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IMPACT OF MATERNAL DIET ON BREAST MILK COMPOSITION

By

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DECLARATION

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we have helped to advance breast milk research in a way that will contribute to better outcomes for babies.

ABBREVIATIONS

Acronym	Definition
AA	Arachidonic Acid
ACC	Acetyl-CoA Carboxylase
AF	Artificial Feeding
ALA	Alpha-linolenic Acid
ANOVA	Analysis Of Variance
ANZCTR	Australian New Zealand Clinical Trials Registry
ATP	Adenosine Triphosphate
BF	Breastfeeding
BFHI	Baby Friendly Hospital Initiative
BHT	Butyrated Hydroxytoluene
BM	Breast Milk
BMI	Body Mass Index
CHMHREC	CSIRO Health and Medical Human Research Ethics Committee
CLA	Conjugated Linoleic Acid
CSIRO	Commonwealth Scientific and Industrial Research Organisation
CV	Coefficient of Variation
DARLING	Davis Area Research on Lactation, Infant Nutrition and Growth
DGLA	Dihomo-gamma-linoleic acid
DHA	Docosahexaenoic Acid
DNL	De Novo Lipogenesis
EBF	Exclusive Breastfeeding
EGF	Epidermal Growth Factor
ELISA	Enzyme-Linked Immunosorbent Assay
ELOVL	Elongation of Very Long Chain Fatty Acids Proteins
EPA	Eicosapentaenoic Acid
FA	Fatty Acid
FAME	Fatty Acid Methyl Ester
FAS	Fatty Acid Synthase
FFM	Free Fat Mass
FFQ	Food Frequency Questionnaire
FIL	Feedback Inhibitor of Lactation
GC	Gas Chromatography
GDM	Gestational Diabetes Mellitus
GLA	Gamma-Linolenic Acid
GLUT1	Glucose Transporter 1
GLUT8	Glucose Transporter 8

HFCS	High Fructose Corn Syrup
HM	Human Milk
HMO	Human Milk Oligosaccharide
IGF	Insulin-Like Growth Factor
KOH	Potassium Hydroxide
LA	Linoleic Acid
LCMS	Liquid Chromatography-Mass Spectrometry
LCPUFA	Long Chain Polyunsaturated Fatty Acid
LS	Lactose Synthase
MFG	Milk Fat Globule
MFGM	Milk Fat Globule Membrane
MUFA	Monounsaturated Fatty Acid
NADP	Nicotinamide Adenine Dinucleotide Phosphate
NAFLD	Non-Alcoholic Fatty Liver Disease
NHS	National Health Service
PUFA	Polyunsaturated Fatty Acid
SAHMRI	South Australian Health and Medical Research Institute
SCD	Stearoyl CoA Desaturase
SD	Standard Deviation
SEM	Standard Error of the Mean
SFA	Saturated Fatty Acid
SGA	Small for Gestational Age
TAG	Triacylglycerol
UNICEF	United Nations Children's Fund
WAZ	Weight for Age Z-Score
WHO	World Health Organisation
WLZ	Weight for Length Z-Score

ABSTRACT

Breast milk (BM) is natural biological fluid that contains many nutritive and protective components and breastfeeding (BF) is associated with improved health outcomes in infancy and reduced incidence of diseases such as obesity and hypertension later in life. The positive impact of BF on health and wellbeing is a result of the constituents within BM. BM is changeable in its composition, with certain components being more variable than others. This variability exists between and within mothers and is prompted by a multitude of factors, one of which is maternal diet. Certain dietary components have been reported to have strong associations with relative concentrations in BM, particularly fatty acids (FAs). Most of the research investigating the impact of maternal diet on BM, however, focuses on habitual intake effects and considerably less work has been done to explore acute changes in BM in response to maternal diet.

This thesis aims to address the above stated gap in the literature by assessing the acute impact of increasing sugar and fat consumption on BM composition, with a focus on macronutrients and certain metabolic hormones. A secondary aim of the thesis was to explore how BM composition naturally fluctuates over the course of a day. Additionally, to further understand the relationship between omega-3 FA intake and relative concentrations in BM, a systematic review investigating the influence of maternal omega-3 long chain polyunsaturated fatty acid (LCPUFA) intake via habitual diet/supplementation and their relative concentrations in milk was carried out.

To address the above concepts, a novel methodology was developed for more robust BM research. Initially, a proof-of-concept study within the UK was completed to test application of a dietary intervention alongside hourly BM sampling and laboratory methods. The data collected during the pilot study informed the design and conduct of a second larger study within Australia. Both trials delivered intervention diets containing relatively modest increases in sugar and fat to exclusively breastfeeding (EBF) mothers. BM samples were collected by mothers at hourly intervals alongside consumption of the study diets within their homes. BM lactose, glucose, protein, total fat/triacylglycerides, cholesterol and individual fatty acids were measured. The metabolic hormones leptin, adiponectin and insulin were also determined. Analyte concentrations were compared to determine the effects of an acute increase in sugar or fat on BM composition. The hourly samples were collected across

12 time-points, also enabling comparisons between time-points to detect any naturally occurring diurnal rhythms in BM components.

Responses to acute changes in maternal intake in BM macronutrients, hormones and FAs were detected in both studies. BM fats and FAs showed significant responses in both cohorts, whereas differing observations were identified for the remaining macronutrients. Total fat/triacylglycerides increased in response to the higher sugar intervention and triacylglycerides (TAGs) increased in response to the higher fat intervention employed in the pilot. The FA profile of BM was also responsive to the interventions. Specifically, total saturated FAs (SFAs) and polyunsaturated FAs (PUFAs) increased in response to the higher sugar diet whereas monounsaturated FAs (MUFAs) tended to decrease. In response to the higher fat diet there were increases in individual SFAs, MUFAs and PUFAs, however, total levels of these classes of FAs were unaffected. In the pilot study we observed some variations across the day in protein and lactose concentrations, however, these findings were not replicated in the second study. In the second study adiponectin and leptin concentrations decreased in response to the higher sugar intervention and leptin and insulin showed significant variations across the day. Further to the results of the feeding trials, a systematic review and meta-analysis of the literature concluded that there was a significant influence of maternal omega-3 LCPUFA intake from either food or dietary supplements on omega-3 concentrations in BM. Whilst, we noted an influence on BM FAs from maternal habitual FA intake, large amounts of heterogeneity interfered with the ability to collate conclusions from a large body of evidence and the systematic review contained only 8 studies.

The findings reported in this thesis support the hypothesis that maternal diet can acutely impact BM composition, and that fat and FA concentrations were particularly sensitive to these dietary changes. Further to this, we demonstrated that habitual FA intake influences the FA composition of human milk via a systematic review and meta-analysis. The acute influence of maternal diet on BM composition is a novel finding and assists in furthering BM research by delivering greater understanding into the dynamic nature of BM composition. The differences in analytes observed over the course of a day also assist in understanding the extent of variability in composition in individual women over the course of the day and should be considered in future when developing sample collection methodologies within BM compositional research.

CHAPTER 1

INTRODUCTION TO THE THESIS AND LITERATURE: BREAST MILK, BREAST FEEDING AND HOW THIS INFLUENCES INFANT GROWTH AND DEVELOPMENT.

1.1 Introduction to the thesis

This project is slightly different to most, in that it has been part of a dual award PhD programme, an alliance that exists between The University of Nottingham (my home university) and The University of Adelaide (my host university). This alliance pre-existed the conception of my project by a few years but continues to create great PhD opportunities for many students at both institutions and creates wonderful collaborations. I am incredibly grateful to this affiliation and all those who keep it running for the experiences I have encountered over my time as a PhD student.

I have always had a great passion for care and an interest in the mother-baby bond. Early in my life I decided that I wanted to work within this remit and set my sights on midwifery. However, after spending a year studying to become a midwife, I changed my mind and transferred to a degree in Biomedical Sciences. Science was a much better fit and so my career as a Scientist began. Around a year after completing my undergraduate degree, I discovered the advertisement for a PhD studentship studying the impact of maternal diet on breast milk composition, thus the seed to this thesis was sown.

The timeline for my project, including details on locations and how Covid-19 interrupted my studies is shown in Figure 6 in Appendix 5 within a Gannt chart. In brief, I started in 2018 at The University of Nottingham with my principal supervisors Prof Simon Langley-Evans and Dr Matthew Elmes. The conceptualisation of this project and the details of the pilot study occurred in the first few months and within a year data collection for the pilot study was complete. I had a short trip to Australia in March 2020, cut short by the COVID-19 pandemic, and subsequently paused my studies due to border closures in Australia. When the world had resumed some normalcy, I returned to Australia to complete my host stint at the University of Adelaide alongside my supervisor Associate Prof Bev Muhlhausler. I completed the work for my secondary study in Australia over 1.5 years and returned to the UK in August 2022 where I spent just over 4 months writing and finalising my thesis.

1.2 Introduction to breast milk

BM is an incredibly complex substance with ever changing constituents. It is the optimal nourishment for infants and contains many nutritive and non-nutritive compounds. These compounds provide the building blocks and sustenance required for survival and development in early life and have far-reaching benefits that extend into adulthood. This review of the literature will provide an overview of BM composition, how BM is produced, how it varies and how it influences infant growth and development. We will also cover some background information on the structure of the breast and current trends and statistics for BF.

1.2 Terminology and trends in infant feeding

1.2.1 Key terminology

Within the sphere of BM research exists a mass of topic specific terminology. Milk can be referred to as human milk (HM) or as breast milk (BM). For this work we will refer to it as BM for the most part. Breastfeeding is often shortened to BF and AF is used when referring to formula feeding (or artificial feeding). As per the World Health Organisation (WHO) guidelines, women are advised to exclusively breast feed their infants up to the age of 6 months and to continue BF alongside complimentary foods until 2 years of age (WHO, 2021). Exclusive BF is commonly shortened to EBF, and partial BF is used to describe BF that is supplemented with formula or other milks. For some mother-infant dyads EBF at the breast can be difficult and a solution for this is to exclusively pump, meaning baby is fed exclusively with BM but does not suckle at the breast. It is recommended in infant feeding guidelines, including those from the WHO, that complementary feeding is introduced when infants are around 6 months of age and refers to the need for suitable solid foods to be introduced that complement the nutrition provided by BM. Tandem feeding refers to mothers who are feeding more than one infant at a time and can be in relation to multiparous pregnancies or infants of different ages. Established BF refers to an EBF practice that has been successfully initiated and sustained for several weeks.

1.2.2 Contemporary trends in infant feeding

The current information provided by the WHO states that only 44% of infants between 0-6 months worldwide were EBF between the years 2015-2020, whilst a Lancet review published in 2016 reported only 37% of infants below 6 months were EBF (WHO, 2021, Victora et al. 2016). Furthermore, UNICEF reported 48% of infants 5 months or younger were EBF based on global data from 2005-2021. Data for the UK and Australia were not available for inclusion in this report (UNICEF, 2022). Figures from the Infant Feeding Survey in 2010 are the latest reports available for UK BF rates and showed that EBF rates were only 1% at 6 months in the UK (NHS Digital, 2012). More recent records shows that Australian BF rates are much higher, with the 2020-2021 National Health Survey reporting 35% of infants at the age of 6 months were EBF (Australian Bureau of Statistics, 2022).

1.2.3 Factors influencing infant feeding practices

Many factors are involved in the decision to initiate BF and the success with which it continues. Physical factors that influence the onset or continuation of BF can lead to reduced rates of any BF and EBF. Obesity and maternal haemorrhage during birth have both been associated with more difficulty in BF due to delayed initiation, reduced milk supply or late onset of lactogenesis II (commonly referred to as 'milk coming in') (Hashemi-Nazari et al. 2020, Thompson et al. 2010). Giving birth pre-term or to a small for gestational age (SGA) baby can also pose problems with initiation and success of EBF due to separation of the mother-infant dyad and mechanical impediments related to the infants reduced size and strength (Nesjum et al. 2023). In these instances, support is particularly necessary and along with education and maternal confidence has been shown to positively influence the duration of BF (Babakazo et al. 2015). The Baby Friendly Hospital Initiative (BFHI) is a program launched by the WHO and UNICEF that sets a goal for hospitals to have 75% of mothers EBF at time of discharge. Since its inception and implementation, we have seen an 11% increase in Scottish babies receiving any milk at 6 months between 2010 and 2017 (UNICEF, 2018), this shows the incredibly positive influence empowerment and education can have on BF rates. However, such studies lose track of women within a week of giving birth, and it is known that more than a third of UK women cease BF within the first 4-6 weeks (Langley-

Evans, 2021). Interventions to enhance BF rates are effective in promoting BF initiation but not continuation (McGiveron et al. 2015).

1.3 Physiology of lactation

1.3.1 The structure of the lactating breast

The physiological configuration of a breast follows the general anatomy as shown in Figure 1.1. The alveolar, or glandular tissue, of the breast is surrounded by and interspersed with adipose tissue, all of which are held together by Cooper's ligaments to support the breast framework and connect it to the chest wall. The lobes of the alveolar tissue are comprised of many alveolar sacs, each of which are lined by mammary epithelial cells. These cells synthesise BM and expel it into the alveolar lumen where it is stored until the let-down reflex is stimulated. A basket-like web of myoepithelial cells surrounds the alveoli, and these cells contract to force milk into the lactiferous ducts and towards the lactiferous sinus before being ejected through the nipple. Montgomery's tubercules on the surface of the areola create sebaceous fluid that assists with lubrication. The nipple comprises smooth muscle cells that contract when stimulated to allow the infant to locate and latch onto the breast. Stimulation of this area triggers the let-down response through endocrine control, as shown in Figure 1.2 later in this section (Langley-Evans, 2021, Geddes, 2007, Ramsay et al. 2005).

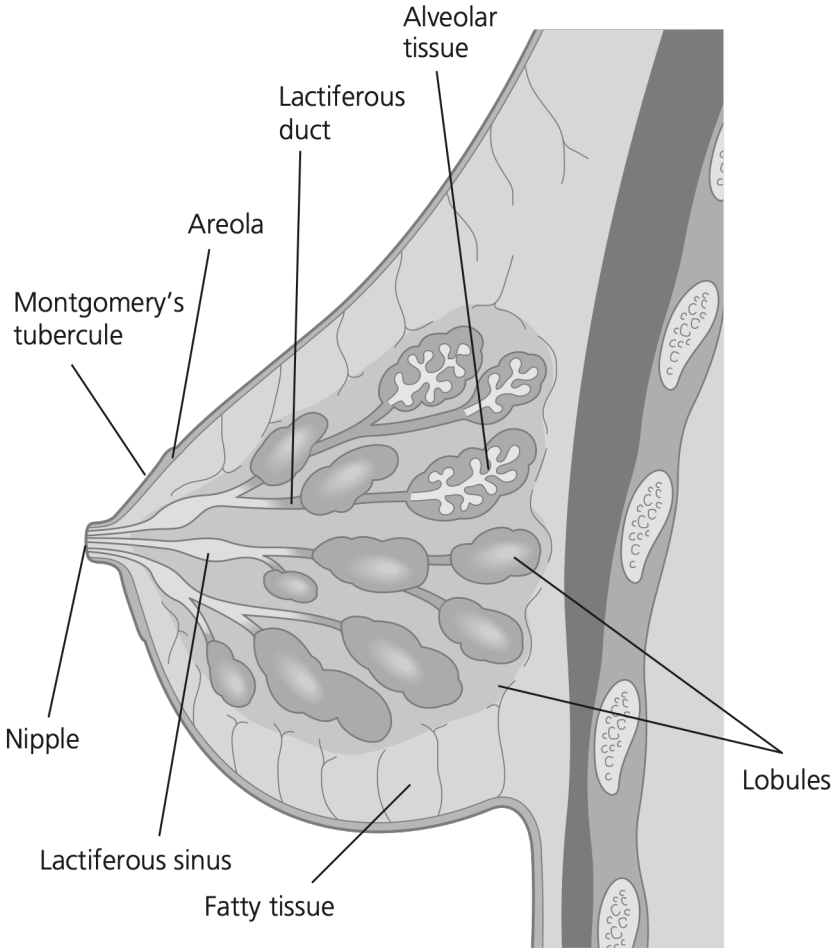


Figure 1.1: A diagrammatic image of the breast taken from Nutrition: A Lifespan Approach by Simon Langley-Evans, 2021.

1.3.2 Development of the breast

The very beginning of breast development occurs *in utero* when ectodermal ridges form, extending from the armpit to the groin (Javed and Lteif, 2013, Moore et al. 2018). Actual breast development starts when these ridges begin to involute, remaining only in the area where nipples will eventually develop. This residual portion of crest forms what is known as the primary mammary bud (an extension of epidermal cells into the mesenchyme below), which differentiates into secondary buds that then become glandular. Within the new gland ducts are formed, which terminate and form an opening to the now depressed area in the epidermis which is where the nipple develops. This process occurs under the influence of sex hormones released from the placenta (Moore et al. 2018).

There is a dormancy period throughout childhood that ends during puberty when breast formation is resumed in response to a rise of oestrogen and progesterone and in the presence of epidermal growth factor (EGF) (Hennighausen and Robinson, 2001). At this time the breast undergoes multiple stages of development, that have been previously described in detail by Marshall and Tanner. These stages are coined Tanner's stages 1-5. In brief, this process starts with elevation of the papilla, through formation of a breast bud and multiple stages of enlargement of the breast mound. This enlargement is caused by increased synthesis of breast tissue and coincides with physical changes to the nipple and areola in both size and colour (Javed and Lteif, 2013, Marshall and Tanner, 1969). On a cellular level, pubertal women undergo increased synthesis of fibrous and fatty tissue within the stroma. This happens alongside increased branching and elongation of the ducts within the breast. In addition to the increased branching, more alveoli are formed, each menstrual cycle stimulates the production of more alveoli (Javed and Lteif, 2013).

During pregnancy the breast fully matures and becomes ready for lactation. Oestrogen and progesterone from the placenta along with prolactin from the anterior pituitary, and other factors such as EGF and placental lactogens, stimulate elongation and branching of the ducts alongside synthesis of further alveolar tissue (Hennighausen and Robinson, 2001, Langley-Evans, 2021). The breast is considered fully developed and ready for lactation around mid-pregnancy, when the alveolar tissue is fully differentiated and the influence of prolactin enables the breast to produce small amounts of the components of the first milk (colostrum) (Neville, Morton and Umemura, 2001).

1.3.3 The process of lactation: Milk production, secretion and ejection

Lactogenesis is known as the onset of milk secretion and exists in 2 stages. Lactogenesis I begins in pregnancy when the epithelial tissue within the mammary gland begins to differentiate and secrete colostrum (Neville, Morton and Umemura, 2001). At the time of lactogenesis I the breast is ready to secrete milk, but this is prevented by the high circulating levels of progesterone and oestrogen coming from the placenta (Neville, Morton and Umemura, 2001, Khun, 1977, Hartmann et al. 1995).

Following birth, progesterone and oestrogen levels drop considerably over the ensuing 30-40 hours. The rapid decline in the levels of these hormones stimulates the onset of lactogenesis II (Hartmann et al. 1995, Pang and Hartmann, 2007, Langley-Evans, 2021). The presence of prolactin, cortisol and insulin are also required to achieve this switch from the first to the second stage of lactogenesis (Pang and Hartmann, 2007). Lactogenesis II is characterised by a significant increase in secreted milk volume (up to 500ml/day), the sudden increase in volume and change in consistency is why this stage is often referred to as BM 'coming in'. There is a marked increase in BM citrate, phosphate, lactose, glucose and calcium concentrations, coupled with a decline in BM sodium and chloride levels as colostrum is replaced by transitional milk (Neville et al. 1988, Neville et al. 1991). These compositional changes happen because of closures in tight junctions in the paracellular pathway and an up-regulation of genes associated with protein synthesis. Increases in important non-nutritive factors such as lactoferrin and immunoglobulins also occur at this time (Lawrence and Lawrence, 2021, Hannan et al. 2023).

During lactation, the hormonal control of milk synthesis is governed by prolactin (produced in the anterior pituitary) and milk ejection is under the influence of oxytocin (produced in the posterior pituitary). The effect of prolactin on BM synthesis is more profound in the early weeks of lactation, as BF is becoming established. It is important for the breast to be stimulated regularly during this time to ensure enough prolactin is released and enough milk is produced to support the current and future feeding demands of the infant (Lawrence and Lawrence, 2021, Langley-Evans, 2021). Oxytocin is responsible for the let-down reflex, that occurs in response to appropriate stimulation by the infant. This hormone is released in a series of pulses that cause the lactiferous ducts to dilate, which leads to alveolar and ductal contractions that force the milk from the lumen down through the lactiferous ducts (Lawrence and Lawrence, 2021, Hannan et al. 2023).

The sequence of events that lead to BM synthesis and ejection are presented in Figure 1.2 and described in more detail below:

1) The process begins with cues from the infant (such as crying or fussing) and the stimulation of the nipple when latching and suckling begins.

2) Receptors in the mother signal to the hypothalamus that these cues are occurring, via the spinal cord.
3) The hypothalamus releases prolactin releasing hormone and the posterior pituitary releases oxytocin. Prolactin releasing hormone triggers the release of prolactin from the anterior pituitary.
4) When the prolactin reaches the breast, it stimulates the alveolar epithelial cells to secrete milk into the lumen and initiates further milk production.
5) When the oxytocin reaches the mammary gland, it works on the myoepithelium surrounding the alveoli, triggering contraction of the muscle cells that force milk out of the lumen, into the ducts and through the nipple (Langley-Evans, 2021, Hannan et al. 2023, Daly et al. 1996).

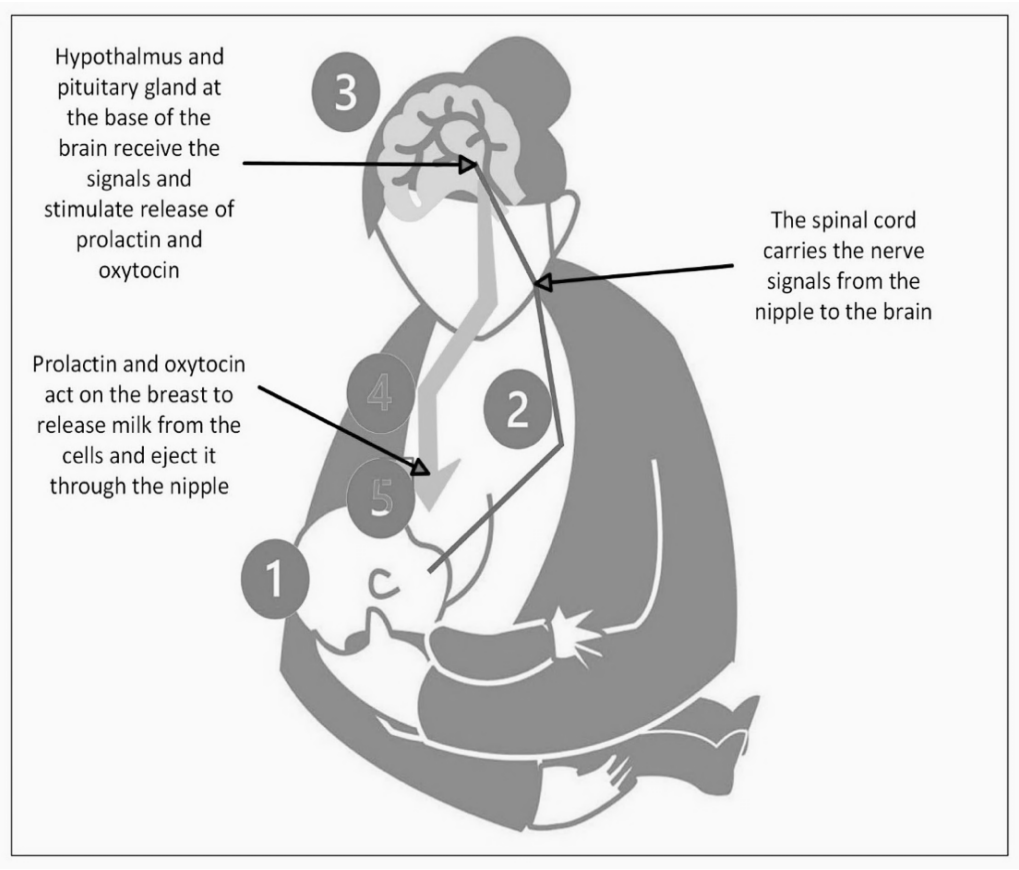


Figure 1.2: Diagrammatical representation of the process described above, showing how milk ejection and synthesis are triggered and controlled.

1.3.4 Continuation of lactation

Lactation continues in a supply-on-demand manner, with milk synthesis and ejection being triggered in tandem by the infant suckling (Lawrence 2022, Hannan et al. 2023, Daly et al. 1996). The repetitive stimulation of the breast, to produce and expel milk, continues for as long as the infant needs and can provide as much milk as is required, with mothers of twins being able to produce twice as much milk as those of singleton infants (Langley-Evans, 2021). Prolactin is important for the production of milk components and the establishment of lactation, however, the rate of milk removal from the breast has also been shown to have a direct correlation to the rate of milk synthesis, and therefore the day-to-day control of milk production. The increase in milk synthesis occurring when milk has been drained from the breast is suggestive of a localised regulator of milk production within the breast (Lawrence

and Lawrence, 2021). A form of whey protein, now known as feedback inhibitor of lactation (FIL), has been detected in BM and is believed to facilitate an autocrine control of BM synthesis by ceasing milk production when it is present in high concentrations within the breast (Lawrence 2022, Wilde et al. 1995). Furthermore, a mechanical feedback reaction caused by stretching of the ducts when milk volumes are high has been identified as another mediating factor in milk secretion (Stewart et al. 2021).

Other factors can influence the ability to establish lactation and the successful continuation of BF. Thyroid hormone imbalances have been associated with disruptions during lactation. Hyperthyroidism (overactive thyroid) has been associated with increased milk production but also with compromised milk ejection reflexes whilst hypothyroidism has been associated with decreased milk supply in a case study of a mother with Hashimoto thyroiditis (West and Marasco, 2009, Stein, Kessler and Hubbard, 2004). Diabetes has been associated with delayed onset of lactogenesis II as well as reduced rates of any BF and earlier cessation of EBF, the cause of this impediment is thought to be in relation to glucose intolerance and insulin resistance associated with diabetes. Similar effects have been reported in mothers with higher BMI, this is also understood to be related to disturbances with insulin and glucose control (Lawrence, 2022, Nommsen-Rivers, 2016, Vanky et al. 2012).

Milk synthesis ceases when stimulation of the breast and milk removal declines or stops. The absence of milk removal increases the pressure within the mammary gland which reduces capillary blood flow, and the lack of stimulation means prolactin and oxytocin release is diminished (Lawrence and Lawrence, 2021). In the absence of suckling and milk removal the mammary gland begins to involute. This occurs in 2 stages - first the epithelial cells undergo apoptosis and begin to die off, however, this process can be reversed if suckling resumes. The second stage involves apoptosis of the surrounding structures along with the epithelial cells, these break down before being re-absorbed. Much of the alveolar tissue formed in pregnancy is replaced by fibrous tissue, this phase is much more permanent (Langley-Evans, 2021, Lawrence and Lawrence, 2021, Hannan et al. 2023).

1.4 Nutritional origins of breast milk

Carbohydrates and fats make up the majority of breast milk's caloric content at 44% and 42% respectively, whilst proteins make up around 14% (Langley-Evans, 2021 and Holland et al. 1991). Some of these are synthesised *de novo* in the breast while others are absorbed via the maternal digestive system and transported to the mammary epithelial cells, this is discussed in more detail below. The introduction of these components to maternal circulation and body stores begins with their ingestion and digestion.

Most of the carbohydrate in breast milk is lactose, although glucose, galactose, oligosaccharides and gangliosides are also present (Ballard and Morrow, 2013). Each of these molecules are created within the mammary cells from glucose and galactose, both of which are derived from maternal circulation.

Breast milk carbohydrates originate in the maternal diet as glucose and galactose or as longer chained sugars. The longer chains are broken down by the enzyme amylase in the mouth and small intestine. If required, they are further digested by more enzymes at the brush borders in the small intestine (e.g. lactase and sucrase). The resulting glucose and galactose molecules pass into the epithelial cells via membrane transporters such as SGLT-1, in the presence of sodium. Any sugars not needed by these cells then pass into the circulation via GLUT-2 transporters (Molnar and Gair, 2015, Barrett and Barman 2019). The glucose and galactose are transported via maternal circulation to the mammary epithelial cells where they are used to produce lactose and the other carbohydrates present in breast milk (Anderson et al. 2015, Langley-Evans, 2021, Bode, 2012).

Proteins in breast milk are a mix of primary, secondary and tertiary protein structures. These are part derived from maternal stores and circulation as well as being made *de novo* in the lactocytes (Lawrence, 2022). Like in most cells, proteins can be created via transcription and translation (Alberts et al. 2002).

The building blocks of milk proteins that are not derived via transcription and translation originate from the maternal diet. Protein digestion begins with the enzyme pepsin within the stomach which breaks proteins down into polypeptides of various lengths. These

polypeptides are further digested in the small intestine by other enzymes called endopeptidases released by the pancreas and intestinal mucosa (Molnar and Gair, 2015, Barrett and Barman 2019). Some chains are broken down into free amino acids in the intestinal lumen, some at the cell brush borders and others within the intestinal epithelial cells. Amino acids are carried into the epithelial cells via multiple different transporters in the presence of sodium and chloride molecules, they then exit the cell via transporters and enter the maternal circulation (Molnar and Gair, 2015, Barrett and Barman 2019). Proteins are introduced to lactocytes via multiple processes including via membrane protein transporters if they are smaller structures or within lipoproteins via transcytosis and paracellular transport (Langley-Evans, 2021. Anderson, 2015).

Fats in milk are stored mostly within TAGs, TAGs consist of 3 fatty acid structures joined to a glycerol backbone and can contain fatty acids of different lengths and different numbers of double bonds (as shown below in Figure 1.5). The FAs present in these TAGs are dependent largely on the maternal diet (Carpenter et al. 2023, Gunstone, 1996).

Fats enter the body in multiple forms, however, many are stored within TAGs. TAG digestion begins properly in the small intestine in the presence of lipase from the pancreas. Lipase enzymes break fats down into fatty acids and glycerides. Bile salts are a key player in fat digestion within the intestine as well as lipases. They are released from the gallbladder and work to emulsify the larger fatty structures into smaller structures to create more surface area for lipases to bind and digest the fats (Molnar and Gair, 2015). Bile salts also help with absorption by surrounding the free fatty acids and ushering them to the brush borders where they combine with the epithelial cells. Within these cells fatty acids aggregate together to form globules containing free fatty acids, TAGs and cholesterol. These collections then become coated with proteins to form structures called chylomicrons. They transport fats across the epithelial cells where they are expelled by exocytosis before entering the circulation via the lymphatic vessels and being transported to lactocytes where they are engulfed by the cell borders (Molnar and Gair, 2015, Barrett and Barman, 2019).

1.4.1 Breast milk synthesis at a cellular level

BM is synthesised locally in the mammary gland by mammary epithelial cells. At the onset of lactation, the cytoplasmic volume and amount of Golgi and endoplasmic reticulum increase to prepare for copious milk production (Hannan et al. 2022, Anderson et al. 2015). The mammary epithelial cells act as hubs for the production and transport of BM composites, as shown in Figure 1.3. Water enters the cells in response to an osmotic gradient created by high intercellular concentrations of lactose that is synthesised from glucose and galactose in the Golgi apparatus (Langley-Evans, 2021, Mardones and Villagran, 2020). Glucose enters the cell from the maternal circulation via membrane transporters (GLUT-1) and is used to synthesise the galactose required to make lactose, although some galactose also enters the mammary cell directly from the maternal circulation (Anderson et al. 2015, Nemeth et al. 2000, Langley-Evans, 2021). Oligosaccharides are created as an extension of this process (Bode, 2012). Proteins and hormones are derived from the maternal circulation as well as being synthesised *de novo* via transcription and translation. Fats are created and elongated by fatty acid synthase as well as being transported into the mammary epithelial cells from the maternal circulation (Langley-Evans, Anderson, 2015, 2021, Visentainer et al. 2018, Demmelmair and Koletzko, 2018, Panuganti, Bazzano and Ley, 2021, Casabiell, 1997, Savino, Liquori and Lupica, 2010, Catli, Dundar and Dundar, 2014).

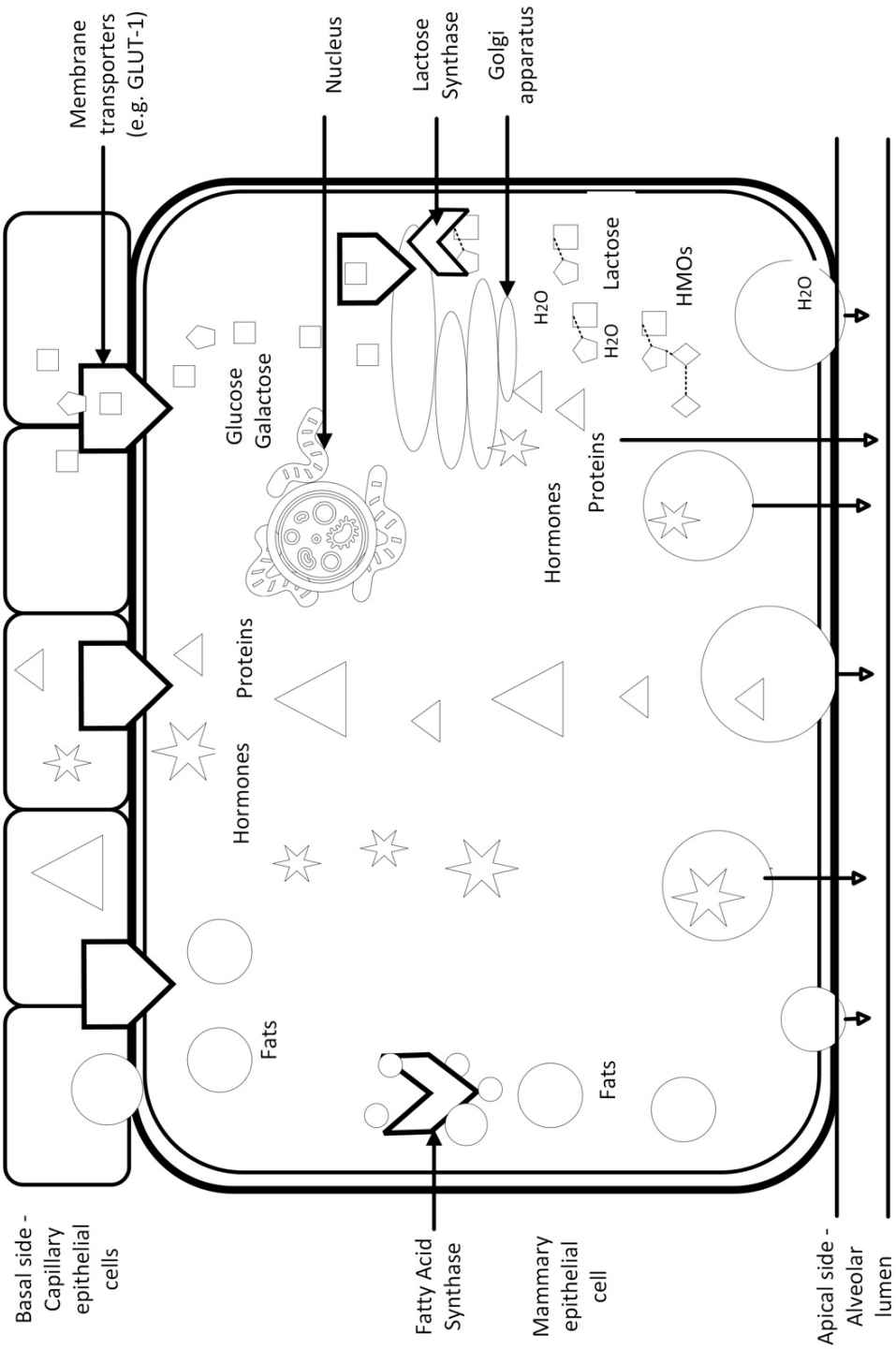


Figure 1.3: A Diagrammatic depiction of a mammary epithelial cell, showing the BM components being synthesised and/or entering the cell and being transferred to the apical side of the cell where they enter the lumen, modified from Langley-Evans 2021 and Anderson et al. 2015.

1.5 BM composition

BM composition encompasses a complex matrix of nutritive (macronutrients and micronutrients) and non-nutritive elements (including hormones, oligosaccharides, immune cells and factors and growth factors). The composition of BM changes throughout the course of lactation. Colostrum, which is the first milk released post-partum, is a sticky yellow substance produced in low quantities and is known as 'liquid gold' due to its high concentrations of protective and immune boosting proteins such as lactoferrin and secretory immunoglobulin A. From colostrum, milk changes into what is known as transitional milk, following the onset of lactogenesis II, the composition alters significantly as milk volume increases and turns white in colour. Finally, the composition settles around 10-14 days postpartum, when milk is deemed to have fully matured (Langley-Evans, 2021, Lawrence, 2022, Neville, Morton and Umenura, 2001). The different stages of BM maturation are compositionally distinctive, a snapshot of the nutritional composition for each stage is shown in Table 1.1.

Table 1.1: Macronutrient composition of BM across colostrum, transitional and mature milk as reported by Langley-Evans, 2021 and Holland et al. 1991.

	Units per 100g of milk		
	Colostrum	Transitional milk	Mature milk
Macronutrients			
Energy (kcal)	56	67	69
Carbohydrate (g)	6.6	6.9	7.2
Carbohydrate (% energy)	44	40	39
Protein (g)	2	1.5	1.3
Protein (% energy)	14	9	8
Fat (g)	2.6	3.7	4.1
Fat (% energy)	42	51	53

1.5.1 Macronutrients

As shown in Table 1.1 the macronutrients in BM (carbohydrates, protein and fats) provide around 40%, 10% and 50% of the energy in milk respectively (Langley-Evans, 2021, Holland et al. 1991). These compositional elements are the main nutritive components, serving as energy sources and building blocks for the developing infant and are believed to exist in ideal quantities for infants as they grow (Lawrence et al. 2022).

1.5.1.1 Carbohydrates

Carbohydrates are a group of organic compounds that are products of photosynthesis and important elements of the human diet (Gerschenson, Rojas and Fissore, 2017). They are comprised of polyhydroxy aldehydes and ketones, each with the general formula (CH2O) (Brody, 1998). They can be monosaccharides (glucose, galactose, fructose), disaccharides (sucrose and lactose), polysaccharides such as glycogen or BM oligosaccharides (HMOs) such as 2'-fucosyllactose (Nelson and Cox, 2001). Carbohydrates can be classified by the type of monomers they are made up of (i.e. glucose and galactose make up lactose), how these units are linked (i.e. α or β glycosidic linkages) and the degree of polymerisation (i.e. number of units per chain). Carbohydrates with saccharide chains of 9 units or less are water soluble whilst those with 10 or more units are not and these are classed as dietary fibres (Brody, 1998, Gerschenson, Rojas and Fissore, 2017). BM carbohydrates include glucose, galactose and lactose as well as longer chain molecules such as oligosaccharides and gangliosides (Ballard and Morrow, 2013).

Lactose

Lactose is a disaccharide comprised of the monosaccharides galactose and glucose, joined by a β(1-4) glycosidic bond (Brody, 1998). Lactose is a major constituent of BM, contributing to around 40% of the total energy (Guo, 2021). In addition to being a source of energy, lactose plays an important role in creating osmotic pressure in BM (Mardones and Villagran, 2020). Water is drawn to lactose molecules during synthesis which in turn controls milk volume and impacts the concentrations of casein (Fox, 2009).

Lactose is created *de novo* within the Golgi apparatus in the mammary epithelial cells, as depicted in Figure 1.3. Glucose and galactose enter the mammary epithelial cells from maternal circulation via membrane transporters (GLUT1 and GLUT8), these transporters have also been reported to facilitate the uptake of glucose and galactose in the Golgi apparatus for lactose synthesis (Zhao, 2014). GLUT transporter expression is up regulated in the mammary epithelium during pregnancy and lactation to facilitate the increased glucose supply needed to produce lactose. This up-regulation is thought to be triggered by a low oxygen tension, resulting from increased metabolic activity and oxygen consumption in the mammary epithelial cells (Zhao, 2014).

Lactose synthase (LS) works to adjoin glucose with galactose to create lactose. LS is a multi-enzyme complex made up of galactosyltransferase and α-lactalbumin, α-lactalbumin is found only in mammary epithelial cells and its presence allows galactosyltransferase to be specific to lactose production by having glucose substrate specificity. LS has a higher affinity for glucose than galactose, therefore the rate limiting factor in lactose synthesis is the glucose availability in the Golgi apparatus (Mardones and Villagran, 2020). In knockout lactating mice that did not produce α-lactalbumin, milk was low in lactose and had a high viscosity, demonstrating the importance of lactose for creating the osmotic pressure in the mammary epithelial cells and ensuring the water content of milk (Stacey et al. 1995, Mardones and Villagran, 2020). As shown in Figure 1.3, lactose is produced in the Golgi apparatus and then travels to the apical side of the cell to be expelled into the lumen within secretory vesicles along with water and electrolytes such as sodium and potassium (Langley-Evans, 2021, Mardones and Villagran, 2020).

Lactose concentrations in BM have been reported to be between 7.35-7.44 g/dL from 3-12 months lactation within the DARLING study (Nommsen et al. 1991) and on average identified to be 7.8 g/dL with a range of 4.9-12.7 g/dL from a large data set of donor breast milk (Wojcik et al. in 2009). These average concentrations of lactose are fairly consistent across the literature which is why it is considered to be the most stable of the BM macronutrients.

Glucose

Glucose is a monosaccharide sugar and the simplest carbohydrate found in BM. Glucose is present at lower concentrations in BM in comparison to lactose (around 255µg/ml) (Cheema et al. 2021). Glucose enters mammary epithelial cells from the maternal circulation via membrane transporters GLUT1 and GLUT8, as shown in Figure 1.3, but can also be synthesised *de novo* via hexoneogenesis (Anderson et al. 2015, Nemeth et al. 2000, Zhao, 2014, Sunehag et al. 2002).

Glucose is primarily a precursor for lactose synthesis in the mammary gland (Zhao, 2014). However, glucose concentrations have been demonstrated to be proportional to the rate of milk secretion when measured in a group of 13 women over the course of lactation. Neville, Hay and Fennessey found that when lactogenesis II was occurring, milk glucose increased at a rate proportional to that of milk volume. Furthermore, they observed a similar response during weaning as glucose concentrations decreased at a rate proportional to the decline in milk production (Neville, Hay and Fennessey, 1990). This group also noted an increase in milk glucose concentrations when plasma glucose was increased and maintained, suggesting that the concentrations of glucose in BM are directly related to concentrations in the maternal circulation.

Glucose concentrations at 1 month postpartum have been identified as an early indicator of BF continuation at 3 months. This is on the basis that glucose is related to milk supply and that achieving a high milk supply early in lactation lays the groundwork for a sufficient supply as lactation continues (Nagel et al. 2021). Higher glucose concentrations in BM have previously been associated with maternal obesity and pre-pregnancy body mass index (BMI) (Ahuja et al. 2011). However, more recent studies have failed to observe the same association (Isganaitis et al. 2019, Young et al. 2017). As mentioned previously, obesity and diabetes are associated with delayed onset of lactogenesis II and with reduced rates of BF. This is believed to be related to interferences with maternal metabolism of glucose and insulin, potentially explaining the higher rates within BM in these women (Lawrence, 2022, Nommsen-Rivers, 2016, Vanky et al. 2012)

1.5.1.2 Protein

Proteins in BM comprise caseins, whey proteins, mucins and other nitrogen containing compounds such as amino acids and various free molecules. BM proteins make up approximately 1% of milk content (Lonnerdal 2003, Guo, 2014). The main caseins within BM are β-casein and κ-casein. Whey proteins consist mostly of α-lactalbumin, lactoferrin and IgA along with other miscellaneous proteins such as lysozyme, serum albumin, enzymes and the mucins found within milk fat globule membranes (Guo, 2021, Mosca and Gianni, 2017, Langley-Evans, 2021). The function of proteins within BM are both nutritive and non-nutritive; they provide important amino acid building blocks and have many active effects such as aiding immunity, assisting nutrient digestion and absorption as well as gut development and functioning (Lonnerdal, 2003).

Milk proteins are created via *de novo* synthesis in the mammary epithelial cells as well as being derived from the maternal circulation as shown in Figure 1.3. Of the 20 amino acids (AAs) present in BM, the 8 essential AAs are derived from the maternal circulation while the remaining, non-essential, AAs are synthesised within the mammary epithelial cells (Lawrence, 2022). Those derived from the maternal circulation enter lactocytes as amino acids via membrane protein transporters or as larger structures, such as lipoproteins, via transcytosis and paracellular transport between cells (Langley-Evans, 2021. Anderson, 2015). For example, immunoglobulin A binds to immunoglobulin receptors on the basal side of mammary epithelial cells, and the protein and receptor form a complex that is internalised by the cell by endocytosis. This complex is then transported to the apical side of the cell and released by exocytosis, taking with it a small portion of the receptor. The regulation of this process in human mammary epithelial cells is not yet fully understood (Anderson et al. 2015, Hunziker and Kraehenbuhl, 1998).

Milk protein concentrations were reported in early work by Lonnerdal et al. in 1976 as 8-9 g/litre in mature milk, and this figure was further refined to ~7-10 g/L in 1992 (Kunz and Lonnerdal, 1992). Since then, a systematic review assessing papers from the mid-1900's to 2019 reported an average range of 0.8g/100ml to 3.3g/100ml (Adhikari et al. 2021), whilst

another systematic review including data only on Chinese mothers reported an average of 1.22g/dL in mature milk (Yang et al. 2018).

As lactation progresses the concentrations of protein in BM declines (Zhang et al. 2021) and as shown above in Table 1.1, concentrations are highest in colostrum. However, the opposite is true for concentrations of two free non-essential AAs (glutamine and glutamate), whose concentrations have been reported to increase over the first 3 months of lactation and they make up more than 50% of the free AAs in milk at this time (Agostini et al. 2000). The ratio of whey to casein proteins within BM also changes throughout lactation, ranging from 80:20 in early lactation to 50:50 in late lactation (Lonnerdal, 2003). The variability in BM protein concentrations is believed to originate from the proteins that are derived from maternal circulation as it is assumed *de novo* synthesis remains relatively constant in the presence of lactation stimulating hormones (Lonnerdal and Atkinson, 1995).

