13], there have been no studies to date that have systematically synthesized the evidence regarding the effect of maternal overweight and obesity on HM macronutrient composition. Therefore, the purpose of this systematic review and meta-analysis was to undertake a synthesis of studies evaluating the effects of maternal obesity, including BMI and other measures of adiposity, on the concentrations of macronutrients in HM.

2. Materials and Methods

2.1. Protocol

The present systematic review protocol was developed based on Preferred Reporting Items for Systematic Reviews and Meta-Analysis Protocols (PRISMA-P) guidelines [14] and Cochrane Handbook for Systematic Reviews of Interventions [15].

2.2. Eligibility Criteria

To be eligible for inclusion, studies had to include overweight and/or obese women who were either breastfeeding (either exclusively or not) or routinely expressing HM (manually or using a breast pump) and a measure of at least one macronutrient (fat, protein, or lactose) in HM. The studies had to state the time postpartum when HM was collected (to enable stage of lactation to be determined) and to report at least one measure of maternal obesity (either self-reported or obtained by study staff) including body mass index (BMI), skinfold thickness, and bioelectrical impedance analysis (BIA), as well as information regarding when the measurement was performed (e.g., pre-pregnancy, at the time of milk collection etc.). Studies only reported as abstracts or not available in English were excluded.

2.3. Search Strategy

The EMBASE, MEDLINE/PubMed, Cochrane Library, Scopus, Web of Science, ProQuest Dissertations and Thesis Global databases were searched. The search terms, medical subject headings (MeSH) terms and truncation symbol (*) used for MEDLINE/PubMed were (milk, human (MeSH) OR human milk* OR breast milk* OR breastmilk* OR lactation) OR (breast feeding (MeSH) OR breast milk express* OR breastmilk express*) AND (body mass index (MeSH) OR overweight (MeSH) OR obesity (MeSH) OR body composition (MeSH)) AND female (MeSH). The search strategy was adapted to the subject headings and syntax of the other electronic databases. The literature search was limited to studies in humans, but no date range restrictions were applied. Reference lists of included studies were scanned for potentially relevant articles. The last search was conducted in December 2018.

2.4. Selection Process

The search results were initially uploaded into EndNote software [16] and, after removal of duplicates, transferred to the Covidence system [17]. The selection of articles for inclusion in the review

was undertaken in two stages. The first stage involved screening the title and abstracts of the search results against the eligibility criteria. In the second stage, the full articles of papers selected in the title/abstract screening stage were screened to confirm that they met the eligibility criteria. At both stages, each article was screened independently by two authors. Disagreement in eligibility status between the first two authors were resolved by a third author or mutual discussion.

2.5. Data Extraction

Two authors independently extracted data from each study based on a standardized extraction form adapted from the Cochrane Pregnancy and Childbirth Group [18]. Data extracted included details of study design, participants (sample size, country/region, stage of lactation, whether infants were term or preterm, and whether women were or were not exclusively breastfeeding), details of HM collection methodology (whether pre-feed, post-feed or full expression HM was collected, time of collection, mode of collection, volume, time since last feed/expression, breast used for collection, and whether mother was fasted before collection), measure of maternal obesity (method and time of measurement), and outcomes (HM macronutrient concentration and analytical method). In the case of missing data, reasonable efforts were made to contact the corresponding authors by email. For the purpose of performing meta-analyses, macronutrient concentrations were converted to g/L. Mean and standard deviations for each macronutrient were averaged into single values, respectively, in case of more than one HM value reported for each lactation stage (colostrum, transitional, and mature). Colostrum was defined as milk produced until four days postpartum, transitional milk as milk between four days and two weeks postpartum, and as mature milk thereafter [19].

2.6. Quality Assessment of Individual Studies

Quality assessment was based on The Newcastle–Ottawa Scale (NOS), designed for assessing non-randomized studies [20]. Reporting details were based on a pragmatic score reported by Andreas et al. (2014) [13], and conducted independently by two authors. The NOS assessed the representativeness of the cohort (“truly represents” and “somewhat represents” the average lactating women in the community), ascertainment of exposure (whether maternal weight/fat mass was measured by study staff or obtained from medical records) and whether the study controlled for any confounding factors (e.g., maternal and infant age, infant sex). We also included additional categories: sample size (small = studies with < 50 participants, medium = studies with between 50–100 participants, and large = studies with > 100 participants); and whether the study (i) stated gestational age of infants, (ii) stated feeding mode (exclusively or partially breastfeeding), (iii) standardized the time when milk sample/s were collected, and (iv) stated method of HM collection (e.g., full expression or pre- and/or post-feed). One other category, whether study included or controlled for maternal pre-existing chronic conditions, such as gestational diabetes mellitus (GDM) and diabetes, was also included in the quality assessment but these data are presented separately since they were not relevant for all included studies.

2.7. Data Synthesis

Findings are presented as structured tables, followed by a description and discussion of the study characteristics that may affect the cumulative evidence. To be eligible for the quantitative synthesis, studies had to report a comparison of HM macronutrient concentrations between normal weight and overweight/obese women following the BMI classification described by the WHO and the standard deviation or other measures of variability for their data. Meta-analyses of eligible studies were conducted using Review Manager software version 5.3. Results are presented by lactation stage (colostrum, transitional, and mature milk) and grouped by overweight and obese women compared to women of normal weight. Data are presented as forest plots, including pooled mean, standard deviation, and 95% confidence intervals. A fixed-effects model was applied, however, if there was a high level of heterogeneity ($I^2 > 50\%$) between studies a random-effects model was applied. For the purpose of this systematic review, carbohydrate concentration was considered to be equivalent to lactose, and

triglyceride levels were considered to be equivalent to fat, since lactose makes up approximately 98% of the total carbohydrates and triglyceride makes up 98% of the total lipids in HM [21,22].

3. Results

3.1. Summary of Studies

The search strategy identified 2712 articles, with 5 additional publications identified through reference lists, providing a total of 2717 studies. After removal of duplicates and screening of titles/abstracts and full text, 31 studies were included in the qualitative synthesis (5078 lactating women). Of these, nine studies were eligible for inclusion in the meta-analysis (872 lactating women). The remaining 22 studies either did not undertake a comparison between normal weight and overweight and/or obese women ($n = 15$), did not provide measures of variability for their data ($n = 3$), did not report values for transitional and mature milk separately ($n = 1$) or did not report or follow the BMI classification system described by the WHO ($n = 3$). A Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) flow diagram is presented in Figure 1.

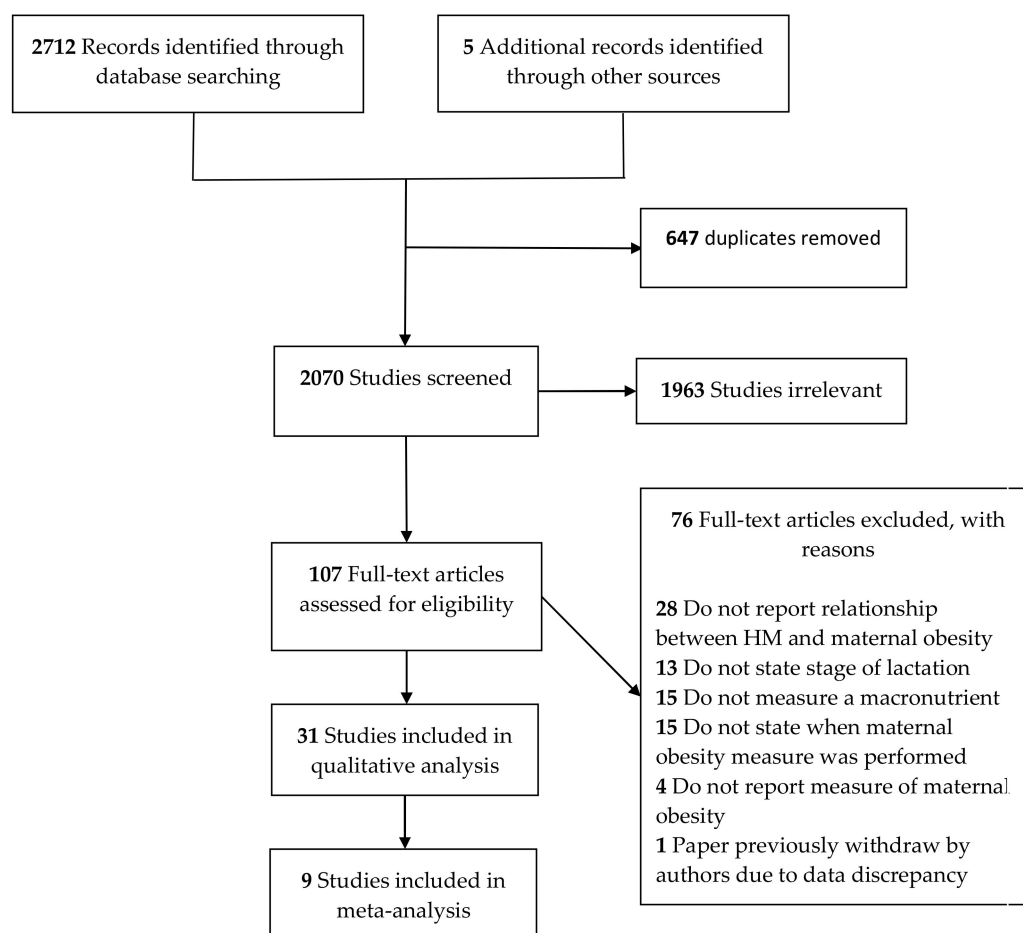


Figure 1. Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) flow diagram highlighting the process of article screening and reasons to exclude.

A summary of included studies is presented in Table 1. The studies included in this review had a broad range of publication dates, between 1986 and 2018, however, most were published after 2000 ($n = 27$). Studies were conducted in 20 different countries/regions with eight studies conducted in the USA. Most studies were cross-sectional ($n = 16$), followed by longitudinal ($n = 13$), and interventional ($n = 2$) in design. Sample sizes ranged from 13 to 2632 women, with the largest studies conducted in Korea and China ($n = 436$ women). Most of the studies collected mature HM samples ($n = 27$), followed by transitional HM ($n = 8$), and colostrum samples ($n = 7$). Besides measures of maternal BMI, eight studies also collected other measures of maternal adiposity, including skinfold and body composition (fat and lean mass). HM fat concentration was reported in 28 of the 31 studies, protein concentrations were reported in 23 studies, while carbohydrate/lactose concentrations were reported in 19 studies.

A summary of all studies reporting comparisons of HM macronutrient concentrations between overweight and/or obese and normal weight women and associations between maternal BMI and/or other measures of obesity and macronutrient concentrations in their milk is presented in Table 2. A summary of results for the qualitative synthesis across all stages of lactation is presented in Table 3.

Table 1. Summary of studies of human milk macronutrient composition included in the systematic review ¹.

Author, Year	Site	Study Design	Sample Size	Stage of Lactation	Measure Maternal Obesity	Nutrients Assessed		
						Milk Fat or TGs	Milk Protein	Milk Lactose or Carbohydrates
Aleali, 2018 [23]	Iran	Longitudinal	51	1, 2, 3, and 4 wk	BMI	✓	✓	✓
Antonakou, 2013 [24]	Greece	Longitudinal	64	1, 3, and 6 mo	BMI	✓		
Aumeistere, 2017 [25]	Latvia	Cross-sectional	28	2–21 mo	BMI			✓
Barbosa, 1997 [26]	Mexico	Longitudinal	40	3 and 6 mo	BMI	✓	✓	✓
Brown, 1986 [27]	Bangladesh	Intervention	58	1–9 mo	Arm circumference, skinfold	✓	✓	✓
Bzikowska-Jura, 2018 [28]	Poland	Longitudinal	40	1, 3, and 6 mo	BMI, BIA	✓	✓	✓
Chang, 2015 [8]	Korea	Cross-sectional	2,632	0–8 mo	BMI	✓	✓	✓
DeLuca, 2016 [29]	France	Cross-sectional	100	1 mo	BMI	✓	✓	✓
Dritsakou, 2017 [30]	Greece	Longitudinal	305	3, 7, and 30 day	BMI	✓	✓	✓
Eilers, 2011 [31]	Germany	Longitudinal	77	3 and 28 day	BMI	✓	✓	
Fujimori, 2015 [32]	Brazil	Cross-sectional	68	2–3 day	BMI	✓	✓	
Gridneva, 2018 [33]	Australia	Longitudinal	20	2, 5, 9, and 12 mo	BMI, BIS		✓	
Jans, 2015 [34]	Belgium	Cross-sectional	48	4 day	BMI	✓	✓	✓
Jans, 2018 [35]	Belgium	Longitudinal	75	3 or 4 day and 1–6 wk	BMI	✓	✓	✓
Kiersen, 2006 [36]	USA	Cross-sectional	20	7–21 day	BMI	✓		
Kuganathan, 2017 [16]	Australia	Longitudinal	59	2, 5, 9, and 12 mo	BMI, BIS		✓	✓
Kurniati, 2016 [37]	Indonesia	Cross-sectional	48	1 mo	BMI, BIA	✓		
Kwon, 2017 [38]	USA	Cross-sectional	44	2–14 wk	BMI	✓	✓	
Lemas, 2016 [39]	USA	Cross-sectional	30	2 wk	BMI	✓	✓	✓
Ley, 2012 [40]	Canada	Longitudinal	170	1 or 7 day and 3 mo	BMI	✓	✓	
Makela, 2013 [41]	Finland	Cross-sectional	163	3 mo	BMI	✓		
Mangel, 2017 [42]	Israel	Cross-sectional	109	1–2 day	BMI	✓	✓	✓
Nommsen, 1991 [43]	USA	Longitudinal	92	3, 6, 9, and 12 mo	Skinfold, % IBW	✓	✓	✓
Panagos, 2016 [9]	USA	Cross-sectional	42	4–10 wk	BMI	✓	✓	✓
Quinn, 2012 [44]	Philippines	Cross-sectional	102	0–18 mo	BMI, skinfold	✓	✓	✓
Rudolph, 2017 [45]	USA	Longitudinal	48	2 wk and 4 mo	BMI	✓		
Schueler, 2013 [46]	USA	Cross-sectional	13	29–38 day	BMI, waist circumference, DXA	✓		
Villalpando, 1992 [47]	Mexico	Cross-sectional	30	4 or 6 mo	BMI, skinfold	✓	✓	✓
Villalpando, 2001 [48]	Mexico	Intervention	10	5–6 mo	BMI, skinfold	✓		
Yang, 2014 [49]	China	Cross-sectional	436	5–11, 12–30, 31–60, 61–120 or 121–240 day	BMI	✓	✓	✓
Young, 2017 [50]	USA	Longitudinal	56	2 wk and 4 mo	BMI	✓	✓	✓

¹ BIA, bioelectrical impedance analysis; BIS, bioelectrical impedance spectroscopy; BMI, body mass index; DXA, dual-energy X-ray absorptiometry; mo, month(s); wk, week(s); TGs, triglycerides; %IBW, pre-pregnancy percent ideal body weight. The checkmark symbol indicates which macronutrient was assessed by each study.

Table 2. Effect of maternal obesity on human milk macronutrient composition: key findings ^{1,2}.

	Gestational Age	HM Method of Collection	Milk Type	Collection Time	Analytical Method	Outcomes/Effect
Comparison of Macronutrients between OW/OB and NW Women						
Aleali, 2018 [23]	Preterm	NS	Transitional and mature	14:00–16:00	MIRIS analyzer, Sweden	No difference in HM fat, protein or lactose between groups
Brown, 1986 [27]	NS	Full expression	Mature	24 h	Gravimetric method, Kjeldahl method and colorimetry	HM fat was higher in women with higher AC and TCSF. No difference in HM protein or lactose between groups
DeLuca, 2016 [29]	Term	Full expression	Mature	9:00–11:00	MIRIS analyzer, Sweden	No difference in HM fat, protein or lactose between groups
Dritsakou, 2017 * [30]	Preterm and term	Full expression	Colostrum, transitional and mature	24 h	MIRIS analyzer, Sweden	HM fat (colostrum, transitional and mature) was higher in OW/OB women. No difference in HM protein and lactose between groups
Fujimori, 2015 [32]	Term	NS	Colostrum	NS	Creamatocrit, Biuret colorimetric	HM fat was higher in OB women. No difference in HM protein between groups
Jans, 2015* [34]	Term	Mid-feed	Colostrum	NS	MIRIS analyzer, Sweden	HM fat was higher in OB women. No difference in HM protein or lactose
Jans, 2018* [35]	Term	Mid-feed	Colostrum, transitional and mature	NS	MIRIS analyzer, Sweden	No difference in HM fat, protein or lactose between groups
Kwon, 2017* [38]	NS	NS	Mature	NS	Lipid extraction, Kjeldahl method	No difference in HM fat or protein between groups
Lemas, 2016* [39]	Term	Mid-feed	Transitional	Morning	Creamatocrit, Bradford protein assay, colorimetric assay	No difference in HM fat, protein or lactose between groups
Makela, 2013* [41]	NS	Pre-feed	Mature	Morning	Lipid extraction	No difference in HM fat content between groups
Mangel, 2017* [42]	Term	Pre-feed	Colostrum	8am–3pm	MIRIS analyzer, Sweden	No difference in HM fat, protein or lactose between groups
Panagos, 2016* [9]	Term	Full expression	Mature	Morning	Julie Z7 Automatic MilkoScope, Germany	No difference in HM fat, protein or lactose between groups
Rudolph, 2017 [45]	Term	Mid-feed	Transitional and mature	Morning	Creamatocrit	HM fat was higher in OW women
Villalpando, 2001 [48]	NS	Full expression	Mature	10:00, 12:00 and 18:00	Gravimetric method	No difference in HM fat between groups
Young, 2017 * [50]	Term	Full expression	Transitional and mature	10:00–13:00	Creamatocrit, Bradford assay, enzymatic method	HM protein (transitional) and lactose (mature) were lower in OW/OB women. No difference in HM fat between groups

Table 2. Cont.

	Gestational Age	HM Method of Collection	Milk Type	Collection Time	Analytical Method	Outcomes/Effect
Correlation between maternal measures of maternal obesity and HM macronutrient concentrations						
Antonakou, 2013 [24]	Term	Pre-feed	Mature	Morning	Creamatocrit	No correlation between HM fat and BMI No correlation between HM lactose and BMI HM fat (1 and 6 mo) and protein (3 mo) positively correlated with BMI, and HM protein (3 mo) with % body fat. No correlation of HM lactose with either BMI or %body fat
Aumeistere, 2017 [25]	NS	NS	Mature	24 h	HPLC	
Bzikowska-Jura, 2018 [28]	Term	Pre- and post-feed	Mature	24 h	MIRIS analyzer, Sweden	HM protein at 0–1 wk, 3–4, and 4–5 mo, and HM fat at 1–2 wk, 2–3, and 7–8 mo positively correlated with BMI. HM lactose at 4–5 and 6–7 mo was negatively correlated with BMI
Chang, 2015 [8]	Term	Full expression	Colostrum, transitional and mature	NS	MilkoScan FT2 Foss Analytical, Denmark	No correlation between BMI and HM fat or protein
Eilers, 2011 [31]	Preterm and term	Pre- and post-feed	Colostrum and mature	16:00–20:00	Creamatocrit, BCA protein assay	No correlation between BMI and HM total protein; whey protein was positively correlated to BMI, fat-free mass, fat-free mass index, and fat mass index
Gridneva, 2018 [33]	Term	Pre- and post-feed	Mature	NS	Bradford protein assay	HM fat was positively correlated to maternal BMI
Kiersen, 2006 [36]	Preterm and term	Full expression	Transitional and mature	NS	Creamatocrit	HM protein positively correlated with %body fat, but not BMI. No correlation of either %body fat or BMI with HM lactose.
Kugananthan, 2017 [16]	Term	Pre- and post-feed	Mature	9:30–11:30	Bradford assay, Enzymatic spectrophotometric method	No correlation between %body fat and HM fat
Kurniati, 2016 [37]	Term	Mid-feed	Mature	6:00–8:00	Creamatocrit	No correlation between BMI and HM fat or protein
Ley, 2012 [40]	Term	early milk Full expression	Colostrum/Transitional and mature	NS	Creamatocrit, BCA protein assay	HM fat (6, 9, and 12 mo) and protein (9 mo) positively correlated with %IBM. No correlations with HM lactose
Nommsen, 1991 [43]	NS	Full expression	Mature	24 h	Folch extraction, Lowry assay, colorimetric assay	HM lactose was inversely correlated with BMI. No relationship between %body fat and HM fat, protein, or lactose
Quinn, 2012 [44]	NS	Mid-feed	Colostrum, transitional and mature	6:00–10:30	Rose-Gottlieb extraction, automated analyzer, phenol-sulfuric acid method	

Table 2. Cont.

	Gestational Age	HM Method of Collection	Milk Type	Collection Time	Analytical Method	Outcomes/Effect
Schueler, 2013 [46]	NS	Pre- and post-feed	Mature	7:00–10:00	Creamatocrit	HM fat positively correlated with total fat mass, BMI, body weight, and %body fat
Villalpando, 1992 [47]	Term	Full expression	Mature	10:00, 12:00, and 18:00	Jeejeebhoy method, Kjeldahl method, automated enzymatic method	HM fat positively correlated with body weight, BMI, and %body fat. No associations between these measures and HM protein or lactose
Yang, 2014 [49]	Term	Full expression	Transitional and mature	9:00–11:00	MIRIS analyzer, Sweden	HM protein and fat were positively correlated, and HM lactose negatively correlated, with BMI
Both comparison and correlation						
Barbosa, 1997 [26]	Term	Full expression	Mature	10:00, 14:00, and 18:00	Gravimetric method, Kjeldahl method, automatic enzyme method	HM fat was positively correlated with BMI and %body fat, and was lower in the lower BMI group (<23 kg/m ²) compared to the higher (≥23 kg/m ²). HM protein and lactose were not correlated with BMI or %body fat and no different between these BMI groups

¹ Analytical methods are reported based on the sequence noted on outcomes, otherwise reporting follows the “fat, protein and lactose” sequence. Maternal obesity values are reported as mean ± SD or mean (range) when SD is not available. Studies included in the meta-analysis are indicated with *. ² Symbol—was used to represent information not stated or unclear. AC, Arm circumference; BCA, Bicinchoninic acid method; BMI, body mass index; HM, human milk; HPLC, high-performance liquid chromatography; % IBM, pre-pregnancy percent ideal body weight; h, hour; MIRIS, Mid-infrared milk analyzer; mo, month (s); NS, not stated; NW, normal weight; OB, obese; OW, overweight; PT, preterm; T, term; TCSF, triceps skinfold thickness; wk, week (s).

Table 3. Summary results of qualitative synthesis across all stages of lactation for studies reporting both comparison and correlation analysis ¹.

Milk Macronutrient	Studies, <i>n</i>	Effect								
		Positive association with Maternal BMI/Adiposity			No Association with Maternal BMI/Adiposity			Negative Association with Maternal BMI/Adiposity		
		All	Comparison	Correlation	All	Comparison	Correlation	All	Comparison	Correlation
Fat	19	<u>11</u>	4	8	8	3	5	0	0	0
Protein	15	<u>6</u>	0	6	<u>9</u>	5	5	0	0	0
Lactose	12	0	0	0	<u>9</u>	4	6	3	0	3

¹ BMI, body mass index; n, total number of included studies. “All” combines the number of studies from both comparison and correlation analysis. One study has reported both comparison and correlation analysis [26] which influenced on the number of studies underlined.

3.2. Fat Concentration

In the meta-analysis ($n = 9$; Figure 2), no difference in fat concentration in colostrum between overweight and obese and normal weight women was seen (mean difference (MD) 1.34 g/L, 95% confidence interval (CI) -3.46 – 6.15 , random-effects model, $p = 0.58$; $I^2 = 75\%$; four studies; 209 overweight and obese women and 203 normal weight women). In transitional milk, however, fat concentration (g/L) was lower in overweight and obese women compared to those of normal weight (MD -4.98 g/L, 95% CI -9.76 – 0.20 , two studies; 35 overweight and obese and 51 normal weight women). In mature milk, fat concentration (g/L) was higher in mature HM from overweight and obese women compared to normal weight women MD 2.73 g/L, 95% CI 0.57–4.89, six studies; 262 overweight and obese women and 259 normal weight women).

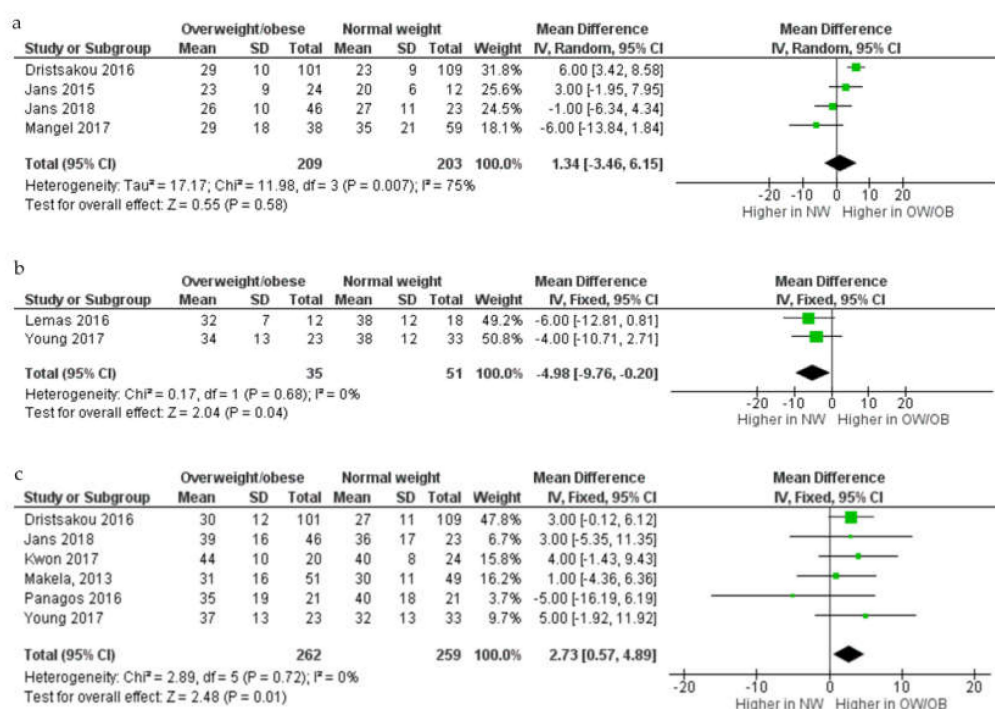


Figure 2. Comparison of fat concentration (g/L) from normal weight and overweight and obese women according to stage of lactation, colostrum (a), transitional (b) and mature (c). CI, confidence interval; Chi^2 and I^2 , measures of heterogeneity; IV, inverse variance; NW, normal weight women; OB, obese women; OW, overweight women; SD, standard deviation.

Of the 19 studies that were not eligible for inclusion in the meta-analysis, seven reported a comparison of fat concentrations between normal weight and overweight and/or obese women while 13 assessed associations between HM fat content and one or more measures of maternal obesity (one study reported both comparison and association).

Six of the seven studies that reported comparisons were conducted in mature milk. Of these one found that HM fat concentration was higher in overweight, but not obese, women [45]; one reported higher fat concentrations in women with a BMI ≥ 23 kg/m² [26]; one found higher fat concentrations in women with higher arm circumference and triceps skinfold thickness measurement [27]; and three found no difference in HM fat concentration between overweight and/or obese and normal women [23,29,48]. Of the two studies conducted in transitional milk, one reported a higher HM fat concentration in overweight women [45] while the other found no difference [23]. The study assessing colostrum samples reported a higher fat concentration in obese women compared to those of normal weight and overweight [32].

Of the 13 studies that assessed associations between HM fat concentration and milk composition, 12 assessed these relationships in mature HM, three in transitional HM, two in colostrum, and two included multiple stages and did not distinguish between them (five studies reported associations for more than one HM type). Eight of the 12 studies in mature milk reported a positive association between women's BMI and/or percentage body fat and fat levels in their milk [8,26,28,36,43,46,47,49], while four found no association [24,31,37,40]. All three studies conducted in transitional HM reported a positive association between maternal BMI and HM fat concentration [8,36,49]. None of the two studies that assessed levels in colostrum found any association with maternal BMI [8,31]. In agreement, Ley 2012 found no association between maternal BMI and fat concentrations in either colostrum or transitional HM [40]. Quinn 2012, which included samples assessed over multiple stages of lactation, also found no association between maternal BMI or percentage body fat and fat concentrations in HM [44].

3.3. Protein Concentration

In the meta analyses ($n = 8$; Figure 3), there was no difference in protein concentration (g/L) in HM between overweight and obese and normal weight women in either colostrum (MD 0.73 g/L, 95% CI −0.68–2.14; four studies; 209 overweight and obese women and 203 normal weight women), transitional (MD −0.65 g/L, 95% CI −1.51–0.21, two studies; 35 overweight and obese women and 51 normal weight women), or mature HM (MD −0.02 g/L, 95% CI −0.40–0.35, fixed-effects model, $p = 0.91$; $I^2 = 33\%$; five studies; 211 overweight and obese women and 210 normal weight women).

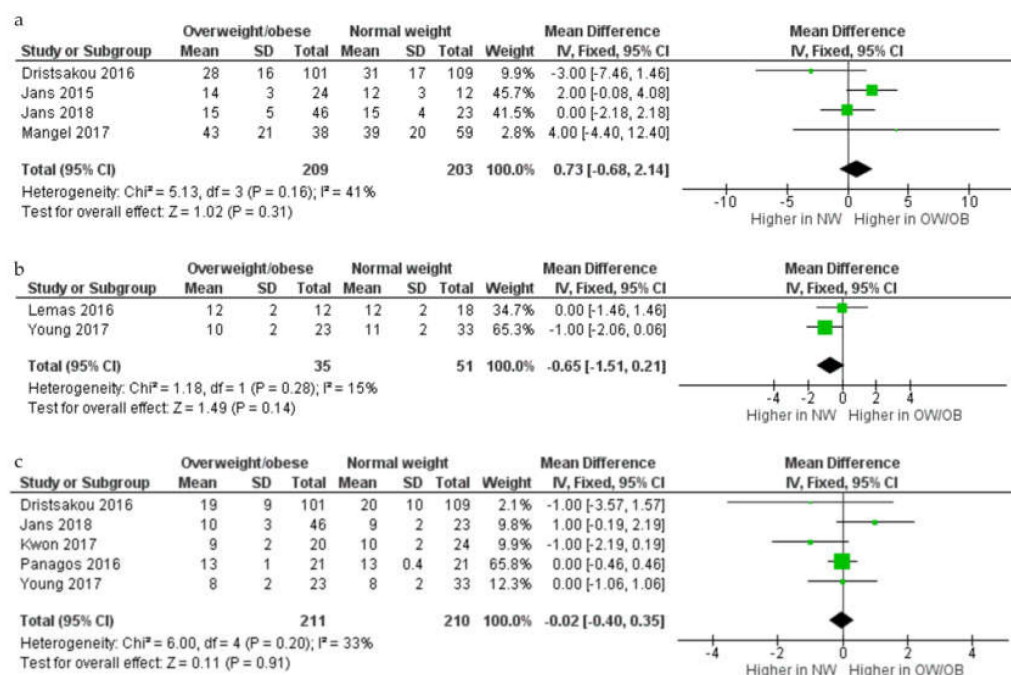


Figure 3. Comparison of protein concentration (g/L) from normal weight and overweight and obese women according to stage of lactation, colostrum (a), transitional (b), and mature (c). CI, confidence interval; χ^2 and I^2 , measures of heterogeneity; IV, inverse variance; NW, normal weight women; OB, obese women; OW, overweight women; SD, standard deviation.

All five studies not eligible for inclusion in the meta-analysis that compared HM protein concentration between normal weight and overweight and/or obese women found no differences in HM protein concentration between these groups [23,26,27,29,32], although this included one study that considered $>23 \text{ kg/m}^2$ as overweight and obese [26]. This was independent of the stage of lactation when the samples were collected ($n = 1$ colostrum, $n = 1$ transitional HM, $n = 4$ mature HM; one study reported comparison for more than one HM type).

Of the 11 studies that reported correlations between HM protein concentration and maternal BMI and/or fat mass, 10 examined these associations in mature HM, two in transitional HM, two in colostrum, one in either transitional or colostrum, and one without clear distinction. Six of the 10 studies in mature HM reported a positive association between maternal BMI and/or percentage body fat and protein/whey concentration [8,16,28,33,43,49] while three found no association [26,31,47]. Of the two studies in transitional milk, one reported a positive association between these variables [49] while the second found no association [8]. Of the two studies in colostrum, one reported a positive association between HM protein concentration and maternal BMI [8], while the second found no association [31]. Ley 2012 also found no association between maternal BMI and either colostrum or transitional HM [40], and Quinn 2012 also reported no association between maternal BMI or percentage body fat and protein concentrations [44].

3.4. Lactose Concentration

In the meta analyses ($n = 7$; Figure 4), lactose concentration (g/L) in colostrum was higher in overweight and obese compared with normal weight women (MD 2.24 g/L, 95% CI 0.85–3.63, four studies, 209 overweight and obese women and 203 normal weight women). There were, however, no differences seen in lactose concentration in either transitional or mature HM samples between overweight and obese and normal weight women (transitional HM: MD 0.43 g/L, 95% CI –3.57–4.43, two studies, 35 overweight and obese women and 51 normal weight women; mature HM: MD –0.01 g/L, 95% CI –2.08–2.06, random-effects model, $p = 0.99$; $I^2 = 59\%$; four studies, 191 overweight and obese women and 186 normal weight women).

