MOTHER-INFANT SIGNALLING DURING BREASTFEEDING AND INFANT GROWTH: AN INVESTIGATION OF PHYSIOLOGICAL, PSYCHOLOGICAL AND ANTHROPOLOGICAL ASPECTS OF INFANT FEEDING

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A thesis submitted for the degree of Doctor of Philosophy

UCL

DECLARATION

I, Nurul Husna Binti Mohd Shukri confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Signature:

Date:

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ABSTRACT

Lactation is a dynamic process involving complex physiological signalling and behavioural negotiation between mother and the infant. The infant can 'signal' his needs to the mother by his behaviour to demand feeding, and the mother can respond by altering the amount or composition of milk. Challenging behaviour amongst breastfed infants has been associated with higher milk cortisol, demonstrating maternal potential to shape infant behaviour by the transmission of bioactive factors in milk. Maternal psychological state is also recognised to be influential, largely affecting milk production. Thus increased stress can disrupt milk flow, whilst milk ejection can be improved by relaxation therapy; previously shown in mothers of pre-term infants. However, these mother-infant factors are inter-related, making it difficult to define cause and effect using an observational study design. Therefore, I aimed to investigate biological and anthropological aspects of mother-infant signalling during breastfeeding using an experimental approach. Maternal psychological state was manipulated using relaxation therapy in mothers breastfeeding their full-term infant to test the primary hypothesis that the intervention would reduce maternal stress, favourably affect breast milk composition and positively influence infant behaviour and growth; and the secondary hypotheses that milk composition (including hormones) and infant characteristics (temperament, appetite, gender) would associate with infant growth. Pregnant women, recruited from antenatal clinics in Malaysia, were randomised postnatally into control (no treatment) and intervention (audio relaxation recording) groups. Home visits were performed at 2-3, 6-8 and 12-14 weeks to assess infant anthropometry, maternal stress and infant behaviour, and to collect fore- and hindmilk samples for composition including cortisol, ghrelin and leptin. The relaxation therapy was effective in reducing maternal stress during lactation, favourably affecting breast milk composition and positively influencing infant sleeping behaviour and growth. Infant temperament, appetite and breast milk hormones were also found to be associated with infant growth. Overall, this thesis presents results based on the primary and secondary hypotheses, explores potential pathways for intervention effects, and discusses the findings from a biological and anthropological perspective. It also highlights the practical relevance and potential applications of the results in terms of supporting breastfeeding mothers.

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LIST OF ABBREVIATIONS

BAI : Beck Anxiety Inventory

BEBQ : Baby Eating Behaviour Questionnaire

BF : Breastfeeding

CHO : Carbohydrate

EF : Enjoyment of Food (Appetite trait)

EPDS : Edinburgh Postnatal Depression Scale

FFM : Fat free mass

FM : Fat mass

FMI or FFMI : Fat Mass Index or Fat Free Mass Index

FR : Food Responsiveness (Appetite trait)

GA : General appetite

GLM : General Linear Model

HV : Home visit

HV1 /2 /3/ 4 : Home visit 1/2/3/4

IFQ : Infant Feeding Questionnaire

IIFAS : Iowa Infant Feeding Attitude Scale

IRMS : Isotope-Ratio-Mass Spectrometry

PSS : Perceived Stress Scale

RCT : randomised controlled trial

RIBQ : Rothbart's Infant Behaviour Questionnaire (Revised)

SD : Standard deviation

SE : Slowness in Eating (Appetite trait)

SR : Satiety Responsiveness (Appetite trait)

TBW : Total body water

Mother-Infant Signalling During Breastfeeding

Sepilok, Sabah, MALAYSIA.

CHAPTER 1

1. INTRODUCTION

1.1. Background

Mother's milk is a sophisticated living product consisting of nutrients and various bioactive factors such as immune components, hormones, naturally occurring opiates, enzymes and many other active molecules. These components are specific to the species, as well as the offspring's characteristics, in order to ensure optimal growth, development and survival [1, 2]. Epidemiological studies and randomised trials in humans have shown that breast milk protects against diarrhoea and infection in infants, reduces the risk of obesity, type 2 diabetes and cardiovascular disease later in life, and is associated with an increase in cognitive development [3-6]. It has also been reported to decrease the risk of breast and ovarian cancers in mothers [6, 7]. Globally, it is estimated that breastfeeding could save more than 800 000 children's lives and prevent over 20 000 deaths from breast cancer in women annually [6, 7]. Overall, breastfeeding is not only important for infant survival, fitness and health in children, but also saves women's lives, and hence it is a valuable investment for human capital development. From nutritional, economic and evolutionary perspectives, breast milk is (also) an ideal food for the infant, and is considered as the gold standard for infant feeding.

The volume and composition of human milk changes within a feed, during the day and over the course of lactation, as well as varying between mothers and between breasts in an individual. This complex physiological change is important in meeting the infant's demands and needs, as well as providing the best source of nutrition and energy for early infant growth; formula milk can never provide a substitute in this respect [6, 7]. It is also important to

acknowledge that breastfeeding is a dynamic process which involves complex biological signalling and behavioural negotiation between the mother and the infant [8, 9]. These processes are hypothesised to shape infant behaviour and feeding, including appetite regulation, and hence may also influence infant growth. Biological signalling is thought to involve bioactive factors (e.g. hormones and opiates) in breast milk, but is largely unexplored in human studies. Although many initiatives have been undertaken to improve breastfeeding rates, at both national and international levels, many biological and psychosocial aspects of breastfeeding remain poorly understood. One of the aims of this thesis is to explore how breast milk may influence infant growth through physiological and psychosocial signalling between the mother and infant during the lactation period. This may provide a greater understanding of maternal-infant factors which influence the success of breastfeeding, and which may, therefore, be useful targets for future interventions.

Whilst most of the focus is on biological and physiological aspects, infant feeding can also be viewed from an anthropological perspective. This is largely unexplored in humans [8] and is another focus of this thesis. Focusing on maternal investment strategy and potential trade-offs during lactation, the thesis considers breastfeeding in the context of the 'tug-of-war' or the parent-offspring conflict between the mother and infant. Reducing the tension of this conflict is important since both the mother and infant are in highly energetically demanding periods; lactation for the mother and early growth for the infant. In this context, firstly, this thesis aims to review the general Trivers' theory of parent-offspring conflict during human postnatal life and experimentally test the tug-of-war during breastfeeding. Secondly, it will investigate whether it is possible to reduce the tension of the tug-of-war, by manipulating maternal environmental stress and investigating the effects on maternal capital and infant behaviour and growth. By combining available evidence with my research findings, I hope this evolutionary approach might provide insights into potential behavioural interventions or strategies to reduce parent-offspring conflict, and provide better understanding on the possible psychosocial and physiological mechanisms of the tug-of-war during the lactation period.

In summary, this thesis aims to examine physiological, psychological and anthropological aspects of mother-infant signalling during breastfeeding using a robust methodological study design, identifying mother-infant factors that influence early infant behaviour and growth, and highlighting the practical relevance and potential applications of the results in terms of supporting breastfeeding mothers.

1.2. Overview of the thesis

Here, I provide an outline of the thesis:

Chapter 2: This chapter starts with a description of the evolution of lactation in general and an overview of human lactation. Following that, I provide a detailed discussion about mother-infant signalling, which includes the physiological and psychological factors that are involved during breastfeeding. The anthropological perspective of mother-infant signalling is also incorporated, focusing on tug-of-war mechanisms during lactation. The chapter ends with a systematic review of intervention studies aimed at improving breastfeeding outcomes, along with research gaps and the planning of an intervention study, the MOM Study.

Chapter 3: This chapter describes the research methodology of the MOM Study, including study design, research questions, outcome measures and hypotheses, and also providing an anthropological perspective on the hypotheses. Data collection procedures and research tools used in the study are described in detail, followed by a summary of the planned statistical analyses.

Chapter 4: This chapter provides descriptive characteristics and socio-demographic background factors of the whole MOM Study population and a comparison with the general Malaysian population. This chapter also includes a comparison of the results for socio-demographic background factors and perception of infant feeding between mothers who were excluded and included for randomisation in the MOMS trial.

Chapter 5: This chapter addresses the primary hypotheses of the study and includes the main outcomes of the randomised controlled trial with a comparison of primary and secondary outcomes between randomised groups.

Chapter 6: Chapter 6 presents the secondary outcome results for the whole MOM study population. The main purpose is to address the secondary hypotheses, which involve milk hormones and infant behaviour. Exploratory results are presented to describe the associations between mother-infant factors and infant growth outcomes.

Chapter 7: This final discussion chapter provides a summary and overview of both primary and secondary outcome results, and combines them to discuss potential pathways for mother-infant signalling during breastfeeding. The overall strengths and limitations of my research and the implications of the trial for breastfeeding support and intervention are included. Finally, I provide a conclusion and suggest directions for future research.

To date, I have submitted one paper on the study protocol for publication. Results from the study have also been presented at a number of international and local conferences, and some abstracts have been published in scientific journals. The list of abstracts are included in Appendix 3.

CHAPTER 2

2. LITERATURE REVIEW

Introduction

This chapter consists of 3 (main) parts: i) overview of lactation, ii) description of mother-infant factors (physiological and psychological factors) which influence lactation and iii) a systematic review of intervention studies using relaxation therapy to improve breastfeeding outcomes. In part (i), I briefly describe the evolution of lactation, including comparative lactation across mammals, before focusing on the physiological aspects of human lactation. Next, along with a description of the development of the human mammary gland, I also consider the health effects and prevalence of breastfeeding. In part (ii), I present the major physiological and psychological factors that are involved in breastfeeding, and the possible mechanisms of mother-infant signalling in early human life. This includes an anthropological perspective on parent-infant conflicts and maternal investment during the lactation period. In part (iii), I present a systematic review of intervention studies aimed at improving breastfeeding outcomes, focusing on milk production (volume and/or composition). To end this chapter, I discuss research gaps and the limitations of published data leading to an outline and justification for my study project.

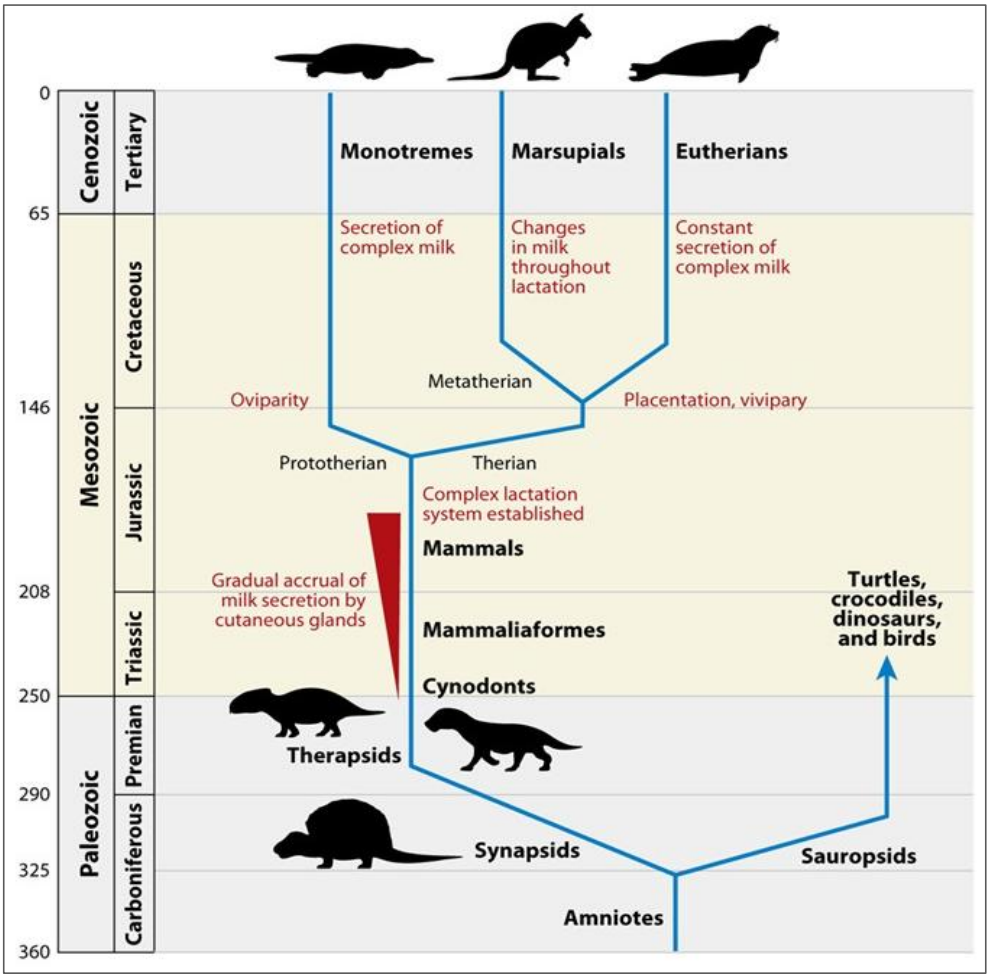
2.1. INFANT FEEDING IN EARLY LIFE

2.1.1. Evolution of lactation: a brief summary

Lactation occurs in all mammals, and plays an important role in providing nourishment (including protective substances) to the young for its survival and fitness in very early life. Lactation was gradually established long before mammals came into existence approximately 300 million years ago (MYA), among a group of animals called synapsids [2, 10], as shown in Figure 2.1. Synapsids secreted an antimicrobial liquid from epidermal glands to protect and moisturise their eggs; in other words, its role was microbe-killing rather than for nourishment [2, 10]. Oftedal [2] hypothesised that this epidermal or apocrine-like gland later gradually evolved into a mammary gland that secreted a nutrient-rich liquid, containing immunologically active substances, known as milk, when these animals radiated and evolved

into different forms from the class Therapsida to Mammalia, starting approximately 200 MYA (the Triassic period). Thus, the mammary gland was a successful innovation for evolution that has evolved not only to protect but nourish the offspring through the secretion of milk, and which also provides additional benefits in terms of behavioural aspects by promoting bonding between two parties (mother and infant) that could enhance neonatal survival [11]. During mammalian evolution, adaptation and natural selection diversified mammals into different species according to their life-history strategy (which is discussed later) that results in a wide variation in morphology and anatomy of mammary glands. For example, egg-laying monotremes (e.g. platypus and echidnas) have mammary glands without nipples; hairs appeared near the nipple in marsupials (e.g kangaroo and koala) and an udder with many teats is a feature of the ruminants' mammary gland (e.g cow, buffalo and sheep) [2, 10]. In the next section, I briefly describe comparative lactation across mammals in terms of lactation strategy, before focusing on human lactation (per se).

Figure 2.1 Evolution of mammals and lactation. (Figure taken from: Lefèvre, Sharp [10])



2.1.2. Comparative aspects of lactation

Lactation is the natural process for feeding the live born infants of all mammals. As shown in the above figure (2.1), the reproductive strategy is diversified between the major lineages: monotremes, marsupials and eutherians. Monotremes lay eggs and feed solely milk to their altricial offspring during the lactation period; marsupials have a short gestation period and thus give birth to highly altricial infant, and their milk composition changes constantly depending on the infant development; and eutherians (also known as placental mammals) transfer maternal resources to their offspring both during the intrauterine and postnatal period, and produce relatively constant milk throughout lactation (apart from colostrum) [10]. The lactation strategy is also different and varied between and within species. This includes variation in milk composition and volume between individuals and across time points within the same individual [1, 10]. Variation in lactation strategies such as milk production, milk energy transfer, changes in milk composition, as well as duration of lactation, reflect the species' evolutionary origin, life-history pattern, current ecology and the patterns of development and growth of the offspring, and these factors are usually correlated [1, 2, 12]. For example, arctic hooded seals (*Cystophora cristata*) feed their pups for a very short period (4-5 days), but transfer a very high amount of fat, to a total of approximately 7.5 kg of milk per day [13]. Most seals feed their pups on unstable ice sheets as they are safe here from predators, especially polar bears, therefore lactation needs to be done in a short period, and a high quantity of energy needs to be transferred quickly to sustain the offspring in the extremely cold environment. The hooded seal has the highest fat percentage (61%) in the milk among mammals, as high fat stores are needed for insulation and thermogenesis [13]. Due to the short lactation, high energy is also needed to sustain the pups post-weaning, before they establish their swimming skills and are capable of hunting for food.

Besides extreme environments, highly concentrated milk (high fat and protein) is also found in animals who are unable to nurse their offspring frequently, such as rabbits, hares and echidnas [12]. These animals produce high-energy density milk to provide their offspring with enough energy for several days as they forage away from their nest for one or several days, and avoid returning frequently as it will attract predators. In addition, these animals have a faster growth rate, and the higher protein in milk plays an important role for offspring growth. For example, rabbits have approximately 14% of protein in the milk and their offspring take about 6 days to double their birth weight [14]; in humans, for comparison, the milk contains only about 0.9% of protein, and the offspring take longer (20 weeks) to double their birth

weight. Other factors, including physical attributes, also influence milk composition. For example, bats cannot carry a large amount of dilute liquid milk as it can constrain their flying capability, so they produce small volumes of highly concentrated milk [12].

In general, highly concentrated milk is produced by animals that grow fast during the postnatal period and have short life spans, as higher energy is needed for accelerated growth [12, 15]. In contrast, primates including humans have a slower growth rate during infancy and milk is relatively dilute, with a low concentration of protein and fat. This is suited to the longer period of infancy in primates, and therefore the duration of lactation is also longer and the maternal bodily reserves are not depleted so quickly in order to invest in their offspring's growth and development [12, 15]. Nevertheless, within species, milk production also varies among mothers. For example, in humans, milk composition such as fatty acid profile [16] and the amount of minerals or vitamins such as iodine [17], vitamin C [18] and vitamin D [19] are influenced by maternal diet. In addition to maternal factors, lactation performance is also influenced by the communication between the mother and the infant during lactation, such as the pattern of 'demand' by the infant and how the mother responds, which influences milk synthesis and composition [8]. This signalling during breastfeeding is the major focus of my research project and will be discussed further in this chapter.

2.1.3. Overview of human lactation

The mammary gland originates in humans from the beginning of foetal life and development continues gradually until puberty, primarily by producing a branched system of ducts. At puberty, changes in hormones lead to a considerable increase in the growth of the ducts until they occupy the major part of the fat pad volume in the breast [20]. In early pregnancy, the increasing levels of progesterone, prolactin and placental lactogen hormones produce a further increase in the growth of the duct system. During this phase of mammogenesis, the mammary gland proliferates, creating multiple ducts and the lobulo-alveolar structure of alveoli to prepare for lactogenesis at a later stage of gestation [20, 21]. Lactogenesis I occurs during the second trimester of pregnancy, when there is an increase in the production of mRNA for milk proteins and enzymes in the mammary gland, which is necessary for milk formation and secretion [22]. At this stage, fat droplets increase in size in the mammary cells and become enormous at the end of the pregnancy. In late pregnancy, other milk components such as lactose, casein and α-lactalbumin are produced due to increasing levels of prolactin (the milk secretion hormone) in the blood, which causes secretion of colostrum in small

amounts [21, 23]. However, as milk is not removed by sucking, milk components are reabsorbed into the blood stream via the paracellular pathway [21, 23].

At birth, progesterone, estrogen and placental lactogen drop dramatically and unblock the action of prolactin, resulting in the onset of lactogenesis II, with secretion of copious milk. The secretion of milk during parturition gradually increases until the mother can sense the fullness of the breast at about 40 to 96 hours following delivery [21, 24]. In the first 2-3 days after delivery, the mammary gland produces colostrum, which is low in fat and high in protein, specifically immune components and protective substances. The composition of colostrum is constantly varying and it is secreted in small amounts (~30 ml per day) during the first two days postpartum; this is suited to the neonate's capability for digestion and the maturity of the gastrointestinal tract [24, 25]. Following colostrum, transitional milk is produced as milk volume and fat concentration increase; this continues up to 3 or 4 weeks until mature milk is produced. Subsequent to this, mature milk is produced according to the physiological changes in the mammary gland as well as in response to demands made by the infant [12].

Breast milk volume and milk composition is never constant, as it changes within a feed, diurnally and over the course of lactation and, as indicated previously, it also varies between mothers and between breasts in an individual [12, 26-28]. This complex physiological change is important in fulfilling the infant's demands and needs, as well as providing the best source of nutrition and energy for infant growth [7]. In 2001, the World Health Organization (WHO) recommended that mothers should exclusively breastfeed their infants for the first six months and continue breastfeeding up to two years alongside complementary feeding [29]. Extensive research has been performed on breast milk composition (especially the nutrient content) and the importance of breastfeeding for both the mother and infant [30, 31].

2.1.4. The importance of early nutrition for infant health

Early infancy is a critical and sensitive period of development and growth [32-34]. Nutrition, in particular during early life, has an important impact on long term health and development [33], termed 'nutritional programming', and defined by Lucas (2005) as "the concept that a stimulus applied at a critical or sensitive period may have long-term or lifetime effects on the structure or function of an organism" (p.2) [32]. The stimulus can either be endogenous (e.g. hormones, metabolites) or exogenous (e.g. environmental, nutrients, drugs) or both [32].

Evidence from extensive animal studies has shown that nutrition can programme later blood pressure, obesity, diabetic tendency, atherosclerosis, cognitive function and longevity [33], all of which are considered important public health issues in humans. Data from randomised trials and from epidemiological studies in humans have also shown that early nutrition has major consequences for health outcomes in infancy particularly on infection (gastrointestinal and respiratory tract), but also later in childhood and into adult life, including obesity, cardiovascular disease risk and cognitive function and bone health [32, 35, 36]. Another example of programming is the association between accelerated growth during infancy and an increased risk of obesity in later life in high-income population; this can be produced by over-nutrition (eg. by using enriched formulas) in early infancy, whereas slower growth among breast-fed infants has been reported to have beneficial effects on obesity, blood pressure and lipid profile in later life [32, 35, 37].

The benefits of breastfeeding for the short and longer-term health of the infant are well documented in systematic reviews and meta-analyses [3-6]. However, findings from infant feeding studies in human are primarily observational due to the fact that it is unethical to randomise infants to be breast-fed or formula-fed [38]. Since observational studies cannot demonstrate causal relationships between the type of feeding and health outcomes, and are affected by confounding and reverse causation, it is vital to adjust as far as possible for such factors [38]. One of the main potential confounders is socio-economic status since breastfeeding is typically associated with higher educational and income levels, especially in high-income countries [6]. In general, evidence has shown that breastfeeding protects against diarrhoea, infection and dental malocclusion, reduces the risk of overweight and type 2 diabetes later in life, and is associated with an increase in cognitive development [3-6]. The mechanism for these health effects has been extensively investigated in recent years, and it is probable that different mechanisms operate for different outcomes. For example, beneficial effects of human milk on the risk of infection or cognitive outcome and brain structure are most likely to reflect specific components of the milk (nutrient or non-nutrient), whereas effects on later cardiovascular health and obesity risk may be due to the difference in growth patterns early in infancy. Hence, the growth pattern of the breastfed infant is considered to strongly influence both short and longer-term health outcomes [6]. Reviews have also reported that breastfeeding provides benefits for maternal health: protection against breast cancer, reduced risk of ovarian cancer and type II diabetes, and also improved birth spacing [3, 6, 39]. In developing countries, breastfeeding is of vital importance in reducing child

mortality, especially in early life [6]. This has been shown by an increase in neonatal mortality risk associated with delayed initiation of breastfeeding [40-42], especially in populations (such as in India and Nepal) that practice colostrum withholding due to their cultural beliefs [43].

2.1.5. Health burden and breastfeeding rates

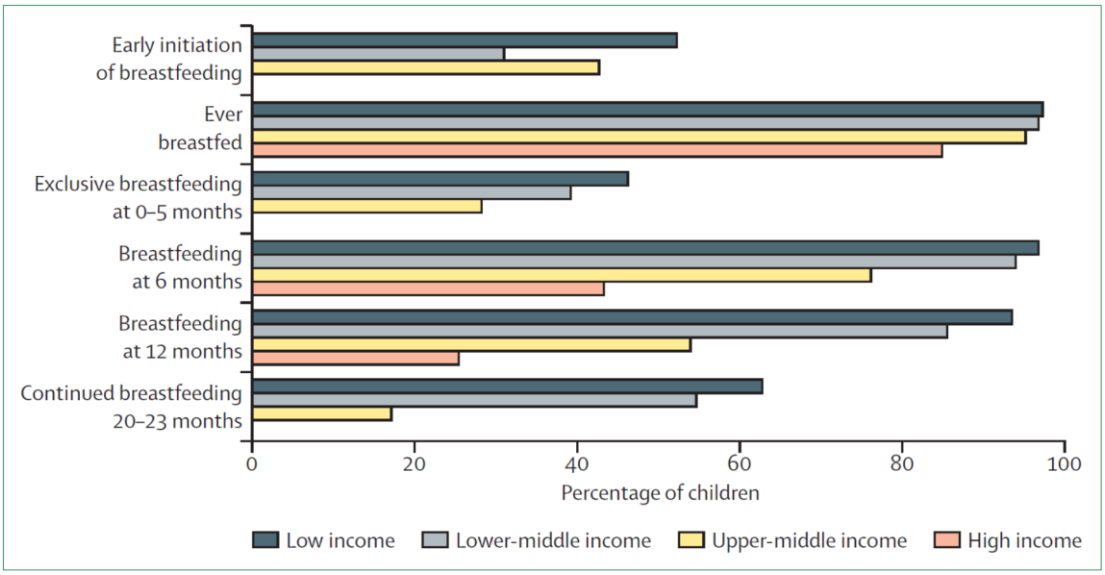
In countries with high mortality rates, especially those with poor levels of hygiene (in particular lack of access to clean water), high rates of infant deaths (or infant mortality) could be reduced as well as prevented by increasing rates and duration of breastfeeding [44]. Suboptimal breastfeeding, including non-exclusive breastfeeding in the first 6 months of life, was estimated to be responsible for 1.4 million child deaths and 44 million disability-adjusted life years; this analysis included infants in developed countries [26, 45]. A recent *Lancet* review indicates that increasing breastfeeding rates worldwide could reduce the ill-health burden globally: it is estimated that it could save more than 800 000 young children's lives annually [6, 7]. Moreover, increasing breastfeeding rates could also reduce hospital admissions and cut treatment costs for childhood illness which could have been prevented by breastfeeding [7]. Therefore, the promotion and support of breastfeeding are considered an international public health priority [7].