1.5.1.3 Fat

Fats, or lipids, are a diverse collection of substances characterised by their insolubility in water. There are many chemical structures, classes and sub-classes of lipids (Gurr et al. 2016, Lawrence, 2022). Fatty acids are the functional units of fat and are chained structures of hydrocarbons with a carboxyl end group that are classified based on the number of carbons within the chain and the presence, number and location of double bonds between these hydrocarbons. They can be separated into 3 groups: saturated (SFA), monounsaturated (MUFA) and polyunsaturated fatty acids (PUFA). SFA contain no double bonds and are abundant in animal products such as meat and cheese while MUFAs contain only one double bond and are present in plant oils and seeds such as olive oil and almonds. Finally, PUFAs contain two or more double bonds and can be further classified into two main groups: omega-3 and omega-6 PUFAs, depending on the location of the first double bond from the methyl end (Murff and Edwards, 2014). Omega-3 FAs are present in flaxseed and algae and omega-6 PUFAs are abundant in soy and sunflower oil (Gunstone, 1996).

The number of double bonds and their positions lead to distinctions in membrane fluidity which dictate the difference in melting point between SFA, MUFAs and PUFAs (Hac-Wydro and Wydro, 2007). The structure of both MUFAs and PUFAs depend on what side of the hydrocarbon the double bonds are formed. These are classified into cis or trans bonds (i.e. cis double bonds exist on the same side which cause a bend in the carbon chain, shown in Figure 1.5 below, whereas the opposite is true for trans bonds (Gunstone, 1996, Clark, Douglas and Choi, 2018). Trans fats are unsaturated FAs that contain one or more double bond in the trans configuration. They can be present in animal products, but they primarily enter our diets via processed foods in the form of hydrogenated vegetable and fish oils. These fats have a negative reputation as they have been associated with increased weight gain, atherosclerosis, inflammation and cancer risk (Koletzko and Decsi, 1997, Dhaka et al. 2011, Lopez-Garcia et al. 2005).

There are two essential FAs that humans are unable to synthesise due to the position of their double bonds, these are linoleic acid (LA) (C18:2n-6) and alpha-linoleic acid (ALA) (C18:3n-3) or linolenic acid (Glick and Fischer, 2013). These FAs are considered as a ratio in our bodies due to their competition for the fatty acid desaturase enzyme ($FADS_2$) that is required for elongation to Arachidonic acid (AA) (C20:4n-6) from LA (C18:2n-6) and Docosahexaenoic acid (DHA) (C22:6n-3) and Eicosapentaenoic acid (EPA) (C20:5n-3) from ALA (C18:3n-3) (shown in figure 1.6) (Simopoulos, 2010). A ratio favouring omega-6 PUFAs (or LA (C18:2n-6)) in the diet can interfere with the desaturation and elongation of ALA (C18:3n-3), thus favouring the production of AA (C20:4n-6) and limiting the production of EPA (C20:5n-3) and DHA (C22:6n-3). The intake of LA (C18:2n-6):ALA (C18:3n-3) has been shown to have a dose-dependent response, whereby decreasing the consumption of LA (C18:2n-6) lead to increased omega-3 LCPUFAs in plasma phospholipids in the absence of any changes in dietary intakes of ALA (C18:n-3) or omega-3 LCPUFAs (Wood et al. 2014).

The ratio of omega-6:omega-3 fatty acids in the diet has been reported to be as high as 16:1 in the typical US diet by Simopoulos in 2002. Higher omega-6:omega-3 ratios in humans have been associated with health problems due to the downstream derivatives of AA (C20:4n-6) and EPA (C20:5n-3). These derivatives are known as eicosanoids and, while they all have

important functions in relation to inflammatory response, those derived from AA (C20:4n-6) are generally more pro-inflammatory whereas derivatives of EPA (C20:5n-3) and DHA (C22:6n-3) are associated with anti-inflammatory effects. An imbalance in the levels of pro- and anti-inflammatory eicosanoids can lead to chronic inflammation and the propagation of inflammatory disease states, including cardiovascular disease, diabetes, cancer and obesity (Patterson et al. 2012, Calder, 2015, Ricciotti and FitzGerald, 2011, Simpopoulos, 2010).

In humans, *de novo* FA synthesis occurs via a biosynthetic pathway beginning with acetyl-coenzyme A (CoA) and catalysed by acetyl Co-A carboxylase (ACC) and fatty acid synthase (FAS) (Rangan and Smith, 2002, Galal, 2019). Acetyl-CoA is carboxylated by ACC to form malonyl-CoA which acts as a building block for the formation of fatty acids. The cycle repeats, adding an additional 2 carbons each time under the action of FAS (Gunstone, 1996, Demmelmair and Koletzko, 2018). In most cells this process ends when the carbon chain reaches 16 in length (Palmitic acid), however, due to the presence of thiosterase II in the mammary epithelium, chains are cleaved and released when they reach 8-14 carbons (Gunstone, 1969, Galan et al. 2019, Demmelmair and Koletzko, 2018). The presence of thiosterase II results in high concentrations of medium chain (6-12 carbons) FAs in BM.

Fatty Acids can be further classified according to their chain length with short chains having 5 carbons or less, long chains consisting of 13-21 carbons and very long chains consisting of 22 or more (Waisundara, 2018, Neville and Picciano, 1997). Fatty acids of longer chain length are created by elongation of the product of the pathway described above, in mammary epithelial cells this product is a FA of 8-14 carbons. Multiple stages of elongation and desaturation occur to create different classes of long and very long chain FAs, involving many different enzymes, as shown in Figure 1.4 overleaf. Stearoyl-CoA desaturase-1 (SCD1) acts by desaturating carbon chains (adding a double bond) and is responsible for the synthesis of MUFAs such as oleic acid (C18:1) (Gunstone, 1996, Song et al. 2018). The production of long and very long chain PUFAs involves multiple elongation and desaturase enzymes and steps. This process is particularly important in understanding the relationship between omega-3 and omega-6 FAs in humans. As mentioned previously, LA (C18:2n-6) and ALA (C18:3n-3) are precursors to AA (C20:4n-6), EPA (C20:5n-3) and DHA (C22:6n-3). As shown in Figure 1.4, the steps to convert LA (C18:2n-6) to AA (C20:4n-6) and ALA (C18:3n-3) to EPA (C20:5n-3) /DHA

(C22:6n-3) involve the same elongation and desaturase enzymes which is what creates competition between these two essential FAs and their derivatives, and ultimately determines the levels of these respective classes of PUFA in blood and tissues (Murff and Edwards, 2014, Zhuang et al. 2022).

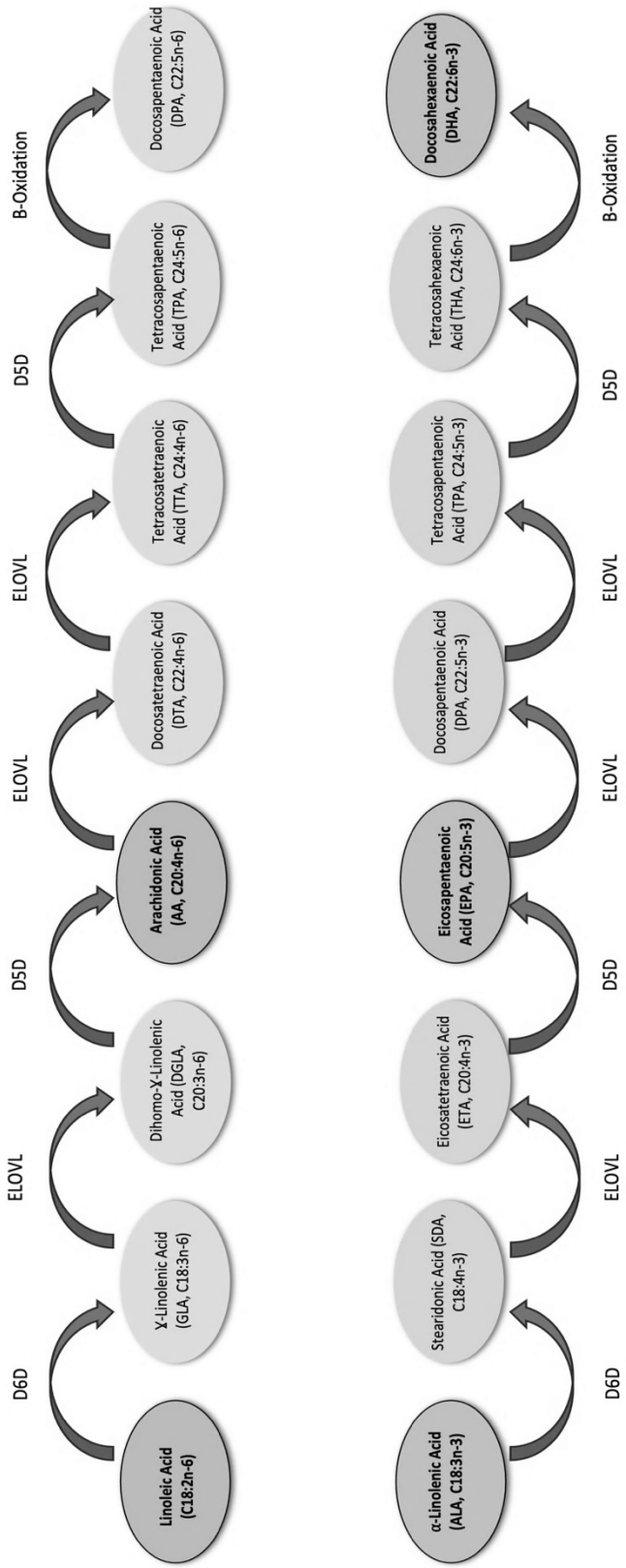

D6D
ELOVL
D5D
ELOVL
D5D
B-Oxidation
Linoleic Acid (C18:2n-6)
γ-Linolenic Acid (GLA, C18:3n-6)
Dihomo-γ-Linolenic Acid (DGLA, C20:3n-6)
Arachidonic Acid (AA, C20:4n-6)
Docosatetraenoic Acid (DTA, C22:4n-6)
Tetracosatetraenoic Acid (TTA, C24:4n-6)
Tetracosapentaenoic Acid (TPA, C24:5n-6)
Docosapentaenoic Acid (DPA, C22:5n-6)
D6D
ELOVL
D5D
ELOVL
D5D
B-Oxidation
α-Linolenic Acid (ALA, C18:3n-3)
Stearidonic Acid (SDA, C18:4n-3)
Eicosatetraenoic Acid (ETA, C20:4n-3)
Eicosapentaenoic Acid (EPA, C20:5n-3)
Docosapentaenoic Acid (DPA, C22:5n-3)
Tetracosapentaenoic Acid (TPA, C24:5n-3)
Tetracosahexaenoic Acid (THA, C24:6n-3)
Docosahexaenoic Acid (DHA, C22:6n-3)

Figure 1.4: The conversion of LA (C18:2n-6) to AA (C20:4n-6) and ALA (C18:3n-3) to EPA (C20:5n-3) /DHA (C22:6n-3), showing the multiple stages of desaturation and elongation involving D-5 desaturase (D5D), D-6 desaturase (D6D), elongation of very long chain fatty acids proteins (ELOVL). The FAs of interest are shown in bold; the others are intermediate FAs within this process.

FAs are stored within TAGs, which are structures comprised of a glycerol backbone with 3 fatty acids joined by ester bonds (shown in Figure 1.5). TAGs consist of FAs that are synthesised *de novo*, (also known as *de novo* lipogenesis (DNL)), as well as from those consumed within the diet. DNL is upregulated following consumption of carbohydrate rich meals but is lower during fasting and in response to a high fat diet. Therefore, our FA composition is highly dependent on dietary choices (Carpenter et al. 2023, Gunstone, 1996, Song et al. 2018). By way of example, diets high in the sugars fructose and sucrose are known to strongly induce DNL, leading to higher amounts of stored fat that is associated with ill health outcomes such as insulin resistance, cardiovascular disease and non-alcoholic fatty liver disease (NAFLD) (Song et al. 2018, Moore, Gunn and Fielding, 2014).

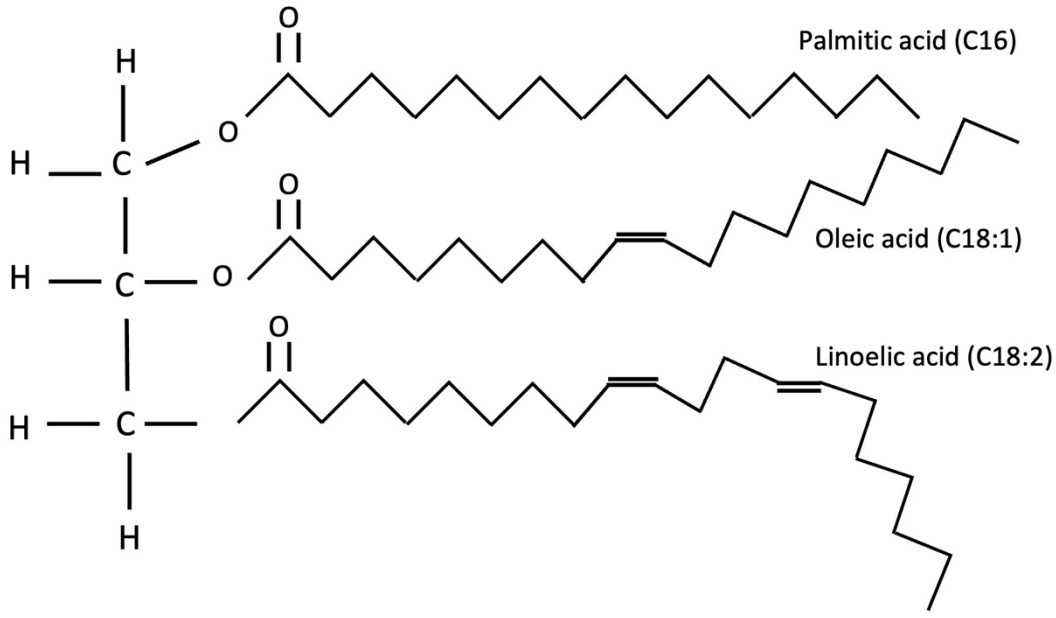


Figure 1.5: The chemical structure of a TAG comprised of 1 palmitic acid chain (C16), 1 oleic acid chain (C18:1) and 1 linoleic acid chain (C18:2) as well as 1 glycerol backbone.

Fats in BM provide close to 50% of the total energy. The major forms of fat present in BM are TAGs, making up around 98% of the fat constituents. BM also contains fat within phospholipids and cholesterol (Langley-Evans 2021, Guo. 2019). TAGs, lipid molecules and fat-soluble vitamins are encased within spherical droplets called milk fat globules (MFG) which consist of a core and outer tri-layer named the milk fat globule membrane (MFGM) (Lopez and Menard, 2011). MFGs in BM have a mean diameter of around 4.2-5.1µm. They form within the mammary epithelial cells by the coalescing of lipid droplets which acquire a plasma membrane before they are released into the lumen, taking some of the maternal cell membrane components with them (Thum et al. 2021, Langley-Evans, 2021). The contents of these droplets are derived from maternal circulation as well as being synthesised *de novo* within mammary epithelial cells.

A systematic review assessing data from >3300 women reported milk fat concentrations at 2.2-3.4 g/dL for term infants and 2.6-3.7g/dL from pre-term infants. These ranges show the average fat concentrations from 1 to 12 weeks of life and indicated that there is a gradual increase in fat concentration as milk matures (Gidrewicz and Fenton, 2014). BM fats are known as the most variable macronutrient in BM, a leading influence on this variability is the maternal diet and/or maternal fat stores (Demmelmair and Koletzko, 2018). Longer chain fatty acids are derived solely from maternal circulation, contributing to the particularly high variability of their concentrations in BM (Visentainer et al. 2018, Demmelmair and Koletzko, 2018). The FA composition of human milk, whilst variable, is generally comprised of 34-47% SFA (primarily in the form of palmitic acid (C16), 31-43% MUFA, and 13-30% PUFA (12-25% as omega-6 PUFAs and 0.8-3.6% as omega-3 PUFAs) (Delplanque et al. 2015). The fatty acid composition of BM has been shown to vary in response to a wide range of factors including maternal diet, maternal BMI and sociodemographic measures. The association between maternal intake of omega-3 FAs and their concentrations in milk has been reported in multiple studies, with most recording a strong positive correlation (Liu et al. 2016, Jorgensen et al. 2001, Jensen et al. 200). This includes the CHILD study, in which BM DHA (C22:6N-3) concentrations were positively associated with maternal fish intake and fish oil supplementation. Genetic factors have also been shown to influence BM fatty acid composition, particularly variations in the FADS gene cluster. In one study, for example, AA

(C20:4N-6) concentrations were lower in BM of mothers carrying the minor allele of fatty acid desaturase 1 (FADS1

(Miliku et al. 2019).

1.5.2 Micronutrients

Table 1.2: Micronutrient composition of BM across colostrum, transitional and mature milk as reported by Langley-Evans, 2021 and Holland et al. 1991.

	Units per 100g of milk		
	Colostrum	Transitional milk	Mature milk
Micronutrients			
Calcium (mg)	28	25	34
Phosphorous (mg)	14	16	15
Sodium (mg)	47	30	15
Zinc (mg)	0.6	0.3	0.3
Vitamin A (µg)	177.5	91.2	62
Riboflavin (mg)	0.03	0.03	0.03
Niacin (mg)	0.8	0.6	0.7
Vitamin B6 (µg)	Trace	Trace	0.01
Folate (µg)	2	3	5
Vitamin C (mg)	7	6	4

BM contains many minerals and vitamins. Table 1.2 above shows the concentrations of a representative group of these micronutrients and how they vary across colostrum, translational and mature milk. Generally, the micronutrient profile of BM meets the nutritional demands of infants, however, the concentrations of vitamins D and K have received attention as their levels are occasionally too low to meet infant needs (Greer, 2001). It is common practice in western countries for infants to receive an injection of vitamin K after birth to prevent haemorrhagic disease of the newborn that can occur because of vitamin K deficiency (Ng and Loewy, 2018). EBF babies are at risk of vitamin D deficiency and

supplementation of the mother and infant is believed to counteract this, especially in populations at higher risk of vitamin D deficiency such as those living in areas with lower sunlight exposure or those in cultures/religions that encourage extensive skin coverage (Tan, Abrams and Osborn, 2020).

The concentrations of some micronutrients in BM, including the B vitamins, vitamin A, D and iodine, have been found to vary in relation to maternal diet and body stores (Valentine and Wagner, 2013). BM concentrations of several micronutrients, including zinc, calcium and beta-carotene have been associated with infant growth and body composition as well as neurodevelopmental outcomes. However, two recent systematic reviews of studies that have reported these associations identified high levels of heterogeneity between studies, a high probability of bias and acknowledged a need for more research (Reyes et al. 2023, Lockyer, McCann and Moore, 2021). As such, both the source of micronutrients in BM, and the potential role of these micronutrients in influencing infant growth and development remains unclear.

1.5.3 Bioactive compounds

The composition of BM goes far beyond the nutritional profile created by the macro and micronutrients. The many protective benefits of BM, originate from the bioactive compounds that are present. These include oligosaccharides, proteins, hormones, antioxidants, live cells, growth factors and immune factors. Bioactive compounds originate from the maternal circulation as well as being derived from within the mammary epithelial cells, furthermore, the MFGM structure plays an important role in transporting protective proteins from epithelial cells to the infant (Ballard and Morrow, 2013, Liao et al, 2011).

Infants' immune systems are immature at birth, which increases their susceptibility to disease. Nutritional and environmental exposures during the early infant period play a critical role in the development of the immune system. Many bioactive compounds in BM are involved in this process, including proteins like lactoferrin and alpha-lactalbumin, macrophage cells, oligosaccharides and other compounds such as mucins, antioxidants and

cytokines. The specific roles of these components have not been fully elucidated. However, macrophage cells are believed to activate T-cells thus protecting the infant from infections, cytokines are associated with antibody production and B-cell activation, whilst oligosaccharides support immune cell responses as well as preventing pathogens from attaching to cells (Duijts et al. 2010, Ballard and Morrow, 2013, Lonnderdal, 2016, Gila-Diaz et al. 2019).

Growth factors such as epidermal growth factor (EGF) and insulin-like growth factor (IGF) are another group of bioactive compounds present in BM that work together to support the growth and maturation of other organ systems. EGF plays a key role in gastrointestinal development whilst IGF is believed to support tissue growth and erythropoiesis (Ballard and Morrow, 2013, Gila-Diaz et al. 2019).

1.5.3.1 Hormones

Hormones are chemical messengers that communicate between cells and tissues within the body. These messengers are produced by cells, tissues and organs, all of which make up the endocrine system. The human body produces many different hormones that can be broadly categorised into three groups: lipid derived hormones (e.g. oestrogen and testosterone), amino acid derived hormones (e.g. adrenaline and noradrenaline) and peptide hormones (leptin, adiponectin and insulin) (Molnar and Gair, 2021, Obradovic, 2021, Wilcox, 2005 Achari and Jain, 2017).

Many hormones have been identified in BM (e.g. ghrelin, cortisol, epidermal growth factor) (Savino, Liquori and Lupica, 2010). The concentrations of metabolic hormones ingested by infants, such as leptin and adiponectin, have been shown to influence infant metabolic development and body composition. Infant intake of leptin concentrations measured in whole milk has been positively associated with infant fat mass during the first 12 months of life (Gridneva et al. 2018). The same group also noted a positive association between adiponectin intake and adiposity as well as a negative association with infant lean mass.

Leptin

Leptin is derived primarily from adipocytes and concentrations of this hormone in human plasma correlate positively with total body fat stores (Sinha et al. 1996). Leptin plays a central role in regulating energy homeostasis and works by suppressing hunger cues in response to a positive energy balance within the body (Keim, Stern and Havel, 1998, De Graaf et al. 2004, Savino, Liquori and Lupica, 2010). In addition to adipose tissue, leptin has also been shown to be synthesised by both the placenta and the mammary epithelium in the perinatal period (Masuzaki et al. 1997, Smith-Kirwin et al. 1998). In addition to being synthesised within the mammary gland, leptin is transferred from the maternal circulation to milk, this is believed to be the primary source of leptin in BM (Casabiell, 1997, Weyermann et al. 2006).

Leptin concentrations are higher in colostrum than transitional and mature milk when infants are born at full term, in mature milk leptin concentrations are around 1.8ng/ml (Panuganti, Bazzano and Ley, 2021, Khodabakhshi, 2015, Ilcol et al. 2006). Milk leptin is higher in overweight or obese mothers and is positively associated with gestational weight gain while being negatively associated with postpartum weight loss (Sims et al. 2020, Sadr Dadres et al. 2019). Serum leptin concentrations are higher in breast fed infants than those fed infant formula (Savino et al. 2002). Milk leptin concentrations at 1 month were negatively correlated with infant BMI at 18 and 24 months (r=-0.493 and r=-0.456 respectively), although this correlation was not found within milk at 3, 6 or 9 months of age (Miralles et al. 2006, Panuganti, Bazzano and Ley, 2021). In addition, a systematic review assessing the influence of BM composition on infant anthropometrics in the first 2 years, reported that BM leptin concentrations had negative associations with multiple anthropometric measures, including infant weight, weight for length-z scores (WLZ), weight for age Z-scores (WAZ) head circumference, % fat, total fat mass and free fat mass (Brockway et al. 2023). This is contradictory to the associations reported by Gridneva and colleagues, as noted above. They observed positive correlations between leptin and infant adiposity, however, they calculated infant intake of this hormone whilst the majority of other studies refer to actual leptin concentrations in BM.

Adiponectin

Adiponectin is another peptide hormone and, like leptin, is primarily derived from adipose tissue in human adults. Adiponectin has anti-inflammatory and anti-atherogenic properties, it promotes insulin sensitivity and glucose uptake (Schondorf et al. 2005). Adiponectin is also reported as being protective against obesity, type-2 diabetes and coronary heart disease (Panuganti, Bazzano and Ley, 2021). It has been detected in BM and is thought to derive from maternal circulation as well as being synthesised within the mammary epithelium (Savino, Liquori and Lupica, 2010, Catli, Dundar and Dundar, 2014).

Adiponectin levels have been reported to decrease as lactation progresses and are present at much higher concentrations in BM than leptin, at around 19ng/ml (Panuganti, Bazzano and Ley, 2021, Bronsky et al. 2011, Gridneva et al. 2018, Martin et al. 2006). Adiponectin levels in milk were positively associated with post-pregnancy maternal BMI by Martin et al. in 2006. However, no such association was identified in a large cohort of 430 mothers, or in a systematic review on 26 studies (Chan et al. 2018, Andreas et al. 2014). Adiponectin is present at high concentrations in cord blood, and concentrations of this hormone in infant serum in the first days of life have been positively correlated with birth weight and infant adiposity (Inami et al. 2007, Savino, Liquori and Lupica, 2010, Weyermann et al. 2006). Concentrations of adiponectin in BM are mostly associated negatively with infant weight and adiposity. Furthermore, in the 2023 systematic review completed by Brockway and colleagues, BM adiponectin concentrations were negatively associated with infant weight, length, WLZ, WAZ, and head circumference (Brockway et al. 2023). However, a systematic review focusing on infant intake rather than concentrations report positive associations with high weight gain and infant adiposity and negative associations with FFM (Norrish et al. 2023).

Insulin

Insulin, another peptide hormone, is synthesised in pancreatic cells in response to increased plasma glucose (Fu, Gilbert and Liu, 2013). Insulin plays a critical role in blood glucose metabolism, stimulating glucose uptake by cells, promoting lipogenesis and inhibiting lipolysis (Sonksen and Sonksen, 2000). Insulin has been identified in BM and exists in much higher concentrations in BM than in maternal serum (Young et al. 2017). Insulin is present in

higher concentrations in colostrum and transitional milk and declines as lactation progresses (Ley, Hanley and Mathew, 2012, Panuganti, Bazzano and Ley, 2021).

Maternal BMI and metabolic conditions, including insulin resistance, have been associated with higher levels of insulin in BM (Sims et al. 2020, Ley, Hanley and Mathew, 2012, Young et al. 2017, Chang et al. 2018), although this is not consistent across the literature (Panuganti, Bazzano and Ley, 2021). Insulin concentrations in BM, and their associations with maternal BMI, are thought to influence the infant microbiome (Panuganti, Bazzano and Ley, 2021, Lemas et al. 2016) which could in turn impact the developing infant's metabolism and immune development. Chan et al reported a U-shaped association between insulin concentrations in BM and infant WLZ scores in 2018. Another study found higher BM insulin concentrations to be associated with lower infant weight and lean mass at 1 month old (Fields and Demerath, 2012). In contrast, multiple further studies have failed to note any significant response in infant growth and composition to HM insulin levels (Cheema et al. 2021, Fields et al. 2017, Goran et al. 2017).

1.5.3.2 Oligosaccharides

BM oligosaccharides, or human milk oligosaccharides (HMOs) as they are commonly referred to, are unconjugated complex sugars that can vary in length but tend to have between 2 and 22 sugar units. Up to 200 different HMOs have been identified to date (Ninonuevo et al. 2006, Gridneva et al. 2019). The building blocks that make these sugar chains are glucose, galactose, n-acetylglucosamine, fucose and n-acetyl-neuraminic acid (Ninonuevo et al. 2006, Hanau et al. 2020, Bode, 2015). HMOs are synthesised within the mammary epithelial cells as an extension to lactose synthesis in the Golgi apparatus (Bode, 2012). Their functions in BM are highly protective. They are antimicrobial, play a role in preventing the attachment of pathogens to cell surfaces and they can positively influence immune cell responses. They also play an important role in the development of a healthy gut microbiome in infants by acting as bifidus factors and supporting the growth of commensal bacteria (Bode, 2015, Bode, 2012).

Oligosaccharides are present in volumes of around 10-15g per litre in BM and at much lower concentrations in bovine milk (Bode, 2015, Hale and Hartmann, 2007). The concentrations and composition of HMOs vary across the course of lactation and between women. Total oligosaccharide concentrations generally decrease as lactation progresses, aside from a sharp spike around 2 weeks postpartum, and decline from ~9g/L within the first 14 weeks to ~4g/L at 1 year postpartum. This general decrease in total HMO concentrations is driven by fluctuations in the 1,2 linked fucosyloligosaccharides, whereas the concentrations of 1,3/4 linked fucosyloligosaccharides increase slightly from ~1g/L during the first week to ~2g/L at 1 year postpartum (Chaturvedi et al. 2001, Gridneva et al. 2019, Bode et al. 2015). Genetic factors, parity and BF exclusivity have all been reported to influence concentrations and composition of HMOs within individual mothers' milk (Bode, 2012, Alderete et al. 2015, Chaturvedi et al. 2001).

1.6 Variations in BM Composition

Understanding how BM varies is paramount to understanding its complex nature. BM is a fluid that is ever evolving, changing both within and between women in response to many stimuli. BM composition varies longitudinally, for example depending on the length of a feed, time of day and stage of lactation, as well as to individual factors such as infant gestational age, maternal BMI and diet (Dror and Allen, 2018, Ballard and Morrow, 2013, Samuel et al. 2020).

1.6.1 Longitudinal changes in BM composition

1.6.1.1 Stage of lactation

As shown in Tables 1.1 and 1.2, BM composition changes as lactation progresses. The compositional differences between colostrum and mature milk are particularly distinct, however further compositional shifts occur the longer BF is sustained. Colostrum, which is produced before and for the first few days after women give birth, is a thick, sticky substance and contains low concentrations of lactose and high concentrations of protein and immune and growth factors. Furthermore, sodium, chloride and magnesium are higher,

and calcium and potassium are lower in colostrum than in mature milk (Langley-Evans, 2021, Ballard and Morrow, 2013, Kulski and Hartmann, 1981). As previously described, secretory activation (or lactogenesis II) occurs in the first week after delivery and initiates the closing of tight junctions between mammary epithelial cells. This causes lactose concentrations to increase, together with the ratio of sodium to potassium in BM, and signals the transition towards mature BM, which is attained at approximately 2 weeks after delivery (Ballard and Morrow, 2013, Kulski and Hartmann, 1981).

Slight decreases in protein and increases in fat have been observed as lactation progresses and lactose is thought to increase up to 4-7 months before declining gradually thereafter. Oligosaccharide concentrations in BM decrease over time, whereas FA concentrations remain relatively stable (Bauer and Gerss, 2011, Andreas, Kampmann and Le-Doare, 2015, Moya-Alvarez et al. 2022, Langley-Evans, 2021).

1.6.1.2 Across the day and throughout a feed

Interestingly, BM also changes across more acute timeframes, with differences in composition being observed across the day and even within a feed. Fat concentrations are reportedly higher in the morning compared to the evening (Kociszewska-Najman et al. 2012, Cannon et al. 2015). Some studies, including a study by Cannon et al. in 2015, have also reported changes in BM lactose concentrations across the day, but other studies have failed to identify this diurnal variation (Italiner, et al. 2020). Similarly, protein concentrations have been shown to vary across the course of the day in some (Lammi-Keefe, Ferris and Jensen 1990, Lopez et al. 2011), but not all studies (Cetinkaya et al. 2017). Therefore the extent of this variation, and the factors driving it, is unclear. There also appear to be minimal changes in lactose and protein concentrations across a feed (Mitoulas et al. 2002, Khan et al. 2013, Leghi et al. 2020). In contrast, BM fat concentrations are consistently reported to increase as feeds progress, with changes of 24g/L and higher being documented between fat concentrations at the beginning vs the end of a feed (Freed et al. 1986, Khan et al. 2013). Positive correlations between BM fat concentrations and the degree of breast emptying have been reported (Daly et al 1993, Saarela, Kokkonen and Koivisto, 2005, Mizuno et al. 2009). While the mechanism underlying this association is not completely understood, it is

speculated to be due to the increasing size of milk fat globules or an increase in their release from the mammary gland as more milk is emptied from the breast (Mizuno et al. 2009).

1.6.2 Inter-individual variations in BM composition

1.6.2.1 Gestational age

BM from a woman who has delivered a pre-term infant differs to milk from a women who delivered at full term. A systematic review reported that lactose concentrations were lower, while protein concentrations were higher in pre-term compared to term BM in the immediate post-partum period. The same systematic review concluded that fat content was similar between pre-term and term BM, however individual studies have reported differences in fat concentrations and energy content between pre-term and term milk (Gidrewicz and Fenton, 2014, Bauerr and Gerss, 2011, Mills et al. 2019).

1.6.2.2 Maternal body composition

BM composition also varies in response to maternal factors. Mothers who are overweight or obese were noted to have higher fat and protein concentrations and lower total carbohydrates in their BM compared to women of normal weight (Daniel et al. 2021, Leghi et al. 2021, Sims et al. 2020, Binder et al. 2023). Glucose, however, has been reported in higher concentrations in the milk of mothers with obesity than in those with normal weight (Ahuja et al. 2011). As mentioned previously maternal BMI can also influence the concentrations of certain hormones including leptin, insulin and possibly adiponectin (Sims et al. 2020, Martin et al. 2006, Chan et al. 2018, Geddes et al. 2021).

1.6.2.3 Maternal diet

As previously discussed, BM constituents are synthesised both *de novo* within the mammary epithelial cells and through the transport of composites from maternal circulation. Therefore, maternal nutritional status and habitual diet have significant influence on BM composition. Few studies have looked specifically at the association between maternal carbohydrate intake and the carbohydrate content in breast milk. However, a small intervention study in

America comparing normal vs high carbohydrate diets found no difference in breast milk carbohydrate concentrations. In contrast, a larger study on 136 mothers in Vienna noted a statistically significant association between habitual maternal carbohydrate intake and carbohydrate content in BM (r=0.999) (Binder et al. 2023). The authors failed to comment on their findings related to carbohydrates and it seems that there is a considerable lack of evidence looking into the association between maternal diet and total milk carbohydrate concentrations. A study designed to assess the impact of high sugar intake on milk sugar content provided mums with high sugar drinks as well as artificially sweetened drinks. They showed that ingestion of fructose acutely increased the fructose concentrations in BM, however, lactose and glucose remained unaffected (Berger et al. 2018). Another study found that maternal carbohydrate and energy intakes appear to illicit changes in the oligosaccharide composition of breast milk (Seferovic, 2020). The relationship between maternal sugar intake and breast milk composition requires more attention and more studies such as that carried out by Berger et al. in 2018.

Protein concentrations in milk have also been shown to be responsive to maternal protein consumption (Forsum and Lonnerdal, 1980, Binder et al. in 2023). The older of these studies implemented a 4-day dietary intervention providing various levels of protein and found that milk protein concentrations increased with higher levels of protein consumption (Forsum and Lonnerdal, 1980). This study contained only 3 participants, however, which undermines the impact of their findings greatly. The study completed by Binder and colleagues was more robust, including over 130 women, and used a 24-hour recall method to determine habitual dietary intake. They noted that protein intake was associated with higher milk protein concentrations ($p<0.05$). They also reported lower protein concentrations in women with low BMI vs normal or higher BMI and increased protein concentrations in milk of women with high BMI ($p<0.05$). This group have also reported that the women with higher BMI scores consumed significantly more protein, making it difficult to ascertain which factor is the driving force behind these differences in milk protein concentrations between the groups. Furthermore, the authors themselves acknowledge this study included infants at varying ages, which may impact the findings reported (Binder et al. 2023).

Contrastingly, other studies have failed to identify such associations including Bravi's systematic review compiling 36 studies on breast milk composition and maternal diet. It is worth noting that there were much fewer studies reporting on associations between dietary and milk protein concentrations compared to the other milk components, showing there is a need for more research in this area (Boniglia et al. 2003, Minato et al. 2019, Bravi et al. 2016).

The relationship between maternal fat intake and BM fat concentrations has received considerable attention. 18 of the 36 papers included in Bravi's systematic review on maternal diet and BM composition focused on FAs and 6 reported on total fat. Conversely, 5 studies reported on protein and only 1 of the included studies researched lactose concentrations (Bravi et al. 2016). Bravi et al reported a considerable degree of heterogeneity between the studies included as well as those that did not meet the inclusion criteria. Of those that were included, 3 reported a link between maternal fish consumption and BM DHA (C22:6N-3) concentrations. This is supported by other studies investigating relationships between consumption of different food types and fatty acids in BM, who noted significant associations between fatty fish and milk DHA (C22:6N-3) (r_s=0.46, p=0.01) and margarine consumption and milk ALA (C18:3N-3) (r_s=0.44, p=0.02) (Jonsson et al. 2016). In a cross-over trial Nasser et al. reported significant differences in BM lauric, stearic, palmitoleic, ALA (C18:3N-3) and arachidonic acid (AA (C20:4N-6) concentrations, when high fat and low-fat diets were provided over 4 days.

Long chain fatty acids (LCPUFAs) including DHA (C22:6N-3) and AA (C20:4N-6) have received considerable attention in the literature due to their perceived role in brain and retina development in infancy (Uauy, et al., 2001, Innis, Gilley & Werker, 2001). Many studies have observed positive relationships between habitual maternal DHA (C22:6N-3) intake and DHA (C22:6N-3) concentrations in BM ($r=0.477$; $P<0.001$) (Krešić et al. 2013, Liu et al. 2016) There are, however, other studies that have failed to observe significant correlations between the two (Iranpour et al. 2013, Jirapinyo et al. 2008). Maternal supplementation of LCPUFAs has been shown to significantly increase relative concentrations in BM in both observational and interventional study designs (Quin et al. 2020, Ueno et al. 2020, Argaw et

al. 2021, Ay et al. 2018, Bergmann et al. 2008, Jensen et al. 2000). This finding has also been reported in a systematic review (Amaral et al. 2017).

A study on Malaysian women found significant associations between increased trans fatty acids in milk and habitual intake of foods such as cheeseburgers (r_s=0.24, p<0.05), buns (r_s=0.21, p<0.05) and mayonnaise (r_s=0.26, p<0.05) (Daud et al, 2013). Cholesterol levels were measured in breastmilk following high PUFA, high phytosterol and low cholesterol diets as well as low PUFA, low phytosterol and high cholesterol diets. Significant associations were found between high phytosterol diet and increased phytosterols in plasma (r=0.62, P=0.0001) and phytosterols in plasma and milk (r=0.39, P=0.02) (Mellies, Guy and Glueck, 1978).

Most of the studies referenced above assessed the influence of maternal diet on BM composition using observational habitual dietary data or through interventions spanning multiple days, weeks or months. There is a notable lack of studies reporting on acute or 'in real time' effects, leaving a large gap in our understanding of how maternal daily dietary choices impact BM composition.

1.7 Breast milk research methodologies

Research into human milk has been ongoing for decades, and many researchers and groups have dedicated their work to understanding this nutritional substance.

The methodologies underpinning this work vary due to the many factors involved with breastfeeding and breast milk. These factors include, but are not limited to, maternal and infant characteristics and demographics (as discussed above), milk collection procedures, milk storage conditions and milk collection timings. Research shows that methodologies can have significant impacts on study outcomes and conclusions (Leghi et al. 2020, Chang et al. 2012, Kim et al. 2019). When designing a breast milk research study all factors must be considered and every effort to minimise confounding the results must be made, however, it is difficult to account for so many potential impacts whilst also ensuring a practical and appropriate methodology for the mother, baby and researcher.

Breast milk collection procedures and how they impact milk macronutrient measurements were assessed via a systematic review by Leghi and colleagues in 2020. This review included data from 101 studies. They characterised the collection methods applied into 3 categories: collection of milk from all feeds over 24h (n=32), collection of milk at 1 timepoint (n=62) and other methods (n=7). They recorded differences in the fat and protein concentrations measured across the methodologies but found no difference within lactose concentrations. The studies within this review varied in other aspects of methodology, i.e. gestational age of infants included, breastfeeding exclusivity of dyads included and analytical methods. The significant differences noted by this group suggest that collection methods do matter, however, the above listed factors may have influenced these outcomes and thus more research is required to address this methodology consideration.

Studies on milk storage conditions also show significant differences within macronutrient concentration measurements. Two groups considering this impact have reported differences in macronutrient content of milk when it has been stored and thawed in different ways. The process of freezing and thawing/warming milk seems to impact the macronutrient composition. Both studies recommend babies are fed breast milk fresh and without storage or freezing to minimise the potential impact from their reported differences. A group in Korea reported differences in milk fat, total protein and carbohydrate when milk was frozen vs fresh, they also found differences in milk composition after various thawing methods in comparison to fresh milk (Kim et al. 2019). Similarly, a group in Taiwan observed significant differences in the fat, protein and carbohydrate concentrations in milk after being stored, frozen and thawed vs when the milk was fresh (Chang et al. 2012). Again, there were other factors such as infant age, milk collection timings and breastfeeding exclusivity that may have confounded these results, however, it does appear that freezing milk has an impact to some extent on the macronutrient composition.

When designing milk compositional studies, the above information should be considered and built into the methodology. It is, however, difficult to prevent the changes observed following storage, freezing and thawing as analysis of fresh milk samples is rarely feasible in a research setting. Timings and other factors such as maternal and infant characteristics can be

controlled slightly more, and efforts to minimise extreme variations in these factors would likely reduce some confounding of study findings.

1.8 How breast milk influences infant health and development

1.8.1Infant growth and anthropometry

A recent systematic review of the literature reported on 57 studies researching associations between BM composites and infant growth outcomes (Brockway et al. 2023). They found evidence to support a positive association between BM protein concentrations and infant length. Furthermore, BM carbohydrate concentrations were positively associated with infant weight. Brockway and colleagues reported mixed conclusions in relation to BM fat. Although the consensus across many studies suggests that fat concentrations are negatively associated with infant BMI, weight gain and WAZ other studies report positive associations between BM fat, infant weight and body fat measures. It is also important to note that the authors of this review reported a high degree of heterogeneity between studies and their utilised methods, and that this made it difficult to draw robust conclusions from the entirety of the literature (Brockway et al. 2023).

While there is variability in the findings across the scientific literature, many individual studies investigating different composites of BM and their influence on infant anthropometry and growth have reported strong and significant associations. A randomised controlled trial with 208 mothers designed to alter BM long chain polyunsaturated fatty acids (LCPUFAs) through supplementation observed positive associations between infant skin fold thickness at 12 months and milk DHA (C22:6N-3) (β=1.43, 95% CI=0.01, 2.84), EPA (C20:5N-3) (β=6.53, 95% CI=0.29, 12.77), and total omega-3 LCPUFAs (β=0.91, 95% CI=0.00. 1.82). The same study reported negative associations between total omega-6 LCPUFAs concentrations with infant weight (β=-687.65, 95% CI=-1,218.36, -156.95), BMI (β=-1.65, 95% CI=-2.71, -0.59) and lean body mass at 4 months (β=-491.69, 95% CI=-857.20, -126.18) (Much et al. 2013).

In rodent studies, work done by Draycott et al has shown a negative relationship between

offspring birth weight when maternal diets were higher in fat across pregnancy and lactation ($p<0.001$). They also observed offspring sensitivity to the omega-6:omega-3 ratio within maternal diets (Draycott et al. 2020). Omega-3 FAs within the diet of dams has been shown to influence offspring outcomes during an intervention involving supplementation with DHA (C22:6N-3) that led to higher percentage body fat in male and female offspring at 6 weeks (Muhlhausler et al. 2011). A systematic review investigating the effect of omega-3 LCPUFA supplementation in humans by Muhlhausler and colleagues found the volume of studies on the subject were low and reported variable conclusions supporting positive, negative and neutral associations with infant fat mass (Muhlhausler, Gibson and Makrides, 2010).

Work done by Gridneva and colleagues in the University of Western Australia reported strong associations with infant limb fat areas and certain BM components within a recent pilot study (Gridneva et al. 2022). These significant relationships were reported with calculated infant intakes, interestingly they found absolute concentrations did not correlate as strongly. This group have identified the importance of calculating infant intakes when assessing the influence of BM components on infant outcomes (Gridneva et al. 2023). Calculated daily intakes are calculated by measuring the concentrations of milk components as well as recording data on 24-hour milk intake and milk production (Gridneva, 2018). They found a positive and time dependent association with daily intakes of lysozyme ($p=0.001$) and HMOs ($p=0.004$) in BM and mid-arm fat in infants. Furthermore, they reported larger mid-arm ($p=0.008$) and mid-thigh ($p<0.001$) fat areas in infants with increased breastfeeding frequency (Gridneva et al. 2022).

The same group published a systematic review reporting on the correlations between infant body composition and BM macronutrients and bioactive compounds (Norrish et al. 2023). Only three studies found significant correlations between protein intake and infant outcomes. Two of the three reported positive associations between infant intake of protein and infant adiposity, one found this relationship changed to a negative association after three months of age. The third study's findings contradicted the other two, showing a positive relationship between infant protein intake and fat free mass. Lactose intake was positively associated with infant adiposity in most of the included studies, two studies also reported a positive

relationship with FFM. Two studies showed a change in direction of the relationship between lactose intake and fat free mass, similar to that observed with protein. Total carbohydrate intake results varied across the included studies. One study failed to identify any significant relationships, another noted positive associations with infant abdominal adiposity and a third reported negative associations with carbohydrate intake and fat free mass and positive associations between intake and fat mass. Total fat intake was positively associated with infant weight gain at 3 months in only one study. In another study multiple lipid species were positively associated with infant weight, head circumference and WLZ, these correlations were all time dependent. Similar to what was observed by Gridneva and Norrish, another study found that carbohydrate and protein intake from BM was associated positively with infant anthropometric measures. Namely, carbohydrate intake was associated with higher weight gain at 0-6 weeks (β=0.04$\pm$0.01, p<0.001) but lower weight gain at 3-12 months (β=-0.03$\pm$0.01, p=0.025). Protein intake was associated with higher weight gain (β=0.22$\pm$0.04, p<0.001) and skin fold gains (β=0.28$\pm$0.07, p<0.001) at 0-6 weeks but lower skin fold gains at 3-12 months (β=-0.19$\pm$0.07, p=0.006). They also observed a significant positive relationship between breast milk intake at 4-6 weeks and infant weight gain between 0-6 weeks (r=0.52, p=0.0001), however, report that this effect is reversed between 3 and 12 months (Olga et al. 2023).