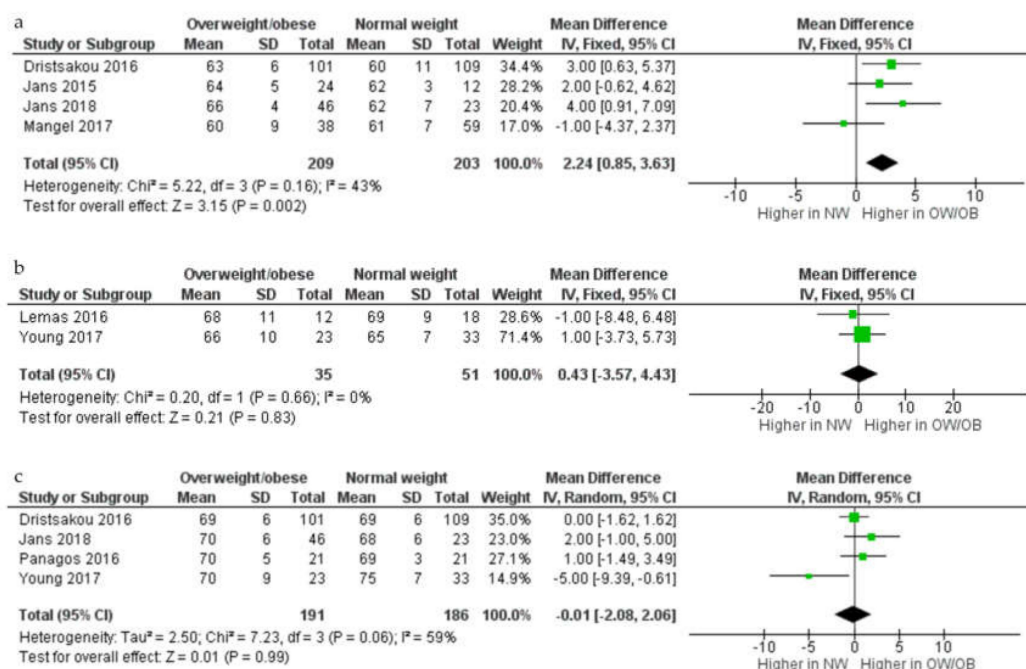


Figure 4. Comparison of lactose concentration (g/L) from normal weight and overweight and obese women according to stage of lactation, colostrum (a), transitional (b), and mature (c). CI, confidence interval; Chi^2 and I^2 , measures of heterogeneity; IV, inverse variance; NW, normal weight women; OB, obese women; OW, overweight women; SD, standard deviation.

All four studies not eligible for inclusion in the meta-analysis that compared HM lactose concentration between normal weight and overweight and/or obese women found no difference in lactose concentration between overweight/obese and normal weight women in either transitional

($n = 1$) or mature ($n = 4$) HM (one study reported comparison for more than one HM type) [23,26,27,29], however this included one study in which a BMI >23 kg/m² was classified as overweight and obese.

Of the nine studies that assessed associations between maternal BMI and/or percentage body fat and lactose concentration in mature HM, two reported a negative association between lactose concentration and maternal BMI [8,49], while six found no association with either maternal BMI or body fat [16,25,26,28,43,47]. Of those studies assessing mature milk, one study reported a negative association between maternal BMI and lactose concentrations in transitional milk [49], while the other found no association for colostrum and transitional milk [8]. In addition, one study that assessed lactose concentrations across lactation (with no distinction between milk type) reported a negative association with maternal BMI [44].

3.5. Quality Assessment

The quality assessment is outlined in Table 4 with overall quality of the studies being relatively low. The ability to control for confounders and to state infant's feeding mode were the categories that scored the lowest across the studies.

Women with pre-existing conditions known to influence HM macronutrient composition, including GDM and diabetes, were excluded in most of the studies ($n = 18$) [23,24,26,28,29,31,34–36,39,40,42,43,45–48,50]. Two studies included women with GDM [30,32], while whether or not women with these conditions were included was unclear [27,49] or not stated [8,9,16,25,33,37,38,41,44] in 11 studies.

Table 4. Quality and reporting assessment for studies measuring macronutrient concentrations in human milk ¹.

Reference	Representativeness Cohort	Measure of Maternal Obesity	Controls for Confounders	Sample Size (Small, Medium or Large Study)	State Gestational Age	State Feeding Mode	Standard Time of HM Collection	State HM Collection Method
Aleali, 2018 [23]	X	✓	x	Medium	✓	x	✓	x
Antonakou, 2013 [24]	✓	✓	✓	Medium	✓	✓	✓	✓
Aumeistere, 2017 [25]	X	x	x	Small	x	✓	✓	x
Barbosa, 1997 [26]	X	✓	x	Small	✓	x	✓	✓
Brown, 1986 [27]	✓	✓	✓	Medium	x	x	✓	✓
Bzikowska-Jura, 2018 [28]	✓	✓	x	Small	✓	✓	✓	✓
Chang, 2015 [8]	✓	✓	✓	Large	✓	x	x	✓
DeLuca, 2016 [29]	✓	✓	✓	Medium	✓	✓	✓	✓
Dritsakou, 2017 [30]	X	x	x	Large	✓	✓	✓	✓
Eilers, 2011 [31]	✓	x	x	Medium	✓	x	✓	✓
Fujimori, 2015 [32]	✓	x	x	Medium	✓	✓	x	x
Gridneva, 2018 [33]	✓	✓	✓	Small	✓	✓	x	✓
Jans, 2015 [34]	✓	✓	x	Small	✓	x	x	✓
Jans, 2018 [35]	✓	✓	x	Medium	✓	x	x	✓
Kiersen, 2006 [36]	X	x	x	Small	✓	x	x	✓
Kugananthan, 2017 [16]	✓	✓	x	Medium	✓	✓	✓	✓
Kurniati, 2016 [37]	✓	✓	x	Small	✓	✓	✓	✓
Kwon, 2017 [38]	✓	x	x	Small	x	x	x	x
Lemas, 2016 [39]	✓	✓	✓	Small	✓	✓	✓	✓
Ley, 2012 [40]	✓	x	✓	Large	✓	x	x	x
Makela, 2013 [41]	✓	✓	x	Large	x	x	✓	✓
Mangel, 2017 [42]	✓	x	x	Large	✓	x	✓	✓
Nommsen, 1991 [43]	✓	✓	✓	Medium	x	✓	✓	✓
Panagos, 2016 [9]	✓	✓	✓	Small	✓	x	✓	✓
Quinn, 2012 [44]	✓	✓	✓	Large	x	✓	✓	✓
Rudolph, 2017 [45]	✓	x	x	Small	✓	✓	✓	✓
Schueler, 2013 [46]	✓	✓	✓	Small	x	✓	✓	✓
Villalpando, 1992 [47]	X	✓	x	Small	✓	✓	✓	✓
Villalpando, 2001 [48]	X	✓	x	Small	x	✓	✓	✓
Yang, 2014 [49]	✓	✓	✓	Large	✓	✓	✓	✓
Young, 2017 [50]	✓	✓	✓	Medium	✓	✓	✓	✓

¹ Sample size is defined as small = studies with <50 participants; medium = studies with between 50–100 participants; and large = studies with >100 participants. The checkmark symbol indicates yes and cross symbol indicates no.

4. Discussion

This paper describes the first (to our knowledge) systematic synthesis of the current literature investigating the effect of maternal overweight and obesity on HM macronutrient levels. Overall, the meta-analysis indicated that there were differences in the concentrations of fat and lactose in HM of women who were overweight or obese, however the direction of change was dependent on the stage of lactation. While the qualitative analyses broadly supported the findings of the meta-analysis, there was considerable variability, making it difficult to draw robust conclusions.

The higher fat concentration of mature milk in women who were overweight and obese was the most consistent observation across studies. In addition, the majority of studies that examined associations also found a positive correlation between maternal BMI and/or fat mass and HM fat concentration, providing further support of higher levels of fat in HM in women with a higher BMI. The fat concentration of HM is known to be highly dynamic, varying both across the day and even within a feed [51,52], however the role of external factors in regulating fat synthesis in the breast are not as well understood [43]. It is possible that the metabolic dysregulation commonly reported in women with a higher maternal BMI/fat mass, in particular dyslipidemia and higher circulating triglyceride levels, was associated with an increase in the fat concentration of HM [32]. An alternate possibility is that the higher fat concentration was due to higher dietary fat and/or protein intakes in women who were overweight and obese. A Korean study ($n = 238$ women) demonstrated that fat levels in breast milk and maternal diet were highly correlated, and higher fat intakes are commonly reported in association with overweight and obesity [53]. In the DARLING study, a landmark study assessing determinants of macronutrients in HM across the first 12 months, maternal protein intake was also positively related to HM lipid concentration after, but not before, 16 weeks postpartum [43]. Irrespective of the cause of the higher fat concentration, there is the potential for this to impact on infant growth/body composition and long-term obesity risk. A study in the UK involving 614 mother-infant dyads reported that fat percentage in mature HM was inversely correlated with increases in body weight, BMI, and adiposity between 3 and 12 months, and with infant BMI and adiposity at 12 months postpartum [11].

The meta-analysis indicated that the fat concentration in transitional milk was lower in overweight and obese compared to normal weight women. It is important to note, however, that this only included two studies (86 participants) and was not supported by the findings of the studies included in the qualitative analysis. In addition, no impact of maternal overweight and obesity on the fat concentration of colostrum was identified. The fact that maternal overweight and obesity had less impact on HM fat concentration at earlier stages of lactation is consistent with the findings of the DARLING study [43], which indicated that milk composition is more sensitive to maternal factors, including body weight, body composition, and diet later in lactation than during the first few months postpartum. It may be that the rapid mobilization of pregnancy fat stores and dynamic shifts in HM composition that occur in the first few days to weeks postpartum, make it difficult to detect the influence of other external factors on HM composition until later in lactation [43].

The impact of maternal overweight and obesity on HM protein concentration remains inconclusive; while the meta-analysis suggested that there were no differences in HM protein at any lactation stage, some studies from the qualitative synthesis reported a positive association between HM protein and maternal BMI and fat mass across lactation. Protein concentration in HM has previously been shown to decrease progressively across lactation [54–56], and studies have also suggested influences of several other factors, including the infant's gestational age [42], maternal smoking [57], parity [58] and mode of delivery [59], on HM composition. As a result, differences in these factors between studies may have accounted for some of the variability in findings. In previous studies, obesity has been associated with impairments in amino acid metabolism [60], and with elevated amino acid concentrations in both the circulation [61] and HM [62,63]. This raises the possibility that the metabolic changes associated with obesity may affect HM composition, however, there is currently insufficient evidence to draw robust conclusions and further studies are required [64–66]. Such studies are of particular relevance given the important role that protein concentrations in HM have in programming of metabolism and growth

in early life [67]. Thus, previous studies have reported that HM protein concentrations in mature milk are positively correlated with birth weight [30] and infant BMI at 12 months postpartum [11], while a study in Australia with 20 breastfeeding dyads showed that both HM casein concentration and calculated daily intake of casein in the infant (based on HM concentration and infant milk intake) were positively associated with fat mass and fat mass index, and lower fat-free mass, in the infant at 5, 9, and 12 months postpartum [33].

The concentration of lactose, the major carbohydrate in HM [21], did not appear to be influenced by maternal overweight and obesity in mature or transitional HM. Lactose is known to maintain adequate osmotic pressure in HM [68], and therefore maintaining consistent levels of this component may be important so as not to disrupt this vital function [16]. There was some evidence, albeit limited, that lactose concentrations in colostrum were higher in women who were overweight and obese, however, given the low number of studies and inconsistencies between studies included in the qualitative analysis, it is difficult to draw clear conclusions. Differences in HM lactose concentration in colostrum could potentially be explained by differences in maternal dietary intake, but this seems unlikely given that no previous studies have shown any associations between HM lactose concentrations and maternal diet [43].

The major strength of this manuscript is the inclusion of a meta-analysis which enabled us to quantitatively synthesize much of the available evidence. Other strengths of this manuscript are the comprehensive literature search, which included different study designs (both observational and interventional), lactating women from a wide range of countries, both urban and rural areas, a broad range of ethnic and sociodemographic backgrounds, and the ability to evaluate relationships at different lactation stages (colostrum, transitional, and mature HM). In addition, eligibility criteria were applied to ensure that a number of critical elements were reported, i.e., when HM samples were collected (stage of lactation) and when maternal obesity measurements were performed/collected (pre-pregnancy or time of HM collection). Further, the inclusion of other measurements of maternal obesity besides BMI, in particular assessment of body fat by BIA, enabled us to examine relationships between HM macronutrient concentrations and more direct measures of maternal adiposity, although it should be noted that relatively few studies had undertaken such measures.

Despite these strengths, there were also limitations, the most significant being the small number of studies eligible for both the quantitative and qualitative analyses. Further to this, the quality of the included studies was generally low, and there was a lack of standardization of collection methods and procedures. Physiological and methodological aspects of HM, such as gestational age, method, and time of collection, can significantly influence macronutrient levels [51,52,69] and therefore affect the results of comparisons/associations in individual studies. For instance, fat concentration in HM increases significantly (up to three-fold) from the beginning to the end of a breastfeed or expression [70]. In addition, HM composition varies between preterm and term births [69], and whether the relationship between maternal overweight and/or obesity and HM composition could potentially vary in HM from mothers of term and preterm infants is unknown. In addition, the fact that lactating women from overweight and obese groups may also have other metabolic conditions, such as gestational or pre-gestational diabetes, could influence the results, given emerging evidence of differences in HM composition between women with and without diabetes [71]. Almost all studies utilized BMI to classify women as overweight and obese. While this is a practical measure, it does not always accurately reflect maternal fat mass/body composition, and there is evidence that correlations between some HM components and maternal obesity measures may be stronger for fat mass than for BMI [16]. Further, for the majority of studies reporting associations/correlations between maternal obesity and HM macronutrient concentration, most participants were in the normal BMI range, which may have limited the range of values for assessing correlations, and therefore the results of the correlation analyses. We identified some studies that used a BMI classification not consistent with the WHO guidelines, making it difficult to compare with other studies. The timing of the maternal obesity measurement also varied between studies (i.e., measurements obtained either pre-pregnancy or postpartum), and this

may have affected the relationships, since maternal weight (and therefore BMI) may be considerably higher postpartum compared to prior to pregnancy.

5. Conclusions

In summary, our meta-analysis of existing studies has suggested that measures of maternal obesity were associated with changes in fat and lactose concentrations in HM at different lactation stages, whereas there did not appear to be any influence of maternal BMI and/or obesity measures on HM protein concentration. However, the overall quality of the evidence in this area is relatively low and more high-quality studies are needed to better understand the relationships between maternal adiposity and HM composition. This is particularly relevant considering the potential implications of higher HM fat concentration on growth and fat deposition during the first few months of infancy, and long-term BMI and adiposity. The mechanisms through which maternal overweight and obesity can influence HM composition, i.e., as a consequence of maternal metabolic disturbances and/or dietary factors, also remains unclear and further studies are required to investigate this.

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CHAPTER 6. THE IMPACT OF MATERNAL OBESITY ON HUMAN MILK METABOLIC HORMONE CONCENTRATIONS: A SYSTEMATIC REVIEW

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6.1 Abstract

Metabolic hormones, leptin, insulin, ghrelin and adiponectin, are present in human milk (HM) and their concentrations have been associated with short- and long-term health outcomes in infants. The increasing number of women who are overweight and obese during pregnancy and while breastfeeding has led to concerns about the impact of maternal obesity on HM composition, particularly on the components known to regulate growth and fat deposition in the infant. This study aimed to provide an updated synthesis of studies that had evaluated associations between maternal overweight and obesity on HM metabolic hormone concentrations. EMBASE, MEDLINE/PubMed, Cochrane Library, Scopus, Web of Science and ProQuest databases were searched for relevant articles. Two authors conducted screening, data extraction and quality assessment independently. Of 2717 articles, 25 were included in the systematic review (2026 lactating women). The qualitative synthesis strongly indicated that the concentrations of leptin (at all lactation stages) and insulin (transitional and mature HM) were higher in HM from overweight and obese women and, that time of obesity measurement did not influence findings. The association between maternal overweight and obesity and other HM components remains inconclusive. The current body of evidence suggests that maternal overweight and obesity results in altered concentrations of key metabolic hormones in HM, and highlights the need to understand both the mechanisms through which overweight and obesity influence HM composition as well as the short- and long-term impacts of the growth, adiposity and obesity risk in the infants.

Systematic review registration: PROSPERO CRD42018115867

Keywords: Systematic review; maternal obesity; BMI; adiposity; human milk composition; metabolic hormones; infant health

6.2 Introduction

The metabolic hormones, leptin, insulin, ghrelin and adiponectin, have previously been identified in human milk (HM) [7-10]. These hormones have all been shown to be transferred from HM to the infant circulation, and therefore to have the potential to exert effects on appetite control, weight gain and fat mass [12,90,225]. The presence of metabolic hormones in HM, which are absent from infant formula, has been suggested to be one factor contributing to the more rapid growth and fat deposition reported in formula-fed compared to breastfed infants [226]. This, in turn, has been cited as a potential biological mechanism for the protective effect of breastfeeding for the long-term risk of obesity and poor metabolic health [227,228]. In addition, the concentrations of metabolic hormones have also been related to growth and fat deposition in the infant in the early postpartum period and to long-term risk of obesity [12,90]. Consequently, variations in the HM concentrations of metabolic hormones between individual women have the potential to play a critical role in infant feeding and health outcomes.

As global rates of overweight and obesity continue to rise [182], there has been increasing interest in understanding the impact of maternal obesity on HM composition, particularly for those components known to regulate growth and adiposity in the infant [76]. Maternal body mass index (BMI), although not a direct measure of maternal adiposity [229,230], is the most studied marker in this context due to the feasibility of undertaking this measurement in population studies. A systematic review of studies investigating associations between maternal BMI and HM hormone concentrations, published in 2014, suggested that the literature available at that time supported an association between maternal BMI and HM leptin, but that evidence for other hormones was inconclusive. The authors of the review also commented on

the need for further studies with larger sample sizes and utilising appropriate collection methodologies, in order to build the evidence base [192]. In the period since this review, several additional studies investigating the role of maternal BMI and adiposity markers on metabolic hormones in HM have been published, however, an updated synthesis of this evidence has not yet been conducted. Therefore, the aim of this systematic review was to undertake a comprehensive evaluation of published literature investigating the impact of maternal overweight and obesity and leptin, insulin, adiponectin, ghrelin, resistin, GLP-1, PYY and glucose concentrations in HM in order to provide an updated synthesis of the evidence.

6.3 Methods

6.3.1 Protocol

The present systematic review protocol was developed based on the Preferred Reporting Items for Systematic Reviews and Meta-Analysis Protocols (PRISMA-P) guidelines[212,231] and Cochrane Handbook for Systematic Reviews of Interventions [232].

6.3.2 Eligibility criteria

To be eligible for inclusion, studies had to include overweight and/or obese women who were either breastfeeding (exclusively or partially) or routinely expressing HM (manually or using a breast pump) and a measure of at least one metabolic hormone (leptin, adiponectin, insulin, ghrelin, resistin, glucagon-like peptide 1 (GLP-1), peptide YY (PYY), apelin or obestatin) and/or glucose in HM. The study had to state the stage of lactation when the sample was collected (i.e. weeks postpartum or specify whether colostrum, transitional or mature HM was

collected). The studies had to report at least one measure of maternal obesity – either self-reported or obtained by study staff - including body mass index (BMI), skinfold thickness and bioelectrical impedance analysis (BIA), and information as to when the measurement was performed (e.g. pre-pregnancy, at the time of milk collection, etc.). Studies only reported as abstracts or not available in English were excluded.

6.3.3 Search strategy

The EMBASE, MEDLINE/PubMed, Cochrane Library, Scopus, Web of Science, ProQuest Dissertations and Thesis Global databases were searched. The search terms and medical subject headings (MeSH) terms used for MEDLINE/PubMed were (milk, human [MeSH] OR human milk* OR breast milk* OR breastmilk* OR lactation) OR (breast feeding [MeSH] OR breast milk express* OR breastmilk express*) AND (body mass index [MeSH] OR overweight [MeSH] OR obesity [MeSH] OR body composition [MeSH]) AND female [MeSH]. The search strategy was adapted to the subject headings and syntax of the other electronic databases. The literature search was limited to studies in humans, but no date range restrictions were applied. Reference lists of included studies were scanned for potentially relevant articles. The last search was conducted in December 2018.

6.3.4 Selection process

The search results were initially uploaded into EndNote software [19] and, after removal of duplicates, transferred to the Covidence system [233]. The selection of articles for inclusion in the review was undertaken in two stages. The first stage involved screening the title and abstracts of the search results against the eligibility criteria. In the second stage, the full articles

of papers selected in the title/abstract screening stage were screened to confirm that they met the eligibility criteria. At both stages, each article was screened independently by two authors. Disagreement in eligibility status between the first two authors were resolved by a third author or mutual discussion.

6.3.5 Data extraction

Two authors independently extracted data from each study based on a standardised extraction form from the Cochrane Pregnancy and Childbirth Group [234]. Data extracted included details of study design, participants (sample size, country/region, stage of lactation, whether infants were term or preterm and whether women were exclusively breastfeeding or not), details of HM collection methodology (whether pre-feed, post-feed or full expression HM was collected, time of collection, mode of collection, volume, time since last feed/expression, breast used for collection and whether mother was fasted before collection), measure of maternal obesity (method and timing of measurement) and outcomes (HM metabolic hormone concentrations, analytical method and type of milk (i.e. skim or whole) analysed). Colostrum was defined as milk produced until 4 days postpartum, transitional milk as milk between 4 days and 2 weeks postpartum and as mature milk thereafter [6]. In the case of missing data, reasonable efforts were made to contact the corresponding authors by email.

6.3.6 Quality assessment

Quality assessment was based on The Newcastle-Ottawa Scale (NOS), designed for assessing non-randomised studies [235]. Reporting details were assessed on a designed pragmatic score based on the one reported by Andreas, et al., 2014 [192], conducted independently by two authors. The NOS assessed the representativeness of the cohort (“truly represents” and

“somewhat represents” the average lactating women in the community), ascertainment of exposure (whether maternal weight/fat mass was measured by study staff or obtained from medical records) and whether the study controlled for any confounding factors (e.g. maternal and infant age, infant sex). We also included additional categories : sample size (small = studies with <50 participants, medium = studies with between 50 and 100 participants and large = studies with > 100 participants), whether the study (i) stated gestational age of infants, (ii) stated feeding mode (exclusively or partially breastfeeding), (iii) standardised the time when milk sample/s were collected and (iv) stated method of HM collection (e.g. full expression or pre- and/or post-feed). One other category, whether study included or controlled for maternal pre-existing chronic conditions such as gestational diabetes mellitus (GDM) and diabetes, was also included in the quality assessment but these data are presented separately since they were not relevant for all included studies.

6.3.7 Data synthesis

Findings are presented as structured tables, followed by description and discussion of the study characteristics which may affect the cumulative evidence. To be eligible for the quantitative synthesis, studies had to report a comparison of HM metabolic hormone concentrations between normal weight and overweight and/or obese women following the BMI classification described by the WHO and the standard deviation or other measures of variability for their data.

6.4 Results

6.4.1 Summary of studies

Our search strategy identified a total of 2717 articles, of which 25 were included in the systematic review and qualitative synthesis (2026 lactating women). Of these, 21 studies were not eligible for inclusion in the quantitative synthesis, reasons included: (i) did not undertake or report comparison of values between overweight and/or obese and normal weight women (n=17) or (ii) did not provide the standard deviation or other measures of variability for their data (n=4). The remaining 4 studies would be eligible for inclusion in the meta-analysis [89,236-238], however, the available data were insufficient to perform comparisons for each of the respective metabolic hormones at each lactation stage. A Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) flow diagram is presented in Figure 6.1.

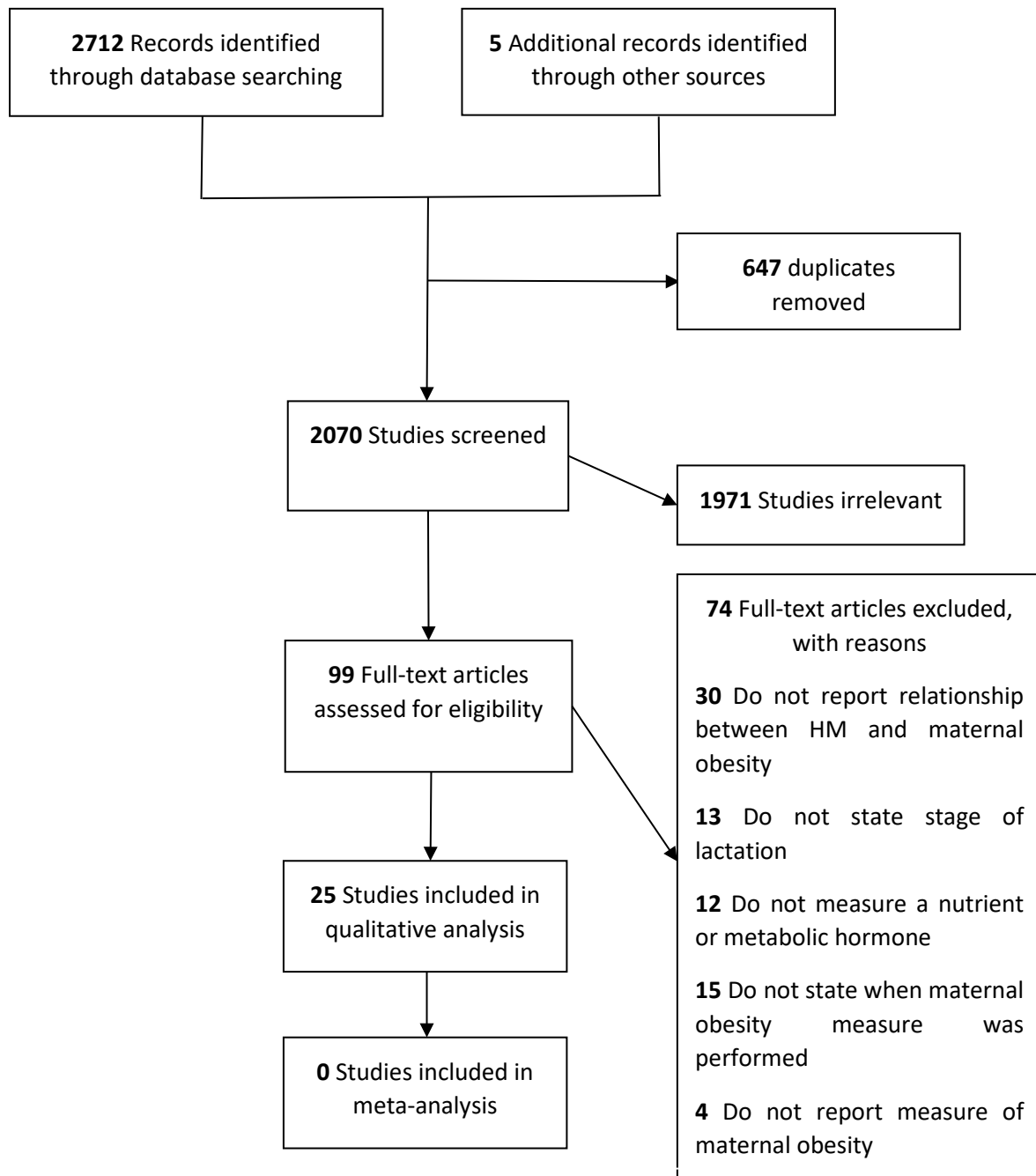


Figure 6.1. PRISMA flow diagram highlighting the process of article screening and reasons to exclude.

An overview of the included studies is presented in Table 6.1. Overall, publication dates ranged from 2002 to 2018 and sample sizes ranged from 13 to 430 lactating women. Studies were conducted in 14 countries and included cross-sectional (n=14), longitudinal (n=10) and

randomised controlled trial (n=1) study designs. The studies included HM samples collected at different stages of lactation; 10 studies collected colostrum, 5 collected transitional HM and 16 studies collected mature HM, while 3 additional studies collected samples across multiple stages of lactation. Maternal BMI was used as a measure of maternal obesity in all studies and body composition measures (bioelectrical impedance spectroscopy (BIS), skinfold and dual-energy X-ray absorptiometry (DXA)) were applied in 4 studies. Leptin was the hormone that was measured most frequently (n=16 studies), followed by adiponectin (n=13), insulin (n=9), glucose (n=5), ghrelin (n=5) and GLP-1, PYY and resistin (n=1).

Table 6.1. Summary of studies of human milk metabolic hormones included in the systematic review^{1,2,3}

Author, Year	Site	Study design	Sample size	Stage of lactation	Measure maternal obesity	Nutrients assessed
Anderson, 2016 [194]	Philippines	Cross-sectional	117	0-24mo	Postpartum BMI, skinfold	Adiponectin
Andreas, 2016 [26]	UK	Longitudinal	95	1wk and 3mo	Postpartum BMI	Leptin, insulin, ghrelin, resistin
Bielicki, 2004 [163]	Switzerland	Longitudinal	33	2-3, 4-5d and 4-6wk	Postpartum BMI	Leptin
Bronsky, 2006 [167]	Czech Republic	Cross-sectional	59	2d	Pre-pregnancy and at time of delivery BMI	Leptin, adiponectin
Chan, 2018 [22]	Canada	Cross-sectional	430	3-4mo	Pre-pregnancy BMI	Leptin, insulin, adiponectin
DeLuca, 2016 [189]	France	Cross-sectional	100	1mo	Pre-pregnancy BMI	Leptin
Dundar, 2010 [94]	Turkey	Cross-sectional	25	2d	Pre-pregnancy BMI	Adiponectin, ghrelin
Eilers, 2011 [81]	Germany	Longitudinal	77	3 and 28d	Pre-pregnancy BMI	Leptin
Fields, 2012 [156]	USA	Cross-sectional	19	1mo	Pre-pregnancy BMI	Glucose, leptin, insulin
Fujimori, 2015 [237]	Brazil	Cross-sectional	68	2-3d	Pre-pregnancy BMI	Glucose
Fujimori, 2017 [193]	Brazil	Cross-sectional	45	2-3d	Pre-pregnancy BMI	Leptin, adiponectin
Kuganathan, 2017 [19]	Australia	Longitudinal	59	2, 5, 9 and 12mo	Postpartum BMI, BIS	Leptin, adiponectin
Kwon, 2018 ¹ [238]	USA	Cross-sectional	37	2-14wk	Pre-pregnancy BMI	Glucose, leptin, insulin, adiponectin, ghrelin
Lemas, 2016 [89]	USA	Cross-sectional	30	2wk	Pre-pregnancy BMI	Leptin, insulin
Ley, 2012 [91]	Canada	Longitudinal	170	1 or 7d and 3mo	Pre-pregnancy BMI	Glucose, insulin, adiponectin
Luoto, 2012 [209]	Finland	RCT	181	Up to 3d	Pre-pregnancy BMI	Adiponectin
Martin, 2006 ² [9]	USA	Longitudinal	22	0-7mo	Postpartum BMI	Adiponectin

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Nunes, 2017 [67]	Brazil	Longitudinal	69	1-2d and 30d	Pre-pregnancy and postpartum BMI	Leptin, insulin, adiponectin
Quinn, 2015 [210]	Philippines	Cross-sectional	102	0-36mo	Postpartum BMI, skinfold	Leptin
Savino, 2016 [78]	Italy	Cross-sectional	58	61d	Postpartum BMI	Leptin
Schueler, 2013 [170]	USA	Cross-sectional	13	29-38d	Postpartum BMI, DXA	Leptin, GLP-1, PYY
Schuster, 2011 [77]	Germany	Longitudinal	23	1, 2, 3, and 4wk, 2, 3, 4, 5 and 6mo	Pre-pregnancy and postpartum BMI	Leptin
Uysal, 2002 [236]	Turkey	Cross-sectional	50	90-100d	Postpartum BMI	Leptin
Young, 2017 [17]	USA	Longitudinal	48	2wk and 4mo	Postpartum BMI	Glucose, leptin, insulin, adiponectin, ghrelin
Yu, 2018 [18]	China	Longitudinal	96	3, 42 and 90d	Pre-pregnancy and postpartum BMI	Leptin, insulin, ghrelin, adiponectin

¹Considered n = 15 for obese group according to supplementary table/raw data provided by authors.

²Only included data from RHMB longitudinal cohort as study only provides maternal weight results for this specific cohort.

³BIS, bioelectrical impedance spectroscopy; BMI, body mass index; D, day(s); DXA, dual-energy X-ray absorptiometry; Mo, month(s); RCT, randomised controlled trial; Wk, week(s).

A synthesis of all studies reporting comparisons of HM metabolic hormone concentrations between overweight and/or obese and normal weight women and associations between maternal BMI and/or other measures of obesity and metabolic hormone concentrations in their milk is presented in Table 6.2.