However, despite many initiatives designed to promote and protect breastfeeding, either at the population or individual level, it is widely recognised that breastfeeding rates worldwide are still disappointingly low and below target levels [7]. UNICEF 2012 [46] and the latest *Lancet* series reported that less than half of the world's population exclusively breastfeed their infants (0-5 month), with a global rate of 36% [6]. The *Lancet* series estimated that high-income countries have lower rates of breastfeeding (1 in 5 children) than lower-and middle-income countries (a third), with a prevalence of breastfeeding up to 12 months of less than 20% [6]. The lowest exclusive breastfeeding rates were reported in the UK and Ireland (1-3%) [6]. For exclusive breastfeeding rates between 0 to 5 months, the overall rate in developing countries was only 37%, despite it being higher than the average rate in high-income countries (Figure 2.2); nevertheless, the overall rate increased by about 11% from 1993 to 2013 [6]. Since my research project was based in Malaysia, I am particularly interested in the rates of breastfeeding in this country. Contrary to the increased rates of exclusive breastfeeding in many low- and middle-income countries, the rate decreased in Malaysia by 10% from 29% in 1999 to 19% in 2006 [47]. The latest Malaysia National Health Survey 2006 also estimated that the rate of exclusive breastfeeding up to six months is only 15% [47].

Nevertheless, data on exclusive breastfeeding is generally difficult to obtain especially if obtained retrospectively since this can lead to potential recall bias or inaccurate results depending on the type of question(s), or how it was asked. Thus, it is very important to carefully define all breastfeeding terms (or WHO feeding indicators) to mothers when collecting information to ensure their understanding about the definition of exclusive breastfeeding, and other related terms such as ever-breastfeeding, predominant breastfeeding and mixed-feeding [6, 38]. In addition, well-structured questions are important to objectively ask mothers about their infants' diet within a particular time (e.g. past 24-hours, past weeks or months, since birth) in order to ascertain exclusivity of breastfeeding, and also detailed prospective information about their breastfeeding practice (e.g ever/never feeding formula and/or water, practice of night feeding, or feeding duration and frequency).

Most attempts to improve breastfeeding rates focus on providing additional support, such as improving maternity leave, adopting the practice of the UNICEF Baby-Friendly Hospital Initiative, and the existence of various breastfeeding support groups and organisations; yet many aspects of the breastfeeding process are poorly understood. To increase this understanding, for my research project, I investigated the mechanisms of biological and behavioural signalling during early human life, focusing on breastfeeding in the first 3-4 months. My project aimed to provide a greater understanding of maternal-infant factors which influence the success of breastfeeding. This may, in turn, allow identification of modifiable factors that can be useful targets for future interventions to increase breastfeeding rates and duration. In the next section, I first explain the mother-infant factors that influence breastfeeding in early human life, including the physiological (part 1) and psychological (part 2) signalling between the mother and infant, before describing my study in the next chapter. An anthropological perspective of mother-infant signalling is also incorporated in this section.

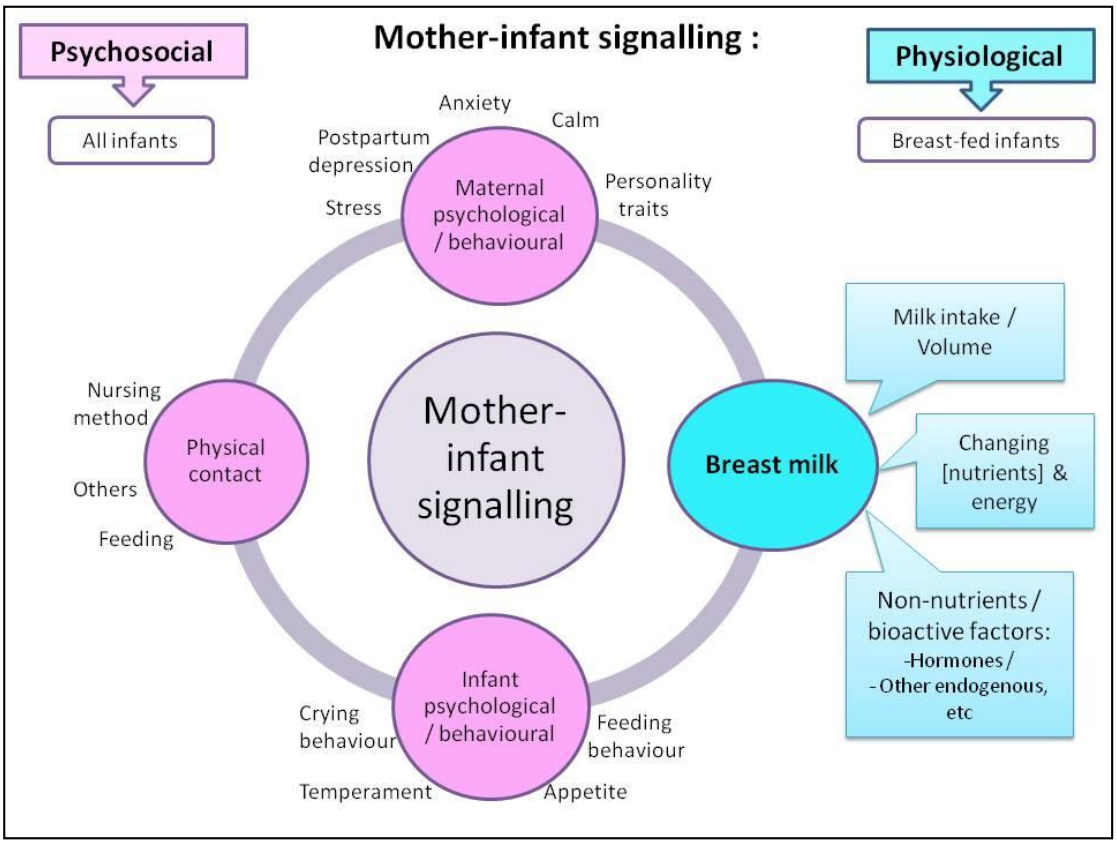
Figure 2.2 Breastfeeding indicators by country income group (n=153) in 2010 (Figure taken from: [6])



2.2. SECTION II: MOTHER-INFANT FACTORS INFLUENCING INFANT FEEDING (Part 1)

Infant feeding involves complex interaction and communication ('signalling') between the mother and infant, as it is one of the main intensive aspects of parenting. This is especially so during the early postpartum period, since feeding a newborn infant demands a great commitment from a mother, especially new mothers, in understanding and responding to infant cues and gestures. Thus, the signalling between the mother and infant, specifically during breastfeeding, is one of the prominent inter-relational mother-infant factors in early life, the signals for hunger and satiety being amongst the most prominent and rigorous cues provided by a newborn infant. The mechanism of mother-infant signalling can be broadly categorised into psychosocial and physiological factors, and examples are shown in Figure 2.3.

Figure 2.3 Possible mechanisms of mother-infant signalling during the postnatal period



The psychosocial aspects such as physical contact, maternal psychology and infant behaviour may occur in all mother-infant pairs, regardless of their feeding method. However, the 'physiological' mechanism is unique to breastfed infants and their mothers. Milk synthesis and milk composition does not solely reflect maternal physiological and psychological processes, but it also reflects a complex physiological and behavioural negotiation between the mother and the offspring [9]. As mentioned previously, lactation strategy varies different between species and also individuals, and it can also vary between offspring of an individual mother, as it depends on how the mother allocates her energy for her current and future offspring. In this section, I explain the potential mechanism of mother-infant signalling during breastfeeding that reflects maternal strategy in lactation, including signalling that could lead to parent-offspring conflicts during this period, focusing on physiological signalling during breastfeeding (part 1). Prior to that, I briefly describe life history theory as it relates to reproduction, focussing on the possible trade-offs during lactation. Next, in part 2, I discuss the psychological aspects of mother-infant signalling and effects on breast milk outcomes (milk composition and volume).

2.2.1. Life history theory

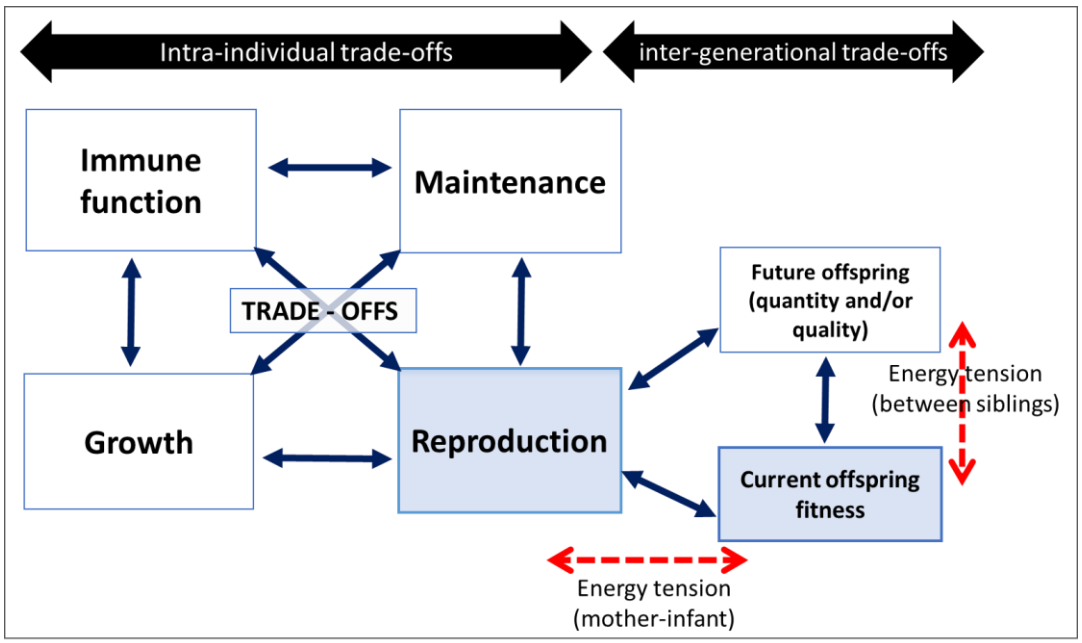
Life history theory seeks to explain how a living organism maximises its fitness and optimises its reproduction over the life course by making 'decisions' in allocating energy (time and effort) between life functions (growth, reproduction, storage and maintenance (including repair)), within diverse environmental conditions [48]. As a basic principle in energy allocation, resources used for one purpose are diverted from another and thus diminish the resources available for another purpose [48]. In this case, within the environmental constraints, an individual makes decisions (mostly unconsciously) to trade-off energy between life functions throughout the lifetime [48]. Thus, in the context of female reproductive effort, in particular pregnancy and lactation, the use or resources or maternal capital are very high, since a mother has to face multiple trade-offs in allocating energy for her life functions (intra-individual) and also subsequently for her offspring fitness (inter-generational) [48, 49]. The two main trade-offs that a mother has to face are: i) between current and future reproduction and ii) the quantity and quality (fitness) of the offspring [48, 49], as illustrated in Figure 2.4. In this case, the maternal investment strategy for her reproductive success is very critical, since the cost of reproduction is energetically demanding [50]. In the following sections, I will only focus on the maternal investment during breastfeeding, and also trade-offs that could occur between the mother and infant during this period.

2.2.2. Lactation cost and strategy

Lactation is very expensive and the energy cost is higher than that of gestation [50], requiring an additional of 2.62Mj/day [51]. This energetic cost is determined by milk energy density, gross composition, and volume and efficiency of synthesis, which comprises 500-680 kcal/day in early lactation (the first three months) [50-53]. The cost is also influenced by infant age, frequency of feeding and number of offspring currently being breastfed (e.g twin babies or tandem nursing) [50]. Thus, during the lactation period, the maternal investment of energy is critical as it reflects her strategy for maximising fitness payoffs [54, 55]. Lactation strategy is also strongly influenced by trade-off decisions that reflect maternal conditions and the effort of an individual mother to maximise her available options to invest for her offspring's fitness [12, 50].

To compensate for the high cost of lactation within the environmental constraints, in addition to using maternal body stores, as part of lactation strategy, lactating mothers have been reported to have a tendency to increase their energy intake, and/or reduce their physical activity to conserve energy and/or decrease the basal metabolic rate to reduce the cost of maintenance [50, 52]. These strategies may also be affected by the biosocial context such as family and social support [56, 57], practicing postpartum confinement (common in many Asian countries including Malaysia) [58, 59], and having a specialised traditional postpartum diet (including increased meal size or frequency) that is perceived to enhance milk production [56]. Thus, lactation strategy varies among mothers within the same population, and also between populations, as long as the energetics of lactation can be achieved [50, 52, 60].

Figure 2.4 Principles of energy allocation and trade-offs



The shaded boxes (reproduction and current offspring) represent the trade-offs that occurred mostly during lactation, especially among the first-time mothers (which the main focus in my thesis).

2.2.3. Parent offspring conflict

From an evolutionary perspective, investment of very high energy to maximise the fitness of the current offspring can probably result in fewer future offspring, which in turn results in less maternal genes inherited and passed to future generations. Thus, mothers may restrict some of their resources invested in the current offspring for investment in future offspring because they will also benefit the maternal genes [61]. Therefore, mothers will invest for their current offspring at a possibly minimal cost, and invest equally in each current and future offspring,

as long as the offspring will be fit to survive and reproduce in future [8, 55]. In contrast, offspring will demand more resources than mothers would provide optimally for their own fitness, if this increases offspring fitness [8, 61], as also described by Trivers' theory [62]. Although the offspring will demand high maternal resources, they will also strive to be fit (stay healthy) so they will not drain their mother's resources to an extent that could harm the mother, which might prevent them from getting future resources. Overall, current offspring actually compete for energy with their parents and also with future siblings, in a 'tug-of-war', as also shown in Figure 2.4. This competition -termed genetic-conflict- starts during gestation, since fetal genes will select and demand a high nutrient transfer, whereas the mother's genes will limit the transfer to her optimal level [63]. The competition continues between mother and infant during lactation to compete for energy [8], termed parent-offspring conflict, and largely occurs subconsciously [55].

Thus, apart from the maternal condition and her decision to breastfeed, lactation performance is also influenced by the offspring, either physiologically or behaviourally, such as through vocalisation or begging behaviours [8]. This is contrary to the general assumption that breastfeeding is a completely harmonious relationship, providing health benefits to both mother and infant [8, 63]. In this context, lactation essentially represents a conflict or 'tug-of-war' between the supplier and consumer, where the mother and infant are competing with each other over how much maternal resource will be invested in the breast milk [8]. In fetal nutrition for example, the fetal genes send hormonal signals without considering the mother's signals, so that even if she is diabetic, the fetus signals just as much as to a normal mother, and ends up gaining excess weight [63]. In another example, studies have reported that around the time of weaning, the baby plays a role in increasing milk volume by intense suckling, even if the mother is undernourished [64, 65], which has been associated with delayed re-conception [66, 67]. Nevertheless, some studies have reported that this lactational amenorrhoea is regulated by maternal energy balance, not by infant suckling [68, 69], suggesting that the mother is more in control in managing her energy capital [70]. Overall, the question of whether the mother or the offspring is more 'in control' in determining how much energy will be allocated to breast milk still remains.

2.2.4. Possible mechanisms of mother-infant signalling during breastfeeding

During the lactation period, the infant's demand can be shown by his/her behaviour, appetite, vocalisation and/or through non-nutritive suckling; and the mother's response through breastfeeding behaviour, the amount of milk produced and/or the composition of milk both in terms of nutrient and non-nutrient components [8]. As shown in Figure 2.2, there are 3 main pathways of physiological signalling between the mother and the infant through the mediation of breast milk, and these are inter-related with the other mother-infant factors.

Firstly, it might be assumed that the mother would respond to infant signals by increasing milk supply, but it is also possible that she could restrict her supply, by restricting nipple access, hence down-regulating milk synthesis and affecting milk volume. Secondly, the macronutrient composition of milk changes during a feed, which may affect infant responses such as satiety. This can be illustrated by the varying fat content within a single feed as fat concentration is usually the lowest at the beginning (foremilk) and highest (two- to three-fold) at the end of a feed (hind milk) [28, 71, 72]. Thus, infants may respond by stopping suckling when they achieve satiation or feel full, something which may not occur in the same way in an infant fed formula with a fixed composition. Satiety in breast-fed infants may also be affected by certain non-nutrient components in breast milk, although this is less well understood. This is a third possible way in which the mother could respond to infant signals during breastfeeding: via certain bioactive factors or endogenous opiates in the milk that may manipulate infant behaviour and/or feeding pattern. All of these possible mechanisms of mother-infant signalling could underlie the tug-of-war during breastfeeding, and these may take place at a more intense level during early life, when energy demands for growth are higher. To date, we still do not know who is in command in the tug-of-war; how the mother allocates her energy economically to maximise her offspring's fitness as well as for her own reproductive future; and, for example, if maternal energy is abundant, will the tension in the tug-of-war reduce?

This mother-infant signalling during breastfeeding is complex and largely unexplored, thus, it is very intriguing to understand how the process happens and how to make it mutually beneficial. For example, in a well-nourished mother with a good milk supply, the infant may not find it necessary to vocalise and waste energy demanding milk, and the mother will then be less distressed and perhaps may produce more milk for the infant; this would be an example where both parties 'understand' each other well, but it is easy to see how the process could be less well balanced. Therefore, my research investigated this mother-infant

relationship during the first 3 months of lactation, focusing on the non-nutritive factors in breast milk and the influence of maternal psychological state on milk intake and composition, in order to better understand the process of establishing breastfeeding.

2.2.5. Mother-infant signalling through non-nutritive components in breast milk

As well as providing nutrition for the infant, breast milk is also rich in non-nutritive components, such as protective substances and hormones that are important for immunity and metabolic regulation, providing health benefits to infants. However, to study the breastfeeding outcomes of mother-infant behaviours, I am focusing on hormonal constituents in breast milk that may act as messengers in breastfed infants.

Breast milk hormones are transferred from maternal serum/plasma into breast milk, and/or synthesized by the mammary gland. During breastfeeding, these hormones are ingested and hence may be transferred into the infant circulation or act locally in the gut, potentially affecting infant feeding behaviour or pattern. It is important to study these non-nutritive factors in milk and their effect on infant behaviour and feeding pattern in early life, as it may influence early infant growth. For example, begging behaviour occurring for a long period such as greater vocalisation or non-nutritive suckling is considered as a cost since energy is used up to demand more milk, which may then influence infant growth [8]. However, it is also possible that crying could be a price worth paying, even if this involves a non-honest signal (to manipulate the mother) through vocalisation, if the return is greater than the cost of crying.

A longitudinal infant study (n=316) [73] found that breast-fed and mixed-fed infants were reported by their mothers to have more challenging infant temperament (greater distress, less smiling, laughing and vocalisation) compared to formula-fed infants. It was suggested that infant temperament and behaviour in early infancy is one of the factors that influences the duration of exclusive breastfeeding. Challenging temperament and behaviour such as sadness and fussiness among breast-fed infants has also been associated with elevated levels of cortisol in breast milk. However, it is possible that breast-fed infants are fed less amount of milk than formula-fed infants, and breast milk is also digested faster than formula milk, and therefore they demand more milk or more frequent feeding through vocalisation. Also, the study compared breast-fed and formula-fed infants, where confounding could exist since the breast-fed mothers might be more sensitive to their infant cues.

Hormones in breast milk have been hypothesized to be involved in feeding regulation in early infancy which could lead to the programming of energy balance later in life, and influence infant growth [74, 75]. In addition, naturally occurring opiates or other bioactive substances in breast milk such as ß-casomorphins may also influence feeding behaviour by acting as sedatives, and hence blunting or lowering infant appetite [76]. Another example is melatonin in breast milk [77, 78] which has been associated with improved infant sleep regulation and reduced colic [77], both of which could help to conserve infant energy or use it more efficiently. These behaviours could be economically energy favourable for mothers too, especially in malnourished or young mothers who need extra energy to survive or grow themselves. In addition, these biological signalling mechanisms could influence long term energy investment for maternal future reproduction as infant sleeping and feeding pattern have been associated with lactational amenorrhea [66, 79]. However, regardless of maternal condition, it is still very important for the offspring to receive optimal nutrition for their growth and development during the critical period in early life. Thus, transferring hormones to regulate infant appetite could be beneficial for both parties, so that the infant might only give honest signals when hungry rather than manipulative signals to demand extra food.

An increasing number of studies have reported on bioactive factors in breast milk, including hormonal constituents, but the associations between these substances and infant outcomes such as behaviour and metabolic regulation are still poorly understood [80]. Although many different hormones and/or bioactive factors in breast milk have been proposed to influence infant outcomes, in the following section I am focusing on specific hormones - cortisol, ghrelin and leptin - for which there is more evidence compared to other bioactive factors.

2.2.5.1. Cortisol and its function

Glucocorticoids (GC), the human endogenous form of cortisol, are steroid hormones that regulate several metabolic functions including the metabolism of macronutrients. For example, to maintain blood glucose in the fasting state, cortisol is required for gluconeogenesis to synthesize glucose from non-carbohydrate sources (amino and fatty acids), as well as increase glycogen breakdown in the liver [81]. In addition to its metabolic action, cortisol is also a stress responsive steroid hormone that is involved in stimulating and suppressing other stress-related hormones [82]. Certain stressful events activate the central nervous system and stimulate the hypothalamus to release corticotropin-releasing-hormone,

which then causes the release of adrenal-corticotropin-hormone (ACTH) from the anterior pituitary that eventually stimulates the adrenal glands to release cortisol [83].

In lactation, cortisol also plays an important role in triggering lactogenesis II during parturition and early lactation [22]. Its effects during lactation include regulating tight junction permeability [84], as well as preventing apoptosis and the involution of breast tissue [68, 85]. A study measuring cortisol in rhesus macaque milk (n=44) found a significant positive correlation of cortisol with protein (r=0.441, p=0.03) and fat concentrations (r=0.398, p=0.07) in milk [86]. The authors suggested that cortisol may also be involved in regulating or assimilating the amount of fat and protein in milk, acting as a 'gate-keeper' to regulate the concentration of macronutrients in milk. However, their findings are only based on a single milk collection, and the rhesus monkeys were given a sedative prior to milk sampling, which could significantly affect the concentrations of certain components in breast milk, especially cortisol.

Cortisol is present in human milk in an average range of 0.02 to 3.2 mcg/dl [87-89] and evidence suggests that it is not synthesized by the mammary tissue, but transferred from maternal plasma [90, 91]. In maternal plasma, cortisol is bound to albumin and corticosteroid-binding-globulin in order to be transported to mammary secretions [90], and a study also found significant correlation between cortisol concentrations in maternal plasma and in the milk [92]. There is also the possibility that ingested cortisol from mother's milk may be involved in neonatal physiology since cortisol receptors are found on the mucosal cells of the GI tract in rats [93, 94] and humans [95]. Nevertheless, there is no clear evidence describing the function of human milk cortisol in relation to infant development and behaviour.

2.2.5.2. The influence of breast milk cortisol on infant behaviour and temperament

Studies have hypothesized that mothers have the potential to shape infant behaviour in early life by the transmission of biologically active compounds including cortisol in milk during breastfeeding [86, 87, 96], termed lactocrine programming [97]. Using gene expression profiles derived from sloughed epithelial cells in human infant stools, cortisol receptors are found to be higher in breast-fed than formula-fed infants [95], suggesting that breast-fed infants may have an enhanced ability to process such signals. This is supported by animal studies that found cortisol receptors in the gut to be highest during infancy and decline towards adult values post-weaning [94, 98]. A primate study revealed that cortisol

concentrations were higher in breast milk of mothers of male infants than mothers of female infants, although no significant difference was found in maternal plasma cortisol. They also found that breast milk produced for sons had higher fat and lower glucose levels than milk produced for daughters. A significant positive correlation was also found between cortisol concentrations in breast milk and 'confident' temperament among male offspring rhesus, but not daughters; based on temperament factors including 'bold', 'active', 'curious' and 'playful' [86]. The study suggested that mothers may invest more in sons due to their greater potential reproductive fitness in later life [86].

In contrast, in human studies, elevated levels of cortisol in breast milk have been associated with challenging infant temperament [9, 87, 99]. For example, breast milk cortisol levels are positively correlated with negative affectivity of infant temperament, especially fear and sadness [87]. This is consistent with previous findings [99] from this group reporting that higher maternal plasma cortisol was associated with increased fearful temperament in breastfed infants, with no apparent association among formula-fed infants. Nevertheless, the breast milk samples used in these observational studies were taken at random times from a single feed and cortisol concentrations may vary within a feed or diurnally [100]. Hence, the question of whether there is indeed any association between cortisol levels in breast milk and infant behaviour requires further investigation with a more robust methodological design.