The review completed by Norrish and colleagues also assessed relationships of infant anthropometry and bioactive compounds, including metabolic hormones, in BM and found positive associations between infant intakes of these compounds and various growth measurements. Adiponectin intake was positively associated with high weight gain and infant adiposity. It was also negatively associated with FFM. Associations were varied between leptin intake infant outcomes. Two studies reported positive associations with leptin intake and infant whole-body adiposity. Another found no association with leptin intake and visceral or abdominal adiposity yet a further study found contrasting associations between leptin intake and weight gain at different ages. In skim milk, leptin intakes were associated with infants with higher weight gain compared to those with normal weight gain at 2 months, whilst the opposite was true for infants at 3 months. Some studies have reported positive associations with infant BMI and milk leptin while others have reported negative or no

associations (Gridneva et al. 2018, Miralles et al. 2006, Chan et al. 2018, Kon et al. 2014, Uçar et al. 2000). Chan et al. reported inverse relationships with milk leptin concentrations and infant WLZ scores at 4 months (β=-0.67, CI=-1.17, -0.17) and 1 year (β=-0.58, CI=-1.02, -0.14) (Chan et al. 2018). They also studied the relationship between milk adiponectin and infant body composition at 4 months and 1 year postpartum and found no significant correlations. In contrast, Woo et al. found higher milk adiponectin concentrations were negatively correlated with infant WLZ (β=-0.02±0.04, p<0.0001) and WAZ (β=-0.29±0.08, p=0.0002) scores in predominantly BF infants between 0-6 months of age (Woo et al. 2009). Chang et al reported a U-shaped association between insulin concentrations in BM and infant WLZ, such that intermediate concentrations were associated with lower WLZ at 4 months (β=-0.51, CI=-0.87, -0.15) and 1 year (β=-0.35, CI=-0.66, -0.04) (Chang et al. 2018).

1.8.2 Disease and allergy

BM is believed to be a protective as well as nutritive substance and BF is readily encouraged due to its ability to reduce the risk of disease in infancy and later in life. A longitudinal study conducted recently in Ireland found that breastfed infants had a significantly reduced incidence of ill health including hospital visits (CI=-0.06, 0.03) and morbidities such as respiratory infections (CI=-0.12, 0.08), asthma (CI=-0.03, 0.01), eczema (CI=-0.08, 0.04), vomiting (CI=-0.03, 0.0) and skin issues (CI=-0.04, 0.0) (Murphy et al. 2023). Similarly, a systematic review found that EBF babies between 6-23 months had significantly lower risks of any mortality, including infection related mortality. Risk for each gradually increased from EBF to partial BF, some BF and finally to no BF, which was associated with the highest risk for both outcomes (Sankar et al. 2015). An older systematic review and meta-analysis found that BF was protective against short-term ill-health such as gastroenteritis, lower respiratory tract infections, atopic dermatitis, asthma, necrotising enterocolitis as well as longer term illnesses including childhood leukaemia, sudden infant death syndrome, obesity and type-1 and -2 diabetes (Ip et al. 2007).

Furthermore, BF has been reported to be significantly protective against allergy and atopic disease. A large observational study in Sweden found exclusive BF was protective against

asthma (OR=0.66, 95% CI=0.51, 0.87), atopic dermatitis (OR=0.85, 95% CI=0.71, 1.0), and allergic rhinitis (OR=0.73, 95% CI=0.54, 0.99), and partial BF was associated with a reduced risk of asthma (OR=0.69, 95% CI=0.52, 0.91 (Kull et al. 2002). Another observational study in the US found that infants who were partially BF for 3 months were more likely than infants who were EBF for 3 months to develop food allergy symptoms (Matthias et al. 2019).

1.8.3 Visual, cognitive and behavioral development

BF has been linked to improved visual acuity and cognitive outcomes (Birch et al. 1993 and 2007). A large, randomised trial found that an intervention to improve BF rates and duration led to increased numbers of infants being EBF at 3 months and an overall higher prevalence of any BF. These outcomes were also significantly positively associated with multiple cognitive development measures including full scale IQ (mean difference=+5.9, CI=-1, 12.8) at 6 years of age (Kramer et al. 2008). Similar findings have been reported in a meta-analysis that found BF was associated with better cognitive development in infants compared to those who were fed formula milk (mean difference=2.66, 95% CI=2.15,3.17) (Anderson et al. 1999). BF for 4 months or longer has been associated with improved motor skills, adaptability, sociability and communication in infants between 1 and 3 years of age. Babies who were breastfed for less than 4 months were significantly more at risk for an atypical score in all of the above listed outcomes at 1 year (OR=1.49, 95% CI=1.16, 1.92) 2 years (OR=1.29, 95% CI=1.01, 1.65) and 3 years of age (OR=1.34, 95% CI=1.06, 1.70). Interestingly, this group also noticed that boys were more susceptible to this effect than girls (Oddy et al. 2011). There are concerns, however, that factors such as maternal education and socioeconomic status may confound such observations. Women who EBF are more likely to be educated to degree level and come from a higher socioeconomic background and educated, therefore they may in turn spend more time in educational play with their infants.

Furthermore, the levels of individual components such as FAs within BM have been reported to have an influence on infant development. By way of example, a study on 960 mother-infant dyads from the Mother's and Children's Environmental Health study reported significant relationships between infant mental and psychomotor development scores at 6

months of age and the omega-6:omega-3 ratios and LA (C18:2N-6):ALA (C18:3N-3) ratios in the maternal diet (Kim et al. 2017). Another study observed higher levels of DHA (C22:6N-3) in the brain cortex of BF infants when compared to AF infants, suggesting this FA is important for early cognitive development (Makrides et al. 1994). However, further studies have failed to find associations between specific FAs and infant cognitive outcomes. Innis et al. in 2001 found significant associations with DHA (C22:6N-3) in infants' blood and visual development but did not measure DHA (C22:6N-3) in BM. Another study found a similar association between infant erythrocyte DHA (C22:6N-3) measures and visual acuity, but this association was not present between BM DHA (C22:6N-3) and visual outcome measures (Lauritzen et al. 2004). Hurtado et al. in 2015 did not find any significant relationship between BM FAs and visual acuity. As a result of these mixed findings, the effects of BM LCPUFAs on cognitive development remain unclear (Keim et al. 2012, Hurtado et al. 2015).

Fewer studies report on the specific BM components that influence infant behaviour and behavioural development but a relatively small study on pre-term infants found positive associations between early BM concentrations of mead acid and AA (C20:4N-6) and very early measures of infant behaviour including (Lundqvist-Persson et al. 2010).

1.8.4 Early programming of health and disease

The developmental origins of health and disease (DOHaD) hypothesis utilizes experimental and epidemiological evidence to comprehend the mechanisms underlying how early environmental influences impact health outcomes in later life. This concept continues from forerunners such as the Barker hypothesis that proposed that the environmental, nutritional and psychosocial exposures experienced during critical windows of development have a lasting impact on an individuals' health. It also proposes that adverse exposures can serve to predispose the infant to the development of a range of non-communicable diseases later in life, including obesity, cardiovascular disease and diabetes (Gluckman, Hanson and Buklijas, 2009, Kim, 2004). The DOHaD concept integrates a focus on epigenetics and the first 1000 days of life. Researchers around the world are dedicating their work to maximising on the

preventative potential of this life stage for later health and disease outcomes (Roldao 2023, Geddes and Prescott, 2013, Black et al. 2008).

The Barker hypothesis states that infant systems are plastic and subject to programming at this stage of life. The concept of early life programming is essentially a cause-and-effect phenomenon and consists of a cause or stimuli (e.g. nutrition) and a physiological adaptation to the stimuli that lasts into later life (e.g. insulin resistance). As previously mentioned, BF has been associated with improved disease, allergy and developmental outcomes. It is the first nutritive substance in life and therefore one of the main environmental influencers on infant development. By extension BM has the potential to protect from disease in adulthood, thereby, improving morbidity and mortality in entire generations (Langley-Evans, 2015, Geddes and Prescott, 2013, Gluckman, Hanson and Buklijas, 2009, Lucas, 2007).

Changes in the mother and infants' environment during lactation can impact the composition of milk being produced and consumed. This concept has been studied in rat models, as well as in some human studies and evidence suggests these can impact health outcomes in later life. Lisboa et al have published a narrative review on this topic and discuss both rodent and human studies. They summarise how factors including maternal stress, drug use and early weaning can influence milk composition and lead to poor outcomes in adulthood such as increased body fat, obesity, altered lipid metabolism, hypertension and hormonal disturbances (Lisboa et al. 2021, Bonomo et al. 2007, Eberle, 2021, Souzo et al. 2020, Miranda et al. 2020).

Further to the above listed influences on the infant's environment, maternal nutrition and nutritional status act as major influencers on BM composition, which can act as an imprinting factor for later development of comorbidities (Pico et al. 2021). The obesogenic, or cafeteria, diet during lactation in rodents leads to increased fat accumulation, altered lipid profiles and impaired responses to glucose tolerance tests (Pomar et al. 2017). In another rodent study spanning pregnancy and lactation, offspring from obese dams that had been fed an obesogenic chow diet had dysregulated metabolic phenotypes at 3 months with a predisposition for non-alcoholic fatty liver disease (NAFLD) (Oben et al. 2010). Furthermore,

in this study a similar phenotypic response was observed in the offspring of lean adult mice that were suckled by obese mice, showing an independent effect from obesity during lactation. Research done by George and colleagues in 2019 also reports on a lactation specific influence from maternal obesity, leading to unfavourable outcomes such as increased adiposity, insulin resistance and higher fasting blood glucose (George et al. 2019, George et al. 2019). Pico et al report that, in animal models, an obesogenic dietary pattern in pregnancy rather than an excess of adiposity (or obesity) seems to be the predominant indicator of adverse outcomes in infants. This is based on a study that showed regression of altered programming effects in offspring when a normal diet was resumed during pregnancy, after obesity had been induced by a high-fat diet (Zambrano et al. 2010). The same influence from diet was ascertained in another rodent study spanning lactation, however, they noted that pre-existing obesity in the parent is also important in determining the influencing aspect of milk composition (Rolls et al. 1986).

Maternal weight and BMI have been positively associated with BM leptin concentrations in human studies (Cagiran Yilmaz and Ozcelik 2021, Miralles et al. 2006). Miralles and colleagues also noted that BM leptin concentrations were negatively related to infant BMI at 18 and 24 months of age (r=-0.493 and r=-0.456 respectively). As discussed earlier in this chapter, leptin plays a central role in the regulation of appetite and energy balance. It is suggested that leptin in BM can offer protection from obesity through this action and may suggest why BF is associated with a lower risk of obesity and weight gain (Roldao, 2023, Miralles et al. 2006).

1.8.5 Impact of BF on mothers

BF is often encouraged and prioritised for its protective and beneficial influence on infant health, growth and development. However, the mother is also a beneficiary of this feeding choice. BF is believed to assist with and improve the rate of uterine involution postpartum, as well as reducing the rate of bleeding in the immediate postpartum period (Yahya, Sabati and Mousa, 2019). It is believed that there is a beneficial relationship between BF and postpartum weight loss in BF mothers. This advantage can be strengthened when mothers EBF their infants and when BF duration is maintained for longer (Baker et al. 2008). BF

supports a strong bond between mother and baby, whilst this of course occurs in the absence of BF, the brain responses in BF mothers to their baby's cry showed increased sensitivity in the first month postpartum than in mothers who were not BF (Kim et al. 2011).

Further to these short-term benefits, BF has been positively associated with a protection from multiple health issues and diseases. Metabolic diseases such as diabetes and cardiovascular disease are reported to occur less in mothers who BF, similarly to the association with weight loss, longer BF duration is related to higher protection from metabolic ill health in later life (Ram et al. 2009). Type-2 diabetes, hypertension, hyperlipidaemia, cardiovascular disease and obesity have all been recorded in lower incidences in women who have previously breast fed than those who have not (Schwarz et al. 2010, Schwarz et al. 2009, Natland et al. 2012). Pregnancy triggers a change in metabolism that is required to sustain the pregnancy and to prepare for supporting the life of the infant after birth via BF. These changes include increased fat deposition, altered lipid metabolism, altered glucose metabolism and insensitivity to insulin (Zeng, Liu and Li, 2017, King, 2000). The effects of lactation seem to support the reversal of these required changes in pregnancy, suggesting a built-in cycle that occurs in reproduction, designed to return women to a healthy metabolic state after pregnancy and lactation. This cycle was named 'The Reset Hypothesis' by Stuebe and Rich-Edwards in 2009, who highlight the importance of this return to pre-pregnancy weight and metabolic status for women's future health (Stuebe and Rich-Edwards, 2009).

Breastfeeding is associated with a reduced risk in cancers with a hormonal origin in women. Epidemiological evidence supports a reduced rate of ovarian cancer in women who BF (OR= 0.76, 95% CI=0.71,0.80), intensified by longer duration of BF and shorter time since last BF period (Babic et al. 2020). According to two meta-analyses, similar associations are evident between BF and the risk of developing breast or endometrial cancer. These papers cite significant inverse relationships between BF and breast/endometrial cancer in women, further to this, they also report that longer durations of BF have further protective effects against the development of these cancers (Zhou et al. 2015, Zhan et al. 2015).

1.9 Scope and importance of further research

The importance of making informed decisions about an infants' environment and nutrition is paramount as it is during this time that foundations for their lifelong physiological and metabolic functions are laid (Langley-Evans 2015). Much is known about breast milk, how it varies and how its constituents can influence infant health and development. As discussed above we are aware that maternal diet can influence breast milk composition, with milk fat concentrations and composition being particularly responsive (Demmelmair and Koletzko, 2018, Krešić et al. 2013, Liu et al. 2016, Jonsson et al. 2016). This effect is true for multiple elements of the maternal diet and whilst particular emphasis on fat intake impacting milk fat has dominated, some studies have reported that other dietary components such as sugar and protein can also affect milk composition (Binder et al. 2023). Most of the research investigating the impact of maternal diet on breast milk composition has looked at habitual intake or interventions over time. Berger and colleagues recently reported an acute response in breast milk carbohydrates to an intervention involving high fructose corn syrup, suggesting milk composition does respond rapidly to changes in sugar consumption. Further emphasis on the acute changeability of BM composition is an important aspect of BM science that has been neglected until recently but could provide incredible insights into BM as a substance, thus increasing the potential positive impacts BM and BF can have on health.

In addition, maternal BMI has been related to the hormone composition of breast milk, with studies reporting positive associations between maternal BMI and leptin (Sims et al. 2020) and adiponectin (Martin et al. 2006), although these associations have not been observed by all groups (Chan et al. 2018, Andreas et al. 2014). Furthermore, leptin appears to be positively associated with weight gain during pregnancy and negatively associated with post-partum weight loss (Sadr Dadres et al. 2019).

Maternal diet has also been explored in relation to BM hormone concentrations. A recent study reported positive correlations between habitual dietary protein and fibre intake and ghrelin concentrations in milk ($\beta=2.6 \pm 1.1$ and $\beta=1.8 \pm 0.5$ respectively) while another group noted associations between protein intake and adiponectin and leptin concentrations in milk (Ramiro-Cortijo et al. 2023, Binder et al. 2023). Whilst we are aware of some correlations

between BMI and maternal diet and the composition of BM, this has not been studied in enough detail and requires a deeper look to really understand any relationships.

There is also evidence available to support the idea that variations in breast milk composition, regarding both macronutrient and hormone concentrations, can influence infant health and growth outcomes (Gridneva et al. 2018, Gridneva et al. 2022, Norrish et al. 2023, Brockway et al. 2023, Much et al. 2013). The studies researching this question are attempting to further elucidate the idiosyncrasies at play within breastfeeding mother and infant dyads. Are there particular maternal phenotypes that produce negative impacts on infant health via breast milk? Are there particular dietary habits that might negatively, or positively, influence breast milk composition? To what extent does breast milk change in response to maternal dietary behaviours, and to what extend do these changes impact infants?

Further complications exist in this field of research including the considerable heterogeneity between studies and the complexities associated with researching this ever-changing substance. Risk of bias and funding from large breast milk substitute companies add to the complexities and contribute to the difficulties encountered when reviewing and comparing research in this area. We recognise the importance of filling the gaps in our knowledge listed above and doing so in a way that is mindful of the complexities in this area of nutritional science.

The work presented in this thesis will explore the relationship between maternal diet and BM composition, focusing on the acute impact of increased sugar and fat consumption on milk macronutrient concentrations, with some exploration into metabolic hormones and fatty acids. I also aim to gain insight into the natural diurnal changes that may occur in milk macronutrients over the day. The interventions utilised in this work will represent relatively normal fluctuations in maternal diets towards ‘unhealthy’ choices and have been designed to mimic realistic dietary choices in BF women. The data I collect from measuring a range of nutrients and hormones will enable a novel investigation into how daily dietary choices acutely impact BM composition. Both the interventional and observational data will provide us with more information into how BM composition varies, in ways that have not previously

been explored. This will improve understanding and direction for BF mothers as well as those who support and educate them.

Aims, objectives and hypotheses.

The work outlined in this thesis is completed with the aim of addressing the current gap in our knowledge on how maternal diet acutely impacts breast milk composition. This question lacks appropriate and considerable attention in the current literature body yet could be an important piece in the puzzle of fully understanding human milk and how it varies in response to diet. As a health professional and scientist whose focus it is to improve health outcomes for mums and babies, this work holds great importance and signifies the beginning of a contribution to the field that I hope to be helpful, impactful and productive.

Primary objective:

- To determine if increased maternal consumption of sugar and fat influence BM macronutrient concentrations over a 12-hour study period.

Secondary objectives:

- To observe the natural fluctuations in BM macronutrient concentrations throughout the day.

- To explore the impact of increased sugar and fat consumption on concentrations of metabolic hormones and fatty acids in BM.

I hypothesise that BM lactose, protein, fats and hormones will respond to acute increases in sugar and fat consumption and that concentrations of these constituents will vary according to the time of day.

CHAPTER 2

THE PILOT STUDY: CHANGES IN BREAST MILK MACRONUTRIENT COMPOSITION IN RESPONSE TO INCREASED SUGAR AND FAT CONSUMPTION AND TIME OF DAY.

Ward, E., Yang, N., Muhlhausler, B. S., Leghi, G. E., Netting, M. J., Elmes, M. J., & Langley-Evans, S. C. (2021). Acute changes to breast milk composition following consumption of high-fat and high-sugar meals. Maternal and Child Nutrition, 17(3).

2.2 Introduction

BM is a fascinating and incredibly adaptive substance that sustains early human life through its nutritional and immunological composition (Ballard and Morrow, 2014). Human milk composition is highly variable and changes in response to many factors, including but not limited to; diet, BMI and age of infant (Mohammad et al. 2009, Bachour et al. 2012, Hsu et al. 2014). Mothers with high BMI have been reported to produce BM with a pro-inflammatory fatty acid profile (Panagos et al. 2016) as well as higher fat and lactose concentrations across lactation (Leghi et al. 2020). BM composition also changes across the duration of lactation; protein and calcium levels decrease whilst lactose concentrations increase as lactation progresses (Hsu et al. 2014).

In infancy there is a high degree of plasticity within developing organs and systems (Hochberg, 2011) and therefore the nutritional environment a child is exposed to can have lasting effects on physiological and psychological health (Langley-Evans and Muhlhausler, 2017). BF during this period has been associated with a number of health benefits for the infant in comparison to formula feeding, including improved cognitive ability (Evenhouse and Reilly, 2005) and reduced risks of both diabetes and obesity (Victora et al. 2016, Owen et al. 2005).

Research into the influence of habitual maternal diet on BM composition shows differing relationships between nutrient consumption and relative concentrations in BM. Several studies have suggested that compensatory mechanisms act to maintain adequate concentrations of protein irrespective of maternal nutrition (Lonnerdal, 1986), and amino acid patterns in BM are not correlated with amino acids in the maternal diet (Ding et al. 2010). BM protein synthesis is, however, thought to be influenced by maternal amino acid and energy availability in livestock (Bionas, Hurley and Loor 2012). Similarly, BM lactose content does not appear to be strongly influenced by changes in dietary intakes in lactating women (Smilowitz et al. 2013). Lactose is known to be synthesised *de novo* (Anderson et al. 2007) and glucose and galactose are thought to be derived from maternal plasma as well as generated through hexoneogenesis in human breast tissue (Sunehag et al. 2002). BM fats, however, are derived from a combination of maternal stores, dietary intake and *de novo* synthesis and appear to be more closely related to maternal dietary intakes (Koletzko 2016).

Trans-fatty acid and PUFA profiles in BM have particularly strong correlations with their intakes in the maternal diet (Samur et al. 2009, Jonsson et al. 2016).

Despite a general acceptance of the importance of maternal diet in determining BM composition, there is a marked shortage of evidence regarding the short-term changes in BM composition in response to diet. In addition, studies evaluating effects of maternal diet, or relationships between specific dietary components and their levels in BM, have typically collected samples at a single time-point during the day (Kim et al. 2017) or pooled samples collected over a 24-hour period (Aumeistere et al. 2019), rather than taking a time-course approach. Therefore, to the best of our knowledge, there are no studies investigating the short-term effects of maternal dietary changes on BM composition at multiple time-points throughout one day. This gap in the evidence base is important as advice for BF mothers regarding their diet is extremely limited yet could be of huge importance in optimising infants' developmental outcomes.

The aim of this study was to determine the effect of acutely increasing the intake of fat or sugar in the maternal diet on the protein, lactose and lipid concentrations in BM over the subsequent 12-hour period.

2.3 Methods

2.3.1 Selection Criteria

This pilot study was based on a protocol previously developed by our group (Leigh, Netting and Muhlhausler, 2020). To be eligible to participate in this study mothers had to be healthy, without history of diabetes including gestational diabetes, non-smokers, exclusively BF a singleton infant born at term, have no major dietary restrictions (e.g., vegan) or allergies (e.g., dairy) and be comfortable with collecting BM over multiple time-points. Infants needed to be between 6 and 24 weeks of age at the time of enrolment.

2.3.2 Participant Recruitment

Mothers (n=9), living in the East Midlands region of the UK, were recruited for this study using advertisements via social media platforms. Women who expressed interest were offered a screening visit, during which mothers' heights and weights were measured, infants were weighed and questionnaires focusing on health, pregnancy, birth and infant feeding patterns were completed to determine eligibility and for demographic information. A discussion to ensure women had established breast feeding and were solely feeding their infants with BM was included as part of the screening visit, women with difficulties breast feeding were not deemed eligible for inclusion. Written informed consent was obtained from all mothers during the screening visit, prior to study visits.

2.3.3 Study Design

All screening and sample collection sessions took place in participants' homes. All sample collection sessions spanned a full day (~8am -~7pm) and were conducted at least 1 week apart.

At visit 1, participants were supplied with a 'control' diet, this was designed with the help of a dietitian to ensure that the diet provided sufficient calories, macro- and micronutrients to meet the requirements of BF women (Department of Health, 1991). On visit 2 participants received a 'higher sugar' diet and on visit 3 they received a 'higher fat' diet. The control diet comprised 40% carbohydrate (CHO), 22% protein and 38% fat, by energy the high-sugar diet was 50% CHO, 22% protein and 28% fat and the high-fat diet was 37% CHO, 18% protein and 45% fat. The high-sugar diet provided 66g more sugar and the high-fat diet 28g more fat compared to the control diet. Further details of these diets are presented in Appendix 1. The diets supplied consisted of breakfast, lunch, dinner and two snacks. Breakfast was consumed between 8am and 9am, the specific time breakfast began (e.g. 8.10am) started the 12-hour sample collection period. Lunch and dinner were consumed at 5 and 10 hours from breakfast respectively and snacks were allowed to be consumed at any time between meals. Women were instructed to consume all food provided. These meals were standardised across participants where possible with exceptions made to accommodate the pescatarian diet (to replace chicken in the evening meal) and some dietary preferences (replacement of peanut butter with a similar spread). Beverages throughout the intervention days were restricted to

water and caffeine free tea/coffee. Similar meals and snacks were provided at each of the sessions, with the different fat and sugar content achieved by modifications such as replacement of low-fat dairy with high-fat dairy in the breakfast and lunch meals and inclusion of high fat/sugar snacks.

2.3.4 Dietary Assessment

Participants were supplied with a 5-day estimated food record diary to complete between the screening visit and visit 1. This included at least 1 weekend day. Women were instructed to record intake of all beverages, meals and snacks and brand names were encouraged to be recorded where possible. Questions related to habitual diet including whether women usually trimmed visual fat from meat prior to cooking/consumption, habitual use of low/full fat milks or spreads and cooking habits were also included to help determine overall energy consumption. Dietary data were analysed using Nutritics (Nutrition Analysis Software, Research Edition v5.098) and are presented in Appendix 2.

2.3.5 BM Collection

Participants were asked to express (manually or by breast pump) at least 2ml of BM into sterile 15ml tubes from either breast. The first sample was expressed before breakfast after an overnight fast (between 8 am and 9 am) and this was followed by hourly collections until the last sample which was collected one hour after the evening meal. Participants were instructed to collect samples as close to the hourly time frame as possible. Most samples were collected as stand-alone expressions and if a sample coincided with feeding, they were collected from the opposite breast whilst the infant was suckling. Samples were immediately frozen in participants' home freezers and transferred on ice at the end of the day to the laboratory where they were stored at -20°C until initial sample preparation processing and then at -80°C until analysis.

2.3.6 BM Analysis

Sample preparation

All samples were defrosted for processing within one week of being transported to the laboratory. Processing consisted of centrifugation for 10 minutes at 3000RPM at 4°C. This

created a visible 'cream' layer at the top of the sample which was discarded. The aqueous component of the sample was pipetted and aliquoted before being frozen at -80°C.

Measurement of protein concentrations

BM sample protein concentrations were determined using a standard Lowry assay protocol (Lowry et al. 1951). Briefly, BM samples were diluted and plated in duplicate onto a 96-well plate. 150µl of 0.1M NaOH was added followed by 50µl of reagent 1 (2% Na_2CO_3, 1% $CuSO_4$, 2% KNa tartrate) and the samples allowed to stand for 5 minutes at room temperature. Reagent 2 (0.1M NaOH and Folin-Ciocalteau reagent) was then added and the samples allowed to stand for a further 20 minutes at room temperature. Standards were created using bovine serum albumin dissolved in water. Plates were read at 655nm on a BioRad plate reader. The average intra- and inter-assay CVs were 5.4% and 14.2% respectively.

Measurement of lactose concentrations

Lactose concentrations were determined using liquid chromatography-mass spectrometry (LCMS). BM samples were diluted in the mobile phase (80% Acetonitrile and 20% H_2O), then filtered using a syringe filter (0.45µm, 40 hydrophilic nylon syringe filter, Millipore Corporation) and transferred into sealed glass vials. The standard lactose analysis reported previously (Liu et al. 2019) was used. The liquid chromatography equipment (1100 Series, Agilent) consisted of a degasser (G1322A, Agilent), a pump (G1312A, Agilent) and an auto-sampler (G1313A, Agilent). The system was interfaced with a Quattro Ultima mass spectrometer (Micromass, UK Ltd.) fitted with an electrospray ion source. The Luna 5u NH2 100A column (250×3.20 mm, 5µm, Phenomenex) was used to separate lactose at room temperature. Chromatographic separation was conducted using a mobile phase of 80% acetonitrile. The flow rate was set at 0.7 mL/min, the volume injected was 5µL. Peaks were determined by comparing retention times to the standard solution made from the lactose monohydrate standard. A calibration curve using a range of six standard solutions (10-150mg/L of lactose in 80% acetonitrile) was created for quantification of lactose level in the BM samples. Samples and standards were run in duplicate or triplicate in a randomised order. The average intra- and inter-assay CVs were 28% and 14.1% respectively.

Measurement of TAG and cholesterol concentrations

BM samples were diluted for TAG analysis but not for cholesterol analysis. These assays were completed using Thermofisher Infinity TAGs or cholesterol kits as per the method reported by Elmes et al. (2011). The average intra- and inter-assay CVs were 2.8% and 13.8% respectively for TAGs and 2.6% and 13.8% for cholesterol.

2.3.7 Statistical Analysis

Results are presented as mean ± standard error of the mean. Statistical significance was determined using repeated and single measures ANOVA with Tukeys HSD post hoc tests where appropriate, using IBM SPSS Statistics for Windows Version 26. Correlations between the maximal changes in concentrations (i.e. change from concentration at baseline and the hour with peak concentration) of each macronutrient across the 12-hour period and maternal habitual diet and maternal BMI were assessed by Pearson's Correlation tests using XLSTAT Version 2019.4.2.63912. A p-value of $p<0.05$ was considered to be statistically significant.

Table 2.1: Demographic characteristics of mothers and infants recorded at the start of the study.

	Mean	SEM	Range	N (%)
Mothers, n=9				
Age (yrs.)	33.6	1.1	10.0	
Height (cm)	166.4	2	18.0	
Weight (kg)	73.8	4.7	44.3	
BMI	26.7	1.8	15.3	
Infants, n=9				
Age (wks.)	13.9	1.8	16	
Weight (kg)	6.1	0.4	3.6	
Delivery Type				
Vaginal				4 (44.4)
(Forceps/Ventouse)				2 (22.2)
Caesarean Section				3 (33.3)

2.4. Results

2.4.1 Participant characteristics

Of the 10 women who were originally enrolled in this study, 9 completed all study appointments and BM sampling times. One participant withdrew from the study due to difficulty with sample collection and was excluded from the analyses. The sociodemographic information for the participants and their infants is shown above in Table 2.1. Four mothers were of normal weight, three were overweight and 2 were obese according to the WHO criteria (World Health Organisation, accessed 20/01/21). All mothers were of Caucasian English descent except one who was Caucasian Irish. All mothers were exclusively BF their infants and had been since birth. Three of the 9 mothers were multiparous and had one older child, none were BF more than one child. The remaining mothers were primiparous. One woman was a pescatarian, whilst the others all consumed meat regularly.

2.4.2 Dietary Analysis

The energy and macronutrient composition of the women's habitual diets, assessed over a 5-day period, is presented in Appendix 2. There was considerable variation in total energy intake between women ($p<0.05$), but there were no significant differences in their habitual intakes of carbohydrates, total fat, protein or total sugars.

2.4.3 Effect of higher-fat and higher-sugar intakes on BM composition

Consumption of a diet higher in sugar/fat, compared to the control diet, was associated with significantly greater BM TAG concentrations (Figure 2.1). Consumption of a higher sugar diet was associated with the greatest increase in TAG content, with a mean difference in TAGs of 5.36g/dL when compared to the control day ($p<0.001$).

A mean difference in TAGs of 1.49g/dL was observed between the control and higher fat day ($p<0.001$). Although BM TAGs had increased in response to consumption of the higher fat diet, they were significantly lower when compared to the higher sugar day with a mean difference of 3.87g/dL ($p< 0.001$).

BM cholesterol concentrations increased to a greater extent in response to the higher sugar diet (0.07g/dL) than both the control (0.04g/dL) and higher fat diet (0.05g/dL) (Figure 2.2) but were not different between the higher-fat and control diet ($p>0.05$).

No effect was observed on BM protein content in response to consumption of the higher sugar diet, but protein content was lower on the higher -fat diet when compared to the control (Figure 2.3, $p=0.05$). BM lactose concentrations were higher following the consumption of the higher fat diet compared to the control diet ($p=0.006$) but were not affected by the higher sugar diet (Figure 2.4).

2.4.4 Variations in BM composition across the day

There were significant differences in the concentrations of BM macronutrients and lipids between collection points across the day, independent of dietary treatment. Concentrations of TAGs were lower at the start of the day and higher at the end of the day ($p<0.001$) and concentrations at several individual time-points across the day were also significantly different (Figure 2.1).

BM cholesterol concentrations also varied across the course of the day and were significantly higher at the 12-hour collection point than at all other time-points ($p<0.001$) (Figure 2.2).

As depicted in Figure 2.3, BM protein concentrations fluctuated across the day, and were significantly greater at hours 2, 7 and 10 than at all other time-points (Figure 2.3, $p<0.001$). Lactose concentrations similarly fluctuated across the 12-hour collection period, increasing after breakfast and lunch before decreasing towards the end of the sampling period. BM lactose concentrations were lowest at the 12-hour time-point, and highest at the 3 and 10-hour time-points (Figure 2.4, $p<0.001$).

2.4.5 Correlations between change from baseline in BM components and habitual maternal intake of macronutrients/maternal BMI.

There were no significant relationships between the maximum changes in concentration from the start of the day to the peak concentration across the day in BM protein, lactose or cholesterol concentrations, and either maternal BMI or habitual dietary intake. On the day

the women consumed the control diet, there was a significant negative correlation between women's habitual fat intake and the maximal change in BM TAG levels from baseline to end of day (r=-0.816, p=0.048).

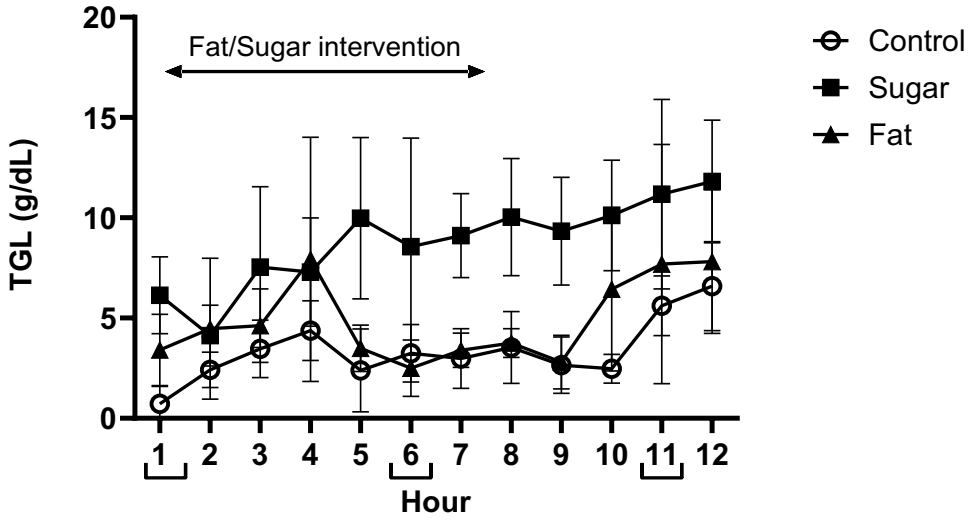


Figure 2.1: Data presented as mean±SEM. Mealtimes are represented on the x-axis with brackets [B] breakfast, [L] lunch and [D] dinner. Repeated measures ANOVA indicated that milk TAG concentration was influenced by diet intervention (fat, sugar or control) ($p<0.001$), as well as showing a response to time of day ($p<0.001$) ($n=6$).

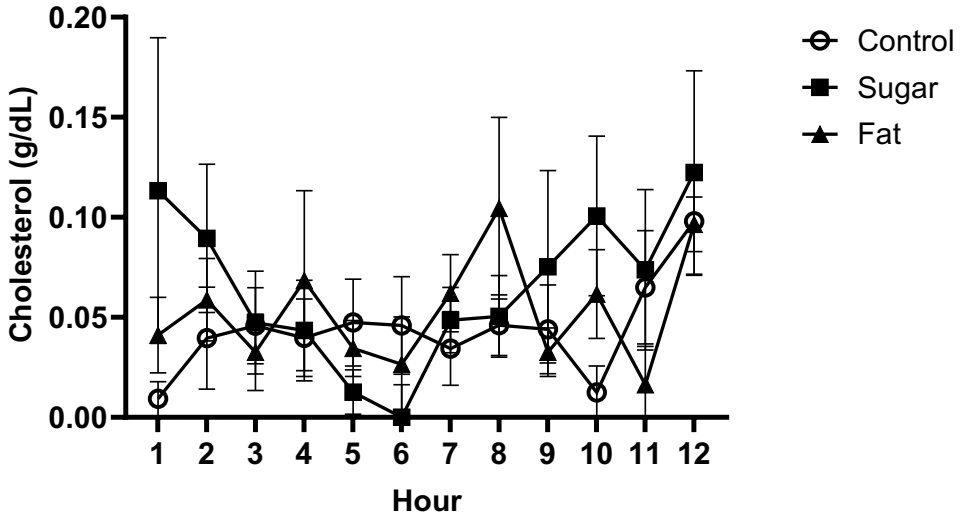


Figure 2.2: Data presented as mean±SEM. Mealtimes are represented on the x axis with brackets [B] breakfast, [L] lunch and [D] dinner. Repeated measures ANOVA indicated that milk cholesterol was influenced by increased sugar and fat consumption ($p<0.001$ and $p=0.002$ respectively), as well as showing a response to time of day ($p<0.001$) (n=6).

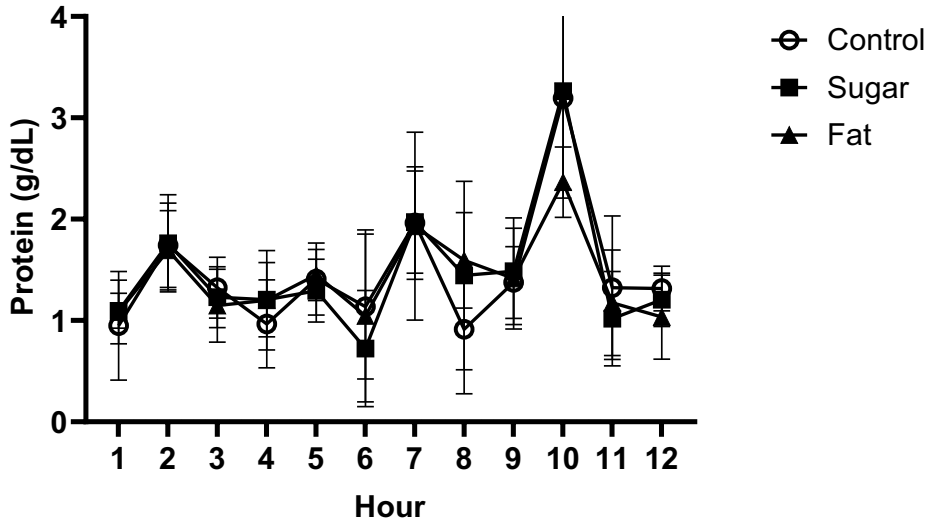


Figure 2.3: Data presented as mean±SEM. Mealtimes are represented on the x axis with brackets[B] breakfast, [L] lunch and [D] dinner. Repeated measures ANOVA indicated that milk protein was influenced by increasing fat ($p=0.05$) as well as showing a strong response to time of day with peaks at hours 2, 7 and 10 ($p<0.001$) (n=9).

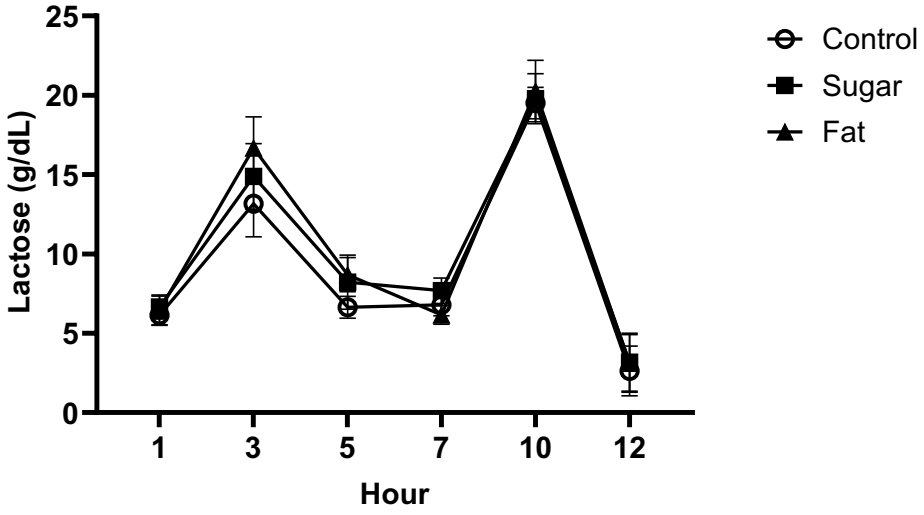


Figure 2.4: Data presented as mean±SEM. Mealtimes are represented on the x axis with brackets [B] breakfast, [L] lunch and [D] dinner. Repeated measures ANOVA indicated that milk lactose was influenced by increasing fat ($p=0.006$), as well as showing a strong response to time of day with peaks at hours 3 and 10 and a trough at hour 12 ($p<0.001$) (n=9).

2.5 Discussion

The findings of this study suggest that the levels of all macronutrients in BM are to some extent responsive to a woman's diet. We have demonstrated that there was a significant increase in BM TAGs over 12 hours when women consumed a higher sugar diet compared to when they consumed either a control diet or a diet higher in fat. BM cholesterol concentrations were also higher on the day that women consumed the higher-sugar diet than on the days when the control or higher-fat diets were consumed. In addition, we also observed significant variations in the levels of protein, lactose and lipid concentrations in BM over the course of the day, independent of the diet consumed.

The role of maternal diet as a determinant of BM composition has been an important research question for decades with the prevailing conclusion being that some BM constituents, in particular fatty acids, are influenced by maternal dietary intake (Liu, Liu and Wang 2019) whilst others (such as protein and lactose) are not (Ding et al. 2010, Smilowitz et al. 2013). The findings of our current study add to these discussions by demonstrating that concentrations of TAGs, cholesterol, protein and lactose are all influenced by relatively small and transient changes in the maternal diet. Specifically, we found that the immediate diet of mothers can influence each of these components in BM over the course of the subsequent 12 hours.

The extent of the responsiveness of BM TAGs to maternal sugar intake compared to fat intake was unexpected, particularly since BM TAGs have previously been reported to increase in response to a high-fat diet (Mohammad et al. 2009). However, this discrepancy may be due to the fact that the intervention diets in the previous study (Mohammad et al. 2009) were consumed for a longer period (4-7 days) than in our study, providing a much longer period of exposure. A potential explanation for the increased TAG in response to sugar consumption

could be that the sugar stimulated an increase in lipogenesis. Parks et al. 2008 completed a feeding study on adults which showed a significant increase in lipogenesis following acute ingestion of fructose with an increase postprandially on lipemia and TAG synthesis. To the best of our knowledge, the changes in BM TAG and cholesterol concentrations in response to increased sugar intake have not been reported elsewhere, suggesting a need for further study in the area.

The fluctuations in BM protein and lactose concentrations across the 12-hour sample collection period, independent of diet, suggest that there is a circadian variation in the concentrations of these factors in BM. The variations across the 12-hour sampling period reported here challenge the prevailing view that fat concentrations in BM are more variable across the day than concentrations of protein and lactose. A study assessing circadian rhythms in BM reported no significant difference in protein, fat or carbohydrate throughout the day (Cetinkaya et al. 2017), however, these researchers collected BM samples at only three points over one day, which may not have been frequent enough to detect the patterns that we observed. A further study by Kent et al. 2006 reported that BM fat concentrations were higher during the day (10am-10pm) than during the night (10pm-10am) but again samples were not collected at regular intervals. Therefore, the differences between our observations and previous studies could simply be due to the sampling strategy we employed (frequent sampling at regular intervals).

Circadian rhythms in BM amino acids have been reported in a previous study by Sanchez et al. 2013, in which BM samples were collected every 3 hours over a 24-hour period in colostrum, transitional and mature milk, with particularly substantial variations observed in concentrations of tryptophan, methionine, tyrosine, phenylalanine, glycine and aspartic acid. None of the patterns reported by Sanchez et al, however, correspond to the pattern we observed in total BM protein. It is possible that the cumulative effect of the fluctuations in all amino acids and active proteins combined to create the patterns we observed over the 12-hour sampling window. Another study also reported a circadian rhythm in BM tryptophan concentrations, indicating that these peaked at 3am and resulted in increased concentrations of the melatonin metabolite (6-sulfatoxymelatonin) present in infants' urine. The same study reported that BF was also associated with improvements in sleep parameters when

compared to formula feeding, which the authors attributed to the circadian variations in melatonin in BM (Cubero et al. 2005).

While this study adopted a novel approach to investigating the influence of maternal diet on BM composition, it is also important to acknowledge its limitations. First, the small sample size makes it difficult to draw robust conclusions and, now that we have proof-of-concept of an acute effect of maternal diet on BM composition, it will be important to repeat the study in a larger number of women in order to confirm the findings. This study was powered to detect relatively large changes in BM composition (40% change in lactose concentration; 2-fold change in TAGs) therefore more subtle changes in BM composition across the day or in response to dietary change may not have been detected. The results here are derived from a small group of Caucasian women from one region in England and are not representative of other populations which may show different responses. Although the frequency of sampling across the day is a strength of the study, we did not collect samples beyond 7pm meaning there could be further compositional changes in response to our interventions and/or diurnal rhythms. Employing a multi-day intervention could also further support the relationship observed between sugar consumption and BM TAGs and would allow us to determine if habitual diets high in sugar influence BM in the longer term as well as acutely.

Furthermore, by improving details in future work such as using human milk standards for macronutrient analysis and instructing mothers more stringently on BM collection methods (i.e., directing women to collect foremilk only and collecting data on infant feeding times) we could better account for variations resulting from non-dietary influences. This is particularly necessary in relation to the lactose methodology as the inter-assay CV calculations show significant variability. The design of this method was based upon techniques used and developed for sensory science and using a standard that was not specific to BM. This variability is perhaps due to the method not being adapted to accommodate the complex matrix of BM and we recognise the need to approach future lactose analysis with a more appropriate method. It is important to note that the results presented here serve the primary function of informing the design of further studies which will aim to clarify and expand on the observations recorded.

In conclusion, this study has shown for the first time that BM can be influenced by maternal fat and sugar intake over a period of 12 hours. This is an observation that should inform future studies and could be taken into account when discussing diet with BF mothers. Furthermore, the observations of potential circadian rhythms in BM protein and lactose concentrations give insight into BM's nutritional composition over a day. These modulations may have an influence on metabolic and hormonal processes which could affect infant development and have lasting effects, a concept which, if further explored, could maximise the benefits of BF and its influence on infant health.

CHAPTER 3

THE MAIN STUDY: CHANGES IN BREAST MILK NUTRITIONAL AND HORMONAL COMPOSITION IN RESPONSE TO INCREASED SUGAR CONSUMPTION BY AUSTRALIAN WOMEN.

3.1 Introduction

BM is a powerful nutritional substance. It provides all the nutrients and bioactive factors required to support healthy infant growth and development and is therefore classified as the optimal source of infant nutrition (WHO, 2023, Victora, et al. 2016). Human milk also contains a wide range of non-nutrient elements, including but not limited to, growth factors, immune cells, bacteria, oligosaccharides and metabolic hormones (York, et al. 2021, Togo, et al. 2019, Bode, 2012, Suwaydi, et al. 2021). The concentrations of these hormones in milk are variable but thought to influence infant metabolism, body composition and the risk of developing obesity and associated metabolic diseases (Sinkiewicz-Darol et al. 2022, Gridneva et al. 2018, Chan et al. 2018, Savino et al. 2009).