Table 6.2. Effect of maternal obesity on metabolic hormone concentrations in HM: key findings^{1,2,3}

Reference	Gestational age	Milk type	Collection time	HM method of collection	Analytical method	Sample type	Outcomes/Effect
Comparison of HM metabolic hormone concentrations between OW/OB and NW women							
Fujimori, 2015 [237]	T	Colostrum	-	-	Enzymatic	Skim	HM glucose was higher in OB women
Kwon, 2018 ¹ [238]	-	Mature	-	-	Bio-Plex Multiplex Immunoassay System, Bio-Rad; Glucose assay	Skim	HM leptin and insulin were higher in OB women. No difference in HM glucose, ghrelin or adiponectin between groups
Correlation between maternal measures of maternal obesity and HM metabolic hormone concentrations							
Anderson, 2016 [194]	-	Colostrum/ transitional/ mature	6:00-10:00	Mid-feed	ELISA	Skim	No correlation between BMI and %body fat and HM adiponectin
Andreas, 2016 [26]	T	Transitional and mature	10:00-13:00	Pre- and post-feed	Bio-Plex Multiplex Immunoassay System	Skim	HM leptin (1wk and 3mo) and insulin (3mo) positively correlated with BMI
Bielicki, 2004 [163]	PT and T	Colostrum and mature	Morning	Pre-feed	RIA	Whole	HM leptin (2-3d) positively correlated with BMI
Bronsky, 2006 [167]	PT and T	Colostrum	After 7:00	Post-feed	ELISA	Skim (leptin), whole (adiponectin)	HM leptin positively correlated with body weight and BMI; HM adiponectin positively correlated with body weight
Dundar, 2010 [94]	T	Colostrum	8:00-10:00	-	ELISA (adiponectin); RIA (ghrelin)	Whole	HM total ghrelin negatively correlated with BMI. No correlation between BMI and HM adiponectin

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Fields, 2012 [156]	T	Mature	8:00-10:00	Full expression	Glucose oxidase method; immunoassay kits	Skim	HM leptin positively correlated with BMI. No correlation between BMI and HM insulin or glucose
Kuganathan, 2017 [19]	T	Mature	9:30-11:30	Pre- and post-feed	ELISA	Whole (leptin and adiponectin) and Skim (leptin)	Whole and skim HM leptin positively associated with fat mass and BMI. No associations between these measured and HM adiponectin
Ley, 2012 [91]	T	Colostrum/ transitional and mature	-	- early milk Full expression (mature)	Enzymatic assay; RIA; Immunoassay	Skim	Mature HM insulin positively associated with BMI. No association between BM and HM glucose or adiponectin
Luoto, 2012 [209]	-	Colostrum	-	-	Fluorometric assay	-	No correlation between BMI and HM adiponectin
Martin, 2006 [9]	T	Colostrum/ transitional/ mature	10:00-13:00	Full expression	RIA	Skim	HM adiponectin positively correlated with BMI
Nunes, 2017 [67]	T	Colostrum and mature	-	Pre- or post-feed	ELISA	Skim	HM leptin (colostrum and mature) and insulin (mature) positively associated with BMI. No correlation between BMI and HM adiponectin
Quinn, 2015 [210]	-	Colostrum/ transitional/ mature	06:00-8:00	Mid-feed	ELISA	Skim	HM leptin positively associated with BMI and %body fat
Savino, 2016 [78]	T	Mature	07:00-09:00	Pre-feed	RIA	Skim	HM leptin positively correlated with BMI
Schueler, 2013 [170]	-	Mature	07:00-10:00	Pre- and post-feed	RIA	Skim	HM leptin positively correlated with weight, BMI, %body fat and fat mass. HM GLP-1 positively correlated with body weight. No association between these measures and HM PYY
Schuster, 2011 [77]	T	Mature	-	-	RIA	Skim	HM leptin positively correlated with BMI
Yu, 2018 ² [18]	T	Colostrum and mature	08:00-09:00 (colostrum) 14:00-16:00 (mature)	Pre-feed (colostrum) Pre- and post-feed (mature)	ELISA	Skim	HM leptin, insulin and adiponectin positively correlated with BMI. HM ghrelin negatively correlated with BMI

Both comparison and correlation analysis

Chan, 2018 [22]	PT and T	Mature	24-h	Pre- and post-feed	Multi-array assay, Mesoscale Discovery	Skim	HM leptin and insulin were higher in OB/OW women and positively correlated with BMI. Adiponectin was lower in OB women.
DeLuca, 2016 [189]	T	Mature	09:00-11:00	Full expression	RIA	Whole	HM leptin was higher in OB women and positively correlated with BMI
Eilers, 2011 [81]	PT and T	Colostrum and mature	16:00-20:00	Pre- and post-feed	RIA	Skim	HM leptin (28d) was higher in OW/OB women and positively correlated (3 and 28d) with BMI
Fujimori, 2017 [193]	T	Colostrum	-	-	ELISA	Skim	HM leptin and adiponectin were higher in OB women and positively correlated with BMI.
Lemas, 2016 [89]	T	Transitional	Morning	Mid-feed	High-sensitivity ELISA; RIA	Skim	HM leptin and insulin were higher in OB women and positively correlated with BMI
Uysal, 2002 [236]	-	Mature	08:00-11:00	Pre-feed	RIA	Skim	HM leptin positively correlated with BMI but no difference between groups
Young, 2017 [17]	T	Transitional and mature	Morning	Mid-feed	Hexokinase ultraviolet assay; ELISA (leptin); RIA; - ghrelin	Skim	HM leptin and insulin (transitional and mature) were higher in OW/OB women and positively correlated with BMI. Transitional HM adiponectin positively associated with BMI. No association between BMI and HM glucose or ghrelin.

¹Considered n = 15 for obese group according to supplementary table/raw data provided by authors.

²Colostrum and mature were pooled for correlations with maternal BMI

³ELISA, enzyme-linked immunosorbent assay; NW, normal-weight; OB, obese; OW, overweight; PT, preterm; RIA, radioimmunoassay; T, term

A summary of results for the qualitative synthesis across all stages of lactation is presented in Table 6.3.

Table 6.3. Summary results of qualitative synthesis across all stages of lactation for studies reporting both comparison and correlation analysis¹

Milk metabolic hormone	Studies, n	Effect					
		Positive association with maternal BMI/adiposity		No association with maternal BMI/adiposity		Negative association with maternal BMI/adiposity	
		Pre-pregnancy	Postpartum	Pre-pregnancy	Postpartum	Pre-pregnancy	Postpartum
Leptin	19	10	12	2	2	0	0
Insulin	9	6	3	3	2	0	0
Glucose	5	1	0	4	0	0	0
Adiponectin	13	2	3	6	4	1	0
Ghrelin	5	0	0	1	1	0	3
Resistin	1	0	0	0	1	0	0
GLP-1	1	0	1	0	0	0	0
PYY	1	0	0	0	1	0	0

¹N, number of included studies

6.4.2 HM leptin concentrations

The qualitative synthesis included 16 studies measuring HM leptin (n=5 colostrum, n=3 transitional, n=14 mature and n=2 pooled samples collected across different lactation stages). All of the five studies in colostrum reported a positive correlation between leptin concentrations and maternal pre-pregnancy [67,81,167,193] and/or postpartum [67,163,167] BMI. Two of these studies also included a comparison between normal weight and overweight and/or obese women [81,193], but only 1 reported higher levels of leptin in colostrum from obese women [193]. Similarly, all three studies in transitional HM reported positive correlations between HM leptin concentrations and maternal pre-pregnancy [89] or postpartum [17,26] BMI. Two of these studies also compared HM leptin between normal weight and overweight and/or obese women, and both reported higher levels of HM leptin in obese and overweight women [17,89]. Twelve

of the 14 studies conducted in mature milk reported a positive association between HM leptin and maternal pre-pregnancy [22,67,81,156,189] or postpartum BMI [17,19,26,67,77,78,170,236] and %body fat and fat mass [19,170]. Of the two remaining studies, one assessed the relationship of HM leptin with both pre-pregnancy and postpartum BMI and reported a significant positive association only for postpartum BMI [77]. The other study reported no association between postpartum BMI and HM leptin concentrations [163].

Two studies assessed relationships between HM leptin and maternal BMI and %body fat in samples collected across different stages of lactation [18,210]. Of these studies, Quinn and colleagues [210] reported a positive association between HM leptin and maternal postpartum BMI and %body fat, while Yu and colleagues found a positive association between HM leptin and both maternal pre-pregnancy and postpartum BMI [18]. Of the six studies that reported comparisons between normal weight and overweight and/or obese women, five showed higher HM leptin levels in women who were overweight or obese [17,22,81,189,238] and one found no difference [236].

6.4.3 HM insulin and glucose concentrations

The qualitative synthesis included 9 studies measuring HM insulin (n=1 colostrum, n=3 transitional, n=7 mature and n=2 pooled samples). Only one study assessed insulin in colostrum and found no association with either maternal pre-pregnancy or postpartum BMI [67]. A second study collected both colostrum and transitional HM samples and did not assess relationships separately between the two. This study also did not report an association between HM insulin

concentrations and maternal pre-pregnancy BMI [91]. Of the three studies conducted in transitional HM, two reported positive associations between HM insulin and maternal pre-pregnancy [89] or postpartum [17] BMI, while one found no association with maternal postpartum BMI [26]. A comparison of HM insulin concentrations between normal weight and overweight and/or obese women was also performed in two of these studies and both reported higher levels of HM insulin in overweight and obese women [17,89].

Mature milk was assessed in seven studies and, of those, six reported associations/correlations between HM insulin and one or more measures of maternal obesity while three reported comparisons between normal weight and overweight and/or obese women (2 studies reported both). Five of the 6 studies that assessed correlations reported positive associations between HM insulin and maternal pre-pregnancy [22,67,91] and/or postpartum [17,26,67] BMI, while one study found no association with pre-pregnancy BMI [156]. All three studies that undertook comparisons between normal weight and overweight and/or obese women reported higher insulin levels in overweight and obese women [17,22,238]. In addition, one study that pooled colostrum and mature HM samples reported a positive association between HM insulin concentrations and maternal pre-pregnancy and postpartum BMI [18].

There were five studies that had assessed HM glucose (n=1 colostrum, n=1 transitional HM, n=4 mature, n=1 pooled samples and n=2 measured more than one milk type). The only study assessing glucose in colostrum reported higher levels in obese women compared to overweight and normal weight women [237]. However, a second study which included both colostrum and transitional HM (and did not distinguish between them), found no association between HM

insulin and maternal pre-pregnancy BMI [91]. The one study that collected only transitional milk showed no differences in HM glucose between overweight and obese and normal weight women and no correlation between HM glucose concentrations and maternal pre-pregnancy BMI [17]. Out of the four studies measuring glucose in mature milk, three found no difference in HM levels between normal weight and overweight and/or obese women [17,91,238] and two found no association between HM glucose and maternal pre-pregnancy BMI [17,156].

6.4.4 HM adiponectin concentrations

The qualitative synthesis included 13 studies measuring adiponectin (n=5 colostrum, n=1 transitional, n=6 mature and n=4 pooled samples/broad lactation stage range). Out of the five studies measuring adiponectin in colostrum, two reported a positive association between HM adiponectin and maternal pre-pregnancy BMI [193,239], while three found no association with pre-pregnancy [67,94,209] or postpartum [67] BMI. One study that included both colostrum and mature HM in the analysis found a positive correlation between HM adiponectin and maternal postpartum BMI [18], whereas a separate study that measured colostrum and transitional HM (and did not analyse relationships separately by milk type) found no association between HM adiponectin and maternal pre-pregnancy BMI [91]. The one study in colostrum that reported comparisons showed higher levels of adiponectin in obese and overweight mothers [193]. The only study assessing transitional HM only reported a positive association between HM adiponectin and maternal postpartum BMI [17]. Of the six studies measuring mature HM, five studies reported correlations/associations between HM adiponectin and measures of maternal obesity/BMI and three studies reported comparisons between normal weight and overweight and/or obese women. Of these studies, none identified associations between HM adiponectin

and maternal pre-pregnancy [22,67,91] or postpartum [17,19,67] BMI or fat mass [19]. Of the three studies that undertook comparisons, one showed lower levels of adiponectin in obese women [22] while, two found no difference in HM adiponectin between normal weight and overweight and/or obese women [17,238]. Of the two studies collecting HM samples across all three milk types (but which did not undertake analyses separately in each milk type), one reported a positive correlation between HM adiponectin and maternal postpartum BMI [9] while the other found no association with either postpartum BMI or %body fat [194].

6.4.5 HM ghrelin concentrations

The qualitative synthesis included 5 studies assessing HM ghrelin (n=1 colostrum, n=2 transitional, n=3 mature, n=1 pooled samples and n=2 measured more than one milk type). The only study in colostrum found a negative correlation between ghrelin concentrations and maternal pre-pregnancy BMI [94]. A second study which pooled colostrum and mature samples also found a similar negative correlation between HM ghrelin and maternal postpartum BMI [18]. Of the studies assessing ghrelin in transitional HM, one study reported a negative correlation with maternal postpartum BMI [17], while a second study found no association [26]. One of these studies also performed a comparison on HM ghrelin concentrations between overweight and/or obese and normal weight women, and reported lower levels of HM ghrelin in women who were overweight and obese [17]. Of the studies assessing ghrelin in mature HM, one study reported a negative correlation with maternal postpartum BMI [17] while, a second study found no association [26]. Of the two studies reporting comparisons between overweight and/or obese and normal weight women, one found lower HM ghrelin levels in overweight and obese women [17] while the other reported no difference [238].

6.4.6 HM resistin, GLP-1 and PYY concentrations

The qualitative synthesis included two studies assessing resistin (transitional and mature), GLP-1 (mature) and PYY (mature). These studies showed no relationship between resistin concentrations in transitional and mature HM and maternal postpartum BMI [26]. In agreement, mature PYY levels were also not associated with maternal postpartum BMI and %body fat [170]. However, GLP-1 concentrations in mature HM were positively correlated with maternal postpartum body weight, but not BMI and %body fat, in the same study [170].

6.4.7 Quality assessment

The risk of bias assessment is outlined in Table 6.4 and highlights the main quality points relevant to this review. The overall quality of the studies was relatively low and the ability to control for confounders and to state infant's feeding mode were the categories that scored the lowest across the studies.

Women with pre-existing conditions known to influence HM composition, including GDM and pre-gestational diabetes, were excluded in most of the studies (n=13) [17,26,77,78,81,89,91,156,163,170,189,209,238]. Four studies included women with GDM [18,67,193,237], while 8 studies did not state if this maternal condition had been accounted for [9,19,22,94,167,194,210,236].

Table 6.4. Quality and reporting assessment for studies measuring metabolic hormone concentrations in human milk^{1,2}

Reference	Representativeness cohort	Measure of maternal obesity	Controls for confounders	Sample size (small, medium or large study)	State gestational age	State feeding mode	Standard time of HM collection	State HM collection method	State type HM for analysis
Anderson, 2016 [194]	✓	✓	✓	Large	x	x	✓	✓	✓
Andreas, 2016 [26]	✓	✓	✓	Medium	✓	x	✓	✓	✓
Bielicki, 2004 [163]	✓	✓	x	Small	✓	x	✓	✓	✓
Bronsky, 2006 [167]	✓	x	x	Medium	✓	x	✓	✓	✓
Chan, 2018 [22]	✓	✓	x	Large	✓	✓	✓	✓	✓
DeLuca, 2016 [189]	✓	✓	✓	Medium	✓	✓	✓	✓	✓
Dundar, 2010 [94]	✓	✓	x	Small	✓	x	✓	x	✓
Eilers, 2011 [81]	✓	x	x	Medium	✓	x	✓	✓	✓
Fields, 2012 [156]	✓	✓	x	Small	✓	✓	✓	✓	✓
Fujimori, 2015 [237]	✓	x	x	Medium	✓	✓	x	x	✓
Fujimori, 2017 [193]	✓	x	x	Small	✓	x	x	x	✓
Kuganathan, 2017 [19]	✓	✓	x	Medium	✓	✓	✓	✓	✓
Kwon, 2018 [238]	✓	x	x	Small	x	x	x	x	✓
Lemas, 2016 [89]	✓	✓	✓	Small	✓	✓	✓	✓	✓
Ley, 2012 [91]	✓	x	✓	Large	✓	x	x	x	✓
Luoto, 2012 [209]	✓	x	x	Large	x	✓	x	x	x
Martin, 2006 ¹ [9]	✓	✓	x	Small	✓	x	✓	✓	✓
Nunes, 2017 [67]	✓	✓	x	Medium	✓	x	x	✓	✓
Quinn, 2015 [210]	✓	✓	x	Large	x	✓	✓	✓	✓
Savino, 2016 [78]	✓	✓	x	Medium	✓	✓	✓	✓	✓

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Schueler, 2013 [170]	✓	✓	✓	Small	x	✓	✓	✓	✓
Schuster, 2011 [77]	✓	✓	x	Small	✓	x	x	x	✓
Uysal, 2002 [236]	✓	✓	x	Medium	x	✓	✓	✓	✓
Young, 2017 [17]	✓	✓	x	Small	✓	✓	✓	✓	✓
Yu, 2018 [18]	✓	✓	✓	Medium	✓	✓	✓	✓	✓

¹Only included data from RHMB longitudinal cohort as study only provides maternal weight results for this specific cohort.

²Sample size is defined as small = studies with <50 participants, medium = studies with between 50 and 100 participants and large = studies with > 100 participants.

6.5 Discussion

This review describes an updated synthesis of the current literature investigating the effect of maternal overweight and obesity on HM metabolic concentrations. Overall, the qualitative synthesis suggested that the concentrations of leptin (across all lactation stages) and insulin (transitional and mature HM) were higher in HM from overweight and obese women compared to those of normal weight. Although adiponectin concentrations in HM were investigated in 13 studies, their findings were conflicting, and it is not possible to draw robust conclusions. There were considerably fewer studies that assessed associations between HM concentrations of glucose, ghrelin, resistin, GLP-1 and PYY and maternal BMI and/or fat mass, and the results were inconsistent. Thus, while leptin and insulin concentrations in HM were reported to be elevated in women who are overweight and obese, the impact of maternal overweight and/or obesity on the HM concentrations of other metabolic hormones remain unclear.

The higher leptin concentrations in HM from women who were overweight and obese and the positive association with maternal BMI and fat mass across all stages of lactation were the most robust and consistent observations across studies. In addition, the relationships reported were similar irrespective of whether maternal BMI/fat mass was obtained before or after pregnancy. The reason for the higher leptin concentrations in HM in women who are overweight and obese is unclear. While leptin is mainly produced by white adipose tissue, it is also synthesised by mammary epithelial cells [72], and it is therefore possible that synthesis in the breast tissue is increased in women who are overweight or obese, as it is in adipose tissue [240]. It is also possible that the higher leptin concentrations are the result of transfer from the maternal circulation. In support of this, circulating leptin concentrations are increased in women who are overweight and obese [77,78] and several studies have reported a direct correlation between

the concentrations of leptin in HM with those in the maternal circulation [23,77,78]. Independent of the cause of the elevated leptin concentrations, this could have impacts on the breastfed infant. Leptin concentrations in infant circulation have been shown to be directly related to the levels in HM [78,241]. Importantly, HM leptin concentrations measured at 1-4 months postpartum have been inversely associated with infant weight, weight-for-length Z-score (WLZ) or infant BMI Z-score (BMIZ) at 1 [156], 4 [22,154] and 12 months postpartum [22], and infant length, body fat, total fat mass and trunk fat mass at 6 months postpartum [12]. This suggests that the concentration of leptin in HM could play an important role in infant growth and fat deposition in the early postpartum period, with longer-term consequences for growth and body composition in the infant.

While fewer studies had assessed relationships between HM insulin in mature HM and measures of maternal obesity, all but one of these studies reported a positive association between these factors. As with leptin, the reason that maternal overweight and obesity would be associated with higher HM insulin concentrations is unclear. One possibility, however, is that this is a result of metabolic disturbances in the mother that are linked with maternal obesity, in particularly glucose intolerance and/ or insulin resistance. This is supported by the results of studies showing that hyperglycemia, higher fasting serum insulin concentrations, insulin resistance and lower insulin sensitivity were all associated with higher HM insulin concentrations [17,91]. Insulin concentrations in HM are also higher in other conditions associated with hyperinsulinemia such as, maternal diabetes and GDM [242-244], even in women receiving dietary interventions to improve glycemic control or insulin therapy [18]. In addition, concentrations of insulin in HM seem to be directly correlated with concentrations in

maternal serum [17]. The potential impact of higher HM insulin on infant outcomes such as, adiposity, growth, metabolism and regulation of appetite is not yet fully understood. Previous studies have reported that insulin in mature HM was negatively correlated to infant weight, lean mass [156] and weight gain at one month [67], infant WLZ and BMIZ at 4 and 12 months [22], while no associations have been reported for infant body composition [12] and WLZ [18] at 1-6 months postpartum. Thus, whether insulin concentrations in HM have any long-term impact on the infant, in relation to growth, fat deposition and risk of obesity or metabolic disease, remains to be determined. The concentrations of glucose in HM did not differ between overweight and/or obese and normal weight women at any stage of lactation. Indeed, the extremely small number of studies highlights the need for more research in this area, particularly given that hyperglycemia [245,246] and insulin resistance [17,246] are common issues for women who are overweight and obese.

The concentrations of adiponectin and ghrelin in HM did not appear to be influenced by maternal obesity at any stage of lactation, although it is important to note that the number of studies that have evaluated these relationships is limited, particularly for ghrelin. Adiponectin in HM appears to be derived from both the maternal circulation and synthesis in mammary epithelial cells [87]. The lack of robust evidence of a relationship between maternal overweight and obesity and maternal BMI was somewhat unexpected in light of previous findings that circulating adiponectin concentrations are negatively associated with maternal weight and BMI [86]. It is possible that adiponectin concentrations in HM are more influenced by mammary gland production than circulating maternal levels, or it may be that the current studies were

underpowered to investigate these differences and that further studies are required. While ghrelin concentrations in HM do not appear to be impacted by maternal overweight and/or obesity, concentrations of both total and active ghrelin in colostrum [94] are positively correlated with their respective concentrations in maternal serum, suggesting that a significant proportion of the ghrelin in colostrum is derived from the maternal circulation. Interestingly, however, ghrelin concentrations in mature HM are higher than in maternal plasma [10], indicating a major contribution from synthesis within the mammary glands. It is also important to note that other factors, including the type of milk used for analysis (whole vs skim) [10] and time relative to a feed when sample is collected [26,172] also influence ghrelin concentrations in the samples, and therefore variability in these measures across studies could also have contributed to some of the variability in the findings, and limit the ability to draw robust conclusions.

The concentrations of resistin, GLP-1 and PYY were only assessed by one study each which reflects the limited evidence and makes it impossible to draw a definitive conclusion as to the relationship of these HM components to maternal BMI and fat mass. Further studies to interrogate these relationships are important, particularly given the established roles of these hormones in regulating appetite and metabolism [247-249] and contributing to short- and long-term growth, fat deposition and health outcomes in infants. Based on the current evidence, only GLP-1 concentrations in HM was associated with maternal postpartum body weight. This anorexigenic hormone was also associated with changes across a single feed in the same study, showing higher concentrations in post-feed samples, which was also observed in HM fat [170]. This could suggest a role of HM fat as a mediator of changes in GLP-1 concentrations. Also,

the authors hypothesized that mammary gland could be mediating GLP-1 synthesis due to rapid changes in its levels from the feed. However, more studies assessing HM and circulating levels of GLP-1 are required to confirm these hypotheses. While concentrations of resistin and PYY in HM did not show any association with maternal BMI, HM resistin was positively correlated with levels in the maternal circulation, and resistin concentrations in both HM and serum were positively correlated with serum leptin concentrations [250]. Further studies are required to better elucidate relationships between HM concentrations of resistin, GLP-1 and PYY in HM and maternal obesity.

The major strength of this study was the comprehensive and systematic approach to reviewing the existing literature, and this included lactating women from several countries, both urban and rural areas, with distinct ethnic and sociodemographic backgrounds. In addition, we were able to capture different study designs (observational and intervention), stage of lactation (colostrum, transitional and mature HM) and gestational age (preterm and term). Our inclusion criteria also enabled us to assess studies which had applied more direct measures of maternal adiposity (e.g. BIS and DXA) as well as, their timing of measurement (pre-pregnancy and/or postpartum) to better elucidate their relationships with HM composition. The inclusion of quality assessment which focused on specific factors relevant to HM standardisation also contributed greatly to the evidence synthesis.

While there were several strengths, there were also limitations. Most notable was the insufficient number of studies eligible for performing a quantitative synthesis and the low number of studies eligible for inclusion in the qualitative analysis. In addition, the overall

quality of the included studies was relatively low, and many failed to report factors other than maternal BMI and/or fat mass, which also had the potential to influence HM composition, complicating the interpretation of the data. Differences in the collection method and the type of milk analysed (whole or skim) also have the potential to affect the results. For instance, ghrelin concentrations in mature HM were reported higher at the beginning of the feed compared to the end, which aligns with its main role on appetite regulation [26,172]. In addition, previous studies have reported higher leptin concentrations in whole HM compared to skim HM as fat and cellular components are excluded from the skim fraction [72,251]. The impact of both method of collection and type of HM needs to be further explored, particularly when assessing appetite-regulating hormones. Further, many studies only used BMI to classify lactating women as overweight or obese, rather than more direct measures of adiposity. This may also have impacted the findings, since a study that evaluated relationships between HM composition and both of these measures suggested that concentrations of HM leptin were more closely related to %body fat, rather than BMI [19]. Further, emerging evidence show that HM composition is influenced by maternal metabolic dysfunction such as diabetes and gestational diabetes [149] therefore, presented findings can be impacted since overweight and obese lactating women could also be suffering from these conditions.

6.6 Conclusion

This study found that maternal BMI and adiposity measures were associated with higher HM leptin and insulin concentrations at different lactation stages, while the impact of maternal overweight and/or obesity on HM glucose, adiponectin, ghrelin, resistin, GLP-1 and PYY concentrations remains inconclusive. The relatively low quality of the evidence in this area,

and the limited number of studies highlight a need for further research. In the context of the current obesity epidemic, an improved understanding of the potential impact of maternal overweight and obesity on HM hormone concentrations, as well as the biological mechanisms through which these effects may be mediated, is critical.

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Abbreviations:

Bioelectrical impedance spectroscopy (BIS)

BMI Z-score (BMIZ)

Body mass index (BMI)

Confidence interval (CI)

Dual-energy X-ray absorptiometry (DXA)

Glucagon-like peptide 1 (GLP-1)

Human milk (HM)

Mean difference (MD)

Newcastle-Ottawa scale (NOS)

Preferred reporting items for systematic reviews and meta-analysis (PRISMA)

Preferred reporting items for systematic reviews and meta-analysis protocols (PRISMA-P)

Peptide YY (PYY)

Weight-for-length Z-score (WLZ)

World Health Organization (WHO)

CHAPTER 7. THE SHORT-TERM IMPACT OF DIETARY FAT AND SUGAR INTAKE ON BREAST MILK COMPOSITION: A CLINICAL TRIAL PROTOCOL

Gabriela E. Leghi, Merryn J. Netting, and Beverly S. Muhlhausler

The manuscript entitled “The short-term impact of dietary fat and sugar intake on breast milk composition: A clinical trial protocol” was published in the peer reviewed journal *Nutrition and Health*. 2020;26.

Additional file 1 is listed as Appendix 9. SPIRIT Checklist – Chapter 7

Additional file 2 is listed as Appendix 10. Detailed nutritional information for breakfast meals – Chapter 7

Statement of Authorship

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Principal Author

Name of Principal Author (Candidate)	Gabriela Esteves Leghi		
Contribution to the Paper	Contributed to the research idea, experimental design and Ethics application. Drafted and constructed manuscript and had primary responsibility for final version.		
Overall percentage (%)	75		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	05.05.2020

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

- the candidate's stated contribution to the publication is accurate (as detailed above);
- permission is granted for the candidate to include the publication in the thesis; and
- the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Signature		Date	05.05.2020

The short-term impact of dietary fat and sugar intake on breast milk composition: A clinical trial protocol

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Abstract

Background: Breast milk is uniquely designed for the infant and contains the key nutrients and bioactive factors required to support optimal infant health and development. While previous studies have reported that maternal obesity can influence milk composition, whether this relationship is driven by maternal or dietary factors remains unclear. **Aim:** The aim of this study is to assess the impact of test meals varying in fat and sugar content on post-prandial concentrations of macronutrients and metabolic hormones in the breast milk. **Methods:** This open label crossover study will include 25 lactating women. On the three days of the intervention, women will be randomized to receive a breakfast meal with a fat and sugar content consistent with the Australian Guide to Healthy Eating (9 g fat, 25 g of sugar) or a breakfast meal containing higher levels of fat (28 g fat, 18 g of sugar) or sugar (5 g fat, 56 g of sugar). All breakfast meals will be similar in composition (cereal, milk, yogurt, toast and spread) and matched for total energy content. This study will measure breast milk concentrations of metabolic hormones (leptin, insulin, adiponectin, ghrelin and glucagon-like peptide-1) and macronutrients in the following 12 hours. **Results and Conclusion:** The results of this study will provide novel direct evidence of the impact of variations in dietary fat and sugar content to alter the macronutrient and/or metabolic hormone concentrations in breast milk. Data on the effect of maternal diet on milk composition is critical given the established importance of nutritional exposures in early infancy for an individual's life-long health outcomes.

Keywords

Maternal nutrition, diet, breast milk composition, macronutrients, hormones, lactation

Background

Breast milk is uniquely designed for the infant and contains all key nutrients and bioactive factors required to support optimal infant health and development (Ofstedal, 2012; Walker, 2010). Knowledge of breast milk composition has increased significantly in recent years and it is now clear that, in addition to macronutrients (fat, protein and carbohydrates), breast milk also contains a wide range of other bioactive compounds, including metabolic hormones involved in regulating appetite, metabolism and gut function. These metabolic hormones have been shown to be transferred to the breastfed infant, and the concentrations of several of these factors have been related to measures of infant growth and long-term outcomes (Fields and Demerath, 2012; Fields et al., 2016; Woo et al., 2009). For instance, leptin and adiponectin concentrations in breast milk at different stages of lactation have been related to measures of infant growth and fat mass up to 2 years of age (Brunner et al., 2014; Fields et al., 2017).

It is increasingly clear that there are significant variations in the concentrations of both macronutrients and metabolic hormones in breast milk between individual women and in relation to time of the day and stage of lactation (Bauer and Gerss, 2011; Cannon et al., 2015; Khan et al., 2013; Perrin et al., 2017). However, our understanding of the maternal factors that determine these concentrations is still incomplete. The number of women

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who are overweight or obese while pregnant and breastfeeding has increased markedly in the past few decades, consistent with the global increase in obesity prevalence (WHO, 2017). In Australia, around 50% of pregnant women are overweight or obese when attending their first antenatal appointment (Athukorala et al., 2010; Dodd et al., 2011), and similar statistics have been reported in the USA and UK (Poston et al., 2016). The increasing body weights of pregnant and breastfeeding women has led to interest in the impact of maternal overweight and obesity on the health of the mother during and after pregnancy and potential effects on her infant after birth, including changes in breast milk composition (Young et al., 2012). Although previous studies have shown that breast milk composition is altered in women who are overweight and obese in comparison to women of normal weight (Andreas et al., 2014, 2016; Fujimori et al., 2015; Kuganathan et al., 2017), it is not clear if these changes are due to the alterations in maternal metabolism that typically accompany overweight and obesity, differences in maternal dietary quality or a combination of both.

Maternal diet is often regarded as a critical determinant of breast milk composition, yet evidence-based information on the relationship between dietary intakes and breast milk composition is surprisingly sparse, particularly for metabolic hormones. Findings of a recent systematic review demonstrated that, except for direct correlations between maternal intake and breast milk content for vitamin C and docosahexaenoic acid (DHA), the relationship between dietary intake and the milk content of specific macro- and micro-nutrients remains poorly defined. In addition, most, if not all, of the studies included in the systematic review had limitations, including significant heterogeneity and inconsistent collection and analytical methodologies (Bravi et al., 2016). It is also unknown whether, and how quickly, levels of metabolic hormones in breast milk can be altered in response to changes in maternal dietary intake. Furthermore, no studies have directly assessed the impact of patterns of consumption common in poor-quality Western diets on breast milk composition, particularly the impact of high intakes of fat and refined sugar. An improved understanding of the effect of maternal diet on milk composition is critical given the established importance of nutritional exposures in early infancy for an individual's life-long health outcomes (Goran et al., 2017).

Aim

This study aims to determine the impact of providing breastfeeding women with a breakfast containing differing amounts of fat and sugar on the post-prandial concentrations of macronutrients and metabolic hormones in their breast milk over the subsequent 12-hour period.

Contribution to the field

This will be the first study to assess macronutrient and metabolic hormone concentrations after dietary intervention and will identify the timeframe over which changes in macronutrient and hormone concentrations occur. The results of this research will provide proof-of-concept evidence that improving the quality of the maternal diet while breastfeeding could alter the composition of breast milk. This has not been investigated in the context of the current obesity epidemic and could potentially have implications for dietary recommendations for breastfeeding women. Improving breast milk quality through dietary interventions will ultimately impact the long-term health outcomes of the child.

Study objectives

Main objective

To assess the effect of consumption by breastfeeding women of a high-fat or high-sugar breakfast on concentrations of metabolic hormones (leptin, insulin, adiponectin, ghrelin and glucagon-like peptide-1 (GLP-1)) and key macronutrients (fat, protein, lactose) in breast milk over the subsequent 12 hours.

Specific objectives

Primary: To assess and compare breast milk concentrations of metabolic hormones (leptin, insulin, adiponectin, ghrelin and GLP-1) in the 12 hours following consumption of either a high-fat or high-sugar breakfast compared to concentrations after consumption of a control breakfast.

Secondary: To assess changes in breast milk concentrations of macronutrients (fat, protein, lactose) in the 12 hours following consumption of either a control, high-fat or high-sugar breakfast.

Methods: Participants, interventions and outcomes

Protocol

The study protocol has been developed based on Standard Protocol Items: Recommendations for Interventional Trials (SPIRIT) guidelines (Chan et al., 2013). A SPIRIT checklist file is attached (Additional file 1).

Study design

This study is an open label crossover design with women assigned to receive the high-fat, high-sugar and control breakfasts in a random order. There is a one-week wash-out period between the different breakfasts.

Study setting

This study will be performed in the Women's and Children's Hospital (WCH), community centres or participants'

Table 1. Distribution of breakfast meals in % of total energy.

Breakfast meal	Protein (%)	Fat (%)	Saturated fat (%)	Carbohydrate (%)	Sugar (%)
High-fat	14	48	23	35	14
High-sugar	14	9	4	74	43
Control	25	17	9	54	19

Note: Based on estimated food values and calculations provided by FoodWorks, Xyris, version 7 and excludes dietary fibre and other dietary sources.

homes. Women will be screened for eligibility either antenatally or postnatally, either at the WCH, the South Australian Health and Medical Research Institute (SAHMRI) or in community centres.

Study population

Participants must meet the following inclusion criteria: (a) singleton pregnancy; (b) term delivery; (c) currently breastfeeding; and (d) between six weeks and 16 weeks post-partum at the time of the first study visit.

The exclusion criteria are as follows: (a) known major congenital abnormalities or health issues in the infant that could significantly affect feeding behaviour; (b) complementary feeding introduced before first study session; (c) maternal diseases known to affect gastric absorption; (d) maternal diabetes; and (e) restrictive diets (e.g. gluten free, dairy free, milk free, vegan).

Interventions

The dietary interventions will be provided in the form of a specially designed breakfast meal. There will be three test meals: The first will have a fat and sugar content consistent with the Australian Guide to Healthy Eating (AGHE) (control, 9 g fat and 25 g sugar), the second will have higher levels of fat (high-fat, 28 g fat) and the third, higher levels of sugar (high-sugar, 56 g sugar). The breakfast meal will be provided at ~7:30 am after women have fasted from midnight on the previous day. The breakfast meals will contain similar food types (cereal, milk, yogurt, toast and spread) and will be matched for total energy content as closely as possible. The macronutrient composition, sugar and saturated fat contents (as a percentage of total energy) of the three breakfast meals can be found in Table 1 and detailed composition information can be found in Additional file 2.

High-fat breakfast meal. The high-fat breakfast meal will consist of 30 g muesli, 100 ml full fat milk, 90 g Greek yogurt, 42.5 g (1 slice) soy and linseed bread and 8 g butter. This breakfast provides 28 g of fat, 18 g of sugar and has an energy content of 2160 kJ.