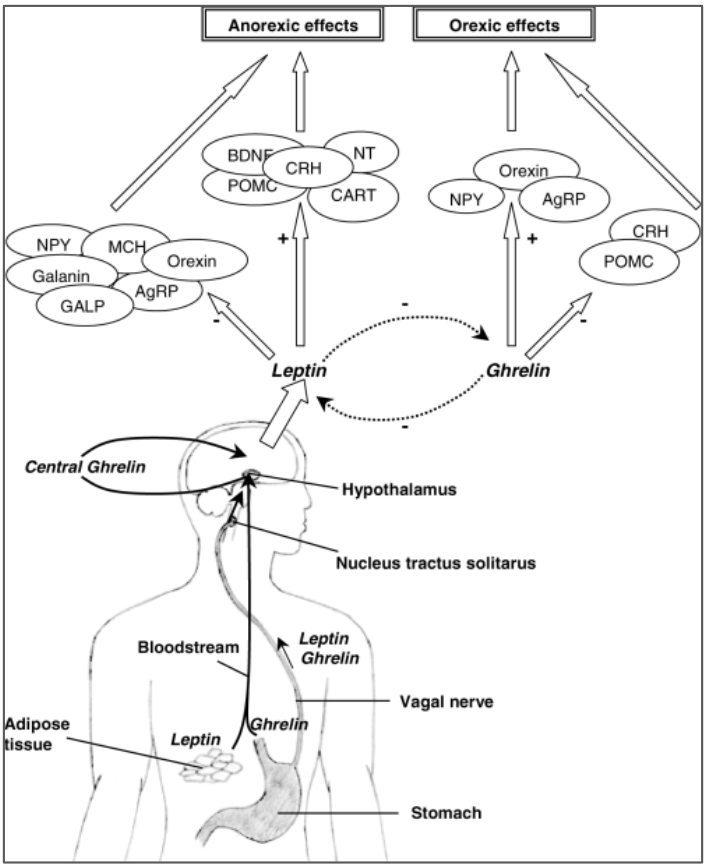
In the context of the tug-of-war, there is a possibility that mothers who are relaxed may produce different concentrations of cortisol or other hormones to influence infant behaviour, appetite and sleeping pattern that may favour the maternal energy budget. For example, mothers who are relaxed may produce a low concentration of cortisol, to signal to the baby that he does not need to signal so strongly by vocalising or crying for his milk supply. This would reduce his own energy expenditure on signalling and allow him to reserve more energy for growth. However, the next question is, how responsive is the baby to maternal signals? Therefore, to investigate infant responses to maternal signals, it is also interesting to investigate infant temperament and behaviour (e.g sleeping and crying behaviour) and also infant feeding behaviour (appetite and feeding duration/frequency). In addition to milk cortisol, there are many other hormones and bioactive factors that may influence infant behaviour, particularly appetite and feeding behaviour. To address this, in the next section, I am focusing on additional specific hormones (ghrelin and leptin) and their relation to infant feeding behaviour and growth.

2.2.6. Influence of ghrelin and leptin on infant feeding

Studies have suggested that breast-fed infants may have better appetite regulation and self-control of feeding due to the presence of bioactive factors in breast milk, particularly hormones such as ghrelin and leptin [101-103]. Ghrelin and leptin have been reported to have opposite functions in regulating energy balance in humans through the stimulation of hypothalamic neurons [103] as illustrated in Figure 2.5. In general, studies have reported that ghrelin stimulates appetite and increases body weight (orexic effects on energy balance), whilst leptin stimulates satiety and hence decreases food intake (anorexic effects) and controls body weight [103-105]. Ghrelin and leptin are found in biologically active forms in breast milk, with a wide range of concentration reported at different time points during lactation in different studies, summarised in the next subsections. Since breast milk composition is not static within a feed or throughout lactation, the same variability might be seen in the concentrations of these hormones, thus it is plausible that they could be involved in regulating infant feeding and influencing growth in early and later life. In contrast, the constant content and lack of biologically active non-nutritive components (especially hormones) in infant formula, exacerbated by the high calorie content, may be responsible for the tendency for overfeeding and more rapid weight gain in formula-fed infants, with an increased subsequent risk of overweight [35].

From an anthropological perspective, transferring hormones to regulate infant feeding or appetite could be beneficial for the mother if the infant responds correspondingly. In this case, for example, the mother signals to the baby by transferring hormones to regulate infant appetite, and the infant may respond by demanding food only when hungry (that is, only giving an honest signal) and unlatching from the breast when feeling full (short-term effect on satiety) or by showing less demanding behaviour when his requirements are achieved (long-term effect on feeding behaviour or appetite regulation). Thus, this may develop an efficient breastfeeding process between the mother and infant. The following sections discuss the published data on ghrelin and leptin that are only related to infant feeding in early life.

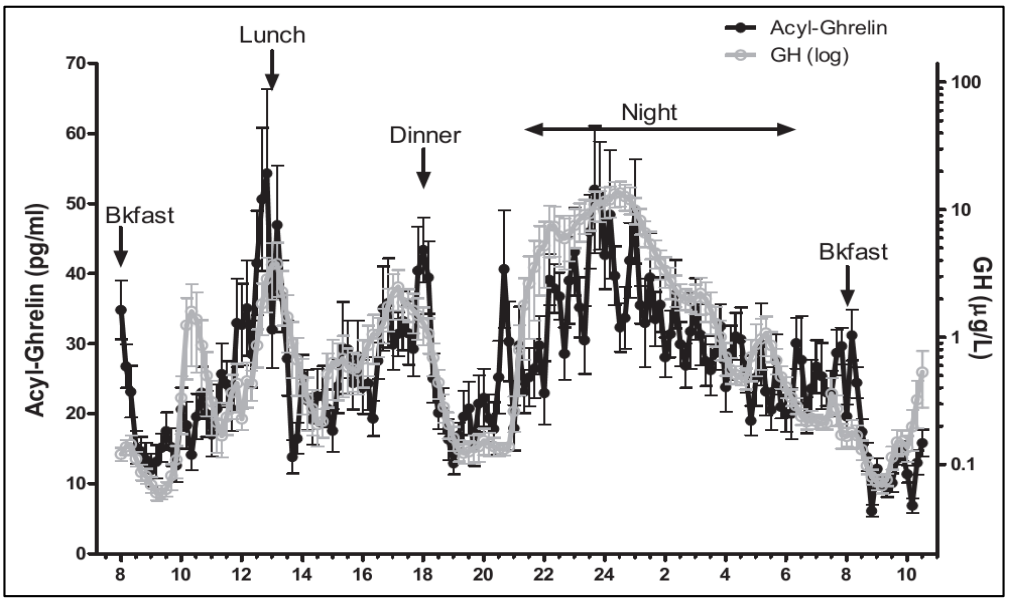
Figure 2.5 The pathways of leptin and ghrelin to the hypothalamus, which have been suggested to give opposite effects on energy balance. Figure taken from Klok et al. [103].



2.2.6.1. Studies on ghrelin

Ghrelin is a peptide hormone -consisting of 28 amino acids- which is produced predominantly in the stomach. There are two major forms of ghrelin in human blood: acyl-ghrelin (active form) and des-acyl ghrelin (inactive form) [106]. Ghrelin must be in its active form (acylated ghrelin) in order to act on its receptor, the growth hormone secretagogue receptor (GHSR). Once it binds to its receptor, this stimulates the release of growth hormone. The ghrelin receptors were found to be present in many parts of the human body including the brain, gastrointestinal tract, lung, ovaries and heart [106, 107]. In addition to stimulating the growth hormone secretion, studies suggested that ghrelin derived from different parts of human body may effects differently, or plays different roles in human body such as energy homeostasis, gastric acid secretion, glucose metabolism and sleep modulation [106, 107]. Ghrelin derived from the stomach was predominantly being reported to stimulate appetite (as a short term effect) and influence food intake regulation [104-106]. This has been demonstrated by the sharp rise and drop in plasma ghrelin levels before and after every meal (Figure 2.6), suggesting that ghrelin delivers a hunger signal to the brain that influences appetite [108].

Figure 2.6 Mean plasma acyl-ghrelin (pg/ml) and serum GH levels (mg/l) during a feed (n=8).



Source from Nass et al. (2008) [108].

Studies have reported inconsistent results on ghrelin levels in mothers and infants, including in breast milk (Table 2.1). Some studies found that the total ghrelin levels in breast milk and maternal plasma increased across lactation [109, 110] whereas some found contrary results: ghrelin levels decreased across lactation [111, 112]. However, many studies [109, 113-116] measured the total ghrelin level solely, without measuring acyl-ghrelin separately, despite the fact that only active ghrelin can bind to the receptors in the brain in order to release growth hormone [117].

Ghrelin levels in maternal plasma have been reported to be correlated with the ghrelin levels in breast milk and/or breast-fed infants [109], suggesting that acyl-ghrelin in breast milk may largely come from maternal plasma. However, a few studies found ghrelin mRNA in the human mammary gland [116, 118], suggesting it is also produced in the breast tissue [116], and then transferred into breast milk. In addition, some studies have reported an inverse association between maternal plasma acyl-ghrelin and breast milk ghrelin across lactation [109], and found that active ghrelin levels were significantly higher in breast milk than in maternal and infant plasma [112]. Overall, these inconsistent findings on ghrelin levels in breast milk and maternal or infant plasma could be due to the sample collection method that was unstandardized (or not well-described) in many studies. The use of non-specific assay kits for ghrelin in breast milk may also contribute to inaccuracy or inconsistent data in many studies.

In addition to acting as a hunger signal, ghrelin also has been suggested to have a long-term function in maintaining blood glucose levels by stimulating the release of growth hormone [106], especially during starvation [119]. Related to this, studies have reported negative correlations between ghrelin levels in breast milk and infant growth or weight gain [114, 115], suggesting that ghrelin may be involved in the regulation of infant body weight in early life by stimulating more growth hormone production in infants with lower weight gain. On the other hand, there is also a possibility that higher ghrelin was required in those lower weight gain infants to increase appetite (by sending more hunger signals) in an attempt to compensate for poor weight gain. However, none of these studies investigated the breast milk intake or feeding and appetite behaviour in infants, in relation to ghrelin levels in breast milk or infant plasma.

Studies [113, 115] have also compared plasma ghrelin levels between breast-fed and formula-fed infants in early life (age 4 months and below), and found a higher mean ghrelin level in formula-fed infants. Savino et al. (2011) indicated that the ghrelin levels in infant formula (2007±1725 pg/ml) and cow's milk (2816±219 pg/ml) were significantly higher ($p=0.05$) than in breast milk samples (828±323 pg/ml). However, there are statistical limitations on their findings since the sample size was small ($n=20$), the standard deviation was very large and the p-value was equal to but not lower than 0.05. These studies [113, 115] also measured the total ghrelin levels, without separating the active ghrelin in milk. Thus, it is questionable whether ghrelin in infant formula or cow's milk is still in active form, and if it is, whether it remains active and functional once it is ingested. In addition, the function of ghrelin in human milk might be different to that in infant formula or cow's milk, as cow's ghrelin consists of 26 amino acids (human = 28 amino acid). Various studies have reported different numbers of amino acids in ghrelin from many other animals but the effect if any on function is yet to be investigated [117].

Overall, as ghrelin is assayed in human milk, and its receptors are expressed in the gastrointestinal tract [106], there is potential for bioavailable ghrelin to reach the infant's blood. However, the short-term effect of ghrelin on infant appetite (in stimulating hunger) is still unclear due to inconclusive findings in the literature, and few studies have investigated infant feeding behaviour or milk intake in relation to ghrelin levels in breast milk or infant plasma or the consequent effect on infant growth. To date, only one study ($n=62$) measured active and total ghrelin together with leptin and fat (triglycerides and cholesterol) levels in

breast milk within a single feed, in two visits (at infant age of two and five months) [101]. The study found that active and total ghrelin, and total cholesterol levels in foremilk were significantly higher than in hind milk. In contrast, triglycerides and leptin were higher in hind milk than in foremilk. These results suggested a possible role of ghrelin and leptin in regulating infant hunger (through higher ghrelin at the beginning of a feed) and satiety (through higher leptin at the end of a feed) during breastfeeding. At a later visit, the study found that the formula-fed infants had the fastest growth rates with the highest increase in BMI (14.6%). In contrast, the lowest increment of BMI (3.5%) was reported in breast-fed infants, whereas the mix-fed infants (breast milk+ infant formula) had a moderate increase in BMI; 11.8% [101]. Thus, it is possible that the changes in ghrelin, leptin and lipid levels in breast milk during a single feed may help breast-fed infants to self-control their milk intake during feeding. In contrast, formula-fed infants may have a lack of self-control of appetite and satiety regulation, which may lead to a tendency to overfeed. This is exacerbated by the fact that the content of infant formula is mostly higher in calories. However, infant appetite or feeding behaviour was not assessed in the study described above and thus the effect of these hormones on infant feeding regulation is still inconclusive.

Table 2.1 Reported ghrelin levels in mothers and infants, and in breast milk.

Type of ghrelin & analysis method	**Maternal plasma (pg/ml)**	**Breast milk (pg/ml)**			**Infant: plasma (pg/ml) /age/sample size**		**Ref.**
	Lactating	Colostrum	Transitional milk	Mature	Breast-fed	Formula-fed	
Total Ghrelin ; (RIA)	Day 1: 95 ± 16 Day 10: 111 ± 13 Day 15: 135 ± 16 (n=17)	70.3 ± 18 (n=17)	83.8 ± 18 (n=17)	97.3 ± 13 (n=17)	-	-	[109]
Active Ghrelin ; (RIA: acidification)	Day 1-3: 124 ±17 (n=16) Day 4-10: 95 ±13 (n=16) Day 22-28: 71 ±7 (n=16)	Cross-over sample: 450 ±25 (n=49) Longitudinal sample: 505 ±51 (n=16)	Cross-over sample: - Longitudinal sample : 707 ±65 (n=16)	Cross-over sample: 801 ±43 (n=49) Longitudinal sample: 804 ±62 (n=16)	52 ± 7 Age: 4-30 days (n=49)	-	[110]
Total Ghrelin (RIA)	Day 1-3: 483 ±33 (n=16) Day 4-10: 908 ±72 (n=16) Day 22-28: 711 ± 711 (n=16)	Cross-over sample: 880 ±80 (n=49) Longitudinal sample: 867 ±51 (n=16)	Cross-over sample: - Longitudinal sample: 1750 ±196 (n=16)	Cross-over sample: 3250 ±378 (n=49) Longitudinal sample: 1982 ±293 (n=16)	866 ± 42 Age: 4-30 days (n=49)	-	
Active Ghrelin (RIA: acidification)	Mo 1: 23.6 ±12 (n=25) Mo 4: 347.3 ±207 (n=19)	-	-	Mo 1: 1042 ±148 (n=25) Mo 4: 1659 ±156 (n=19)	Mo 1: 65 ±44 (n=25) Mo 4: 130.6 ±137 (n=19)	-	[112]
Total Ghrelin (RIA)	Mo 1: 3579 ±1060 (n=25) Mo 4 : 2315 ±1393 (n=19)	-	-	Mo 1: 3095 ±1507 (n=25) Mo 4: 2876 ±1626 (n=19)	Mo 1: 4847 ±687 (n=25) Mo 4: 5188 ±951 (n=19)	-	

*Mo = month, FM = foremilk, HM = hind milk, GDM = gestational diabetes mother

Continue (Table 2.1)

Type of ghrelin & analysis method	Maternal plasma (pg/ml)	Breast milk (pg/ml)			Infant: plasma (pg/ml) /age/sample size		Ref.
	Lactating	Colostrum	Transitional milk	Mature	Breast-fed	Formula- fed	
Active Ghrelin (ELISA: acidification)	Early lactation: Healthy: 41 ±3 (n=10) GDM: 32.4 ±3 (n=10)	Later lactation: Healthy: 50 ± (n=10) GDM: 42.4 ± (n=10)	Control: 39 ±2 (n=10) GDM: 28 ±2 (n=10)	-	Control: 48 ±5 (n=10) GDM: 37.7 ±2 (n=10)	-	[111]
Total Ghrelin (ELISA)	Early lactation: Control: 542 ±60 (n=10) GDM: 384 ±44 (n=10)	Later lactation: Control: 584 ± (n=10) GDM: 426 ± (n=10)	Control: 466 ±52 (n=10) GDM: 338 ±49 (n=10)	-	Control: 505 ±52 (n=10) GDM: 359 ±51 (n=10)	-	
Total Ghrelin (RIA)	1319 ± 140 (n=20)	-	-	828.17 ± 323 (n=20)	1045 ± 263 (n=37)	1247 ± 328 (n=19)	[113]
Total Ghrelin (RIA)	-	-	-	-	Mo<4: 1974 ±620 (n=20) Mo 4-8:3030 ±1301 (n=17)	Mo<4: 2609 ±739 (n=15) Mo 4-8:2965 ±1101(n=10)	[115]
Total Ghrelin (RIA)	-	-	-	Median: Preterm infant's milk: 2500 (n=10) Term infant's milk: 1575 (n=10)	-	-	[116]
Total Ghrelin (ELISA)	-	-	-	-	Median: 205 (n=40)	-	[114]
Active Ghrelin (RIA: acidification)	-	-	-	FM: 11.9 ±2.5 (1mo) & 15.3 ±3.9 (3 mo) (n=26) HM: 8.5 ±1.6 (1mo) & 11.6 ±2.7 (3 mo) (n=26)	-	-	[101]
Total Ghrelin (RIA)	-	-	-	FM: 289 ±63 & 235 ±84 HM: 199(Median) & 158 ±20.1 (n=26)	-	-	

*Mo = month, FM = foremilk, HM = hind milk, GDM = gestational diabetes mother

2.2.6.2. Studies on leptin

Leptin is produced in proportion to energy storage in the body, primarily adipose tissue. Once leptin has been released into the circulation, it crosses the blood-brain barrier and binds to receptors in the hypothalamus providing information about the body's energy storage status [103]. This subsequently stimulates the production of anorexic hormone peptides such as cholecystokinin and obestatin in hypothalamus that affect metabolic regulation and energy balance [103]. Leptin can also act on leptin receptors (LEPR or OBR) expressed in several parts of the human body including the gastric epithelial cells and intestinal mucosa cells [120, 121]. Studies have suggested that leptin from breast milk can be absorbed into the infant circulation via these receptors. In addition, it has been reported that the ingested breast milk leptin is still biologically active after absorption into the blood [122, 123]. Hence, leptin in breast milk is suggested to play a role as a satiety signal to infants during breastfeeding, which has a short-term effect on self-regulation of milk intake during feeding [104, 124]. Studies also suggest that leptin acting with other anorexic peptides has short-term effects on food intake by controlling the meal size and/or the frequency of food intake in adults [125, 126].

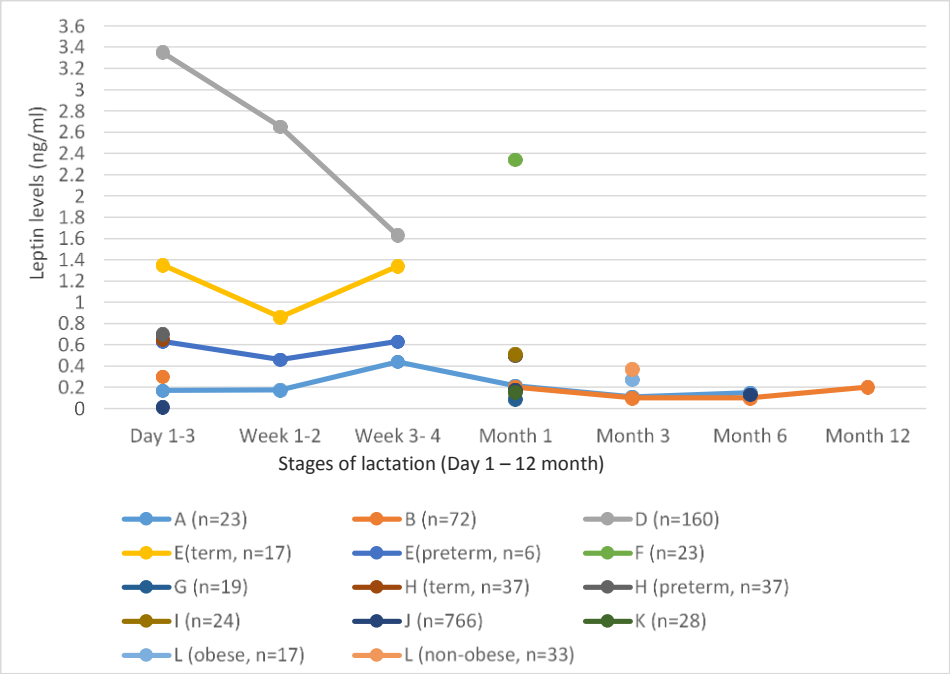
Long-term effects are shown by the influence of leptin on feeding behaviour and body weight homeostasis, by the suppression or control of food intake and by an increase in energy expenditure and metabolic rate [103, 125]. Studies have reported inverse associations between breast milk leptin and infant growth (weight gain, BMI and body composition) in early infancy [127-131]. Thus, it has been suggested that leptin may contribute to the protective effect of breastfeeding against obesity in later life, via its influence on metabolic and appetite regulation and energy balance in early life [132]. Thus some studies have suggested that leptin may be involved in early life nutrition programming of obesity in later risk [132, 133].

Leptin is present in human milk, but not in infant formula, and is suggested to be synthesized (in small amounts) in mammary epithelial cells [122, 134]. Several studies have reported significant associations between leptin levels in breast milk and maternal plasma and/or maternal adiposity [128, 131, 135-139], with higher concentrations in the maternal plasma than in breast milk, suggesting that the leptin in breast milk may also come from maternal plasma. Therefore, infants breast-fed by mothers with higher adiposity may be exposed to higher leptin levels compared to formula-fed infants, as leptin is lacking in infant formula.

However, the reported leptin levels in plasma and breast milk vary between individuals, and are very inconsistent, as shown in Table 2.2.

In terms of leptin in breast milk, many studies have reported lower levels of leptin in breast milk than maternal plasma, and its presence in breast milk is generally at a low range, with a slight decrease or stabilising trend across lactation (0.01-3.35 ng/mL in colostrum and 0.01-17.8 ng/mL in mature milk) [127, 131] as illustrated in Figure 2.7 with detailed data showed in Table 2.2. Contrary to most other findings, Doneray et al. [129] found that the leptin level in breast milk was significantly higher than in maternal plasma, and their reported leptin levels in human milk were also much higher than in most other studies [129]. They also reported a significant increase in leptin levels in breast milk from early lactation (5.69 ± 4.58 ng/mL in colostrum) to later stages (23.84 ± 17.79 ng/mL in mature milk), which differs from other studies. However, their sample size was small (n=15), which may have led to a wider variability in their results, as their standard deviation values were large for both leptin in colostrum and mature milk [129]. A few studies have investigated the changing concentrations of leptin in breast milk within a feed, and found no significant difference between fore and hind milk [123, 139, 140], however, the measurements were only done at one point during the lactation period, and their sample size was small (n=13-19).

Figure 2.7 Reported leptin levels (ng/ml) in human milk across lactation (Study A-L).



Overall, the leptin levels in maternal plasma and breast milk in reported studies are highly variable. The inconsistent findings may have several causes. Firstly, some studies collected the blood or breast milk samples while mothers were fasting for at least three hours, whereas other studies collected samples after mothers had eaten. This may influence the leptin levels in plasma as well as in breast milk since leptin may have a short-term effect on food intake and metabolic regulation. Secondly, some studies only analysed the leptin concentration in whole milk whilst evidence shows that the concentration of leptin in whole milk is higher than in skim milk [122]. Studies suggest that the higher leptin level in whole milk is influenced by milk fat globules which contribute to elevated leptin levels during some assay analysis [122, 141]. The usage of different assay kits may also influence the result, as some kits are specifically optimised for only measuring leptin levels in blood, but some studies may have used these same assay kits for measuring leptin in milk as well.

2.2.6.3. Summary of studies on ghrelin and leptin

There has been much speculation about the importance of leptin and ghrelin for infant feeding regulation and appetite, as well as infant growth, but their function in infants has not been extensively investigated and is still poorly understood in humans. Although the studies on the levels of these hormone in breast milk are increasing, the effects of the hormones on milk intake or feeding behaviour (such as appetite, duration or frequency of feeding) are largely unexplored. The changing concentrations of these hormones in breast milk within a feed and across lactation have also not been extensively investigated. To date, two studies [101, 142] investigated the change in both leptin and ghrelin levels in breast milk within a single feed at few points of lactation and the influence on infant weight gain. However, milk intake was not measured, and the influence on infant appetite and feeding behaviour was not investigated.

Overall, future studies are needed to investigate the changes in these hormones within a feed in relation to infant feeding behaviour (duration, frequency or milk volume) in order to understand the short-term effects of these hormones on infant appetite regulation. For the long-term effect of these hormones on infant body weight homeostasis, more research is needed to investigate the pattern of these hormone levels throughout the lactation period in relation to milk intake and infant growth. Therefore, as part of my study, I aimed to explore the concentrations of leptin and ghrelin in relation to both feeding behaviour and infant growth by measuring changes in the concentrations of these hormones within a feed and at different time points across lactation.

Table 2.2 Reported values of leptin levels in mothers and infants, and in breast milk.