This is particularly relevant, given that early infancy represents a critical developmental window and rapid growth trajectory, during which nutritional influences can shape the development of multiple physiological systems, including the brain and digestive system (Schwarzenberg and Georgieff, 2018, Brenna & Carlson, 2014, Vandenplas et al. 2020, Hryciw, 2022). However, the composition of human milk varies substantially (Ballard and Morrow, 2013), creating the potential to affect the short and long-term outcomes of infants. Consequently, improving our understanding of the modifiable factors that can influence BM composition has the potential to assist in identifying approaches to optimise infant development.

It is generally understood that fat is the most variable macronutrient in BM, followed by protein and finally lactose, which is relatively stable and unresponsive to maternal factors (Lonnerdal, 1986, Mitoulas et al. 2002). There is emerging evidence which suggests that maternal diet during BF has the capacity to influence BM composition. This has now been reported in several studies including a recent observational study which reported that women with higher levels of adherence to a Mediterranean diet had lower concentrations of saturated fatty acids and higher concentrations of monounsaturated and omega-3 polyunsaturated fatty acids compared to those with lower levels of adherence (Di Maso et al. 2022). Overall, however, the evidence supporting the role of the maternal diet in determining BM composition is still limited, and few high-quality studies have been

conducted in this area to date. Inconsistencies in BM collection methods and data reporting across these studies make it difficult to draw reliable conclusions. This is particularly the case when it comes to understanding the acute impact of maternal dietary choices on BM composition, since studies conducted to date have focused almost exclusively on the effects of habitual intakes or shifts in maternal dietary intake over longer periods of time.

As reported in Chapter 2, our pilot study conducted in 9 BF women, focused on this question and suggested that an acute increase in maternal sugar consumption resulted in increases in BM TAG concentrations across the day, when compared to a lower sugar diet (Ward et al. 2021). In this study, we sought to verify these findings in a larger study population, and to further investigate the effects of increased maternal sugar consumption on other BM components beyond macronutrient composition, specifically glucose and metabolic hormones.

3.2 Methods

3.2.1 Ethical Consideration

The study was conducted in accordance with the Declaration of Helsinki. All participants who expressed interest in participating in the study provided verbal consent prior to completing the screening questionnaire. Those who were deemed to be eligible to participate following the screening questionnaire provided full written informed consent at the time of the first study day visit, prior to the initiation of study procedures and BM collection. Ethical approval for this study was obtained from the CSIRO Health and Medical Human Research Ethics Committee (CHMHREC) (Ethics reference number: 2022_007_HREC). This trial was also registered with Australian New Zealand Clinical Trials Registry (ANZCTR) (Trial ID: ACTRN12622000651785)**.**

3.2.2 Inclusion criteria

Mothers who were exclusively BF singleton infants between 6-24 weeks old at the time of the first appointment, and who were born at term were invited to participate. Women were eligible for the study if they were healthy, had a BMI of 30 or lower, were between the ages

of 21-35 and had no major dietary restrictions (e.g. vegan) or allergies (e.g. dairy). Women also needed to indicate that they were comfortable with collecting BM at regular intervals over a 12-hour period.

3.2.3 Participant recruitment

Participants were recruited via online social media platforms and contacted through a database comprising of contributors to previous clinical trials who had provided consent to be contacted for future research studies. All women were recruited from the city of Adelaide, Australia and surrounding areas. Women who expressed an interest in participating were contacted to assess their eligibility and complete the screening questionnaire. Following confirmation of eligibility and if mothers reported being comfortable with the study design, they were enrolled and dates for the study day visits were arranged.

The sample size for this study was calculated based on the pooled data collected for milk triglycerides in our UK pilot study (Ward et al 2021) where we observed a 5.36 g/dL increase in triglycerides following the higher sugar diet compared to the control diet ($p<0.001$). A power calculation was performed using the tool available at Statulator.com for a paired samples design and the calculation was amended (90% power and 1% significance level) to allow for a smaller effect size than that observed in the pilot. On this basis 13 mother/baby dyads would be needed for appropriate power.

3.2.4 Study Design

The study protocol has been previously described in detail in Chapter 2. Briefly, BF mothers were asked to consume two study diets (one control and one higher sugar) on two separate days at least one week apart. All mothers received the control diet first. These diets were identical to the diets developed for the pilot study with the only differences coming from the different food brands between Australia and the UK. Every effort was made to ensure the macronutrient composition was matched when brands were different - details can be found in Appendix 1. Both diets comprised a full day (~8am-7pm) of breakfast, lunch and dinner and women were instructed to collect BM samples at hourly intervals on each of these study days (12 samples per woman per day). The additional sugar on the intervention day was included in all meals except for dinner as this was consumed close to the end of the sampling

period. All diets were prepared in a dedicated study kitchen within the CSIRO clinic at SAHMRI in Adelaide, Australia. Meals and study materials were delivered to the participants' homes on the evening prior to the study day. Study diet consumption was calculated by weighing any food left over.

3.2.5 Milk collection

BM samples were collected at hourly intervals within the study day period, starting before breakfast and finishing after dinner. Women were instructed to collect from whichever breast was fullest at the time of collection in an attempt to standardize all collections to foremilk and to collect at least 2ml by hand or manual pump. If collections coincided with an infant feeding period, then mothers were instructed to feed their infant first and then collect the BM sample from the opposite breast. Participants were instructed to place their BM samples in the fridge (4°C), until collection on the following day. After collection, the BM samples were transported to the laboratory and aliquoted into 3-6 aliquots of ~2ml volume, and were then frozen at -20°C before transporting to the laboratory at University of Western Australia, where they were stored at -80°C.

3.2.6 Macronutrient analysis

Breastmilk fat concentrations were measured using a modified version of the creamatocrit technique previously validated for use in human milk (Lucas et al. 1978, Du et al. 2017). In brief, whole milk samples were thawed and vortexed gently before duplicates were drawn up into glass capillary-tubes (41A2502, Kimble-Chase, USA) and plugged at one end with sealant (43510, Kimble-Chase, USA). Capillary tubes were then centrifuged using a flat-bed centrifuge (CEN 96221, Phoenix Scientific Industries Ltd, USA) for 10 minutes at 2000g. This process separated the fat from the aqueous portion of milk and allowed for total fat concentrations to be determined using the Creamatocrit Plus device (Medela, AG). Capillary tubes were then cut, and the de-fatted milk was retained for protein and lactose analysis.

Total protein concentrations were determined in duplicate by a modified Bradford method using a commercial protein assay kit (5000006, Bio-Rad Laboratories, USA) for use in human

milk as described by Mitoulas et al. in 2002. A human milk protein standard was derived from a sample of BM in the laboratory using the Kjeldhal method, as described by Attwood and Hartmann (1992). The average intra- and inter-assay CVs were 2.9% and 11.9% respectively.

Lactose and glucose concentrations were analysed in duplicate by enzymatic methods validated for use in human milk as previously reported (Mitoulas et al. 2002, Cheema et al. 2021, Suwaydi, 2023). Enzymes used for the lactose and glucose assays were bought from Megazyme (K-LOLAC and K-GLUHK-220A Megazyme, Wicklow, Ireland). Standards for both of these assays were made up from materials provided by Megazyme. In brief, lactose analysis involved diluting defatted milk in distilled water. Samples and standards were added in duplicate to 96 well plates before 15µl of a solution of NADP/ATP buffer was added to all wells. A first read was carried out on a plate spectrophotometer (Enspire Multimode Plate Reader, Waltham, MA, USA) at 340nm after 3 minutes. 2µl of a second solution (HK/G-6-PDH/6-PGDH) was added to all wells and a second read was completed at 340nm after 10 minutes. Finally, 2µl of β-galactosidase was pipetted into all wells and read on the spectrophotometer after 15 minutes. Total lactose concentrations were determined by subtracting the second reading from the third. The average intra- and inter-assay CVs were 1.7% and 15.7% respectively.

Details of the glucose methodology have been previously reported in detail (Suwaydi, 2023). Briefly, samples were diluted in distilled water before being added to 96 well plates along with standards in duplicate. 210µl of a buffer solution was added to all wells before a first read on the plate spectrophotometer (Enspire Multimode Plate Reader, Waltham, MA, USA) at 340nm. 12µl of an enzyme solution was then added to all wells and plates were read after at least 10 minutes, or when the reaction had plateaued. Glucose concentrations were determined by subtracting read 1 from read 2. The average intra- and inter-assay CVs were 3.6% and 20.9% respectively.

Repeats were completed for all analytes if duplicate measurements had a CV of over 11 or if they appeared as an outlier (more than 2 standard deviations from the group mean) when viewing the full dataset. These were repeated in triplicate when there was enough sample to

do so and if not were repeated in duplicate. Some samples did not have enough for any repeats and were therefore removed from the final data set.

3.2.7 Hormone analysis

Milk hormone analyses were completed using commercial ELISA kits, these were previously utilised for analysis of human milk (Suwaydi et al. 2023). Milk adiponectin, insulin and leptin concentrations were determined by thawing whole milk samples before homogenizing them using a bead homogenizer (BeadBugTM 6 Position Homogenizer (BencBMark Scientific, Sayreville, NJ, USA). For adiponectin and insulin homogenised milk was then pipetted into the 96 well plates provided within the commercial ELISA kits (RD191023100, Lot: E21-040, BioVendor, Brno, Czech Republic and RIS006R, Lot: X21-136S01, BioVendor, Brno, Czech Republic respectively). Steps were followed in accordance with the manufacturer's instructions and plates were read on the spectrophotometer (Enspire Multimode Plate Reader, Waltham, MA, USA). The average intra- and inter-assay CVs were 3.3% and 6.9% respectively for adiponectin and 4.5% and 8.7% for insulin.

Leptin measurements were completed using plates, solutions and diluent from R&D systems (RDSDY398, Human Leptin DuoSet ELISA, RDSDY999 Substrate Reagent Pack, RDSDY995 R&D Systems Reagent Diluent Concentrate, R&D systems, Mineapolis, USA). 50µl of the capture antibody from the DuoSet ELISA kit was added to plates before allowing them to incubate overnight at room temperature. The following day, plates were washed x3 using a plate washer and then a blocking solution of reagent diluent was added before incubating for at least 1 hour at room temperature. Plates were washed three further times and then 50µl of standards, samples and quality control were added in duplicate before incubating for a further 2 hours at room temperature. Plates were then washed again before adding 50µl of detection antibody and incubated for a further 2 hours at room temperature. Next, 50µl of Streptavidin-HRP was added and incubated for at least 20 minutes at room temperature in the dark. Plates were washed again for a final time before adding 50µl of the substrate colour reagent before a final incubation for 20 minutes at room temperature in the dark. The reaction was then stopped by adding 25µl of sulphuric acid and absorbance was read at 450nm using a plate spectrophotometer (Enspire Multimode Plate Reader, Waltham, MA, USA). The average intra- and inter-assay CVs were 3% and 21.8% respectively.

Similarly to the macronutrient methods, all initial measurements were completed in duplicate, and any requiring repeats were repeated in duplicate or triplicate depending on the amount of milk available.

3.2.8 Statistical analysis

Data were analysed using R Statistical Software (RStudio 2023.06.1+524 "Mountain Hydrangea"). All data were presented as mean ± standard error of the mean unless otherwise specified. Linear mixed effects models were used to determine differences in milk components between the days (diets) and between hours for both days. Visualisation of data was completed using GraphPad Prism version 10. A P value of <0.05 was considered to be statistically significant. As the analysis primarily identified the main effects of diet or interactions of diet and time of day, the following graphs are not annotated to show statistically significant time-points. For example, a main effect of diet on milk lactose concentrations would reflect a difference between the high sugar diet and control diet across the full set of hourly samples.

3.3 Results

3.3.1 Study demographics

22 women were initially enrolled for this study. One did not comply with the intervention sufficiently, all other women consumed 98% or more of the study diets. Another mother was deemed ineligible after BMI was confirmed (as shown in Figure 3.1 below). Details on the 20 exclusively BF mothers who completed the study are presented in Table 3.1. All mothers included had a postpartum BMI of 30.5 or less with an average of 26 across the group. Maternal ages ranged from 27 to 35 with a mean of 31.7 years and the infants ages ranged from 9 to 24 weeks at time of the first visit, with a mean of 16 weeks. 7 were multiparous and the remainder were feeding their first baby. None of the participants included were tandem feeding.

Table 3.1: Demographic information of participants and their infants (n=20).

	Mean	St. Deviation	Range	N (100%)
Mothers, (n=20)				
Age (years)	31.7	2.4	8.0	
Height (cm)	167.4	5.7	25.0	
Weight (kg)	72.7	6.8	28.0	
Ethnicity				
Caucasian				100
BMI	26.0	2.5	8.5	
Parity	1.6	0.7	2.0	
Infants, (n=20)				
Age (weeks)	16.1	3.8	15.0	
Weight (kg)	6.4	1.2	4.6	

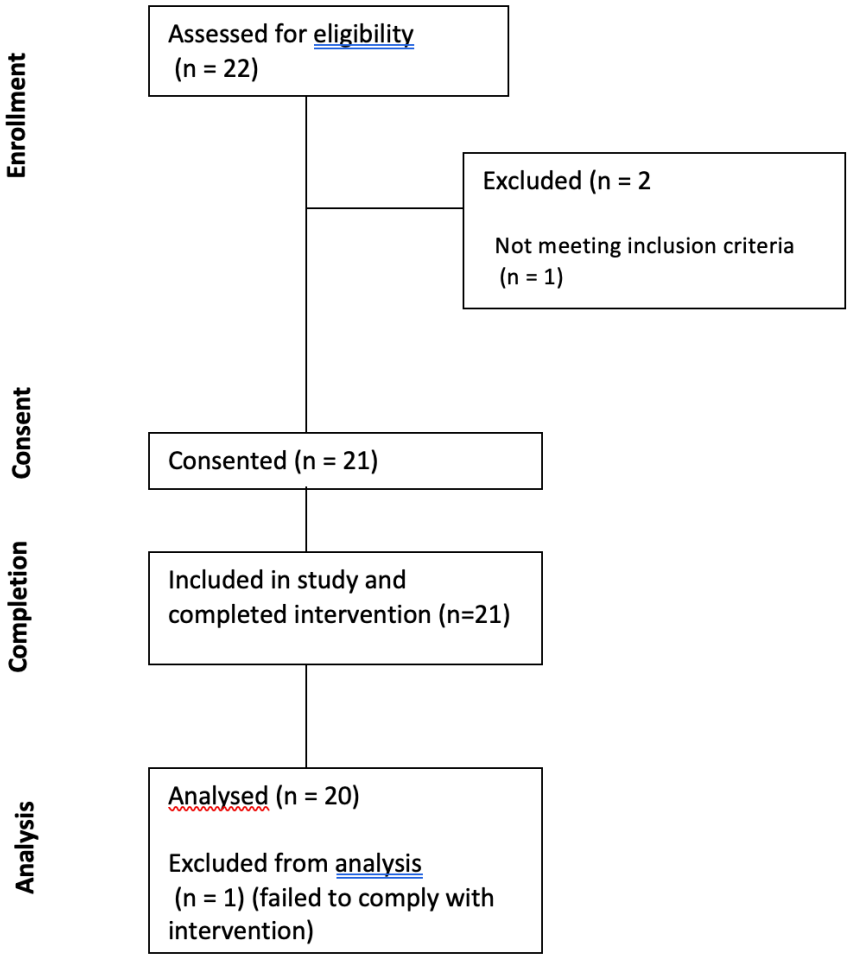


Figure 3.1: A flow diagram illustrating participant recruitment, inclusion and final sample size included in analysis.

3.3.2 Effect of sugar consumption on macronutrient concentrations

Considering concentrations across the whole study days, milk fat, lactose and glucose concentrations were significantly increased when mothers were consuming the higher sugar diet ($p<0.001$) whereas milk protein concentrations were significantly lower on the day that the women were consuming the higher sugar diet ($p<0.001$) (Figure 3.2 - Figure 3.5). Milk fat increased by 6.5g/L (13.4%), lactose increased by 7g/L (8.7%) and glucose by 0.2g/L (9.6%). Protein concentrations were 1.3g/L (10.6%) lower on day 2.

Table 3.2: A table showing the average concentrations ± SEM of BM macronutrients and hormones on the control and higher sugar day (significantly different results in bold).

	Mean±SEM	
	Control	**Sugar**
Fat	48.2±4.4	**56.8±5.1**
Protein	11.7±0.6	**10.5±0.5**
Lactose	76.8±2.9	**83.6±3.8**
Glucose	1.6±0.1	**1.8±0.1**
Adiponectin	13.0±0.8	**12.5±0.9**
Insulin	10.4±1.5	10.7±1.6
Leptin	0.3±0.02	**0.2±0.03**

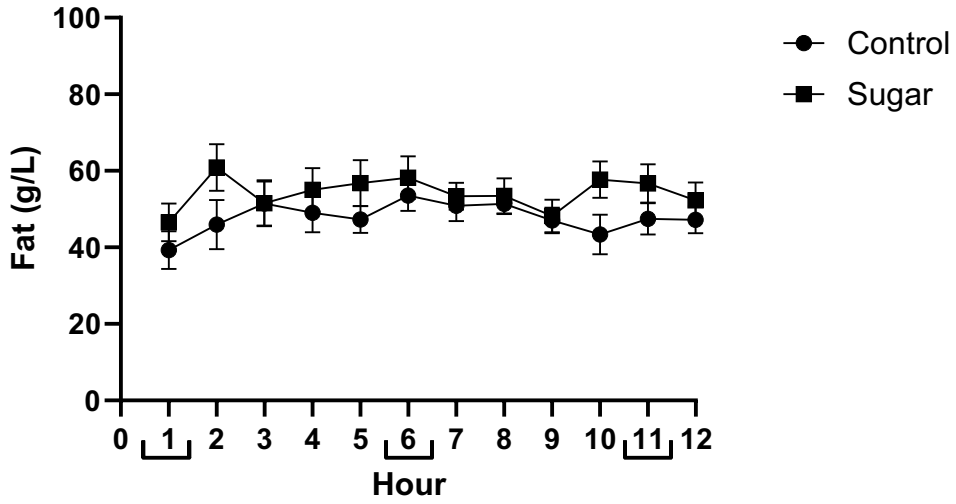


Figure 3.2: Milk fat concentrations across the day. Two diets were consumed, one control and one higher sugar. Meals were consumed at hours 1, 6 and 11 approximately, the sugar intervention was administered across hours 1-9. Data presented as mean ± SEM (n=20). Linear mixed effects modelling showed significantly higher fat concentrations during the sugar day ($p<0.001$) and a significant difference between time-point 1 on the control day and time-point 2 on the sugar day.

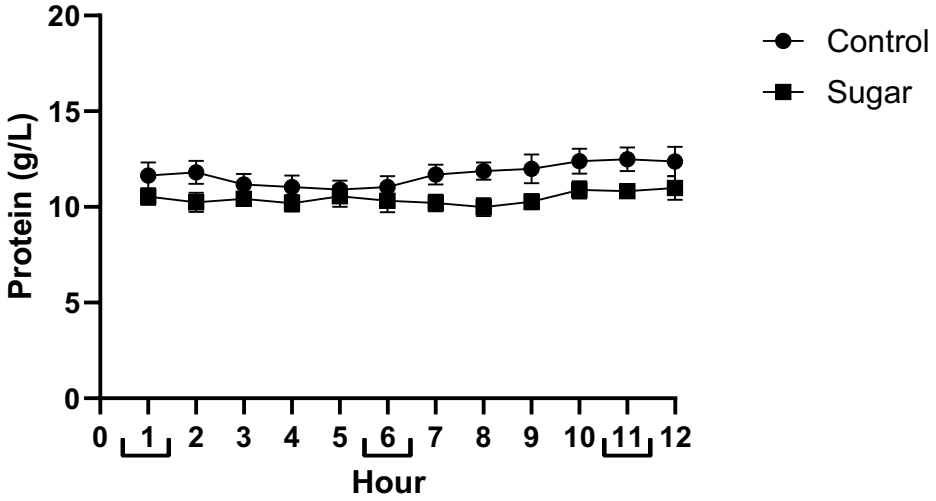


Figure 3.3: Milk protein concentrations across the day. Two diets were consumed, one control and one higher sugar. Meals were consumed at hours 1, 6 and 11 approximately, the sugar intervention was administered across hours 1-9. Data presented as mean ± SEM (n=20). Linear mixed effects modelling showed significantly lower concentrations of protein during the sugar day ($p<0.001$) but no differences between time-points across the day.

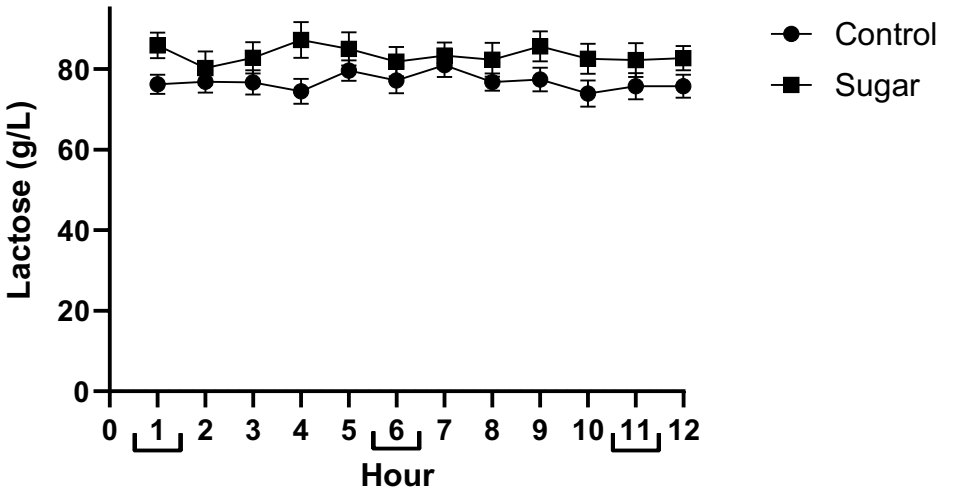


Figure 3.4: Milk lactose concentrations across the day. Two diets were consumed, one control and one higher sugar. Meals were consumed at hours 1, 6 and 11 approximately, the sugar intervention was administered across hours 1-9. Data presented as mean ± SD (n=20). Linear mixed effects modelling showed significantly higher concentrations of lactose during the sugar day ($p<0.001$) but no differences between time-points across the day.

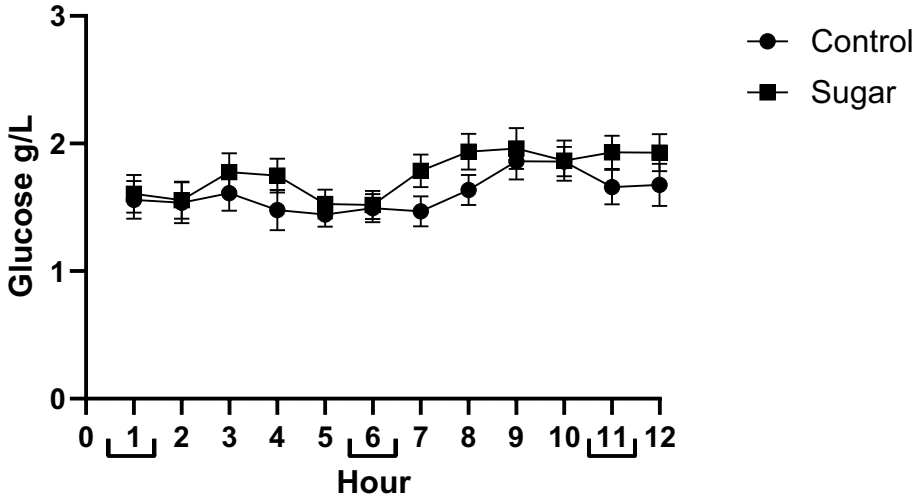


Figure 3.5: Milk glucose concentrations across the day. Two diets were consumed, one control and one higher sugar. Meals were consumed at hours 1, 6 and 11 approximately, the sugar intervention was administered across hours 1-9. Data presented as mean ± SEM (n=20). Linear mixed effects modelling showed significantly higher concentrations of glucose during the sugar day ($p<0.001$) but no differences between time-points across the day.

3.3.3 Effect of sugar consumption on hormone concentrations

Differences in hormone concentrations between the control and higher sugar days were observed (Figure 3.6 - Figure 3.8). Adiponectin and leptin concentrations were significantly lower when mothers were consuming the sugar diet ($p=0.0024$ and $p=0.001$ respectively) with adiponectin being 0.68ng/ml (9.8%) and leptin 0.02ng/ml (21%) lower than the control day. Insulin concentrations were similar across both days.

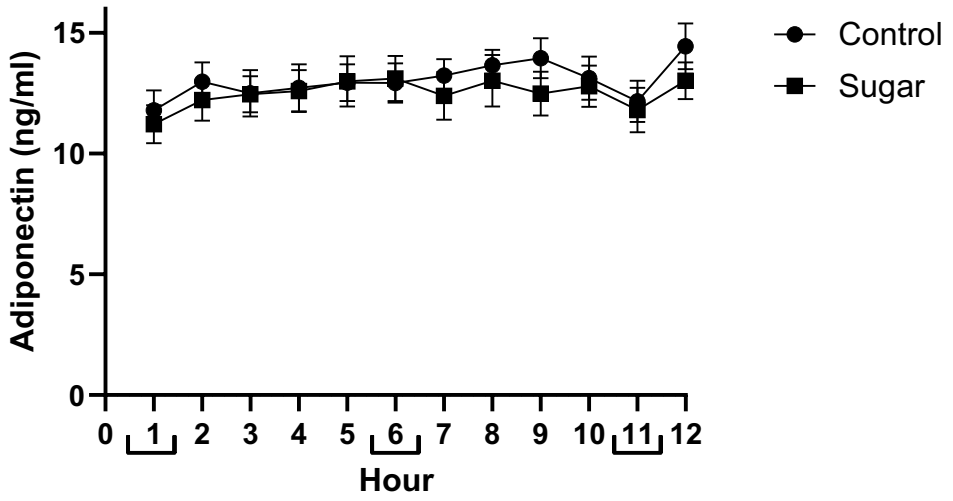


Figure 3.6: Milk adiponectin concentrations across the day. Two diets were consumed, one control and one higher sugar. Meals were consumed at hours 1, 6 and 11 approximately, the sugar intervention was administered across hours 1-9. Data presented as mean ± SEM (n=20). Linear mixed effects modelling showed significantly lower adiponectin concentrations during the sugar day (p=0.0024) but no differences between time-points across the day.

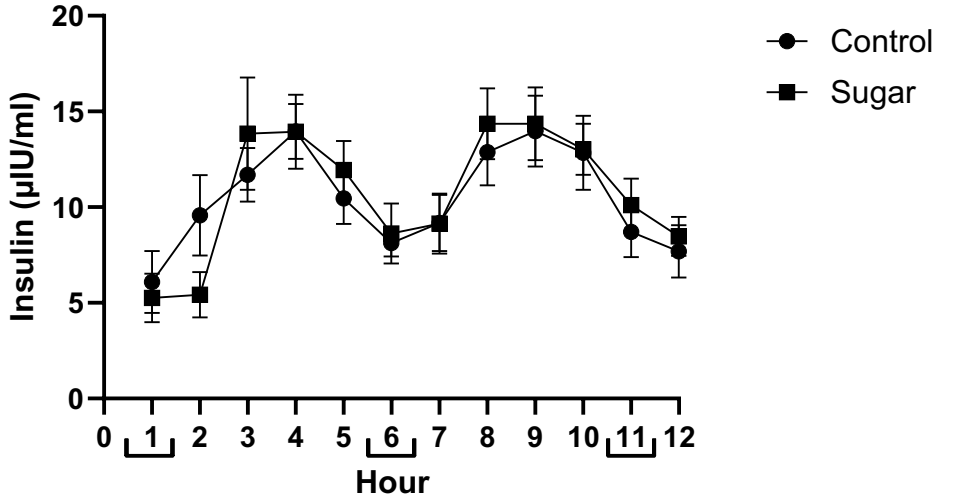


Figure 3.7: Milk insulin concentrations across the day. Two diets were consumed, one control and one higher sugar. Meals were consumed at hours 1, 6 and 11 approximately, the sugar intervention was administered across hours 1-9. Data presented as mean ± SEM (n=20). Linear mixed effects modelling showed no difference in insulin

concentrations between the days but did indicate significant differences between time-points across the days ($p<0.05$).

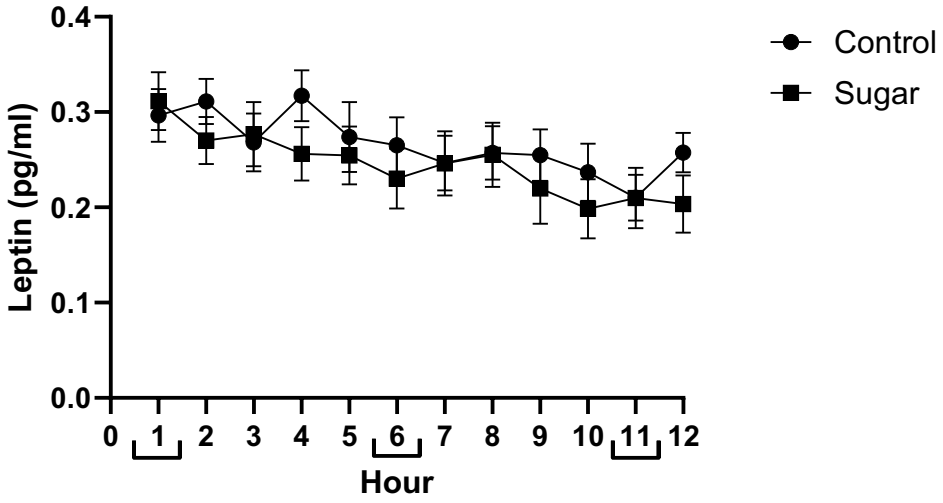


Figure 3.8: Milk leptin concentrations across the day. Two diets were consumed, one control and one higher sugar. Meals were consumed at hours 1, 6 and 11 approximately, the sugar intervention was administered across hours 1-9. Data presented as mean ± SEM (n=20). Linear mixed effects modelling showed significantly lower leptin concentrations during the sugar day ($p=0.001$) as well as showing significant differences between time-points across the days ($p<0.05$).

3.3.4 Variation across 12 hours in macronutrient concentrations

Although there were significant changes across the full day, the repeated measures analysis reported few differences between specific hours of the day across both diets. Milk lactose, protein and glucose did not show any significant variations in relation to time of day independent of the maternal diet, although glucose exhibited peaks at hours 3 and 4, 8 and 9 that appeared to be in response to mealtimes (Figure 3.3 - 3.5). Milk fat concentrations did not show any significant variations within either day with the exception of a difference being recorded between day 1 hour 1, and day 2 hour 2 ($p=0.041$; Figure 3.2).

3.3.5 Variation across 12 hours in hormone concentrations

Hormone concentrations showed more considerable variation across time of day. Leptin and insulin concentrations varied across each of the individual study days as well as between days. The within-day variations in insulin concentrations appeared to be related to maternal food consumption, as peaks followed 2-3 hours after food consumption. Insulin concentrations varied across individual days as well as between days, exhibiting a notable rise and fall pattern across the 12-hour sampling period (Figure 3.8, $p<0.05$). Figure 3.7 shows an overall decline in leptin concentrations across both days ($p<0.05$). Adiponectin concentrations did not vary significantly across either study day (Figure 3.6).

3.4 Discussion

In the current study, we verified the findings from our previous pilot study (Chapter 2; Ward et al. 2021) demonstrating that milk fat content increased in response to a higher sugar diet compared to a lower sugar control diet. We further extended these findings to demonstrate that a higher sugar intake was also associated with higher lactose and glucose concentrations, and lower levels of protein and of the metabolic hormones, adiponectin and leptin. These observations support our hypothesis that acute changes in maternal diet, specifically increased maternal sugar consumption, are associated with short-term changes in both macronutrient composition and hormone/metabolite concentrations in BM.

In line with our pilot study, we observed that increasing maternal sugar intake was associated with a significant (13.4%) increase in the total fat concentrations in BM. Furthermore, this increase was observed 2 hours following the initial sugar ingestion within the breakfast meal, and was maintained throughout the rest of the day, suggesting an immediate and sustained effect on BM fat content. Fats in human milk are derived from maternal circulation (longer chains) as well as being produced *de novo* within lactocytes (medium and intermediate chains) (Visentainer et al. 2018, Demmelmair and Koletzko, 2018). Given that increased sugar consumption is known to stimulate *de novo* lipogenesis (Stanhope et al. 2009, Samuel, 2011), the higher BM fat concentrations following higher maternal sugar consumption could potentially be driven by an upregulation of intermediate FA synthesis, either systemically or

specifically within the mammary epithelial cells. This is supported by the increased levels of glucose we observed in response to the sugar diet, suggesting higher sugar consumption leads to higher sugar concentrations in maternal circulation that were transferred to the BM and/or stimulated lipogenesis. It is also important to note that fat concentrations in human milk are highly variable and believed to be related to breast fullness, with higher concentrations being recorded at the end of a feed and levels increasing across the course of a day (Mitoulas et al. 2002, Khan et al. 2013, Daly et al. 1993). This makes it difficult to confidently attribute our findings to the intervention and speaks to the complexities of human milk compositional research. It is also worth noting that the increase in maternal sugar consumption was relatively modest (~66g across the day, not much more than a 500ml bottle of Coca-Cola) suggesting that if the effects observed are associated with this intervention, there could be larger more impactful influences on milk composition in women who consume higher amounts of sugar or those who are consuming similar sugar levels daily.

While the effects of increased sugar consumption on fat concentrations were in line with our pilot study, findings for other macronutrients were different. The current study found that higher maternal sugar intake was associated with higher lactose, and lower protein concentrations, whereas no effects of higher sugar intakes were observed in our previous trial. We also found increases in BM glucose concentrations in response to the higher sugar diet. As previously discussed, however, our pilot study was essentially a proof-of-concept study and was likely underpowered to detect more subtle differences in macronutrient concentrations.

Lactose is formed *de novo* in the breast via the joining of glucose and galactose by the enzyme lactose synthase (Langley-Evans, 2021). Thus, it is possible that the increased blood sugar concentrations resulting from higher sugar consumption provides more substrate for this reaction, leading to higher lactose production in the breast. This is supported by previous findings demonstrating that exogenous glucose infusions result in increases in both glucose and lactose concentrations in human milk (Neville, Hay and Fennessey, 1990). Milk glucose concentrations are related to milk secretion and milk lactose concentrations in mammalian milk are known to be directly related to milk volume as lactose draws water into the Golgi

apparatus via osmosis (Neville, Hay and Fennessey, 1990, Fox, 2009). These functions are crucial and well-regulated, therefore, the idea that their concentrations increase simply due to having more substrate available may be too simplified. Neville, Hay and Fennessey observed an increase in milk glucose concentrations when plasma glucose was increased and maintained over 4 hours and suggested that milk glucose tends to equal plasma glucose. A short-term sugar intervention study that compared the effects of mothers consuming a high fructose corn syrup (HFCS) sweetened beverage with an artificially sweetened beverage noted significant effects on BM fructose concentrations within 2 hours of consumption in the HFCS group. They did not note any significant differences in milk lactose or glucose concentrations between the two groups (Berger et al. 2018). These findings are interesting and different to what was observed in the current study. Berger et al used a crossover design where each mother consumed either the HFCS or artificially sweetened interventions, which could explain the differences in results as our study allowed each woman to be her own control.

The same group have also recorded positive relationships between BM fructose concentrations and infant body compositional measures including infant weight, lean mass and fat mass (Goran et al. 2017). Increased carbohydrate concentrations in BM have been correlated with changes in infant body composition over the first 12 months of life. A study exploring the relationships between total carbohydrate, lactose and human milk oligosaccharide concentrations and infant anthropometric measures noted a positive relationship between infant length, weight, fat free mass and fat free mass index. They noted a positive relationship between lactose intake (not concentrations) and greater fat mass, fat mass index and % fat mass and fat mass/free fat mass ratio. This study concluded that increased concentrations/intakes of carbohydrates including lactose across the first year of life may increase fat deposition in the infant, which could predispose to obesity later in life (Gridneva et al. 2019).

Protein concentrations in this study were lower when women consumed more sugar. Protein concentrations in human milk are thought to be relatively stable and a systematic review completed by Bravi et al. in 2016 found no maternal dietary effect on BM protein

concentrations within any of the studies they included. These studies tested the impacts of multiple dietary interventions including high carbohydrate/low fat, low carbohydrate/high fat, and changes in protein and total maternal energy intake (Bravi et al. 2016, Mahmoud, Agneta and Morey, 2009, Boniglia et al. 2003). However, a recent study investigating the influence of maternal diet on milk components in 136 lactating women identified significant associations between maternal protein intake and BM protein concentrations, suggesting that changes in maternal protein intake could potentially influence protein concentrations in breast milk (Binder et al. 2023). This is unlikely to explain the findings in the current study, however, given that the difference in protein content on the higher sugar day was only 0.5g (less than half the protein content of 1 egg) lower than on the control day. To the best of our knowledge there have been no studies completed to investigate the impact of maternal dietary choices around sugar and fat on the wider nutritional profile of milk, therefore, further studies are required to determine the mechanisms through which maternal CHO intake could influence BM protein.

For the purpose of this discussion, we have calculated estimated infant intakes of each macronutrient and the % differences in these between the control and sugar day. Calculations assumed infants consumed 750g of BM across the day, based upon previous reporting of average intake for infants around the age of our cohort (Dewey et al. 1991, Norrish et al. 2023). Estimated average intakes of fat, lactose and glucose concentrations were 14.8, 10.1 and 11.8% higher respectively during the sugar intervention and protein intakes were 10.1% lower. These values, whilst crudely calculated, represent the potential nutritional differences in infants consuming BM from mothers consuming higher sugar diets compared to lower sugar control diets. It is important to note that these are mean differences across the population and while increases in response to the sugar intervention were true for the majority of participants, these changes were not observed in all individual women. Fat and carbohydrates in BM have previously been positively correlated with infant weight, adiposity and BMI, raising the possibility that the changes in BM composition induced by higher maternal sugar consumption could influence infant growth and body composition in the longer term (Brockway et al. 2023, Norrish et al. 2023).

Further to the macronutrient response we observed reduced concentrations of adiponectin (9.8%) and leptin (21%) during consumption of the sugar diet when compared to the control. This is similar to the findings of a study conducted by Leghi and colleagues, who reported significant decreases in BM leptin after 2 weeks of a dietary intervention which involved significantly lowering sugar intake (Leghi et al. 2021). The authors commented that these changes could potentially have been secondary to maternal weight loss. Binder and colleagues reported that a higher maternal protein intake was associated with increases in the concentrations of adiponectin and leptin in breast milk (Binder et al. 2023), raising the possibility of an inverse relationship between protein intake and breast milk concentrations of these metabolic hormones. However, given the modest difference in protein content of the higher sugar and control diets, lower protein intake seems an unlikely explanation. Furthermore, both adiponectin and leptin are known to be derived from maternal circulation as well as being made *de novo* within the lactocytes (Smith-Kirwin et al. 1998, Savino, Liquori and Lupica, 2010) and both have been reported as having correlating concentrations in BM with maternal circulation (Savino et al. 2012, Savino et al. 2016). Adiponectin intake has been negatively associated with FFM in infants whilst skim milk leptin has been positively associated with infant anthropometry (Norrish et al. 2023).

While the current study raises interesting questions about the factors regulating leptin and adiponectin concentrations in human milk, the number of studies in this area is extremely limited, and further research is needed to fully elucidate the relationship between maternal diet and the variations in concentrations of these metabolic hormones within BM.

Based on the findings of our pilot study, we had also expected to see variations across the day in macronutrient and hormone concentrations. In the current study, however, we saw no differences in macronutrient concentrations between time-points on either of the study days, apart from a difference in fat concentrations between time-point 1 on the control day and time-point 2 on the sugar intervention day. These observations are also in contrast to the findings of a systematic review focusing on circadian variations in BM components, which concluded that some BM constituents, including total fat, do vary systematically across the day (Italianer et al. 2020). However, the authors of this review noted that there was a high degree of heterogeneity between studies, making it difficult to develop robust conclusions.

Furthermore, while the findings of the current study do not support our observations in Chapter 2, they are more aligned with other studies (Leghi, et al. 2021, Khan et al. 2013, Paulaviciene et al. 2020,).

The differences in findings between the current study and pilot study reported in Chapter 2 could be related to a number of different factors. The most important of these is likely to be the sample size, but it is also possible that observations are explained by differences in the baseline diets of the women or other systematic differences between the British and Australian volunteers. Furthermore, the differences in methodologies for measuring macronutrients may explain some of the variations we have observed across studies. This is particularly likely with lactose. We observed a high variability within assays in the pilot study, which was not evident in this study, suggesting the methods we have utilised within the second study were better suited for lactose measurement in BM. The laboratory methodologies used in this study differ to those used in our pilot study (detailed in Chapter 2). I was invited to attend a host laboratory (The Geddes Hartmann Human Lactation Research Group at UWA, Perth) where I completed the laboratory analyses for this study. The team at UWA are well known for their research into human milk and their methodologies are tried and tested thanks to the many great scientists within the team. Thus, I gratefully accepted the opportunity to complete the analyses according to their procedures despite these being different to those utilised in the pilot study.

Although we did not detect significant variations across the day in any of the macronutrients, we did observe diurnal fluctuations for two of the metabolic hormones – leptin and insulin. Leptin concentrations appeared to decline gradually from morning to evening, whilst insulin concentrations showed an undulating pattern across the day with peaks at hours 3, 4, 8 and 9. Leptin is a satiety inducing hormone that acts to decrease appetite and increase energy expenditure (Obradovic et al. 2021). The decline in leptin concentrations across the day that we have observed contrast with the patterns previously reported in adults, in whom leptin concentrations have been reported to increase across the day and reached up to double their concentration at night than in morning (Langendonk et al. 1998, Sinkiewicz-Darol et al. 2022). Our observations are also in contrast with a previous study reporting on leptin concentrations in BM over 24 hours, which reported a plateau between 06.00 and 17.00 and an increase from 17.00 to 24.00 (Cannon et al. 2015). The downward trend we detected in this study,

however, was a maximal difference of around 1ng/ml which may appear as a plateau or become insignificant if a further 12 hours of sampling had been completed. Furthermore, a recent study on 22 women identified clear circadian rhythms in milk leptin, insulin, adiponectin, glucose and fat noted a decline in leptin concentrations between hours 12.00 and 17.00 suggesting we are not the only group to note this gradual decrease across the day (Suwaydi et al. 2023). This paper collected pre and post feed samples at each feed over 24 hours in conjunction with test weighing to calculate infant intake and used the same analytical methods for macronutrient and hormone concentrations that were utilised for our work.

Interestingly, the peaks in insulin concentrations across the day occurred 2-3 hours post meal consumption, suggesting spikes occurred in response to maternal food consumption. Furthermore, the fluctuations in glucose concentrations visible in figure 3.4, although not statistically significant, appeared to be consistent with the time of rising insulin in milk. This is consistent with insulin serum responses previously reported in adult clinical trials (Sun et al. 2020, Dunstan et al. 2012) where insulin levels peak between 30 and 120 minutes postprandially, depending on what foods were being consumed. Similarly, BM insulin levels have been shown to increase significantly 90 minutes after maternal mealtimes, in line with increases in maternal plasma insulin (Rodel et al. 2022). As noted above, a recent publication identifying clear circadian rhythms in milk hormones have also noted significant variations in milk insulin concentrations across the day. They do not, however, report insulin peaks responding to maternal mealtimes, this may be due to their longer sampling period and lack of maternal mealtime data (Suwaydi et al. 2023).

We did not observe any significant variations in adiponectin concentrations across the 12-hour sampling period. This is in contrast to the findings of Suwaydi et al. 2023, who noted a strong diurnal rhythm with adiponectin concentrations rising between 10.00 and 20.00. Circadian variations in BM adiponectin concentrations are scarcely investigated making it difficult to determine why we did not observe similar fluctuations across the day, although, it could potentially be related to the difference in sample sizes between these studies.

The methodology used in this piece of work has been extensively considered and was optimized based upon the methods used in our pilot study in a bid to elucidate robust and quality observations. We completed a power calculation based on the TAG concentrations measured in the pilot and doubled the sample size to ensure any observations noted in this work would have appropriate statistical power, and to allow for any dropouts or extenuating circumstances. The analytical methodology used in this work has been developed and validated by the Hartmann Geddes Human Lactation Research Group in the University of Western Australia. These methods have been previously reported in numerous peer reviewed clinical trials and are the product of significant work by this team. The decision to visit this laboratory for compositional analyses has beneficially impacted the overall results of this chapter and by extension, the thesis.

Although this work has been thoroughly designed and the concept modified from our pilot study, some limitations remain. Due to the intensive sampling strategy employed we did not request pre- and post-feed samples. Significant differences between pre- and post-feed concentrations of milk fat, glucose, adiponectin and insulin concentrations have been noted in other studies. Higher concentrations of milk glucose and adiponectin and lower concentrations of milk fat and insulin concentrations have been reported in pre-feed samples (Mitoulas et al. 2002, Suwaydi, 2023). We did, however, request that BM be collected from the fullest breast at each sampling time in a bid to control the impact of extensive breast emptying where possible. Furthermore, milk sample collection along with test weighing infants to determine infant consumption has been proven to be more powerful than simply recording absolute concentrations of milk components. This is particularly impactful when attempting to extrapolate our findings to potential infant outcomes (Gridneva et al. 2023), hence our inclusion of crude infant intake estimations discussed above. A further limitation could be that we did not repeat the whole sampling procedure multiple times for each participant. Repeating these methods for each participant may provide further insight into the strength of associations between the acute response in milk macronutrients and changes in maternal consumption. The nature of human milk studies makes it difficult to strike a balance between methodological robustness and validity without creating a negative impact on the mother and infant dyad or disrupting their often-demanding routines. These are

important considerations in future work and were given significant consideration in the design of this trial.

In conclusion, we have identified small but statistically significant differences in human milk fat, protein, lactose, glucose, adiponectin and leptin concentrations in response to acute modifications in maternal sugar consumption. We have also detected circadian patterns in both leptin and insulin across the 12-hour sampling period. While these observations are interesting it is difficult to transpose these findings into potential impacts on infant health and development. However, if extrapolated over time, or researched in women with high habitual sugar intakes, it may show significant impacts on milk composition and potentially infant outcomes. Nevertheless, this research represents another piece in the puzzle to further understanding how maternal diet impacts BM composition and requires more work to unravel the mechanisms behind these responses as well as how impactful they may be on the feeding infant. Future recommendations based upon this report include a similar study design with multiple data collection days per participant as well as including test weighing of infants.