High-sugar breakfast meal. The high-sugar breakfast meal will consist of 30 g high-fibre breakfast cereal, 100 ml

semi-skimmed milk, 100 g strawberry yogurt, 65 g (1 slice) fruit bread, 15 g raspberry jam, 1 teaspoon honey and 1 teaspoon of white sugar. This breakfast provides 5 g of fat, 56 g of sugar and has an energy content of 2072 kJ.

Control (AGHE) breakfast meal. The control breakfast meal will consist of 45 g whole grain cereal, 200 ml semi-skimmed milk, 160 g vanilla low-fat yogurt, 1 wholemeal crumpet, 70 g fresh strawberries and 8 g butter. This breakfast provides 9 g of fat, 25 g of sugar and has an energy content of 2029 kJ.

Outcomes

Primary outcome. Breast milk concentrations of metabolic hormones (leptin, insulin, adiponectin, ghrelin and GLP-1) in the 12 hours following consumption of either a control (AGHE), high-fat or high-sugar breakfast.

Secondary outcome. Breast milk concentrations of macronutrients (fat, protein, lactose) in the 12 hours following consumption of either a control (AGHE), high-fat or high-sugar breakfast.

Timeline and procedures

The study visits. Women who are interested and eligible for the study will be invited to attend three separate appointments, with at least one week between appointments. Women will be asked to fast from midnight on the day before and to arrive for the study visit at ~7:00 in the morning. A baseline breast milk sample will be collected and women will then be provided with either a high-fat, high-sugar or control breakfast meal (in random order determined by a computer-based randomization schedule). Women will record details of their last breastfeed/expression prior to arrival (time and collection breast) before collection of the baseline sample. The women will then be asked to remain in or around the clinic room for meals during the day. All participants (regardless of the test breakfast they were allocated to) will be provided with morning tea, lunch, afternoon tea and snacks throughout the day. The nutrient profile of the meals and snacks will be consistent with the AGHE. Women will be asked to record everything they eat during the day. Breast milk samples will be collected at regular intervals throughout the day (1, 2, 4 and 6 hours after the breakfast meal) for the assessment of macronutrients and metabolic hormone concentrations. After the collection of the 6-hour sample, women will be able to return home, where they will be asked to collect a further milk sample at ~8 pm (~12 hours after the breakfast meal). Women will be provided with standardized meals (snacks and dinner) consistent with the AGHE to take home once they leave the clinic. Participants will be reminded to complete their food records until the last breast milk sample is collected. At the time of booking the first session women will be asked to

Table 2. Summary of data to be collected.

Time	Activity	Samples collected	Data collected
7:00	Arrival	Breast milk (~5–10 ml)	Food Frequency Questionnaire Maternal weight and height (first session)
7:30	Breakfast meal	–	–
8:00	Completion of Breakfast meal	–	–
	Measurement of infants	–	Weight and length
9:00	1 hour after breakfast	Breast milk (~5–10 ml)	–
	Case Report Form	–	Sociodemographic, clinical and infant information (first session) 24 h-recall
10:00	2 hours after breakfast	Breast milk (~5–10 ml)	–
10:10	Morning tea	–	Food intake recorded
12:00	4 hours after breakfast	Breast milk (~5–10 ml)	–
	Lunch	–	Food intake recorded
14:00	6 hours after breakfast	Breast milk (~5–10 ml)	Meals for the day to take home will be provided
	Women return home	–	–
~ 20:00	~ 12 hours after breakfast	Breast milk (~5–10 ml)	–

Note: Proposed time points for data collection may be subject to small alterations according to each participant (to be recorded).

provide details of any food allergies or intolerances, so that their breakfast meals can be adjusted accordingly if required. This information will be recorded in the study Case Report Form and documented in the study database to ensure that this information is readily accessible.

Participant contact/data collection

Baseline. Baseline clinical and sociodemographic characteristics of the mother, including age, education, household income, alcohol intake, smoking status, use of dietary supplements and presence of chronic diseases, will be collected by study research staff in the Case Report Form at the first clinic session. If women report that they are taking dietary supplements and/or medications (other than supplements that are designed specifically for pregnancy/breastfeeding) they will be provided with details of the Medicines Information Centre in South Australia (SA), where they can confirm that the supplements/medications are safe for use during pregnancy, if they have not already done so. The body weight and height of the women will also be measured. Infant information, including sex, gestational age at birth, birth weight and length, current supplements, medications or chronic health conditions will be obtained by parent report and medical records, and infant weight and length measured by study staff.

Dietary intake. Women will be asked to complete a standardized food frequency questionnaire using the Cancer Council of Victoria's Dietary Questionnaire for Epidemiological Studies (DQES) version 3.1 Food Frequency Questionnaire at their first study visit. The DQES has been previously validated for women of reproductive age (Hodge et al., 2000) and applied to lactating women from the Australian population (Huddy et al., 2016; van der Pligt et al., 2016). Participants will also complete a 24-hour food recall at each study visit describing intake on the day before the study breakfast and will maintain a food record

on the day of the study visit. Dietary intake data for 24-hour recalls and food records will be collected and analysed using the Australian version of the Automated Self-Administered 24-hour (ASA24) Dietary Assessment Tool, version 2016, developed by the National Cancer Institute, USA (Subar et al., 2012). Study staff will be available to assist participants during completion of the dietary assessments.

Sample collection during the study. At each of the three study visits, women will be asked to collect breast milk samples (~5–10 ml) at baseline (after their arrival and before eating breakfast) and at 1, 2, 4 and 6 hours after breakfast. Breast milk samples (pre-feed) will be collected from the breast opposite to the one used to feed the infant at the previous feed. Women will be reminded of the timing of sample collection by study staff and will be given a private area in which they will be able to express breast milk either manually or using a hand breast pump (provided). Women will be also asked to collect milk samples ~12 hours after the breakfast (~8 pm). Milk samples collected at home can be stored in their home freezer. Women will be provided with a cooler bag and ice brick to bring samples to their next clinic session, and we will arrange collection of samples from their final session from the women's home by study staff. Table 2 summarizes data collection and procedures for the study visit.

Sample size

This study will include 25 women/infant pairs. A total of 30 women will be enrolled to allow for a 20% drop out or non-completion of the study per protocol. This number of women with the sample size selected (25 women) will provide 80% statistical power to detect a 5% reduction in protein (mean = 13.21, SD = 2.52), 27% reduction in fat (mean = 4.14, SD = 2.0) and 30% reduction in leptin

(mean = 429, SD = 232) and adiponectin (mean = 10.2, SD = 5.26) concentrations in breast milk, based on the mean and SD obtained for these measures by our group in preliminary studies.

Recruitment

Study staff will recruit antenatally and postnatally for this study. Women will be approached by research staff at one of their antenatal appointments or in the postnatal ward after delivery or at postnatal appointments at the WCH and provided with a brief outline of the study. Women indicating an interest in the study will be screened for eligibility by asking a series of yes/no questions related to the study inclusion/exclusion criteria. Interested women who are screened postnatally can be enrolled into the study as soon as eligibility is confirmed. Women in the postnatal ward will only be approached by a member of our study staff after they have provided consent to a WCH staff member. This consent will be documented on the screening log for the study. Women in the postnatal ward may also be screened and recruited by a WCH staff member. The study will also be advertised on social media, the Australian Breastfeeding Association and flyers/brochures distributed to community centres.

Women screened antenatally who have signed a Screening Consent Form and Consent to Contact form will be contacted by study staff at ~5 weeks post-partum to re-confirm their willingness to take part in the study and re-confirm eligibility. Women who are still interested and eligible will be provided with a copy of the Participant Information and Consent Form and given the opportunity to speak with friends/family and have any questions answered by study staff.

The information sheet will describe the purpose of the study, the procedures to be followed and the risks and benefits of participation. The investigator, or research staff, will conduct the informed consent discussion and will check that the information provided is understood and answer any questions about the study. Consent will be voluntary and free from coercion. Women willing to participate will sign the written consent form prior to the first session. Following documentation of informed consent, a member of the study research staff will collect baseline data, including contact details, demographic data, diet and infant/maternal health-related background data, using the study Case Report Form. The women will be randomized by study staff to determine the order of their intervention days. A computer-generated randomization schedule using balanced blocks will be generated by an independent statistician who is not involved in any other aspect of the study. The schedule will be kept by the independent statistician and the treatment allocation of each randomization code can be provided to the investigator or unblinded individual if required.

A record of all women screened, eligible and enrolled or not enrolled will be maintained to enable accurate

Consolidated Standards of Reporting Trials (CONSORT). Women may withdraw from the study for any reason at any time. If the women are willing to provide a reason for withdrawal, this will be recorded. We will aim to advertise study participation with brochures, posters and social media. All advertising material has been reviewed and approved by the Women's and Children's Health Network (WCHN) Human Research Ethics Committee (HREC).

Methods: Data collection, management and analysis

Data collection and management

Data will be collected by trained research staff onto paper Case Report Forms. Data entry and management will be coordinated by the research staff with cross-checking of at least 10% of the entries. The Case Report Forms will be stored in a locked office. Only research staff directly involved in the study will have access to the information. Data will only be released to persons authorized to receive those data. Originals/copies of study documents will be retained on site or in archives. Documents and electronic data will be retained for at least 30 years after study completion as per the data retention schedules for research involving minors, after which documentation will be destroyed using confidential document disposal. The study electronic data will be secured by servers with access only granted to authorized study personnel.

Statistical methods

The change in breast milk concentrations of each individual macronutrient and hormone over the 12 hours following the breakfast will be assessed using repeated-measures analysis of variance (ANOVA). The area under the concentration curve will be calculated to determine the total change in levels of each hormone/macronutrient across the 12-hour period. The pattern of change and total change in the level of each hormone/macronutrient across the 12-hour period following the three different breakfast meals will be assessed using a repeated-measures two-way ANOVA and paired *t*-test, respectively.

Methods: Study oversight and monitoring

Steering Committee

A Steering Committee consisting of the investigators will review and monitor the progress of the study (recruitment, compliance, data quality and loss to follow-up).

Adverse events

While the risks of participating in this study are very low, we accept the possibility that the mother or infant may have an adverse reaction to one or more of the study breakfasts. All adverse events will be documented in the Case Report

Form and separate Adverse Event Form during the course of the study. All adverse events occurring during the period from screening visit/signed informed consent to last visit/study completion will be registered. An independent data monitoring committee will assess the severity, causality, expectedness and seriousness for all adverse events. The Investigator is responsible for reporting adverse events in accordance with current guidelines and regulations to the HREC and, if applicable, relevant regulatory authorities.

Ethics and dissemination

Protocol amendments

Any modifications to the protocol that may impact on the conduct of the study, be of potential benefit to the participant or may affect patient safety, including changes of study objectives, study design, patient population, sample sizes, study procedures or significant administrative aspects, will require a formal amendment to the protocol. Such amendment will be agreed upon by the investigators and WCHN HREC prior to implementation and will be notified to the health authorities in accordance with local regulations. Administrative changes of the protocol are minor corrections and/or clarifications that have no effect on the way the study is to be conducted. These administrative changes will be agreed upon by the investigators and will be documented. WCHN HREC may be notified of administrative changes at the discretion of the Steering Committee.

Confidentiality

All study-related information will be stored securely at the study site. All participant information will be stored in areas with limited access. All laboratory specimens, reports, data collection and administrative forms will be identified by ID numbers, study ID and name code only to maintain participant confidentiality. All local databases will be secured with password protected access systems. Forms, lists, logbooks, appointment books and any other listings that link participant ID numbers to other identifying information will be stored in an area with limited access.

Access to data

All investigators involved in the study will have access to the full data set.

Dissemination policy

The results of this study will be presented at seminars to contribute to publicly available knowledge about the effect of consuming specific dietary factors on short-term changes in breast milk composition. These results, significant or not, will be published to allow non-biased data to be assessed following authorship eligibility guidelines. There are expected to be minimal intervals between the

completion of data collection and the release of the study results.

Summary

Decades of research have demonstrated that breast milk composition varies between women and populations. More recently, the levels of specific components in breast milk have been shown to relate to infant growth and fat deposition, raising the possibility that they may have impacts on long-term health outcomes (Fields et al., 2017; Goran et al., 2017; Prentice et al., 2016). However, our understanding of the factors that influence breast milk composition remains limited. Breast milk composition, including levels of fat, protein and metabolic hormones, is altered in women who are overweight and obese compared to those of normal weight (Andreas et al., 2014, 2016; Kuganathan et al., 2017), but the reason for these differences, and whether they may be related to maternal dietary factors, remains unclear. Furthermore, in epidemiological studies, it is difficult to separate the effects of maternal obesity per se from those of other maternal factors, including maternal diet. Therefore, the potential impact of the consumption of poor-quality high-fat, high-sugar diets on breast milk composition has yet to be determined.

Data on the effect of maternal diet on milk composition is critical given the established importance of nutritional exposures in early infancy for an individual's life-long health outcomes. The results of this study will provide novel direct evidence of the relationship between specific dietary components (i.e. fat and sugar) and macronutrient and metabolic hormone concentrations in breast milk. If post-prandial changes are observed, this will provide important proof-of-concept evidence that changes to the maternal diet during breastfeeding can rapidly influence the composition of the breast milk. This information is vital to allow studies to be translated into clinical practice. Furthermore, these studies are essential to increase awareness of the importance of dietary quality while breastfeeding on milk composition and, ultimately, to deliver more targeted dietary advice to lactating women.

Abbreviations

AGHE	Australian Guide to Healthy Eating
BMI	Body Mass Index
GLP-1	Glucagon-like peptide-1
HREC	Human Research Ethics Committee
SAHMRI	South Australian Medical Research Institute
WCH	Women's and Children's Hospital
WCHN	Women's and Children's Health Network

Author contributions

GEL and BSM worked on the conception and design of the study in consultation with co-author MN. GEL worked on design of breakfast studies with assistance from MN and BSM. GEL wrote the first draft of this protocol with

substantial contribution from all authors. All authors read and approved the final manuscript.

Declaration of conflicting interests

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

Ethics approval and consent to participate

This protocol and the template informed consent forms were reviewed and approved by the Women's and Children's Health Network, Human Research Ethics Committee (HREC/17/WCHN/182) with respect to scientific content and compliance with applicable research and participant regulations. The investigator or research staff will conduct the informed consent discussion and will check that the information provided is understood and answer any questions about the study. Consent will be voluntary and free from coercion. Women willing to participate will sign the written consent form prior to the first session. All procedures will be performed in accordance with the Declaration of Helsinki.

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Supplemental material

Supplemental material for this article is available online.

Trial registration

This clinical trial was registered with the Australian and New Zealand Clinical Trial Registry (ACTRN 12618000613202), <https://www.anzctr.org.au/Trial/Registration/TrialReview.aspx?ACTRN=12618000613202>.

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CHAPTER 8. THE EFFECT OF IMPROVING MATERNAL DIETARY QUALITY DURING LACTATION ON HUMAN MILK COMPOSITION AND PRODUCTION

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8.1 Abstract

Maternal diet is considered an important determinant of HM composition. However, no studies have determined whether, and to what extent, improving dietary quality during breastfeeding can affect the composition of HM. The primary aim of this study was to assess the effect of improving dietary quality in lactating women over a 2-week period on the concentrations of macronutrients and metabolic hormones in HM. The secondary aims were to assess 24-h milk production, maternal body composition and infant growth. Fifteen women completed a 1-week baseline period followed by a 2-week dietary intervention phase targeted towards reducing fat and sugar intake. Maternal anthropometric and body composition and infant growth measurements were performed weekly. Total 24-h milk production was measured before and after the dietary intervention and HM samples were collected daily. Maternal intakes of energy, carbohydrate, sugar, fat and saturated fat were significantly at the end of the dietary intervention. HM insulin, leptin and adiponectin concentrations were 10-25% lower at the end of the dietary intervention, but HM concentrations of fat, protein and lactose were unaffected. Maternal body weight and fat mass were significantly reduced at the end of the dietary intervention, but there were no effects on 24-h milk production or infant growth. These results suggest that significant reductions in maternal energy, fat and sugar intake over a 2-week period are associated with significant reductions in HM insulin, leptin and adiponectin concentrations. These changes may be secondary to decreases in maternal weight and fat mass and, if sustained, have the potential to positively influence infant growth and adiposity.

Clinical trial registration: Australian New Zealand Clinical Trials Registry (ACTRN12619000606189).

Keywords: human milk, lactation, diet, macronutrients, fat, protein, lactose, hormones, leptin, adiponectin, insulin

8.2 Introduction

HM composition is uniquely species-specific and, as such, provides the optimal nutrition to support the growth and development of human infants [3]. However, the levels of essential nutrients, immune factors and hormones vary considerably between mothers, and these differences may affect the short and longer-term health of the child [12,52]. Emerging evidence suggests that the variation in HM composition may be related to maternal, geographical, and other environmental factors [15,16,18].

Maternal diet is considered an important determinant of HM composition. Despite this, however, very few studies have directly assessed the effect of maternal diets on HM composition. The relationship between specific dietary components and the levels of HM components is also poorly defined and a systematic evaluation of the existing literature suggested that the only nutrients for which there was reasonable evidence of an association between maternal dietary intake and HM concentrations were individual fatty acids and vitamin C [27]. In addition, there is limited understanding of how changes to the maternal diet while breastfeeding can impact on HM macronutrient composition. The only study to date to directly investigate the impact of a dietary intervention on HM macronutrient composition reported that HM fat and total energy content were increased in lactating women who consumed a high-fat, low-carbohydrate (30% carbohydrate, 55% fat) diet for 8 days. In this same study, however, HM fat and energy content was not altered when women consumed a low-fat, high-carbohydrate (60% carbohydrate, 25% fat) diet for the same period, and HM production, lactose and protein concentrations were not affected by either of the dietary interventions [199]. It is important to note, however, that this study did not include only women who were exclusively breastfeeding. To the best of our knowledge, no studies have investigated the effect

of a maternal dietary intervention during lactation on HM concentrations of metabolic hormones.

Many women in Western countries consume poor quality diets that contain excess amounts of fat and added sugars [252,253], and animal studies suggest that this dietary pattern has the potential to impact the macronutrient, fatty acid and metabolic hormone composition of the mother's milk [254-256]. However, no human studies have determined the effect of improving dietary quality during breastfeeding on the composition of HM. Therefore, the primary aim of this study was to determine the effect of reducing energy, fat and sugar intakes in lactating women over a 2-week period on the concentrations of macronutrients and metabolic hormones in HM. The secondary aims of the study were to assess the effect of this dietary intervention on the 24-h milk production, maternal body composition and infant growth.

8.3 Methods

8.3.1 Study design

This study was an open label dietary intervention trial. Women completed a 1-week baseline period, during which they consumed their habitual diet. Following the baseline week, women completed a 2-week dietary intervention phase targeted towards reducing fat and sugar intake, during which they were provided with all meals and snacks. This study was approved by the Human Research Ethics Committee of The University of Western Australia (RA/4/20/4953) and registered with the Australian New Zealand Clinical Trials Registry (ACTRN12619000606189).

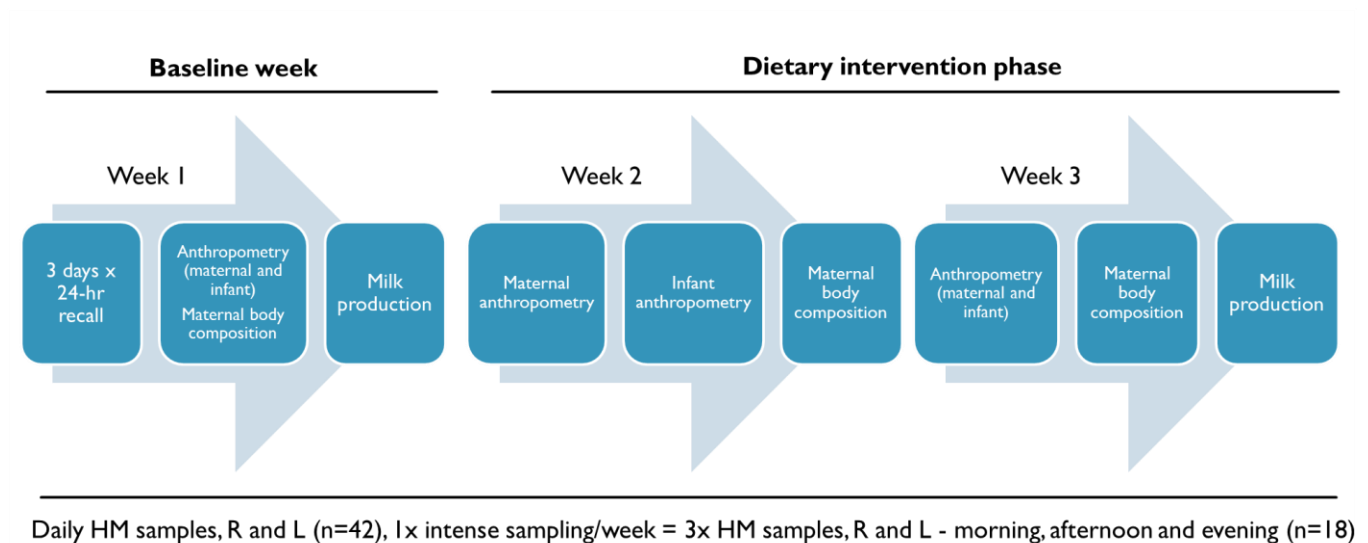


Figure 8.1. Summary of collected data across the baseline week (1 week) and dietary intervention phase (2 weeks)¹

¹HM, human milk; L, left; R, right.

8.3.2 Study participants

Women were approached to participate in the study through community centres, social media and existing networks. Women were eligible to participate in the study if they were exclusively breastfeeding a term singleton infant who was between 6 and 20 weeks postpartum at the time of the first study visit and growing normally (according to WHO standards) [215]. Women and infant pairs were excluded from participation if there were any known major infant congenital abnormalities or health issues that could affect feeding behaviour, pregnancy complications (e.g. gestational diabetes, preeclampsia, fetal growth restriction), maternal diabetes, maternal diseases known to affect gastric absorption, restrictive diets (e.g. gluten free, dairy free, milk free, vegan) or maternal smoking.

8.3.3 Dietary assessment

After enrolment, women completed three 24-h dietary recalls (2 week and 1 weekend day) for 1 week before the dietary intervention using the Automated Self-Administered 24-Hour Dietary Recall (ASA24), which has been previously validated in pregnant women [257] and childbearing women participating in a weight loss program [258] against measures of true intakes and interviewer administered 24-h recall. The online interface guides women to report intake via seven recall 'passes' (i.e. meal-based quick list, meal gap review, detail pass, forgotten foods, final review, last chance, usual intake). Images assist with portion size estimation and food codes from the Australian Food Supplement and Nutrient Database (AUSNUT) 2011-2013 are automatically assigned. Habitual average dietary intakes of the women were assessed using this information.

8.3.4 Dietary intervention

After the first week of the study, during which women were instructed to follow their habitual diet, women participated in a 2-week dietary intervention phase, which was aimed at improving dietary quality by reducing intakes of discretionary foods, saturated fats and added sugars. All foods/snacks were provided to women (via a home delivery service) to facilitate compliance, and participants were asked to record any foods/drinks they consumed other than those provided as well as any of the provided foods that they did not consume. Before the start of the dietary intervention, women were asked to provide details of any food allergies or intolerances, so that their meal plans could be adjusted accordingly if required. A dietitian tailored a nutritionally balanced and portion controlled 1553-1975 kcal diet (based on the participant's energy requirements and personal preferences) according to Australian Dietary Guidelines. Overall, the diets had at least a 500kcal deficit compared to their estimated energy requirements, < 22% of total energy from total sugar, <10% of total energy from saturated fat

and > 30g of fiber. The dietitian contacted women via their preferred means twice weekly to assess compliance with the diet. Total dietary intake per participant was calculated based on the daily consumption of provided diet and daily consumption of any extra food or beverages using the nutrition analysis software FoodWorks 10.

8.3.5 Anthropometry measurements

All maternal and infant measurements were performed immediately before, and at 1 week and 2 weeks after the start of the dietary intervention (weeks 2 and 3). Maternal weight was measured using a calibrated electronic scale (accuracy ± 0.1 kg; Seca, Chino, CA, USA). Height was measured against a calibrated marked wall (accuracy ± 0.1 cm; Stadiometer). Infant weight was measured before breastfeeding using a calibrated electronic scale (accuracy ± 2 g; Medela Inc., McHenry, IL, USA). Infant crown-heel length was measured using non-stretch tape and a length board, headpiece and footpiece both applied perpendicularly to the hard surface (accuracy ± 0.1 cm). Infant head circumference was measured with a non-stretch tape (accuracy ± 0.1 cm). Maternal and infant body mass index (BMI) were calculated as kg/m^2 . Infant growth rate (weight-for-length z-score, weight-for-age z-score and length-for-age z-score) were calculated according to WHO standards [259].

8.3.6 Body composition measurements

Maternal body composition was assessed immediately before and at 1 week and 2 weeks after the start of the dietary intervention (weeks 2 and 3) and all measurements were performed after a breastfeeding session. At each time point, body composition was measured by 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 (fat mass, percentage fat mass and fat-free mass) were taken within 1–2 min and averaged for data analysis. Within-participant coefficient of variation for maternal percentage fat mass was 0.21%.

8.3.7 Human milk sample collection

Women were instructed to collect HM samples (~5 mL) each morning before the first morning feed and at least 2 hours after the previous feed daily throughout the study. In addition, women were also instructed to carry out intensive milk sampling once a week throughout the study. This involved collecting three HM samples: before the first feed in the morning, one in the afternoon and one in the evening for one 24-h period. As per the study protocol, the total number of HM samples collected across the study period for each woman was 54 samples (27 from each breast). For each sample collection, women were asked to collect pre-feed samples aseptically from both breasts either by hand expression or using a breast pump. Participants stored the samples in their home freezer until weekly collection by study staff. Samples were transported in cooler bags with ice to maintain low temperature until reaching the biochemical lab where samples were stored at -80°C for later analysis.

8.3.8 24-h HM production

Mothers measured milk production and milk intake using a standardised 24-h milk production protocol [260,261] at 2 time points in the study before (week 1) and after the dietary intervention (week 3). Briefly, they weighed their fully clothed infant on calibrated electronic scales (Medela Baby Scale, accurate to 2 g) before and after each feed from each breast for one 24-h period plus one additional breastfeed. Milk intake was calculated by subtracting the weight of the infant at the start of the feeding session from their weight at the end of the session and

amounts of HM (g) consumed by the infant were recorded for each feeding session. Milk samples were collected before and after every feed. Current feeding frequency of the infants was self-reported by mothers.

8.3.9 Calculated intake of human milk macronutrients

The infants' milk intake across a 24-h period and fat, protein and lactose concentrations in their mothers' milk were used to calculate the infant's average intake of each macronutrient from a breastfed. Average intakes were calculated by averaging the HM concentrations of each respective macronutrient measured in the right and left breasts across a 24-h period, multiplied by the corresponding 24-h milk intake (g) [79].

8.3.10 Sample preparation

All samples were thawed for 2-h at room temperature, mixed on Intelli-Mixer (RM-2, ELMI, Ltd, Riga, Latvia) using UU mode for 15s at 50rpm and, after 3 gentle top-to-bottom inversions, aliquoted into 1.5 mL microtubes (Sarstedt, Numbrecht, Germany) prior to analysis. Whole milk (500µl) samples were thawed and transferred into a homogeniser tube containing zirconium beads (3.0mm diameter, D1032-30, Benchmark Scientific, USA). The milk samples were homogenised for 3 cycles at 2500 rpm, 5 second per cycle with a 3 second pause using a microtube homogeniser (BeadBug 6, Benchmark Scientific, USA). Whole milk was used to measure fat, leptin, adiponectin and insulin concentrations and HM samples were defatted for measurement of protein and lactose concentrations. The standard assays were carried out using a JANUS workstation (PerkinElmer, Inc., Waltham, MA, USA) and measured on an EnSpire (PerkinElmer, Inc., Waltham, MA, USA).

8.3.11 Macronutrient measurements

Fat concentration of milk samples (including milk samples from the 24-h milk production) was measured by a modified creatocrit method [216]. Spun capillary-tubes were read on Creatocrit Plus™ device (Medela, AG) to determine the fat percentage. The read capillary-tubes were then cut, and the skim milk portion collected for the protein and lactose assay.

Protein concentration in the milk sample was measured by a modified Bradford method using a commercial protein reagent (Bio-Rad Laboratories, USA) [217]. Protein standards were prepared from an aliquot of HM and the protein concentration determined by the Kjeldahl method, as described by Atwood and Hartmann (1992) [218] and the protein assay as described by Mitoulas et al (2002) [169]. The recovery of a known amount of protein added to the milk samples was $101 \pm 2.1\%$ (n=6). The detection limit of this assay was 0.03g/L and the inter-assay CV was 18% (n=22).

Lactose concentration in the milk sample was determined by an enzymatic method [169,219]. The recovery of a known amount of lactose added to the milk samples was $99.3 \pm 1.6\%$ (n=6). The detection limit of this assay was 6g/L and the inter-assay CV was 10% (n=22).

8.3.12 Metabolic hormone measurements

The concentration of adiponectin in the homogenised milk sample was determined by the Biovendor human adiponectin ELISA kits (RD191023100, Lot E19-046, Biovendor-Laboratorni medicina a.s., Czech Republic). The recovery of a known amount of adiponectin added to the milk samples was $95 \pm 7\%$ (n=7). The detection limit of this assay was 0.61ng/ml.

The concentration of insulin in the homogenised milk sample was determined by the Biovendor human insulin ELISA kits (RIS006R, Lot X19-342, Biovendor-Laboratorni medicina a.s., Czech Republic). The recovery of a known amount of adiponectin added to the milk samples was $97 \pm 7\%$ ($n=7$). The detection limit of this assay was $4.88\mu\text{IU/ml}$.

The concentration of leptin in the homogenised milk sample was determined by the R & D systems leptin DuoSet ELISA kit (DY398, Lot P209801, R&D Systems Inc, USA). The recovery of a known amount of leptin added to the milk samples was $99 \pm 1.7\%$ ($n=7$). The detection limit of this assay was 0.19ng/ml .

8.3.13 Statistical analysis

The data was analysed by fitting a linear mixed effects model to each of the response variables: fat, protein, lactose, leptin, insulin and adiponectin concentrations. Day, collection breast (left or right), time of day, 24-h milk production, maternal and infant anthropometry and maternal body composition were input as explanatory variables for each of the respective models. In all models, the mothers were fitted as random effects as to account for the within mother variation. Model selection was carried out using backwards selection. All statistical analysis, including descriptive statistics, was computed using the statistical programming language R [220]. A p value of <0.05 was considered statistically significant.

8.4. Results

8.4.1 Study participants

Of the 66 women currently breastfeeding or expressing HM who initially expressed interest in participating and requested further information, 38 confirmed their interest and completed the eligibility assessment. Of the 18 who were eligible and enrolled in the study, 15 women

completed the study (Figure 8.2). Clinical and sociodemographic characteristics of the study participants (mothers and infants) are shown in Table 8.1. Study participants were predominantly of Caucasian background (n=13), with one woman identifying as Hispanic and one not providing information on ethnicity. Most participants had completed a University degree (n=12). The age of the infants at the time of study entry ranged from 1.6 to 4.9 months, and there were approximately equal numbers of males and females.

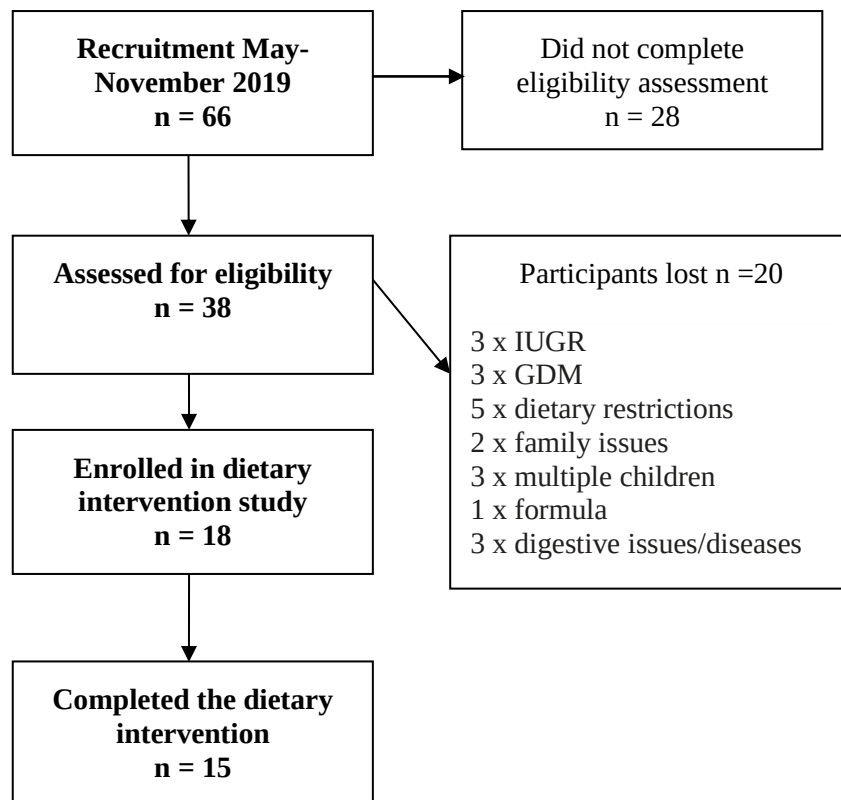


Figure 8.2. Flow chart of participants from recruitment to completion of study¹

¹GDM, gestational diabetes; IUGR, Intrauterine growth restriction

Table 8.1. Demographic characteristics of participating mothers and infants at start of dietary intervention study¹

Characteristics	Mean $\pm$ SD	Range
Mothers		
Age (years)	32 $\pm$ 3	27-37
Pre-pregnancy weight ² (kg)	69.4 $\pm$ 10.8	55-89
Pre-pregnancy BMI ² (kg/m ²)	25.1 $\pm$ 4.1	17.2-32.7
Current weight (kg)	71.4 $\pm$ 10.3	54.4-80.7
Current BMI (kg/m ²)	25.8 $\pm$ 4.1	17.1-33.0
Infants		
Age (months)	3.1 $\pm$ 0.8	1.6-4.9
Sex ² (M/F)	8/6	-
Birth weight ² (kg)	3.6 $\pm$ 0.4	2.9-4.3
Birth length ² (cm)	51.1 $\pm$ 1.7	48-53
Current weight (kg)	6.1 $\pm$ 1.0	4.6-7.6
Current length (cm)	60.3 $\pm$ 3.3	55.2-65.5

¹BMI, body mass index; F, female; M, male²Missing information from one participant

A total of 797 HM samples were provided and analysed in duplicate for each of the macronutrients. Eleven women provided a complete set of 54 HM samples and the remaining 4 women provided between 47 and 53 HM samples. Of the 797 HM samples, 257 were selected for the assessment of metabolic hormones (18 samples per participant with 13 samples missing).