Sample size	Maternal plasma (ng/ml)	Breast milk (ng/ml)			Infant plasma (ng/ml)	Study ID and reference
		Colostrum	Trans milk	Mature		
Postnatal age: 10 ± 5 week	-	-	-	FM: 0.43 ± 0.10 HM: 0.42 ± 0.11 (n=479)	-	Study A: [140]
Postnatal age: 6 week (n=152) 4 mo (n=120)	Median (IQR) 6 week: 9.52 (8.56) 4 mo: 8.3 (10.64)	-	-	Median (IQR) 6 week: 0.11 (0.19) 4 mo: 0.09 (0.18)	-	Study B: [127]
Postnatal age: 29-38 days (n=13)	-	-	-	FM: 0.9 ± 0.7 HM: 1.0 ± 0.8	-	Study C: [139]
Mother (n=23) Infant (n=23)	-	-	-	Median (IQR) 2.34 (5.7)	Median (IQR) 3.04 (3.68)	Study D: [143]
Mother (n=19)	-	-	-	0.092 ± 0.047	-	Study E: [130]
Mother (n=23)	Week 1:3.83 Mo 3: 5.06 Mo 6:4.67	Week 1: Median: 0.17 Range: 0.01–0.65	Week 2: 0.17–0.18 Range: 0.01–0.56	Week 3: 0.01–0.87 Week 4: 0.01–0.42 Week 8: 0.11 Mo 6: 0.15	-	Study F: [131]
Mother (n=72)	-	0.3 ± 0.04	-	Mo 1:0.2 ± 0.03 Mo 3:0.1 ± 0.01 Mo 6:0.1 ± 0.02 Mo 12:0.2 ± 0.04	-	Study G: [144]
Mothers of Term infants (n=37) Preterm infants (n=37)	-	Term: 0.70 ±0 .79 Preterm: 0.65 ± 0.67	-	Term: 0.50 ± 0.50 Preterm: 0.50 ± 0.40	-	Study H: [141]
Mother (n=36) Infant (n=36)	Median (IQR) 3.02 (2.85)	-	-	Median (IQR) 0.51 (0.34)	Median (IQR) 3.42 (2.65)	Study I: [145]

*Mo = month, FM = foremilk, HM = hind milk, IQR = Inter quartile range

Sample size	Maternal plasma (ng/ml)	Breast milk (ng/ml)			Infant plasma (ng/ml)	Study ID and reference
		Colostrum	Trans milk	Mature		
Mother (n=15) Infant (n=15)	Day 1: 1.45–7.75 Week 3-4: 1.5–29	5.69 ± 4.58 Range: 1.5–15.4	-	23.84 ± 17.79 Range 2.6–46.3	Day 1: 3.69 ± 2.06 Week 3-4: 5.35 ± 4.48	Study J: [129]
Mother (n=766) Infant (n=766)	Median: 0.013 Range: 0.006-0.025	-	-	6 week: 0.175 (range 0.08 – 0.34) 6 Mo: 0.130 (range 0.06-0.723)	-	Study K: [146]
Mother (n=28)	12.8 ± 1.7 Range: 6.7 - 37.5	-	-	0.156 ±.039 Range : <0.0001 to 0.853	-	Study L: [128]
Mother (n=160)	Day 1-3: 16.6 ± 1.7 Day 4-14: 14.2 ± 1.9 Day 15-30: 13.8 ± 1.6 Day 91: 10.2 ± 1.4	Longitudinal sample: 3.35 ± 0.25 Cross-over sample: 3.28 ± 0.21	Longitudinal sample: 2.65 ± 0.21	Longitudinal sample: 1.63 ± 0.18	-	Study M: [137]
Mother (n=33) Infant: Term (n=17) Preterm (n=6)	Mothers of term infant: 13.24 ±2.5 Mothers of pre-term infant: 4.46 ±1.05	All: 1.15±0.12 (1.04) Term: 1.34±0.14 (1.35) Pre-term: 0.63±0.18 (0.31)	All: 0.79±0.10 (0.56) Term: 0.92±0.12 (0.86) Pre-term: 0.46±0.10 (0.32)	Term: 1.34 ± 0.14 Pre-term: 0.63 ± 0.18	-	Study N: [147]
Infant: Obese (n=17) Non-obese (n=33)	-	-	-	Obese: 0.27 ± 70.2 Non-obese: 0.37 ± 70.4	-	Study O: [135]
Mother (n=18) Infant (n=18)	7.48 ± 1.3 Range: 2.0-25	-	-	FM: 3.63±1.2 HM: 3.10±0.8 Mean: 3.36±1.0 Range: 0.8 – 15	Mean: 4.64±0.8 Range: 1.0-12 Age: 40.4±7 days	Study P: [123]

*Mo = month, FM = foremilk, HM = hind milk

2.3. SECTION II: PSYCHOLOGICAL ASPECTS OF BREASTFEEDING (Part 2)

Having their first baby is a pleasurable and joyful moment for most new mothers, but some mothers may find difficulty in coping and adapting to this new phase of life, especially those with lack of support during this time [148]. Thus, numerous situations or events that they have undergone through pregnancy and the postpartum period which are perceived as stressful may lead to chronic or severe maternal psychological distress, with the prevalence of postpartum depression reported to be around 13% [149, 150]. Studies have indicated several main risk factors for depression among new mothers: maternal stress, anxiety, sleep disturbance and pain, including any breastfeeding problems that cause pain [151], and history of psychological disturbance; all of which are inter-related [149, 152]. In addition, a systematic review also indicated that infant distress in the first three months, such as colic and crying, could increase maternal stress and anxiety, which could in turn lead to postpartum depression [153]. However, since most studies are observational, they also suggested that the pathway could be reversible. For example, if a mother is stressed, this could lead to disengagement with her infant, which could result in increase in infant crying or demands for care or feeds, which consequently, could raise the maternal stress level further.

If this mother-infant relationship is prolonged, it could lead to depression in the mother, which might subsequently have detrimental effects on the mother's health and later infant development [154]. This is supported by studies reporting that mothers with psychological distress have difficulties in interacting with or responding to their infants including less contact or touching, being less sensitive to infant cues, and tending to have a negative perception towards infant signals [155, 156]. In evolutionary approaches of parental investment, it is predicted that the mother who is having postnatal depression may reduce, or to a certain degree, stop investing for the infant, or neglect the infant if the costs outweigh the benefits [157]. This is especially when the mother is isolated and/or having a lack of paternal and/or social support, and thus the cost of parenting could not being tolerated by the mother alone [157].

Among breastfeeding mothers, those with depressive symptom tend to be less sensitive in touching their infant and more likely to have poor positioning during breastfeeding, which can result in poor infant latching to the breast, with subsequent adverse effects on milk yield (due to poor milk ejection), infants milk intake and weight gain [151, 158, 159]. The combination of both

depressive symptoms and difficulty in breastfeeding can also influence the duration of (exclusive) breastfeeding [151, 160-162]. Thus, to investigate factors that could influence breastfeeding practice and behaviour, and also mother-infant interaction during breastfeeding, as part of my study I also investigated maternal psychological state during the postpartum period and the physiological effects in mothers during breastfeeding, such as maternal hormones and milk composition.

2.3.1. The influence of maternal psychology on breastfeeding

It is well recognised that maternal psychological state influences milk ejection known as the let-down reflex [163]. During lactation, the let-down reflex is activated by the hormone oxytocin which is secreted by the posterior pituitary gland, usually stimulated or triggered by infant suckling. The let-down reflex can also be stimulated when a mother intends to or expects to breastfeed, e.g. thinking, hearing, touching and/or smelling her baby, or any pleasurable experiences between mother and infant [164]. Hence, good relationships between the mother and the baby, as well as regular skin-to-skin contact are important to maintain breastfeeding. In contrast, if the mother is unwell or distressed, secretion of oxytocin may be suppressed, which would lead to difficulty in milk ejection [163, 165]. Human studies have reported that emotional distress in mothers inhibits the let-down reflex leading to disruption of milk flow and reduced milk volume, hence affecting breastfeeding success [158, 163, 166-168]. Conversely, milk ejection can be improved by relaxation therapy, and this has been shown in previous randomised studies that used relaxation techniques such as guided-imagery and music therapy [169-171]. These studies found that mothers of premature infants that listened to guided relaxation/imagery recordings (as a relaxation therapy) produced significantly more milk than control groups [169, 170], but to my knowledge, this type of intervention has not been formally tested in mothers who are breastfeeding their healthy full-term infant.

2.3.2. Mother-infant behavioural factors that influence breastfeeding

Some studies suggested that chronic or substantial psychological distress during the postpartum period, such as severe stress or depression, upregulate and/or dysregulate the hypothalamic-pituitary-adrenal axis (HPA) and lead to elevated maternal cortisol [40, 172, 173]. Thus, as indicated earlier in this chapter, maternal psychological distress has been associated with maternal cortisol during the postpartum period [172, 174]. This has been suggested to interfere

with the regulation of oxytocin and prolactin, which may influence breastfeeding performance [40, 172]. It is suggested that the repeated inhibition of the let-down reflex may lead to incomplete emptying of the breast, which consequently leads to the down-regulation of milk secretion. However, studies on maternal hormone regulation during breastfeeding are not well described in humans and the findings are inconsistent.

From an anthropological perspective, postpartum distress may also raise tension in the tug-of-war, affecting the maternal energy budget, since lactation is costly. This is because psychological distress increases energy expenditure [175-179], so chronic stress may reduce energy allocation in breast milk. Individuals with major depression use excess energy and need to trade-off resources between maintenance or immune function over growth and reproduction, depending on the type of depression [175, 176, 180]. In a well-nourished population and in mothers with minor psychological distress, trade-offs might be difficult to detect, but are still high likely to occur since mother and baby still compete for resources [181], especially during the lactation period. This is especially true because the infant also plays a major role in triggering milk ejection through suckling or vocalisation, which up-regulates milk synthesis. Therefore, as indicated earlier, infants could possibly take advantage of this by doing non-nutritive suckling and or giving non-honest signals of hunger to demand food. According to the tug-of-war theory, infant signals to the mother will only be honest if they are costly, but if the infant signals strongly or too much, ultimately, because the mother is providing all the food, she has to pay for those signals [8].

In animals for example, crying can also increase the risk of predation, hence the mother must feed the offspring to reduce the threat [8]. If this is effective in getting extra milk, the infant could blackmail his/her parent through non-nutritive suckling and crying [8]. This begging behaviour creates high tension in the tug-of-war, especially if maternal capital is limited. As crying increases metabolic rate [8, 182], infants who cry more were reported to spend less time asleep, and therefore, use more energy per day [182]. Crying should be an honest signal especially in early life when the growth rate is high, and could make the mother provide milk, as it triggers let-down-reflex, including when the mother is malnourished [8, 65]. Therefore, honest signalling could increase a needy offspring's nutritional intake, which is beneficial for both parties [183, 184].

However, if a well-nourished infant begs vigorously, it perhaps wastes energy for both parties, as this could increase maternal stress or anxiety, and at the same time, the infant might use extra energy for vocalisation instead of growth. Moreover, as growth cost decline at later age, especially starting six months [185], blackmail become easier for infants especially if they found it is effective in getting more energy. On the other hand, if a mother is more relaxed and less stressed, she may be able to allocate more energy to invest in her offspring, since the energy consumed by stress may be reduced. Therefore, I am intrigued to investigate whether manipulating maternal psychological state by making the mother more relaxed can reduce the costs of lactation, pushing the tug-of-war toward positive energy balance, and hence increasing investment in milk production. In terms of infant responsiveness, if a well-nourished infant is passive in the tug-of-war, this will be a huge benefit to the mother because she simply has to fund the growth of the infant, without paying an extra budget to compensate the energy that the infant might have wasted during vocalisation.

Overall, although severe stress and anxiety are experienced by a relative minority of mothers during the postpartum period, more minor levels of stress or anxiety (which may occur in many mothers) may still have a negative influence on breastfeeding outcomes. Therefore, reducing these symptoms may be important to get the best breastfeeding outcomes, including to prolong breastfeeding duration. A previous study on mothers of full-term infants has shown that guided imagery relaxation therapy was effective in diminishing postnatal anxiety and depression in primiparous mothers in the first to fourth week of lactation [186], but the effect on milk production was not measured and the influence on breastfeeding performance was not studied. Although a few studies have found that relaxation therapy increased milk volume as mentioned previously [169, 170], the studies were performed only among mothers of pre-term infants, and none of those studies measured outcomes in infants, such as milk intake, weight gain, and infant behaviour.

2.4. PART III: SYSTEMATIC REVIEW OF INTERVENTION STUDIES USING RELAXATION THERAPY DURING BREASTFEEDING

I conducted a systematic review in August 2016 to search for relaxation therapy intervention trials among breastfeeding mothers according to PRISMA guidelines [187]. The main purpose of this review was to investigate the effectiveness of interventions using relaxation therapy to improve breastfeeding outcomes, and to assess the consequent impact on infant growth and behaviour. The main research question was: Does relaxation therapy (verbal protocol/ guided imaginary recording/ meditation/ music therapy) help to improve breastfeeding outcomes and have consequent effects on infants?

2.4.1. Methods

Studies that were considered for this review were intervention studies (including non-randomised studies) using relaxation therapy during the postnatal period, which involved only breastfeeding mothers. The primary outcomes were breastfeeding and/or infant outcomes. Specifically, the breastfeeding outcomes were breast milk volume or milk intake, breast milk macronutrient content (levels of fat, protein and carbohydrate), breast milk energy, and breast milk cortisol levels. The infant outcomes were infant growth including weight gain and BMI, and infant behaviour such as feeding, sleeping and crying duration, and temperament. The secondary outcomes were maternal psychological state during the lactation period and/or other bioactive factors in breast milk (other than cortisol). Databases that were used for the literature search were Embase, Medline, CINAHL Plus, AMED, Web of Science, and the Cochrane Library. The search terms, keywords and search strategy are shown in Table 2.3. For initial literature research, no limits were applied for language or publication date during the search (the end date was August 2016). Articles were eligible for inclusion in the review if they were full-text articles published in English that reported an experimental study design testing the effectiveness of relaxation therapy on breastfeeding and/or infant outcomes.

2.4.2. Results

Based on the search strategy used (Table 2.3), 147 references were identified and all were exported into Endnote. The number of references found from each database was: Embase =70; Medline=26; CINAHL Plus=13; AMED=26; Web of Science=3; and Cochrane Library=7. As shown in Figure 2.8, after duplicates were removed, and titles and abstracts were screened based on the eligibility criteria, 5 articles were eligible to be included in the analysis. Of those 5 articles, 3 studies reported primary outcomes related to breastfeeding: milk volume and milk composition (either macronutrient [169, 170] or cortisol levels [171]), and the other two presented data on the secondary outcomes: breast milk secretory IgA [188] and maternal psychological state [189]. None of the studies reported on infant growth or behaviour outcomes.

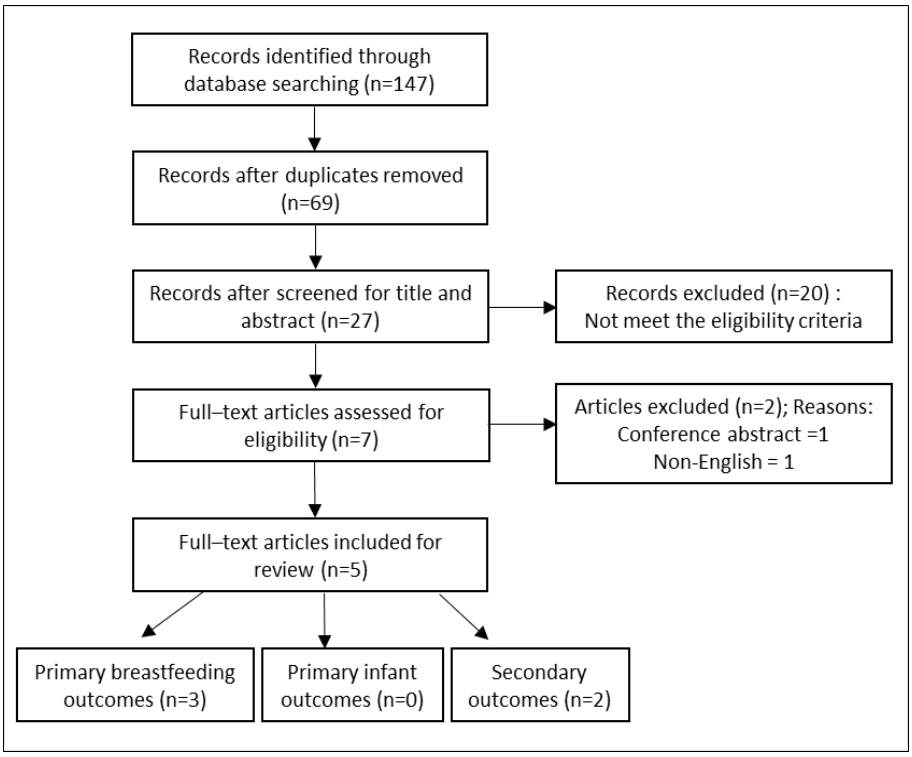
Table 2.3 MeSH and keywords used in article the literature search and the search strategy used.

No.	Search strategy	Map term to subject heading (MeSH)	Keywords
1	MeSH **OR** keywords (key findings for breastfeeding)	Breastfeeding, lactating, lactation, human milk, breast milk, breastfeed, breastfed	breastfeeding or "breast feeding" or breastfed or lactation or "breast milk" or "human milk"
2	MeSH **OR** keywords (key findings for relaxation therapy)	Relaxation therapy, relaxation techniques, meditation, imagery, verbal protocol, guided-imagery, music therapy	relaxation therap* or meditation or guided imagery or music therapy or verbal protocol
3	1 **AND** 2 (combination both of key findings)	(Breastfeeding, lactating, lactation, human milk, breast milk, breastfeed, breastfed) **OR** ((breastfeeding or "breast feeding" or breastfe* or lactation or "breast milk" or "human milk") **AND** (Relaxation therapy, relaxation techniques, meditation, imagery, verbal protocol, guided-imagery, music therapy) **OR** (relaxation therap* or meditation or “guided imagery” or “music therapy” or “verbal protocol”)	

Of the 5 studies considered in this review, 1 was European, 3 were North American (USA) and 1 was Indian. The studies included 311 mother-infant pairs, of which 64 infants were full-term and 274 were premature infants. Studies that presented the primary outcomes of breastfeeding only involved mothers of pre-term infants [169-171] whereas the two studies that presented the secondary outcomes (maternal psychological state [188] and other bioactive factors in breast milk [189]) only involved mothers of full-term infants. Three out of five studies were randomised

controlled trials (RCT) [169, 170, 188]. The quality of the RCT studies are in the next section (subheading no. 2.44). Three studies used a guided-imagery recording or meditation as a relaxation therapy [170, 188, 189], one study used music therapy [171] and only one study compared both guided imagery recordings and music therapy [169]. Table 2.4 shows a detailed description of all studies including the length of relaxation therapy used and their results.

Figure 2.8 PRISMA Flow diagram of data extraction



2.4.3. Intervention tools

Four studies that used a guided-imagery recording or meditation as a relaxation therapy involved mothers practising a progressive muscle relaxation technique such as taking deep and rhythmic breaths. The duration of the voice protocol recording in these selected studies was as short as 12 minutes and as long as 20 minutes. The guided-imagery in two RCTs [169, 170] included descriptions of pleasant surroundings, positive and supportive messages about breastfeeding and mother-infant bonding, and all mothers in the intervention group received the same guided imagery recording. The only difference was the addition of lullabies or guitar songs to the recording, and also the use of additional visual images of the mother's baby in two additional

intervention groups in the study by Keith et al.[169]. Another two studies involved meditation to aid or stimulate relaxation in general [188, 189], of which one study had a well-planned meditation program of 8 different sessions targeted to increase mindfulness and self-empowerment including maternal self-efficacy [188], whereas the other study [189] individualised the tape recorded meditation according to maternal preference thus each mother received a different recording. Only one study used music therapy (without an accompanying verbal protocol) which was based on the raga played on the flute specifically for the Indian population in the study [171]. All mothers were advised to listen to the therapy once daily, and the duration of use varied from 4 days to 8 weeks. Apart from NICU [169, 170], two studies involved mothers attending relaxation therapy sessions at the study centres [171, 188], and one study [189] involved a researcher performing relaxation training (meditation) during a home visit where it was audiotaped, and then mothers were asked to listen to the audiotape twice daily for the next two weeks.

2.4.4. Quality of the randomised controlled trial studies

Three RCTs [169, 170, 188] were assessed for their quality using a critical appraisal tool for therapy articles by the Centre for Evidence-Based Medicine, University of Oxford. One study [169] used a random schedule for randomisation while another two studies [170, 188] did not mention the method of randomisation; none of these studies indicates whether the randomisation was prepared by an independent person. All of the RCTs, however, indicated that both control and intervention groups were similar at baseline since there were no significant differences between groups in baseline characteristics. Apart from the intervention, all mothers in the studies were treated the same throughout the study period. The loss to follow-up or incomplete data (e.g failure to collect milk samples) in these studies were around 20-25%, and none of the studies mentioned pre-protocol. Researchers and mothers involved in the RCTS were not blinded, and mothers in the control group were aware of the relaxation therapy treatment of the mothers in the intervention group. Therefore, there was a possibility that mothers in the control group might seek similar therapy during the study period – and this concern was not acknowledged in any of the RCTs. The sample size of two RCTs [169, 170] was adequate to detect hypothesised differences in the primary outcome(s) between groups and their effect sizes (mean differences) were also large. Another RCT had a very small sample size and they were not able to detect differences in any of the primary outcome results [188].

Table 2.4 Summary of studies included in the review

Study design	Randomisation	Participants	Methodology : Intervention tool / groups	Sample collection / assessment	Outcomes
RCT: Feher et al., 1989 [170]	Randomised: Method not stated	Urban USA, mothers of mixed parity (n=55), breastfeeding. Infant: Pre-term infants in the NICU for at least 10 days	Tool: A 20-minute audio cassette tape based on relaxation and visual imagery techniques. Instruction: Listen once daily prior to breastfeeding for 7-13 days. Analysis: T-test	Milk volume: Volume of single expression at 1 week after enrolment. Milk fat content: creamatocrit	Frequency of listening: 50% women listened to the tape >5 times before expressing a milk sample. **Milk volume:** Intervention: 90.1 ± 60 ml; Control: 55.4 ± 48.2 ml; 63% higher in the Intervention group, $p<0.05$. **Creamatocrit (fat):** Intervention: 7.2 ± 2.9 %; Control: 6.8 ± 2.4 %; $p>0.05$. **Dose-response:** Milk volume & frequency of listening.
RCT: Keith et al., 2012 [169]	Random schedule	Urban USA, mothers of mixed parity (n=162), breastfeeding. Infant: Pre-term infants (born before 38 weeks) in the NICU or critically ill.	Groups: A: Control; B: Verbal protocol (12 min) + lullabies; C: Verbal protocol + guitar music background + images of the infant. D: Verbal protocol only. Instruction: use as often as possible while pumping milk for 14 days. Analysis: Repeated measure ANOVA.	Milk volume: No. of times pumped and volume of milk produced. Milk fat: creamatocrit Collection of 1ml sample of composite breast milk of expressed milk, collected daily from day 1 to 14, close to noon time.	Frequency of listening: Not reported **Milk volume:** Group B, C & D had significantly higher milk volume than the control group (A); $p<0.05$. **Creamatocrit (fat):** Group C & D had significantly higher fat content at day 1-6, compared to A & B ($p<0.05$). **Dose-response:** Not reported
RCT: Perez-Blasco et al., 2013 [188]	Randomised: Method not stated	Urban – Valencia, Spain, mothers of mixed parity (n=26), breastfeeding. Infant: Healthy infants	Tool: 2-3 sessions of 10-minute guided- meditations. Instruction: 2-hour session per week, for 8 weeks. Analysis: ANCOVA	Only secondary outcomes: Psychological state (DASS 21), mindfulness and self-efficacy at baseline and end-point after 11 weeks.	**Psychological state:** Intervention group had significant higher reduction in anxiety and stress ($p<0.05$). **Self-efficacy, self-compassion and mindfulness:** All showed higher score in the intervention group ($p<0.05$).

Study design	Randomisation	Participants	Methodology: Intervention tool / groups	Sample collection / assessment	Outcomes
Quasi-experimental: Ak et al., 2015 [171]	Random permuted blocks for task. No control group.	Bangalore, India, parity was not mentioned, breastfeeding mothers (n=30). Infant: Pre-term infants (born before 34 weeks) in the NICU.	Tool: a 30-minute rendition of the raga flute song (music therapy). Instruction: Listen for 4 times within 4 days. Analysis: Paired t-test	Milk volume: milk pumped for 15 minutes at minute 15 of the therapy. Milk collection: twice a day at around 11am and 4pm (for 4 days). Salivary cortisol: before and after music therapy on the last day. Stress level using PSS at Day 1 & 4.	Frequency of listening: 4 days **Milk volume:** Music therapy: 7.12 ±1.6 ml; Non-music therapy: 6.68 ±1.4 ml (p=0.033). Milk volume increase significantly from Day 1-4 during music therapy period (p=0.024). **Salivary cortisol:** Music therapy: 3.31 ±3.5 nmol/L; Non-music therapy: 2.99 ±4.0 nmol/L. Significant reduction was reported after music therapy period, p=0.001. **Stress level:** Mean PSS score at day 1 (42.4 ±3.3) was significantly higher than at day 4 (33.5 ±3.5), p=0.01.
Quasi-experimental: O'Connor et al., 1998 [189]	Not randomised, no control group.	Urban USA (Ohio), mothers of mixed parity (n=38), breastfeeding. Infant: Healthy infants	Groups: 1) Relaxation training & audiotaped; 2) Conversation about life; 3) None. Group 2 and 3 received the relaxation audiotape after HV2. Instruction: Group 1: Listen to the tape or perform the relaxation training twice daily for 2 weeks after HV1. Group 2 & 3: Listen to the audiotape daily after HV2. Analysis: ANOVA	Milk collection: 10 ml breast milk sample at baseline (HV1), after two weeks (HV2) and after 6-8 weeks (HV3). Psychological state using SCL-90-R to measure overall stress, anxiety and depression at HV 1-2.	Frequency of listening: 36% of Group 1 practiced less than once & 60% practice 1-2 times daily for 2 weeks after HV1. **Milk sIgA:** No significant difference between groups at all HV. **Psychological test:** No significant difference between groups at all HV. Mothers who were stressed at HV2 (n=14) had significant increase in sIgA level at HV3 (+0.16 g/L), compared to those who were not stressed (n=22); sIgA (-0.09 g/L); p=0.03.

*HV=home visit

2.4.5. Discussion

Overall, there are very few studies investigating the effects of relaxation therapy on breastfeeding outcomes, in particular, breast milk volume and composition. Two RCTs [169, 170] found that listening to relaxation therapy significantly increased milk volume by more than two-fold compared to that produced by mothers in the control group; demonstrating a large effect size. A dose-response effect was also reported showing a significant positive association between frequency of listening to the therapy and milk produced from a single pumping session [170]. There was also a non-randomised study [171] that claimed that listening to music therapy was effective in increasing the amount of milk expressed in their population, but the difference in milk volume compared to the amount expressed while not listening to the therapy was very small (0.5 ml or a 7% difference) and unlikely to be clinically relevant. In terms of milk composition, one study [169] reported a significant increase in fat content in the breast milk of mothers in two of the intervention groups (Group D: who received the imagery protocol only; and Group C: who received the imagery protocol accompanied by visual images of the mother's infant) than in the control group or the intervention group that received the voice protocol accompanied by lullabies (Group B) [169]. The authors suggested that the lullabies might have distracted the mothers from focussing on the guided-imagery protocol and thus affected the milk produced during milk expression. The other RCT reported higher fat content in breast milk of mothers in the intervention group than the control group, but the result was not significant [170].