CHAPTER 4

IMPACT OF MATERNAL FATTY ACID CONSUMPTION FROM DIET AND SUPPLEMENTS ON BREAST MILK OMEGA-3 LONG CHAIN POLYUNSATURATED FATTY ACIDS- A SYSTEMATIC REVIEW AND META-ANALYSIS

4.1 Introduction

Human milk fat concentrations are one of the most variable constituents in BM (Visentainer et al. 2018, Demmelmair and Koletzko, 2018) and it has been demonstrated that concentrations of individual FAs in BM are influenced by maternal dietary choices (Montez de Sousa et al. 2022, Bravi et al. 2016). The concentrations of omega-3 LCPUFAs in BM have been a particular focus of attention, due to the established importance of these fatty acids in supporting the development of the infant's brain and visual systems (Innis, Gilley & Werker, 2001, Koletzko and Rodrigues-Palmero, 1999). In early studies, higher levels of the omega-3 LCPUFA DHA (C22:6N-3) were observed in the brain cortex of breast-fed infants than in formula fed infants by Makrides et al. in 1994. A meta-analysis conducted in 1999 found that BF was associated with better cognitive development than AF (Anderson et al. 1999). DHA (C22:6N-3) intake from AF has since been shown to have lasting impacts on brain structure and function (Lepping et al. 2019) as well as reduced incidence of lower respiratory tract infections and possible associations with reduced asthma symptoms (Adjibade et al. 2021).

Given their important role in infant development, the relationship between maternal dietary omega-3 FAs and their concentrations in BM has been the subject of many studies. There is an extensive body of literature that considers the impact of omega-3 supplementation (Khandelwal et al. 2023, Schaefer et al. 2020). The BM concentrations of LCPUFAs are suggested to be particularly responsive to habitual maternal LCPUFA intake (Krešić et al. 2013, Liu et al. 2016, Bravi et al. 2021), with positive associations also being reported in response to fish consumption (Jorgensen et al. 2001). Although there are some studies that have failed to detect this significant relationship between habitual intake and concentrations in milk (Jirapinyo et al. 2008). Increasing maternal omega-3 LCPUFA intake via supplementation has also been shown to positively impact milk DHA (C22:6N-3) concentrations (Jensen et al. 2000). These observations likely reflect the fact that short chain fatty acids are synthesised *de novo* in mammary tissue, whilst longer chain fatty acids are taken up from the maternal circulation or enter milk in the membranes of endocytic vesicles (Demmelmair & Koletzko, 2018, Langley-Evans, 2021).

Further to their increased concentrations in milk, positive associations have been observed between breastfed infants' IQ and visual acuity at 4 years when maternal intake of omega-3 LCPUFAs in pregnancy and lactation was higher (Helland et al. 2003, Lauritzen et al. 2004). The relationship between maternal omega-3 supplementation during lactation and infant outcomes is, however, far from clear, with a systematic review that attempted to synthesise this body of literature reporting a lack of consistency in the results obtained. While the heterogenic nature of the included studies was identified as a limitation which made it difficult to compare studies, the overall conclusion of the review was that there was no compelling evidence of a positive association between omega-3 supplementation in pregnancy and/or lactation and infant cognitive outcomes (Nevins et al. 2021).

The current systematic review and meta-analysis aimed to evaluate the scientific literature investigating the effects of maternal intake of omega-3 LCPUFA, derived from both habitual dietary and supplemented intake, on the FA composition of BM. Previous systematic reviews in this research area, such as those discussed previously, have focused on the impact of *either* maternal diet or supplement use on BM FA. Evaluating whether both habitual and supplemental intake of omega-3 LCPUFA intake influences BM omega-3 LCPUFAs will allow us to determine which source, if any, has a stronger influence on BM FA composition, and this represents an important and novel aspect of this review.

4.2 Methods

4.2.1 Study Design and Search Strategy

This systematic review was prospectively registered in Prospero (CRD42022339642). The protocol was not appended to the registration. A detailed search of the scientific literature was carried out in March 2022 to identify all published studies investigating the impact of fatty acids derived from either the diet or supplementation on breastmilk omega-3 LCPUFA composition. A computerised search was carried out by one reviewer on three separate databases: Medline, Web of Science and Scopus. The search terms, including medical subject heading (MeSH) terms and truncations used for Medline were: Exp lactation/ OR lactation.ti,ab. OR exp breast feeding/ OR (breast-fe* or breastfe*) OR exp milk, human/ OR

lactating mother*.ti,ab. OR lactating woman.ti,ab. OR lactating women.ti,ab. AND exp fatty acids/ OR fatty acid*.ti,ab. OR (omega-3 or omega-3).ti,ab. OR LC n-3 FA*.ti,ab. OR N-3 FA*ti,ab. OR N-3 fatty acid*.ti,ab. OR Docosahexaenoic acid*.ti,ab. OR Docosahexanoic acid*.ti,ab. OR Docosahexenoic acid*.ti,ab. OR Eicosapentaenoic acid*.ti,ab. OR Eicosapentanoic acid*.ti,ab. OR Eicosapentenoic acid*.ti,ab. OR Fatty acids omega-3*.ti,ab. OR N-3 OR N-3 PUFA*.ti,ab. OR DHA OR EPA. The literature search was limited to studies in humans, but a date range restriction was not applied. Versions of the above search method were used for Web of Science and Scopus searches. Further details of these are supplied in Appendix 3 as well as a completed PRISMA checklist.

4.2.2 Eligibility Criteria

Articles that met the following selection criteria were included:

- Studies which recruited healthy mothers between the ages of 18-35, who had a reported healthy BMI.
- Studies which recruited mothers with healthy, full-term, singleton infants.
- Studies which quantifiably reported maternal fatty acid intake (through diet and/or supplementation) as well as the FA composition of BM (specifically omega-3 long chain PUFAs).
- Studies which failed to specify infant gestational age at birth or did not report maternal age or BMI were excluded, as were studies conducted on animals, reviews, meeting abstracts, letters or commentaries and studies that were not published in English.

4.2.3 Study Selection

Research articles that were identified in the search were imported into EndNote X9. Following the removal of duplicates, the titles of all papers were screened against the inclusion criteria. For those papers that were deemed to be potentially eligible, the abstracts were then independently reviewed by 3 authors (EW- Ellen Ward, ME- Matthew Elmes and SLE- Simon Langley-Evans), with all conflicts resolved by mutual discussion. Full-text review

of the remaining articles was conducted by EW, and reviewed by SLE and ME, with any conflicts again resolved by collective discussion and decision.

4.2.4 Data Extraction and Analysis

Data was extracted from the reviewed papers utilising a standard template that was specifically modified for this review. This allowed the reviewers to ensure a consistent approach to collecting information on study design (population, sample size, research setting), objectives and outcome measures. The main outcomes of interest include quantitative data on omega-3 LCPUFAs and LCPUFAs in maternal diet and human milk, ratio of omega-6/omega-3 PUFAs/LCPUFAs, if these were reported, in conjunction with the results of any correlation between maternal fatty acid intakes (from diet or supplements) and BM fatty acid concentrations. All relevant data is presented in Table 4.1 and any correlation or regression analyses reported are presented in Table 4.2 in Appendix 4. All eligible data from the 5 studies that reported mean differences between control and intervention groups, or lower and higher intake groups was included in the meta-analysis.

4.2.5 Quality Assessment

Assessment of study quality and risk of bias was completed using a previously published tool used by Leghi et al. in 2020 which was derived and adapted from Andreas et al. 2014. For the purposes of this review, some additional criteria were also included; (i) whether the study specified if fore, hind, full feed or other sample type was collected, (ii) if women were required to be EBF (and whether or not this was reported), (iii) if time of milk collection was standardised, both in relation to time of day and time postpartum, (iv) if FA intake was measured appropriately or (in the case of supplement studies) that supplement dose was controlled appropriately, (v) if the study controlled for relevant confounders (including but not limited to maternal and infant age, stage of lactation, infant sex) and (vi) to what extent the demographic of the study population was representative of the average lactating woman in the community. The full quality assessment tool is displayed in Table 4.3 in Appendix 4. Each manuscript was assessed independently by two authors (EW and SLE) and any differences resolved by discussion.

4.2.6 Meta-Analysis

To be eligible for inclusion in the quantitative analysis studies had to report mean differences between supplemented/higher FA intake and control/lower FA intake. Meta-analyses were completed using the Review Manager (v5) web-based software. Results are presented as forest plots showing pooled mean differences, standard deviation and 95% confidence intervals. All mean differences were entered as % of total FAs and heterogeneity and the overall effect size of combined studies was reported. A random effects model was applied given the high heterogeneity between studies. Separate funnel plots were created to assess publication bias for each measure (DHA (C22:6N-3), EPA (C20:5N-3) and total omega-3 PUFAs) A p value <0.05 was considered significant.

4.3 Results

4.3.1 Summary of included studies

The initial search strategy yielded a total of 7044 papers after removal of duplicates. Of these, 6853 were removed during the title/abstract review stages, 191 full-text papers were reviewed and assessed against the eligibility criteria resulting in a total of 8 papers (a total number of 535 BF women) were included in the review. Of these 8 papers, 5 reported quantitative mean differences in milk omega-3 PUFA concentrations and were included in the meta-analyses. Further detail on the review process and reasons for exclusion is provided in a Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) flow diagram below (Figure 4.1). The 8 studies included in the review spanned several countries and were all published between 2001 and 2020. Sample sizes varied greatly, ranging from 10 (Del Prado et al. 2001) to 233 (Helland et al. 2006). The design of the studies also varied, with 4 studies applying an intervention in the form of LCPUFA supplementation (Ay et al. 2018, Helland et al. 2006, Patin et al. 2006 and Weseler et al. 2008), while the remaining 4 were observational studies which aimed to relate reported maternal dietary omega-3 LCPUFA intakes to BM omega-3 LCPUFA concentrations (Del Prado et al. 2001, Nishimura et al. 2014, Ueno et al. 2020 and Xiang et al. 2005). One of these observational studies also assessed FA transfer from the mother to the BM by supplying participants with labelled LA (C18:2N-6) at the beginning of the study (Del Prado et al. 2001). A summary of all the included studies,

including participant demographics, study design and outcome measures is presented in Table 4.1, Appendix 4, and results of the studies that were eligible for quantitative analyses are presented in Figures 4.2 and 4.3 below.

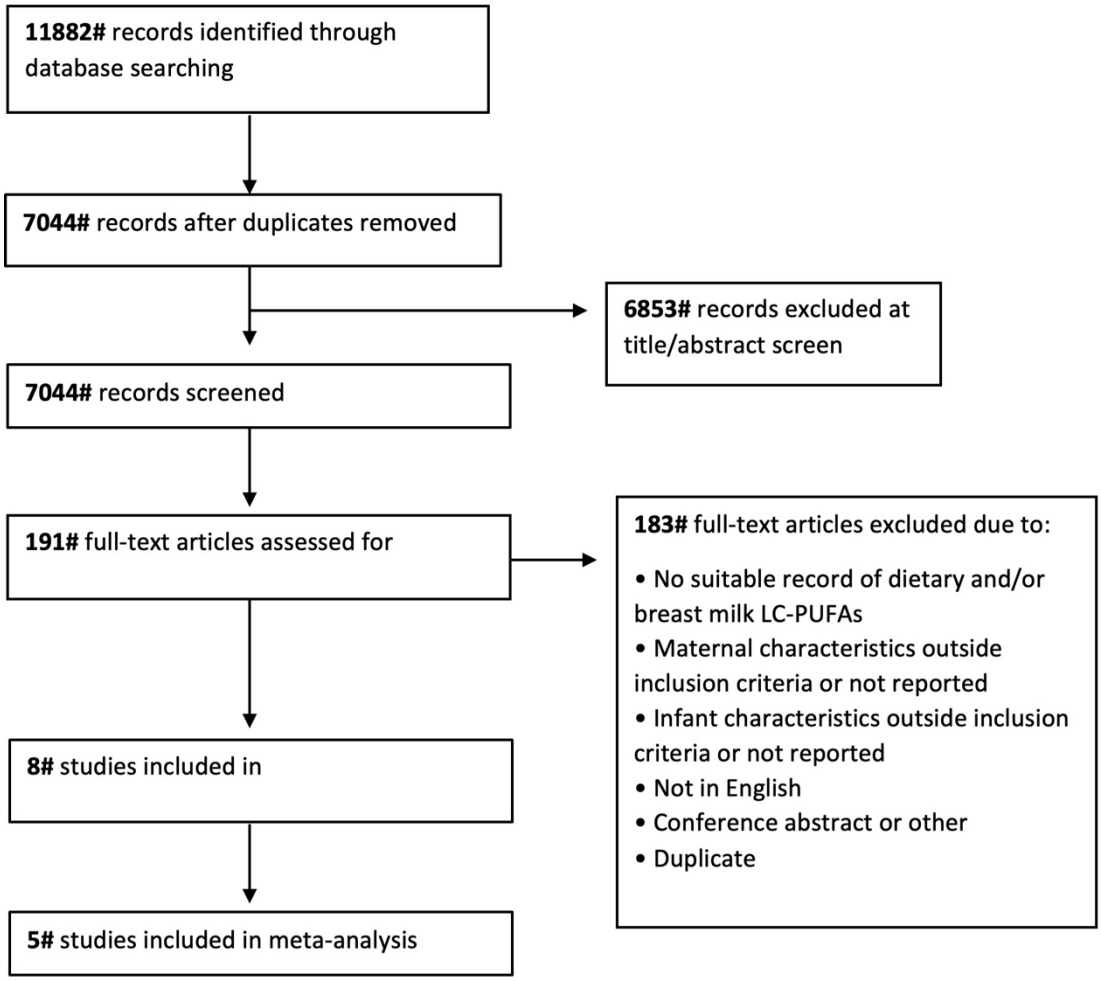


Figure 4.1: A flow chart depicting the process of elimination of papers at each stage leading to the final 8 studies included in the systematic review and 5 studies in the meta-analysis.

4.3.2 Quality Assessment

Results of the quality assessment are presented in Table 4.3, Appendix 4. The quality assessment tool was divided into 2 sections; section 1 reviewed methodology surrounding

sample collection and dietary intake, whereas section 2 assessed whether consideration had been made for confounding variables as well as the cohort's representativeness and sample size. The highest scoring paper in this review received 6 out of a possible 8 (Nishimura et al. 2014), with small sample size and failing to report the type of milk collected identified as quality issues. The quality of the remaining papers was assessed as being low to moderate, ranging from 3.5 – 5/8 with a score of 4/8 being received by half of the studies. There were 2 studies that failed to receive any scores in section 2, but still received a 4 overall (Del Prado et al. 2001 and Patin et al. 2006). This suggests they were aware of the complexities of collecting human milk but did not place emphasis on robust recruitment of a study cohort, or the appropriate adjustment of the data to include confounding variables.

4.3.3 Supplement interventions vs habitual diet observations

Half of the studies included in this review provided participants with an omega-3 LCPUFA supplement, with the aim of relating this to the LCPUFA concentrations in BM. However, each of these 4 studies used different study designs, complicating the comparison. The studies by Helland et al. and Weseler et al. were randomised controlled trials. Whilst Helland et al. had a standard supplement vs control design, Weseler et al. included 4 different groups (3 test supplement groups (DHA (C22:6N-3) +EPA (C20:5N-3), Low AA (C20:4N-6), High AA (C20:4N-6)) and 1 control). A further study used a supplement vs control design, however, the control group did not receive a placebo, instead comprising of participants who agreed to be in the study but were not willing to be assigned to the intervention (Ay et al. 2018). The final study involved a dietary intervention of increased intake of sardines to supplement participants' PUFA intake – however, this same intervention was applied to all participants, with results based on a comparison of participants with the lowest vs highest adherence to the intervention, rather than a treatment vs control group comparison (Patin et al.2006).

The remaining 4 papers included in the review were observational in design with a focus on assessing habitual LCPUFA intakes of the women and relating this to the concentrations of the respective FA in BM. The assessment of maternal FA intake was conducted using different tools across the studies; Xiang and colleagues used a 3-day dietary record, Nishimura et al. utilised a Food Frequency Questionnaire (FFQ), while Ueno et al. used both a standard FFQ and a tailored questionnaire specifically designed to determine participants' habitual use of

DHA (C22:6N-3) supplements. The final study assessed maternal FA intakes by using both a 24h dietary recall and 2-day test-weighing approach (Del Prado et al. 2001).

4.3.4 Methodologies for BM collection and fatty acid analysis

There was significant heterogeneity between methodologies used to collect BM samples. Only 4 of the 8 included studies reported and/or required that participants were EBF at the time of collection (Del Prado et al. 2001, Nishimura et al. 2014, Patin et al. 2006 and Xiang et al. 2005). Of the 8 studies, 6 reported the type of milk collected (fore/hind milk). The type of milk collected was inconsistent across the publications, ranging from after infants fed (n=3) (Nishimura et al. 2014, Patin et al. 2006 and Xiang et al. 2005), to mid feed (n=2) (Helland et al. 2006 and Weseler et al. 2008) and before and after a feed which was presumably pooled for analysis (Del Prado et al. 2001). Ay et al. and Ueno et al. failed to specify what type of milk or when relative to the feed, milk was collected. Most of the studies (n=5) did, however, report the time postpartum (n=6) and/or the time of day (n=5) that BM samples were collected. Ueno et al. simply reported collecting milk at multiple stages over the first 6 months postpartum and requested participants provide one sample per day for 7 days which was pooled before analysis. Nishimura et al. reported collecting milk between 5 and 12 weeks postpartum. The study by Ay et al. collected samples at multiple stages of lactation but were more specific, colostrum was collected just after birth as well as a sample of mature milk at 6 months postpartum. Similarly, the studies by Helland et al. and Weseler et al. involved the collection of BM samples at 4 and 12 weeks and at 3-, 5- and 11-weeks postpartum respectively. Xiang et al reported collecting study material when infants were 3 months old. The remaining studies did not specify at what point postpartum BM samples were collected, but rather reported collection of samples at defined points after the start of the intervention or observational study. Thus, Patin et al. collected samples on days 0, 15 and 30 of their intervention and Del Prado collected milk at 9 time-points across a 72h period.

There was also considerable variation across studies on the volume of BM collected. Helland et al. and Ueno et al. did not report the volume collected, whereas Patin et al. reported a minimum requirement of 40ml. The remaining studies requested BM sample volumes of

between 2-10ml. Interestingly, two of the included studies requested BM samples from both breasts (Patin et al. 2006 and Weseler et al. 2008), yet only Weseler et al. reported that these samples were pooled prior to analysis. Of the studies that reported the method of BM collection (n=6), 5 used hand expression, while one instructed women to use a breast pump (Ueno et al. 2020). The method of assessment of BM FA composition was consistent across all studies included in this review, namely total lipid extraction, followed by separation into fatty acid methyl esters and analysis by gas chromatography or gas-liquid chromatography.

4.3.5 LCPUFA intake

The studies involving omega-3 LCPUFA supplements (n=4) provided participants with varying doses and FA composition, which were supplied at different time-points and for differing durations pre- and post-partum. The study by Ay et al. provided mothers with a capsule containing 378mg of DHA (C22:6n-3) and 504mg of EPA (C20:5n-3) (950mg of total omega-3 PUFAs) that were consumed once a day from the beginning of the 3rd trimester in pregnancy until the end of the 6th month of lactation. The control group in this study did not receive a placebo but comprised of women who did not want to take the supplements. In the study by Helland et al. women in the intervention group received 10ml cod liver oil per day containing 1183mg DHA (C22:6n-3) and 803mg EPA (C20:5n-3) (2632mg of total omega-3 PUFAs), while the control group received corn oil containing 4747mg of LA (C18:2n-6) and 92 mg of ALA (C18:3n-3). Patin et al. took a different approach in that women were instructed to consume 500g per week of fresh sardines (containing 6.44% DHA (C22:6n-3), 1.41% EPA (C20:5n-3) and 12.06% total omega-3 PUFAs). The final supplementation study by Weseler et al. included 4 different groups, 1 control and 3 that received various PUFA based interventions in the form of a powder-based drink. The DHA (C22:6n-3)+EPA (C20:5n-3) group received 320mg DHA (C22:6n-3), 80mg EPA (C20:5n-3) and 80mg of other omega-3 PUFAs while the 2 remaining intervention groups (Low AA (C20:4n-6) group and High AA (C20:4n-6) group) received the same as the DHA (C22:6n-3)+EPA (C20:5n-3) group plus additional AA (C20:4n-6) (200mg and 400mg respectively). The control group received a control product containing no LCPUFAs.

Of the 4 studies investigating the habitual dietary intake of FA, Del Prado et al. and Xiang et al. presented results as g/day whereas Nishimura et al. presented this data as % of total FAs

consumed and Ueno et al. as mg or g/1000 kcal. Habitual DHA (C22:6n-3) intake was the only omega-3 LCPUFA that was reported across all 4 studies. The mean dietary intake of DHA (C22:6n-3) ranged between 0.01-0.12g/day as reported by Xiang et al. who provided data from a Chinese cohort and a Swedish cohort, the DHA (C22:6n-3) intake was significantly lower in the Chinese population. The total DHA (C22:6n-3) was reported to provide 0.054% of total FA in diet by Nishimura et al. and lastly DHA (C22:6n-3) was reported to make up 235.9mg/1000kcal in all maternal diets reported by Ueno et al. with a higher intake observed for DHA (C22:6n-3) supplement users (265.4mg/1000kcal) compared to non-supplement users (225.1mg/1000kcal). A detailed summary of the PUFA intakes reported across studies is provided in Table 4.1, Appendix 4.

4.3.6 Milk LCPUFA concentrations

The DHA (C22:6n-3) concentration within BM was reported in all studies, whereas EPA (C20:5n-3) was reported by 7 and total omega-3 LCPUFA concentrations were reported by 6 of the 8 included studies. The majority reported milk FAs as % of total fatty acids (n=6) and the remainder as g/100g of total fatty acids (n=2). In the habitual dietary intake studies the mean concentration of DHA (C22:6n-3) in BM ranged from 0.1-0.55%, EPA (C20:5n-3) from 0.02-0.16% and total omega-3 from 0.57-2.44% of total FAs. BM omega-3 LCPUFA concentrations were higher in those studies that involved maternal FA supplementation, with mean DHA (C22:6n-3) concentrations ranging from 0.31-1.37%, EPA (C20:5n-3) 0.07-0.43% and total omega-3 from 0.8-3.35 % of total fatty acids. Not surprisingly the highest reported values for DHA (C22:6n-3), EPA (C20:5n-3) and total omega-3 PUFAs within this data set came from the study that supplied the highest number of omega-3 FAs in their intervention (1183mg of DHA (C22:6n-3), 803mg of EPA (C20:5n-3), 2632mg of total omega-3 PUFAs) (Helland et al. 2006).

All of the supplementation studies reported a significant increase in BM DHA (C22:6n-3) concentrations in those groups receiving the omega-3 supplements. Furthermore, all studies that investigated the relationship between maternal dietary LCPUFA intakes and the BM FA concentrations reported significant positive relationships between these measures (n=3). A

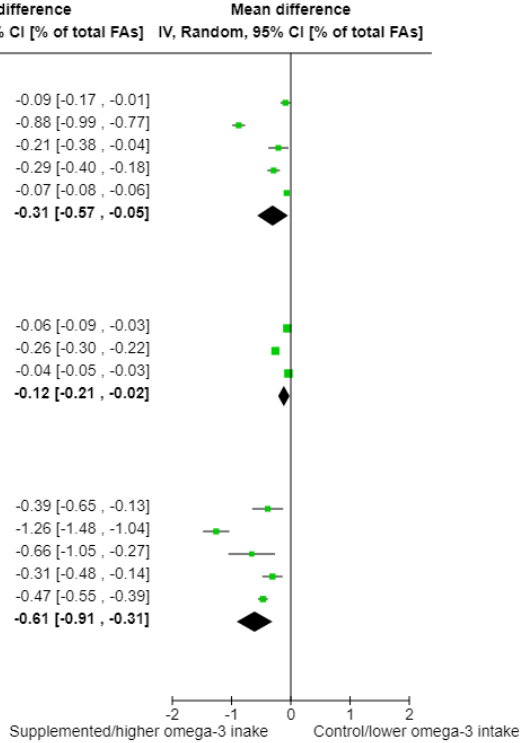
significant positive correlation was found between dietary FA intake postpartum and the omega-3/omega-6 ratio in BM by Nishimura and colleagues. Similarly, positive correlations were reported between habitual consumption of DHA (C22:6n-3) supplements and increased frequency of supplement use with BM DHA (C22:6n-3) concentrations (Ueno et al. 2020) and between habitual dietary DHA (C22:6n-3) intake and BM DHA (C22:6n-3) concentrations (Xiang et al. 2005). Details of the reported correlations between omega-3 PUFA intake and BM FA concentrations are presented in Table 4.2, Appendix 4.

4.3.7 Meta-Analysis

As several studies reported mean differences in DHA (C22:6n-3) (n=5), EPA (C20:5n-3) (n=3) and total omega-3 PUFAs (n=5) in BM either between control and supplemented groups or between low and high habitual intake groups a meta-analysis was undertaken (Figure 4.2). The meta-analysis clearly highlighted that the mean concentrations of DHA (C22:6n-3), EPA (C20:5n-3) and total omega -3 PUFAs in BM were significantly higher following increased consumption from either diet or supplements (all with p values of $p<0.0001$). The heterogeneity across the studies was high for each of the fatty acids analysed (92-98%). It is important to note that 4 out of the 5 studies included in the meta-analysis were supplement interventions, and the 1 study that reported differences in habitual intake showed considerably less difference in mean concentrations between treatment groups. Despite this it appears that increasing DHA (C22:6n-3) intake significantly increases BM omega-3 concentrations regardless of their source. The asymmetry of the funnel plots in Figure 4.3 shows evidence of some publication bias across all three analyses (DHA (C22:6n-3), EPA (C20:5n-3) and total omega-3 PUFAs). Reports on the effects of maternal intake on DHA (C22:6n-3) concentrations in milk appeared to be most subject to bias.

Figure 4.2 (below): A forest plot showing the mean differences in milk DHA (C22:6n-3) in studies comparing supplementation (or increased omega-3 sources intake) and control (or baseline) data.

Study or Subgroup	Control/lower intake Mean [% of total FAs]	SD [% of total FAs]	Total	Supplement/higher intake Mean [% of total FAs]	SD [% of total FAs]	Total	Weight	Mean difference IV, Random, 95% CI [% of total FAs]
1.1.1 DHA								
Ay et al. 2018	0.22	0.1	26	0.31	0.23	36	20.3%	-0.09 [-0.17 , -0.01]
Helland et al. 2006	0.49	0.32	138	1.37	0.58	145	20.0%	-0.88 [-0.99 , -0.77]
Patin et al. 2006	0.46	0.11	31	0.67	0.47	31	19.0%	-0.21 [-0.38 , -0.04]
Weseler et al. 2008	0.24	0.08	8	0.53	0.13	8	20.0%	-0.29 [-0.40 , -0.18]
Xiang, Harbige and Zetterstrom. 2005	0.18	0.02	23	0.25	0.01	19	20.7%	-0.07 [-0.08 , -0.06]
Subtotal (95% CI)			**226**			**239**	**100.0%**	**-0.31 [-0.57 , -0.05]**
Heterogeneity: Tau² = 0.09; Chi² = 230.71, df = 4 (P < 0.00001); I² = 98%								
Test for overall effect: Z = 2.30 (P = 0.02)								
1.1.2 EPA								
Ay et al. 2018	0.08	0.003	26	0.14	0.08	36	33.5%	-0.06 [-0.09 , -0.03]
Helland et al. 2006	0.15	0.13	138	0.41	0.22	145	32.2%	-0.26 [-0.30 , -0.22]
Xiang, Harbige and Zetterstrom. 2005	0.02	0.01	23	0.06	0.01	19	34.3%	-0.04 [-0.05 , -0.03]
Subtotal (95% CI)			**187**			**200**	**100.0%**	**-0.12 [-0.21 , -0.02]**
Heterogeneity: Tau² = 0.01; Chi² = 105.17, df = 2 (P < 0.00001); I² = 98%								
Test for overall effect: Z = 2.40 (P = 0.02)								
1.1.3 Total omega-3								
Ay et al. 2018	1.26	0.31	26	1.65	0.71	36	19.5%	-0.39 [-0.65 , -0.13]
Helland et al. 2006	1.86	0.81	138	3.12	1.09	145	20.3%	-1.26 [-1.48 , -1.04]
Patin et al. 2006	2.34	0.68	31	3	0.89	31	16.4%	-0.66 [-1.05 , -0.27]
Weseler et al. 2008	0.57	0.17	8	0.88	0.18	8	21.3%	-0.31 [-0.48 , -0.14]
Xiang, Harbige and Zetterstrom. 2005	1.48	0.13	19	1.95	0.14	23	22.5%	-0.47 [-0.55 , -0.39]
Subtotal (95% CI)			**222**			**243**	**100.0%**	**-0.61 [-0.91 , -0.31]**
Heterogeneity: Tau² = 0.10; Chi² = 50.91, df = 4 (P < 0.00001); I² = 92%								
Test for overall effect: Z = 3.97 (P < 0.0001)								
Test for subgroup differences: Chi² = 10.36, df = 2 (P = 0.006), I² = 80.7%								



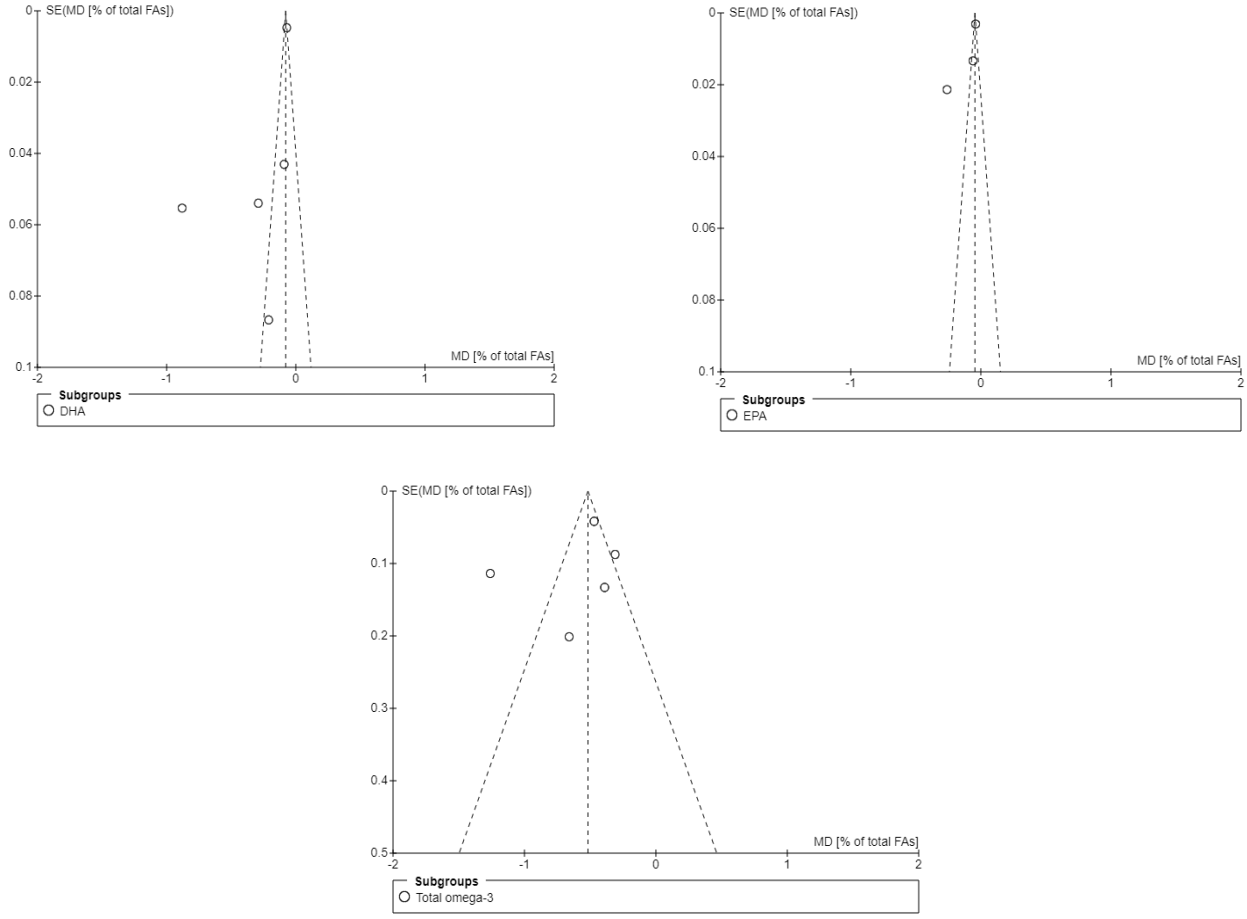


Figure 4.3: Three funnel plots showing the mean differences of DHA (C22:6N-3), EPA (C20:5N-3) and total-omega-3 PUFAs against their standard error of the mean.

4.4 Discussion

The key aim of this systematic review and meta-analysis was to assess the strength of the evidence supporting a positive correlation between maternal intake of omega-3 LCPUFAs and the omega-3 LCPUFA concentrations in BM. We also proposed to compare if this differed according to whether the omega-3 LCPUFA were derived from supplements vs habitual diet. The novelty of the work lay in considering how diet or supplementation during lactation alone influenced milk composition. Synthesis of the evidence from the 8 included studies, indicated that there were significant differences in mean DHA (C22:6n-3), EPA (C20:5n-3),

and total omega-3 LCPUFA concentrations between supplemented and control groups as well as differences between groups with higher vs lower habitual omega-3 FA intakes. The study reporting the highest DHA (C22:6n-3), EPA (C20:5n-3) and total omega-3 LCPUFA milk concentrations transpired to be the intervention study supplying the largest volume of omega-3 PUFAs to their participants (Helland et al. 2006) providing evidence of a dose-dependent relationship between maternal omega-3 intakes and BM concentrations, this study also had a considerably higher sample size than the others (n=233).

The recognised importance of omega-3 LCPUFA for infant growth and development has prompted numerous reviews that have focused on synthesising the literature relating to the effects of maternal omega-3 supplementation during pregnancy and/or lactation on long-term infant outcomes (Muhlhausler, Gibson and Makrides 2010, Dziechciarz, Horvath and Szajewska 2010, Nevins et al. 2021). However, relatively few have specifically examined the effects of maternal omega-3 intakes during lactation on BM FA concentrations. The results of the current review are consistent with the findings of a previous systematic review by Do Amaral et al. in 2017 who found that all the studies they included reported a positive relationship between omega-3 consumption and their concentrations in BM. It is interesting to note that these authors also commented on the considerable heterogeneity in study design, reporting high variability between sample sizes, sample collection timing and the type of intervention used. The differences in sample size and timing of interventions were mentioned as possible reasons for the lack of statistical significance or differences in findings reported across some of their included studies. Similarly, previous reviews focusing on the relationship between high habitual dietary intake of omega-3 FAs and BM omega-3 concentrations (Bravi et al. 2016, Bzikowska, 2019) reported that ingestion of omega-3 LCPUFAs, in particular DHA (C22:6n-3), increases relative concentrations in BM.

A key feature of the current study, as compared to those that have been published previously, is that, by including both supplementation and observational studies in the same systematic review, we were able to investigate whether there was any evidence of a difference in the relative influence of FA derived from the maternal diet vs dietary supplements (and therefore higher doses) on BM FA composition. Overall, the findings of our study are in line with previous findings that BM DHA (C22:6n-3) concentrations are highly responsive to maternal

DHA (C22:6n-3) intakes (Ay et al. 2018, Helland et al. 2006, Patin et al. 2006, Weseler et al. 2008), and that a dose-response relationship exists between maternal intakes and BM omega-3 PUFA concentrations, irrespective of the source of these FAs. The concentrations of DHA (C22:6n-3) in BM increased in response to omega-3 PUFA supplementation, ranging from 480mg-2632mg per day in this review as well as in response to increased consumption of an oily fish (sardines) which was supplied as the intervention by Patin et al. 2006. Similarly, Ueno et al. 2020 reported a significant positive relationship between intake of grilled fish and milk DHA (C22:6n-3) concentrations in a univariate linear regression analysis. The same study also reported higher BM DHA (C22:6n-3) concentrations from mothers who reported consumption of supplements as part of their habitual diet compared to those who did not. Furthermore, higher DHA (C22:6n-3) concentrations in BM were found in a Swedish population compared to a Chinese population by Xiang et al. in 2005, which was reflective of their relative reported DHA (C22:6n-3) intakes. Nishimura et al. in 2014 reported a significant relationship between the dietary omega-3/omega-6 ratio and relative concentrations in milk within a regression coefficient analysis.

While most prior studies have reported positive relationships between maternal dietary omega-3 LCPUFA intakes and BM concentrations, it is important to note that this has not been a consistent finding across all studies. By way of example, Kreisc et al. 2013 completed a study in Croatia investigating Mediterranean diet and BM fatty acid profile, which yielded a strong significant correlation between DHA (C22:6n-3) intake and the DHA (C22:6n-3) concentrations in BM. In contrast, however, a study conducted in four different regions of Thailand reported no relationship between DHA (C22:6n-3) intake and BM concentrations (Jirapinyo et al. 2008). These studies were not included in the current systematic review, as they failed to meet our eligibility criteria, therefore the quality of the study designs may have impacted their observed results. Their observations may also be due to the significant variability that exists between BM DHA (C22:6n-3) levels in different countries as observed in the study by Xiang et al. 2005 when comparing Chinese and Swedish BM FA composition. Another study comparing the fatty acid profiles between nine countries observed DHA (C22:6n-3) concentrations ranging from 0.17-0.99% with the highest levels in Japan and lowest in Canada and the USA, again likely related to differences in the habitual diet of these

respective countries (Yuhas et al. 2006). This observation was mirrored in a systematic review and meta-analysis by Bahreynian, Feizi and Kelishadi in 2020, who found a high degree of heterogeneity in BM omega-3 FA concentrations across different populations. These observations highlight the level of variability in BM LCPUFA concentrations within and between women, and the difficulty in extrapolating findings in one population to other regions or ethnicities.

In this review we applied stringent inclusion criteria to eliminate studies which had failed to consider influencing factors, resulting in a smaller number of final studies than we anticipated. This included an age range of 21-35 and women with a healthy BMI. Both high BMI and maternal age have been reported as factors associated with higher fat content in milk (Kedem et al. 2012, Dritsakou et al. 2021, Daniel et al. 2021, Leghi et al. 2021). Concerns regarding the heterogeneity across studies investigating human milk composition have been expressed by many previously published reviews (Bravi et al. 2016, Do Amaral et al. 2017, Leghi et al. 2020), which was also reflected in this review. The lower number of included studies than expected was largely attributable to the broad and pragmatic approach taken by many researchers in selecting their participants. Recruiting women and infants for lactation studies is challenging for a number of reasons and this can lead researchers to drop strict and robust criteria in order to obtain a sample with statistical power to test their hypotheses. In this work we placed extra emphasis on controlling for confounding factors in a bid to correct the concern around heterogeneity that has been reported in previous systematic reviews in this research area (Bravi et al. 2016, Do Amaral et al. 2017, Leghi et al. 2020).

It is important to consider the appropriateness of the cohort and methodologies utilised in BM compositional studies when interpreting the findings of this systematic review and meta-analysis as these are known to affect the variability of results (Leghi et al. 2020). This variation was a key consideration that was incorporated within the design of our review through the eligibility criteria and the quality assessment tool. By separating the quality assessment tool into two sections with separate areas of consideration we were able to review and report on each of the papers included in the study more critically. Unfortunately, the consensus of this review is that most sample sizes, data collection strategies and considerations of

confounding variables tend to be pragmatic rather than robustly applied. As a result, the overall quality of the research included in this review was low to moderate and there was some evidence of publication bias. It is important to remember these points when considering conclusions from the collective data.

In summary, we observed significant positive associations between the habitual dietary and supplemented intake of DHA (C22:6n-3), EPA (C20:5n-3) and total omega-3 PUFAs and their subsequent concentrations in BM. In addition, the available evidence presented in this review, comparing various intervention and observational studies, supports the suggestion of a dose-response relationship between maternal omega-3 LCPUFA intakes and BM concentrations, irrespective of whether these fatty acids are derived from diet or supplements. This review should be used as a point of reference for future studies to encourage efforts for better control of confounding variables and an improvement in uniformity across BM compositional studies and their designs.

CHAPTER 5

CHANGES IN BREAST MILK FATTY ACID COMPOSITION IN RESPONSE TO AN ACUTE INCREASE IN SUGAR OR FAT: A UK PILOT STUDY AND SECONDARY STUDY IN AUSTRALIA

5.1 Introduction

Fats in BM provide around 50% of the total energy and are believed to be important contributors to the developmental benefits associated with BF (Guo, 2021, Koletzko, 2016). As noted in Chapter 1, most fats in human milk exist within TAGs, and these are secreted within a larger structure called a milk fat globule along with other non-polar lipids such as phospholipids. Each of these structures are comprised of FAs and have variable FA profiles. By extension, the FA profile of BM is also highly variable and is influenced by a broad range of intrinsic and extrinsic factors including maternal diet, BMI and duration of feeds (Lopez and Menard, 2011, Demmelmair and Koletzko, 2018, Aumeistere, 2019, Mitoulas et al. 2002).

Correlations between maternal diet and BM FA composition have been reported by researchers across multiple countries (Koletzko, Thiel and Abiodun, 1992 and Yuhas, Pramuk and Lien, 2006, Aumeistere, 2019). Recent research that recruited 223 European mothers showed positive correlations between maternal adipose tissue FA composition and the FAs present in their BM. In this same study, the researchers also reported correlations between maternal adipose tissue and plasma FA concentrations with those in BM, suggesting that the FAs in maternal stores reflect those in her circulation and eventually in the BM (Giufridda et al. 2022). Other studies have recorded associations between maternal FA intake during pregnancy and lactation and the FA composition of milk (Nishimura et al. 2014, Antonakou et al. 2013, Liu et al. 2016). The systematic review of the literature reported in Chapter 4 of this thesis confirmed that the fatty acid composition of milk is responsive to maternal FA consumption via habitual diet and supplementation. Within this review we also discovered a dose-response relationship between maternal FA intakes from food and/or dietary supplements and the FA content of breastmilk.

In addition to FA intakes, associations between carbohydrate intake and BM FA concentrations have been reported (Neville and Picciano, 1997). Diets high in sugars, particularly the disaccharide fructose, are associated with increased TAG concentrations in the blood. These dietary patterns are associated with increased lipogenesis in humans which contributes to the development of undesirable health outcomes, including non-alcoholic fatty liver disease (Moore, Gunn and Fielding, 2014, Kelishadi, Mansourian and Hediari-Beni,

2014, Chong, Fielding and Frayn, 2007). In support of this, a study by Chong et al (2007) provided women with a meal containing labelled fructose and showed that 0.4% of the FAs within the circulating very low-density lipoprotein TAGs measured 4 hours post-ingestion were derived from this fructose, with the largest increase observed for palmitic acid (C16). In this same study, a significant increase in *de novo* glycerol synthesis was also measured after fructose consumption (Chong et al. 2007). The data reported in Chapters 2 and 3 also suggested that the TAG concentration in milk could be increased by acute exposure to a diet with a moderately higher sugar content.

The substantial variation in BM FA concentrations between women, and the potential for this to be modified by dietary factors, is particularly significant in the context that many of these FAs have been reported to play an important role in infant growth and development. SFAs including decanoic acid (C10) and lauric acid (C12) have been observed in altered concentrations in the presence of cold symptoms in mum and baby, furthermore lauric acid and palmitic acid are known to have antimicrobial effects and have been reported to change within milk to boost the immune reaction within infants (Gardner et al. 2017, Thormar et al. 1987, Chen, Takeda and Lamb, 2005). The omega-3 long chain PUFAs have been linked to improved infant outcomes including enhanced cognitive and visual maturation (Harris et al. 2014, Visentainer et al. 2018). Omega-3 LCPUFAs have also been shown to reduce the risk of pre-term birth, and high maternal intakes during pregnancy have been associated with a reduced risk of allergies, asthma and obesity in children (Helland et al. 2001, Visetainer et al. 2018, Koletzko, 2016). It is important to note, however, that not all studies have observed protective effects from an increased supply of omega-3 LCPUFA throughout pregnancy/lactation and some systematic reviews report a lack of robust evidence to support associations with long-term health outcomes in children (Khandelwal et al. 2020, Dziechciarz, Horvath and Szajewska, 2010, Muhlhausler, Gibson and Makrides, 2010).

Despite the large volume of research reporting on BM FA and their relative concentrations in maternal diet, few studies have investigated acute fluctuations in milk FA concentrations in response to maternal dietary interventions. In 2010, a clinical trial in which women consumed either a low or high fat diet reported differences in milk FA composition after the

4-day dietary intervention period (Nasser et al. 2010). Similarly, Francois et al. 1998 reported that certain FA concentrations increased in BM within 6 hours of ingesting oils known to be rich sources of these FA (including DHA (C22:6n-3), ALA (C18:3n-3) and palmitoleic acid (C16:1)). Even fewer studies have looked at the impact of acute increases in fat and sugar consumption on breast milk FA composition as a whole. That an acute change in the non-fat component of human diet could influence milk FA composition is of particular interest given the data reported in Chapters 2 and 3, which showed a very modest increase in maternal sugar intake could influence the concentrations of TAGs and other proximate nutrients in milk. The principal aim of this chapter was to determine whether BM FAs respond to acute variations in maternal fat and sugar intake over the course of 12 hours.

5.2 Methods

5.2.1 Participant recruitment and sample collection

5.2.1.1 Pilot study (UK)

Full and detailed methods of recruitment and sample collection for this study have been previously reported in Chapter 2. Briefly, mothers were recruited via online platforms and screened for eligibility before being enrolled in the study. Participants received 3 diets as part of the study, 1 control, and 2 intervention diets (one 66g higher in sugar and one 28g higher in fat compared to the control diet, see Appendix 1 for more detail). Diets were consumed over a 12-hour period and BM samples were collected hourly. Participants were asked to express at least 2ml of BM from their fullest breast at each sampling point into sterile 15ml tubes either by manual expression or using a breast pump. If a sample collection time-point coincided with a feed, then women were instructed to collect samples from the opposite breast whilst the infant was suckling. BM samples were frozen in participants home freezers for up to 24h and transferred on ice to the laboratory where they were stored at -20°C for up to 7 days before being processed.