8.4.2 Dietary intake before and during dietary intervention

The daily intakes of total energy and key macronutrients during the baseline week and dietary intervention period are presented in Table 8.2. Women consumed significantly less total energy (-32%), carbohydrate (-21%), sugar (-29%), fat (-54%) and saturated fat (-63%) during the dietary intervention phase compared to baseline. The percentage of energy for protein and carbohydrate were increased, and the percentage of energy for fat and saturated fat were decreased, while the intake of dietary fibre was unchanged.

Table 8.2. Overview of daily intake of key nutrients before and during the dietary intervention phase¹

	Baseline week²	Intervention phase	P value
Energy (kcal)	2525±579	1711±101	< 0.001
Protein (%en)	17±3	22±1	< 0.001
Protein (g)	105±24	93±7	0.055
Carbohydrate (%en)	40±5	47±2	< 0.001
Carbohydrate (g)	254±62	200±12	0.008
Fibre (g)	29±7	33±3	0.107
Total sugars (g)	116±45	82±5	0.016
Fat (%en)	40±6	27±2	< 0.001
Fat (g)	114±38	52±6	< 0.001
Saturated fat (%en)	14±9	9±1	0.044
Saturated fat (g)	46±17	17±2	< 0.001

¹Values are shown as mean ± standard deviation; Daily intake for the dietary intervention phase show values for all 14 days of the intervention. Percentage of energy (%en) is provided for protein, carbohydrate, fat and saturated fat

²Missing information from one participant

8.4.3 Effect of the dietary intervention on HM macronutrient and metabolic hormone composition

There were no changes in the concentrations of HM fat, protein and lactose in either the first or second week after commencement of the dietary intervention (Table 8.3).

The concentrations of insulin in HM were significantly lower during both weeks of the dietary intervention phase (-25%) compared to the baseline period. HM concentrations of leptin (-20%) and adiponectin (-10%) were both significantly lower at the end of the dietary intervention (week 3 of the study) compared to levels at baseline (Table 8.3).

Table 8.3. Overview of human milk macronutrient and metabolic hormone concentrations before and after dietary intervention¹

<i>Macronutrient</i>	Baseline period	Intervention phase		P value
	Week 1	Week 2	Week 3	
Fat (g/L)	34±13	34±16	35±14	0.63
Protein (g/L)	8±3	8±2	8±3	0.30
Lactose (g/L)	65±22	61±20	63±23	0.75
Leptin (ng/ml)	0.25±0.17 ^a	0.23±0.18 ^a	0.20±0.17 ^b	<0.0001
Insulin (μIU/ml)	28.70±20.79 ^a	18.91±12.28 ^b	21.49±13.88 ^b	<0.0001
Adiponectin (ng/ml)	12.63±6.44 ^a	11.80±5.24 ^a	11.35±5.56 ^b	0.048

¹Values are shown as mean ± standard deviationSuperscripts show values that are significantly different from each other ($p<0.05$).

8.4.4 Effect of the dietary intervention on HM production and intake of macronutrients

There was no significant effect of the dietary intervention on HM production or the dose of macronutrients consumed by the infant over time (Table 8.4).

Table 8.4. Overview of milk production before and after dietary intervention¹

<i>Milk production</i>	Baseline period	Intervention phase	P value
	Week 1	Week 3	
Total milk production (mL)	760±204	773±181	0.74
Fat dose (g)	24.8±8.1	26.6±8.0	0.30
Protein dose (g)	6.1±1.9	5.59±1.8	0.43
Lactose dose (g)	49.5±18.8	47.7±18.6	0.71

¹Values are shown as mean ± standard deviation

8.4.5 Effect of the dietary intervention on maternal anthropometrics and infant growth

Maternal body weight (-1.8%, $p=0.002$), BMI (-5.8%, $p=0.002$), fat mass (-6.3%, $p=0.007$) and fat mass to fat-free mass ratio (-6.6%, $p=0.031$) were all significantly lower at the end of the dietary intervention compared to baseline (Table 8.5). The average maternal BMI shifted from overweight during the baseline period to normal range after the dietary intervention phase. Infant weight, length and head circumference all increased significantly across the 2-week dietary intervention compared to baseline (Table 8.5). Infant rate of growth was stable across

the study (week 1 vs week 3) according to weight-for-length z-score (0.10 ± 0.88 vs 0.13 ± 0.96 , $p=0.83$), weight-for-age z-score (-0.17 ± 0.69 vs -0.14 ± 0.63 , $p=0.29$) and length-for-age z-score (-0.27 ± 0.99 vs -0.12 ± 0.56 , $p=0.08$).

Table 8.5. Maternal body composition and infant growth measurements across the study¹

Anthropometry and body composition	Baseline period	Intervention phase		PE ± SE ¹	<i>p</i>
	Week 1	Week 2 ²	Week 3		
<i>Mothers</i>					
Weight (kg)	71.4±10.3	70.6±10.3	70.1±9.8	-1.39±0.35	0.002
BMI (kg/m ²)	25.8±4.1	25.6±4.1	24.3±4.0	-0.50±0.13	0.002
Fat-free mass (kg)	44.2±4.7	45.2±4.6	44.5±4.0	0.32±0.58	0.60
Fat mass (kg)	27.2±7.1	25.4±6.7	25.5±6.5	-1.70±0.54	0.007
Fat mass (%)	37.6±5.4	35.5±5.0	35.9±4.9	-	-
Fat mass to fat-free mass ratio	0.61±0.1	0.56±0.1	0.57±0.1	-0.05±0.02	0.031
<i>Infants</i>					
Weight (kg)	6.1±1.0	6.3±1.0	6.5±1.0	0.43±0.05	<0.001
Length (cm)	60.3±3.3	61.1±3.0	61.9±3.1	1.53±0.15	<0.001
Head circumference (cm)	40.4±2.0	40.8±1.7	41.4±1.6	0.99±0.14	<0.001

¹Effects of predictors taken from univariate linear mixed effects models comparing baseline period (week 1) to end of intervention phase (week 3). PE, parameter estimate; SE, standard error.

²Missing information from one mother and her infant

8.4.6 Associations between HM composition and production at baseline and maternal body composition

There were no relationships between the concentrations of fat, protein or lactose in HM at baseline and maternal body weight, BMI, fat mass, fat-free mass or fat mass to fat-free mass ratio at this same time point (Table 8.6). There was no relationship between any maternal anthropometric measurements and HM production (Table 8.6).

Table 8.6. Associations between human milk macronutrient and metabolic hormone concentrations and maternal weight and body composition at baseline¹

Predictor	Fat (g/L)		Protein (g/L)		Lactose (g/L)		TMP	
	PE ± SE	<i>p</i>	PE ± SE	<i>p</i>	PE ± SE	<i>p</i>	PE ± SE	<i>p</i>
Weight (kg)	-0.20±0.16	0.23	-0.01±0.06	0.92	0.04±0.45	0.94	-2.59±4.70	0.59
BMI (kg/m ²)	-0.38±0.41	0.38	-0.00±0.15	0.99	0.51±1.11	0.65	-10.3±11.6	0.39
Fat-free mass (kg)	-0.30±0.36	0.42	-0.08±0.13	0.56	-0.62±0.97	0.53	8.28±9.50	0.40
Fat mass (kg)	-0.29±0.23	0.23	0.02±0.09	0.80	0.35±0.65	0.60	-8.66±6.21	0.18
Fat mass to fat-free mass ratio	-11.9±12.6	0.36	2.58±4.62	0.59	32.1±33	0.35	-542±284	0.08

¹Effects of predictors taken from univariate linear mixed effects models. PE, parameter estimate; SE, standard error

Leptin concentrations at baseline were positively associated with baseline maternal body weight, BMI, fat mass and fat mass to fat-free mass ratio (Table 8.7). There were no relationships between the concentrations of insulin and adiponectin in HM at baseline and maternal body weight, BMI, fat mass, fat-free mass or fat mass to fat-free mass ratio at this same time point.

Table 8.7. Associations between metabolic hormone concentrations and maternal weight and body composition at baseline¹

Predictor	Leptin (ng/ml)		Insulin (µIU/ml)		Adiponectin (ng/ml)	
	PE ± SE	<i>p</i>	PE ± SE	<i>p</i>	PE ± SE	<i>p</i>
Weight (kg)	0.01±0	<0.001	0.75±0.38	0.07	-0.02±0.16	0.88
BMI (kg/m ²)	0.03±0	<0.001	1.73±0.97	0.10	-0.39±0.38	0.33
Fat-free mass (kg)	0.01±0	0.12	1.09±0.90	0.24	0.13±0.35	0.71
Fat mass (kg)	0.02±0	<0.001	1.11±0.55	0.06	-0.11±0.23	0.64
Fat mass to fat-free mass ratio	0.98±0.18	<0.001	44.26±30.84	0.17	-10.41±11.90	0.40

¹Effects of predictors taken from univariate linear mixed effects models. PE, parameter estimate; SE, standard error.

8.4.7 Associations between infant growth measures and intake of macronutrients and 24-h HM production

Infant length (0.005, $p=0.049$) and head circumference (0.004, $p=0.022$) across all study weeks were positively correlated with 24-h milk production (parameter estimate, p value). Infant head circumference was the only measurement to be positively correlated with the amount of fat consumed by the infant (0.091, $p=0.026$). There was no association between any measures of infant growth and amounts of protein and lactose consumed over the study period.

8.4.8 Associations between baseline concentrations of macronutrients and metabolic hormones and baseline maternal dietary intake

Lactose concentrations in HM were inversely associated with maternal dietary intake of total energy, protein, total sugar, carbohydrate and fibre (Table 8.8). HM protein at baseline was inversely associated with maternal protein intakes.

Adiponectin concentrations in HM at baseline were positively associated with baseline maternal intakes of total energy and carbohydrate, while leptin concentrations in HM were positively associated with maternal intake of cholesterol and negatively associated with maternal intake of fibre.

Table 8.8. Associations between human milk macronutrient and metabolic hormone concentrations and maternal dietary intake at baseline¹

Predictor	PE ± SE	P	PE ± SE	p	PE ± SE	p
	Milk Fat (g/L)		Milk Protein (g/L)		Milk Lactose (g/L)	
Univariate models						
Energy (kcal)	-0±0	0.54	-0±0	0.08	-0.004±0	0.02
Protein (g)	-0.14±0.07	0.05	-0.06±0.02	0.01	-0.43±0.17	0.02
Carbohydrate (g)	0.02±0.03	0.60	-0.01±0.01	0.16	-0.18±0.06	0.01
Fibre (g)	-0.20±0.24	0.42	-0.013±0.08	0.15	-1.32±0.55	0.03
Total sugars (g)	0.02±0.04	0.57	-0.01±0.01	0.27	-0.24±0.09	0.02
Fat (g)	-0.04±0.05	0.41	-0.02±0.02	0.26	-0.18±0.12	0.16
Saturated fat (g)	-0.07±0.11	0.55	-0.06±0.03	0.09	-0.50±0.25	0.07
	Leptin (ng/ml)		Insulin (µIU/ml)		Adiponectin (ng/ml)	
Univariate models						
Energy (kcal)	0±0	0.90	-0.002±0	0.44	0.001±0	0.04
Protein (g)	0.003±0	0.13	0.09±0.20	0.66	0.09±0.07	0.21
Carbohydrate (g)	-0.0004±0	0.64	-0.08±0.07	0.29	0.06±0.02	0.03
Fibre (g)	-0.008±0	0.18	-0.26±0.64	0.69	0.41±0.21	0.07
Total sugars (g)	-0.0004±0	0.69	-0.06±0.10	0.60	0.05±0.04	0.18
Fat (g)	-0.0001±0	0.91	-0.07±0.12	0.56	0.06±0.04	0.17
Saturated fat (g)	0.0004±0	0.89	-0.21±0.28	0.46	0.16±0.09	0.12
Cholesterol (mg)	0.0006±0	0.02	0.01±0.03	0.75	-0.006±0.01	0.58
Multivariate model for leptin						
Fibre (g)	-0.009±0	0.04	-	-	-	-
Cholesterol (mg)	0.0007±0	0.01	-	-	-	-

¹Effects of predictors taken from univariate linear mixed effects models. PE, parameter estimate; SE, standard error. A series of multivariate models were fit in addition to the above univariate models (one multivariate model per hormone) and only data with statistical significance has been presented.

8.5 Discussion

The results of this study indicate that improving maternal dietary quality during lactation, by lowering intakes of energy, fat, saturated fat, carbohydrate and sugar for as little as 2 weeks, is associated with significant reductions in maternal body weight and fat mass, and in concentrations of the metabolic hormones leptin, insulin and adiponectin in HM. The dietary intervention did not, however, affect either the concentrations of fat, protein or lactose in HM, 24-h milk production or infant growth rate over the same 2-week period.

This study is the first to directly assess the impact of a dietary intervention specifically aimed at reducing saturated fat and sugar intake in lactating women on the composition of their milk. The lack of significant changes in the concentrations of fat, protein or lactose in the current study is consistent with the results of the only previous study to assess the impact of a low-fat, high-carbohydrate diet in lactating women, which also found no changes in HM macronutrient composition across an 8-day period [199]. Interestingly, that study showed that consuming a high-fat diet, low-carbohydrate diet for 8 days resulted in increased fat and energy content in HM in lactating women between 6 and 14 weeks postpartum, although HM sampling did not include fat concentrations from a 24-h collection. This aligns with data from an animal study in which feeding rat dams a high-fat, high-sugar diet during lactation is associated with increased fat content of the milk, but no changes in the levels of protein [254]. Thus, while higher maternal fat intakes may be associated with increased fat content in HM, the results of both the current and the previous human study described above suggest that reducing fat intake for a similar period is not associated with reductions in HM fat content [15]. The level of fat in HM is the primary determinant of its energy content [262], and it may be that the maintenance of HM fat content in response to decreased maternal fat intakes is a mechanism to maintain energy supply to the infant in the face of maternal nutrient restriction. This is supported by the results of studies in which women are subjected to intermittent fasting (e.g. during Ramadan) or periods of energy restriction, which indicate that fat content of HM is maintained even when maternal nutrient intake is significantly decreased [178,179,263,264].

In contrast to the macronutrients, HM concentrations of the metabolic hormones, leptin, insulin and adiponectin, were all decreased (by 10-25%) at the end of the dietary intervention

compared to baseline. To our knowledge, no previous studies have directly investigated the effects of a maternal dietary intervention aimed at reducing maternal fat and sugar intakes during lactation on the concentrations of metabolic hormones in HM. However, studies in lactating rats have reported that consumption of high-fat diets is associated with increased concentrations of leptin [265,266] and/or insulin [267] in the milk, but the effects of reducing fat intakes on HM composition have not been reported. In humans, dietary counselling in pregnant women, aimed at achieving fat and fibre intake aligned with dietary recommendations, has been associated with increased concentrations of adiponectin in colostrum, but effects on hormone concentrations in transitional and mature HM were not assessed [209]. Previous studies have shown that metabolic hormones in HM can be derived from both the synthesis in mammary epithelial cells and maternal circulation [72,87,240]. One possibility is that the decreases in metabolic hormones in HM in the current study were secondary to decrease in maternal circulating concentrations. In support of this, decreases in intake of energy and sugar, such as those in the current study, are associated with lower circulating glucose and insulin concentrations [268,269], both of which are directly related to insulin concentrations in mature HM [91]. Similarly, HM adiponectin and leptin concentrations are directly related to the levels of these respective hormones in the maternal circulation [17,78,86], and HM leptin has also been shown to be positively correlated to maternal body fat mass in both this and prior studies [19,22]. The relative contribution of reduced concentrations of metabolic hormones in the maternal circulation and reduced synthesis within the mammary gland to the reduced levels of insulin, leptin and adiponectin at the end of the dietary intervention in this study is, however, unclear. The long-term consequences of these changes on the infant is also of interest, given that metabolic hormones concentrations in HM in the early postpartum period have been related to

growth and fat deposition in the infant in the first few months after birth and to long-term risk of obesity [12,90].

We took the opportunity presented by the study to explore the relationship between maternal dietary intake and HM composition during the baseline period. While these analyses are exploratory, given the small sample size, it is nevertheless noteworthy that HM lactose concentrations were associated with a range of maternal dietary intakes, including maternal energy, protein, carbohydrate, sugar and fibre intakes, a result not detected in other studies. These findings were unexpected, given that lactose concentrations in HM are generally considered to be highly stable, since they are tightly regulated by the mammary gland [33]. There is evidence from previous studies, however, that lactose concentrations are positively related to infant milk intakes [21], and are also positively related to infant weight and fat mass between 3 and 12 months postpartum [11]. While studies evaluating relationships between maternal dietary intake and the concentrations of fat and protein in HM are limited, the existing study have only identified an association between maternal protein intake and protein concentrations in HM. A landmark study which assessed HM macronutrient composition in the first 12 months, reported that maternal protein intake was associated to HM fat after 16 weeks postpartum [38]. The authors commented that the physiological basis for this relationship were not well-documented. No associations between fat and protein intake and the concentrations of fat, protein or other nutrients in HM have been reported from other studies [25,188,270]. This is also the first study to report associations between maternal intake of specific dietary components, including energy, fibre and cholesterol, with the concentrations of leptin and adiponectin in HM. While associations between maternal dietary components and HM lactose, leptin and adiponectin are interesting findings, whether these relationships are direct,

or driven by effects of these dietary nutrients on other maternal metabolic pathways, remains to be determined.

Similar to HM macronutrient content, the findings of the current study suggest that a short-term reduction in energy and fat intake did not reduce 24-h milk production which aligns with the results of the study described earlier, in which women consumed a low-fat, high-carbohydrate or high-fat, low carbohydrate diet for 8 days [199]. As a result, the average amount of fat, protein and lactose supplied to the infant was not altered by the dietary intervention. This was reflected in the maintenance of infant growth across the dietary intervention period, and the positive association between 24-h milk production and infant growth supports the suggestion that HM milk intake is a critical driver of infant growth in infants who are exclusively breastfed [263]. Overall, these findings suggest that milk production, as well as HM macronutrient composition, is maintained in the face of a short-term reduction in maternal energy, fat and sugar intakes.

We are confident that compliance with the dietary intervention was high, as evidenced both by the dietary intake data collected from women before and during the intervention and the significant reductions in body weight (-1.8%) and body fat mass (-6%) that was observed over the study period. These results are consistent with a systematic review reporting that dietary interventions were effective for reducing body weight and fat mass in lactating women [271]. Importantly, the results of this study also add to this previous literature, by suggesting that women can safely reduce energy intakes during lactation without negative effects on milk production, HM macronutrient content or infant growth. It is important to note, however, that

the dietary intervention here was applied for just 2 weeks, and the effects of longer dietary interventions on milk composition and production remains to be determined.

The rising incidence of overweight and obesity has led to increasing interest in the impact of maternal obesity on the composition of HM. A recent systematic review and meta-analysis concluded that, out of the macronutrients, only fat content was higher in mature HM in women who were overweight and obese and was also positively correlated with maternal BMI and fat mass in the majority of studies [272]. We did not identify any associations between maternal BMI and HM fat content in the current study, however this may be due to the small sample size (n=15) and the relatively narrow range of maternal BMIs in the study population.

A major strength of this study is the use of a home-delivery service for the delivery of meals and snacks during the dietary intervention, which ensured a high degree of compliance with the dietary intervention. Another strength is the extensive sampling regimen, with daily collection of samples across the study period and from both breasts, in order that any rapid shifts in composition could be monitored. In addition, no women in the study sample had health conditions, such as diabetes and gestational diabetes, or were smokers, all of which have been shown to impact HM composition and could therefore have confounded the results [149,224]. While the study had many strengths, there were also limitations, the principal one being the small sample size and relatively short duration of the dietary intervention, and thus it is not possible to draw definitive conclusions as to the effect of longer durations of reduced fat and sugar intakes on HM composition, and further studies are needed.

8.6 Conclusion

In conclusion, the results of the study suggest that improving maternal dietary quality during lactation, by reducing energy, fat, carbohydrate and sugar intakes for a 2-week period, significantly reduced both maternal body weight and fat mass and reduced concentrations of leptin, insulin and adiponectin in HM, all of which have the potential to impact infant growth in the short-term and reduce the risk of obesity and poor metabolic health in the longer-term. HM macronutrient concentrations, milk production and infant growth were unaffected. While further studies are required to assess the impact of longer durations of maternal dietary interventions during lactation and their impact on infant health, these results suggest that there are no adverse impacts of a short duration of modest maternal energy restriction on milk production or macronutrient content. These findings should provide reassurance to women who seek to lose body weight and fat mass while breastfeeding, although further studies are required to assess any potential effects of the reduced leptin, adiponectin and insulin concentrations in HM on the growth and/or adiposity of the infant.

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Conflicts of interest: The authors declare that they have no competing interests.

Abbreviations:

Body mass index (BMI)

Human milk (HM)

Gestational diabetes (GDM)

Intrauterine growth restriction (IUGR)

CHAPTER 9. GENERAL DISCUSSION

9.1 Summary of Major Findings

Human milk (HM) contains numerous essential nutrients and bioactive factors known to promote infant health and survival [1,2]. The World Health Organization (WHO) recommends that infants should be exclusively breastfed until 6 months' postpartum, with continued breastfeeding to 24 months of age and beyond [3]. Concentrations of macronutrients and metabolic hormones in HM have important roles in regulating infant growth and fat deposition [11,13]. As a result, variations in these levels between women have the potential to influence the long-term health of the infants, including their risk of obesity and metabolic disease. Consequently, there has been increasing interest in understanding the factors which determine HM composition.

The major findings and outcomes of this thesis are detailed below and summarised in Figure 9.1:

- Systematic evaluation of different collection methodologies applied in studies around the world assessing HM composition indicated that fat and protein concentrations in HM varied between samples obtained using different collection methods (pre-feed, post-feed or full expression/pooling of pre and post-feed samples), within the same lactation stage, but there were no obvious differences in lactose concentrations (Chapter 2, Leghi et al., Systematic Reviews, 2018 and Chapter 3, Leghi et al., The Journal of Nutrition, 2020).

- On the basis of these findings, evidence-based recommendations as to the most appropriate HM sampling protocols to balance logistical requirements and participant burden (associated with sample collection aimed at obtaining compositional measures that were most closely aligned to the “gold standard” method) were developed (Chapter 3, Leghi et al., *The Journal of Nutrition*, 2020). It was concluded that the most appropriate alternative methods were: collection of pre- and post-feed samples for all feeds across a 24-hr period followed by collection of pre- and post-feed samples 3 times a day and collection of one pre- and post-feed samples at the same time point for all participants. The importance of reporting and standardising not only collection method, but also other factors that could potentially influence HM composition, including analytical method, infant gestational age and infant mode of feeding was also emphasised.
- Chapter 4 provided evidence that pre-feed samples collected at any point across a 3-week period and from either the left or right breast provided comparable measures of fat, protein and lactose concentrations in mature HM in an individual.
- Maternal BMI and adiposity measurements were associated with higher HM fat (mature HM) and lactose concentrations (HM colostrum), whereas protein concentration in HM did not differ between overweight and/or obese and normal weight women (Chapter 5, Leghi et al., *Nutrients*, 2020).
- In addition to changes in fat and lactose content, concentrations of leptin (all lactation stages) and insulin (transitional and mature HM) were higher in HM from overweight and obese women (Chapter 6).
- Systematic development of a clinical trial protocol to assess the impact of test meals varying in fat and sugar content on post-prandial concentrations of HM macronutrients and metabolic hormones (Chapter 7, Leghi et al., *Nutrition and Health*, 2020).

- Improving maternal dietary quality during lactation, by reducing intake of energy, fat and sugar for a 2-week period, was associated with reduced concentrations of leptin, insulin and adiponectin in HM, while macronutrient concentrations in HM and milk production were unaffected (Chapter 8).

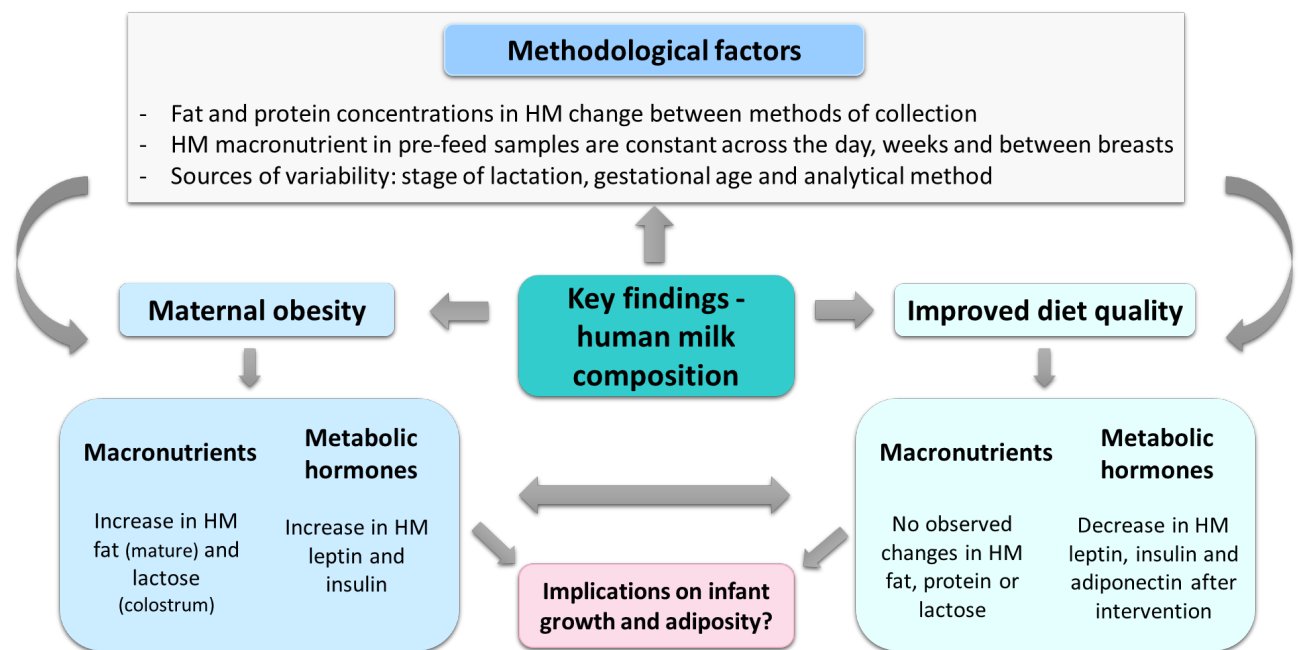


Figure 9.1. Summary of key thesis findings. The figure highlights key methodological factors responsible for changes in HM macronutrient content, including sampling method and approach. Maternal obesity and a dietary intervention aimed at reducing sugar and fat intakes were associated with shifts in HM fat and lactose and, HM leptin, insulin and adiponectin concentrations, respectively. There may be a relationship between HM changes as a result of maternal obesity and dietary intake as nutritionally poor diets and maternal overweight and obesity often coincide in lactating women. The implications on infants' short- and long-term health outcomes as a result of altered concentrations of macronutrients and metabolic hormones in HM require further elucidation.

9.2 Implications and Limitations

9.2.1 Sampling Methodology

The systematic review of collection methods used in HM research to date highlighted the complexity in developing evidence-based recommendations for sample collection and the overall poor quality of reporting in this field to date. The substantial variability between studies in other factors potentially impacting HM composition, including stage of lactation, gestational age and analytical method, was a major impediment to being able to undertake meaningful comparisons between collection methods. While we had expected there to be a certain degree of variability in study design and sampling approach, we were surprised by the degree of heterogeneity – and poor reporting of study detail. In addition to differences in collection methods and other factors likely to impact HM composition, the majority of studies identified in the systematic review were very small and are therefore unlikely to be representative of the general populations of the regions in which they were conducted. It was also noted that very few authors provided a rationale for selecting a particular HM collection method, leading to speculation that the selection was based on convenience or personal preference, rather than scientific evidence. One trend identified in the systematic review, however, was a shift away from the “gold standard” method of collecting full breast expressions at all feeds over a 24-h period in more recent studies. The reason underlying this change is unclear but may be due to growing focus on ethics of research and concerns over participant burden associated with full breast expression. In any case, this shift makes the development of a standardised collection method for use in HM studies even more urgent and relevant.

The implications of the findings and limitations highlighted above are that it is difficult, based on the current available data, to combine data on HM composition from different studies. This

is currently an impediment to being able to advance knowledge in the HM field, particularly when it comes to obtaining reliable information on contemporary HM composition. This is particularly important as the current data on HM composition is based on results from only a small number of women and was conducted prior to major shifts in typical dietary composition (especially fatty acid composition) of the Western diet, and therefore likely to be outdated. This has relevance to maternal and infant health, since HM provides the sole source of nutrition to the breastfed infant, and thus the level of macronutrients, micronutrients and bioactive factors is important for predicting infant health outcomes. In addition, HM composition is used as the basis for the development of alternate infant feeds, so accuracy is important. To address this knowledge gap, it is suggested that large population-based studies utilise the same recommended sampling methods (one of the three identified in this thesis), to be able to obtain reliable information on contemporary HM composition. Ultimately, if all studies utilised this same standardised sampling protocol, it would allow for the results of different studies to be combined and therefore accelerate global research in the field. It is also important to note, however, that the most appropriate sampling regimen may also depend on the research question being asked, the population being studied and the primary HM component of interest— so there may not be a “one size fits all” approach.

A key consideration in the design of any human studies, including those involving collection of HM, is to reduce participant burden. To this end, the work in the systematic review was extended to assess the impact of other aspects of HM sampling protocols on HM composition, including daily or weekly variations or differences between breasts. The findings presented in Chapter 4 indicated that there were no significant differences in the concentrations of fat, protein or lactose in pre-feed samples collected over a 3-week period either according to time

of day of collection, day, week or collection breast. This suggests that other factors, such as infant feeding pattern, time since last feed and breast fullness, are likely to be more important determinants of the levels of these HM components, particularly fat content. This information is particularly important for studies undertaken in the community, including those in remote settings, where women often collect their own HM samples and the capacity to control time of day and time in between feeds is limited.

While the results of this Chapter have relevance to the design of studies where the primary aim is to obtain data on HM macronutrient composition of an individual, it is important to highlight some limitations to this study. Firstly, it is not clear whether the same results would be obtained if women were using different collection methods, since only pre-feed samples were collected. A direct comparison of results obtained from pre-feed, mid-feed and post-feed samples was, therefore, also not possible and would be a valuable area for future investigation. In addition, it is unclear whether the same consistency in composition would also be observed for other HM components, including metabolic hormones or immune factors, and it is possible that more frequent sampling would be needed if the primary objective of a study was to measure these components. The generalisability of these findings is also unclear, since the trial in this thesis was restricted primarily to healthy Caucasian women from a developed country, and whether these same results apply to different ethnic, geographical and socio-economic groups is not known. While a large number of samples was collected from each woman across the study period, the number of participants was relatively small, and increasing the sample size would provide greater confidence that a less frequent sampling regimen is appropriate for obtaining a

relatively reliable estimate of the HM composition of an individual. It is also important to highlight that, since only pre-feed samples were collected, the compositional analyses are not likely to be fully representative of what the infant is receiving over the course of the day, and if this is the key objective of the study then an alternative collection method (ideally pooled pre- and post-feed samples in line with the findings of Chapter 3) may be required.

9.2.2 Impact of maternal overweight and obesity on HM composition

While there was evidence prior to this thesis that the concentrations of at least some components in HM are altered in women who are overweight and obese [19,193], the extent of these differences across the full complement of nutritional factors and metabolic hormones in HM was unclear. The systematic reviews reported in Chapters 5 and 6 successfully addressed these research gaps, and indicated that maternal overweight and obesity were associated with altered concentrations of fat, lactose, leptin and insulin in HM. Both systematic reviews also identified some other limitations in the area, including the relatively low quality of the evidence and limited number of studies eligible for inclusion in the quantitative synthesis. This highlights the heterogeneity present in studies assessing impact of maternal obesity on HM composition and the difficulty in synthesising and interpreting the current evidence and move to more robust conclusions. In addition, a number of studies did not control for the presence of maternal pre-existing conditions which could influence HM composition such as diabetes and gestational diabetes [149]. A particularly notable limitation to this area of research was that very few studies had attempted to examine relationships between HM composition and measures of maternal obesity beyond BMI. While BMI is a convenient measure, it does not always provide a reliable indicator of body fat mass [229,230], and therefore the use of more direct measures of

maternal adiposity may have produced different results. This is particularly relevant given that one study that assessed relationships between HM leptin and both maternal BMI and maternal percentage body fat, found that the relationship was notably stronger for fat mass [19]. In addition, obtaining information on both fat mass and the presence of comorbidities would help in understanding whether the differences in composition are likely due to maternal factors related to obesity (e.g. degree of adiposity/metabolic disturbances) vs other coincidental factors (e.g. diet).

While these results indicated that HM macronutrient and metabolic hormone concentrations are altered in women who are overweight or obese, they provide no insights into the underlying mechanisms. Given the association between feeding patterns and frequency with HM macronutrient composition [20,21], one possibility is that the compositional differences were due to differences in infant feeding patterns, feeding frequency and/or milk production in women who are overweight and obese. This is supported by findings that duration of any breastfeeding is significantly lower in obese women [272] which could be extrapolated to potential reduced feeding frequency as well. This is particularly relevant for the reported alterations in HM fat content since previous studies have shown that the fat content of HM increases as greater volumes of HM are removed from the breast [47,273], which could occur at an individual feed if the infant is feeding less frequently. Obese women have also been reported to experience greater difficulties in initiating breastfeeding and maintaining supply [272]. It may be, therefore, that the higher fat content in HM of overweight and obese women is a mechanism to maintain the supply of energy and essential lipids to the infant in response to lower milk production. Alternatively, it may be that maternal metabolic disturbances, in particular dyslipidemia and higher circulating lipid concentrations in overweight and obese women [237],

are major contributors to the higher fat content in their milk. Overweight and obesity are also often associated with maternal insulin resistance and hyperinsulinemia and higher circulating leptin, which is likely to be the primary determinant of the higher leptin and insulin levels in their milk, given that the major source of these metabolic hormones is thought to be the maternal circulation, rather than synthesis within the breast [17,78]. To improve understanding of the factors which underly the compositional changes in HM of women who are overweight and obese, further studies, which assess maternal fat mass, maternal circulating lipid and hormone levels, maternal metabolic status and factors associated with infant feeding (feeding frequency, milk production) in both lean and obese women are required.