From this review, there is evidence from three studies suggesting that relaxation therapy may be effective in producing significantly increased milk volume and from one study suggesting therapy may have beneficial effects on milk fat levels. However, there are several things that I would like to highlight regarding methodological issues with these studies. Firstly, the studies did not define the stages of lactation during sample collection which would be expected to influence the results for milk volume and composition, since milk production changes over the course of lactation, especially during the early stages (first three weeks) [24, 28]. In addition, it is also important to define the status of exclusivity of breastfeeding since it may affect the milk yield, as mothers that have established breastfeeding may be likely to produce higher milk volume due to greater frequency of breast emptying or higher demand from the infant [28]. Next, it is very important to standardise the milk collection procedure, for example specifying the time of the last feed or last pumping, and time of day, as all of these can

influence both milk volume and composition due to high variability of milk synthesis between mothers [190]. This was not mentioned in most studies.

With regard to fat content, neither of the RCTs [169, 170] specified the milk sampling procedure and the methods used could have been non-physiological if samples were obtained on a single occasion by either mechanical or hand expression. Thus, future studies should consider performing mid-feed, fore or hind-milk sampling in order to assess breast milk composition, especially milk fat, given that the breast milk content is not static, but changes within a feed and diurnally, as described earlier in this chapter. In addition, for future studies, it would be desirable to use the doubly labelled water method to estimating the energy transfer of breast milk and/or milk intake, as this method is physiological (suckled breast milk), non-invasive (it does not interfere with the breastfeeding process), and suited to free-living infants [191]. A study performed by Lucas et al. (1987) [71] using this method reported lower metabolisable energy content of breast milk than that previously reported from expressed breast milk samples at week 5 and 11, with figures of 57 and 61 kcal/100 ml respectively.

Similarly, standardising the timing of sample collection is important when measuring cortisol levels in humans as the concentration changes throughout the day [83]. Only one study investigated the effect of the relaxation therapy on maternal salivary cortisol, and it was measured only on the last day of the music therapy session. They concluded there was a reduction of cortisol levels after listening to the music therapy. However, the results were not convincing for several reasons: i) the study was not randomised and all mothers were exposed to the music therapy several times at different sessions, and therefore, although the sample collection was done on the last day, mothers that were assigned not to listen to the therapy might feel relaxed during breastfeeding since they had already been exposed to the therapy previously; ii) in the results section of the article, the value given for salivary cortisol was, in fact, higher among mothers that listened to the music therapy compared to those that did not, and yet they did not report the changes in salivary cortisol after listening to the therapy, therefore the mean difference was not reported; iii) there were statistical limitations since the sample size of the study was small (n=30), and the SD values were large. Therefore, future studies with better study design and a larger sample size are required to further investigate the effect of relaxation therapy on salivary or milk cortisol.

One study also reported the effect of the relaxation intervention on sIgA level in breast milk and found no significant difference between intervention groups [189]. Similar to the Ak et al., [171] study design, all mothers received the intervention at different time points, hence all mothers were exposed to the relaxation therapy and might have carry-over effects even at the point where they were not receiving therapy. Thus, the changes in breast milk composition and psychological state due to the effects of the intervention could not be ascertained between groups.

Two studies reported the effectiveness of the intervention in reducing maternal stress [171, 188] and anxiety [188]. As discussed above, due to limitations in study design, the results of the Ak et al., study were not convincing, as they compared maternal psychological state before and after the study period for all mothers, without having a control group. Thus, causality cannot be determined. The RCT of Perez-Blasco et al., [188] reported an improvement of overall maternal psychological state: reduction in stress and anxiety and higher scores in self-efficacy, self-compassion and mindfulness among mothers in the intervention group. However, since the intervention involved different meditation programs during each session, they did not identify which program could have contributed the most or been most effective in reducing maternal distress or increasing mindfulness during the postpartum period.

2.4.6. Conclusion

In conclusion, there are limited studies and inconclusive evidence on the effectiveness of relaxation therapy on both primary and secondary outcomes of this review. The strongest evidence was for an effect in increasing milk volume expressed by mothers of preterm infants in two RCTs. Only one study found an effect on milk fat content. Two studies reported an effect on maternal stress. Nevertheless, all studies included in the review had limitations either relating to study design or the sample collection procedure. With regard to the intervention, mothers in the control group in all studies were aware of the availability of the relaxation tools that were used in the intervention group(s), and thus, there is the possibility that some mothers may have sought similar relaxation tools and used them during the study period. None of these studies acknowledge the potential influence of parity on breast milk outcomes or maternal psychological state, therefore, and this should be considered for future studies as a potential confounder, in addition to socio-economic status. Many of the studies had a small sample size and, due to a higher potential of selection bias (selecting breastfeeding mothers from higher social economic status for the study), it is important to acknowledge that

it may not be appropriate to generalise the study results to all breastfeeding mothers from all socioeconomic class; none of the studies addressed this issue. Finally, none of the studies reported the effects of the relaxation therapy on infant growth or behaviour. Since manipulating maternal psychological state may affect breastfeeding outcomes, it is also intriguing to ascertain the consequent effects on infant growth and behaviour. As discussed earlier, certain components in breast milk or the production of different milk volumes as a result of intervention therapy could potentially influence infant appetite, behaviour and growth during infancy.

2.5. SUMMARY

2.5.1. Limitations of published scientific studies

Breast milk contains abundant non-nutritive components including bioactive constituents, many of which are yet to be discovered and studied. With limited scientific evidence, we still do not understand how these biological components get into the milk and how milk composition (both nutrient and non-nutrient component concentrations) changes during a feed, between breasts and over time [80]. Once milk is ingested by the infant, we also do not know the mechanistic and functional outcomes of those biologically active components and their role or significance in infant health and development. Apart from physiological changes, maternal psychological factors can also influence breastfeeding performance and infant outcomes [80]. Most previous studies are observational and can only show associations, because findings might also be influenced by various confounding factors [38, 80]. Infants cannot be ethically randomised into breastfeeding and formula feeding groups, which prevents the use of an experimental study to investigate the effects of maternal factors and different feeding methods on infant health and development [38].

Milk sampling is also an important issue, as there is a lack of consistency in sample collection in previous studies. As mentioned earlier, the composition of human milk varies across lactation, and there is no constant composition between and within individuals as it varies diurnally and across a single feed. One possible explanation for the inconsistent findings between published studies is the different methods of sample collection, such as single spot or pooled milk sampling, as well as the lack of standardisation of timing, either during the day or according to the stage of lactation [190]. This is especially important when measuring milk energy density in breast milk, as the changing fat content in fore- and hind milk affects the

total energy content. Presumably, this will also influence the content of other fat soluble substances in breast milk, such as fat-soluble vitamins or certain other bioactive substances. To date, there is no universal sampling protocol or gold standard method for human milk sampling since various issues need to be considered including ethical aspects as well as limitations of the population and conditions in the field [190]. Thus, comparing data from different studies is challenging and problematic especially for data that is based on a single milk sample collection.

Regarding the influence of breast milk hormones on behaviour and appetite regulation in breast-fed infants, the evidence is currently unconvincing due to the limitations of the studies. There is a lack of studies combining measurement of these hormones with milk intake and infant outcomes, such as behaviour, appetite and growth, because mother-infant physiological and psychological factors during breastfeeding are commonly studied separately. There are very few studies investigating the changing concentrations of breast milk hormones within a feed and throughout lactation. Besides that, the use of different assay kits may also influence the results, as some kits are specifically designed to be used only for measuring hormones levels in the blood, but studies often use the same assay kits for measuring hormones in milk as well. As discussed earlier, the use of different methods (e.g. ELISA vs RIA) non-specific assay kits, particularly for ghrelin and leptin in some previous studies, may have contributed to variability in results between studies. Plus, some studies measured the total ghrelin level without measuring acyl-ghrelin (active ghrelin) separately. This may lead to misleading information about the influence of breast milk hormones on infant feeding pattern and appetite regulation. Therefore, ideally future studies should pay greater attention to the milk sampling protocol and the usage of assay kits.

2.5.2. Planning of an intervention study

Although there are many interesting and unexplored issues in the signalling between mother and infant during breastfeeding, as discussed above the complexity of the inter-relationships between factors makes it problematic to define cause and effect using an observational study design. Therefore, I used an experimental approach to investigate causal relationships between maternal psychological state (manipulated using a relaxation intervention) and breast milk volume and composition including cortisol concentrations, breast milk intake, and infant outcomes (behaviour and growth). This study aimed to fill some of the research gaps identified in my literature review, by combining both psychological and physiological mother-infant aspects during breastfeeding and by using a more robust methodological design.

As presented in part III, the evidence on the effectiveness of relaxation therapy is limited and inconclusive, and none of the studies has investigated the consequent effects on infant growth and behaviour. Studies looking at the effects on breast milk outcomes have only been performed in mother of pre-term infants, and no study has yet been done investigating the effects of relaxation therapy on breastfeeding outcomes of mothers of full-term infants. Thus in my study, guided-imagery relaxation therapy was used as an intervention in first-time mothers of healthy full term infants. I hypothesised that mothers who were more relaxed and less anxious would have increased milk production and decreased breast milk cortisol, with favourable effects on infant behaviour, milk intake and growth. One aim of the study was to identify modifiable factors which can be used to encourage and support exclusive breastfeeding. The details of the planning and data collection are discussed in Chapter 3.

In terms of an anthropological perspective, my study is also the first to test Trivers' (1974) parent-offspring conflict theory [62] in humans using a randomised controlled trial, which would be the first experimental human work on this hypothesis. Previous life-history studies on human biology are all observational, and therefore only predictions can be made [192-194]. My study could increase the understanding of energy provisioning in the evolutionary conflict between mother and infant, identifying who is more in control in the tug-of-war during lactation. By manipulating maternal psychological state, I aimed to reveal the mechanisms of signalling by both parties in the tug-of-war. This project therefore applies evolutionary theory to a broad area of infant nutrition, which could be key to understanding parent-offspring coadaptation to balance maternal reproduction success and infant fitness.

CHAPTER 3

3. METHODOLOGY

A) Methods

3.1. Introduction

This chapter consists 2 parts: A) Methodology of the study design and B) Research materials used in the study and detailed procedure. Part A describes the methodological aspects of the trial conducted, which includes study design, hypotheses and research questions, including an anthropological perspective on the study hypotheses. Following that, I provide details of the sample size and population, study location, and the procedures for data collection including a flow chart of the study design and the home visit procedures for data collection. Outcome measures are summarised in this chapter, but the elaboration of each measure including the research tools are described in Part B.

3.2. Study design

This was a randomised controlled trial that involved first-time healthy breastfeeding mothers and their full-term infants (n=64 mother-infant dyads). The objective of this trial was to investigate the causal effects of maternal psychological state (manipulated using a relaxation intervention) on breast milk (volume and composition including cortisol concentrations) and infant outcomes (behaviour and growth). More generally, the study investigated mother-infant signalling during breastfeeding and aimed to provide a greater understanding of maternal-infant factors which influence the success of breastfeeding, and which might be useful targets for future interventions. The trial was named the Mother-Offspring Milk Study (MOM Study). Table 3.1 provides the summary of the study hypotheses, outcome measures and research tools used in the trial

3.3. Research questions and hypotheses

In generating hypotheses, I developed several main research questions: Primary research questions for the trial analyses:

i. Do mothers (in the intervention group) who listen to the relaxation recording have reduced stress and anxiety?
ii. Do mothers who are more relaxed have increased breast milk volume and/or altered breast milk composition, including lower breast milk cortisol concentrations?
iii. Does the changing of breast milk composition influence infant growth?
iv. Does breast milk cortisol shape infant behavioural phenotype in early life?

Main research questions for the observational cohort analyses:

i. Are breast milk bioactive factors associated with infant behaviour and growth?
ii. Is there any bias in maternal investment in terms of milk volume and composition according to offspring gender?

The research questions generated the following hypotheses:

a) Primary hypotheses: The use of a relaxation tape by breastfeeding mothers starting at week 2 postpartum will result in:

i) reduced maternal stress and anxiety
ii) lower milk cortisol concentrations
iii) increased breast milk energy
iv) favourable effects on infant behaviour (less crying, more sleeping)
v) higher milk intake by the infant
vi) more optimal growth in the infant, specifically higher lean mass and lower fat mass at HV4 (infant's age of 14-16 months)

Measures of (i) - (v) were assessed at baseline (week 2) and at 12 weeks in control and intervention groups, while measures of (ii) and (iii) were assessed pre and post a single breast-feed in both groups at 2 week only, given that there will possibly be some contamination of the intervention effects at later HV. Measures of (vi : body fat and fat-free mass) were assessed at HV4 (infant's age 14-16 weeks).

To test the hypotheses, the following primary outcome measures for trial analyses were recorded at baseline and at 12 weeks:

i) maternal stress and anxiety scores assessed using questionnaires
ii) breast milk cortisol concentrations
iii) breast milk macronutrient composition
iv) infant behaviour measured using a 3-day diary
v) infant weight gain and body composition measured using stable isotopes
vi) physiological changes (maternal saliva cortisol, breast milk cortisol and macronutrient composition) before and after a breastfeeding session
vii) breast milk intake assessed non-invasively using stable isotope techniques

The following are the secondary outcomes for the trial analyses where I compared the results between randomised groups:

i) Breast milk leptin and ghrelin
ii) Maternal depression assessed by the Edinburgh Postnatal Depression Scale
iii) Infant behaviour measured using the Rothbart's questionnaire (RIBQ)
iv) Infant appetite assessed using the Baby Eating Behaviour Questionnaire (BEBQ)

b) Additional hypotheses for observational cohort analyses:

I. Infant temperament, appetite and breast milk composition are associated with infant growth, and these associations also differ by gender.
II. Non-nutrient factors in breast milk (specifically hormonal constituents; ghrelin and leptin) are associated with infant appetite and behaviour and hence infant growth.

The main outcome measures for the observational cohort analyses were:

i) non-nutrient factors in breast milk – leptin and ghrelin of the whole study population
ii) infant temperament (RIBQ) of the whole study population
iii) infant appetite (BEBQ) of the whole study population
iv) Infant weight, BMI, and weight gain of the whole study population

3.4. Anthropological perspectives of my study

I have presented my research questions and hypotheses from a biological and physiological perspective. However, they can also be framed using an anthropological perspective.

3.4.1. Purpose of the study

My project aimed to investigate anthropological aspects of mother-infant signalling during breastfeeding by focusing on the tug-of-war mechanism in order to increase the understanding of energy provisioning in the evolutionary conflict between mother and infant. A novel feature of my study was to manipulate the maternal energy budget and demonstrate the knock-on effects in the infant using an experimental approach, testing several hypotheses that emerge from Trivers' theory (1974) (as explained in Chapter 2). This experimental project investigated effects on both mother and infant, independent of other factors and thus aimed to identify who is more in control in the tug-of-war during lactation within the study period. This project therefore applies evolutionary theory to a broad area of infant nutrition, which could be key to understanding parent-offspring coadaptation to balance maternal reproductive success and infant fitness and provide a greater understanding of maternal-infant factors that could be incorporated into life-history theory. As indicated previously, previous life-history studies on human biology have all been observational, and could therefore show associations [192-194], but could not prove causation. Although anthropologists have occasionally used experimental approaches, this has generally been in the field of psychology, e.g Henrich et al. [195], but limited in physiology. Therefore, my project is the first human study to test experimentally Trivers [62] parent-offspring conflict theory during lactation, combining both anthropological and biological aspects of mother-infant signalling during breastfeeding, and using a more robust methodological design. The next paragraphs describe the hypotheses that were generated to answer my research questions within the context of the evolutionary biology of infant feeding.

Table 3.1 Hypotheses, outcome measures and research tools used in the trial

No	Hypotheses	Outcome measures		Research tools / Sample analysis
		Baseline & End-point: 12 - 14 week	Pre- and post-breastfeeding: at baseline, week 6 and 12	
1	Based on the primary hypotheses, i-vi were considered as primary outcomes for the trial: **Primary hypotheses:** The use of relaxation tape therapy during breastfeeding starting at 2 week postpartum will result in:			
i	reduced maternal stress and anxiety	maternal stress and anxiety scores assessed using questionnaires	Maternal emotions (happiness/stress/anxious)	▪ Stress: PSS ▪ Anxiety: BAI ▪ Maternal emotions: MBQ
ii	lower milk cortisol concentrations	breast milk cortisol levels	breast milk cortisol levels in fore- and hind milk	ELISA Kits
iii	increased breast milk energy (higher calories)	macronutrient composition (fat, carbohydrate & protein levels)	Milk fat, carbohydrate & protein in fore- and hind milk	MIRIS human milk analyser
iv	favourable effects on infant behaviour (less crying, more sleeping)	infant behaviour measured using a 3-day diary	None	3-day infant diary
v	higher milk intake by the infant	breast milk intake assessed non-invasively	None	Isotope ratio-mass spectrometry
vi	more optimal growth in infant (higher lean mass and lower fat mass)	infant weight gain and body composition	None	▪ Weight and length scales ▪ Isotope ratio-mass spec.
2	Secondary outcomes for the trial:	Maternal depression (EPDS) Infant temperament & appetite	Milk ghrelin and leptin	Questionnaires and ELISA kits for milk hormones
3	**Observational cohort hypotheses:**	**Outcome measures**		**Research tools**
i	Infant temperament, appetite and breast milk composition are associated with infant growth, and these associations also differ by gender.	infant temperament		RIBQ (questionnaire)
		infant appetite assessed		BEBQ (questionnaire)
		maternal depression (additional information)		EPDS (questionnaire)
ii	Non-nutrient factors in breast milk (specifically hormonal constituents; ghrelin and leptin) are associated with infant appetite and behaviour and hence infant growth.	non-nutrient factors in breast milk (leptin and ghrelin)		ELISA assay kits

3.4.2. Hypotheses and research questions

a) Primary trial research questions:

i) Do mothers (in the intervention group) who listen to the relaxation tape have reduced stress or anxiety and increased breast milk volume?

Hypothesis:

Mothers who are more relaxed and less anxious will have increased breast milk production.

This study investigated whether manipulating maternal psychological state by making the mother more relaxed is economically energy-favourable during the early postpartum period. Psychological distress is energetically expensive [175] and may affect maternal energy allocation for investment in breast milk. Andrews et al. (2015) reported that people with major depression use excess energy, and need to trade-off resources between maintenance or immune function over growth and reproduction, depending on the type of depression. I hypothesised that the mother-infant tug-of-war can be pushed toward a positive energy balance by preventing/reducing postnatal psychological stress, resulting in greater energy investment in milk production, thus improving the success of breastfeeding.

ii) Do mothers who are more relaxed produce breast milk with lower cortisol concentrations?

Hypothesis:

Mothers who are more relaxed and less stress will produce less cortisol in breast milk.

Animal studies have reported that cortisol helps maintain glucose homeostasis and regulates the tight junction permeability that is important in preventing apoptosis [84]. A study measuring cortisol in rhesus macaque milk found a significant positive correlation of cortisol with protein and fat [86]. The authors suggested that cortisol may be involved in regulating or controlling the amount of fat and protein in milk that then influences infant growth. Thus, it is possible that breast milk cortisol may play a role as an energy 'gate-keeper' to allocate different concentrations of macronutrients in breast milk, depending on the availability of maternal resources and/or signals from the infant (vocalisation/suckling). Nevertheless, to my knowledge, there is no clear evidence describing the function of human milk cortisol in relation to infant development, apart from preventing the involution of breast tissue [196, 197]. Therefore, one of the objectives of this study was to investigate the associations of milk cortisol concentrations with macronutrient content in breast milk.

iii) Does breast milk cortisol shape infant behavioural phenotype in early life?

Hypothesis: Infants breast-fed by mothers who are more relaxed will have better feeding behaviour and temperament.

As indicated in Chapter 2, studies reported that maternal cortisol levels or milk cortisol were positively correlated with negative affectivity [87, 99] and increased fearfulness in breast-fed infants, with no apparent association among formula-fed infants. In the context of the tug-of-war, there is a possibility that mothers who are relaxed may produce different concentrations of cortisol or other hormones to influence infant behavior, appetite and sleeping pattern that may favour the maternal energy budget. My aim was to investigate one potential physiological pathway of mother-infant signalling that may mediate these effects.

Summary hypothesis: mothers who are more relaxed and less anxious will have increased milk production and decreased breast milk cortisol, with favourable effects on infant behaviour, milk intake and growth. Postnatal stress can waste energy whereas mothers who are relaxed can conserve more energy. Therefore, the intervention in this trial may reduce maternal stress, or increase relaxation, allowing mother to invest more in breast milk and facilitating exclusive breastfeeding, which may in turn benefit infant outcomes. This study will be novel, as it is the first to experimentally intervene in the mechanism of the tug-of war in humans by manipulating the mother's psychological state during the lactation period. The practical aim is to explore the potential of using aspects of the mother-infant signalling process to make breastfeeding more successful.

b) Observational cohort research questions:

Is there any bias in maternal investment in term of milk volume and composition according to offspring gender?

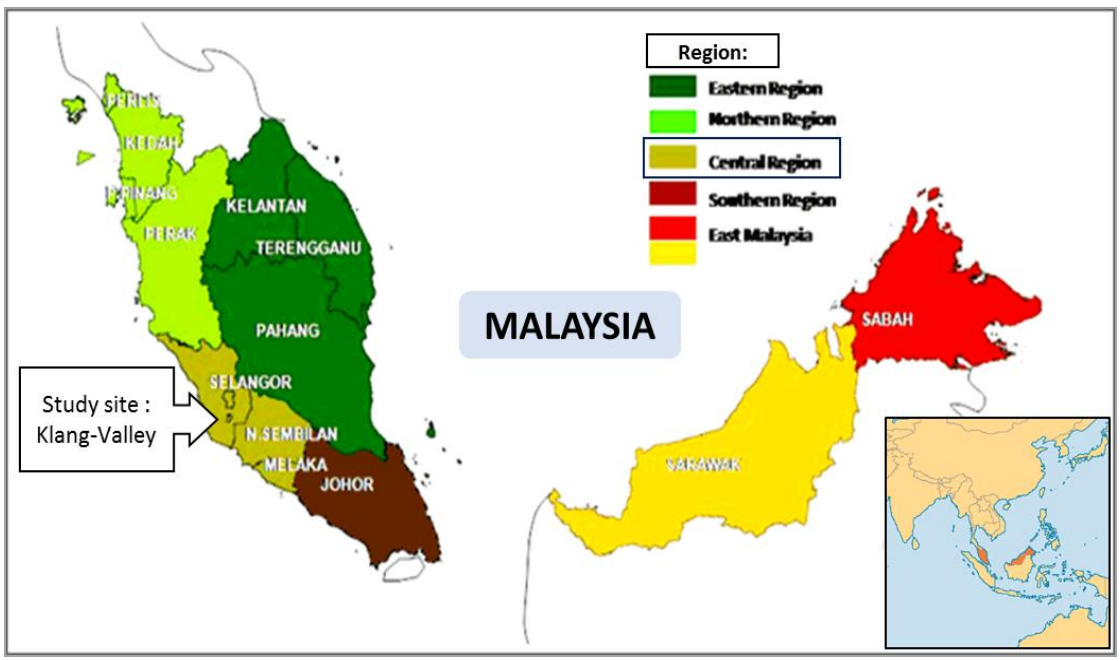
Hypothesis: There are differences in milk volume/composition in relation to infant gender and temperament.

Animal studies [96, 198-202] and limited human studies [191, 203-205] have reported inconsistent findings on gender differences in milk intake and composition, most probably due to different methods of milk sampling and confounding variables such as infant parity, milk intake and body mass. Studies have suggested that mothers may invest more in sons, especially in better condition, potentially due to their greater potential reproductive fitness later in life [201, 206]. However, in less favourable condition, mothers might invest more in daughters due to their faster maturation rate, enabling the next generation to start reproducing earlier [199]. It remains uncertain whether there is a sex-bias in maternal investment, and whether mothers may have physiological adaptations to respond to offspring gender. Thus, another objective of my study was to investigate the gender differences in milk composition, including milk hormones. I tested this in observational cohort analysis due to the low statistical power in detecting milk composition differences between genders.

3.5. Sample population and study location

The target sample population was first-time mothers and their new born infants living in the central region of Malaysia (Selangor, N.Sembilan and Melaka). Malaysia is located in South East Asia and has a total population of 31.6 million (in 2016). The country is represented by multi-ethnic groups which consist of Malay/Bumiputera (67.4%), Chinese (24.6%), Indians (7.3%) and others (0.7%) [207]. The study site where recruitment and data collection were mainly carried out was in the Klang-Valley area, located in the middle of the central region of Malaysia, as shown in Figure 3.1. Klang-Valley also comprises the federal territories of Malaysia (Kuala Lumpur and Putrajaya), areas that have been recognised as the most developed states in Malaysia. Thus, this region has the highest population density with the most diverse ethnic groups [207]. According to the Malaysia Health Indicator 2014 [208], the area has the highest birth rates and lowest infant mortality and morbidity rates. In addition, the awareness of breastfeeding is also increasing in the urban areas in Malaysia, particularly in Selangor [209-211]. All these factors were taken into consideration when selecting the study location. Furthermore, because my laboratory and office facilities in the Universiti Putra Malaysia were located in Klang-Valley, it was convenient in terms of recruitment and data collection, as well as storing biological samples within the required time after a HV session.

Figure 3.1 Map of Malaysia showing the study location area



In Malaysia, Malay is the primary language and English is the secondary language used, but the majority of the population can understand and speak English, especially those living in the urban areas. Therefore, in this study, questionnaires and the intervention tool were available in both languages.