At the time of initial processing, all samples were thawed (on ice) and 1ml was pipetted onto spot cards specifically designed to stabilise LCPUFAs for subsequent FA analysis (Liu, Muhlhausler and Gibson, 2014).

These milk spot cards were allowed to dry before being packaged in air-tight containers and stored at room temperature until shipment. Liu, Muhlhausler and Gibson tested the stability of LCPUFAs from BM (the FAs most vulnerable to oxidisation) on these spot cards and discovered they remained stable for 4 weeks at room temperature. After 4 weeks, the cards were stored at -80°C until analysis to prevent any degradation of the LCPUFAs.

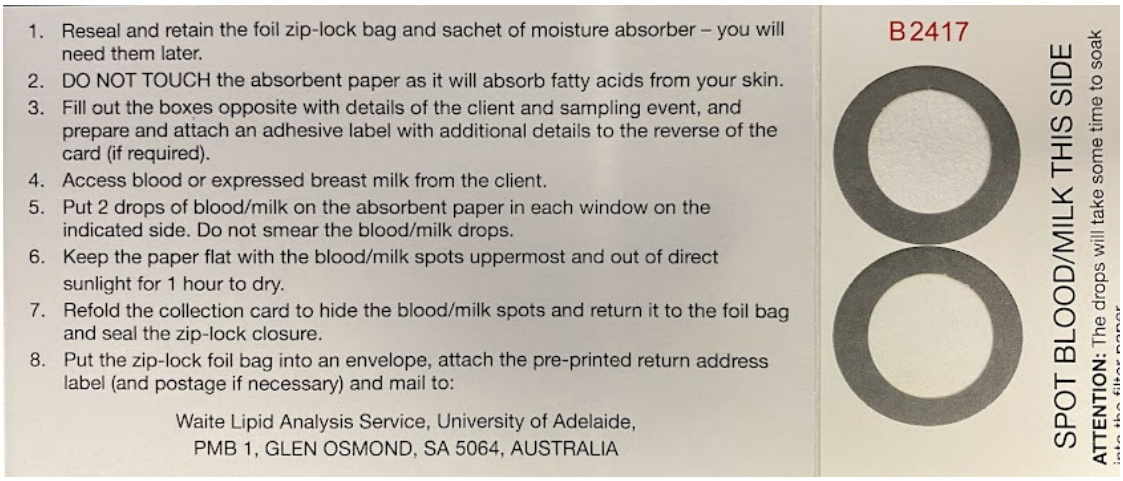


Figure 5.1: An image of the spot cards used for BM FA analysis.

5.2.1.2 Secondary study (Australia)

Full methodology for participant recruitment and sample collection has been previously reported in detail in Chapter 3. In brief, mothers were recruited via online advertising and through a pre-existing participant database within the CSIRO Health & Biosecurity clinical trials facility. Phone call screenings determined eligibility before participants were enrolled. Women were supplied with 2 diets, 1 control and 1 higher in sugar (66g extra sugar) (more detail is provided in Appendix 1). Diets were consumed over a 12-hour period and BM samples were collected hourly. Participants were asked to collect at least 2ml of BM from whichever breast was fullest at the time of collection into wide based collection pots either by hand or using a manual breast pump. Similarly, to the pilot study, if collections coincided

with an infant feeding period, then mothers were instructed to feed their baby and collect the BM sample from the opposite breast. BM samples were refrigerated in participants homes and collected within 24h before being transported on ice to the laboratory and separated into 3-6 aliquots of ~2ml in volume, they were then frozen at -20°C until analyses were completed.

5.2.2 Fatty acid analysis

The protocol utilised for quantifying FAs in this study was based upon a method previously optimised for use in human blood by the Health and Biosecurity team at CSIRO (Rodrigues et al. 2015). The processes for initial sample processing/preparation differed slightly between the pilot study and secondary study (as detailed below) due to the different form the samples were stored in.

5.2.2.1 Pilot study (UK)

Spot cards were removed from the freezer and thawed at room temperature. A specialised device was used to extract a circular punch of milk spotted paper and this was inserted into an 8ml clear vial. 1ml of 0.2M KOH/MeOH (0.005% BHT) was added, ensuring the blood spot card punches were submerged in the solution and vials were capped tightly. Vials were then heated to 90°C using a heat block for 10 minutes. Vials were allowed to cool slightly before 2ml of methanol was added. Next 200µl of acetyl chloride was added slowly while gently vortexing and vials were re-capped and heated for a further 60 minutes at 90°C, vortexing every 10 mins. After 60 minutes, the vials were removed and allowed to cool to room temperature prior to the addition of 1.5ml of hexane and 150µl of distilled water. Vials were then shaken for 30 seconds (IKA vibrax-VXR orbital shaker) before centrifugation at 3000rpm for 10 minutes at room temperature. The hexane-containing top layer was gently extracted from each vial and transferred into a 1.5ml gas chromatography (GC) vial which was capped. A second hexane extraction was repeated as per the steps outlined previously and the top layer was added to the formerly capped GC vials. The supernatant was then dried under a stream of nitrogen and re-dissolved in 100µl of iso-octane before being pipetted into a glass vial insert and returned to the GC vial.

5.2.2.2 Secondary study (Australia)

FA quantification was completed as described above for the pilot study, with minor modifications. Briefly, whole milk samples were removed from the freezer and thawed at room temperature. 100µl of milk was added to 12ml glass vials along with 1ml of 0.2M KOH/MeOH (0.005% BHT). From this point in the method samples were treated as described above until after the first centrifugation. Here samples underwent only one hexane extraction before the top layer was siphoned off and added to a GC vial. The supernatant was dried under a stream of nitrogen, then re-dissolved in 250µl of iso-octane before being pipetted into a glass vial insert and returned to the GC vial.

5.2.2.3 Estimation of fatty acid composition using gas chromatography

FA composition was assessed using GC. Briefly, a 1µl aliquot of each sample was injected into a gas chromatographic column (DB – FastFAME, 20m x 0.18 mm from Agilent Technologies), using a PerkinElmer Clarus 590 GC with a split 50:1 injector. FAs were identified by comparison with authentic Sepelco 37 component FAME mix standards (Sigma-Aldrich, Australia). The area under the curve was measured for each peak using TotalChrom software (PerkinElmer) and individual fatty acids were calculated as a percentage of total identifiable FAs (see Figure 5.1 for an example of the GC traces produced).

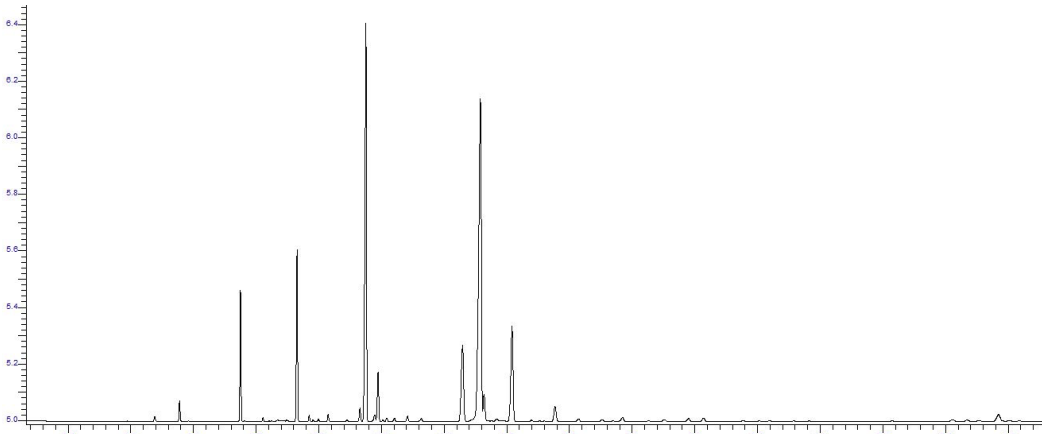


Figure 5.1: An example of the GC traces produced as per the method described above, showing the larger shorter chain FAs peaks and the smaller peaks produced by the longer chain fatty acids.

5.2.3 Statistical Analysis

Data were analysed using R Statistical Software (RStudio 2023.06.1+524 "Mountain Hydrangea"). All data were presented as mean ± standard error of the mean unless otherwise specified. Data for individual FAs were only presented where significant responses to diets were observed. Linear mixed effects models were used to determine differences in average FA concentrations across the day between diets. Visualisation of data was completed using GraphPad Prism version 10. A p value of <0.05 was considered to be statistically significant. As the analysis primarily identified the main effects of diet or interactions of diet and time of day, the following graphs are not annotated to show statistically significant time-points.

5.3 Results

5.3.1 Baseline fatty acids in pilot (UK) cohort versus in second (Australian) cohort

The concentrations of total SFAs, MUFAs and PUFAs as well as individual FAs differed slightly but significantly between women in the UK pilot study compared with those in the larger Australian study. For the Australian study, levels of docosanoic acid (C22), tricosanoic acid (C23), erucic acid (C22:1), docosatetraenoic acid (C22:4) and eicosatrienoic acid (C20:3n-3) in the BM were below the limit of detection for the FA analysis (Table 5.1). The Australian women produced milk that was richer in saturated fatty acids (decanoic acid (C10), lauric acid (C12), palmitic acid (C16), stearic acid (C18)), than that of the UK women (who had more myristic acid (C14) in their milk). The BM Australian women had a higher MUFA content (myristoleic acid (C14:1), palmitoleic acid (C16:1), oleic acid (C18:1) and eicosenoic acid (C20:1), but lower concentrations of PUFA (linoleic acid (LA) (C18:2n-6) and ALA (C18:3n-3)) in comparison with the UK women.

Table 5.1: A summary of baseline fatty acids measured across both studies control days, significant differences between the cohorts are shown with an * and p-values are included.

	Pilot Study (UK cohort, n=9)		Second Study (Australian cohort, n=20)	
	% of total Fas	SEM	% of total Fas	SEM
SFA*	39.48	1.82	41.10**	0.84
C10	0.34	0.07	0.67	0.07
C12	3.08	0.45	3.94	0.37
C14*	4.96	0.50	5.45*	0.27
C15*	0.35	0.03	0.41*	0.02
C16*	22.45	0.76	21.98**	0.53
C17	0.28	0.02	0.39	0.02
C18*	7.39	0.69	8.05*	0.35
C20	0.20	0.02	0.12	0.03
C22	0.12	0.01	ND	ND
C23	0.05	0.01	ND	ND
C24	0.27	0.02	0.09	0.02
MUFA*	45.24	1.52	45.90***	0.55
C14:1*	0.21	0.02	0.22**	0.01
C15:1	0.21	0.02	0.06	0.01
C16:1*	2.32	0.15	2.08*	0.10
C17:1	0.22	0.04	0.17	0.02
C18:1 (total)*	40.23	1.34	41.70**	0.55
C18:1n-7*	1.48	0.13	1.29*	0.16
C20:1*	0.42	0.02	0.37	0.03
C22:1	0.10	0.01	ND	ND
C24:1	0.07	0.01	ND	ND
PUFA*	14.95	0.80	12.90*	0.53
C18:2 trans*	0.10	0.02	0.11*	0.02
C18:2n-6*	12.03	0.69	10.72*	0.44
C18:3n-6	0.05	0.02	0.02	0.01
C20:2n-6	0.24	0.02	0.09	0.03
C20:3n-6	0.28	0.03	0.12	0.03
C20:4n-6	0.39	0.03	0.25	0.04
C18:3n-3*	1.44	0.10	1.43***	0.12
C20:3n-3	0.04	0.01	ND	ND
C20:5n-3	0.08	0.01	0.02	0.01
C22:6n-3	0.31	0.04	0.14	0.04

5.3.2 Fatty acids in response to higher fat diet in UK pilot study

5.3.2.1 Saturated fatty acids (SFA)

There was no change in BM total SFA content in response to the higher fat diet, compared to the control diet. However, four of the individual fatty acids were present at higher concentrations when women consumed the higher fat diet. Margaric acid (C17), arachidic acid (C20), docosanoic acid (C22), lignoceric acid (C24) concentrations were 8, 10.1, 26.7 and 5.7% higher respectively, when women consumed a high fat diet compared to the control diet (Figure 5.2).

5.3.2.2 Monounsaturated fatty acids (MUFA)

Total MUFA exhibited no significant response to the higher fat diet. However, two of the individual MUFAs were present at significantly higher concentrations during consumption of the high fat diet when compared to the control diet and three had significantly lower concentrations when consuming the high fat diet. Eicosenoic acid (C20:1) and nervonic acid (C24:1) had concentrations 10.6 and 18.2% higher respectively on the day women consumed the higher fat diet whilst myristoleic acid (C14:1), pentadecenoic acid (C15:1), palmitoleic acid (C16:1) had concentrations 9.3, 23.1 and 5.6% lower on the day women consumed the higher fat diet compared to the control day (Figure 5.3).

5.3.2.3 Polyunsaturated fatty acids (PUFA)

Total PUFA showed no significant response to the higher fat diet. However, one of the individual PUFAs was present at a significantly higher concentration and two at significantly lower concentrations during consumption of the high fat diet when compared to the control diet. ALA (C18:3n-3) concentration was 6.5% higher when women consumed the higher fat diet compared to the day they consumed the control diet while gamma-linolenic acid (GLA) (C18:3n-6) and DHA (C22:6n-3) were 27.3 and 7.7% lower respectively (Figure 5.4).

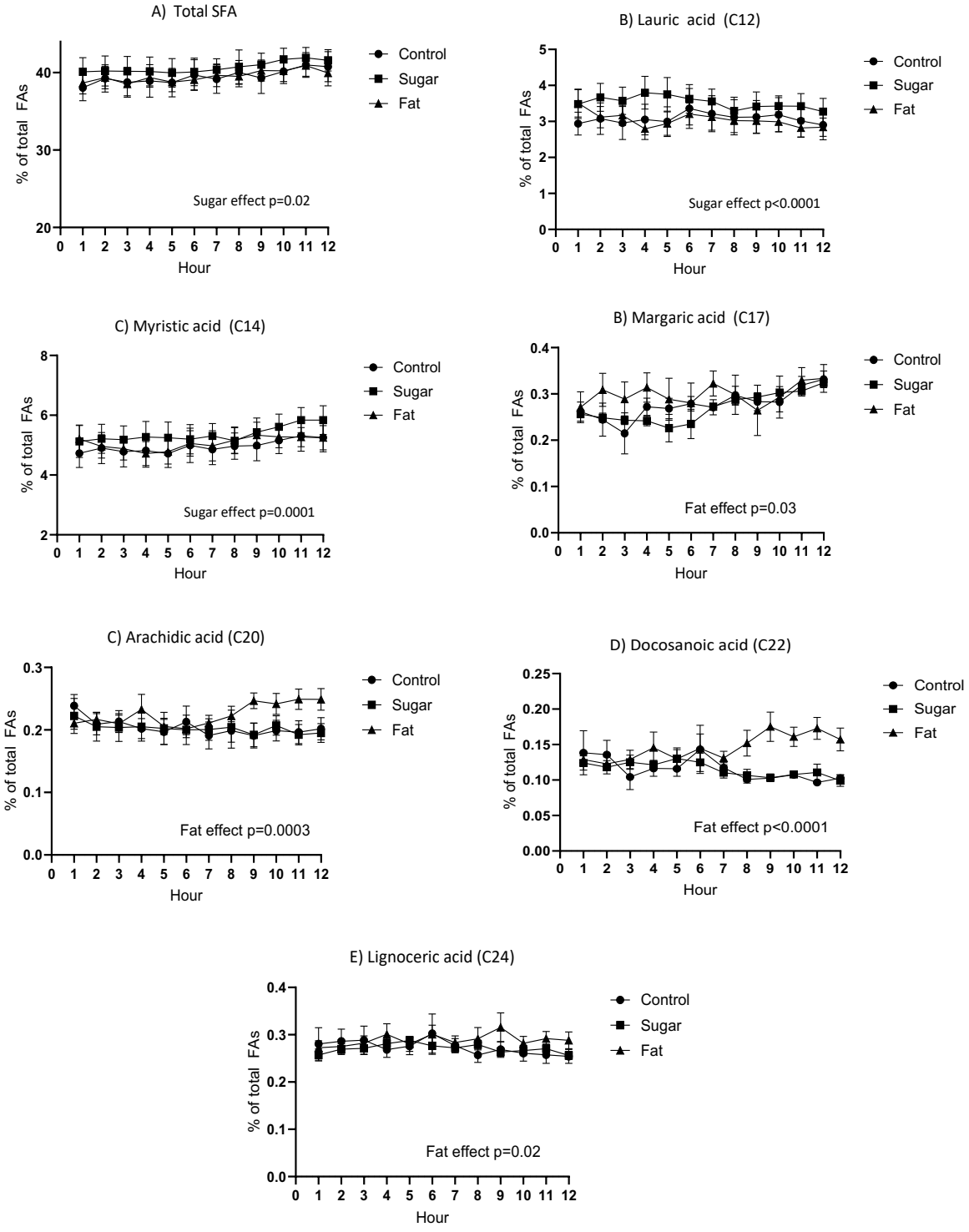


Figure 5.2: Concentrations of A) Total SFAs, B) Lauric acid, C) Myristic acid, D) Margaric acid, E) Arachidic acid, F) Docosanoic acid, G) Lignoceric acid across the day during the pilot study. Three diets were consumed, one control, one higher sugar and one higher fat. Meals consumed at hours 1, 6 and 11 approximately; dietary interventions were administered across hours 1-9. Data presented as mean ± SEM (n=9). Main effects with p values are displayed within graphs.

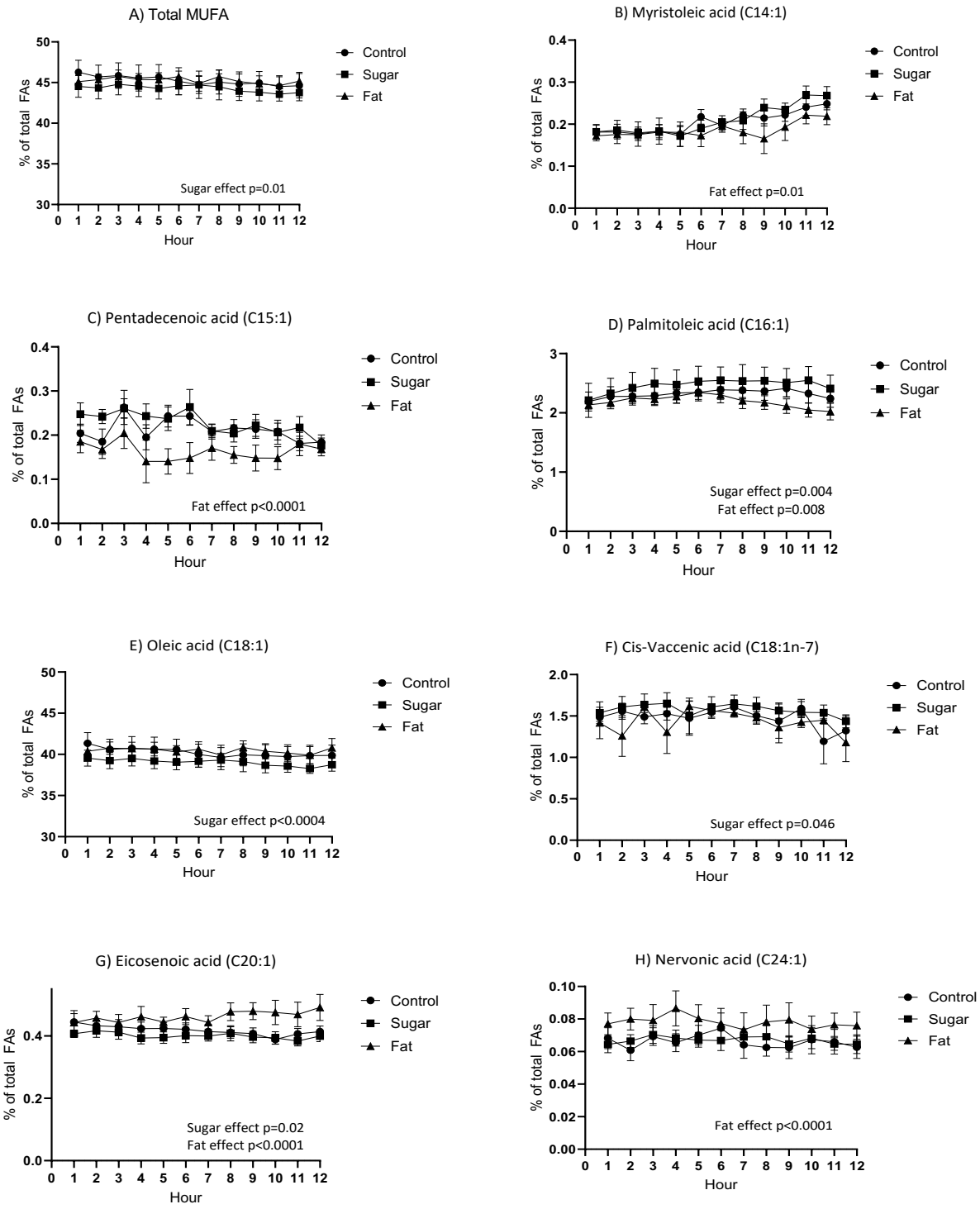


Figure 5.3: Concentrations of A) Total MUFAs, B) Myristoleic acid, C) Pentadecenoic acid, D) Palmitoleic acid, E) Oleic acid, F) Cis-Vaccenic acid, G) Eicosenoic acid and H) Nervonic acid across the day during the pilot study. Three diets were consumed, one control, one higher sugar and one higher fat. Meals consumed at hours 1, 6 and 11 approximately; dietary interventions were administered across hours 1-9. Data presented as mean ± SEM (n=9). Main effects with p values are displayed within graphs.

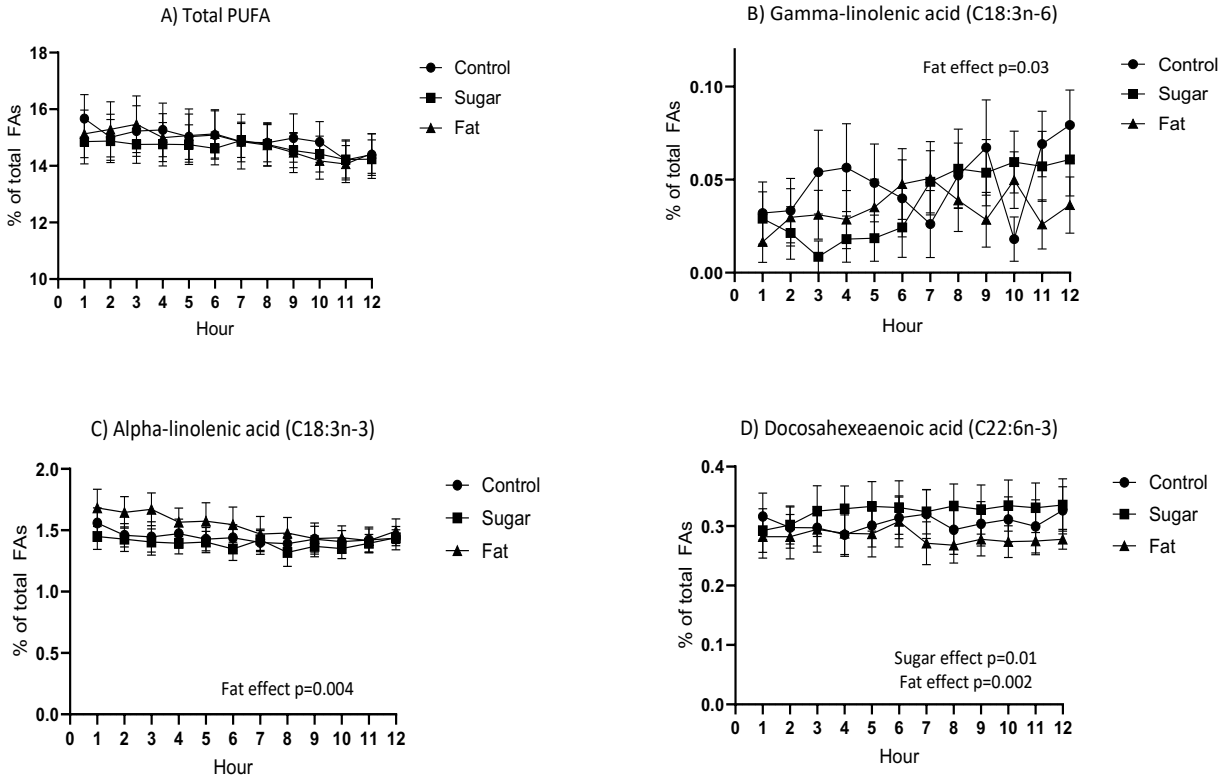


Figure 5.4: Concentrations of A) Total PUFAs, B) Gamma-linolenic acid, C) Alpha-linolenic acid and D) Docosahexaenoic acid across the day during the pilot study. Three diets were consumed, one control, one higher sugar and one higher fat. Meals consumed at hours 1, 6 and 11 approximately; dietary interventions were administered across hours 1-9. Data presented as mean ± SEM (n=9). Main effects with p values are displayed within graphs.

Table 5.2: A summary of fatty acids measured on the control, high fat and high sugar days in the pilot study (n=9). Significantly different measurement indicated by *. * = p<0.05, ** = p<0.01, *** = p<0.001.

	Pilot Study (UK cohort, n=9)		**Second Study (Australian cohort, n=20)**	
	% of total Fas	SEM	% of total Fas	SEM
SFA*	39.48	1.82	41.10**	0.84
C10	0.34	0.07	0.67	0.07
C12	3.08	0.45	3.94	0.37
C14*	4.96	0.50	5.45*	0.27
C15*	0.35	0.03	0.41*	0.02
C16*	22.45	0.76	21.98**	0.53
C17	0.28	0.02	0.39	0.02
C18*	7.39	0.69	8.05*	0.35
C20	0.20	0.02	0.12	0.03
C22	0.12	0.01	ND	ND
C23	0.05	0.01	ND	ND
C24	0.27	0.02	0.09	0.02
MUFA*	45.24	1.52	45.90***	0.55
C14:1*	0.21	0.02	0.22**	0.01
C15:1	0.21	0.02	0.06	0.01
C16:1*	2.32	0.15	2.08*	0.10
C17:1	0.22	0.04	0.17	0.02
C18:1 (total)*	40.23	1.34	41.70**	0.55
C18:1n-7*	1.48	0.13	1.29*	0.16
C20:1*	0.42	0.02	0.37	0.03
C22:1	0.10	0.01	ND	ND
C24:1	0.07	0.01	ND	ND
PUFA*	14.95	0.80	12.90*	0.53
C18:2 trans*	0.10	0.02	0.11*	0.02
C18:2n-6*	12.03	0.69	10.72*	0.44
C18:3n-6	0.05	0.02	0.02	0.01
C20:2n-6	0.24	0.02	0.09	0.03
C20:3n-6	0.28	0.03	0.12	0.03
C20:4n-6	0.39	0.03	0.25	0.04
C18:3n-3*	1.44	0.10	1.43***	0.12
C20:3n-3	0.04	0.01	ND	ND
C20:5n-3	0.08	0.01	0.02	0.01
C22:6n-3	0.31	0.04	0.14	0.04

5.3.3 Fatty acids in response to the higher sugar diet: UK pilot study

5.3.3.1 Saturated fatty acids (SFA)

Total SFA concentrations were 3% higher during the day the high sugar diet was consumed compared to the control day. Furthermore, lauric acid (C12) and myristic acid (C14) concentrations were 14.5 and 8.3% higher respectively on the day women consumed the higher sugar diets (Figure 5.2).

5.3.3.2 Monounsaturated fatty acids (MUFA)

Total MUFA concentrations were 2.1% lower on the day the women consumed higher sugar compared to the day they consumed the control diet. There were also differences in the concentrations of individual MUFAs; palmitoleic acid (C16:1) and cis-vaccenic acid (C18:1n-7) concentrations in BM were 6.2% and 6.6% higher respectively on the day women consumed the higher sugar diet whilst oleic acid (C18:1) and eicosenoic acid (C20:1) concentrations were 3% and 4.22% lower respectively (Figure 5.3).

5.3.3.3 Polyunsaturated fatty acids (PUFA)

Total PUFA concentrations in BM were not different between the days the women consumed the control and higher sugar diet. DHA (C22:6n-3) was the only PUFA to respond significantly to the sugar diet within the pilot study, DHA (C22:6n-3) concentrations were 6.4% higher compared to the control diet (Figure 5.4).

Table 5.2: A summary of fatty acids measured on the control, high fat and high sugar days in the pilot study (n=9). Significantly different measurement indicated by *. * = p<0.05, ** = p<0.01, *** = p<0.001.

	Pilot Study (UK cohort, n=9)		**Second Study (Australian cohort, n=20)**	
	% of total Fas	SEM	% of total Fas	SEM
SFA*	39.48	1.82	41.10**	0.84
C10	0.34	0.07	0.67	0.07
C12	3.08	0.45	3.94	0.37
C14*	4.96	0.50	5.45*	0.27
C15*	0.35	0.03	0.41*	0.02
C16*	22.45	0.76	21.98**	0.53
C17	0.28	0.02	0.39	0.02
C18*	7.39	0.69	8.05*	0.35
C20	0.20	0.02	0.12	0.03
C22	0.12	0.01	ND	ND
C23	0.05	0.01	ND	ND
C24	0.27	0.02	0.09	0.02
MUFA*	45.24	1.52	45.90***	0.55
C14:1*	0.21	0.02	0.22**	0.01
C15:1	0.21	0.02	0.06	0.01
C16:1*	2.32	0.15	2.08*	0.10
C17:1	0.22	0.04	0.17	0.02
C18:1 (total)*	40.23	1.34	41.70**	0.55
C18:1n-7*	1.48	0.13	1.29*	0.16
C20:1*	0.42	0.02	0.37	0.03
C22:1	0.10	0.01	ND	ND
C24:1	0.07	0.01	ND	ND
PUFA*	14.95	0.80	12.90*	0.53
C18:2 trans*	0.10	0.02	0.11*	0.02
C18:2n-6*	12.03	0.69	10.72*	0.44
C18:3n-6	0.05	0.02	0.02	0.01
C20:2n-6	0.24	0.02	0.09	0.03
C20:3n-6	0.28	0.03	0.12	0.03
C20:4n-6	0.39	0.03	0.25	0.04
C18:3n-3*	1.44	0.10	1.43***	0.12
C20:3n-3	0.04	0.01	ND	ND
C20:5n-3	0.08	0.01	0.02	0.01
C22:6n-3	0.31	0.04	0.14	0.04

5.3.4 Fatty acids in response to higher sugar diet: Australian Study

5.3.4.1 Saturated fatty acids (SFA)

Total SFA concentrations were 3% higher during the day the higher sugar diet was consumed. Additionally, seven of the individual SFAs had significantly higher concentrations and one had significantly lower concentrations during consumption of the high sugar diet when compared to the control diet. Decanoic acid (C10), lauric acid (C12), myristic acid (C14), pentadecanoic acid (C15), palmitic acid (C16) and lignoceric acid (C24) concentrations were 23.5, 11.9, 8, 3.9, 2.4, 21.9 and 52.6% higher respectively on the day when the higher sugar diet was consumed whilst stearic acid (C18) concentrations were 5.4% lower (Figure 5.5).

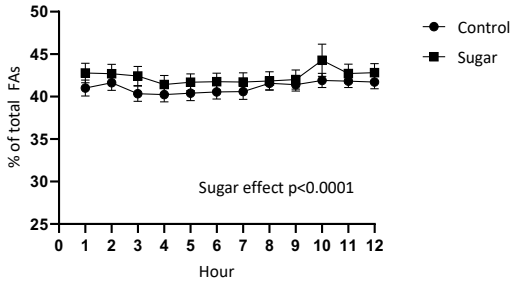
5.3.4.2 Monounsaturated fatty acids (MUFA)

Total MUFA concentrations were 3.2% lower during the day the higher sugar diet was consumed. Additionally, four of the individual MUFAs had significantly higher concentrations and two had significantly lower concentrations on the day women consumed the higher sugar diet, compared to the control diet. Myristoleic acid (C14:1), pentadecenoic acid (C15:1), palmitoleic acid (C16:1) and heptadecanoic acid (C17:1) concentrations were 11.6, 43.6, 9.1 and 22.4% higher respectively, whilst oleic acid (C18:1) and cis-vaccenic acid (C18:1n-7) concentrations were 3.1 and 39.2% lower (Figure 5.6).

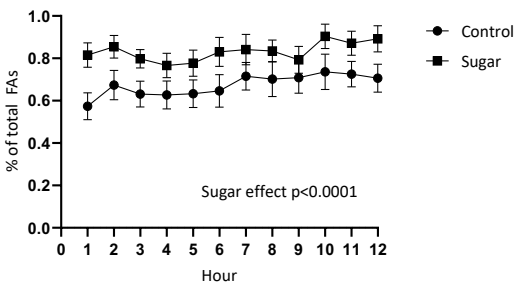
5.3.4 Polyunsaturated fatty acids (PUFA)

Total PUFA concentrations in BM were not different between the higher sugar diet and control diet days. However, five of the individual PUFAs had significantly higher concentrations during the day participants consumed the high sugar diet when compared to the control diet. Cis-linolenic acid (CLA) (C18:2 trans), GLA (C18:3n-6), eicosadienoic acid (C20:2n-6), AA (C20:4n-6) and DHA (C22:6n-3) concentrations were 55.9, 149.3, 72.6, 29.7 and 30.4% higher respectively (Figure 5.7).

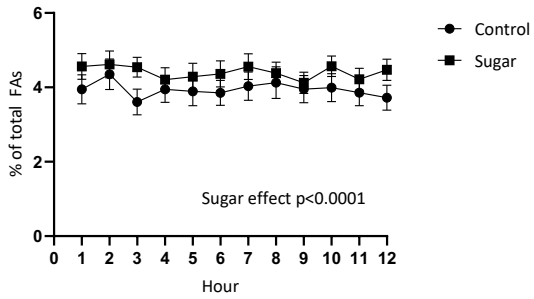
A) Total SFA



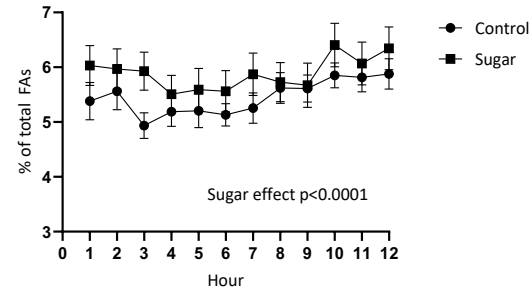
B) Decanoic acid (C10)



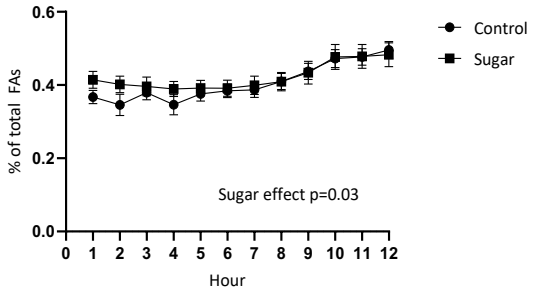
C) Lauric acid (C12)



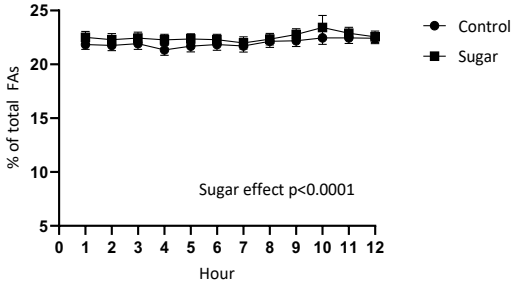
D) Myristic acid (C14)



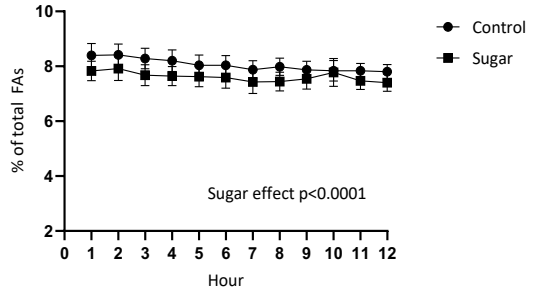
E) Pentadecanoic acid (C15)



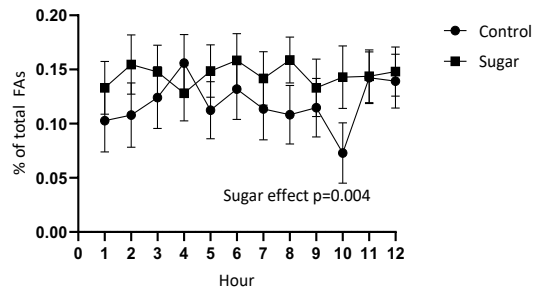
F) Palmitic acid (C16)



G) Stearic acid (C18)



H) Arachidic acid (C20)



I) Lignoceric acid (C24)

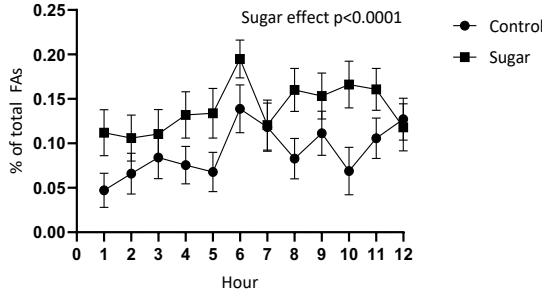


Figure 5.5 (above): Concentrations of A) Total SFAs, B) Decanoic Acid, C) Lauric acid, D) Myristic acid, E) Pentadecanoic acid, F) Palmitic acid, G) Stearic acid, H) Arachidic acid and I) Lignoceric acid across the day during the second study. Two diets were consumed, one control and one higher sugar. Meals consumed at hours 1, 6 and 11 approximately; dietary interventions were administered across hours 1-9. Data presented as mean ± SEM (n=20). Main effects with p values are displayed within graphs.

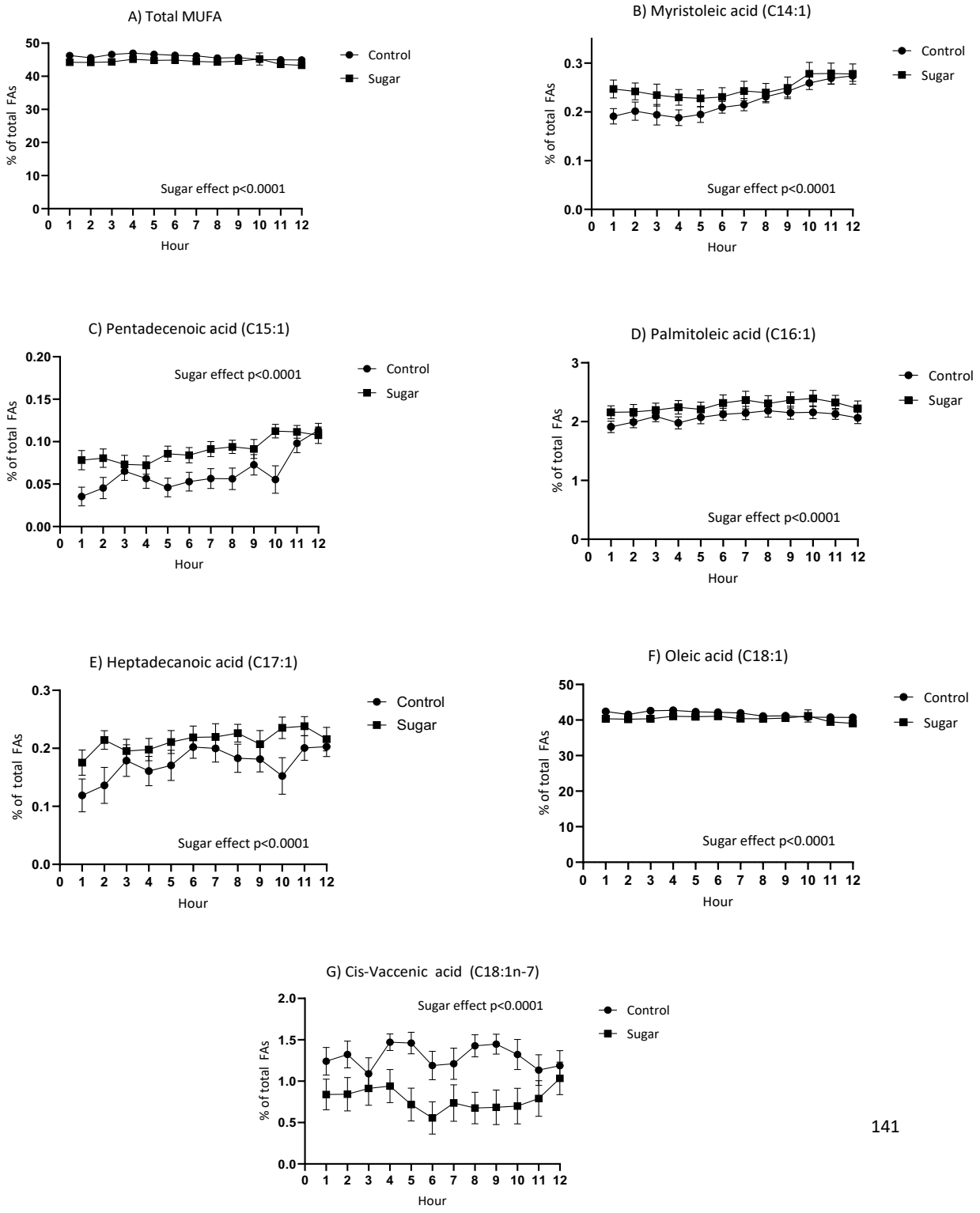


Figure 5.6 (above): Concentrations of A) Total MUFAs, B) Myristoleic acid, C) Pentadecenoic acid, D) Palmitoleic acid, E) Heptadecanoic acid, F) Oleic acid and G) Cis-Vaccenic acid across the day during the second study. Two diets were consumed, one control and one higher sugar. Meals consumed at hours 1, 6 and 11 approximately; dietary interventions were administered across hours 1-9. Data presented as mean ± SEM (n=20). Main effects with p values are displayed within graphs.

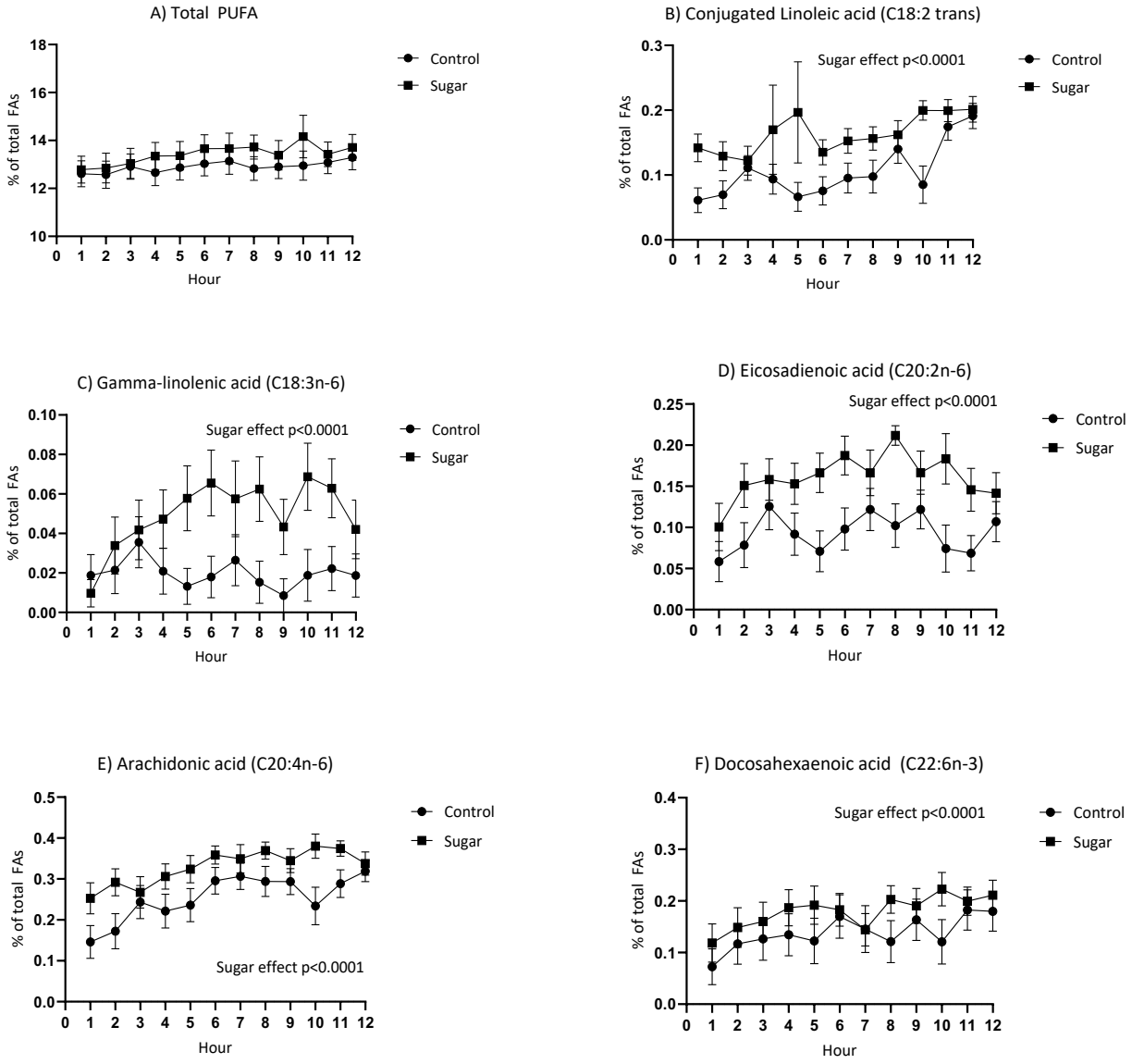


Figure 5.7: Concentrations of A) Total PUFAs, B) CLA, C) GLA, D) Eicosadieonic acid, E) AA and F) DHA across the day during the second study. Two diets were consumed, one control and one higher sugar. Meals consumed at hours 1, 6 and 11 approximately; dietary interventions were administered across hours 1-9. Data presented as mean ± SEM (n=20). Main effects with p values are displayed within graphs.