Irrespective of the source of the differences in composition, an important implication of altered concentrations of HM components from women who are overweight and obese is the potential impact on the short- and long-term impacts of the growth, adiposity and obesity risk in the infants [13,90], and further studies investigating this will be important.

9.2.3 Impact of maternal dietary intake on HM composition

One possible modifiable factor that could affect HM composition is maternal diet. This is particularly relevant since obesity and nutritionally poor diets often coincide in lactating women, making it challenging to identify their relative contribution to HM composition [195]. Despite the fact that maternal diet during breastfeeding has often been cited as a factor impacting HM composition, a review of the existing literature made it clear that very few prior studies had directly assessed the effect of changes in maternal dietary intake on HM composition [27]. The proof-of-concept trial (Chapter 8) showed no effect of a 2-week dietary intervention that significantly reduced maternal fat, sugar and energy intakes on HM

macronutrient concentrations despite significant reductions in maternal body weight and fat mass over the same period. This suggests that fat, protein and lactose concentrations in HM, as primary determinant of its energy content, are maintained at constant levels in the face of the modest maternal energy restriction applied in the study; which may represent a mechanism to ensure that energy supply to the infant is maintained in the context of maternal nutrient restriction, at least over a short period. In contrast, HM concentrations of leptin, insulin and adiponectin were all significantly lower at the end of the dietary intervention.

The observed changes in HM composition from Chapters 5, 6 and 8 suggest that the levels of HM fat and lactose in obese women identified by the systematic review are more likely to be due to maternal factors such as metabolic health or changes in synthesis in the breast, which are not sensitive to short-term changes in maternal energy intake or body weight/fat mass. In contrast, concentrations of metabolic hormones in HM seem to be more responsive to short-term changes in either diet or weight loss, increasing the likelihood that they are secondary to changes in maternal circulating levels. In support of this, circulating leptin concentrations are directly correlated with maternal BMI and fat mass [77,78] and several studies have reported a direct correlation between the concentrations of leptin in HM with those in the maternal circulation [23,77,78].

While Chapter 8 presents an important proof-of-concept study, some limitations need to be noted, including the timeframe chosen for the interventional design. Thus, a 2-week dietary intervention and modest maternal weight loss may not be sufficient to produce changes in maternal nutritional reserves that could potentially affect macronutrient synthesis in the breast if they were maintained for longer. On the other hand, the design of the study, in which the

maternal dietary intervention coincided with decreases in maternal weight/fat mass, did not allow us to separate the effects of dietary changes from the effects of maternal body weight/fat mass loss, and acute feeding studies (such as the protocol described in Chapter 7) will be valuable to determine the effect of dietary factors on HM composition in the absence of changes to maternal weight/fat mass and to complement this research. Another limitation of this study was that we did not collect maternal blood for analysis, which would assist in confirming the hypothesis that the changes observed in the levels of these factors in the milk were secondary to changes in maternal circulating concentrations.

9.3 Directions for future research

Based on the key findings from this thesis, a number of recommendations for future research were identified to build upon current research and address limitations:

- The results presented in Chapter 3 provide research groups in this field with valuable guidance on the details required when designing and reporting studies, particularly in relation to the methodology used for HM collection. Future studies aimed to ensure accurate analysis of milk components with respect to infant outcomes should focus on reporting and standardisation of collection procedures, validation of analytical methodology and inclusion of HM production measures. Large population-based studies which utilise one of the recommended alternative sampling approaches identified in this thesis, and which capture information on other factors that could impact HM composition (e.g. infant sex, gestational age, etc.) will be important to obtain updated information on contemporary HM composition (a major knowledge gap in the field).

- Future studies should also comprehensively assess and control for the impact of maternal pre-existing conditions such as diabetes or gestational diabetes, which can also impact on HM composition [149].
- Findings presented in Chapter 4 imply that other factors, such as infant feeding pattern, time since last feed and breast fullness, are likely to be more important determinants of HM fat content than time of day of collection, which challenges the current dogma that fat content increases progressively across the course of the day. Further studies which capture detailed information on infant feeding behaviour, feeding frequency and milk production/volume, and which collect samples at different times of day and time relative to infant feeds, are required to establish the most important drivers of the variation in fat content of HM across lactation.
- The overall quality of the evidence assessing the impact of maternal obesity on macronutrient and metabolic hormone levels in HM is relatively low and more high-quality studies are needed. As indicated above, studies that both assess maternal adiposity directly (e.g. by DXA or BIS), and capture information on maternal metabolic status, circulating lipid and hormone concentrations and infant feeding behaviour/milk production are required to not only confirm the effects of maternal overweight and obesity on HM composition but to also determine the underlying mechanisms.
- Further research, in particular longitudinal cohort studies, investigating the implications of altered HM fat, lactose, leptin and insulin concentrations on growth and fat deposition during the first few months of infancy, and long-term BMI and adiposity would be a valuable addition to the field. This information would further assist maternal and infant clinical management and public health policies.

- The source of the changes in the concentrations of leptin, insulin and adiponectin in HM after a dietary intervention should be investigated. Future studies in which maternal blood samples are collected and maternal circulating levels of these hormones measured before, during and after a dietary intervention would confirm whether these shifts in HM metabolic hormone concentrations are secondary changes in maternal circulating concentrations. Future research is also required to determine any potential short and long-term impacts of the observed reduced leptin, adiponectin and insulin concentrations in HM.
- In addition to this, further studies focussed on the effect of dietary interventions in breastfeeding women on HM composition should expand the assessment to a wide range of HM components, including key adipokines and hormones, inflammatory substances, growth factors, human milk oligosaccharides and microbiome.
- Further studies are also required to assess the impact of longer durations of maternal dietary interventions aimed at improving diet during lactation on HM composition and production, maternal weight and body composition and infant growth.

9.4 Concluding remarks

In summary, this thesis has made a significant contribution to the field of HM research, by successfully providing: evidence-based recommendations as to a HM sampling protocol to enhance replicability and consistency between studies; advanced understanding and knowledge as to the contribution of maternal overweight/obesity and dietary factors to the concentrations of macronutrients and metabolic hormones in HM; and novel direct evidence of the impact of improved maternal diet quality on HM composition and production. The proposed recommendations on directions for future research build on the novel and significant findings from this thesis.

A better understanding of contemporary HM composition is critical given the established importance of nutritional exposures in early infancy for an individual's life-long health outcomes [13]. This is particularly important in light of the increasing number of pregnant and lactating women who are overweight or obese, the increasing consumption of nutritionally poor diets and the potential for HM to be indirectly contributing to an intergenerational cycle of obesity. Overall, aligned with previous results from Chapters 5 and 6, the findings from Chapter 8 highlight the importance of dietary quality while breastfeeding on HM metabolic hormone composition, and ultimately, the delivery to a more targeted dietary advice to lactating women. These data are extremely relevant to inform and guide public health and nutrition state and federal programs and policies with the ultimate goal to maximise maternal and infant health outcomes as women can improve their diet to positively effect BMI without adverse milk effects.

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APPENDICES

Appendix 1. PRISMA-P Checklist - Chapter 2

Additional file 1 in reference from publication

Section/topic	#	Checklist item	Information reported		Comments
			Yes	No	
ADMINISTRATIVE INFORMATION					
Title					
Identification	1a	Identify the report as a protocol of a systematic review	☒	☐	
Update	1b	If the protocol is for an update of a previous systematic review, identify as such	☐	☒	N/A
Registration	2	If registered, provide the name of the registry (e.g., PROSPERO) and registration number in the Abstract	☒	☐	
Authors					
Contact	3a	Provide name, institutional affiliation, and e-mail address of all protocol authors; provide physical mailing address of corresponding author	☒	☐	
Contributions	3b	Describe contributions of protocol authors and identify the guarantor of the review	☒	☐	
Amendments	4	If the protocol represents an amendment of a previously completed or published protocol, identify as such and list changes; otherwise, state plan for documenting important protocol amendments	☐	☒	N/A
Support					
Sources	5a	Indicate sources of financial or other support for the review	☒	☐	
Sponsor	5b	Provide name for the review funder and/or sponsor	☒	☐	

Appendices

Section/topic	#	Checklist item	Information reported		Comments
			Yes	No	
Role of sponsor/funder	5c	Describe roles of funder(s), sponsor(s), and/or institution(s), if any, in developing the protocol	☐	☒	N/A
INTRODUCTION					
Rationale	6	Describe the rationale for the review in the context of what is already known	☒	☐	
Objectives	7	Provide an explicit statement of the question(s) the review will address with reference to participants, interventions, comparators, and outcomes (PICO)	☒	☐	
METHODS					
Eligibility criteria	8	Specify the study characteristics (e.g., PICO, study design, setting, time frame) and report characteristics (e.g., years considered, language, publication status) to be used as criteria for eligibility for the review	☒	☐	
Information sources	9	Describe all intended information sources (e.g., electronic databases, contact with study authors, trial registers, or other grey literature sources) with planned dates of coverage	☒	☐	
Search strategy	10	Present draft of search strategy to be used for at least one electronic database, including planned limits, such that it could be repeated	☒	☐	
STUDY RECORDS					
Data management	11a	Describe the mechanism(s) that will be used to manage records and data throughout the review	☒	☐	
Selection process	11b	State the process that will be used for selecting studies (e.g., two independent reviewers) through each phase of the review (i.e., screening, eligibility, and inclusion in meta-analysis)	☒	☐	
Data collection process	11c	Describe planned method of extracting data from reports (e.g., piloting forms, done independently, in duplicate), any processes for obtaining and confirming data from investigators	☒	☐	
Data items	12	List and define all variables for which data will be sought (e.g., PICO items, funding sources), any pre-planned data assumptions and simplifications	☒	☐	

Appendices

Section/topic	#	Checklist item	Information reported		Comments
			Yes	No	
Outcomes and prioritization	13	List and define all outcomes for which data will be sought, including prioritization of main and additional outcomes, with rationale	☒	☐	
Risk of bias in individual studies	14	Describe anticipated methods for assessing risk of bias of individual studies, including whether this will be done at the outcome or study level, or both; state how this information will be used in data synthesis	☒	☐	
DATA					
Synthesis	15a	Describe criteria under which study data will be quantitatively synthesized	☐	☒	N/A
	15b	If data are appropriate for quantitative synthesis, describe planned summary measures, methods of handling data, and methods of combining data from studies, including any planned exploration of consistency (e.g., I^2 , Kendall's tau)	☐	☒	N/A
	15c	Describe any proposed additional analyses (e.g., sensitivity or subgroup analyses, meta-regression)	☐	☒	N/A
	15d	If quantitative synthesis is not appropriate, describe the type of summary planned	☒	☐	
Meta-bias(es)	16	Specify any planned assessment of meta-bias(es) (e.g., publication bias across studies, selective reporting within studies)	☒	☐	We will attempt to summarise The Newcastle-Ottawa Scale across studies
Confidence in cumulative evidence	17	Describe how the strength of the body of evidence will be assessed (e.g., GRADE)	☒	☐	

Appendix 2. Search Strategy Example for PubMed – Chapter 2

Additional file 2 in reference from publication

	AND		AND		AND		NOT		NOT	
Breast milk* [ALL]		Breast feeding [MH]		Macronutrient* [ALL]		English[lang]		Animals [MH] NOT Humans [MH]		HIV [MH]
Breastmilk* [ALL]		Breast milk express* [ALL]		Lactose [ALL]						pollutant [ALL]
Milk, Human [MH]		Breastmilk express* [ALL]		Proteins [MH]						
Human milk* [ALL]				Protein [ALL]						
Lactation” [ALL]				Proteins [ALL]						
				Lipids [MH]						
				Lipid [ALL]						
				Lipids [ALL]						

(((((Breast milk* [ALL] OR Breastmilk* [ALL] OR Milk, Human [MH] OR Human milk* [ALL] OR “Lactation” [ALL]) AND (Breast feeding [MH] OR Breast milk express* [ALL] OR Breastmilk express* [ALL]) AND (Macronutrient* [ALL] OR Lactose [ALL] OR Proteins [MH] OR Protein [ALL] OR Proteins [ALL] OR Lipids [MH] OR Lipid [ALL] OR Lipids [ALL]) AND English[lang] NOT (Animals [MH] NOT Humans [MH]) NOT (HIV [MH] OR pollutant [ALL]))))))))

Filters: “Humans” (species); “English” (languages)

Appendix 3. Search Strategy Example for MEDLINE/PubMed – Chapter 3

Supplementary Table 1 in reference from publication

	AND		AND		AND		NOT		NOT	
Breast milk* [ALL]				Macronutrient* [ALL]						
Breastmilk* [ALL]		Breast feeding [MH]		Lactose [ALL]						
Milk, Human [MH]		Breast milk express* [ALL]		Proteins [MH]						
Human milk* [ALL]		Breastmilk express* [ALL]		Protein [ALL]		English[lang]		Animals [MH] NOT Humans [MH]		HIV [MH]
Lactation” [ALL]				Proteins [ALL]						pollutant [ALL]
				Lipids [MH]						
				Lipid [ALL]						
				Lipids [ALL]						

(((((Breast milk* [ALL] OR Breastmilk* [ALL] OR Milk, Human [MH] OR Human milk* [ALL] OR “Lactation” [ALL]) AND (Breast feeding [MH] OR Breast milk express* [ALL] OR Breastmilk express* [ALL]) AND (Macronutrient* [ALL] OR Lactose [ALL] OR Proteins [MH] OR Protein [ALL] OR Proteins [ALL] OR Lipids [MH] OR Lipid [ALL] OR Lipids [ALL]) AND English[lang] NOT (Animals [MH] NOT Humans [MH]) NOT (HIV [MH] OR pollutant [ALL]))))))))

Filters: “Humans” (species); “English” (languages)

Appendix 4. Reasons for exclusion of studies at time of data extraction – Chapter 3

Supplementary Table 2 in reference from publication

Reference of excluded articles	Reasons for exclusion
Agostoni et al., 2001 (1)	Same data reported as Agostoni et al., 2003
Antonakou et al., 2011 (2)	Same data reported as Antonakou et al., 2013
Jackson et al., 1988 (3)	Same data reported as Jackson et al., 1988
Mitoulas et al., 2003 (4)	Same data reported as Mitoulas et al., 2002
Quinn et al., 2011 (5)	Thesis from published study Quinn et al., 2012
Quinn et al., 2012 (6)	Same data reported as Quinn et al., 2013
Verheul et al., 1986 (7)	Methodology paper and results are not separated according to different methods of sample collection
Williams et al., 2016 (8)	Thesis from published study Williams et al., 2017

Supplemental References

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2. Antonakou A, Chiou A, Andrikopoulos NK, Bakoula C, Matalas A-L. Breast milk tocopherol content during the first six months in exclusively breastfeeding Greek women. *Eur J Nutr.* 2011;50:195-202.
3. Jackson DA, Imong SM, Silprasert A, Preunglumpoo S, Leelapat P, Yootabootr Y, Amatayakul K, Baum J. Estimation of 24 h breast-milk fat concentration and fat intake in rural northern Thailand. *Br J Nutr.* 1988;59:365-71.
4. Mitoulas LR, Gurrin LC, Doherty DA, Sherriff JL, Hartmann PE. Infant intake of fatty acids from human milk over the first year of lactation. *Br J Nutr.* 2003;90:979-86.
5. Quinn EA. Life Course Influences on Milk Composition among Filipino Women Living in Cebu, Philippines; 2011.
6. Quinn EA, Largado F, Power M, Kuzawa CW. Predictors of breast milk macronutrient composition in Filipino mothers. *Am J Hum Biol.* 2012;24:533-40.
7. Verheul F, vd Bosch M, Cornelissen P, Waelkens J. Simplified and rapid methods for the determination of protein, fat and lactose in human milk and the energy intake by the breast-fed infant. *Clin Chem Lab Med.* 1986;24:341-6.
8. Williams JE. Of milk and microbes: The interplay of milk cells, macronutrients, maternal diet, and various microbiomes of the mother-infant dyad; 2016.

Appendix 5. Description of study population and methodological features of studies measuring fat concentrations in human milk – Chapter 3

Supplementary Table 3 in reference from publication

Reference	Gestational age	Feeding mode	Time of day	Hand expression vs Pump	Time since last feed	Breast sampled	Samples collected per participant	Analytical method	Milk concentration, g/L
Milk samples collected across 24 h									
Full expression across 24 h									
<u>Anderson et al., 1983 (1)</u>	Preterm and term	BF	24 h	Pump	-	-	3 x 24 h collection	Lipid extraction	3d: Term 18 ±6/Preterm 16±7 7d: Term 31±10/Preterm 35±9 14d: Term 37±10/Preterm 39±8
Boutte et al., 1985 (2)	-	Excl. BF	24 h	Electric/ manual pump	-	Both	1 x 24 h collection	Roese-Gottlieb extraction	Time of collection - Egnell electric pump: 33.5±5.1 Medela manual pump: 34.2±9.6
Brown et al., 1982 (3)	-	-	24 h	Electric pump	-	Both	1 x 24 h collection	Gravimetric	Time of collection - Left 20.5±7.8 Right 22.7±7.6

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Brown et al., 1986 (4)	-	Excl. BF and BF	24 h	Electric pump	-	Both	1 x 24 h collection	Gravimetric	0mo: 31.5±3.6 1mo: 31.2±5.3 2mo: 29.9±5.4 3mo: 28.0±6 4mo: 27.2±3.4 5mo: 25.3±4.3 6mo: 26.6±4.6 7mo: 24.8±4.6 8mo: 27.6±8.1 9mo: 23.0±2.5
<u>Butte et al., 1984a</u> (5)	Term	Excl. BF	24 h	Electric pump	-	Both	1x 24 h collection	Roese-Gottlieb extraction	1mo: 36.2±7.5 2mo: 34.4±6.8 3mo: 32.2±7.8 4mo: 34.8±10.8
<u>Heinig et al., 1993</u> (6)	Term	Excl. BF, BF	24 h	-	-	Both	4 x 24 h collection	Folch extraction	3, 6, 9 and 12mo: 37
<u>Lovelady et al., 1990</u> (7)	Term	Excl. BF	24 h	Electric pump	-	Both	1 x 24 h collection	Folch extraction	9 to 24wk Exercising: 25.5±5.7 Control: 40±2.4
<u>McCrory et al., 1999</u> (8)	Term	Excl. BF	24 h	Electric pump	-	Opposite breast	2 x 24 h collection	Folch extraction	8-16wk Control: baseline 34.1±6 intervention 35.3±8.3

Appendices

									Diet: baseline 35.4±7.4 intervention 36.2±8 Diet + exercise: baseline 37.5±9.3; intervention 35.6±6.1
<u>Motil et al., 1997 (9)</u>	Term	Excl. BF (initially)	24 h	-	-	Opposite breast	1 x 24 h collection	Jeejeebhoy extraction	Adolescents 6mo: 34.5±5.7 12mo: 36.1±7.9 18mo: 33.3±10 24mo: 36.8±13.6 Adults 6mo: 33.7±10.3 12mo: 34.8±8.6 18mo: 39.7±8.6 24mo: 35.7±9.4
<u>Perez-Escamilla et al., 1995 (10)</u>	Term	Excl. BF and BF	24 h	Egnell pump	-	Both	3 x 24 h collection	Folch extraction	4mo: 39±8 5mo: 40±8 6mo: 40±8
Stafford et al., 1994 (11)	-	-	24 h	Egnell pump	2hr	Left breast (non-feeding breast)	1 x 24 h collection	Folch extraction	Time of collection - Mean 24 h: 35.7±6.5
Stellwagen et al., 2013 (12)	Preterm	Excl. BF	24 h	Electric pump	-	-	4 x 24 h collection	Near-infrared spectrophotometer	Time of collection - Pooled all 24 h samples: 38.9±7

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									Individual samples (each feed): 40.3±9.2 All: 39.5±8.7
<u>Stuff et al., 1989</u> (13)	Term	Excl. BF and BF	24 h	Electric pump	-	Both (alternating per feed)	1 x 24 h collection	Gravimetric	16wk: 29.6±7.6 20wk: 32.4±7.4 24wk: 32.1±9.8 28wk: 33.9±12.3 32wk: 33.0±12.5 36wk: 35.9±11.
Pre- and post-feed across 24 h									
<u>Cannon et al., 2015</u> (14)	Term	Excl. BF	24 h	Hand	-	Both	-	Creamatocrit	3-21wk Pre-feed: 31.4±12 Post-feed: 57±20.2
Daly et al., 1993 (15)	-	BF	24 h	-	-	Both	-	Esterified fatty acid assay	4-9mo Pre-feed: 32±15 Post-feed: 83±25
<u>Jackson et al., 1988</u> (16)	Term	Excl. BF and BF	24 h	Hand	-	Same breast as infant fed	-	Creamatocrit	3-36wk Mean 24 h: 34±7 Waking up values: 34.4±7.5 Sleep period: 31.4±6.6
<u>Kent et al., 2006</u> (17)	Term	Excl. BF	24 h	Hand	-	Both	-	Creamatocrit	1-6mo: 41.1±7.8

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<u>Khan et al., 2013</u> (18)	Term	Excl. BF	24 h	Hand or manual pump	-	Both	-	Creamatocrit	1-6mo: 43.2±11.8
<u>Mitoulas et al, 2002</u> (19)	Term	Excl. BF and BF	24 h	Hand or manual pump	-	Both	-	Colorimetric	1mo: 39.9±1.4 2mo: 35.2±1.4 4mo: 35.4±1.4 6mo: 37.3±1.4 9mo: 40.7±1.7 12mo: 40.9±3.3 1-12mo: 37.4±0.6
<u>Perrella et al., 2016</u> (20)	Term	Excl. BF	24 h	Hand	-	Same breast as infant fed	-	Creamatocrit	4mo: 41.1 (22.3-61.6)
Prentice et al., 1981a (21)	-	-	24 h	Hand	-	Both	-	Creamatocrit	1-18mo 19:00-23:00: 32.3 23:00-03:00: 33.2 03:00-07:00: 37.8 07:00-11:00: 39.2 11:00-15:00: 34.4 15:00-19:00: 36.5 All: 35.6
Saint et al., 1986 (22)	Term	Excl. BF and BF	24 h	Hand	-	Both	-	Enzymatic	2-12mo Twi-: 20.5±6.3 right breast, 22.3±5.9 left breast

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									Triplets: 16.5±6.1 right breast, 15.4±6.6 left breast
Valentine et al., 1994 (23)	-	-	24 h	Double breast pump	-	-	-	Gravimetric	1-4wk Pre-feed: 28.6±8.1 Post-feed: 47.8±8.5 24 h pooled composite milk: 39.6±7.3
Post-feed across 24 h									
<u>Agostoni et al., 2001 (24)</u>	Term	Excl. BF and BF	24 h	-	-	-	6 x 24 h collection	Folch extraction	1d: 16.6±9.2 1mo: 45.5±20.8 3mo: 42.8±18.8 6mo: 43.1±29.2 9mo: 39.6±19.4 12mo: 46.4±31.3
<u>Agostoni et al., 2003 (25)</u>	Term	BF	24 h	-	-	-	3 x 24 h collection	Gravimetric	1d: Smoker 17.1±9.8 Non-smoker: 14.3±6.2 1mo: Smoker: 36.4±16.3 Non-smoker: 45.4±21.3 3mo: Smoker: 42.6±21.5 Non-smoker: 43±20.5 6mo: Smoker: 37.5±21.2 Non-smoker: 47.3±28.4

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<u>Bauer et al., 2011 (26)</u>	Preterm and term	-	24 h	Electric pump	-	-	8 x 24 h collection	Creamatocrit	1, 2, 3, 4, 5, 6, 7 and 8wk Extremely preterm (<28wk): 44±9 Severely preterm (28-31wk): 44±8 Moderately preterm (32-33wk): 48±10 Term: 41±7
<u>Marangoni et al., 2000 (27)</u>	Term	-	24 h	-	-	-	6 x 24 h collection	Gravimetric	1d: 23.5 1mo: 44.4 3mo: 47.8 6mo: 45 9mo: 43 12mo: 47.1
<u>Marangoni et al., 2002 (28)</u>	Term	-	24 h	-	-	-	2 x 24 h collection	Folch extraction	1d: 15.8 3mo: 47.6
Morton et al., 2012 (29)	Preterm	-	24 h	Hand and electric pump	-	Both	8 x 24 h collection	Esterified fatty acid assay	1wk: 51.8±20.3 2wk: 60.8±23.5 3wk: 63.1±16 4wk: 65.2±16.6 5wk: 62.8±18.8 6wk: 64.3±17.2 7wk: 63.2±21.8

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									8wk: 57.9±19.7
Milk samples collected at one time point									
Full expression									
<u>Aksit et al., 2002</u> ^{*3} (30)	Term	Excl. BF	9:30-10:30	Manual	At least 2 h	Both	10	Creamatocrit	2mo Beginning: 15.4 1stmin: 27.8 5thmin: 56.8 10thmin: 71 End: 77.5
Barbosa et al., 1997 (31)	Term	-	10:00, 14:00 and 18:00	Egnell pump	2 h	One	6	Jeejeebhoy extraction	BMI <23 3mo: 31.8±9 6mo: 29.1±8.7 BMI ≥23 3mo: 36.7±7.6 6mo: 35.7±9.2
<u>Butte et al., 1984b</u> (32)	Preterm and term	-	8:00-12:00	Egnell pump	At least 2 h	One	6	Roese-Gottlieb extraction	Preterm overall: 39.2±12.5 2wk: 34.5±9.4 4wk: 39.5±12.4 6wk: 39.6±11.8 8wk: 41.2±12.8 10wk: 42.2±13.3

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									12wk: 38.2±14.5 Term overall: 43.1±11.5 2wk: 37.9±10 4wk: 40.4±7.8 6wk: 45.0±11.8 8wk: 47.7±10.6 10wk: 45.1±11.7 12wk: 42.4±15.7
Butte et al., 1992 (33)	Term	-	10:00, 14:00 and 18:00	Electric pump	2 h	One	6	Jeejeebhoy extraction	4mo: 23.4±6.9 6mo: 21.1±6.6
De Pee et al., 1997 (34)	-	-	8:00-11:00	Manual pump	At least 1 h	One	1	Creamatocrit	3-6mo: 29.1±12.2 7-9mo: 35.7±15 10-12mo: 34.5±18.4 13-18mo: 35.8±18
Dewey et al., 1983 (35)	-	BF	2 nd feeding in the morning	Hand	-	One	1	Colorimetric	1mo: 49.2±10.5 2mo: 45.8±9.7 3mo: 45.8±16.5 4mo: 46.2±18.6 5mo: 43.6±16.7 6mo: 43±19.6
Foda et al., 2004 (36)	-	-	Morning	Hand	-	-	4	Folch extraction	Before 3mo

Appendices

									Pre-massage: 35±4 Post-massage: 36±7 After 3mo Pre-massage: 37±4 Post-massage: 45±3
Heon et al., 2016 (37)	Preterm	Excl. BF	Morning	Double electric Medela	-	-	3	Triglyceride enzymatic kit	1, 3 and 6wk Standard care: from 27.1 ± 7.4 to 35.1 ± 10.3 Expression support group: from 24.4 ± 7.3 to 37.1 ± 10.7
Jackson et al., 1994 (38)	Preterm and term	-	Morning	Egnell pump	1 h	Both	12	Folch extraction	IDDM 2d: 8.1±5.0 3d: 14.3±3 7d: 29.7±1.9 14d: 40±2.3 42d: 42.5±2.4 84d: 40.5±2.7 Control 2d: 21.5±3.4 3d: 18.4±2.5 7d: 29.6±2 14d: 38.3±2.3

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									42d: 38.8±2.2 84d: 38.1±2.7 Reference 2d: 11.7±4.3 3d: 23.1±3 7d: 34.8±3 14d: 38.1±3 42d: 39.5±3 84d: 39.3±3.2
<u>Marquis et al., 2003</u> (39)	Term	-	7:00-12:00	Electric pump	2 h	One	2	Creamatocrit	Breastfeeding during pregnancy 2d: 23.5±1.2 1mo: 33.2±1.4 No breastfeeding during pregnancy 2d: 24.9±1.1 1mo: 34.6±1.6
Masters et al., 2002 (40)	-	-	13:00-15:00	Egnell pump	At least 2 h	One	3	Folch extraction	1-12mo Placebo: 30 Washout: 25.9 CLA: 22.7

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Moran-Lev et al., 2015 (41)	Preterm	BF	6:00-9:00 and 21:00-00:00	Breast pump	Overnight for morning sample and 3 h for afternoon	Both	28	MIRIS milk analyzer	1, 2, 3, 4, 5, 6 and 7wk Morning: 33.8±10.2 Evening: 37.5±12.8
Mosley et al., 2007 (42)	-	BF	13:00-15:00	Electric pump	At least 2 h	One (same breast)	7	Folch extraction	6-10mo Placebo: 45 2g CLA: 48 4g CLA: 42
Park et al., 1999 (43)	-	-	15:00-22:00	Electric pump	-	Both or one breast	6	Lipid extraction	1-26mo Low dairy period: 38.3±1.6 High dairy period: 45.6±5
<u>Perrin et al., 2015 (44)</u>	Term	BF	Morning	Pump	-	-	8	Fat Analyzer (CEM Corporation)	11mo: 40±19.6 12mo: 39.4±19.9 13mo: 45.5±18.8 14mo: 54.3±25 15mo: 42.8±26.8 16mo: 39±18.5 17mo: 45.5±20.6 All: 41.5±21
Ritzenthaler et al., 2005 (45)	-	-	Morning	Electric pump	2 h	Same breast	4	Folch extraction	1-10mo: 33±4

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Sauer et al., 2017 (46)	Preterm and term	-	Morning and evening	Electric pump	At least 3 h	One	5	Mojonnier extraction	1, 7, 14 and 21d Preterm: 34.8±10.5 Term: 26.7±4.6 Post-term: 31.9±15 All: 32.6±11.7
<u>Szlagatys-Sidorkiewicz et al., 2013 (47)</u>	Term	Excl.BF	Morning	Electric pump	2 h	-	1	Roese-Gottlieb extraction	17-30d Smoker: 27.8±15.6 Non-smoker: 30±15.4
Williams et al., 2017 (48)	-	-	7:00-11:00	Electric pump	At least 2 h	Same breast	5	Lipid extraction	Time of collection -: 46±2
<u>Young et al., 2017 (49)</u>	Term	Excl. BF	10:00-13:00	Electric pump	-	-	2	Creamatocrit	2wk All: 36±12 Normal weight: 38±12 Overweight/obese: 34±13 4mo All: 34±13 Normal weight: 32±13 Overweight/obese:37±13
Pre-and post-feed									
<u>Da Cunha et al., 2005 (50)</u>	Term	Excl. BF	Morning	Hand	-	-	2	Creamatocrit	15d Pre-feed: 41±16 Post-feed: 54±16

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									All: 48±12
Fornes et al., 1995 (51)	-	Excl. BF	9:00-11:00	Hand	-	-	12	Creamatocrit	15d: 41.7±18.4 30d: 48.3±15.6 45d: 43.8±21.5 60d: 36.4±17.9 75d: 42.1±25.6 90d: 45.4±20.5
<u>Gridneva et al., 2017</u> (52)	Term	-	9:30-11:30	Hand or pump	-	Feeding breast	2	Creamatocrit	2 and 5mo Mean: 42.7±12.1
<u>Grote et al., 2016</u> (53)	Term	BF	Morning	Hand	-	Feeding breast	24	Folch extraction	1mo: 32±12.7 2mo: 31.6±11.8 3mo: 29.2±12.3 6mo: 27.1±12.5
Hahn et al., 2018 (54)	-	Excl. BF	14:00-15:00	-	-	-	2	MIRIS milk analyzer	4wk 20s/Normal BMI: 39±5 20 s/ Overweight BMI: 32±8 30s/Normal BMI: 21±2 30 s/ Overweight BMI: 27±4
Hassiotou et al., 2013 (55)	-	-	Morning	Hand or electric pump	At least 3 h	Both	24–64	Creamatocrit	6-39wk Pooled Feeding breast: 48.7±18.1

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									Non-feeding breast: 33.5±12.4 Pre-feed Feeding breast: 27.2±11.3 Non-feeding breast: 33.9±12.6
<u>Karatas et al., 2011 (56)</u>	Term	BF and formula	21:00-22:00	-	-	-	4	Triglyceride enzymatic kit	1 st visit (1-3mo) BF: 45.4±14.9 BF + formula: 43.8±17.2 2 nd visit (4-6mo) BF: 22.4 (12.4-45.7) BF + formula: 15.3 (11.5-35.2)
McDaniel et al., 1989 (57)	-	-	7:00-9:00	Hand	-	Either left or right	6	Spectrophotometric assay	2-20mo Pre-feed: 11.3±8.2 Post-feed: 32.1±20 All: 21.7±14.1
Michaelsen et al., 1994 (58)	Term	Excl. BF and BF (+formula)	Morning	-	-	Feeding breast	24	Infrared analysis	4-240d Ages combined: 40.6±17.5 Pre-feed: 26.6±14.9 Post-feed: 54.5±25.8
Prentice et al., 1981b (59)	-	-	After 13:00	Hand	-	Both	24	Creamatocrit	Across 12mo: 39.3 (36.2 – 42.8)
Schueler et al., 2011** ⁴ (60)	-	Excl. BF	7:00-10:00	Breast pump	-	Feeding breast	2	Creamatocrit	29-38d