Since this trial was investigating the effect of maternal psychological state on breastfeeding outcome, only primiparous mothers with a singleton pregnancy were invited to participate. The main reason was to ensure that none of the mothers had experience in breastfeeding which helped to set a standard level for all participants at baseline. Multiparous mothers and mothers of twin pregnancies were not eligible to participate in the study due to the possibility of different stress and anxiety levels associated with having more than one child, which could further complicate the results. Table 2 shows the eligibility criteria that were set for mothers and infants in this trial. Based on sample size calculation as described in 3.6, I planned to recruit a minimum of 80 pregnant women.

Table 3.2: The eligibility criteria for mother and infant

First screening	Inclusion criteria	Exclusion criteria
Mother (during pregnancy)	Primiparous mother with singleton pregnancy	Multiparous mother or mother of twin pregnancy
	Free from serious illness / chronic disease.	Having medication due to illness or chronic disease.
	Non-smoker	Smoker
	Understands Malay or English	Does not understand Malay or English
Second screening		
Mother (after birth)	Free of illness that can affect breastfeeding	Mother has illness that prevented her from breastfeeding
	Exclusively breastfeeding at 2 weeks	Mixed- or not breastfeeding at 2 weeks
Infant	Interested in participating in home visit sessions	Not interested in participating in home visit sessions
	Full-term infant (37-42 week of gestation)	Preterm infant (<37 week of gestation)
	Infant birth weight of ≥ 2500 g	Infant birth weight of < 2500 g
	Free from serious illness that could affect nursing or growth	Has illness that could affect nursing or growth

3.6. Sample size calculation

Since there were only two studies tested the effectiveness of a relaxation therapy among breastfeeding mothers (based on the literature search date up to 2013, before the data collection started), it is difficult to estimate the sample size based on limited data. Thus, the conventional formula [212] for two sample t-test was used to determine the number of infants required to detect the hypothesised difference between two groups, control and intervention, as shown below:

$N = 16 (SD^2/D^2)$: (N=number per group, SD=standard deviation, D=Difference between group)

Hence, a sample of 56 infants (28 per randomised group) would allow the detection of a 0.76 SD difference in milk volume between groups at 80% power with a significance level of α=0.05 (D=0.76, SD=1); this is a biologically plausible difference based on previous studies of the effect of relaxation interventions on milk volume production between control and intervention groups of mothers with preterm infants [169]. Since my study involved mothers with healthy full-term infants, the effect size was predicted to be smaller than that in the previous study, as mothers with preterm infants are likely to be more stressed. Therefore, to allow for a smaller effect size as well as for drop-outs or failed measurements, it was planned that 80-100 infants would be recruited. No adjustment of sample size for multiple outcomes (in my primary hypothesis) was done, due to limited data on which to base the sample size calculation.

3.7. Recruitment

Recruitment was performed at selected antenatal clinics in the Klang-Valley area between March and December 2014. In Malaysia, all pregnant women receive a color coding on their antenatal record book to indicate their risk factors during pregnancy, from red (the highest risk), through yellow, green to white (the lowest risk) [213]. During recruitment, I only approached mothers during the third trimester with green and white codes to eliminate those with higher risk, in order to fit the eligibility criteria for this trial. A total of 242 pregnant mothers were approached and were given an information sheet (Appendix 5). The practical details of the study were explained to them. Those that expressed an interest in participating in the study were asked screening questions to determine their eligibility. Those that were

eligible and agreed to participate were enrolled in the study after obtaining written informed consent (Appendix 4). Contact details were obtained from them, including their estimated date of delivery (EDD). All participants were contacted 1-2 weeks after their EDD to check if they had delivered the baby and they were asked the second set of screening questions to confirm their eligibility. Those who were eligible (exclusively breastfeeding at 2 weeks and infant delivered at term, weighing ≥2.5 kg) were assigned a home visit at 2 weeks post-partum (±1 week). Mothers were also advised that if for any reason they did not wish to be contacted, they could inform the researcher in advance. This was so that, if anything went wrong prior to or during the delivery and they were not comfortable to be contacted yet or at all, any unwelcome contact would be avoided during that period.

In addition to recruitment from antenatal clinics, advertisements (Appendix 5b) for the study were also posted on parenting and nutrition websites such as Baby Center and also organizations' website such as the Malaysia Breastfeeding Peer Counsellor Group. Flyers and posters were placed in locations commonly visited by mothers such as private antenatal clinics or hospitals, health care centres and childcare shops around the Klang-Valley area. Those who were interested in the study were contacted and the study was explained to them by phone and email. Screening questions were also asked, and if they were eligible and agreed to participate, a meeting was arranged to proceed with enrolment and the first interview, which mostly took place at antenatal clinics or their own home.

3.7.1. Ethics approval

Ethics approval for the study was obtained from the Medical Research Ethics Committee (MREC), Ministry of Health Malaysia (ID: 13-841-16720) and UCL Ethics Committee (ID:4883) (Appendix 1-2). The MOM Study was also registered with the Malaysian National Medical Research Register (NMMR ID: 16720) and ClinicalTrials.gov (ID: NCT01971216). The research project was performed in collaboration with the Faculty of Medicine and Health Sciences, Universiti Putra Malaysia (UPM). UPM also gave permission for me to use the university's facilities, including processing and storing all biological samples.

3.8. Data collection

In this section, the process of data collection is explained, including the tools used for measurement/assessment. However, detailed descriptions of each tool and measurement are provided in the next chapter. Data collection was carried out from March 2014 to March 2015 and involved approximately 320 home visits with study participants, concurrent with ongoing recruitment throughout the year.

3.8.1. First interview

A questionnaire-led interview was performed straight away after enrolment, after signed consent was obtained. Participants were asked about their social demographic factors, goal for feeding their baby in the first sixth months, and their perception and opinions about infant feeding using the Iowa Infant Feeding Attitude Scale (IIFAS) (Appendix 6)**.** They were also provided with a questionnaire, the Neonatal Questionnaire, for them to complete at home after delivery, giving details of birth experience, early skin-to-skin contact and timing of first breastfeed.

3.8.2. Randomisation

Prior to the first home visit, and after confirming that the mother was still eligible and wished to continue in the study, the mother was randomised to either the control group or the relaxation group. Randomisation was stratified by ethnicity: Malay, Chinese and Indian. Mothers were not informed about this process as doing so would most likely influence their behaviour; in particular, I wanted to avoid mothers in the control group seeking or using some form of relaxation therapy, if the possibility of it being beneficial was raised. However, all mothers were aware that the MOM Study was investigating the effects of maternal mood as well as infant factors on breastfeeding at a general level. The randomisation schedule was generated by computer in blocks of permuted length (2, 4, 6). Assignments were prepared by a member of the research team in London who did not have contact with the subjects and were held in sealed opaque envelopes. Each assignment was revealed by the researcher on the day, just before the first home visit, in order to prepare the intervention materials including a diary log used specifically for mothers in the intervention group. There was a low possibility of contamination between randomised groups since home visit sessions were performed over a large geographical area in the central region of Malaysia and participants in the study did not have contact with each other. Therefore, mothers in the intervention group

were not likely to have the opportunity to reveal the existence of the intervention tool to control mothers. Mothers were only informed about the randomisation process when the trial was completed, when they received a summary of the results.

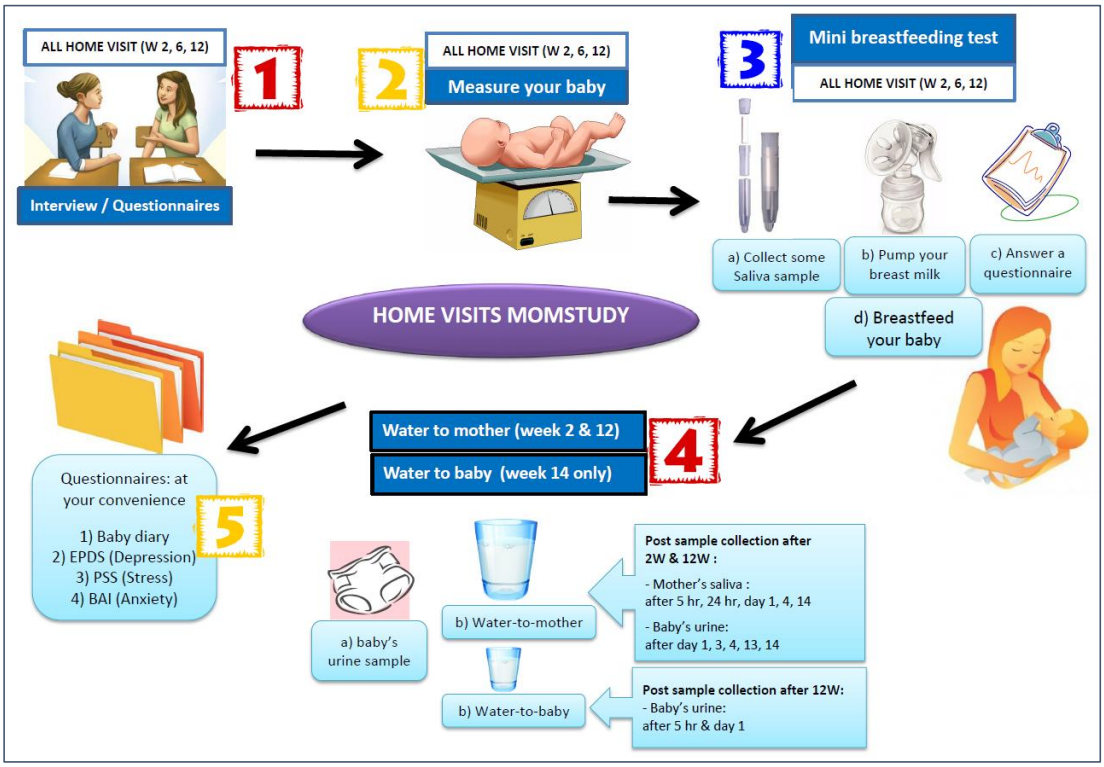
3.8.3. Home visit (HV) after birth

In Malaysia, the majority of the population, especially first time mothers, will practice a traditional postpartum confinement period lasting about 30-45 days, following their cultural tradition according to their ethnicity [58]. During this period, the mother and her infant will be taken care of either by their family members, particularly the infant's maternal grandmother (only a few by the paternal grandmother), or by a confinement lady who will be paid to stay overnight with the mother or come to the house on a daily basis. The caretaker will usually prepare meals for breakfast, lunch and dinner following the cultural confinement diet for the mothers, and will also perform special postpartum traditional massages for the mother, especially during the first 3 weeks of the postnatal period [58, 214]. The caretaker will also help to take care of the infant by changing the nappy, bathing or holding the infant when necessary. This is because the mother is encouraged to minimise physical activity and have ample rest to recover from the birth [58]. This confinement practice was advantageous to the trial since all mothers in the study received approximately standard maternity care at baseline, prior to the first home visit. Each mother and baby was followed-up after birth by conducting four home visits (HV) over a period of 4 months (Figure 3.3). The first home visit (HV1) was done when the infant was aged 2 weeks (±1 week), followed by visits at 6-8 weeks (HV2), 12-14 weeks (HV3) and 14-18 weeks (HV4). The first three HV were done before noon (around 10-11 am) in order to maintain consistency in collecting data, especially to maintain a standardised procedure for the collection of biological samples (breast milk and saliva samples). However, several HV2 were done in the early afternoon (12-3pm) due to time limitations in completing data collection, since I was the only person performing home visits. Furthermore, in some cases, the time of the visit was dictated by the mother's availability. Mothers were allowed to drink throughout the HV session, but they were advised not to eat during the HV session; thus, they were advised to have a meal prior to the session. Each home visit usually took about 2-3 hours to complete and all sessions and measurements were performed by myself.

3.8.4. Home visit I (HV1)

During the first home visit, the mother was first informed about the procedures to be conducted during the visit and was given a leaflet (Figure 3.2) as a guide to the overall plan for the sessions. The mother was also provided with a manual breast pump (Phillips Avent brand) as a token of appreciation and also as a tool for her to express the study breast milk sample if she chose to use it so. I also taught the mother breast massage and hand expression techniques, which gave her the option of an alternative method to express milk according to her preference.

Figure 3.2: Guide leaflet of home visit process



During home visit 1 (HV1), the following data were collected from each mother-infant dyad at 2 weeks postpartum:

i. Collection of data on breastfeeding practices and behaviour using an Infant Feeding Questionnaire (IFQ I), adapted from CDC and UK Infant Feeding Study 2010.
ii. Collection of data on infant appetite using the Baby Eating Behaviour Questionnaire.
iii. Anthropometric measurements on infant (infant weight, length and head circumference) and mother (weight).
iv. Mothers randomised to the relaxation group were provided with an mp3 with the relaxation therapy recording to use during the breastfeeding session. The purpose of the exercises and imagery used in the recording was explained to the mother. Details of the therapy are provided below.

v. A ‘Mini-Breastfeeding test’ was conducted: the mother was asked to collect samples of breast milk and saliva before and after a feed during the HV session. Mothers in the intervention group were asked to listen to the relaxation/imagery recordings during this breast-feed. Prior to the feed, the mother was asked (i) to collect a sample of her saliva to measure cortisol; (ii) to collect a sample of fore milk; (iii) to complete a short questionnaire describing her feelings and emotions (Mini-breastfeeding Questionnaire). She was asked to repeat these measures after completing the feed, including obtaining a sample of hind-milk. The mother’s saliva was collected, and the time and length of the feed were recorded. The infant was weighed before and after the feed if allowed by the mother; in this case the milk intake could be estimated by subtracting the initial from the final weight of the infant, after insensible water loss has been calculated or adjusted for the Malaysian population.
vi. The mother was dosed with the stable isotope, deuterium, to measure breast milk intake as described in 3.17 The mother was given written and oral instructions on sample collection (mother’s saliva and infant’s urine) after dosing.
vii. The mother was given a 3-day infant behaviour diary with instructions to complete it after the visit; this was collected at the next visit.
viii. At the end of the HV1, mothers were given a set of questionnaires to assess maternal psychological state (Edinburgh Postnatal Depression Scale (EPDS), Perceived Stress Scale (PSS), Beck-Anxiety Inventory (BAI) to complete at their convenience.

There was no fixed chronological order of measurement or assessment during the home visit session as it was planned around the infant’s behaviour and needs. If the infant was hungry and/or was expecting a feed, the anthropometric assessment and mini breastfeeding test would be done first and followed up with the questionnaire-led interview. Alternatively, if the baby was sleeping at the beginning of the session, the data collection from the questionnaires would be done first. At the end of the visit, the mother was provided with a folder containing questionnaires and a diary calendar with the schedule for isotope sample collection. Following the visit, text messages were sent to the mother as a reminder to collect these samples on day 1, 3, 4, 13 and 14 post-home visit.

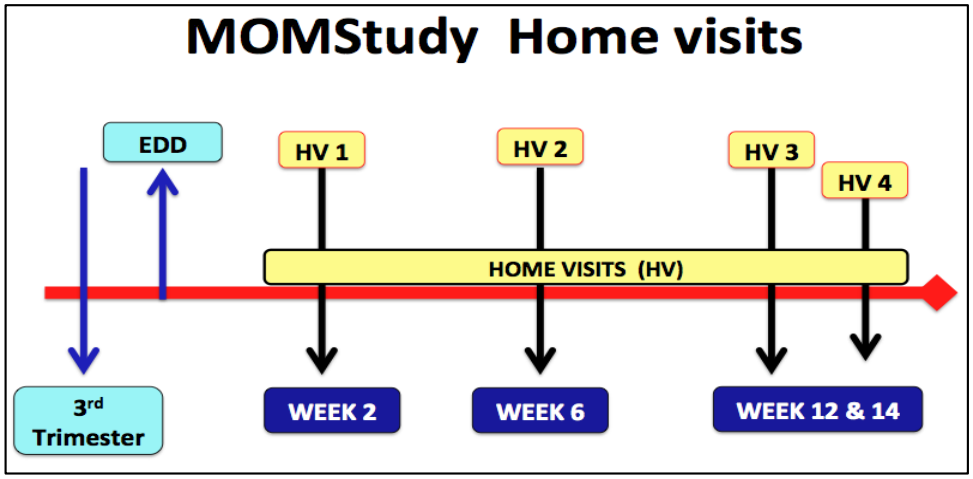
3.8.5. Home visit 2 (HV2)

The same measurements and data were collected without the baseline isotope measurements since the milk intake was not measured. Data on breastfeeding were collected using the IFQ II questionnaire which contains different questions appropriate to the infant’s age and stage of breastfeeding.

3.8.6. Home visit 3 & 4 (HV3 & HV4)

Measurements and data collected at HV2 were repeated in HV3, with a modified breastfeeding questionnaire (IFQ III). The measurement of milk intake by isotope (dose to mother) was repeated to measure the infant's milk intake at 12-14 weeks of age. At the final visit (HV4), infant temperament was assessed using the Revised-Rothbart's Infant Behaviour Questionnaire (RIBQ). At this visit, measurement of infant total body water (for body composition) was performed by dosing the infant with isotope (deuterium) 14 days after the dose was given to the mother. A detailed description of this procedure is provided in 3.17.

Figure 3.3 Overview of home visit time points



3.9. Intervention tool: The relaxation therapy recording

Mothers in the intervention group were provided with a relaxation therapy tape to be used during breastfeeding or expressing milk. The tape recording consists of a guided imagery protocol from a CD designed for breastfeeding mothers [215], which has been used previously in a study of mothers with preterm infants [169, 170]. The guided imagery protocol includes descriptions of pleasant surroundings, positive messages about breastfeeding, supportive messages about mother-infant bonding and a progressive muscle relaxation technique such as taking deep breaths. There are two sets of voice protocol recording which last for about 13-15 minutes and a guideline of using the tape (2 minutes duration). The recording was transcribed and translated into the Malay language, in collaboration with a certified clinical psychologist in UPM (Dr Mukhtar). Mothers in the intervention group were asked to listen to the relaxation audio recording (either Malay- or English-version) while breastfeeding during

every visit session (HV1-3). They were also told that the tape could possibly help the mother to be relaxed during breastfeeding and that by doing so this might or might not have beneficial effects on breastfeeding outcomes. They were also asked to listen to the recording daily for at least 2 weeks starting at each HV (HV1 to HV3). In between visits, they were advised to keep using the relaxation therapy daily, as often as they found it useful, and were given a calendar diary to record when it was used. The frequency of listening to the therapy will later be used to calculate the dose response effects of listening to the therapy with all primary outcomes.

3.9.1. Pilot study

After the relaxation recording of the Malay-version was developed, a pilot study was done among breastfeeding women (n=20) to investigate their overall perception towards the Malay-version of the recording by evaluating the voice, pace and intonation of the recording on a scale from 1 (strongly dislike) to 5 (strongly like). The participant's emotions and feelings were also assessed after listening to the therapy by using a questionnaire that consisted of 7 items on a 10 cm scale with the lowest score (0) being 'very little' and the highest (10) being 'very much'. The majority of participants liked the voice, intonation and pace of the relaxation therapy, with an average score of 83%, 78% and 67% respectively. Other participants indicated 'neutral' and only one person disagreed with each of the criteria. The top three scores for the assessment of emotions and feelings were awarded for 'relaxed'(7.9), 'happy'(7.8) and 'alert'(7.6), and the lowest were 'anxious'(1.1) and 'stressed'(1.0). Thus, overall, the findings showed that the majority of participants had a good perception of the recording therapy, and it appeared to produce the expected relaxation effects in terms of their emotions and feelings [216]. A minor amendment to the voice and content of the Malay-version of the therapy was done to improve the recording based on feedback from the pilot study. A feasibility study was also done among several mothers (n=5) in London & Malaysia, prior to data collection, which helped me to familiarise myself with the practical procedures and organize the home visit sessions most effectively.

3.10. Research flow chart

Flow charts providing an overview of the study and of the overall data and sample collection procedures are shown below.

Figure 3.4 Overview of MOM Study

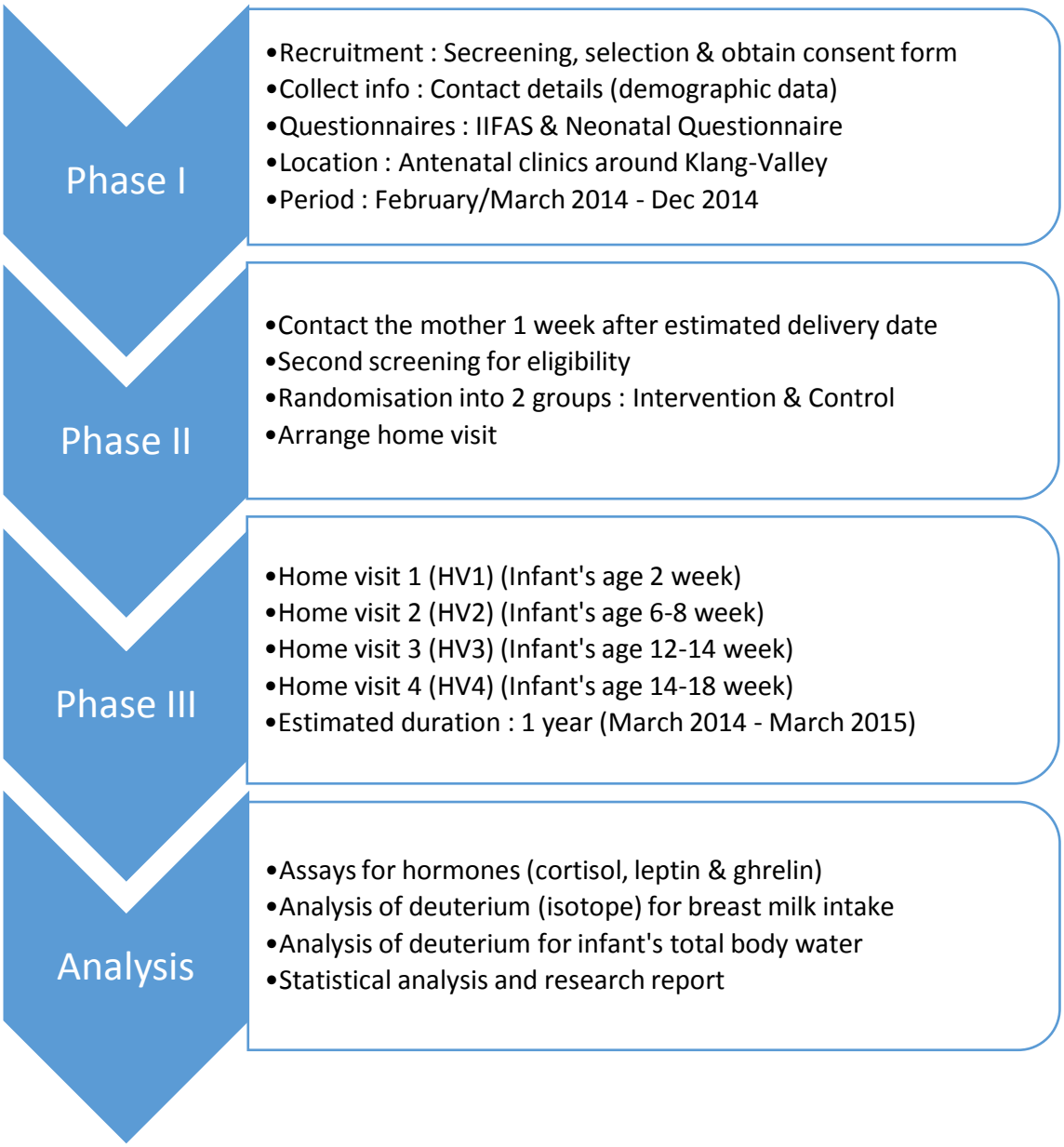
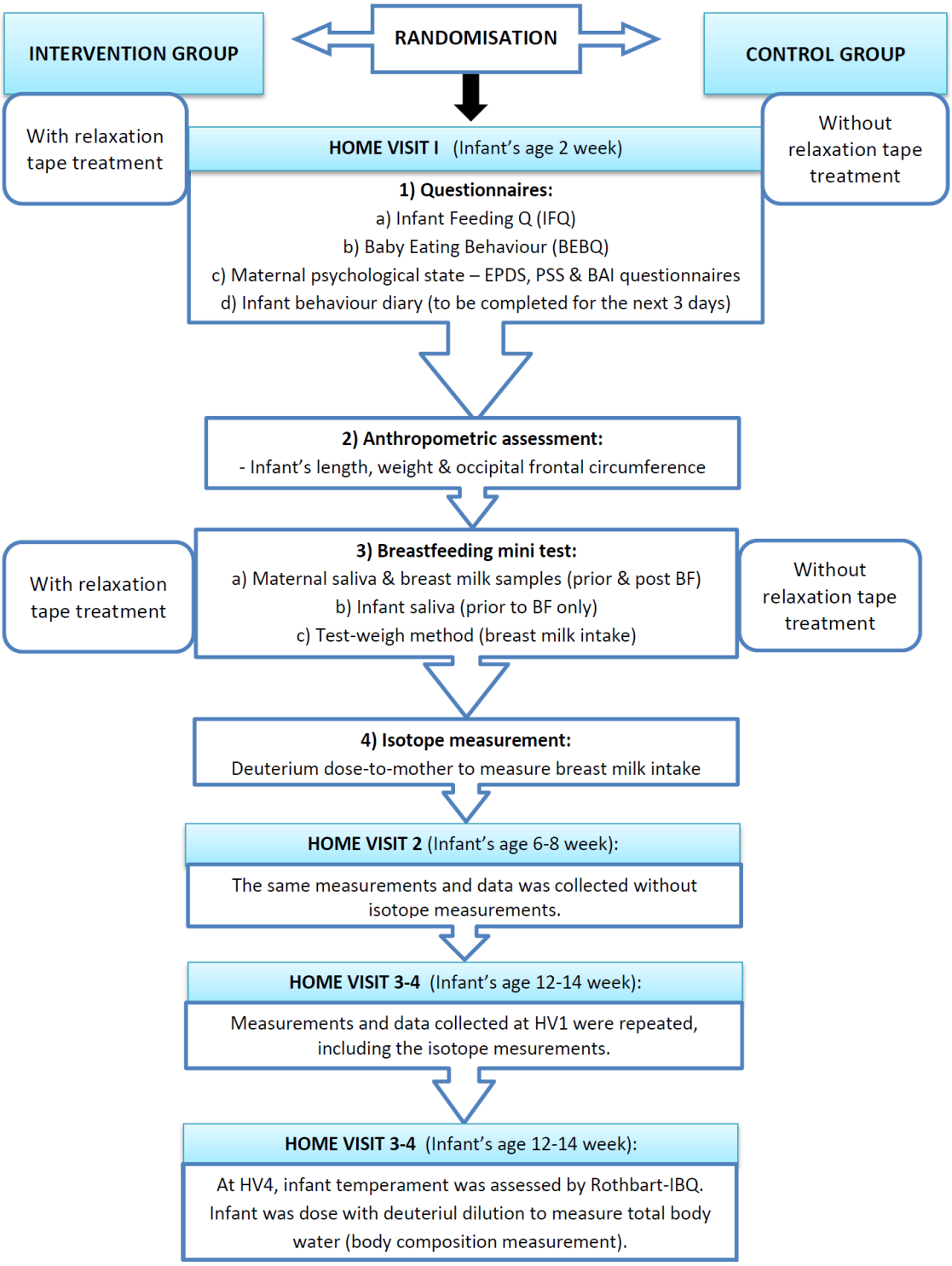


Figure 3.5 Data collection procedures during home visit sessions



B) Research materials and procedures

3.11. Introduction

In this chapter, I provide information about each research tool and the associated outcome measure. Questionnaire measurements are described first followed by clinical measurements. The clinical data comprised anthropometric and biological data, which were all assessed or collected at each home visit (HV). At the end of this chapter, I provide a summary of the planned statistical analyses. Additional information about the statistical analysis for each outcome measure is described in subsequent chapters.