5.4 Discussion

The data presented in this chapter allows us to examine the impact of acute changes in maternal sugar and fat consumption upon the fatty acid composition of BM. Performing the studies at two different locations also gave the chance to determine whether FA composition differed between British and Australian women at baseline, and to determine whether similar responses to the higher sugar diet could be observed between cohorts. We have shown that BM FA composition responded significantly to small, acute changes in the levels of sugar in the maternal diet in both the UK pilot and larger Australian study, and to acute changes in the levels of fat in the British women. Importantly, there was consistency across both studies in fatty acid response to the higher sugar diet. The Australian study replicated observations observed in six of the seven FAs that showed a significant response in the pilot study, strengthening the reliability of these recorded outcomes.

In the UK pilot study, we assessed the acute effects of both a higher fat and higher sugar diet on BM FA composition. Consuming the higher fat diet was associated with increased concentrations of individual SFAs, MUFAs and PUFAs in milk although total SFA, MUFA and PUFA concentrations remained unchanged. The FAs influenced by the higher fat intervention (increased or decreased) were all 14 carbons in length or greater, which may provide some clue as to the mechanism through which the high fat diet mediated changes in milk composition. Within the mammary alveolar cells, *de novo* FA synthesis can occur for FA chains up to and between 8-14 carbons, at which point the presence of thioesterase II intervenes and cleaves the fatty acid chain (Demmelmair and Koletzko, 2018 and Neville and Picciano, 1997). This suggests the acute changes in BM FA composition during the consumption of the higher fat intervention were likely to be the result of modulations in circulating levels of medium and long chain FAs, rather than being due to an up-regulation of *de novo* synthesis in the mammary gland. This is supported by the consensus that human milk FAs are derived, at least in part, from the maternal circulation (Giuffrida et al. 2022, Langley-Evans, 2021). Consequently, the acute exposure to the higher fat diet was sufficient to increase concentrations of the longer chain FAs in milk. Interestingly, while concentrations of ALA (C18:3n-3) were increased in response to the higher fat diet, the content of DHA (C22:6-3) was actually lower. The reason for this is unclear but may be related to increased

competition for conversion of ALA to its long-chain derivatives. Alternatively, it is possible that the higher ALA concentrations could give rise to higher levels of the omega-3 LCPUFA at a later time-point to what was measured in the current study.

Our findings of acute increases in maternal fat intake shifting the FA composition of BM is in line with the results of a similar study by Francois and colleagues. In Francois's study, BF women were provided with one of 6 different breakfast test formulas, each containing different FAs of interest. They measured the FA composition of BM collected ~6 hours after the breakfast meal. The findings of this study showed that the levels of all FAs in the test meal (including EPA (C20:5n-3) and DHA (C22:6n-3) from menhaden oil, linoleic acid (LA) (C18:2n-6) from safflower oil and stearic (C18) and palmitic acid (C16) from cocoa butter) increased in the corresponding BM sample within 6 hours of consumption (Francois et al. 1998). In addition, several studies in which BF women have been provided with dietary FA supplements have also reported increases in the content of these same fatty acids in BM (Helland et al. 2006, Ay, 2018, Weseler et al. 2008).

Similar to the high fat diet, increases in BM concentrations of SFAs and PUFAs were reported in response to the higher sugar diet in both the pilot UK study and larger Australian study, however, the MUFA concentrations were generally lower. The effect of the higher sugar diet on individual fatty acids was similar between the studies, except for cis-vaccenic acid (C18:1n-7), whose concentrations were reduced in response to the higher sugar diet in the Australian study but increased in the pilot UK study. There were also more individual FAs whose concentrations were altered in response to the higher sugar diet in the Australian women, which is likely to be a reflection of the greater sample size. Whilst the majority of FA concentrations were increased in women's BM when they consumed the higher sugar diet, the concentrations of stearic acid (C18), oleic acid (C18:1 (total)) and cis-vaccenic acid (C18:1n-7) as well as total MUFAs were lower, suggesting that the effects observed are not driven solely by a higher overall fat content. The findings of the present study are similar to those of a historical study, conducted by Insull and colleagues in the late 1950s, who demonstrated that a high calorie "fat free" diet, where carbohydrates provided the majority of the calorific content, was associated with increased lauric acid (C12) and myristic acid (C14)

in BM and a reduction in the concentrations of stearic acid (C18) and oleic acid (C18:1) (Insull et al. 1959). They viewed this as a response to the ratio of fat to carbohydrate in the diet. Taken together, this supported the suggestion that dietary factors beyond simply fat content, and specifically the ratio of this to carbohydrate/sugar content, can influence the fatty acid composition of human milk. Many of the milk long chain PUFAs observed also increased in response to the higher sugar diet, including omega-6 PUFAs (GLA (C18:3n-6), eicosadienoic acid (C20:2n-6) and AA (C20:4n-6)) and DHA (C22:6n-3).

The mechanisms underlying the impact of higher sugar consumption on BM FA composition remains to be determined. However, as previously mentioned, higher intakes of sugar, in particular fructose, have been shown to result in an upregulation of lipid synthesis, or lipogenesis (Moore, Gunn and Fielding, 2014, Chong, Fielding and Frayn, 2007). A study on six healthy adults observed an acute upregulation of lipogenesis following fructose consumption as part of a dietary intervention. They also suggested that fructose consumption may have downstream effects that encourage the continuation of TAG synthesis postprandially (Parks et al. 2008). These observations support the idea that acute peaks in sugar consumption can impact lipogenesis in adults. The significant associations between increased sugar consumption and TAGs/total fat in milk reported in Chapter 2 and Chapter 3 suggest that these lipogenic influences are also affecting milk composition. Furthermore, it seems that certain fatty acids are influenced by the increase in sugar consumption with similar patterns in higher intermediate chain FAs and lower MUFAs being recorded in both of our cohorts. An increase in SFAs and decrease in MUFAS, suggest that Stearoyl CoA desaturase 1 (SCD-1) or delta 9 desaturase activity is lower as this enzyme primarily catalyses the synthesis of MUFAs, particularly oleic acid (C18:1), using SFAs as the substrate (Al Johani, Syed and Ntambi, 2017, Yew Tan et al. 2015). However, it is difficult to determine if this mechanism is taking place in response to our intervention as previously increased sugar consumption in healthy adults was associated with increased SCD-1 activity and it was shown to upregulate in the presence of glucose in cell culture and mouse models (Silbernagel et al. 2012, Collins et al. 2010).

Whilst delta-9 desaturase is involved in MUFA synthesis from SFAs, delta 5 and delta 6

desaturase are integral in the synthesis of omega-3 and omega-6 LCPUFAs (Tosi et al. 2014, Nakamura and Nara, 2004). PUFA concentrations increasing in response to higher sugar consumption was unexpected and could potentially be related to an influence on these enzymes. In pregnant women with gestational diabetes mellitus (GDM), plasma concentrations of LA (C18:2n-6), GLA (C18:3n-6), DGLA (C20:3n-6) and AA (C20:4n-6) have been reported to be higher in the first and third trimester compared to pregnant women without GDM. Furthermore delta 6 desaturase estimated activity was higher while delta 5 desaturase activity was lower in the GDM group (Liu et al. 2022). This suggests a link between glucose metabolism, desaturase 5 and 6 activity and, consequently, the concentrations of highly unsaturated fatty acids such as GLA (C18:3n-6) and AA (C20:4n-6) and could offer a possible mechanism behind our altered PUFA concentrations in milk in response to acutely increased sugar intake.

The impact of these altered FA profiles on the infant in the short- or longer-term is difficult to remark upon as few studies have researched the effects of variations in entire FA profiles. Previous studies have reported associations between individual BM FA concentrations and infant growth, including a study by Criswell et al. in 2022 who reported a positive correlation between oleic acid and accelerated growth in infants between 0 and 6 months. A systematic review evaluating the influence of BM macronutrients and infant growth outcomes reported a general inconsistency regarding relationships between BM FAs and infant growth outcomes. They did however note a general positive relationship between BM SFAs and MUFAs with infant growth and found that specific FAs including palmitoleic acid (C16:1) and DHA (C22:6-3) were negatively correlated with infant growth (Brockway et al. 2023). Positive associations between omega-3 LCPUFAs and infant skin fold thickness have been reported along with negative associations between omega-6 LCPUFAs and infant weight, BMI and lean body mass at 4 months of age (Much et al. 2013). As we have observed increases and decreases in FAs both negatively and positively associated with infant growth outcomes it is difficult to hypothesise on the impacts our observations might have.

Overall, the long-term impacts of the changes in FAs observed during the sugar intervention are difficult to elucidate with the information we have currently, and further studies are

required. The changes in milk fatty acid profiles we have recorded are even more profound when we remember the modest nature of the dietary intervention utilised in this study. The higher fat and sugar diets were designed to reflect 'normal' variations in women's diets. Extrapolating these findings to mothers who may consume diets in line with our intervention regularly suggests significant and consistent effects on the FA composition of their milk, which could in turn impact the infant.

The current study had many strengths. First, multiple BM samples were collected from women at regular intervals before and after the consumption of the test diets, generating a large data set and increasing the ability to investigate effects over time. The pilot UK study and larger Australian study were conducted using similar protocols, but in separate study populations, separate countries and at different times, thus effectively creating two separate cohorts meaning similar findings between the studies provides greater confidence as to their biological relevance. Importantly we observed the same basic response to the higher sugar diet, in terms of FA profiles in milk, in both populations even though there were small but statistically significant differences in baseline milk concentrations. Milk from Australian women had higher concentrations of most saturated fatty acids and MUFA, with lower concentrations of some PUFA. However, the higher sugar diet had the effect of increasing milk saturated fats and decreasing MUFA in both cohorts.

While there were many strengths to the study, there are also limitations. The primary focus of this thesis was on macronutrient concentrations in milk and how they respond to changes in maternal diet, whereas the assessment of fatty acid concentrations was a secondary outcome. The GC method used in the secondary study posed some issues and we had difficulty separating the peaks for oleic acid (C18:1) and cis-vaccenic acid (C18:n-7). We decided to dilute samples in order to measure each of these fatty acids accurately which unfortunately led to difficulty detecting and measuring some of the longer chain PUFAs. Ideally, a standardized method should be applied to all samples across both studies. In addition, future work should better consider the methods employed for milk collection in a bid to standardize sample collections as much as possible, i.e. collecting fore and hind milk samples and including measures to record infant intake. This would allow for further

discussion and understanding of the impacts of the changes we have noted in milk FAs in response to increased fat and sugar intake.

To conclude, we have observed significant responses in the FA composition of BM in response to both increased sugar and fat consumption. These effects were observed despite the intervention being acute and relatively mild (the high sugar diet being little more than the equivalent of a single serving of a sugar sweetened beverage and the high fat the equivalent of a pastry with a high fat spread such as butter or peanut butter). This suggests the mechanisms behind this influence occur rapidly and are transferred to the breast milk either via the maternal circulation or through effects on the mammary gland/*de novo* lipid synthesis. Importantly, many of the key findings were consistent between the pilot UK study and the larger Australian study, which increases our confidence in their validity. Taken together with the findings of other previous studies, our observations support the suggestion that changes in maternal dietary intake can acutely alter breast milk FA composition. While the specific implications from these alterations on the infant are unclear, there is emerging evidence to suggest that different FA species have distinct influences on infant outcomes. Consequently, minor variations in habitual diet between women may potentially have long-term implications for their infants. Further work to determine if these observations are replicated in participants over time and with more standardized collection methods will improve the reliability and help to update our understanding of dietary influences on BM FAs.

CHAPTER 6

GENERAL DISCUSSION

6.1 Summary of findings

This thesis has reported significant changes to BM composition in response to acute changes in maternal diet and time of day. Our findings support the hypothesis that BM fat, protein, lactose and hormone concentrations are responsive to acute increases in sugar and fat consumption. Furthermore, we have recorded sensitivities in some of the components to time of day. Across the pilot study, main study and systematic review we observed many significant effects of maternal dietary intake on BM composition, of which the most interesting and unexpected was the finding that acutely, and modestly, increasing maternal sugar consumption influenced milk TAG/total fat concentrations. This was consistent across both the pilot and main study. We also noted changes in the FA composition of BM in response to the higher sugar diet in both cohorts, including an increase in total SFAs and a decrease in total MUFAs.

Due to our multi-faceted hypothesis and various investigations the summary of findings below reports numerous significant observations, however, our over-arching finding is clear: acute and modest changes in maternal dietary intake can rapidly influence BM composition.

6.1.2 Pilot study macronutrient findings

The pilot study consisted of 9 EBF women in the UK who were provided with 3 diets, 1 control, 1 higher in fat and 1 higher in sugar. We observed increased TAG and lactose concentrations in BM in response to the fat diet as well as decreased protein concentrations when compared to the control diet. We also observed an increase in triglyceride and cholesterol concentrations in breast milk in response to the higher sugar diet, compared to the control diet.

Furthermore, all 4 BM components measured in the pilot showed a response to time of day, irrespective of the diet being consumed. TAG concentrations were lowest at the start of the 12-hour sampling period (morning) and highest at the last sample point (evening), cholesterol concentrations were also highest at the end of the day. Protein concentrations undulated across the day with peaks occurring at hours 2, 7 and 10. Lactose concentrations

responded similarly to protein but peaked at hours 3 and 10, and levels of lactose were lowest at the end of the day.

6.1.3 Main study macronutrient and hormone findings

Our main (secondary) study took place in Australia and consisted of 20 EBF women. They were provided with 2 diets, 1 control and 1 higher in sugar. In this study we observed increased total fat, lactose and glucose concentrations in BM in response to the sugar diet and decreased protein, adiponectin and leptin concentrations. We also observed differences in leptin and insulin concentrations in response to the time of day but, in contrast to the pilot study findings, we did not record any diurnal variation in any of the nutritive components that were measured.

6.1.4 Systematic review findings

The systematic review and meta-analysis was designed to determine the influence of maternal omega-3 LCPUFA intake on omega-3 FAs in BM. It was intended to provide a focus purely on the effects of dietary changes during lactation rather than considering pregnancy and lactation together. We employed a refined selection criteria to include only studies that have considered major confounding factors in BM compositional research, such as maternal age, BMI and EBF status. We also developed a highly specific quality assessment tool to further evaluate the consideration of the idiosyncrasies surrounding BM research by each group of investigators. Through these approaches we identified multiple statistically significant findings as well as a substantial problem regarding heterogeneity across the published literature. We collected data on maternal intake of omega-3 LCPUFAs and relative concentrations in BM as well as any reports on calculated correlations between the two. The results of our systematic review found that intake is significantly related to BM FA concentrations, whether they are consumed habitually or via supplementary interventions and the meta-analysis confirmed these findings. Only 1 of the 5 studies included in the meta-analysis reported a relationship between habitual FA intake and relative concentrations in BM. This association appeared weaker than the studies reporting on supplementation, although remained significant. The study reporting the highest intake of omega-3 LCPFUAs

also reported the highest concentrations in BM, suggesting a dose-dependent response exists.

6.1.5 Fatty acids in pilot and main study findings

The FA data from both the pilot and main studies yielded many statistically significant observations. Firstly, we noted a difference in the baseline FA profiles between the UK (pilot) and Australian (secondary study) cohorts. Total SFAs and multiple single SFAs were higher in the Australian group than the UK, however, myristic acid (C14) was higher in the UK group. Total MUFAs as well as individual MUFAs including oleic acid (18:1) were higher in Australian breast milk, whilst total PUFAs including CLA (C18:2 trans) were lower in the Australian group. It is likely that these differences reflect some habitual disparities in the British and Australian diets. These could be due to varying intakes of food that are influenced by climate and seasonality (for example dairy), however, this was not explored in the current study (Larsen et al 2014; Pacheco-Pappenheim et al 2021).

In response to the higher fat diet within the UK pilot study we observed increases in the percentage concentrations of multiple longer chain SFAs (margaric acid (C17), arachidic acid (C20), docosanoic acid (C22), lignoceric acid (C24)). We observed decreases in MUFAs including (myristoleic acid (C14:1), pentadecenoic acid (C15:1), palmitoleic acid (C16:1)) and increased proportions of eicosenoic acid (C20:1) and nervonic acid (C24:1). Lastly, we found increased concentrations of ALA (C18:3n-3) and decreased concentrations of GLA (C18:3n-6) and DHA (C22:6n-3) in the breast milk.

In response to the higher sugar diet in the UK pilot study we observed increased concentrations of total SFA, lauric acid (C12) and myristic acid (C14). We found decreased proportions of total MUFAs as well as oleic acid (C18:1) and eicosenoic acid (C20:1) but increases in palmitoleic acid (C16:1) and cis-vaccenic acid (C18:1n-7). Finally, we found increases in only one PUFA, DHA (C22:6-3), in response to the higher intake of sugar.

The responses to the higher sugar diet in the second study were very similar to those in the pilot study. In addition to the SFA responses reported in the pilot, we found increased proportions of decanoic acid (C10), pentadecanoic acid (C15), palmitic acid (C16) and lignoceric acid (C24) and decreased stearic acid (C18). We observed decreased cis-vaccenic acid (C18:1n-7) concentrations and increases in myristoleic acid (C14:1), pentadecenoic acid (C15:1), palmitoleic acid (C16:1) and heptadecanoic acid (C17:1) in the BM. Finally, we observed increased DHA (C22:6n-3) as well as higher proportions of CLA (C18:2 trans), GLA (C18:3n-6), eicosadienoic acid (C20:2n-6) and AA (C20:4n-6).

6.2 Understanding the key findings

As mentioned above, the primary finding of this thesis is that acute changes in maternal diet can influence BM composition. Namely, we have shown that a relatively modest increase in sugar consumption led to significantly higher concentrations of BM TAGs/total fat. We have theorised in previous chapters that this response may arise from an up-regulation of lipogenesis in the mother, either systemically or within the mammary epithelial cells themselves. It is known that BM fats are created *de novo* in lactocytes as well as being derived from maternal circulation and that the presence of thiosterase II prevents the *de novo* formation of FAs longer than 14 carbons within the breast (Demmelmair and Koletzko, 2018 and Neville and Picciano, 1997). Previous studies in adults support the notion that sugar intake is associated with lipogenesis (Stanhope et al. 2009, Samuel, 2011) and our detection of increased BM glucose in response to the sugar intervention further supports this theoretical mechanism behind the observed increases in BM fat. The findings described in this thesis appear to be novel in that no other study has comprehensively examined the acute response to a small increase in sugar intake across a day, in a purposively sampled group of EBF women.

Additionally, our findings included a difference in the BM FA composition between the control and higher sugar days within both the pilot and main study. Insull and colleagues observed very similar responses in BM FAs when they supplied mothers with a high calorie, ‘fat free’ diet and attributed their findings to a change in the dietary fat to carbohydrate ratio (Insull et al. 1959). No more recent studies appear to have been conducted to evaluate this

research question. Some of the FAs we have measured in greater concentrations are of intermediate chain length and 14 carbons or below, therefore, they may be the products of increased lipogenesis within the mammary epithelial cells. The lower concentrations of total MUFAs recorded with higher sugar feeding, may be caused by displacement due to the increase in SFAs since the FA data are stated as a percentage of total FAs. We are unsure of the mechanisms behind this response in SFAs and MUFAs as increased intakes of sugars (namely glucose) have previously been associated with an up-regulation of SCD-1, the enzyme responsible for elongation of SFAs to create MUFAs (Al Johani, Sayed and Ntambi, 2017, Collins et al. 2010). Further to the above we observed increases in DHA (C22:6n-3) in response to the sugar diet within both studies, although in the main study we observed increases in many more PUFAs. Previous increases in PUFAs have been observed in pregnant women with GDM, alongside increases in delta 6-desaturase, suggesting an up-regulation of PUFA elongation in the presence of altered glucose metabolism (Liu et al. 2022). For a modestly higher sugar diet to have such an effect on regulation of lipogenesis (involving changes to gene expression) over such a short span of time is surprising. Changes in the concentrations of FA that enter milk through transport from maternal circulation was the expected mechanism behind this particular outcome of the study, as transcription and translation of the enzymes involved in lipogenesis would delay the observed response.

Some significant findings from the pilot or main study were not observed across both cohorts. Lactose and protein concentrations responded to the higher sugar diet in the Australian cohort but not in the UK pilot study. In the pilot study we observed significant diurnal patterns in lactose and protein and significant increases in TAGs at the end of the day that were not repeated in the main study. The reasons underlying the different findings across both studies could be due to the different methods we employed for the analysis of BM composition, especially considering the methods used in the second study have been validated and modified specifically for BM analysis. These inconsistencies could also be due to differences in sample sizes between studies or could arise from differences in the cohorts themselves. Despite cohort selection being highly controlled and similar in both groups, the populations of women in the UK versus Australia will inevitably have differences. According to the Global Nutrition Report, there are notable variations in the national average intake of

whole grains, nuts, fish and legumes between the UK and Australian populations (Global Nutrition Report, 2024). Perhaps differences such as these in their habitual diets are contributing to the variations in our observations between the groups. Further work encompassing a habitual dietary data collection and comparison between the two groups would be required to comment on mechanistic explanations as to why these responses were not mirrored in both cohorts. Unfortunately, we did not collect such data for the Australian population.

After completing the pilot study and reviewing how best to design the secondary study, we decided to include quantification of some metabolic hormones in BM, thus this was carried out only in the Australian population. Interestingly, we observed significantly decreased concentrations of leptin and adiponectin in response to the higher sugar diet. There is a marked gap in the literature around how maternal diet, specifically sugar, influences BM metabolic hormones and therefore it is difficult to deduce a possible explanation. A negative relationship between a low-fat, high-carbohydrate diet and circulating adiponectin has been observed in multiple human studies, however, suggesting milk adiponectin could be influenced by changes occurring in response to sugar intake within the maternal circulation (Kasim-Karakas et al. 2006, Song et al. 2016). This explanation is further supported by the studies that have shown a significant relationship between adiponectin concentrations in BM and maternal serum (Savino et al. 2012). Furthermore, Binder et al. in 2023 found a significant relationship between maternal protein intake and BM protein concentrations, and reported an accompanying increase in adiponectin and leptin when protein intake was higher. Since we observed lower protein concentrations in response to the higher sugar diet, we postulated this too could be related to the lower adiponectin and leptin concentrations recorded.

We also observed significant responses to time of day in insulin and leptin concentrations. Insulin concentrations peaked around hours 3 and 4 and again at hours 8 and 9. These peaks appeared to be associated with the peaks in BM glucose concentrations (although variation in glucose was not significant) and mealtimes. Rodel and colleagues observed similar responses in BM insulin to maternal meals (Rodel et al. 2022). Leptin concentrations were

observed to decrease gradually across the day. This finding has been reported in a recent study completed within the Hartmann Geddes Human Lactation Research Group who found similar patterns between hours 12.00 and 17.00 (Suwaydi et al. 2023), although other authors have not reported this downward trend (Cannon et al. 2015). The decreasing leptin concentrations throughout the afternoon may be a contributing factor to the phenomenon of evening cluster feeding that is common in infants, perhaps the drop in leptin within milk initiates an increased appetite leading to cluster feeding behaviours.

Lastly, our findings from the systematic review and meta-analysis confirmed that there is a significant relationship between maternal LCPUFA intake (via habitual diet or supplementation) and BM LCPUFA content. This has been reported previously in other systematic reviews (Do Amaral et al. 2017, Bravi et al. 2016, Bzikowska, 2019). Our data suggests a dose-dependent response exists between intake of FA and relative concentrations in milk (Helland et al. 2006). Furthermore, we observed a significant amount of heterogeneity across the body of evidence, mirroring what has been previously reported in other reviews on BM composition (Bravi et al. 2016, Do Amaral et al. 2017, Leghi et al. 2020). High levels of heterogeneity exist due to the large variation across methodologies being used in BM research. A movement to standardising methodology regarding cohort inclusion criteria, BM collection, laboratory analytical methods and consideration of confounding factors is paramount if we are to improve this issue of heterogeneity.

6.3 Significance of findings for mothers and infants

A robust literature body (discussed in Chapter 1) has established that BM has the potential to influence infant body composition measures, disease, allergy and developmental outcomes. Aside from the general protective associations between BF and infant health and developmental outcomes, there are some links that are specifically relevant to our findings.

BM fat concentrations have not been consistently related to any one specific infant growth outcome, yet impactful associations have been reported between the two. This is summarised in a recent systematic review by Brockway et al. who found one study reported

positive relationships between BM fat and infant body fat, two studies found associations with infant weight, although these were contrasting, and one reported an inverse relationship with BM fat and infant BMI whereas another observed a positive relationship between BM fat and WAZ.

Brockway et al also reported inconsistent conclusions regarding relationships between individual BM FAs and infant growth outcomes. They did however note a general positive relationship between BM SFAs and infant weight, they also found that most MUFAs, aside from palmitoleic acid, were positively associated with infant growth and that DHA (C22:6n-3) tended to have an inverse relationship with outcomes such as infant length and weight (Brockway et al. 2023). This was supported by a study in Norway that reported a positive association between BM MUFAs, particularly oleic acid and rapid infant growth (Criswell et al. 2022). Furthermore, positive associations have been observed between omega-3 LCPUFAs and infant skin fold thickness and omega-6 LCPUFAs were negatively associated with infant weight, BMI and lean body mass (Much et al. 2013).

The changes in the BM FA profile observed in response to our sugar intervention included increased concentrations in FAs positively associated with infant growth (total SFAs), as well as those negatively associated with rapid growth (DHA (C22:6n-3)). We also reported decreases in FAs positively and negatively associated with infant growth (oleic acid and palmitoleic acid). Although it is unclear how the specific FA profile we have observed in response to higher maternal sugar intake might affect infants, there is evidence to suggest changes in SFA/MUFA concentrations in BM will affect the rate of infant growth (Criswell et al. 2022).

Further to the influence of fat and FAs, BM lactose intake via BF in infancy has been positively associated with greater fat mass, fat mass index and % fat mass (Gridneva et al. 2019). This work commented on the ability of carbohydrates in BM, including lactose, to influence infant body composition and increase the risk of developing obesity later in life. Carbohydrates in BM have also been positively correlated with unfavourable infant anthropometry outcomes including infant weight, adiposity and BMI (Norrish et al. 2023). The increase in lactose we reported in the second study could, therefore, positively influence these growth outcomes in infants.

Furthermore, adiponectin, leptin and insulin concentrations have been inversely associated with infant growth parameters such as weight, WAZ, WLZ, head circumference and FFM (Brockway et al. 2023, Woo et al. 2009, Woo et al. 2011, Norrish et al. 2023) and insulin concentrations have been reported to have a U-shaped association with infant WLZ (Chang et al. 2018, Fields and Demarath, 2012). We have observed reduced concentrations in adiponectin and leptin in response to the sugar intervention which could theoretically lead to higher measures of the infant growth parameters that have shown negatively associations with the concentrations of these hormones.

Beneficial influences of individual FAs in BM have been associated with infant cognitive outcomes. A meta-analysis completed in 1999 found significantly better cognitive development in BF infants compared to formula fed infants (Anderson et al. 1999). Early infant mental development and psychomotor skills have been associated with omega-6:omega-3 ratios in BM (Kim et al. 2017), although this observation has not been replicated in other studies (Keim et al. 2012, Hurtado et al. 2015). Specifically, DHA (C22:6n-3) and AA have been reported as playing important roles in brain and retina development in infancy (Uauy, et al., 2001, Innis, Gilley & Werker, 2001). Additionally, maternal intake of FAs has been shown to influence infant outcomes, although the evidence is somewhat contradictory. Positive associations were reported between breastfed infants' IQ and visual acuity at 4 years when omega-3 LCPUFAs intake in pregnancy and lactation was higher (Helland et al. 2003, Lauritzen et al. 2004). However, a systematic review on the subject concluded that there was a lack of evidence to support a positive association between omega-3 supplementation in pregnancy and/or lactation and infant cognitive outcomes (Nevins et al. 2021). Such studies are often confounded by factors including maternal socioeconomic status, educational attainment and genetics. The literature shows positive relationships between these FAs and infant cognitive outcomes, although it is also evident that this relationship is complex and not always obvious. Nevertheless, it is important to identify the potential positive reaches of our findings. We have shown a strong correlation between maternal intake of omega-3 LCPUFAs and concentrations in BM. This observation could be used to underpin future protocols for improving the FA profile of BM in areas where it may be compromised due to

habitual diets and/or to treat those who require highly specified nutrition such as pre-term infants.

Whilst many of the findings connecting BM components and infant outcomes are contrasting, this does not mean that the significant associations identified should be discounted. By extension, it is entirely plausible that the significant alterations in BM components we have measured in response to a relatively small increase in sugar consumption could lead to altered infant outcomes, particularly with prolonged exposure. We have observed increases in BM fat, individual FAs and glucose/lactose in response to maternal sugar consumption, alongside a decrease in protein, adiponectin and leptin concentrations. Each of these BM components have been documented to influence infant anthropometry independently, therefore, the combined effects would likely be much more pronounced and/or nuanced. Whilst these foresights are speculative, they are possible, and it is essential that we give credence to any potential ability to influence infant development at this crucial stage of life.

On the other hand, we must be cautious in how we extrapolate these findings. The changes we have recorded in milk composition are significant statistically and it is possible they could have statistically significant impacts on infant outcomes; however, these effects would likely be nuanced across populations. Furthermore, what is statistically significant may not always be clinically significant and the theory that small changes in milk composition can lead to impactful outcomes in adulthood needs many more decades of research. The potential that infant feeding behaviour could be altered in response to the changes we observed in milk composition is a potential route for nuance to enter this relationship. Some older studies looking into the infants' ability to influence calorie balance suggest that babies drinking formula with less calories drank more than their peers who were fed higher calorie milk (Fomon et al. 1975). Perhaps babies receiving breast milk with more total fat will adjust their feeding durations slightly, thus neutralising the impact of an increase in milk fat concentrations.

In addition to acknowledging the potential impacts of our findings on infant health and the ways in which these may be neutralised by nature, we must also recognise the potential

impact this information could have on mothers. The pressure mothers feel to breastfeed and provide their babies with the best possible nutrition is significant and has been associated with negative breastfeeding experiences. Women have reported feeling judgement, inadequacy, failure and isolation within their feeding experiences (Korth et al. 2023, Thompson et al. 2015). It is not difficult to imagine how a mother might feel if she was to read that small changes in her diet could affect her milk in a way that may or may not impact her baby. The potential public health impact of this information being disseminated before it is well researched and understood could be significant. Therefore, it is important that it is discussed with appropriate context and that strong efforts are made to replicate and strengthen the findings reported in this work.

Lastly, the considerable heterogeneity we observed whilst conducting the systematic review is potentially problematic for the health of infants. The discrepancies between research methodologies prevents comparison and collation of the outcomes reported across this body of evidence. Without the ability to compare results to other similarly conducted studies, or to group observations collectively, it is difficult to effectively build on our knowledge in the area. This is undoubtedly impacting the quality and rate of advancements occurring in the sphere of infant feeding/BM research which in turn is negatively affecting the health of infants worldwide. Standardising the methodologies used within BM research could close huge gaps in our current knowledge that would likely greatly impact infant health and outcomes. Furthermore, the inconsistencies in methodologies and findings could be related to biased sources of funding for this research area. Many compositional studies are funded by breast milk substitute companies which must raise the question, are these findings reliable? And should these findings contribute to the overall picture to the same extent as findings from independently funded studies? (Marangoni et al. 2000, Much et al. 2013, Fischer Fumeaux et al. 2019). It is likely that many studies with funding from BM substitute companies are scientifically valid and that efforts have been made to remove the impact of bias, however, this is difficult to elucidate. This is yet another idiosyncrasy that plays a part in our understanding of breast milk and the research in this area.

6.4 Strengths and limitations

The most noteworthy strength to this thesis is our implementation of a proof-of-concept study before embarking on the larger main trial. The pilot study provided an opportunity to test not only our hypothesis, but also our intervention approach, sampling strategy and overall study design. This provided us with invaluable data that we could use to build a power calculation for the main study, thus ensuring statistical power and validity in the second study conclusions.

The approach taken in this thesis, of focusing on how acute changes in maternal diet will influence BM composition in the short term, was novel and to the best of our knowledge has not previously been investigated in this level of detail and with a focus on realistic increases in the test components (i.e. sugar and fat). Thus, this thesis approached an age-old question around maternal diet and BM composition from a new perspective and observed interesting and unexpected outcomes. This has created a new platform which we can build on to further improve our understanding around the acute influences of diet on BM.

Lastly, the opportunity to work within the Hartmann Geddes Human Lactation Research Group at the University of Western Australia provided a great learning opportunity and improved the reliability of the outcomes in the main study. The analytical methods used for macronutrient and hormone measurement have been previously tried and tested in many studies and were specifically validated for use in human milk by this group. The use of methods that have been practiced and perfected for any study are a significant strength, this is of even more importance when analysing a substance like BM due to its complex matrix of nutritive and non-nutritive components and changeable nature.

Whilst there are many strong points of note in the work presented within this thesis, limitations are inevitable. We recognise in retrospect that despite our best intentions, the BM sampling methods used may not be 'gold standard'. BM changes across a feed and this introduces the possibility that samples will naturally have varying fat concentrations depending on breast fullness, as per the work conducted by Mitoulas et al. 2002. The way to control for this is to request that women collect samples pre- and post-feed to determine the

differences in ‘fore’ and ‘hind’ BM. Furthermore, studies have shown the best way to extrapolate findings from BM compositional studies for understanding infant outcomes is to measure actual infant intake of each composite (Gridneva et al. 2023). Therefore, our omission of test weighing infants throughout study days can also be viewed as a limitation.

Although completing novel research is of great significance, it brings with it increased difficulty in understanding and explaining findings. In our pilot and main study, we observed a significant response to sugar consumption in BM fat content, however, due to the lack of previous research the mechanistic basis of this finding has been difficult to determine, and further studies are required. In this way we have limited parts of our discussion around the main finding, however, there are ways this could be rectified in future work. Suggestions include repeating the interventions in each participant to observe if responses are consistent in individuals/across the group as this would further validate our observation. Furtermore, ollecting in depth habitual dietary data in both studies to determine if habitual eating patterns influence the level of response to the sugar intervention would add another level of understanding and insight into the potential mechanism behind this primary finding.

6.5 Suggestions for improvement

Recognising the limitations of the work reported in this thesis allows us to create suggestions for ways to improve future research in this field. Due to the complexity of BM as a fluid it is paramount that methodology is carefully considered when designing a study centred around BM compositional analysis. Considering the individual influences on BM composition, further care must be given when designing inclusion criteria so that these influences are as standardised as possible across study cohorts. We recommend significant consideration of sample collection methods and study design to incorporate and control for the variations in BM that occur naturally over a feed and a day. This is extended to the consideration that infants do not all ingest the same amount and thus measures of infant intake are required to fully elucidate findings that translate to infant impacts. Further to this, we recommend that methods are tried and validated for use in BM and that BM standards are used where possible to maximise the reliability of measurements.

One of the key findings of the systematic review was that the quality of research studies and the reporting of studies was generally poor. Many papers had to be excluded because key details about sampling were not reported. A high level of heterogeneity and difficulty forming robust conclusions has been reported within many systematic reviews assessing BM and BF literature (Bahreynian, Feizi and Kelishadi in 2020, Bravi et al. 2016, Do Amaral et al. 2017, Leghi et al. 2020, Italianer et al. 2020, Brockway et al. 2023). This observation alone is cause for concern and must be acknowledged. A change in approach, or rather an instigation of a single approach, is imperative to maximise the potential outcomes of research into BM and BF.

Lastly, the nature of BM and BF research is different to other health and nutritional scientific fields. The postpartum period is a unique stage of life that encompasses huge demands on the mother and an incredibly complex period of growth for the baby. When a mother is EBF, the demands of this time are even more heightened. Thus, the design of BF and BM research must take these factors into account for studies to be ethically viable. The usual approach for maximising reliability within a study is to recruit high numbers of participants and to measure as much as possible at as many timepoints as possible. Unfortunately, this is not practical in the field of BM research, making it even more paramount that consistent and fair procedures are adhered to across research groups.

6.6 Future work

The importance of making informed decisions that can influence an infant's nutritional environment is paramount, especially within the first 1000 days, as the foundations for their lifelong metabolic and physiological functions are laid (Geddes and Prescott, 2013, Black et al. 2008). The scope of this project has predominantly been to improve our understanding of the relationship between acute changes in maternal diet and BM composition. There is a dearth of data about the short-term effects of maternal dietary choices and filling this knowledge gap is an essential step in understanding the broader impact of maternal dietary choices on BM composition. The work we have completed has demonstrated some novel and interesting ways that BM responds to maternal diet. To fully understand and maximise on this information more research is required.

A future study following the same design but with repeated sugar intervention days, perhaps one week apart, would allow us to determine if intermittent spikes in sugar consumption continue to have the observed effects on BM composition, or whether there is a degree of habituation. Further to the repeated interventions, increasing levels of sugar consumption would allow detection of a potential dose response. Combining this with the improved sampling methodologies listed above and the inclusion of infant intake measurements would provide translatable data on how acute increases in sugar consumption impact BM composition, and how much of this variation is experienced by babies. The outcomes of a study like this would help to address the difficulty we have had in confidently translating findings to potential infant outcomes.

Furthermore, a longer-term study addressing the influence of maternal sugar intake will give the opportunity to determine if acute increases have a more profound impact on BM composition than chronically elevated sugar consumption. Future work looking at either repeated acute sugar interventions or a longer-term intervention with a higher-sugar diet could also aim to collect more information on the inter-participant variations and provide insights into possible causes of varying levels of response between women. This could include a larger focus on collecting habitual dietary data and information on maternal body composition both before, during and after pregnancy to determine if the responses we have observed are influenced by these factors. Further to this approach, grouping mothers into categories dependent on their nutritional status/body composition would provide insight into whether the changes in BM in response to diet differ according to these factors.

A tracer study providing an acute intervention of labelled sugar and/or fat would be interesting and would be beneficial in exploring the mechanisms behind some of our observations. Similar work has been completed by Chong and colleagues in 2007, who provided a bolus of labelled fructose to mothers and found FAs within TAGs measured 4 hours post-ingestion were derived from this fructose. Implementing a similar technique but labelling glucose would allow us to determine if the same effect is observed with this sugar as well as exploring if the glucose ingested is incorporated into BM lactose within the observation period. This could provide interesting data on how dietary components are

acutely utilised in BM synthesis and would significantly contribute to the information available around daily maternal dietary choices. If we could better elucidate the pathways behind changes in diet and influences on BM components, there would be the potential to beneficially influence BM composition through manipulation of maternal diets.

Lastly, a large, long term follow-up study focusing on habitual sugar intake (with or without an intervention element) would provide an observational look at the same research question. A focus on grouping participants depending on habitual sugar intake and documenting infant outcomes such as body composition and development of insulin sensitivity for example, would allow an insight into how maternal diets high in sugar might impact the developmental origins of disease in populations. Adding BM sampling as per the method outlined in section 6.4 and calculating infant intakes would further strengthen such a study like this and would provide comprehensive information as to the impact of maternal daily dietary choices on BM and on infant outcomes.

6.7 Conclusions

The primary conclusion of this thesis is that acute changes in maternal diet (specifically sugar) influence BM composition (specifically fat concentrations and FA composition). This original observation shows that what a lactating woman eats on any given day influences her milk composition throughout that day. Thus, even short-term dietary choices are being translated to the baby's sole source of nutrition. Whilst the mechanisms behind these observations are only currently at hypothesis level, this work has succeeded in starting a conversation around daily maternal dietary choices and how these may be experienced by baby. This work, along with the rest of the literature on BM composition, has the power to positively influence infant outcomes. We, as BM researchers, hold the potential to protect whole populations from ill health and disease through better understanding this convoluted and incredible substance. It is hoped that the work outlined in this thesis will spark further research around the acutely changeable nature of BM and how this can be appropriated to positively influence infant development and future population health outcomes.

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Appendix 1

Table 2.1: Breakdown of control diet and intervention diets highlighting the 66.7g sugar increase and 28.2g fat increase.

	Control (D1)	Higher Sugar (D2)	Higher Fat (D3)
Energy (Kcal)	2428	2484	2597
Carbohydrates (g)	242.6	311.3	240.5
Sugars (g)	83.7	**150.4**	69.3
Fat (g)	101.5	77.5	**129.7**
Saturated Fat (g)	35.7	27.5	43.4
Protein (g)	136.1	135.6	117.1

High Fat Diet

Breakfast

Bananas, flesh only 80 1x Small

Lurpak Spreadable Slightly Salted 250g 20

Peanut butter, smooth 25 1x Thick spread on slice

Milk, whole, pasteurised, average 140 9.3x Prepacked portion/1 tablespoon

Breakfast cereal, cornflakes, fortified 23 1x null

Snack 1

Croissants 113 1.5x croissant

Lurpak® Spreadable Slightly Salted 1kg 20 0x Pack

Lunch

Princes Tuna Chunks in Spring Water 4 x 14151g2

Hellmanns, Light Mayonnaise 33 1x heaped tablespoon

Sweetcorn kernels, canned in water, drained66 1x NHS serving (3 heaped tablespoons)

Walkers Salt & Vinegar Crisps 45 1x individual bag (18-22 crisps)

Mixed Salad Leaves 24 1x Average serving

Pasta, white, dried 95 1x null

Snack 2

Yogurt, Greek, plain, whole 125 1x average pot

Nuts and raisins, mixed 20 0.5x handful

Blueberries 24 1x null

Dinner

Chicken, breast, grilled without skin, meat on9l0y 1x small fillet

Peppers, capsicum, red, raw 160 1x medium pepper

Onions, raw 60 1x small

Peas, mange-tout, boiled in unsalted water 80 1x NHS serving = 1 handful (20 peas)

Asda Free From Chow Mein Stir-Fry Sauce 16200g 0.5x Pack

Noodles, egg, medium, dried, boiled in unsalt5e0d water 0.2x null

Oil, olive 12.6 1x null

High Sugar Diet

Breakfast

Breakfast cereal, cornflakes, unfortifie2d3g 1 individual pack

Bananas, flesh only 80g 1x Small

Sugar, white 15g 3x teaspoon

Tesco Strawberry Jam 454G 20g 0x Pack

Bread, wholemeal, toasted 32g 1x Medium slice

Milk, semi-skimmed, pasteurised, ave1r3a5gge 1x on cereal (30-35g portion)

Snack 1

Ryvita, multigrain 22g 2x slice

Grapes, red 30g

Cheese, Cracker Barrel Cheddar 66g 3x Average slice from block

Lunch

Princes Tuna Chunks in Spring Water1 142 xg 145g

Hellmanns, Light Mayonnaise 33g 1x heaped tablespoon

Sweetcorn kernels, canned in water, d6r6agined 1x NHS serving (3 heaped tablespoons)

Coca-Cola Original Taste 4 x 200ml C3a3n0sml 1.7x Per 200ml

Mixed Salad Leaves 24g 1x Average serving

Walkers Salt & Vinegar Crisps 45g 1x individual bag (18-22 crisps)

Pasta, white, dried 95g 1x null

Snack 2

Almonds, with skin 18g 18 whole

Yogurt, Greek, plain, whole 150g 1x Larger pot

Honey, raw 21g 1x tablespoon

Blueberries 24g 1x null

Dinner

Chicken, light meat, raw 120g 3.75 slice (breast) average

Garlic, raw 6g 2x Demographic Average

Honey, raw 21g 1 tablespoon

Soy sauce, reduced salt/sodium 12g 2x Average Portion

Peppers, capsicum, red, raw 160g 1x medium pepper

Onions, raw 60g 1x small

Oil, olive 4.2g 1x teaspoon

Peas, mange-tout, boiled in unsalted w80agter 1x NHS serving = 1 handful (20 peas)

Noodles, egg, medium, dried, boiled average serving

Appendix 2

Table 2.3: Habitual dietary intake of pilot study participants (Chapter 2) based on a 5-day food diary with dietary reference values (DRV) as published by the Department of Health UK, 1991.

	Average intake					% of Total Energy Intake		
Participant ID	**Energy (kcal)**	**CHO (g)**	**Protein (g)**	**Fat (g)**	**Sugar (g)**	**CHO (% TE)**	**Protein (% TE)**	**Fat (% TE)**
191	2145	216.89	79.67	94.13	100.86	37.9	14.9	39.5
192	2410	303.1	91.4	89.9	150.0	47.2	15.2	33.6
193	2416	261.1	95.8	107.6	82.8	40.5	15.9	40.1
194	2211	276.6	80.6	83.3	102.5	46.9	14.6	33.9
195	1893	266.0	66.3	61.1	85.3	52.7	14.0	29.0
196	2271	258.6	108.5	89.1	130.2	42.7	19.1	35.3
197	1886	224.13	70.44	73.64	87.54	44.6	14.9	35.1
198	2099	277.18	69.74	76.61	105.56	49.5	13.3	32.8
199	1513	177.05	60.87	62.34	115.76	43.9	16.1	37.1
Mean	2094	251.19	80.37	81.96	106.72			
SEM	91	12.07	4.89	4.76	7.00			
Ref Values	2103-2175	260	50	70	90	50%	-	≤35%

Appendix 3

Search strategy

A slightly different search strategy was used for each database due to their differing search functions, details of these and the MeSH terms used are listed below. Search fields were kept open in an attempt to maximise the number of articles found, using title and abstract (Medline), title, abstract and keywords (Scopus and Web of Science).