Appendices

									Pre-feed: 10.4±13.2 Post-feed: 19.2±17.2
Pre-feed									
<u>Antonakou et al., 2013 (61)</u>	Term	Excl. BF	Morning	Electric pump	At least 2 h	One breast	3	Creamatocrit	1mo: 31.7±16.4 3mo: 28.2±17.1 6mo: 26.3±11.1
Bachour et al., 2012 (62)	-	-	Morning	Manual pump	2 h	-	1	Gravimetric	10-365d Non-smoker: 42.5±2.4 Smoker: 31.1±4.2
Fujita et al., 2012 (63)	-	Excl. BF	Morning	Hand	-	Not breast nursed overnight	1	Creamatocrit	0-20mo: 23 ± 12
Khin-Maung et al., 1980 (64)	-	BF	Before noon	Hand	At least 3 h	Both	3	Roesse-Gottlieb extraction	1-4mo: 31.8±10.3 4-7mo: 34.7±14.9 7-12mo: 35.5±10.5
<u>Picciano et al., 1976 (65)</u>	Term	Excl. BF	5:00-8:00 or 12:00 and 18:00	Hand or manual pump	4-8 h for morning samples; 2-3 h for others	Alternate breasts	7	Spectrophotometric assay	6-12wk Morning: 29.6 Midday: 39.9 Evening: 45.1
Trugo et al., 1988 (66)	Preterm and term	Excl. BF	9:00-10:00	Hand	-	-	1	Creamatocrit	1-5d Term: 22.7±10.3

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									Preterm: 32.3±23.3 6-36d Term: 40.5±11 Preterm: 38.4±13.1
Tyson et al., 1976 (67)	Term	BF	15:00-16:00	Hand	-	Both	-	Die infrared milk analyzer	13-71d: 33.8±4.3
Mid-feed									
Allen et al., 1991 (68)	-	Excl. BF and BF	Mid-morning	Hand or electric pump	-	Both	Average 75	Creamatocrit	21d: 47±3 45d: 53±6 90d: 42±4 180d: 56±4
Dutta et al., 2014 (69)	Preterm	Excl. BF	14:00-16:00	Hand	3min	-	4	Roese-Gottlieb extraction	7d: 33.8±2 28d: 38.2±15 90d: 43.1±11 180d: 58.1±23
<u>Kon et al., 2014 (70)</u>	Term	-	Morning	Hand or electric pump	-	-	3	-	1mo Low weight gain: 40.8±4.5 Normal weight gain: 43.9±8.4 High weight gain: 45.1±5.7 2mo Low weight gain: - Normal weight gain: 44.3±5.2

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									High weight gain: 36.5±3.6 3mo Low weight gain: 25.1±2.6 Normal weight gain: 37.6±4.7 High weight gain: 38±2.5
Lubetzky et al., 2015 (71)	Term	-	Morning and 21:00- 00:00	Hand	-	-	3	MIRIS milk analyzer	3, 7 and 14d Maternal age ≥35y 3d: 53.8±19.4 7d: 65.1±17 14d: 67.9±12.4 Maternal age <35y 3d: 46.4±14.2 7d: 60.8±16.2 14d: 71.5±12.7
Mandel et al., 2005* (72)	Term	BF	6:00-9:00 and 21:00- 00:00	Hand	-	-	2	Creamatocrit	Short lactation (2-6mo): 47.5 Long lactation (>1y): 67
Pines et al., 2016 (73)	-	Excl. BF	Morning	Hand	-	Both	2	MIRIS milk analyzer	1-7mo Left: 35±16 Right: 31±15
Quinn et al., 2013 (74)	-	-	6:00-10:00	Hand	3min	-	1	Roese-Gottlieb extraction	<18mo Male infant: 37.5±14.1

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									Female infant: 39.3±16.5
Quinn et al., 2016 (75)	-	-	6:00-10:00	Hand	1 h	Feeding breast	1	Roesse-Gottlieb extraction	Time of collection - All groups: 52.4±20.1
Post-feed									
Benner et al., 2001 (76)	-	-	9:00-11:30	-	-	-	2	Milkoscan FT120, Type 71200	Time of collection - During Ramadan fasting: 39.7±11.1 After Ramadan (non-fasting): 39.1±11
Cant et al., 1991 (77)	-	-	Morning	-	-	-	2	Lipid extraction	Placebo Group Baseline (2-5.5mo): 41.2±11.8 End after 8mo (10-14mo): 31.8±19.4
<u>Molto-Puigmarti et al., 2011 (78)</u>	Preterm and term	-	8:00-12:00	Mechanical pump	-	One or both	3	Creamatocrit	2-4d Very pre-term: 40.5 ± 16.2 Pre-term: 25.8 ± 18.8 Term: 26 ± 14.8 8-12d Very pre-term: 47.6 ± 16.2 Pre-term: 37.5 ± 12.4 Term: 31.1 ±15.3 28-32d Very pre-term: 46.7 ± 11.9

Appendices

									Pre-term: 29.8 ± 17.5 Term: 30.6 ± 12.9
Rakicioglu et al., 2006 (79)	-	Excl. BF and BF	9:00-11:00	Hand	-	Both	2	Roese-Gottlieb extraction	2-5mo During Ramadan fasting: 36.2 ± 15.3 After Ramadan (non-fasting): 32.3 ± 14.6
<u>Vitolo et al., 1993 (80)</u>	Term	-	8:00-10:00	Hand	-	Both	1	Creamatocrit	1-2d Adolescents: 33 ± 16 Adults: 27 ± 15
Milk samples collected by other methods									
Bassir et al., 1959* (81)	-	-	9:00	Hand	-	Both	3	Creamatocrit	1mo: 22.3 2mo: 33 3mo: 22.3
De Luca et al., 2016 (82)	Term	Excl. BF/BF/formula	9:00-11:00	Medela pump	-	Opposite to feeding breast	1	MIRIS milk analyzer	1mo Normal weight mothers ($18.5 < \text{BMI} < 25 \text{ kg/m}^2$): 34 Obese mothers ($\text{BMI} \geq 30 \text{ kg/m}^2$): 37
Eilers et al., 2011* (83)	Preterm and term	-	16:00-20:00	Electric pump	-	-	2	Creamatocrit	Preterm 3d: 33.6 28d: 36.5 Term

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									3d: 30.6 28d: 31.2
Neville et al., 1984 (84)	Term	Excl. BF	Mid-morning and mid-evening	Electric pump	-	Both	-	Creamatocrit	1-7mo Mid-feed: 33.9±5.4 Pooled (mid-and post-feed): 30.9±4.6
Shehadeh et al., 2006 (85)	Term	Excl. BF and BF	8:00-10:00	Electric pump	-	-	1	Lipid extraction Gerber Method	Group 3mo: 36±16 Group >12mo: 37±19
Woolridge et al., 1990 (86)	Term	-	8:00-20:00 (12 h)	-	-	One or both	3 x 12 h pre-and post-feeds	Creamatocrit	Baseline (4wk) Mean: 45.6±7.4 Pre-feed: 29.9±9.2 Post-feed: 61.2±9.4 After one-breast policy (5 or 6wk) Mean: 52.7±10 Pre-feed: 23.4±9.0 Post-feed: 74.8±12.6 After two-breast policy (5 or 6wk) Mean: 44.3±8.7 Pre-feed: 30.8±9.2 Post-feed: 57.5±10.9

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¹Every effort was made to follow a standardized approach when capturing information from studies into the main results tables. Any variation to the approach was due to the way in which the variable was reported in the respective paper. Values are reported as mean $\pm$ SD or mean (range) when SD is not available. Studies included in the quantitative synthesis were identified with an underline.

² Symbol – was used to represent information not stated or unclear. BF, non-exclusive breastfeeding - addition to complementary feeding; D, day(s); Excl. BF, exclusively breastfeeding; IDDM, insulin dependent diabetes mellitus; MIRIS milk analyzer, mid-infrared human milk analyzer, HMA, Miris AB, Uppsala, Sweden; Mo, month(s); Wk, week(s); Y: year.

³* values provided as cream % and were converted to fat (g/L) using standard adopted formula

⁴** values were kept as originally provided

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Appendix 6. Description of study population and methodological features of studies measuring protein concentrations in human milk – Chapter 3

Supplementary Table 4 in reference from publication

Reference	Gestational age	Mode of feeding	Time of day	Hand expression vs pump	Time since last feeding	Details breast used	Samples collected per woman	Analytical method	Milk concentration, g/L)
Collected across 24 h									
Full expression 24 h									
<u>Anderson et al., 1983 (1)</u>	Preterm and term	BF	24 h	Pump Type -	-	-	3 x 24 h collection	Micro Kjeldahl analysis	3d term: 23±6/preterm 20±7 7d term: 17±2/preterm 16±3 14d term :13±4/preterm 13±2.8
Beijers et al., 1992 (2)	Very preterm and preterm	-	24 h	Electric pump	-	Both	At least 3 x 24 h collection	Kjeldahl/Berthelot method	Time of collection - Very preterm Colostrum: 26.3±6.6 Traditional: 22.5±6.3 Mature: 16.3±2.6 Preterm

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									Colostrum: 25.7±11.1 Traditional: 17.1±3.7 Mature: 13.7±2.8
Bishara et al., 2008 (3)	Preterm	-	24 h	Electric double pump	-	-	1 x 24 h collection	Micro Kjeldahl analysis	21-30d Composite milk: 18.1 Pre-feed: 17.4 Post-feed: 18.3
Brown et al., 1982* ³ (4)	-	-	24 h	Electric pump	-	Both	8	Semi-micro Kjeldahl analysis	Time of collection - Left: 7.8 Right: 7.8
Brown et al., 1986* (5)	-	Excl. BF and BF	24 h	Electric pump	-	Both	1 x 24 h collection	Semi-micro Kjeldahl analysis	Time of collection - 0mo: 10.4 1mo: 8.7 2mo: 8.6 3mo: 8.1 4mo: 8 5mo: 7.9 6mo: 7.8 7mo: 7.4 8mo: 7.5 9mo: 7.1

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<u>Butte et al., 1984a*</u> (6)	Term	Excl. BF	24 h	Electric pump	-	Both	1 x 24 h collection	Kjeldahl analysis	1mo: 10.1 2mo: 8.9 3mo: 8.4 4mo: 8.2
Cregan et al., 2002 (7)	Preterm and Term	BF	24 h	-	-	Both	1 x 24 h collection	Bio-Rad protein assay	5d: 15.49 (method only stated for preterm)
<u>Heinig et al., 1993</u> (8)	Term	Excl. BF, BF	24 h	-	-	Both	4 x 24 h collection	Modified Lowry assay	3mo: 8.7 6mo: 8.1 9mo: 8.4 12mo: 10
<u>Lovelady et al., 1990</u> (9)	Term	Excl. BF	24 h	Electric pump	-	Both	1 x 24 h collection	Modified Lowry assay	9-24wk Exercising: 16.4±2.9 Control: 17.0±2.4
<u>McCrory et al., 1999</u> (10)	Term	Excl. BF	24 h	Electric pump	-	Opposite breast	2 x 24 h collection	Micro Kjeldahl analysis	8-16wk Control: baseline 9.1±1.4; intervention 8.6±1.6 Diet: baseline 9±1; intervention 8.4±1.2 Diet + exercise: baseline 8.62±1.66; intervention 8.35±1.6
<u>Motil et al., 1997*</u> (11)	Term	Excl. BF (initially)	24 h	-	-	Opposite breast	1 x 24 h collection	Micro Kjeldahl analysis	Adolescents: 6mo: 10.4

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									12: 9.2 18: 9 24: 9.3 Adults 6mo: 10.2 12mo: 8.7 18mo: 8.5 24mo: 8.3
<u>Perez-Escamilla et al., 1995 (12)</u>	Term	Excl. BF and BF	24 h	Electric pump	-	Both	3 x 24 h collection	Micro Kjeldahl analysis	4mo: 9.5±1 5mo: 9.1±1.2 6mo: 8.8±1.2
Stellwagen et al., 2013 (13)	Preterm	Excl. BF	24 h	Electric pump	-	-	4 x 24 h collection	Near-infrared spectrophotometer	Time of collection - Pooled all 24 h samples 15.1±2 Individual samples (each feed): 14.8±2.4 All: 14.9±2.3
Stuff et al., 1989* (14)	Term	Excl. BF and BF	24 h	Electric pump	-	Both (alternating per feed)	1 x 24 h collection	Kjeldahl analysis	16wk: 8.4 20wk: 8.2 24wk: 8 28wk: 7.9 32wk: 8 36wk: 8.5

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Pre-and post-feed across 24 h									
<u>Cannon et al., 2015</u> (15)	Term	Excl. BF	24 h	Hand	-	Both	-	Bradford protein assay	3-21wk Pre-feed: 11.2±1.7 Post-feed: 11.3±1.9
<u>Khan et al., 2013</u> (16)	Term	Excl. BF	24 h	Hand or manual pump	-	Both	-	Bradford protein assay	1-6mo: 13.4±2.2
<u>Mitoulas et al., 2002</u> (17)	Term	Excl. BF and BF	24 h	Hand or manual pump	-	Both	-	Bio-Rad protein assay	1mo: 10.5±0.4 2mo: 9.6±0.4 4mo: 9.3±0.4 6mo: 8±0.4 9mo: 8.3±0.5 12mo: 8.3±0.6 1-12mo: 9.2±0.2
<u>Perrella et al., 2016</u> (18)	Term	Excl. BF	24 h	Hand	-	Same breast as infant fed	-	Bradford protein assay	4mo: 9.9 (8.1-11.8)
Saint et al., 1986 (19)	Term	Excl. BF and BF	24 h	Hand	-	Both	-	Modified Lowry assay	2-12mo Twi-: 11.4±1.1 right breast, 12.5±1.4 left breast Triplets: 9.1±1.6 right breast, 9.1±1.2 left breast

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Valentine et al., 1994* (20)	-	-	24 h	Double electric breast pump	-	-	-	Kjeldahl analysis	1-4wk Pre-feed: 10.6 Post-feed: 10.6 24 h pooled composite milk: 10.6
Post-feed across 24 h									
<u>Bauer et al., 2011 (21)</u>	Preterm and term	-	24 h	Electric pump	-	-	8 x 24 h collection	Modified Lowry assay	1, 2, 3, 4, 5, 6, 7 and 8wk Extremely preterm (<28wk): 23±5 Severely preterm (28-31wk): 21±3 Moderately preterm (32-33wk): 19±3 Term: 16±4
Morton et al., 2012 (22)	Preterm	-	24 h	Hand and electric pump	-	Both	8 x 24 h collection	Bio-Rad protein assay	1wk: 20.1±3.5 2wk: 17.8±2.9 3wk: 17.2±2.7 4wk: 15.7±2.3 5wk: 14.7±2.3 6wk: 14.5±2.3 7wk: 13.8±2.2 8wk: 12.7±3.1
<u>Milk samples collected at one time point</u>									

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Full expression									
Barbosa et al., 1997 (23)	Term	-	10:00, 14:00 and 18:00	Electric pump	2 h	One	6	Kjeldahl analysis	BMI <23 3mo: 8.5±1.5 6mo: 8.3±1.4 BMI ≥23 3mo: 9.3±1.1 6mo: 8.9±1.3
<u>Butte et al., 1984b*</u> (24)	Preterm and term	-	8:00-12:00	Electric pump	At least 2 h	One	6	Kjeldahl analysis	Preterm overall: 12.4 2wk: 15.1 4wk: 13.3 6wk: 12.7 8wk: 11.9 10wk: 10.9 12wk: 10.6 Term overall: 10.3 2wk: 13 4wk: 10.9 6wk: 10.2 8wk: 9.5 10wk: 9.2 12wk: 9.1

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Butte et al., 1992* (25)	Term	-	10:00, 14:00 and 18:00	Electric pump	2 h	One	6	Kjeldahl analysis	4mo: 7.8 6mo: 7.7
Dewey et al., 1983 (26)	-	BF	2 nd feeding in the morning	Hand	-	One	1	Lowry assay	1mo: 14.4±2 2mo: 13.3±1.6 3mo: 13.2±1.6 4mo: 13.0±2.4 5mo: 12.5±1.7 6mo: 12.7±3.6
Lonnerdal et al., 1976 (27)	-	-	Ethiopia: 8:00-12:00 Sweden: -	Electric pump	Ethiopia: since midnight previous day Sweden: -	Ethiopia: Both Sweden: One	5	Modified Kjeldahl analysis	0-0.5mo Ethiopia non-privileged - Ethiopia privileged 16.8 Sweden privileged 15.8 0.5-1.5mo Ethiopia non-privileged 13 Ethiopia privileged 15.3 Sweden privileged 9.2 1.5-3.5mo: Ethiopia non-privileged 8.9 Ethiopia privileged 9.8 Sweden privileged 7.5

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									3.5-6.5mo Ethiopia non-privileged 8.5 Ethiopia privileged - Sweden privileged 6.9 6.5mo Ethiopia non-privileged 8.9 Ethiopia privileged - Sweden privileged -
<u>Marquis et al., 2003 (28)</u>	Term	-	7:00-12:00	Electric pump	2 h	One	2	BCA protein assay	Breastfeeding during pregnancy 2d: 27.4±1.1 1mo: 16.2±0.2 No breastfeeding during pregnancy 2d: 30.5±1.8 1mo: 16±0.2
Moran-Lev et al., 2015 (29)	Preterm	BF	6:00-9:00 and 21:00- 00:00	Pump Type -	Overnight for morning sample and 3 h for afternoon	Both	28	MIRIS milk analyzer	1, 2, 3, 4, 5, 6 and 7wk Morning: 10.8±4.7 Evening: 10.6±4.6
Mosley et al., 2007 (30)	-	BF	13:00-15:00	Electric pump	At least 2 h	One (same breast)	7	Bio-Rad protein assay	6-10mo Placebo: 8.4

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									2g CLA: 8.8 4g CLA: 8.7
Neubauer et al., 1993* (31)	Preterm and Term	-	Morning	Electric pump	1 h	Both	6	Micro Kjeldahl analysis	2d IDDM 39.1 Control 27.4 Reference 27.1 3d IDDM 24. Control 17.5 Reference 17 7d IDDM 15.5 Control 15.2 Reference 14.8 14d IDDM 13 Control 12.5 Reference 12.5 42d IDDM 11.3 Control 10.9 Reference 10.6

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									84d IDDM 10.3 Control 9.5 Reference 9.1
<u>Perrin et al., 2015</u> (32)	Term	BF	Morning	Pump Type -	-	-	8	BCA protein assay	11mo: 15.6±1.6 12mo: 16.2±1.7 13mo: 16.8±1.8 14mo: 17.5±2.3 15mo: 17.4±2.1 16mo: 17.3±2.4 17mo: 17.6±2.7 All: 16.6±1.8
Ritzenthaler et al., 2005 (33)	-	-	Morning	Electric pump	2 h	Same breast	4	Spectrophotometric assay	1-10mo: 7.3 ± 0.1
Sauer et al., 2017 (34)	Preterm and term	-	Morning and evening	Electric pump	At least 3 h	One	5	Kjeldahl analysis	1, 7, 14 and 21d Preterm: 19.7±1 Term: 13.2±1.8 Post-term: 9.6±2.4 All: 13.9±2.7
Williams et al., 2017 (35)	-	-	7:00-11:00	Electric pump	At least 2 h	Same breast	5	Bio-Rad protein assay	Time of collection -: 10.1±0.2
<u>Young et al., 2017</u> (36)	Term	Excl. BF	10:00-13:00	Electric pump	-	-	2	Modified Bradford protein assay	2wk

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									All: 11±2 Normal weight: 11±2 Overweight/obese: 10±2 4mo All: 8±2 Normal weight: 8±2 Overweight/obese: 8±2
Pre-and post-feed									
<u>Gridneva et al., 2017</u> <u>(37)</u>	Term	-	9:30-11:30	Hand or pump Type -	-	Feeding breast	2	Bradford protein assay	2 and 5mo: 11.3±2.6
<u>Grote et al., 2016</u> <u>(38)</u>	Term	BF	Morning	Hand	-	Feeding breast	24	Modified turbidimetric Kingsbury method	1mo: 14±0.2 2mo: 12±0.2 3mo: 10±0.1 6mo: 10±0.2
Hahn et al., 2018 (39)	-	Excl. BF	14:00-15:00	-	-	-	2	MIRIS milk analyzer	4wk 20s/Normal BMI: 11 ± 2 20 s/ Overweight BMI: 13±1 30s/Normal BMI: 12±2 30 s/ Overweight BMI: 12±2

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Hassiotou et al., 2013 (40)	-	-	Morning	Hand or electric pump	At least 3 h	Both	24–64	Bradford protein assay	6-39wk Pooled Feeding breast: 11.4±2.9 Non-feeding breast: 10.8±3 Pre-feed Feeding breast: 11.5±3 Non-feeding breast: 10.9±2.7
<u>Kuganathan et al., 2017 (41)</u>	Term	Excl. BF	9:30-11:30	Hand or pump Type -	-	Breast(s) infant fed	2 or 8	Bradford protein assay	2mo: 12.9±6.2 5mo: 11.7±5.7 9mo: 10.8±4.6 12mo: 12.8±6.7 All: 12±5.8
McDaniel et al., 1989 (42)	-	-	7:00-9:00	Hand	-	Either left or right	6	Modified Lowry assay	2-20mo: 10.6±3.9 Pre-feed: 9.8±3.6 Post-feed: 11.5±4.1
<u>Michaelsen et al., 1994 (43)</u>	Term	Excl. BF and BF	Morning	-	-	Feeding breast	24	Infrared analysis	4d: 18±3.5 14d: 13.3±3.4 28d: 11.2±1.9 42d: 10.3±2 56d: 9.5±1.7

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									70d: 9.3±1.9 84d: 8.9±1.8 4mo: 8.6±1.6 5mo: 8.3±1.9 6mo: 7.9±2.1 7mo: 8.1±1.6 8mo: 7.8±1.4
Pre-feed									
Al-Awadi et al., 2001 (44)	-	Excl. BF and BF	Early morning	-	-	-	1	Sum of casein and whey protein (gel-column chromatography)	0-6mo Kuwaiti: 13.16 Non-Kuwaiti: 8.84 6-12mo Kuwaiti: 6.23 Non-Kuwaiti: 4.39 12-18mo Kuwaiti: 5.52 Non-Kuwaiti: 4.81
Bachour et al., 2012 (45)	-	-	Morning	Manual pump	2 h	-	1	Bradford protein assay	10-365d Non-smoker: 14.9±0.6 Smoker: 13.1±0.6
Chavalittamrong et al., 1981 (46)	-	-	9:00-10:00	Hand	3 h	-	1	Colorimetric	0-7d: 15.6

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									8-14d: 11.3 15-21d: 9.9 22-28d: 9.4 29-90d: 8.8 91-180d: 7.8 180-270d: 6 > 270d: 7.4
Donangelo et al., 1989 (47)	-	Excl. BF and BF	9:00-10:00	Hand	-	-	3	Lowry assay	Excl. BF 1-5d: 26±3.1 6-30d: 15.7±1.2 31-280d: 13.1±0.7 Partial BF 31-280d: 26.2±7.0
<u>Dudzik et al., 2008 (48)</u>	Term	Excl. BF	6:00-8:00	Hand	-	-	3	-	3d: 19 21d: 11 100d: 13
Khin-Maung et al., 1980 (49)	-	BF	Before noon	Hand	At least 3 h	Both	3	Micro kjeldahl analysis	1-4mo: 11.9±2.3 4-7mo: 11.5±2.5 7-12mo: 10.6±1.7
<u>Picciano et al., 1976 (50)</u>	Term	Excl. BF	5:00-8:00 or 12:00 and 18:00	Hand or manual pump	4-8-h for morning samples; 2-3 h for others	Alternate breasts	7	Spectrophotometric assay	6-12wk Morning: 12.9

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									Midday: 13.0 Evening: 13.1
<u>Trugo et al., 1988 (51)</u>	Preterm and term	Excl. BF	9:00-10:00	Hand	-	-	1	Lowry assay	1-5d Term: 18.8±9.3 Preterm: 16.6±6.2 6-36d Term: 14.8±2.1 Preterm: 12.9±3.5
Tyson et al., 1976 (52)	Term	BF	15:00-16:00	Hand	-	Both	-	Infrared analysis	13-71d: 16.5±0.9
Mid-feed									
Allen et al., 1991 (53)	-	Excl. BF and BF	Mid-morning	Hand or electric pump	-	Both	Average 75	BCA protein assay	21d: 12±0.4 45d: 11±0.3 90d: 9±0.3 180d: 10±0.3
Dutta et al., 2014 (54)	Preterm	Excl. BF	14:00-16:00	Hand	3min	-	4	Micro Kjeldahl analysis	7d: 21.3±6 28d: 20.4±7 90d: 13.0±2 180d: 13.5±3
<u>Kon et al., 2014 (55)</u>	Term	-	Morning	Hand or electric pump	-	-	3	Kjeldahl analysis	1mo Low weight gain: 17.7±2.2

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									Normal weight gain: 17±1 High weight gain: 16.6±1.6 2mo Low weight gain: - Normal weight gain: 16.8±1.4 High weight gain: 17.7±1.2 3mo Low weight gain: 15.0±0.9 Normal weight gain: 13.2±0.8 High weight gain: 15.0±2.0
Lubetzky et al., 2015 (56)	Term	-	Morning and 21:00- 00:00	Hand	-	-	3	MIRIS milk analyzer	Maternal age ≥35y 3d: 20±19 7d: 10±4 14d: 9±3 Maternal age <35y 3d: 23±17 7d: 12±5

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									14d: 9±4
Pines et al., 2016 (57)	-	Excl. BF	Morning	Hand	-	Both	2	MIRIS milk analyzer	1-7mo Left: 5±2 Right: 5±2
Quinn et al., 2013 (58)	-	-	6:00-10:00	Hand	3min	-	1	PerkinElmer CHN analyzer	Time of collection - Male infant: 13.2±4.7 Female infant: 13.8±4.9
Quinn et al., 2016 (59)	-	-	6:00-10:00	Hand	1 h	Feeding breast	1	PerkinElmer CHN analyzer	Time of collection - All groups: 12.6±3.5
Post-feed									
Benner et al., 2001 (60)	-	-	9:00-11:30	-	-	-	2	Milkoscan FT120, Type 71200	Time of collection - During Ramadan fasting: 16.2±2.8 After Ramadan (non-fasting): 16.5±2.1
Rakicioglu et al., 2006 (61)	-	Excl. BF and BF	9:00-11:00	Hand	-	Both	2	Micro Kjeldahl analysis	2-5mo During Ramadan fasting: 11.5±1.2 After Ramadan (non-fasting): 11.4±1.8
Vitolo et al., 1993 (62)	Term	-	8:00-10:00	Hand	-	Both	1	Biuret method	1-2d Adolescents: 5.5 ± 2.2 Adults: 4.8±2

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Milk samples collected by other methods									
Bassir et al., 1959* (63)	-	-	9:00	Hand	-	Both	3	-	1mo: 13.1 2mo: 8.9 3mo: 10.2
Britton et al., 1986 (64)	Term	Excl. BF	12:00	Hand	-	Both	4	Lowry assay	15d Right breast: 26 Left breast: 18
De Luca et al., 2016 (65)	Term	Excl. BF	9:00-11:00	Electric pump	-	Opposite to feeding breast	1	MIRIS milk analyzer	Normal weight mothers (18.5<BMI<25 kg/m ²): 10 Obese mothers (BMI≥30kg/m ²): 10
Eilers et al., 2011 (66)	Preterm and term	-	16:00-20:00	Electric pump	-	-	2	BCA protein assay	Preterm 3d: 23.9±16 28d: 15.1±26.7 Term 3d: 22.2±8.7 28d: 15.8±55.3
Neville et al., 1984 (67)	Term	Excl. BF	Mid-morning and mid-evening	Electric pump	-	Both	-	Biuret method	1-7mo Mid-feed: 14.1±0.6 Pooled (mid-and post-feed): 13.5±0.5

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Shehadeh et al., 2006 (68)	Term	Excl. BF and BF	8:00-10:00	Electric pump	-	-	1	Micro Kjeldahl analysis	Group 3mo: 12±3 Group >12mo: 10±2

¹Every effort was made to follow a standardized approach when capturing information from studies into the main results tables. Any variation to the approach was due to the way in which the variable was reported in the respective paper. Values are reported as mean ± SD or mean (range) when SD is not available. Studies included in the quantitative synthesis were identified with an underline.

² Symbol – was used to represent information not stated or unclear. BCA assay, bicinchoninic acid assay; BF, non-exclusive breastfeeding - addition to complementary feeding; D, day(s); Excl. BF, exclusively breastfeeding; IDDM, insulin dependent diabetes mellitus; MIRIS milk analyzer, mid-infrared human milk analyzer, HMA, Miris AB, Uppsala, Sweden; Mo, month(s); Wk, week(s); Y: year.

³*values were converted to protein from nitrogen content provided. If protein nitrogen values were not provided, we considered non-protein nitrogen as 20% of the nitrogen value.

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Appendix 7. Description of study population and methodological features of studies measuring lactose concentrations in human milk – Chapter 3

Supplementary Table 5 in reference from publication

Reference	Gestational age	Mode of feeding	Time of day	Hand expression vs pump	Time since last feeding	Details breast used	Samples collected per woman	Analytical method	Milk concentration, g/L)
<u>Milk samples collected across 24 h</u>									
Full expression across 24 h									
<u>Anderson et al., 1983 (1)</u>	Preterm and term	BF	24 h	Pump Type -	-	-	3 x 24 h collection	Enzymatic	3d term 62±9/preterm 62±11 7d term 67±5/preterm 69±6 14d term 67±6/preterm 70±6
Brown et al., 1982 (2)	-	-	24 h	Electric pump	-	Both	8	Method of Folin	Time of collection - Left 78.2±5.4 Right: 79.3±4.5
Brown et al., 1986 (3)	-	Excl. BF and BF	24 h	Electric pump	-	Both	1 x 24 h collection	Colorimeter	0mo: 76.2±3.7 1mo: 77.2±3.6 2mo: 77.0±4.5 3mo: 79.2±3.8

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									4mo: 82.7±2.3 5mo: 83.5±2.4 6mo: 80.8±3 7mo: 81.0±4.8 8mo: 83.3±2.5 9mo: 82.0±1.8
<u>Butte et al., 1984a</u> (4)	Term	Excl. BF	24 h	Electric pump	-	Both	1 x 24 h collection	Automatic analyzer (Yellow Springs Instrument)	1mo: 64.7±2.4 2mo: 65.8±2.5 3mo: 66.5±2.3 4mo: 66.6±2.4
Cregan et al., 2002 (5)	Preterm and Term	BF	24 h	-	-	Both	1 x 24 h collection	Enzymatic	5d: 43.1 (method only stated for preterm)
<u>Heinig et al., 1993</u> (6)	Term	Excl. BF, BF	24 h	-	-	Both	4 x 24 h collection	Modified Dahlquist method	3, 6, 9 and 12mo:74
<u>Lovelady et al., 1990</u> (7)	Term	Excl. BF	24 h	Electric pump	-	Both	1 x 24 h collection	Dahlquist method	9-24wk Exercising: 59.1±6.1 Control: 42.6±3.7
<u>Motil et al., 1997</u> (8)	Term	Excl. BF (initially)	24 h	-	-	Opposite breast	1 x 24 h collection	Automatic analyzer (Yellow Springs Instrument)	Adolescent: 6mo: 60.1±2.8 12mo: 63.5±2.4 18mo: 64.0±2.4 24mo: 64.6±2.1

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									Adult: 6mo: 62.4±3 12mo: 63.0±3.2 18mo: 63.7±2.4 24mo: 64.8±3
Stellwagen et al., 2013 (9)	Preterm	Excl. BF	24 h	Electric pump	-	-	4 x 24 h collection	Near-infrared spectrophotometer	Time of collection - Pooled all 24 h samples: 64.1±9.4 Individual samples (each feed): 63.1±9.6 All: 63.6±9.7
<u>Stuff et al., 1989 (10)</u>	Term	Excl. BF and BF	24 h	Electric pump	-	Both (alternating per feed)	1 x 24 h collection	Automated procedure - measured hydrogen peroxide after galactose oxidation	16-36wk: 66.6±3
Pre-and post-feed 24 h									
<u>Cannon et al., 2015 (11)</u>	Term	Excl. BF	24 h	Hand	-	Both	-	Enzymatic-spectrophotometric assay	3-21wk Pre-feed: 67±6 Post-feed: 66±5.9
<u>Khan et al., 2013 (12)</u>	Term	Excl. BF	24 h	Hand or manual pump	-	Both	-	Enzymatic-spectrophotometric assay	1-6mo: 68±6.8

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<u>Mitoulas et al, 2002</u> (13)	Term	Excl. BF and BF	24 h	Hand or manual pump	-	Both	-	Enzymatic- spectrophotomet ric assay	1mo: 59.7±0.8 2mo: 60.4±1.1 4mo: 62.6±1.3 6mo: 62.5±1.7 9mo: 62.8±1.5 12mo: 61.4±2.9 1-12mo: 61.4±0.6
Saint et al., 1986 (14)	Term	Excl. BF and BF	24 h	Hand	-	Both	-	Enzymatic- spectrophotomet ric assay	2-12mo Twi-: 72.5±4.8 right breast, 74.4±4.7 left breast Triplets: 83.6±6.7 right breast, 85.1±7.6 left breast
Post-feed 24 h									
<u>Bauer et al., 2011</u> (15)	Preterm and term	-	24 h	Electric pump	-	-	8 x 24 h collection	Orcinol carbohydrate assay	1, 2, 3, 4, 5, 6, 7 and 8wk Extremely preterm (<28wk): 76±6 Severely preterm (28-31wk): 75±6 Moderately preterm (32-33wk): 75±5 Term: 62±9
Morton et al., 2012 (16)	Preterm	-	24 h	Hand and electric pump	-	Both	8 x 24 h collection	Enzymatic	1wk: 60.7±9.3 2wk: 67.1±6.8

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									3wk: 68.6±7 4wk: 70.6±7.8 5wk: 71.7±6.9 6wk: 73.5±7.6 7wk: 73.6±7.2 8wk: 75.3±7.8
<u>Collected at one time point</u>									
Full expression									
Barbosa et al., 1997 (17)	Term	-	10:00, 14:00 and 18:00	Electric pump	2 h	One	6	Automatic analyzer (Yellow Springs Instrument)	BMI <23 3mo: 66.6±2.9 6mo: 67.2±2.9 BMI ≥23 3mo: 66.0±3 6mo: 65.1±4.3
Butte et al., 1992 (18)	Term	-	10:00, 14:00 and 18:00	Electric pump	2 h	One	6	Automatic analyzer (Yellow Springs Instrument)	4mo: 66.4±2.7 6mo: 66.8±3.1
<u>Chen et al., 1998</u> (19)	Term	Excl. BF	7:00-10:00	Electric pump	-	One	14	Colorimetric	5d Primiparous vaginal: 60.9 Primiparous cesarean: 58.8 Multiparous vaginal: 65.7