3.12. Questionnaires

Several questionnaires were repeated at each of the HV whilst a few were used only at a specific time point. Below is a list of questionnaires that were used in the study according to the time point, including those that were used during enrolment (Table 3.3). Most questionnaires were available in both languages (English and Malay) to suit the Malaysian population of my study. Several questionnaires (IIFAS, EPDS, PSS and BAI) had already been translated into the Malay language and used among mothers in Malaysia (the validity of the Malay-version of these questionnaires having been tested previously [217-219])**.** Overall, the questionnaires can be categorised into three domains, which are i) breastfeeding practice and attitude, ii) infant behaviour (feeding behaviour and temperament) and iii) maternal psychological state and emotion.

3.12.1. Screening forms

There were 2 stages of screening for eligibility of participants. The first screening was done during recruitment to assess the eligibility criteria of pregnant mothers after they were approached by giving them the MOM Study flyer/poster advertisement (Appendix 6.1). Mothers who showed an interest in the study were asked screening questions and this was followed-up with a detailed explanation of the study. Prior to the first HV (1-2 weeks after birth), all participants were screened again to assess the eligibility criteria for both the mother and baby. If they were eligible and still interested to participate in the study, a HV was arranged.

3.12.2. Socio-demographic questionnaire

The participants were asked about their demographic background factors such as age, living situation, ethnicity, education level, current or most recent occupation, maternal birth order

and the age difference between her and any older siblings. Questions on maternity care and postpartum traditional practices were included in the questionnaire (Appendix 6.2). Mothers were asked who would be taking care of them during the postpartum period (specifically during the confinement period), and for how long and how strictly they would practice the traditional postpartum confinement. Personal data (contact details) were not entered into any electronic database and all participants were identified by a study number.

Table 3.3 List of questionnaires that were used in MOM Study

No	Questionnaires / Forms	Language		Type of Question-naire*	Stages	Method of conduct**
		English	Malay			
Enrolment phase						
1	Screening Questionnaire	X		-	Recruitment	Administered
2	Demographic Questionnaire	X	X	-	Recruitment	Self-completed
Breastfeeding practice and attitude :						
3	Iowa Infant Feeding Attitude Scale	X	X	V	Recruitment	Self-completed
4	IIFAS Add Questionnaire	X	X	A	Recruitment	Self-completed
5	Neonatal Questionnaire	X	X	A	Prior HV	Administered
6a	Infant Feeding Quest. I	X		A	HV 1	Administered
6b	Infant Feeding Quest. II	X		A	HV 2	Administered
6c	Infant Feeding Quest. III	X		A	HV 3	Administered
Infant behaviour :						
7	Baby Eating Behaviour Questionnaire	X	X	V	HV 1,2,3	Self-completed
8	3-day Baby behaviour/crying diary	X	X	V	HV 1 & 2	Self-completed
9	Infant Behaviour Ques.	X	X	V	HV 3	Administered
Maternal psychological state and emotion :						
10	Mini breastfeeding test	X	X	A	HV 1,2,3	Self-completed
11	Perceived Stress Scale	X	X	V	HV 1,2,3	Self-completed
12	Beck Anxiety Inventory	X	X	V	HV 1,2,3	Self-completed
13	Edinburgh Postnatal Depression Scale	X	X	V	HV 1,2,3	Self-completed

* V = Questionnaires that has been validated previously; A = Questionnaire that was adapted from previous studies. ** Self-completed = completed by the mother; Administered = Questionnaire-led interview by the researcher.

3.13. Questionnaires on breastfeeding practice and attitudes

During the third trimester of pregnancy, participants were asked about their perception towards infant feeding and their goals for breastfeeding using the Iowa Infant Feeding Attitude Scale (IIFAS). They were also given a Neonatal questionnaire to complete about their delivery and early experience of breastfeeding. Breastfeeding practice and attitudes at different stages of breastfeeding were assessed at home visit 1, 2 and 3 using Infant Feeding Questionnaires.

3.13.1. Iowa Infant Feeding Attitude Scale (IIFAS)

The questionnaire was administered after the enrolment process. It consists of 17 questions and the participants were asked to give their opinion and perceptions of infant feeding based on a scale of 1 (strongly disagree) to 5 (strongly agree). This questionnaire has been used extensively and has been tested for reliability with Cronbach's alpha ranging from 0.85 to 0.86 [220]. In the IIFAS Add Questionnaire, a few questions were added which enquired about the mother's response to some statements on breastfeeding, her goal for feeding her infant after birth, and plans for work after birth.

3.13.2. Neonatal questionnaire

This questionnaire was adapted from a cohort study in the US, the Infant Feeding Practices Study II (IFPS II), developed by the US Food and Drug Administration (FDA) and the Centers for Disease Control and Prevention (CDC) [221]. Most questions were taken from the first cohort (Infant Feeding Practices I) and had been tested in four pilot studies before being used in the survey. The adapted version of this questionnaire used in the MOM study consisted of three parts: sources of information about breastfeeding; experience of birth and timing of first breast feed. The questionnaire was given to the mother during recruitment for completion after delivery or during the first HV.

3.13.3. Infant Feeding Questionnaires (IFQ)

These self-reported questionnaires are adapted from the IFPS II and have been used in the First-Feed study among breastfeeding women in Glasgow, UK [222]. These questionnaires consist of 5 main parts: a) breastfeeding at present, b) breastfeeding in the future, c) breastfeeding attitudes and difficulties, d) sleeping arrangements and e) health information. There are three different versions of the questionnaires: IFQ I, II and III, each suited to a different infant age and stage of breastfeeding. These questionnaires were only available in English, therefore they were completed through a researcher-led interview during each HV.

3.14. Questionnaires on infant behaviour

Infant feeding pattern and appetite were assessed at different ages by a self-administered Baby Eating Behaviour questionnaire (BEBQ) at HV 1-3 (Appendix 6.8). After HV1 and 2, participants were also asked to record the duration of their infant's feeding, crying and sleeping for three consecutive days in a 3-day infant diary (Appendix 6.10) to assess the overall infant behaviour in early life. Later, at the last visit (HV4), infant temperament was assessed using the Revised-Rothbart's Infant Behaviour Questionnaire (RIBQ) (Appendix 6.14).

3.14.1. Baby Eating Behaviour Questionnaire (BEBQ)

BEBQ is derived from an existing psychometric measure validated for older ages, the Children's Eating Behaviour Questionnaire, supplemented by a review of the literature on milk-feeding behaviours. It has been used in a large birth cohort study in the UK, and appears to be reliable, with Cronbach's alpha values ranging from 0.73 to 0.81 [223]. BEBQ can be used to measure infant appetite and eating behaviour during the period of exclusive milk feeding, which makes it well-suited for the new-born infant. It consists of 18 items designed to measure four traits, including a single item for general appetite (GE): 'enjoyment of food' (4 items), 'food responsiveness' (6 items), 'slowness in eating' (4 items), and 'satiety responsiveness' (3 items). The mothers were asked to respond according to how they would described their infant's feeding at a typical daytime feed based on a scale from 1 (never) to 5 (always).

3.14.2. 3-day Infant Behaviour Diary

Infant feeding and crying behaviour were recorded at 2-4 and 6-8 weeks using a validated 3-day diary. The diary consists of a time scale for 72 hours, which is divided into 15 minutes segments, and has five categories of behaviour: sleeping, crying, fussy, awake and content, and feeding [224]. The description of each behaviour was explained to all mothers, and a written definition was included in the questionnaire. Each category has its own characteristic shading pattern, and the mother was asked to fill in the timescale with the appropriate shading according to the infant's behaviour. The crying element has been validated using audio recordings [225]. This diary has previously been used in many infant research studies including in ICH (my department).

3.14.3. Revised Rothbart's Infant Behaviour Questionnaire (R-IBQ)

Development of temperament in the infant is rapid, varies across infancy, and is reliably observed starting at the age of 2 months [226]. Thus, infant temperament was measured at 14-16 weeks using the validated RIBQ based on a 7-point Likert scale, from 1 (never) to 7 (always). [226]. Three major dimensions were used for the assessment of infant temperament: surgency/extraversion, negative affectivity and effortful control. The reliability and validity of this questionnaire has been reported in many previous studies [226, 227].

3.15. Maternal psychological assessment

Maternal emotions before and after feeding were assessed during the breastfeeding session of HV 1, 2 and 3 using a Mini-breastfeeding questionnaire (Appendix 6.9). The mother was left alone to complete this questionnaire. After the HV sessions (HV1-3), participants were given a set of questionnaires to be completed at their convenience, which were used to assess maternal stress, anxiety and depression using the Perceived Stress Scale, Beck Anxiety Inventory and Edinburgh Postnatal Depression Scale, respectively.

3.15.1. Mini-breastfeeding questionnaire

The Mini-breastfeeding Questionnaire (MBQ) is a self-report questionnaire that consists of 10-items used to measure the mother's emotional state on a visual analogue scale, with the least being 'very little' and the most being 'very much'. Mothers were asked to record their feelings and emotions before and after each breastfeeding session during HV 1-3. A vernier calliper was used to measure the length division that the mother marked on the scale. This questionnaire has previously been used by my research group, with very good rates (90-100%) of completion.

3.15.2. Perceived Stress Scale (PSS)

Cohen's Perceived Stress Scale (PSS) is a psychological self-reported instrument that consists of 14-items for measuring the perception of stress on a scale of five, from 0 (never) to 4 (very often) [228]. It appears to be reliable, and has been validated and used extensively globally, including in three national surveys in the United States [228, 229]. This questionnaire has also been translated and validated for the Malaysian population [219]. Each mother was given both English and Malay versions (Appendix 6.12) to answer (either version) at their convenience after home visit 1,2 and 3 (HV1-3).

3.15.3. Beck Anxiety Inventory (BAI)

Beck Anxiety Inventory (BAI) is a self-report instrument, consisting of 21-items, that is used to measure the severity of different aspects of anxiety such as numbness, fear, anxiety and nervousness, on a scale of four, from 0 (not at all) to 3 (severe)[230]. It has been shown to have high internal consistency and reliability (Cronbach's alpha values = 0.94) [230], and has also been translated into Malay and validated in the Malaysian population with excellent overall alpha values (0.91) [217]. Each mother was given both English and Malay versions (Appendix 6.13) to answer at their convenience after HV 1,2 and 3.

3.15.4. Edinburgh Postnatal Depression Scale (EPDS)

The Edinburgh Postnatal Depression Scale (EPDS) is a self-report questionnaire, consisting of 10-items questions, that is used to screen and identify women with perinatal depression [231]. This questionnaire has been used extensively worldwide, is well-validated and appears to be reliable and sensitive in detecting depression [232]. This questionnaire has also been translated and validated for the Malaysian population [218]. Each mother was given both English and Malay versions (Appendix 6.11) to answer at their convenience after HV 1,2 and 3. After each HV, the mother's score was calculated to identify if she was depressed. The plan was that if a mother was found to be depressed, she would be advised to seek help from a health professional or contact her GP. However, in this trial, none of the participants were identified as depressed throughout the study period.

3.16. Anthropometric assessment

3.16.1. Measurements on mothers

Weight was measured on a clinical weighing scale (Seca Meter, Germany) to the nearest 0.1 kg at the first to third HV. The measurement was repeated three times and the mean value used. The mother's height and pre-pregnancy and late gestation weight were recorded based on the antenatal clinic records.

3.16.2. Measurements on infant

Anthropometric measures (recumbent length, weight and head circumference) on the infant were carried out at all home visits (Figure 3.7). All measurements were repeated three times and the mean value used. The BMI was also calculated from the anthropometric data as follows: weight (kg) / length (m^2).

i) Weight

Weight was measured on a digital infant weighing machine (brand Seca 834, Germany), which was calibrated regularly throughout the data collection period. During the measurement, a towel was placed over the scale before resetting to zero. All infants were weighed naked, with an accuracy of 0.01 kg.

ii) Recumbent length

Infant length was measured to the nearest 1 mm by using an Infant Length Measuring Mat (Rollameter 60, UK) with a fixed headboard on one end and a measuring tape on a movable vertical plate on the other end. During the measurement, the infant's lay supine, with the head against the fixed headboard and the body parallel to the board's axis, following the Frankfurt Plane position as in the Figure 3.6 below. The vertical plate was placed against the base of the infant's feet. The infant's legs were straightened by holding the legs by the ankles with one hand and applying a gentle downward pressure over the legs with the other hand. The mother was asked to check if the infant's head was still in position and touching the headpiece. Once the infant was in a straight line position with feet straight, the vertical plate was mounted to touch the soles of the infant's feet, with toes pointing directly upward. The measurement was read from the red arrow in the reader window.

Figure 3.6 Frankfurt Plane Position

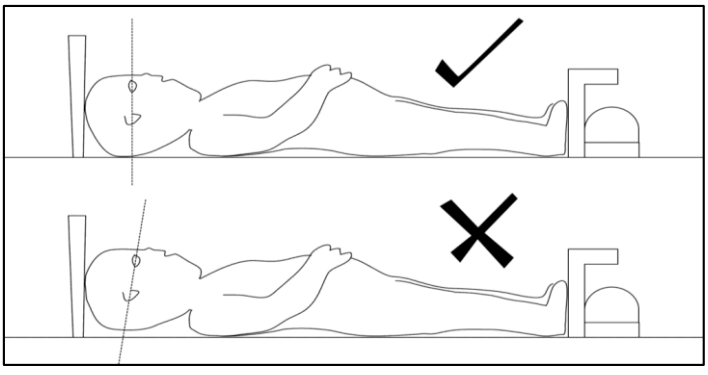


Figure 3.7 Measurement of infant weight (left) and length (right)

iii) Head circumference

A flexible non-stretchable measuring tape (SECA 212 tape) was placed just above the eyebrows and ears, and around the occipital prominence at the back of the head, so that the maximum circumference (largest diameter) was measured, and the measurement was read to the nearest mm. Three measurements were done and a mean of all measurements or best of two was calculated.

iv) Infant body composition

Infant body composition was measured using the isotope dilution method at the final visit (HV4) when the infant was aged 14-18 weeks, by calculating the total body water (TBW) from the isotope samples analysis results. This method is based on the assumption that fat-free mass (FFM) has a relatively constant water content with insignificant water associated with fat stored in adipose tissue [233]. The calculation to estimate the FFM is described in Chapter 5. To perform this measurement, the deuterium oxide (^2H_2O) was administered orally by dripping the solution directly into the infant's mouth using a 10 ml syringe (Figure 3.8), as a previous study showed this to elicit the best cooperation from infants [234]. However, for some infants the deuterium was administered in milk using a sterilised milk bottle according to the mother's preference. Weighed tissues were placed near baby's mouth while dosing to wipe any spillage. After dosing, the syringe or milk bottle and used tissues were re-weighed to estimate the exact dose that was given to the baby. Infant urine samples were collected at baseline (prior to dose) and following 24- and 48-hours post-dose. The dose preparation and sample collection are described in Appendix 7. All samples collected were stored in the freezer at -80°C until analysis. The isotope enrichment samples were measured by isotope ratio mass spectrometry (IRMS) [233, 235].

Figure 3.8 : Administration of isotope (left: bottle feeding method; right: syringe method)

3.17. Collection of biological samples

During HV1 to HV3, mothers were asked to provide samples of breast milk (fore- and hind milk) and saliva before and after a breastfeeding session for measurement of cortisol and macronutrients. Maternal saliva and infant urine samples were also collected to measure breast milk intake using the isotope dilution method. All samples collected were kept cool in an insulated box containing frozen silica pads, before being stored in the freezer within 4 hours after collection.

3.17.1. Collection of breast milk samples

The mother was asked to express about 10-15 ml of breast milk before and after the breastfeeding session (fore- and hind milk), with the time of collection being recorded (Figure 3.9). The mother could choose how to express breast milk - either by hand, using the Philips Avent breast pump provided, or using their own pump. Prior to expressing milk, all mothers were encouraged to massage their breast in order to stimulate milk ejection. Milk samples were stored temporarily in milk storage containers, which were kept in an insulated box containing a frozen silica pad during the visit. After a completed HV, milk samples were then transferred into 15 ml tubes and a portion of the samples were acidified and transferred into 2ml tubes. Before storage, one set of milk samples was acidified by adding 1 N HCL (10% of volume, pH 3-4), as recommended [236]. This is to stabilize the labile side chain of active ghrelin and to prevent its rapid deacylation. All samples were stored at -80^{o}C until analysis.

Figure 3.9 : Milk sample collection and storage

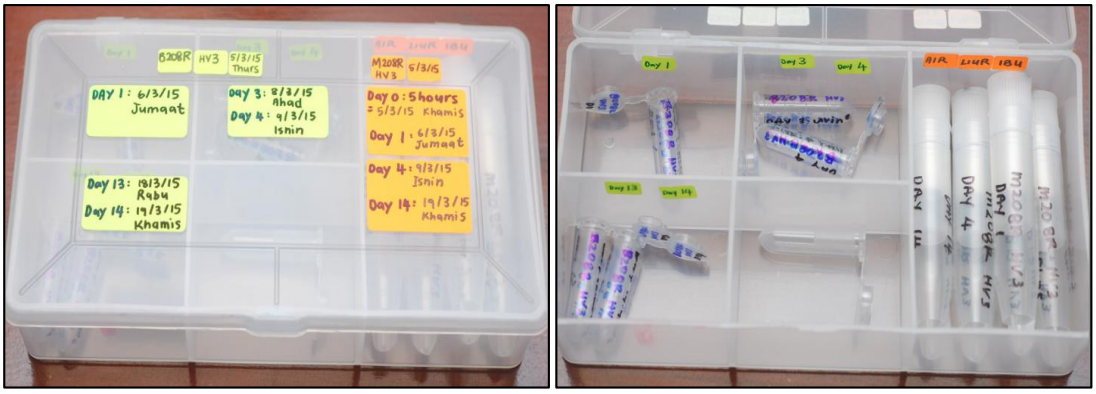
3.17.2. Collection of saliva samples

The mother was asked not to eat or drink for at least half an hour prior to sample collection. The saliva sample was collected using a salivary oral swab (salivette), which the mother gently rolled in her mouth for 2 minutes and then placed into the salivette tube. After home visits, salivettes were centrifuged and saliva samples were transferred into 2 ml tubes, which then were stored at -80 ^{o}C until analysis.

3.17.3. Collection of isotope samples

Breast milk intake was measured at 2-4 weeks (as baseline data at HV1) and 12-14 week (at HV3) using a stable isotope (deuterium) probe, which allows the measurements to be performed without interfering with the breastfeeding process [233, 235]. To dose the mother, she was asked to drink approximately 30 g deuterium diluted in water ($^{2}H_2O$) through a straw. A maternal baseline salivary sample was obtained using a salivette prior to dosing and on days 1, 4 and 14 post-dose. The mother was asked not to eat or drink 30 minutes prior to collecting the saliva sample. Infant urine samples were also collected at day 0 (as baseline sample) and then on days 1, 3, 4, 13 and 14 post-dose (Figure 3.10). Detailed instructions on urine sample collection were given and the technique was demonstrated to the mother during the first home visit session, as described in Appendix 6.7. To estimate infant total body water, additional urine samples were collected after administering 10 g deuterium ($^{2}H_2O$) to the infant as indicated above (3.16); baseline urine and post-dose samples were collected after 24- and 48 hours. All samples were stored in 2ml tubes at -80 ^{o}C until analysis. Total milk intake and total body water were estimated based on the measurement of deuterium enrichment by isotope ratio mass spectrometry (IRMS) [233, 235] at the ICH.

Figure 3.10 Tubes provided to the mother to store urine samples (2 ml tubes) and saliva (salivette).



3.18. Analysis of biological samples

Breast milk samples were analysed to determine the hormone (cortisol, leptin and ghrelin) and macronutrient content. The macronutrient content of breast milk (n=380 samples) was analysed using the MIRIS analyser [237] at UCL, as described in chapter 5. The breast milk cortisol, leptin & ghrelin (n=384 samples for each) were measured using commercially available enzyme-linked immunosorbent assay (ELISA) kits; cortisol saliva human ELISA, leptin human ELISA and ghrelin acylated & total human ELISA kits, respectively. All assays for hormones were performed by my collaborators from the Faculty of Health Sciences, University of Primorska, Slovenia. They are experienced in measuring a variety of hormones (including cortisol, leptin and ghrelin) in biological samples, including human milk. Before storage, one set of milk samples was acidified by adding 1 N HCL (10% of volume, pH 3-4), as recommended [236]. This is to stabilize the labile side chain of active ghrelin and to prevent its rapid deacylation. The samples were then stored at -80^{o}C until analysis.

3.18.1. Analysis of milk hormones

A detailed description of the analysis of milk cortisol, leptin and ghrelin was presented in Chapter 5.

3.19. Data entry

Data that were found to be ambiguous were flagged up and re-checked, and mothers were contacted for clarification where necessary. Data were recorded on Excel datasheet first and later exported to SPSS. Infant weight, height, head circumference and BMI were converted to standard deviation scores (Z-score) using WHO 2006 growth standard data (using the LMS growth add-in for Microsoft excel).

3.20. Statistical analyses

Questionnaires and anthropometric data were analysed using IBM SPSS (version 23). Normality of continuous data was assessed by Q-Q Plot, histogram and was also tested by the Kolmogorov-Smirnov Test. The main analysis was an intention-to-treat analysis comparing the primary outcomes between randomised groups using unpaired t-tests; where baseline and post-intervention data are available, the within-subject change was compared between groups using paired t-tests. Paired & unpaired t-test and repeated measures were used to examine changes in milk intake and other milk components including cortisol levels between groups and between time points respectively. An Independent t-test was used to test for gender differences in milk intake, energy content and hormonal levels. Associations between infant temperament/behaviour and gender and milk volume, milk composition, infant growth and body composition were examined using univariate analyses. Correlation and regression analysis/MANOVA were then used to adjust for confounding factors. Interaction terms were introduced where appropriate in the multivariate analysis. The cut-off used for significance was set at $p<0.05$. Although multiple testing were done to test multiple outcomes within the randomised trial and observational cohort study outcomes, the p-value cut-off point was not adjusted (for multiplicity) since separate univariate analyses were done and all results were independently to each other. Further details and description of specific statistical analyses are described in the following chapters. Internal consistency estimates of reliability for questionnaires (Mini-Bf test, BEBQ and RIBQ) were evaluated using Cronbach's α value.