WOS

(((((((((TS=(lactation)) OR TS=(lactat*)) OR TS=(breast feeding)) OR TS=(breast-fe*)) OR TS=(breastfe*)) OR TS=(breastmilk)) OR TS=(breast-milk)) OR TS=(milk, human)) OR TS=(lactating mother*)) OR TS=(lactating wom?n

AND

((((((((((((((TS=(fatty acids)) OR TS=(fatty acid*)) OR TS=(omega-3)) OR TS=(omega 3)) OR TS=(LC n-3 FA)) OR TS=(n-3 FA)) OR TS=(N-3 fatty acid*)) OR TS=(docasahexaenoic acid*)) OR TS=(docosahex?noic acid*)) OR TS=(eicopasentaenoic acid*)) AND TS=(eicosapent?noic acid*)) OR TS=(fatty acids omega-3)) OR TS=(N-3)) OR TS=(N-3 PUFA)) OR TS=(DHA)) OR TS=(EPA)

AND

((ALL=(Milk, human)) OR ALL=(breast-milk)) OR ALL=(breastmilk)

=**2,013 results**

SCOPUS

TITLE-ABS-KEY (lactation OR "Breast Feeding" OR breast-fe* OR breastfe* OR breast-milk OR breastmilk OR "human milk" OR "lactating mother*" OR "lactating woman" OR "lactating women") AND TITLE-ABS-KEY ("Fatty Acid*" OR omega-3 OR "omega 3" OR "LC n-3" OR "n-3 FA*" OR "n-3 fatty acid*" OR "omega 3 fatty acid*" OR n-3 OR "n-3 PUFA*" OR "docosahexaenoic acid*" OR "docosahexanoic acid*" OR "docosahexenoic acid*" OR "eicosapentaenoic acid*" OR "eicosapentanoic acid*" OR "eicosapentenoic acid*") AND TITLE-ABS-KEY ("human milk" OR breast-

milk OR breastmilk) AND (LIMIT-TO (EXACTKEYWORD , "Human") OR LIMIT-TO (EXACTKEYWORD , "Humans"))

= **2712 results**

OVID

AMED (Allied and Complementary Medicine) <1985 to March 2022>

CAB Abstracts <1910 to 2022 Week 12>

Embase <1974 to 2022 March 28>

Food Science and Technology Abstracts <1969 to 2022 March Week 4>

Global Health Archive on TRIAL <1910 to 1972>

Inspec Archive - Science Abstracts <1898 to 1968>

International Pharmaceutical Abstracts <1970 to February 2022>

Maternity & Infant Care Database (MIDIRS) <1971 to March 15, 2022>

Ovid MEDLINE(R) ALL <1946 to March 28, 2022>

APA PsycInfo <1806 to March Week 3 2022>

Zoological Record <1978 to 2007>

Books@Ovid <March 28, 2022>

Journals@Ovid Full Text <March 29, 2022>

Your Journals@Ovid

APA PsycArticles Full Text

HMIC Health Management Information Consortium <1979 to January 2022>

1 exp Lactation/ 179546

2 lactation.ti,ab. 206076

3 exp Breast feeding/ 135249

4 (breast-fe* or breastfe*).ti,ab. 196008

5 (breast-milk or breastmilk).ti,ab. 65337

6 exp Milk, human/ 52213

7 Lactating mother*.ti,ab.5909

8 Lactating woman.ti,ab. 665

9 Lactating women.ti,ab. 13028

10 1 or 2 or 3 or 4 or 5 or 6 or 7 or 8 or 9 514381

11 exp Fatty Acids/1369615

12 fatty acid*.ti,ab. 884429

13 (omega-3 or omega 3).ti,ab. 67494

14 LC n-3 FA*.ti,ab. 51

15 N-3 FA*.ti,ab. 22812

16 N-3 fatty acid*.ti,ab. 20064

17 Docosahexaenoic acid*.ti,ab. 53877

18 Docosahexanoic acid*.ti,ab. 1341

19 Docosahexenoic acid*.ti,ab. 276

20 Eicosapentaenoic acid*.ti,ab. 38494

21 Eicosapentanoic acid*.ti,ab. 907

22 Eicosapentenoic acid*.ti,ab. 112

23 Fatty acids omega-3*.ti,ab. 1975

24 N-3.mp. [mp=ab, hw, ti, ot, bt, id, cc, tn, dm, mf, dv, kf, fx, dq, rw, nm, ox, px, rx, ui, sy, tc, tm, tx, ct]562704

25 N-3 PUFA*.ti,ab. 20550

26 DHA.ti,ab. 63124

27 EPA.ti,ab. 71269

28 11 or 12 or 13 or 14 or 15 or 16 or 17 or 18 or 19 or 20 or 21 or 22 or 23 or 24 or 25 or 26 or 27 2254043

29 exp Milk, Human/ 52213

30 (breast-milk or breastmilk).ti,ab. 65337

31 29 or 30 95208

32	10 and 28 and 31	7798
33	limit 32 to human	

Table 4.4 Prisma Checklist

Section and Topic	Item #	Checklist item	Page where item is reported
TITLE			
Title	1	Identify the report as a systematic review.	85
ABSTRACT			
Abstract	2	See the PRISMA 2020 for Abstracts checklist.	NA
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of existing knowledge.	86/87
Objectives	4	Provide an explicit statement of the objective(s) or question(s) the review addresses.	87
METHODS			
Eligibility criteria	5	Specify the inclusion and exclusion criteria for the review and how studies were grouped for the syntheses.	88
Information sources	6	Specify all databases, registers, websites, organisations, reference lists and other sources searched or consulted to identify studies. Specify the date when each source was last searched or consulted.	87
Search strategy	7	Present the full search strategies for all databases, registers and websites, including any filters and limits used.	180-182

Section and Topic	Item #	Checklist item	Page where item is reported
Selection process	8	Specify the methods used to decide whether a study met the inclusion criteria of the review, including how many reviewers screened each record and each report retrieved, whether they worked independently, and if applicable, details of automation tools used in the process.	88
Data collection process	9	Specify the methods used to collect data from reports, including how many reviewers collected data from each report, whether they worked independently, any processes for obtaining or confirming data from study investigators, and if applicable, details of automation tools used in the process.	89
Data items	10a	List and define all outcomes for which data were sought. Specify whether all results that were compatible with each outcome domain in each study were sought (e.g. for all measures, time points, analyses), and if not, the methods used to decide which results to collect.	89
	10b	List and define all other variables for which data were sought (e.g. participant and intervention characteristics, funding sources). Describe any assumptions made about any missing or unclear information.	89
Study risk of bias assessment	11	Specify the methods used to assess risk of bias in the included studies, including details of the tool(s) used, how many reviewers assessed each study and whether they worked independently, and if applicable, details of automation tools used in the process.	89

Section and Topic	Item #	Checklist item	Page where item is reported
Effect measures	12	Specify for each outcome the effect measure(s) (e.g. risk ratio, mean difference) used in the synthesis or presentation of results.	89
Synthesis methods	13a	Describe the processes used to decide which studies were eligible for each synthesis (e.g. tabulating the study intervention characteristics and comparing against the planned groups for each synthesis (item #5)).	89
	13b	Describe any methods required to prepare the data for presentation or synthesis, such as handling of missing summary statistics, or data conversions.	NA
	13c	Describe any methods used to tabulate or visually display results of individual studies and syntheses.	89
	13d	Describe any methods used to synthesize results and provide a rationale for the choice(s). If meta-analysis was performed, describe the model(s), method(s) to identify the presence and extent of statistical heterogeneity, and software package(s) used.	89/90
	13e	Describe any methods used to explore possible causes of heterogeneity among study results (e.g. subgroup analysis, meta-regression).	89/90
	13f	Describe any sensitivity analyses conducted to assess robustness of the synthesized results.	NA
Reporting bias assessment	14	Describe any methods used to assess risk of bias due to missing results in a synthesis (arising from reporting biases).	89/90

Section and Topic	Item #	Checklist item	Page where item is reported
Certainty assessment	15	Describe any methods used to assess certainty (or confidence) in the body of evidence for an outcome.	NA
RESULTS			
Study selection	16a	Describe the results of the search and selection process, from the number of records identified in the search to the number of studies included in the review, ideally using a flow diagram.	90
	16b	Cite studies that might appear to meet the inclusion criteria, but which were excluded, and explain why they were excluded.	NA
Study characteristics	17	Cite each included study and present its characteristics.	90/Table 4.1
Risk of bias in studies	18	Present assessments of risk of bias for each included study.	91/Table 4.2
Results of individual studies	19	For all outcomes, present, for each study: (a) summary statistics for each group (where appropriate) and (b) an effect estimate and its precision (e.g. confidence/credible interval), ideally using structured tables or plots.	Table 4.1
	20a	For each synthesis, briefly summarise the characteristics and risk of bias among contributing studies.	90-95

Section and Topic	Item #	Checklist item	Page where item is reported
Results of syntheses	20b	Present results of all statistical syntheses conducted. If meta-analysis was done, present for each the summary estimate and its precision (e.g. confidence/credible interval) and measures of statistical heterogeneity. If comparing groups, describe the direction of the effect.	95/Figure 4.2
	20c	Present results of all investigations of possible causes of heterogeneity among study results.	95/ Figure 4.2
	20d	Present results of all sensitivity analyses conducted to assess the robustness of the synthesized results.	NA
Reporting biases	21	Present assessments of risk of bias due to missing results (arising from reporting biases) for each synthesis assessed.	96/ Figure 4.2
Certainty of evidence	22	Present assessments of certainty (or confidence) in the body of evidence for each outcome assessed.	NA
DISCUSSION			
Discussion	23a	Provide a general interpretation of the results in the context of other evidence.	99-102
	23b	Discuss any limitations of the evidence included in the review.	99-102
	23c	Discuss any limitations of the review processes used.	101
	23d	Discuss implications of the results for practice, policy, and future research.	102
OTHER INFORMATION			

Section and Topic	Item #	Checklist item	Page where item is reported
Registration and protocol	24a	Provide registration information for the review, including register name and registration number, or state that the review was not registered.	87
	24b	Indicate where the review protocol can be accessed, or state that a protocol was not prepared.	NA
	24c	Describe and explain any amendments to information provided at registration or in the protocol.	NA
Support	25	Describe sources of financial or non-financial support for the review, and the role of the funders or sponsors in the review.	NA
Competing interests	26	Declare any competing interests of review authors.	NA
Availability of data, code and other materials	27	Report which of the following are publicly available and where they can be found: template data collection forms; data extracted from included studies; data used for all analyses; analytic code; any other materials used in the review.	NA

Appendix 4

Table 4.1: A summary of all included studies demographics, study design, methodology and results of interest. Results are presented as mean±SD unless otherwise stated. Abbreviations: FA = Fatty acids, DHA = Docosahexaenoic acid, EPA = Eicosapentaenoic acid, PUFA = Polyunsaturated fatty acids, BM = Breast milk, PP = Postpartum, Supp = Supplement, LA = Linoleic acid, DGLA = Dihomo-g-linolenic acid, ARA = Arachidonic acid, ALA = Alpha-linolenic acid, DPA = Docosapentaenoic acid, ND = Not detected, FFQ = Food frequency questionnaire, n-3 = Omega-3, n-6 = Omega-6, CI = Confidence interval, BMI = Body mass index, Kg = Kilograms, SFA = Saturated fatty acid, MUFA = Monounsaturated fatty acid, GLA = Gamma-linolenic acid, IQR = Interquartile range, NSU = Non-supplement user, SU = Supplement user, SEM = Standard error of the mean, n-6DPA = n-6 Docosapentaenoic acid, SA = Stearidonic acid.

Ref	Population	Quality Score	Sample size	Study Design	Exposure measure of interest	Outcome measure of interest	Main results
Ay et al. 2018	Mothers were recruited during prenatal visits between 22-24 weeks in hospital in Turkey, Istanbul.	4	62 (26 control group, 36 supplement group)	Intervention study. FA intake: 1 capsule was provided per day from beginning of the last trimester in pregnancy through to end of 6th month of lactation. Each capsule contained 378 mg DHA and 504 mg EPA in total 950 mg omega-3 PUFA. Control group had no	Supplementation intervention. Daily energy and fat intakes in lactation.	PUFA composition of milk.	Dietary intake (g): Total PUFAs Control 16.2±9.3, Supp 13.6±9.1 Milk composition (% of total FAs): In colostrum: Total PUFA: Control = 19.08±3.56 Supp = 21.35±4.66 (p=0.047) Total Omega-3: Control = 1.32±0.32 Supp = 1.58±0.38 (p=0.083) DHA: Control = 0.36±0.1 Supp = 0.51±0.18 (p=0.000) EPA:

				supplement or placebo. Milk sample collection: All mothers provided 6-10ml BM on two separate occasions (just after birth and at 6 months pp). Milk samples were collected from each subject in the morning between 9.00 and 11.00 am.			Control = 0.09±0.04 Supp = 0.16±0.07 (p=0.000) Total Omega-6: Control = 17.8±3.59 Supp = 19.77±4.52 (p=0.034) Omega-6/Omega-3: Control = 14.31±4.72 Supp = 12.97±3.62 (p=0.723) In mature milk: Total PUFA: Control = 20.2±4.66 Supp = 22.46±5.41 (p=0.08) Total Omega-3: Control = 1.26±0.31 Supp = 1.65±0.71 (p=0.142) DHA: Control = 0.22±0.1

							Supp = 0.31±0.23 (p<0.000) EPA: Control = 0.08±0.003 Supp = 0.14±0.08 (p=0.232) Total Omega-6: Control = 18.93±4.69 Supp = 20.81±5.26 (p=0.094) Omega-6/Omega-3: Control = 16.32±8.13 Supp = 13.79±4.16 (p=0.872)
Del Prado et al. 2001	Mothers were recruited at pre-natal clinics, recruited at 5-6 months postpartum	4	10	Observation study. FA intake: 2.5mg/kg of bodyweight of labelled C13 Linoleic acid was provided in a piece of	Dietary PUFA intake.	PUFA composition of milk.	Dietary intake (%/g/d): LA = 24.9±5.6/11.64±3.49 ARA = 0.2±0.001/0.07±0.04 ALA = 0.9±0.5/0.45±0.3 EPA = ND/ND DHA = 0.1±0.003/0.04±0.02

	in San Mateo, Mexico.			bread at the beginning of the study period. Habitual diet was recorded during the week prior to the study period. Dietary intakes were assessed in mothers' homes by a combination of test weighing the food for 2 days and conducting a 24-hour recall for 1 day. A controlled diet that mimicked their habitual diet was provided for the duration of the study.			Milk composition (% of total FAs): LA = 27.3±2.6 ARA = 0.4±0.1 ALA = 1.01±0.3 EPA = 0.5±0.1 DHA = 0.2±0.04 Daily secretion in milk (g/d): LA = 5.93±1.48 ARA = 0.09±0.03 ALA = 0.242±0.1 EPA = 0.11±0.04 DHA = 0.04±0.02

				Milk sample collection: Milk was collected at 0, 6, 9, 12, 15, 24, 36, 48, and 72 hours after administration of the LA. Milk samples were obtained by expressing 5 mL from the left breast before and after the infants were fed, to include variations in the fat concentration of foremilk and hindmilk. Milk volume was measured for 48hours			

				between 24-72hours by test weighing.			
Helland et al. 2006.	Mothers were recruited at hospital, enrolled when attending 17-19 week scan during pregnancy in Oslo, Norway.	5	233 Cod liver oil group = 145 Corn oil group = 138	Intervention study. FA intake: 10 mL/day of cod liver oil or corn oil was provided. The cod liver oil contained 1183 mg/10 mL of DHA, 803 mg/10 mL of EPA and a total of 2632 mg/10 mL n-3 fatty acids. The corn oil contained 4747 mg/10 mL of LA and 92 mg/10 mL of ALA.	Supplementation intervention. Dietary PUFA intake.	PUFA composition of milk.	Dietary intake was similar between groups at study onset. At 35 weeks of pregnancy there were significant differences in cholesterol, polyenes, LA, ARA, EPA, and DHA reflecting intake of the supplements ($p<0.001$ for all except polyenes which were $p=0.05$). Milk composition (% of total FAs): 4 weeks Total lipid: Cod liver oil = 4.05±1.55 Corn oil = 4.4±1.43 Total PUFA: Cod liver oil = 16.06±3.31 Corn oil = 16.58±3.46

				Mothers completed a FFQ at week 18 and 35 of pregnancy and were advised to continue with their habitual diet for the duration. A questionnaire on breastfeeding and supplement use for infants was administered at 12 weeks. Milk sample collection: Milk samples were collected by manual expression at 4 and 12			Total n-6: Cod liver oil = 12.71±2.82 Corn oil = 14.75±3.21 LA: Cod liver oil = 11.66±2.74 Corn oil = 13.57±3.14 DGLA: Cod liver oil = 0.32±0.09 Corn oil = 0.38±0.09 ARA: Cod liver oil = 0.37±0.07 Corn oil = 0.41±0.07 Total Omega-3: Cod liver oil = 3.35±1.07 Corn oil = 1.83±0.6 ($p<0.001$) ALA: Cod liver oil = 0.96±0.32 Corn oil = 0.9±0.28

				weeks within women's homes. The samples were taken from a morning feed (never the first one), 3–5 minutes after infant started suckling.			DPA: Cod liver oil = 0.36±0.13 Corn oil = 0.19±0.09 p<0.001) DHA: Cod liver oil = 1.37±0.58 Corn oil = 0.49±0.32 p<0.001) EPA: Cod liver oil = 0.43±0.19 Corn oil = 0.13±0.09 p<0.001) N-3/n-6: Cod liver oil = 0.27±0.09 Corn oil = 0.13±0.04 p<0.001) At 12 weeks (Cod liver oil/corn oil) Total lipid: Cod liver oil = 3.81±1.71 Corn oil = 3.81±1.71 Total PUFA: Cod liver oil = 16.09±3.67

							Corn oil = 16.31±3.79 Total n-6: Cod liver oil = 12.86±3.21 Corn oil = 14.36±3.41 LA: Cod liver oil = 11.99±3.15 Corn oil = 13.35±3.33 DGLA: Cod liver oil = 0.26±0.07 Corn oil = 0.26±0.07 ARA: Cod liver oil = 0.33±0.06 Corn oil = 0.37±0.09 Total Omega-3: Cod liver oil = 3.12±1.09 Corn oil = 1.86±0.81 p<0.001) ALA: Cod liver oil = 0.98±0.34

							Corn oil = 0.94±0.32 DPA: Cod liver oil = 0.33±0.12 Corn oil = 0.19±0.09 p<0.001) DHA: Cod liver oil = 1.20±0.57 Corn oil = 0.47±0.40 p<0.001) EPA: Cod liver oil = 0.41±0.22 Corn oil = 0.15±0.13 p<0.001) N-3/n-6: Cod liver oil = 0.25±0.09 Corn oil = 0.13±0.05 p<0.001)
Nishim-ura et al. 2014.	Mothers were recruited in basic health	6	45	Observational study. FA intake: Two FFQs were completed in the	Dietary PUFA intake.	PUFA composition of milk.	Dietary intake (% of total FAs): LA = 17.1387±6.67 ARA = 0.1208±0.10 ALA = 2.1065±0.95 EPA = 0.0186±0.03

	units during pregnancy in Sao Paulo, Brazil.			postpartum period. Three FFQs were completed during pregnancy also but these results are not reported here. Milk sample collection: Samples (5–10 mL) of mature milk (between 5 and 12 weeks postpartum) were obtained by hand expression by the participant in the morning, immediately		Regression coefficients of relation between dietary PUFAs and milk PUFAs.	DHA = 0.0545±0.13 n-3/n-6 = 0.1305±0.05 Milk composition (% of total FAs): LA = 20.7344±4.27 ARA = 0.4746±0.09 ALA = 1.5348±0.40 EPA = 0.0789±0.04 DHA = 0.1023±0.06 n-3/n-6 = 0.0811±0.01 β-regression coefficients (95% CI)/r2 between dietary FA intake postpartum and milk FA: ALA: model 1 = 0.115 (−0.040 to 0.271)/ 0.051 model 2 = 0.140 (−0.044 to 0.299) EPA:

				after the infants first feeding.			Model 1 = −0.044 (−0.338 to 0.251)/ 0.095 Model 2 = −0.055 (−0.422 to 0.313) DHA: Model 1 = 0.015 (−0.121 to 0.151)/ 0.100 Model 2 = 0.021 (−0.170 to 0.212) n-3/n-6: Model 1 = 0.074 (0.006 to 0.143)/ 0.124 Model 2 = 0.086 (0.006 to 0.166)
Patin et al. 2006.	Mothers were recruited in hospital lactation centre postpartum whilst breastfeeding in	4	31	Intervention study. FA intake: 2kg of fresh sardines were provided on days 0 and 15 of the study period. They were instructed to consume them 2x a week in order to have	Supplementation intervention. Dietary PUFA intake.	PUFA composition of milk.	Dietary intake (g): Total PUFAs D0=17.3±8.4 D15=16.4±8.5 D30=17.9±9.7 Milk composition (% of total FAs): Day 0: Total n-6 = 21.55±3.77 LA = 20.72±3.78

	Santos, Brazil.			500g/week. Their usual method of frying was accepted as cooking method. On days 15 and 30 mothers were asked to report how frequent they had consumed sardines in past 15 days. The FA profile of the fried sardines (%) was: SFA = 21.47 MUFA = 24.46 Total n-6 = 42.02 LA = 40.62 GLA = 0.35 ARA = 1.05			GLA = 0.26±0.08 ARA = 0.56±0.14 Total n-3 = 2.34±0.68 ALA = 1.67±0.58 EPA = 0.08±0.07 DPA = 0.24±0.05 DHA = 0.46±0.11 Day 15: Total n-6 = 21.07±4.66 LA = 20.36±5.68 GLA = 0.19±0.08 ARA = 0.53±0.13 Total n-3 = 2.49±0.82 ALA = 1.74±0.53 EPA = 0.07±0.05 DPA = 0.23±0.08 DHA = 0.52±0.28

				Total n-3 = 12.06 ALA = 3.31 EPA = 1.41 DPA = 0.89 DHA = 6.44 N-6/n-3 = 54.07 24hour dietary recalls were completed on days 0, 15 and 30. Milk sample collection: Milk samples (at least 40ml) were collected from both breasts just after infants fed on days 0, 15 and 30 by manual expression.			Day 30: Total n-6 = 21.66±5.14 LA = 20.95±5.10 GLA = 0.21±0.06 ARA = 0.51±0.10 Total n-3 = 2.57±0.68 ALA = 1.82±0.48 EPA = 0.10±0.06 DPA = 0.22±0.07 DHA = 0.47±0.11 Mothers were separated into two groups depending on levels of adherence to intervention; group 1 (regular consumption of sardines) vs group 2 (irregular consumption of sardines and milk PUFA concentrations were compared between groups:

							Total Omega-3: Day 30 Group 1 = 3.00±0.89 Group 2 = 2.62±0.39 (p<0.05) DHA: Day 15 Group 1 = 0.61±0.51 Group 2 = 0.45±0.20 (p<0.05) Day 30 Group 1 = 0.67±0.47 Group 2 = 0.41±0.21 (p<0.05) DPA: Day 15 Group 1 = 0.22±0.08 Group 2 = 0.20±0.03 (p<0.05) Day 30 Group 1 = 0.24±0.11 Group 2 = 0.23±0.07 (p<0.05)

							Milk n-3 and n-6 PUFA concentrations showed correlations with each other using Spearman's correlation coefficient on Day 0, Day 15 and Day 30, a statistically significant correlation existed for Day 15 (r2=0.58) and Day 30 (r2=0.59).
Ueno et al. 2020	Mothers were recruited in medical institutions post-partum (0-6 months) through-out Japan.	3.5	78	Observation FA intake: Maternal diet during lactation was estimated using an FFQ. Another dietary questionnaire was administered to obtain information about	Dietary PUFA intake.	PUFA composition of milk. Associations between milk DHA, dietary FAs and DHA supplementation.	Dietary intake (Median (IQR)) (g/1000kcal): Total PUFA: All = 7.47 (6.44–8.32) Non-supplement users = 7.33 (6.33–8.27) Supplement users = 7.80 (7.20–8.74) Omega-6 PUFA: All = 6.08 (5.16–6.73) NSU = 5.93 (5.07–6.54) SU = 6.36 (5.74–6.88)

				daily DHA supplement intakes and frequency of supplementation, which was categorized as never, infrequent (1–4x week), frequent (5–6x week), daily, or supplemented but not recorded. Milk sample collection: Milk storage bags and manual breast pumps were supplied and mothers were asked to collect milk once a day for 7 days.			Omega-3 PUFA: All = 1.41 (1.21–1.67) NSU = 1.38 (1.14–1.65) SU = 1.48 (1.28–1.77) DHA (mg/1000kcal): All = 235.9 (183.6–352.0) NSU = 225.1 (181.9–351.5) SU = 265.4 (194.6–367.0) Milk composition (Median (IQR)) (% of total FAs): Total PUFAs: All =16.49 (15.75–17.97) NSU = 16.68 (15.82–17.64) SU = 16.38 (15.39–18.69) Total omega–6 PUFAs: All = 14.14 (13.21–15.22)

				Samples were pooled for analysis. Sampling times occurred for 7 days every 2 months, if mothers had more than 1 pooled sample available the most recent one was used.			NSU = 14.22 (13.50–15.12) SU = 13.96 (12.94–15.76) LA: Al = 13.22 (12.19–14.32) NSU = 13.24 (12.57–14.25) SU = 13.01 (12.15–14.64) GLA: All = 0.09 (0.07–0.12) NSU = 0.10 (0.07–0.12) SU = 0.08 (0.06–0.11) EDA: All = 0.23 (0.21–0.25) NSU = 0.24 (0.21–0.25) SU = 0.23 (0.21–0.26) DGLA: All = 0.24 (0.21–0.28) NSU = 0.25 (0.22–0.28) SU = 0.21 (0.19–0.27) ARA:

							All = 0.38 (0.34–0.41) NSU = 0.37 (0.34–0.41) SU = 0.40 (0.33–0.41) Total omega–3 PUFAs: All = 2.48 (2.16–2.92) NSU = 2.44 (2.15–2.84) SU = 2.57 (2.32–2.95) ALA: All = 1.45 (1.30–1.70) NSU = 1.44 (1.31–1.70) SU = 1.47 (1.19–1.69) 20:3: All = 0.05 (0.03–0.05) NSU = 0.05 (0.04–0.05) SU = 0.04 (0.00–0.05) EPA: All = 0.15 (0.11–0.23)

							NSU = 0.16 (0.10–0.24) SU = 0.15 (0.11–0.19) DPA: All = 0.19 (0.16–0.24) NSU = 0.19 (0.16–0.25) SU = 0.20 (0.17–0.23) DHA: All = 0.62 (0.47–0.78) NSU = 0.55 (0.41–0.76) SU = 0.74 (0.60–0.90) (p=0.011) n–6/n–3: All = 5.74 (4.94–6.55) NSU = 5.81 (4.94–6.82) SU = 5.48 (4.98–6.06) Univariate and multivariate linear regression analyses of DHA in BM: Univariate:

							Maternal dietary intake of grilled fish (β ± SEM: 0.006 ± 0.003; standardized β: 0.267; r2 = 0.071, P = 0.018) and Infant age (β ± SEM: −0.001 ± 0.001; standardized β: −0.260; r2 = 0.068, P = 0.022) predicted milk DHA concentration. The frequency of supplement use (never, infrequent (1–4x/week), frequent (5–6x/week), daily) was associated with milk DHA concentration (β ± SEM: 0.067 ± 0.030; standardized β: 0.259; r2 = 0.067, P = 0.027), and The fixed effect of DHA supplementation was a significant factor (β ± SEM: 0.144 ± 0.064; standardized β: 0.248; r2 = 0.062, P = 0.028). Multivariate:

							A multivariate model (with maternal dietary intake of grilled fish and the fixed effect of DHA supplementation as potential confounders) predicted that milk DHA concentration was associated with maternal dietary intake of grilled fish (β ± SEM: 0.006 ± 0.003; standardized β: 0.234; r2 = 0.232, P = 0.036) after adjustment for the use of DHA supplements, maternal and infant age, maternal current BMI, and infant birth weight
Weseler et al. 2008.	Mothers were recruited at hospitals during prenatal checks at	5	34 (8 in control group, 8 in DHA + EPA group, 9 in low	Intervention study. FA intake: Mothers were randomly assigned into 1 of 4 test groups at 3 weeks	Supplementation intervention.	PUFA composition of milk.	Milk composition (g/100g): Total fatty acids in milk: Control = 33.7 ± 16.7 DHA + EPA Group = 38.2 ± 8.6 Low ARA Group = 23.2 ± 7.3 High ARA Group = 36.5 ± 15.2

	34-35 weeks in Maastricht and Heerlen, The Netherlands.		ARA group and 8 in high ARA group).	postpartum. Powder-based test drinks were supplied with instructions to consume 2x per day (200ml) or 38g of powder in 180ml of water. The consumption of 2 portions of the test drinks per day resulted in an additional daily intake of 320 mg DHA 80 mg EPA, and 80 mg of other (n-3) fatty acids (all 3 treatment groups), and 200 mg/d ARA (low ARA group)			n-6 PUFA Baseline Control = 1.12 ± 0.05 DHA + EPA Group = 1.08 ± 0.08 Low ARA Group = 1.08 ± 0.08 High ARA Group = 1.01 ± 0.17 2 weeks Control = 1.06 ± 0.10 DHA + EPA Group = 1.05 ± 0.12 Low ARA Group = 1.06 ± 0.19 High ARA Group = 1.08 ± 0.19 (p=0.01-0.05) 8 weeks Control = 0.89 ± 0.10 (p=0.001-0.01) DHA + EPA Group = 0.90 ± 0.11(p≤0.001) Low ARA Group = 0.99 ± 0.19 High ARA Group = 1.04 ± 0.14 ARA Baseline

				or 400 mg/d ARA (high ARA group). Milk sample collection: At week 3 (baseline), 5 and 11 postpartum women were visited at home and were asked to have collected a morning sample midway during a feed, ~10 mL milk from each breast by manual expression.			Control = 0.52 ± 0.03 DHA + EPA Group = 0.45 ± 0.08 Low ARA Group = 0.50 ± 0.11 High ARA Group = 0.47 ± 0.08 2 weeks Control = 0.45 ± 0.06 (p=0.001-0.01) DHA + EPA Group = 0.43 ± 0.09 Low ARA Group = 0.53 ± 0.08 High ARA Group = 0.55 ± 0.09 (p≤0.001) 8 weeks Control = 0.41 ± 0.06 (p≤0.001) DHA + EPA Group = 0.40 ± 0.04 Low ARA Group = 0.49 ± 0.10 High ARA Group = 0.56 ± 0.07 (p≤0.001) N-3 PUFA Baseline Control = 0.64 ± 0.08 DHA + EPA Group = 0.74 ± 0.15

							Low ARA Group = 0.62 ± 0.16 High ARA Group = 0.68 ± 0.29 2 weeks Control = 0.65 ± 0.20 DHA + EPA Group = 0.99 ± 0.35 (p=0.01-0.05) Low ARA Group = 1.02 ± 0.42 (p=0.001-0.01) High ARA Group = 0.80 ± 0.19 8 weeks Control = 0.57 ± 0.17 DHA + EPA Group = 0.88 ± 0.18 Low ARA Group = 0.92 ± 0.26 (p=0.01-0.05) High ARA Group = 0.84 ± 0.22 DHA Baseline Control = 0.30 ± 0.06 DHA + EPA Group = 0.34 ± 0.10

							Low ARA Group = 0.30 ± 0.11 High ARA Group = 0.34* ± 0.19 2 weeks Control = 0.25 ± 0.08 DHA + EPA Group = 0.55 ± 0.23 (p=0.001-0.01) Low ARA Group = 0.60 ± 0.26 (p=0.01-0.05) High ARA Group = 0.46 ± 0.13 (p=0.05=0.1) 8 weeks Control = 0.24 ± 0.08 DHA + EPA Group = 0.53 ± 0.13 (p=0.001-0.01) Low ARA Group = 0.54 ± 0.17 (p=0.01-0.05) High ARA Group = 0.50 ± 0.14 (p=0.05=0.1)
Xiang, Harbige and Zetterst	Mothers were recruited at 3 months	4	42 23 in Chinese group	Observational study. FA intake:	Dietary PUFA intake.	PUFA composition of milk.	Dietary intake (mean±SEM) (g/day): Total n-6: Chinese = 14.10±1.47 Swedish = 10.06±0.99 (p<0.05)

-rom. 2005	postpartum from Beijing, China and Stockholm, Sweden.		and 19 in Swedish group.	Dietary records were collected for 3 days, mothers were instructed on measuring and recording all food and fluid intake themselves. Milk sample collection: 2–5 ml of milk was collected by manual expression just after the breast was suckled by the infant for 2 min.		Correlation between DHA intake and milk DHA.	LA: Chinese = 14.06±1.47 Swedish = 9.91±0.98 ($p<0.05$) GLA: Chinese = ND Swedish = 0.01±0.01 EDA: Chinese = 0.003±0.001 Swedish = 0.01±0.00 ($p<0.01$) DGLA: Chinese = ND Swedish = 0.05 ±0.01 ARA: Chinese = 0.03±0.01 Swedish = 0.08±0.01($p<0.01$) ADA: Chinese = 0.01±0.00 Swedish = 0.0009 ±0.0005 ($p<0.05$)

							Total n-3: Chinese = 1.81±0.11 Swedish = 1.65±0.12 ALA: Chinese = 1.8±0.11 Swedish = 1.35±0.12 ($p<0.01$) SA: Chinese = ND Swedish = 0.03±0.01 EPA: Chinese = ND Swedish = 0.09±0.02 DPA: Chinese = 0.001±0 Swedish = 0.07±0.01 ($p<0.001$) DHA: Chinese = 0.01±0 Swedish = 0.12±0.02 ($p<0.001$)

							Milk composition (mean±SEM) (g/100g): Total n-6: Chinese = 24.54±1.07 Swedish = 12.19±0.47(p<0.001) LA: Chinese = 22.69±1.05 Swedish = 10.93±0.45(p<0.001) GLA: Chinese = 0.18±0.01 Swedish = 0.18±0.02 EDA: Chinese = 0.58±0.02 Swedish = 0.22±0.01(p<0.001) DGLA: Chinese = 0.46±0.03 Swedish = 0.30±0.01(p<0.001) ARA: Chinese = 0.51±0.02 Swedish = 0.38±0.02(p<0.001)

							n-6 DPA: Chinese = 0.10±0.00 Swedish = 0.16±0.01(p<0.001) Total n-3: Chinese = 1.48±0.13 Swedish = 1.95±0.14 (p<0.01) ALA: Chinese = 1.19±0.11 Swedish = 1.60±0.13 (p<0.05) SA: Chinese = 0.09±0.01 Swedish = 0.04±0.01(p<0.001) EPA: Chinese = 0.02±0.01 Swedish = 0.06±0.01 (p<0.01) DHA: Chinese = 0.18±0.02 Swedish = 0.25±0.01 (p<0.01)

							Ratios: LA/LNA: Chinese = 22.97±2.65 Swedish = 7.50±0.52 (p<0.001) ARA/DHA: Chinese = 3.14±0.19 Swedish = 1.56±0.09 (p<0.001) n-6/n-3 PUFAs: Chinese = 19.31±1.95 Swedish = 6.65±0.39 (p<0.001) n-6/n-3 LC-PUFAs: Chinese = 6.36±0.43 Swedish = 3.32±0.24 (p<0.001) Correlation between DHA intake and milk DHA: Chinese: r2 = 0.71 (p<0.001) Swedish: r2 = 0.54 (p<0.05)

Table 4.2: A table showing the reported correlation/regression analyses and/or mean differences between groups in relation to omega-3 LC-PUFA intake and milk omega-3 LC-PUFA concentrations from intervention and observation studies (n=525). Results are presented as % of total fatty acids and as mean±SD unless otherwise stated. Abbreviations: DHA = Docosahexaenoic acid, EPA = Eicosapentaenoic acid, NS = Non-significant, NA = Not applicable, CI = Confidence interval, FA = Fatty acids, ALA, Alpha-linolenic acid, n-3 = Omega-3, n-6 = Omega-6, ARA = Arachidonic acid, IQR = Interquartile range, NSU = Non-supplement user, SU = Supplement user, SEM = Standard error of the mean.

Reference	Supplementation Study	Correlation/relationship analysis completed	Mean difference reported between 2 or more groups	Results	Significance
Ay et al. 2018	X		X	Control vs DHA+EPA Total Omega-3: Control = 1.26±0.31 DHA+EPA = 1.65±0.71 DHA: Control = **0.22±0.1** DHA+EPA = **0.31±0.23** EPA: Control = 0.08±0.003 DHA+EPA = 0.14±0.08 (p=0.232)	Total Omega-3 = NS (p=0.142) **DHA = (p<0.000)** EPA = NS (p=0.232)

Del Prado et al. 2001	NA	NA	NA	NA	NA
Helland et al. 2006	X		X	Cod liver oil vs corn oil 4 weeks Total Omega-3: Cod liver oil = **3.35±1.07** Corn oil = **1.83±0.6** ALA: Cod liver oil = 0.96±0.32 Corn oil = 0.9±0.28 DPA: Cod liver oil = **0.36±0.13** Corn oil = **0.19±0.09** DHA: Cod liver oil = **1.37±0.58** Corn oil = **0.49±0.32** EPA:	Repeated measurements showed significant differences between the two groups: **Total Omega-3 = (p<0.001)** ALA = NS **DPA = (p<0.001)** **DHA = (p<0.001)** **EPA = (p<0.001)** **N-3/n-6 = (p<0.001)** Repeated measurements showed significant differences between the two groups*time for the below: **Total Omega-3 = (p<0.011)**

				Cod liver oil = **0.43±0.19** Corn oil = **0.13±0.09** N-3/n-6: Cod liver oil = 0**.27±0.09** Corn oil = **0.13±0.04** 12 weeks Total Omega-3: Cod liver oil = **3.12±1.09** Corn oil = **1.86±0.81** ALA: Cod liver oil = 0.98±0.34 Corn oil = 0.94±0.32 DPA: Cod liver oil = **0.33±0.12** Corn oil = **0.19±0.09** DHA: Cod liver oil = **1.20±0.57** Corn oil = **0.47±0.40**	ALA = NS **DPA = (p<0.006)** **DHA = (p<0.00)** **EPA = (p<0.01)** **N-3/n-6 = (p<0.001)**

				EPA: Cod liver oil = **0.41±0.22** Corn oil = **0.15±0.13** N-3/n-6: Cod liver oil = **0.25±0.09** Corn oil = **0.13±0.05**	
Nishimura et al. 2014		X		**β-regression coefficients (95% CI)/r^2** between dietary FA intake postpartum and milk FA: ALA: model 1 = 0.115 (−0.040 to 0.271)/ 0.051 model 2 = 0.140 (−0.044 to 0.299) EPA: Model 1 = −0.044 (−0.338 to 0.251)/ 0.095	ALA: NS EPA: NS DHA: NS **N-3/N-6 RATIO:** Model 1 = 0.074 (0.006 to 0.143)/ 0.124 Model 2 = 0.086 (0.006 to 0.166)

				Model 2 = −0.055 (−0.422 to 0.313) DHA: Model 1 = 0.015 (−0.121 to 0.151)/ 0.100 Model 2 = 0.021 (−0.170 to 0.212) n-3/n-6: Model 1 = 0.074 (0.006 to 0.143)/ 0.124 Model 2 = 0.086 (0.006 to 0.166)	
Patin et al. 2006	X		X	Group 1 (regular consumption of sardines) vs Group 2 (irregular consumption of sardines. Total Omega-3: Day 30 Group 1 = **3.00±0.89** Group 2 = **2.62±0.39**	**Total Omega-3 Day 30 = (p<0.05)** **DHA Day 15 & Day 30 = (p<0.05)** **DPA Day 15 & Day 30 = (p<0.05)**

				DHA: Day 15 Group 1 = **0.61±0.51** Group 2 = **0.45±0.20** Day 30 Group 1 = **0.67±0.47** Group 2 = **0.41±0.21** DPA: Day 15 Group 1 = **0.22±0.08** Group 2 = **0.20±0.03** Day 30 Group 1 = **0.24±0.11** Group 2 = **0.23±0.07**	
Ueno et al. 2020		X	X	Habitual non-supplement users vs habitual supplement users.	Total Omega-3 = NS **DHA = (p=0.011)**

				(Median (IQR)) Total Omega-3: All = 2.48 (2.16–2.92) NSU = 2.44 (2.15–2.84) SU = 2.57 (2.32–2.95) DHA: All = 0.62 (0.47–0.78) NSU = **0.55 (0.41–0.76)** SU = **0.74 (0.60–0.90)** EPA: All = 0.15 (0.11–0.23) NSU = 0.16 (0.10–0.24) SU = 0.15 (0.11–0.19) DPA: All = 0.19 (0.16–0.24)	EPA = NS DPA = NS n–6/n–3 = NS Univariate linear regression analyses of DHA in milk: **Frequency of supplement use = (p= 0.027)** **Fixed effect of DHA supplementation was a significant factor = (p=0.028)** Multivariate linear regression analysis of DHA in milk: **Maternal dietary intake of grilled fish = (p= 0.036)**

				NSU = 0.19 (0.16–0.25) SU = 0.20 (0.17–0.23) n–6/n–3: All = 5.74 (4.94–6.55) NSU = 5.81 (4.94–6.82) SU = 5.48 (4.98–6.06) Univariate linear regression analyses of DHA in milk: The frequency of supplement use (never, infrequent (1–4x/week), frequent (5–6x/week), daily) was associated with milk DHA concentration = **(β ± SEM: 0.067 ± 0.030; standardized β: 0.259; r2 = 0.067.** The fixed effect of DHA supplementation was a significant	

				factor **= (β ± SEM: 0.144 ± 0.064; standardized β: 0.248; r2 = 0.062.** Multivariate linear regression model (with maternal dietary intake of grilled fish and the fixed effect of DHA supplementation as potential confounders) predicted that milk DHA concentration was associated with maternal dietary intake of grilled fish **(β ± SEM: 0.006 ± 0.003; standardized β: 0.234; r2 = 0.232, P = 0.036)** after adjustment for the use of DHA supplements, maternal and infant age, maternal current BMI, and infant birth weight	

Weseler et al. 2008	X		X	Control vs DHA+EPA vs Low ARA vs High ARA (change from baseline) (g/100g) Total Omega-3: 2 weeks Control = 0.65 ± 0.20 DHA + EPA Group **= 0.99 ± 0.35** Low ARA Group **= 1.02 ± 0.42** High ARA Group = 0.80 ± 0.19 8 weeks Control = 0.57 ± 0.17 DHA + EPA Group = 0.88 ± 0.18 Low ARA Group **= 0.92 ± 0.26** High ARA Group = 0.84 ± 0.22 DHA 2 weeks	Total Omega-3: 2 weeks **DHA + EPA Group = (p=0.01-0.05)** **Low ARA Group = (p=0.001-0.01)** 8 weeks **Low ARA Group = (p=0.01-0.05)** DHA 2 weeks **DHA + EPA Group = (p=0.001-0.01)** **Low ARA Group = (p=0.01-0.05)** 8 weeks

				Control = 0.25 ± 0.08 DHA + EPA Group **= 0.55 ± 0.23** Low ARA Group **= 0.60 ± 0.26** High ARA Group = 0.46 ± 0.13+ 8 weeks Control = 0.24 ± 0.08 DHA + EPA Group = **0.53 ± 0.13** Low ARA Group = **0.54 ± 0.17** High ARA Group = 0.50 ± 0.14	**DHA + EPA Group = (p=0.001-0.01)** **Low ARA Group = (p=0.01-0.05)**
Xiang, Harbige and Zetterst-rom. 2005		X		Correlation between DHA intake and milk DHA: Chinese: **r^2 = 0.71** Swedish: **r^2 = 0.54**	Chinese group = (p<0.001) Swedish group = (p<0.05)

Table 4.3: Quality assessment for studies included in this review.

	Stated what kind of milk	Mode of feeding exclusive BF	Standardised time of sample collection	Standardised time postpartum of sample collection	Supplementation controlled/appropriate measure of FA intake	Controls for confounders	Representativeness of cohort	Sample size	Section 1 score	Section 2 score	Score (8)
Ay et al. 2018	X	X	✓	✓	✓	X	✓	Small	3	1	4
Del Prado et al. 2001	✓	✓	✓	X	✓	X	X	Small	4	0	4
Helland et al. 2006	X	X	✓	✓	✓	✓	✓	Large	3	2	5
Nishimura et al. 2014	X	✓	✓	✓	✓	✓	✓	Small	4	2	6
Patin et al. 2006	✓	✓	X	✓	✓	X	X	Small	4	0	4

Ueno et al. 2020	X	X	X	X	✓	✓	✓	Medium	1	2.5	3.5
Weseler et al. 2008	✓	X	✓	✓	✓	X	✓	Small	4	1	5
Xiang et al. 2005	X		X	✓	✓	X	✓	Small	3	1	4

Appendix 5

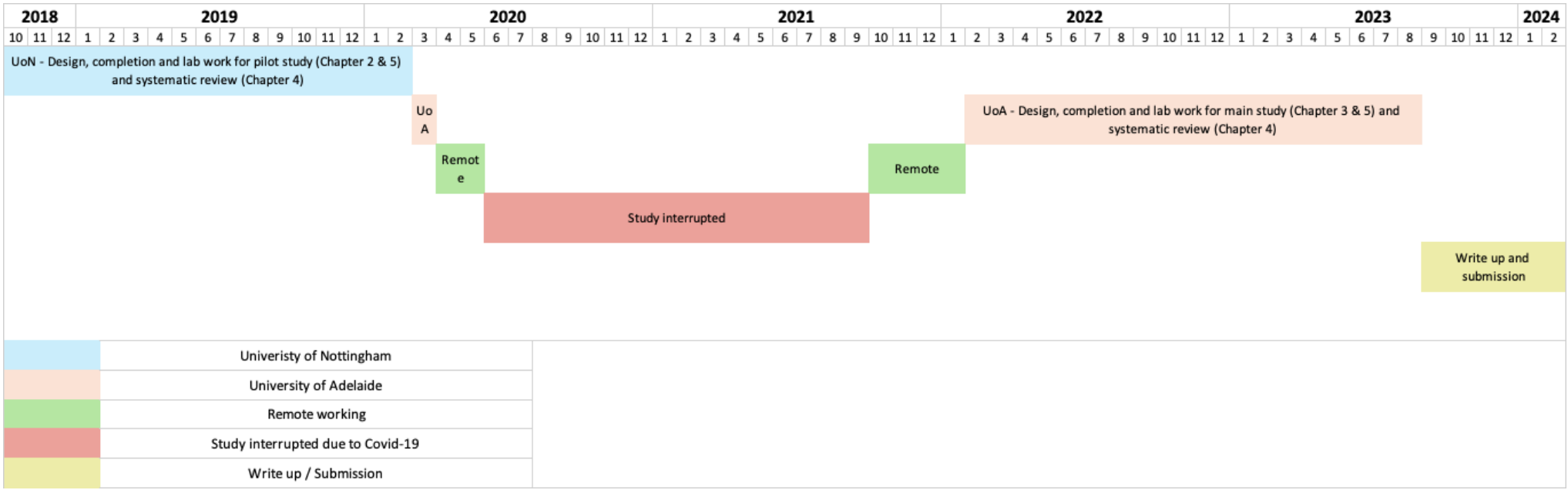

Figure 6: A GANTT chart showing the timeline of the work completed in this thesis.