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Dewey et al., 1983 (20)	-	BF	2 nd feeding in the morning	Hand	-	One	1	Enzymatic	1mo: 70.5±5.6 2mo: 72.1±6.2 3mo: 71.3±7.9 4mo: 76.1±4 5mo: 76.2±3.3 6mo: 77.5±2.7
Foda et al., 2004 (21)	-	-	Morning	Hand	-	-	4	HPLC	Before 3mo Pre-massage: 71±4 Post-massage: 73±3 After 3mo Pre-massage: 73±3 Post-massage: 73±4
<u>Goran et al., 2017</u> (22)	Term	Excl. BF	8:00-10:00	Double electric pump	-	-	2	LC-MS/MS	1mo: 78±8 6mo: 75±7 All: 76±6
Jackson et al., 1994 (23)	Preterm and term	-	Morning	Electric pump	1 h	Both	12	Automatic analyzer (Yellow Springs Instrument)	IDDM 14d: 60.9 42d: 63.5 84d: 64.4 Control 14d: 63.4

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									42d: 65.8 84d: 69 Reference 14d: 64.3 42d: 69.1 84d: 70.3
Lonnerdal et al., 1976 (24)	-	-	Ethiopia: 8:00-12:00 Sweden: -	Electric pump	Ethiopia: since midnight previous day Sweden: -	Ethiopia: Both Sweden: One	5	Automatic Analyzer (Technicon)	0-0.5mo Ethiopia non-privileged - Ethiopia privileged 64.6±8.9 Sweden privileged 59.3±5.8 0.5-1.5mo: Ethiopia non-privileged 74.2±5.1 Ethiopia privileged 66±6.3 Sweden privileged 70.2±5.6 1.5-3.5mo: Ethiopia non-privileged 74.3±4.8 Ethiopia privileged 76.4±1.6 Sweden privileged 73.3±4.7 3.5-6.5mo: Ethiopia non-privileged 74.9±3.1

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									Ethiopia privileged - Sweden privileged 76.4±3.5 6.5mo: Ethiopia non-privileged 77.8±4.8 Ethiopia privileged – Sweden privileged –
<u>Marquis et al., 2003</u> <u>(25)</u>	Term	-	7:00-12:00	Electric pump	2hr	One	2	Enzymatic	Breastfeeding during pregnancy 2d: 53.2±0.9 1mo: 62.4±0.6 No breastfeeding during pregnancy 2d: 51.1±0.9 1mo: 63.8±0.4
Moran-Lev et al., 2015 (26)	Preterm	BF	6:00-9:00 and 21:00-00:00	Breast pump	Overnight for morning sample and 3 h for afternoon	Both	28	MIRIS milk analyzer	1, 2, 3, 4, 5, 6 and 7wk Morning: 41.2±11.9 Evening: 40.4±12
Mosley et al., 2007 (27)	-	BF	13:00-15:00	Electric pump	At least 2 h	One (same breast)	7	Spectrophotometric method	6-10mo Placebo: 60.3 2g CLA: 60.8 4g CLA: 63.8

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Neubauer et al., 1993 (28)	Preterm and Term	-	Morning	Electric pump	1 h	Both	6	Automatic analyzer (Yellow Springs Instrument)	2d IDDM: 32.8 Control: 44.7 Reference: 48.8 3d IDDM: 54.7 Control: 55.7 Reference: 57 7d IDDM: 55.9 Control: 60.1 Reference: 62.3 14d IDDM: 60.9 Control: 63.4 Reference: 64.3 42d IDDM: 63.5 Control: 65.8 Reference: 69.1 84d IDDM: 64.4

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									Control: 69 Reference: 70.3
<u>Perrin et al., 2015 (29)</u>	Term	BF	Morning	Pump Type -	-	-	8	HPLC	11mo: 57.0±7 12mo: 59.6±8 13mo: 55.5±6.7 14mo: 58.6±8 15mo: 57.1±8.2 16mo: 54.9±5.2 17mo: 56.3±9.3 All: 56.9±7.6
Ritzenthaler et al., 2005 (30)	-	-	Morning	Electric pump	2 h	Same breast	4	Spectrophotometric assay	1-10mo: 70.4 ± 0.8
Sauer et al., 2017 (31)	Preterm and term	-	Morning and evening	Electric pump	At least 3 h	One	5	HPLC	1, 7, 14 and 21d All: 62.6±9.8 Preterm: 62.9±10.9 Term: 59±13.9 Post-term: 63.9±3.1
Thurl et al., 1993 (32)	-	Excl. BF	Morning	Electric pump	-	-	2	Enzymatic- commercial kit lactose/ galactose	Samples were divided in 4 parts during collection 8d 1: 59.9 (56.2-63) 2: 60.2 (55.3-64.4)

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									3: 61.2 (58.5-63.6) 4: 51.7 (41-62.5) 57d 1: 66.4 (62.4-75.1) 2: 65.2 (57.8-72.4) 3: 67.1 (63.3-71.4) 4: 68.4 (63.4-72.1)
Wack et al., 1997 (33)	-	-	10:00-12:00	Hand or breast pump	-	One	7	HPLC	0-60d: 66±4 61-120d: 70±4 121-180d: 70±3 181-240d: 71±4 241-300d: 70±4 301-360d: 71±4 >360d: 71±4
Williams et al., 2017 (34)	-	-	7:00-11:00	Electric pump	At least 2 h	Same breast	5	Spectrophotometric assay	Time of collection -: 64.1±0.4
<u>Young et al., 2017</u> (35)	Term	Excl. BF	10:00-13:00	Electric pump	-	-	2	Enzymatic- spectrophotometric assay	2wk All: 65±9 Normal weight: 65±7 Overweight/obese: 66±10 4mo All: 73±8

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									Normal weight: 75±7 Overweight/obese: 70±9
Pre-and post-feed									
<u>Gridneva et al., 2017 (36)</u>	Term	-	9:30-11:30	Hand or pump Type -	-	Feeding breast	2	Enzymatic-spectrophotometric assay	2 and 5mo: 65.8±5.1
<u>Grote et al., 2016 (37)</u>	Term	BF	Morning	Hand	-	Feeding breast	24	HPLC	1mo: 72.4±13.5 2mo: 80.3±11.6 3mo: 78.0±13.9 6mo: 79.2±17.3
Hahn et al., 2018 (38)	-	Excl. BF	14:00-15:00	-	-	-	2	MIRIS milk analyzer	4wk 20s/Normal BMI: 61±7 20 s/ Overweight BMI: 65±4 30s/Normal BMI: 67±5 30 s/ Overweight BMI: 66±5
<u>Kuganathan et al., 2017 (39)</u>	Term	Excl. BF	9:30-11:30	Hand or pump Type -	-	Breast(s) infant fed	2 or 8	Enzymatic-spectrophotometric assay	2mo: 67.5±9.1 5mo: 68.1±8.1 9mo: 68.4±8.8 12mo: 69.7±9.1 All: 68.4±8.8
McDaniel et al., 1989 (40)	-	-	7:00-9:00	Hand	-	Either left or right	6	Boehringer Mannheim kit	2-20mo Pre-feed: 79.1±7.5

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									Post-feed: 77.1±6.1 All: 78.1±6.8
Michaelsen et al., 1994 (41)	Term	Excl. BF and BF	Morning	-	-	Feeding breast	24	Milkoscan 104	4-240d Ages combined: 71±3.5
Pre-feed									
<u>Dudzik et al., 2008 (42)</u>	Term	Excl. BF	6:00-8:00	Hand	-	-	3	-	3d: 69 21d: 73 100d: 66
Khin-Maung et al., 1980 (43)	-	BF	Before noon	Hand	At least 3 h	Both	3	Titration with Benedict's solution	1-4mo: 71.9±2.4 4-7mo: 70.5±2.4 7-12mo: 71.0±3.6
Mid-feed									
Allen et al., 1991 (44)	-	Excl. BF and BF	Mid-morning	Hand or electric pump	-	Both	Average 75	Automatic analyzer (Yellow Springs Instrument)	21d: 60.2 (56.4-64.6) 45d: 62.2 (58.8-65) 90d: 64.3 (59.2-68.7) 180d: 65.3 (54.4-73.2)
Dutta et al., 2014 (45)	Preterm	Excl. BF	14:00-16:00	Hand	3min	-	4	Enzymatic	7d: 61.8±23 28d: 66.6±18 90d: 70.1±13 180d: 58.8±17

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Lubetzky et al., 2015 (46)	Term	-	Morning and 21:00-00:00	Hand	-	-	3	MIRIS milk analyzer	Maternal age ≥ 35 y 3d: 49 ± 13 7d: 55 ± 12 14d: 58 ± 9 Maternal age < 35 y 3d: 50 ± 9 7d: 51 ± 8 14d: 52 ± 7
Pines et al., 2016 (47)	-	Excl. BF	Morning	Hand	-	Both	2	MIRIS milk analyzer	1-7mo Left: 35 ± 5 Right: 36 ± 4
Quinn et al., 2013 (48)	-	-	6:00-10:00	Hand	3min	-	1	Phenol-Sulfuric Acid (DuBois) technique	Time of collection - Male infant: 73 ± 6.5 Female infant: 73.3 ± 5.1
Quinn et al., 2016 (49)	-	-	6:00-10:00	Hand	1 h	Feeding breast	1	Phenol-sulfuric acid (DuBois) technique	Time of collection - All groups: 73.7 ± 4.9
Post-feed									
Benner et al., 2001 (50)	-	-	9:00-11:30	-	-	-	2	Milkoscan FT120, Type 71200	Time of collection - During Ramadan fasting: 52.8 ± 12.7 After Ramadan (non-fasting): 52.5 ± 11.4

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Rakicioglu et al., 2006 (51)	-	Excl. BF and BF	9:00-11:00	Hand	-	Both	2	Automatic analyzer (Yellow Springs Instrument)	2-5mo During Ramadan fasting: 58.8±3 After Ramadan (non-fasting): 59.0±3.5
Milk samples collected by other methods									
De Luca et al., 2016 (52)	Term	Excl. BF	9:00-11:00	Electric pump	-	Opposite to feeding breast	1	MIRIS milk analyzer	1mo Normal weight mothers (18.5<BMI<25 kg/m ²): 68 Obese mothers (BMI≥30kg/m ²): 68
Neville et al., 1984 (53)	Term	Excl. BF	Mid-morning and mid-evening	Electric pump	-	Both	-	Automatic analyzer (Yellow Springs Instrument)	1-7mo Mid-feed: 68.4 Pooled (mid-and post-feed): 68.2
Shehadeh et al., 2006 (54)	Term	Excl. BF and BF	8:00-10:00	Electric pump	-	-	1	Remainder after subtracting water, protein, fat, and ash contents from 100	Group 3mo: 76±10 Group >12mo: 76±18

¹Every effort was made to follow a standardized approach when capturing information from studies into the main results tables. Any variation to the approach was due to the way in which the variable was reported in the respective paper. Values are reported as mean ± SD or mean (range) when SD is not available. Studies included in the quantitative synthesis were identified with an underline.

² Symbol – was used to represent information not stated or unclear. BF, non-exclusive breastfeeding - addition to complementary feeding; D, day(s); Excl. BF, exclusively breastfeeding; HPLC, High pressure liquid chromatography; IDDM, insulin dependent diabetes mellitus; LC-MS/MS, Liquid chromatography–mass spectrometry; MIRIS milk analyzer, mid-infrared human milk analyzer, HMA, Miris AB, Uppsala, Sweden; Mo, month(s); Wk, week(s); Y: year.

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Appendix 8. Ethics approval – Chapter 7

22nd March 2018



A/Prof B Muhlhausler
Head, Food and Nutrition Group
School of Agriculture, Food and Wine
Waite Campus, University of Adelaide
PMB 1
GLEN OSMOND SA 5064

Research Secretariat
Level 2, Samuel Way Building
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Tel 08 8161 6390
Tel 08 8161 6521
www.wch.sa.gov.au

Dear A/Prof Muhlhausler

Re: The short-term impact of dietary fat and sugar intake on breast milk composition: The Diet and Breast Milk Composition Study. HREC/17/WCHN/182. Ethics expiry date: 31/03/2021.

Lead HREC for the above study for the following institutions/sites:

Women's and Children's Health Network

I refer to your letter dated 8th March 2018 in which you responded to matters raised by the WCHN Human Research Ethics Committee at its 6th December 2017 meeting. I am pleased to advise that your protocol has been granted full ethics approval and meets the requirements of the *National Statement on Ethical Conduct in Human Research*.

Specifically, the following documents have been noted/approved:

Document	Version	Date
Diet and Breast Milk Composition Study Flyer	1	15 November 2017
Diet and Breast milk Composition Trial Consent to contact	1.0	05 March 2018
Diet and Breast milk Composition Trial Antenatal Screening Form	1.0	16 November 2017
Diet and Breast milk Composition Trial CRF	1.0	21 November 2017
Diet and Breast Milk Composition Study Brochure	1.0	21 February 2018
Diet and Breast Milk Composition Trial Adverse effects	1.0	16 November 2017
Patient Information Sheet/Consent Form: Diet and Breast milk composition Trial PIS and Consent form	1.0	05 March 2018
Protocol: Diet and Breast Milk Composition Trial Protocol	1.0	21 February 2018
Diet and Breast milk Composition Trial Postnatal Ward Screening Log	1.0	20 February 2018
HREA Application: AU/1/4C6239		22 November 2017
Response to Request for Further Information: Response Letter		08 March 2018
Diet and Breast milk Composition Trial Postnatal Screening Form	1.0	21 November 2017

This letter constitutes advice on ethical consideration only. You must not commence this research project at a site until you have obtained separate research governance approval from the site concerned. A copy of this letter should be forwarded to all site investigators for submission to the relevant Research Governance Officer.

At the WCHN, or any other SA Health site, separate authorisation from the Chief Executive or delegate of that site must be obtained through a Site Specific Assessment (SSA) request. For information on this process at the WCHN, please contact the WCHN Research Governance Officer, Ms Camilla Liddy (telephone 8161 6688, email camilla.liddy@sa.gov.au).



Government
of South Australia
SA Health

I remind you approval is given subject to:

- immediate notification of any serious or unexpected adverse events to participants;
- immediate notification of any unforeseen events that might affect continued ethical acceptability of the project;
- submission of any proposed changes to the original protocol. Changes must be approved by the Committee before they are implemented;
- immediate advice, giving reasons, if the protocol is discontinued before its completion;
- submission of an annual report on the progress of the study, and a final report when it is completed to the WCHN Research Governance Officer. It is your responsibility to provide these reports, without reminder. The proforma for the report may be found on the WCHN Research Governance and Ethics website.

Approval is given for three years only. If the study is more prolonged than this, an extension request should be submitted unless there are significant modifications, in which case a new submission may be required. Please note the expiry date in the title above and include it in any future communications.

Yours sincerely

TAMARA ZUTLEVICS (DR)
CHAIR
WCHN HUMAN RESEARCH ETHICS COMMITTEE

Appendix 9. SPIRIT Checklist – Chapter 7

Additional file 1 in reference from publication



STANDARD PROTOCOL ITEMS: RECOMMENDATIONS FOR INTERVENTIONAL TRIALS

SPIRIT 2013 Checklist: Recommended items to address in a clinical trial protocol and related documents*

Section/item	Item No	Description	Addressed on page number/comment
Administrative information			
Title	1	Descriptive title identifying the study design, population, interventions, and, if applicable, trial acronym	Title, page 1
Trial registration	2a	Trial identifier and registry name. If not yet registered, name of intended registry	Abstract, page 2
	2b	All items from the World Health Organization Trial Registration Data Set	N/A
Protocol version	3	Date and version identifier	Trial Registration, page 2
Funding	4	Sources and types of financial, material, and other support	Declarations, Funding, pages 15-16
Roles and responsibilities	5a	Names, affiliations, and roles of protocol contributors	Authors' contributions and authors' information, page 16

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5b	Name and contact information for the trial sponsor	N/A
5c	Role of study sponsor and funders, if any, in study design; collection, management, analysis, and interpretation of data; writing of the report; and the decision to submit the report for publication, including whether they will have ultimate authority over any of these activities	N/A
5d	Composition, roles, and responsibilities of the coordinating centre, steering committee, endpoint adjudication committee, data management team, and other individuals or groups overseeing the trial, if applicable (see Item 21a for data monitoring committee)	N/A

Introduction

Background and rationale	6a	Description of research question and justification for undertaking the trial, including summary of relevant studies (published and unpublished) examining benefits and harms for each intervention	Background, pages 3-4
	6b	Explanation for choice of comparators	N/A
Objectives	7	Specific objectives or hypotheses	Study objectives, pages 4-5
Trial design	8	Description of trial design including type of trial (eg, parallel group, crossover, factorial, single group), allocation ratio, and framework (eg, superiority, equivalence, noninferiority, exploratory)	Methods: participants, interventions, and outcomes, page 5

Methods: Participants, interventions, and outcomes

Study setting	9	Description of study settings (eg, community clinic, academic hospital) and list of countries where data will be collected. Reference to where list of study sites can be obtained	Study setting, page 5
Eligibility criteria	10	Inclusion and exclusion criteria for participants. If applicable, eligibility criteria for study centres and individuals who will perform the interventions (eg, surgeons, psychotherapists)	Study population, pages 5-6
Interventions	11a	Interventions for each group with sufficient detail to allow replication, including how and when they will be administered	Interventions, pages 6-7
	11b	Criteria for discontinuing or modifying allocated interventions for a given trial participant (eg, drug dose change in response to harms, participant request, or improving/worsening disease)	N/A

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	11c	Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (eg, drug tablet return, laboratory tests)	N/A
	11d	Relevant concomitant care and interventions that are permitted or prohibited during the trial	N/A
Outcomes	12	Primary, secondary, and other outcomes, including the specific measurement variable (eg, systolic blood pressure), analysis metric (eg, change from baseline, final value, time to event), method of aggregation (eg, median, proportion), and time point for each outcome. Explanation of the clinical relevance of chosen efficacy and harm outcomes is strongly recommended	Outcomes, page 7
Participant timeline	13	Time schedule of enrolment, interventions (including any run-ins and washouts), assessments, and visits for participants. A schematic diagram is highly recommended (see Figure)	Timeline and procedures, pages 7-10
Sample size	14	Estimated number of participants needed to achieve study objectives and how it was determined, including clinical and statistical assumptions supporting any sample size calculations	Sample size, page 11
Recruitment	15	Strategies for achieving adequate participant enrolment to reach target sample size	Recruitment, pages 11-12
Methods: Assignment of interventions (for controlled trials)			N/A
Allocation:			
Sequence generation	16a	Method of generating the allocation sequence (eg, computer-generated random numbers), and list of any factors for stratification. To reduce predictability of a random sequence, details of any planned restriction (eg, blocking) should be provided in a separate document that is unavailable to those who enrol participants or assign interventions	N/A
Allocation concealment mechanism	16b	Mechanism of implementing the allocation sequence (eg, central telephone; sequentially numbered, opaque, sealed envelopes), describing any steps to conceal the sequence until interventions are assigned	N/A
Implementation	16c	Who will generate the allocation sequence, who will enrol participants, and who will assign participants to interventions	N/A

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Blinding (masking)	17a	Who will be blinded after assignment to interventions (eg, trial participants, care providers, outcome assessors, data analysts), and how	N/A
	17b	If blinded, circumstances under which unblinding is permissible, and procedure for revealing a participant's allocated intervention during the trial	N/A

Methods: Data collection, management, and analysis

Data collection methods	18a	Plans for assessment and collection of outcome, baseline, and other trial data, including any related processes to promote data quality (eg, duplicate measurements, training of assessors) and a description of study instruments (eg, questionnaires, laboratory tests) along with their reliability and validity, if known. Reference to where data collection forms can be found, if not in the protocol	Data collection and management, page 12
	18b	Plans to promote participant retention and complete follow-up, including list of any outcome data to be collected for participants who discontinue or deviate from intervention protocols	N/A
Data management	19	Plans for data entry, coding, security, and storage, including any related processes to promote data quality (eg, double data entry; range checks for data values). Reference to where details of data management procedures can be found, if not in the protocol	Data collection and management, page 12
Statistical methods	20a	Statistical methods for analysing primary and secondary outcomes. Reference to where other details of the statistical analysis plan can be found, if not in the protocol	Statistical methods, page 12
	20b	Methods for any additional analyses (eg, subgroup and adjusted analyses)	N/A
	20c	Definition of analysis population relating to protocol non-adherence (eg, as randomised analysis), and any statistical methods to handle missing data (eg, multiple imputation)	N/A

Methods: Monitoring

Data monitoring	21a	Composition of data monitoring committee (DMC); summary of its role and reporting structure; statement of whether it is independent from the sponsor and competing interests; and reference to where further details about its charter can be found, if not in the protocol. Alternatively, an explanation of why a DMC is not needed	Methods: study oversight and monitoring, pages 12-14
	21b	Description of any interim analyses and stopping guidelines, including who will have access to these interim results and make the final decision to terminate the trial	N/A

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Harms	22	Plans for collecting, assessing, reporting, and managing solicited and spontaneously reported adverse events and other unintended effects of trial interventions or trial conduct	Adverse events, page 13
Auditing	23	Frequency and procedures for auditing trial conduct, if any, and whether the process will be independent from investigators and the sponsor	N/A
Ethics and dissemination			
Research ethics approval	24	Plans for seeking research ethics committee/institutional review board (REC/IRB) approval	Ethics approval and consent to participate, page 15
Protocol amendments	25	Plans for communicating important protocol modifications (eg, changes to eligibility criteria, outcomes, analyses) to relevant parties (eg, investigators, REC/IRBs, trial participants, trial registries, journals, regulators)	Protocol amendments, page 13
Consent or assent	26a	Who will obtain informed consent or assent from potential trial participants or authorised surrogates, and how (see Item 32)	Recruitment, pages 11-12
	26b	Additional consent provisions for collection and use of participant data and biological specimens in ancillary studies, if applicable	N/A
Confidentiality	27	How personal information about potential and enrolled participants will be collected, shared, and maintained in order to protect confidentiality before, during, and after the trial	Confidentiality, page 13
Declaration of interests	28	Financial and other competing interests for principal investigators for the overall trial and each study site	Competing interests, page 15
Access to data	29	Statement of who will have access to the final trial dataset, and disclosure of contractual agreements that limit such access for investigators	Access to data, page 14
Ancillary and post-trial care	30	Provisions, if any, for ancillary and post-trial care, and for compensation to those who suffer harm from trial participation	N/A

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Dissemination policy	31a	Plans for investigators and sponsor to communicate trial results to participants, healthcare professionals, the public, and other relevant groups (eg, via publication, reporting in results databases, or other data sharing arrangements), including any publication restrictions	Dissemination policy, page14
	31b	Authorship eligibility guidelines and any intended use of professional writers	N/A
	31c	Plans, if any, for granting public access to the full protocol, participant-level dataset, and statistical code	N/A

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Informed consent materials	32	Model consent form and other related documentation given to participants and authorised surrogates	Available on request
Biological specimens	33	Plans for collection, laboratory evaluation, and storage of biological specimens for genetic or molecular analysis in the current trial and for future use in ancillary studies, if applicable	N/A

*It is strongly recommended that this checklist be read in conjunction with the SPIRIT 2013 Explanation & Elaboration for important clarification on the items. Amendments to the protocol should be tracked and dated. The SPIRIT checklist is copyrighted by the SPIRIT Group under the Creative Commons “[Attribution-NonCommercial-NoDerivs 3.0 Unported](#)” license.

Appendix 10. Detailed nutritional information for breakfast meals – Chapter 7

Additional file 2 in reference from publication

Breakfast meal	Food	Brand	Quantity	Energy (kJ)	Fat content (g)	Sugar content (g)
High in fat			273.5g	2160	28.2	18.5
	Apricot Date & Almond Muesli	Coles	30g	524	4.7	5.2
	Full Cream Milk	Devondale	100mL	289	3.5	6.2
	Greek Yogurt Natural	Jalna	90g	530	9.1	6.2
	Soy & Linseed Bread	Helga's	42.5g	575	4.4	0.9
	Butter	Western Star	8g	242	6.5	0
High in sugar			325.4g	2072	5.0	56.1
	High-Fiber Fruity Breakfast Cereal	Sanitarium Weet Bix	30g	431	0.3	6.3
	Semi-Skim Milk	Devondale	100mL	199	1.3	5.2
	Strawberry Yogurt	Coles	100g	368	0.9	14.6
	Raisin Toast	Tip Top	65g	748	2.5	9.8
	Raspberry Jam	Coles	15g	165	0	10.1
	Honey	AUSNUT	1 teaspoon	94	0	5.9
	White sugar	AUSNUT	1 teaspoon	67	0	4.2
Breakfast meal	Food	Brand	Quantity	Energy (kJ)	Fat content (g)	Sugar content (g)
Control			539g	2029	9.5	24.7
	Whole Grain Flakes Cereal	Be natural	45g	670	1.9	5.4
	Skim Milk	Devondale	200mL	295	0.2	9.9
	High protein vanilla yoghurt	Danone	160g	397	0.3	6.1

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Wholemeal Crumpet	Golden	1 round crumpet	349	0.5	0.6
Fresh strawberry	AUSNUT	70g	76	0.1	2.7
Butter	Devondale	8g	242	6.5	0

*Breakfast meals were planned based on Australian products and calculated using FoodWorks, Xyris, version 7, and were accurate as at November 2018.

** AUSNUT: Australian Food, Supplement and Nutrient Database 2011-2013



THE UNIVERSITY OF
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Appendix 11. Ethics approval – Chapter 8

Our Ref: RA/4/20/4953

04 December 2018

Dr Donna Geddes
School of Molecular Sciences
MBDP: M310

Dear Doctor Geddes

HUMAN RESEARCH ETHICS APPROVAL - THE UNIVERSITY OF WESTERN AUSTRALIA

Assessing the effect of a short-term dietary intervention on human milk composition

Ethics approval for the above project has been granted in accordance with the requirements of the *National Statement on Ethical Conduct in Human Research* (National Statement) and the policies and procedures of The University of Western Australia. Please note that the period of ethics approval for this project is five (5) years from the date of this notification. However, ethics approval is conditional upon the submission of satisfactory progress reports by the designated renewal date. Therefore initial approval has been granted from 04 December 2018 to 03 December 2019.

You are reminded of the following requirements:

1. The application and all supporting documentation form the basis of the ethics approval and you must not depart from the research protocol that has been approved.
2. The Human Ethics office must be approached for approval in advance for any requested amendments to the approved research protocol.
3. The Chief Investigator is required to report immediately to the Human Ethics office any adverse or unexpected event or any other event that may impact on the ethics approval for the project.
4. The Chief Investigator must submit a final report upon project completion, even if a research project is discontinued before the anticipated date of completion.

Any conditions of ethics approval that have been imposed are listed below:

Special Conditions

None specified

The University of Western Australia is bound by the *National Statement* to monitor the progress of all approved projects until completion to ensure continued compliance with ethical principles.

The Human Ethics office will forward a request for a Progress Report approximately 30 days before the due date.

If you have any queries please contact the Human Ethics office at humanethics@uwa.edu.au.

Please ensure that you quote the file reference – RA/4/20/4953 – and the associated project title in all future correspondence.

Yours sincerely

Appendices

Mark Davies
Manager, Human Ethics

<i>Name</i>	<i>Faculty / School</i>	<i>Role</i>
Dr Donna Geddes	School of Molecular Sciences	Chief Investigator
Dr Ching Lai	School of Molecular Sciences	Co-Investigator
Professor Beverly Muhlhausler	University of Adelaide	Co-Investigator
Professor Mary Wlodek	University of Melbourne	Co-Investigator



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Appendix 12. Participant information form – Chapter 8

Project title: Assessing the effect of a short-term dietary intervention on human milk composition

Name of Researchers:

Professor Donna Geddes

A/Professor Beverly Muhlhausler

Professor Mary Wlodek

Ms Gabriela Esteves Leghi

Invitation:

You are invited to participate in a project, which aims to determine the effect of changing your diet during breastfeeding on the levels of nutrients and hormones in your breastmilk. You are asked to take part in this project because you are currently breastfeeding an infant who is between 4 weeks and 3 months of age and are interested in improving the quality of your diet.

Aim of the Study (What is the project about?)

Breast milk is made up of nutrients including fat and sugar. It also contains hormones that help control how hungry or full we feel and how our body uses energy. When a mother breastfeeds, these hormones are transferred from the breast milk to the baby and may play a role in how baby grows and develops. We know that the levels of these hormones in breast milk varies between mothers, but we know very little about what determines the levels of these hormones in breast milk, including how these are affected by the mother's diet. The purpose of this study is to look at whether improving dietary quality (especially reducing intakes of fat and added sugars) for 2 weeks can affect the amounts of nutrients and other factors, including hormones, in the breast milk. This research will help us to understand the effect of different dietary choices during breastfeeding on the composition of the breast milk and, in the future, may help inform dietary guidelines for breastfeeding women.

What does participation involve?

If you consent to being involved, then you will be part of this study for 3 weeks. At the time of enrolment into the study, we will ask you a series of questions about the general health of you and your infant, and some basic information about you and your household (such as where you live and whether anyone in your home smokes). We will then measure your weight and height and also measure the weight, length and head circumference of your infant. At this appointment, we will also ask you to collect a sample of breast milk (~5ml) and will give you containers to collect breast milk samples in your home for the remainder of the study.

For the first week of the study, we will ask you to continue to follow your normal diet. On three days of this week (two week and one weekend day), you will need to complete a questionnaire about what you have eaten on the previous day. This questionnaire takes about 20 mins to fill out and can be completed on-line. We will also ask you to continue to collect a sample of breast milk (~5ml) before your first morning feed every day of this week. On one day, we will ask you to collect 3 breast milk samples (morning, afternoon, evening) to see how levels of nutrients change over a single day. These samples can be stored in your home freezer until collection by study staff. We will also ask you to record how much milk your infant is consuming for one 24 hour period. This will involve weighing your infant before and after each breastfeed from 8 am on one day to 8 am on the next, and recording the difference in weight for each feed. We will provide you with special scales to do this.

After this first week you will have Lite'n'Easy meals and snacks delivered to your home for the following 2 weeks. We will ask you to consume as much of these meals and snacks as you can, and to record any of this food that you don't eat as well as any extra food or drinks you have during this time. The meals are all designed to be nutritionally balanced and contain healthy amounts of fat and sugar. During these two weeks, we will ask you to continue to collect daily breastmilk samples (~5ml before the first morning feed) and to measure your infants milk intake on one day in each of the 2 weeks. One of our study staff will visit you weekly during this time to collect the breast milk samples and weigh and measure you and your infant.

At the end of the 2 week dietary intervention phase we will ask you to attend one final appointment, where we will undertake the final measurement of you and your infant, and collect the final breast milk sample.

The breast milk samples will be used to determine how levels of nutrients and hormones change over time, and how they are influenced by the switch in diets. Measuring infant milk intake during this time will let us assess whether there are any effects of the diets on milk production.

Voluntary Participation and Withdrawal from the Study

Participation in this research study is entirely voluntary. If at any time you change your mind about being involved in this study you may withdraw your consent without giving an explanation and without affecting the care of you or your infant. If you withdraw from the study after we have obtained samples for assessment, we will ask that you allow these results to remain as part of the research, to ensure that the results are as useful as possible. However, you are free to require that all information that can be linked to your identity be removed from the project.

Your privacy

Your samples and results will be kept securely at all times. Anyone who has access to your identified samples and records is bound by law and by professional codes of conduct to keep your information confidential. Results from this research will be published in various ways, including conference papers, reports and journal articles. They will not be published in a form that could identify you. Your information will remain confidential except in the case of a legal requirement to pass on personal information to authorised third parties. This requirement is standard and applies to information collected both in research and non-research situations. Such requests to access information are rare; however, we have an obligation to inform you of this possibility.

Possible future research

Breast milk composition has many components which could be related to maternal diet. If there is any remaining breast milk after measuring nutrients and hormones, we may measure some of these other factors in your breast milk (including immune cells and vitamins), if you provide consent for us to do so. Researchers involved in these additional projects are bound by law and by professional codes of conduct to keep your information confidential. To further protect your identity, the samples will be coded and not labelled with any personal information (e.g. name of you or your baby). We may also use some of the information collected in this study to look at other factors that can affect the breast milk composition. The studies are very unlikely to produce any clinically significant findings, but will help us to understand more about breast milk composition and how it is influenced by diet.



Possible Benefits

There is unlikely to be any benefit to you taking part in this study. However, it will help us better understand how the mother's diet affects the levels of different factors in her milk, and may ultimately help to develop more evidence-based dietary guidelines for breastfeeding women in the future.

Possible Risks and Risk Management Plan

We do not foresee any risks associated with your involvement in this study.

Contacts

If you would like to participate or discuss any aspect of this study please feel free to contact the Chief Investigator Donna Geddes on 0415 419881 or by email donna.geddes@uwa.edu.au

Sincerely,

Chief Investigator

Approval to conduct this research has been provided by the University of Western Australia, in accordance with its ethics review and approval procedures. Any person considering participation in this research project, or agreeing to participate, may raise any questions or issues with the researchers at any time. In addition, any person not satisfied with the response of researchers may raise ethics issues or concerns, and may make any complaints about this research project by contacting the Human Ethics office at UWA on (08) 6488 4703 or by emailing to humanethics@uwa.edu.au. All research participants are entitled to retain a copy of any Participant Information Form and/or Participant Consent Form relating to this research project.



Chief Investigator A/Prof Donna Geddes
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Appendix 13. Participant consent form – Chapter 8

Participant Consent Form

Assessing the effect of a short-term dietary intervention on human milk composition

I, _____ have read the information provided and any questions I have asked have been answered to my satisfaction. I agree to participate in this research project, realizing that I may withdraw at any time without reason and without prejudice.

I understand that all identifiable information that I provide is treated as confidential and will not be released by the investigator in any form that may identify me unless I have consented to this. The only exception to this principle of confidentiality is if this information is required by law to be released.

I agree for any remaining breast milk samples to be stored for use in additional research projects that are approved by the UWA ethics committee ☐ Yes ☐ No

 Participant signature

 Date

Approval to conduct this research has been provided by the University of Western Australia, in accordance with its ethics review and approval procedures. Any person considering participation in this research project, or agreeing to participate, may raise any questions or issues with the researchers at any time.

In addition, any person not satisfied with the response of researchers may raise ethics issues or concerns, and may make any complaints about this research project by contacting the Human Ethics Office at the University of Western Australia on (08) 6488 3703 or by emailing to humanethics@uwa.edu.au

All research participants are entitled to retain a copy of any Participant Information Form and/or Participant Consent Form relating to this research project.