Table 3.4. Description of data collected according to key categories findings

Key finding	Parameters	Measure	Tool	First meet	HV1 (n=64)	HV2 (n=63)	HV3 (n=63)	HV4 (n=62)	Main stat. analysis
Socio-demographic	Maternal characteristic	Date of birth Age Ethnicity Parity / Birth order	Demographic Q	√					Descriptive Univariate (T-test)
	Socio economic status	Education level Occupation Household income Mother's care taker	Demographic Q	√					Descriptive
	Infant characteristic	Gender Gestation age Birthweight Birth length	Screening Q II		√				Descriptive Univariate (T-test)
Maternity	Birth planning	Place of delivery Care taker after birth Maternity leave BF goal Confinement practice	Demographic Q IIFAS Add Q	 √	√				Descriptive
	Birth experience	Method of delivery Birth attendant Medication (labour) Hospital stay BF advice Skin-to-skin contact First breastfeeding	Neonatal Q		√				Descriptive

Key finding	Parameters	Measure	Tool	First meet	HV1 (n=64)	HV2 (n=63)	HV3 (n=63)	HV4 (n=62)	Statistical Analysis
Breastfeeding	Perception	Formula feeding BF perception Father's role	IIFAS	√					Descriptive Univariate (T-test)
	Attitude	Method of feeding Benefits of BF Goal of BF	IIFAS Add Q	√					Descriptive Univariate (T-test)
	Early postnatal experience	BF support Early BF experience BF problems Neonatal health	IFQ I		√				Descriptive Univariate
	Establishing BF	Duration of BF Breast pump usage Target of exclusive BF BF attitudes Sleeping arrangements Postpartum confinement	IFQ II			√			Descriptive Univariate
	Regular BF practice	Duration of BF BF attitudes Breast pump usage Milk expression schedule Feeding expressed milk Target of BF duration	IFQ III				√		Descriptive Univariate

Key finding	Parameters	Measure	Tool	First meet	HV1 (n=64)	HV2 (n=63)	HV3 (n=63)	HV4 (n=62)	Statistical Analysis
Short-term psychological state	Emotion & feeling during BF	Stress, anxiety and tired Happy, relax and calm Alert, tired and sleepy	Mini-BF test scale		√ (P-P)	√ (P-P)	√ (P-P)		Univariate (T-test)
Long-term psychological state	Stress Anxiety Depression	Stress level Anxiety level Depression identification	PSS BAI EPDS		√ √ √	√ √ √	√ √ √		Univariate (T-test)
Baby behaviour	Appetite	Food responsiveness Enjoyment of food Satiety responsiveness Slowness in eating	BEBQ		√	√	√		Univariate (T-test) Descriptive Univariate
	Daily behaviour	Sleeping, crying, awake and feeding duration	Baby 3-d diary		√	√			(T-test) Bivariate
	Temperament	Surgency Negative affect Effortful control	RIBQ					√	(correlation) Multivariate (Regression)
Anthro-pometric	Maternal size	Pre-preg. weight & height	Record data	√					Univariate
		Weight	Weighing scale	√	√	√	√		(T-test)
	Infant body size	Weight Height Head circumference	Weighing scale Roll-meter Measuring tape		√ √ √	√ √ √	√ √ √	√ √ √	Univariate (T-test) Bivariate (correlation) Multivariate (Regression)
	Infant body composition	Total body water (isotope technique)	Mass-spec					√	Univariate (T-test)

Key finding	Parameters	Measure	Tool	First meet	HV1 (n=64)	HV2 (n=63)	HV3 (n=63)	HV4 (n=62)	Statistical Analysis
Physiological changes	Hormones	Salivary cortisol Milk cortisol Milk leptin Milk ghrelin	ELISA kits		√ (P-P) √(P-P) √(P-P) √(P-P)	√ (P-P) √(P-P) √(P-P) √(P-P)	√ (P-P) √(P-P) √(P-P) √(P-P)	√ (P-P) √(P-P) √(P-P) √(P-P)	Univariate (T-test) Bivariate (correlation) Multivariate (Regression)
	Milk intake	Isotope samples	Mass-spec		√		√		T-test
	Breast milk composition	Fat content Protein content Carbohydrate content Total calories	MIRIS analyser		√ (P-P) √(P-P) √(P-P) √(P-P)	√ (P-P) √(P-P) √(P-P) √(P-P)	√ (P-P) √(P-P) √(P-P) √(P-P)	√ (P-P) √(P-P) √(P-P) √(P-P)	Univariate (T-test) Bivariate (correlation) Multivariate (Regression)

BF=breastfeeding; * P-P : Measured at prior and post feeding within a BF session

CHAPTER 4

4. THE BASELINE CHARACTERISTICS OF THE STUDY POPULATION

4.1. Introduction

This chapter provides descriptive characteristics of the whole MOM Study population (n=88) including the results of the questionnaires measuring the mothers' attitudes towards breastfeeding during pregnancy, and a comparison of the results from the study population with National data. The whole study population is categorised into two groups: 'exclusion' and 'inclusion', and their results are compared. The exclusion group comprised mother-infant dyads who were not eligible for the second phase of the study and thus excluded from the randomisation; whereas the inclusion group comprised those who were eligible for randomisation for the next phase of the study.

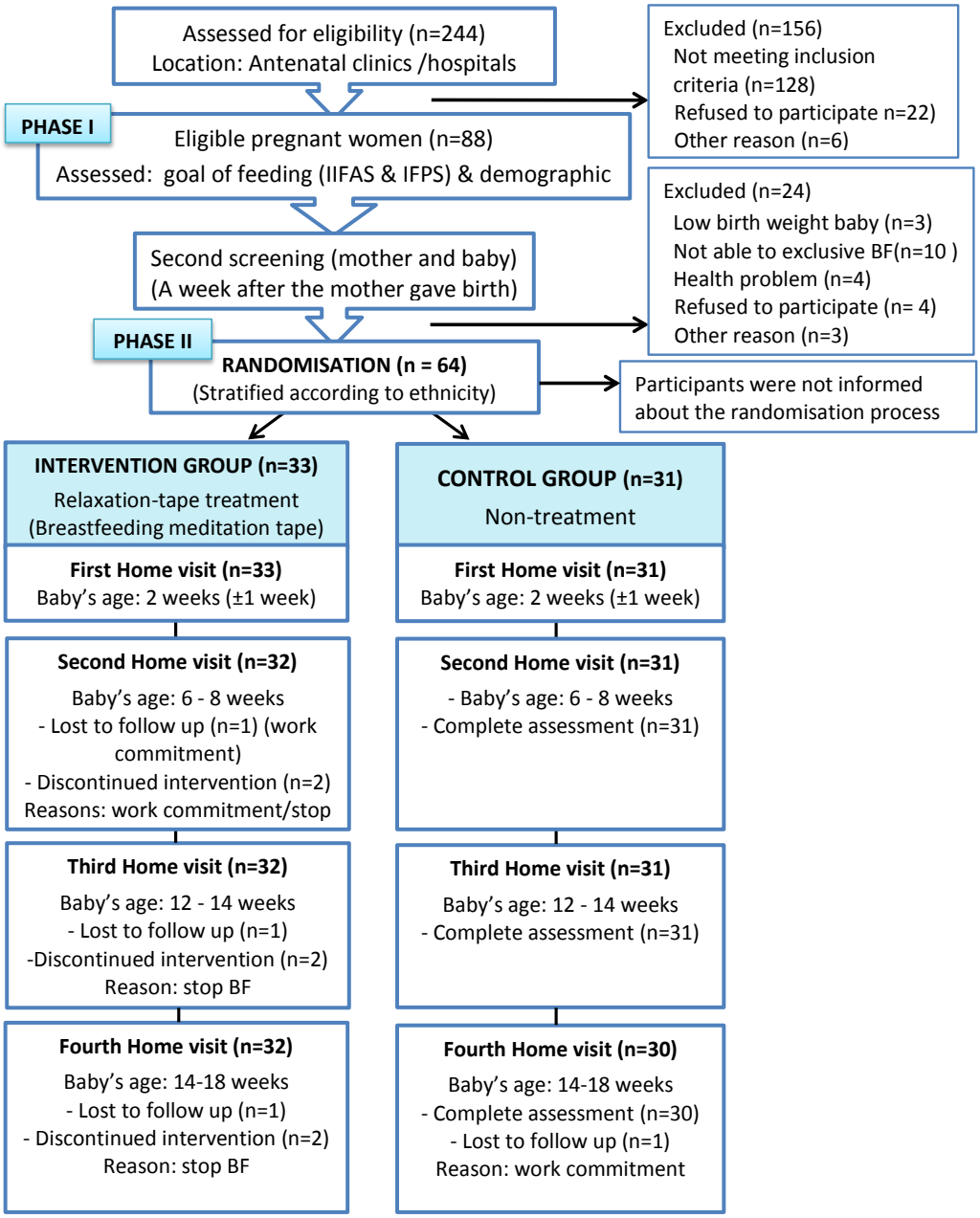
The main aims in this chapter were i) to compare my study population with the general Malaysian population and ii) to identify any differences between the inclusion and exclusion groups. Results for the randomised trial (n=64) are presented in the next chapter.

4.2. Study population and follow-up visits

Recruitment into the study finished in December 2014 and the data collection was completed in March 2015. Figure 4.1 shows the flow chart of the different phases of the study including the follow-up visits. A total of 244 mothers were approached during the recruitment stage and only 36% (n=88) were eligible for and/or interested in participating in the study. Of the 64% of women that were excluded from the study, 82% (n=128) did not meet the inclusion criteria, 14% (n=22) refused to participate and 4% (n=6) did not respond to the researcher. The second screening was done after the mothers delivered their babies, and only 64 healthy

mothers (who were exclusively breastfeeding) and their infants (full-term baby; ≥ 2.5 kg at birth) remained eligible. Of the 27.5% (n=24) that were non-eligible at the second screening, 42% (n=10) were not able to exclusively breastfeeding, 12.5% (n=3) infants were low birth weight, 16.5% (n=4) mothers refused to participate, and the others either had health problems (16.5%) or other personal reasons (12.5%; for example, some of them indicated that they were not confident that they would be able to exclusively breastfeed).

Figure 4.1 Flow chart of subjects through the study



4.3. Methods

4.3.1. Socio-demographic background information

All study participants (n=88) completed a socio-demographic questionnaire as described in Chapter 3 (3.12). The descriptive characteristics of the study population were compared to those of a Malaysian population, of similar age, region and gender [207, 208, 238-240].

4.3.2. **Attitudes towards breastfeeding**

The IIFAS questionnaire was self-completed by 87 mothers during the third trimester of pregnancy to assess their perception towards infant feeding. The questionnaire was described in 3.13. The mothers rated each question on a 5-point Likert scale ranging from 1 (strongly disagree) to 5 (strongly agree). Questions favouring formula feeding were reverse-scored, and the sum scored was calculated to identify the total score. Total attitude scores could range from 17 (indicating positive attitudes toward formula feeding) to 85 (reflecting positive attitudes towards breastfeeding). A score of 51 indicated a neutral attitude. The additional part of IIFAS assessing the agreement with some breastfeeding statements (questions adapted from the IFQ), rated by the mothers on the same 5-point Likert scale, was also summed to calculate the total score. Total scores could range from 5 (reflecting disagreement with the suggested advantages of breastfeeding) to 25 (reflecting agreement with the suggested advantages of breastfeeding). The intended feeding period indicated by the mothers during pregnancy was recorded in weeks and was categorised into 5 groups (the duration of breastfeeding from 6 months to 2-3 years). The mother's confidence level that she would achieve her goal was rated from 1 (strongly unconfident) to 5 (strongly confident).

4.3.3. **Statistical analysis**

Outcome measurements for the socio-demographic factors and breastfeeding goals were presented as frequency and/or percentages, and were followed up with univariate analysis (Chi-square or Fisher Exact test) in order to compare groups. Outcome measurements that involved continuous numbers were checked first for normality by using Q-Q Plots and histograms. For normally distributed data, mean ± standard deviation (SD) was presented along with the statistical result (T-test or ANOVA) for group comparisons. Alternatively, median ± interquartile range (IQR) was presented along with the non-parametric test (Mann-Whitney or Kruskal-Wallis test) result. Associations between variables were also assessed using Pearson Product or Spearman Rank correlation. The overall plan of statistical analyses.

4.4. Baseline results

Data collected for the whole study population (n=88) during phase I of the study comprised socio-demographic factors and maternal attitude towards breastfeeding, including information about maternal perception towards infant feeding and also her breastfeeding goal.

4.4.1. Socio-demographic background

Table 4.1 shows the socio-demographic characteristics of the study population and the reference population data for the central region of Malaysia (Klang-Valley), collected as part of the national data. 43% of the infants were male, and there was no significant difference in the number of male and female infants between the inclusion and exclusion groups (X^2=1.2, p=0.275). The study sex ratio is consistent with the national data from 2014, in which the proportion of males and females was 48% vs 52% [208].

The majority of the study population were Malay (89.8%), followed by Chinese (5.7%) and Indian (4.5%). Although Malay is the majority population in Malaysia [207], this study has an over-representation of Malay mothers. The mean maternal age of the study population was 27 years (±3 years) with the majority in the 26 to 30 years group (64.8%), which is almost double the percentage of the national data within this band. All mothers in the study (100%) were married, consistent with the national data (99% of married women within the age range of 20-35 years in the Klang-Valley area were neither divorced nor widowed [207]). The majority of the study population were highly educated (69.4%), with the highest proportion achieving tertiary level (58% Bachelor degree and 11.4% Postgraduate degree) and others (30.7%) having completed their education at school (14.8%) or pre-university college (15.9%). In contrast, the majority of the women within the same age category in Klang-Valley attained their highest education at school or pre-university college (63%), and only 32% continued their studies up to tertiary level [238]. The household income of the study population was approximately similar to the Klang-Valley population, with the majority having a total household income between RM1500 and RM8000 (£240-1265) [239].

Table 4.1 Descriptive characteristics of participants

Descriptive characteristics		Exclusion (n=24)	Inclusion (n=64)	Total (all) (n=88)		National data
		n	n	n	(%)	%
Baby's gender	Male	12	25	37	(42.5)	48.4 [a]
	Female	11	39	50	(57.5)	51.6 [a]
Mother's ethnicity	Malay/Bumiputera	18	61	79	(89.8)	58.6 [b]
	Chinese	3	2	5	(5.7)	28.4 [b]
	Indian	3	1	4	(4.5)	12.9 [b]
Age group	20-25	1	21	22	(25)	35.9 [b]
	26-30	19	38	57	(64.8)	36.1 [b]
	31-35	4	5	9	(10.2)	28 [b]
Marital status	Married	24	64	88	(100)	99 [b]
Educational levels	School	3	10	13	(14.8)	63 [c]
	Certificates/Diploma	6	8	14	(15.9)	
	Bachelor degree	12	39	51	(58)	32 [c]
	Postgraduate	3	7	10	(11.4)	
Household monthly income	<1500 (<£240)	1	0	1	(1.2)	1.4 [d]
	1500-3000	7	19	26	(31)	16.8 [d]
	3001-5000	9	16	25	(28.4)	28.1 [d]
	5001-8000	2	19	21	(23.9)	25.2 [d]
	8001-10000	0	6	6	(7.1)	9.7 [d]
	>10000 (>£1580)	1	4	5	(6)	18.8 [d]
Birth hospital	Public hospital	13	38	51	(58)	78[e]
	Private hospital	9	26	35	(39.8)	22[e]
Main maternity care person	Husband	4	26	30	(35)	-
	Parents	13	31	44	(51.2)	-
	In-laws	1	3	4	(4.6)	-
	Sibling/relatives	4	0	4	(4.6)	-
	Confinement lady	0	2	2	(2.3)	-
	Self (No one)	0	2	2	(2.3)	-
Levels of practicing traditional post-partum confinement	Very strong	5	6	11	(13.1)	-
	Strong	8	21	29	(34.5)	-
	Medium	6	35	41	(48.8)	-
	Low	0	2	2	(2.4)	-

[a] [208]; [b] [207]; [c] [238]; [d] [239]; [e] [240]

Consistent with the National reference data [240], more mothers in the study gave birth at government hospitals rather than private hospitals (58% vs 39.8%). Almost all mothers (99%) indicated that, after birth, they would be practicing the traditional postpartum confinement for an average duration of 44 days, with a range from a minimum of 7 to a maximum of 100 days. About half of the mothers (48.8%) indicated that they would follow the traditional postpartum practice at a medium level, while almost half would practice it strongly (34.5% indicated strong level and 13.1% indicated very strong/strict). Only 2.4% indicated that they would follow the tradition only minimally (low level) post-delivery.

Within the whole study population, there were no significant differences between groups (inclusion vs exclusion) for socio-demographic variables except age (Appendix 8: SPSS output). Mothers in the inclusion group were significantly younger than those in the exclusion group (26.7±2.8 v 28.5±2.5, p=0.007, CI: 0.5, 3.1) and there was also a significant difference in age category between included and excluded mothers (X^2=9.1, p=0.008). The birth order of the mothers was also significantly different between groups, as those in the inclusion group were more likely to have older siblings than excluded mothers (median birth order 1±1 IQR v 3 ±2 IQR, p=0.01). After delivery, 35% of mothers were mainly being taken care of by their husband and 51.2% were mainly being taken care of by their parents. The main maternity care person was significantly different between groups (Fisher's Exact: X^2=12.8, p=0.01) as more husbands were the main primary maternity care person in the inclusion group (68%) compared to the exclusion group (22%). Maternal age was associated with educational levels (n=88, r=0.22, p=0.04) and household income (n=84, r=0.24, p=0.03).

4.5. Attitudes towards breastfeeding

Both inclusion and exclusion groups had a similar perception towards breastfeeding with IIFAS mean scores of 67 ± 6SD and 66.4± 6SD respectively (p=0.42, CI:-4.2,1.8), showing a positive perception towards breastfeeding. Considering individual items in the IIFAS (Table 4.2), the inclusion group scored significantly higher for two items; *Formula feeding is better choice if a mother plans to work outside home* and *Women should not breastfeed in public places* (p=0.016 and p=0.028 respectively), indicating greater disagreement with these statements. Scores for these items were also positively associated (r=0.46, p<0.001), indicating similarity in the perceptions towards breastfeeding in public or at work. No significant differences were found for any other items as shown in Table 4.2.

The agreement with the breastfeeding statements (adapted IFQ questionnaire section) was not significantly different between groups (U: 583, p=0.231), with a median score of 18±8 IQR and 19±7 IQR (full score is 25). This shows that the majority of the mothers were in overall agreement with the statements on the advantages of breastfeeding. There were also no significant differences between groups for all 5 questions from the adapted IQR questions (all p>0.05). All mothers in the study planned to exclusively breastfeed their infants for at least 5 months with a median duration of planned breastfeeding of 24 months. The majority of the mothers (63%) were confident that they would be able to achieve their goal for breastfeeding with 44% and 19% scoring 4 (confident) and 5 (strongly confident) respectively on a scale of 5. There was also no significant difference in confidence level for achieving the goal of breastfeeding between groups (X^2 =4.3, p=0.36). The overall IIFAS score was significantly associated with the adapted IFQ section in the questionnaire (r=0.294, p=0.006) and with the confidence level for achieving the breastfeeding duration goal (r=0.285), p=0.008).

4.6. Breastfeeding attitudes and demographic factors

The IIFAS score was significantly positively associated with the study population education level (r=0.31, p=0.003), household income (r=0.32, p=0.003), and maternal age (r=0.28, p=0.008). ANOVA was performed to ascertain the differences of IIFAS score between educational levels of the mothers and found that there was a significant difference in IIFAS score according to the level of education (F (3,83)=8.4, p=0.02). The ANOVA post-hoc results revealed that mothers who attained the highest education at postgraduate level had significantly higher IIFAS mean score than those who had received a certificate/diploma. However, total scores for the IIFAS and adapted IFQ questions were not significantly different between inclusion and exclusion groups with regard to other socio-demographic factors.

Table 4.2: Mean scores for individual questions in IIFAS

IIFAS Questions	All (n=88)		Exclusion (n = 24)		Inclusion (n=64)			
	Mean	SD	Mean	SD	Mean	SD	P-value	C.I
1. The nutritional benefits of breast milk last only until the baby is weaned from breast milk. ǂ	2.7	1.4	2.6	1.5	2.8	1.3	0.546	-0.9, 0.5
2. Formula-feeding is more convenient than breastfeeding. ǂ	3.7	1.1	3.6	1.1	3.8	1.1	0.447	-0.7, 0.3
3. Breastfeeding increases mother-infant bonding.	4.9	0.5	5.0	0.0	4.9	0.5	0.334	-0.1, 0.3
4. Breast milk is lacking in iron. ǂ	4.2	1.0	4.3	1.0	4.2	1.0	0.440	-0.3, 0.7
5. Formula-fed babies are more likely to be overfed than breast-fed babies.	3.0	1.1	3.0	1.0	3.0	1.1	0.825	-0.6, 0.5
6. Formula-feeding is the better choice if a mother plans to work outside home. ǂ	3.9	1.0	3.5	1.1	4.1	0.9	**0.016** *	-1.0, -0.1
7. Mothers who formula-feed miss one of the great joys of motherhood.	4.0	1.1	4.3	0.9	3.9	1.2	0.186	-0.2, 0.9
8. Women should not breast-feed in public places such as restaurants. ǂ	3.6	1.1	3.2	1.2	3.8	1.1	**0.028** *	-1.1, -0.1
9. Babies fed breast milk are healthier than babies who are fed formula.	4.6	0.8	4.6	0.8	4.6	0.8	0.989	-0.4, 0.4
10. Breast-fed babies are more likely to be overfed than formula-fed babies. ǂ	3.5	1.1	3.6	1.1	3.5	1.0	0.665	-0.4, 0.6
11. Fathers feel left out if a mother breast-feeds. ǂ	3.9	0.9	3.8	1.1	3.9	0.9	0.886	-0.5, 0.4
12. Breast milk is the ideal food for babies.	4.9	0.4	4.9	0.3	4.9	0.4	0.925	-0.2, 0.2
13. Breast milk is more easily digested than formula.	4.5	0.8	4.3	1.1	4.6	0.7	0.065	-0.8, 0.02
14. Formula milk is as healthy for an infant as breast milk. ǂ	4.2	0.8	4.3	0.9	4.2	0.8	0.571	-0.3, 0.5
15. Breastfeeding is more convenient than formula feeding.	3.9	1.0	4.0	1.1	3.9	1.0	0.892	-0.5, 0.5
16. Breast milk is less expensive than formula.	4.7	0.6	4.7	0.6	4.7	0.6	0.804	-0.3, 0.2
17. A mother who occasionally drinks alcohol should not breast-feed her baby. ǂ	2.3	1.2	2.3	1.3	2.4	1.1	0.847	-0.6, 0.5

* p-value < 0.05

ǂ Unfavourable to breastfeeding (reversed score applied)

4.7. Discussion

242 pregnant women were approached during recruitment, but only 36% were eligible to be included in the study. The majority were mostly interested in participating in the study, but many did not meet the specific eligibility criteria of being primiparous and intending to stay within the Klang-Valley area throughout the first four months postpartum. Half of the approached women (48%) that were excluded from the study were planning to go back to their hometown (outside the Klang-Valley area) during the postpartum period. In Malaysia, the majority of mothers, especially first-time mothers, will return to their hometown to stay with their family during the postpartum period mostly to practice the traditional postpartum confinement [58, 241, 242], as described in section 3.8 in chapter 3. In the current study, 99% of mothers in the study population indicated that they would be practicing the traditional postpartum confinement after birth for an average of 44 days. Similarly in previous studies in Malaysia, Malays usually practice the traditional confinement for an average of 40-44 days, whereas Chinese and Indian mothers usually practice it for a 30-day period [58, 241].

The overall socio-demographic characteristics of the study population were similar to the national data except that the ethnicity was not representative of the Malaysian multi-cultural population. The main recruitment took place in government antenatal clinics in Bangi, where the majority of the population is Malay (67%) [243], and this could have contributed to the over-representation of Malay in the study. In addition to online advertisements, flyers and posters were distributed in a few private clinics in areas where the population ethnicity is more diverse, but the response rate was very low. There is also the possibility that more Malay participated in the study because exclusive breastfeeding rates were higher among Malay compared to other ethnicities as reported in previous studies [209, 244-246] and also the latest National Health and Morbidity Survey (III) [47]. Another possibility was that the flyer and advertisement posters circulated were only in Malay and English, not in Tamil or Mandarin, thus the posters could have been circulated more among Malays than Chinese or Indian communities in Selangor.

The study population was also more educated compared to the age- and region-matched population, as 70% of the mothers in the study were educated to tertiary level. It seems likely that the highly educated mothers might have a greater interest in breastfeeding research and that they were more willing to follow the quite complicated and demanding study protocol.

The need to stay in the central region area for the duration of the study may also have contributed to the recruitment of mothers with a higher educational level since this area is one of the most urbanised in Malaysia [207]. The women who were eligible were mostly brought up in the central region so their parent's or family's houses, where they stayed for the confinement period, were located there. However, although the majority of the mothers in the study were highly educated, only 13% of them were in the higher income groups. This is probably because all mothers in the study were primiparous, and therefore the majority of them were still young (below 30 years) and might still be in the early stages of their careers. In addition, the wealthiest mothers might be more likely to attend a private antenatal clinic rather than the government antenatal clinic where the main recruitment took place. Consistently, maternal age was significantly associated with educational levels and household income in this study.

Within the study population, the socio-demographic characteristics were similar between inclusion and exclusion groups except that the inclusion group mothers were younger. This also contributed to them being more likely to have one or more older sibling. Being younger in the family, or having older siblings, perhaps resulted in them having additional support from family members to establish breastfeeding. A study performed in Malaysia's central region reported that family members play an important role in encouraging and supporting breastfeeding [209]. Thus, being younger and having older siblings might have contributed to these mothers being able to maintain exclusive breastfeeding during the first-two weeks after delivery. On the other hand, many mothers in the inclusion group indicated that their husband would be their primary maternity care person rather than their mother or mother in law. This suggests that having a husband to support them may have been important in helping the mothers to maintain exclusive breastfeeding. This is supported by a study conducted in a similar area in Malaysia, Selangor, [245] which reported that having a supportive husband is one of the main factors that influences the success of breastfeeding or duration of exclusive breastfeeding. Hence, social support especially from family members, such as the husband or the grandmother of the infant, may play an important role in rearing an infant [247], which have been reported in many other populations [194, 248, 249]. This support could possibly encourage the mother to breastfeed longer.

The overall attitudes and perceptions toward breastfeeding were also similar in the inclusion and exclusion groups, with the average population mean score of 66.7, which indicates a positive attitude toward breastfeeding given the maximum possible score is 85. This is not surprising given the highly selected group of participants: mothers who intended to exclusively breastfeed their babies for at least 4 months. This is consistent with a previous study performed in Kuala Lumpur (n=690), Malaysia (the capital city, which is also located in the central region), which found that mothers who intended to breastfeed had the highest IIFAS score (mean: 64.1±6.2) compared to groups of mothers who were undecided (60.9±5) or those who planned to feed formula (59.5±7.5) to their infants [246]. The study also found that mothers who had received tertiary education had significantly higher IIFAS score than those who had received a primary education, similar to the trend in the present study. Having a higher level of education and good perception toward breastfeeding (based on the IIFAS score) is likely to have resulted in the mothers being highly motivated to exclusively breastfeed and being highly confident of achieving their breastfeeding goal. This is supported by the association of the IIFAS score in the present study with agreement with the statements about the advantages of breastfeeding and also their confidence level in achieving a long duration of breastfeeding.

Although the overall IIFAS mean score was not significantly different between inclusion and exclusion groups, two individual items were significantly different between groups and there was also a strong association between these questions ($r=0.46$, $p<0.001$). Both questions involved maternal perception about feeling comfortable and thinking that it is practical to breastfeeding outside the home, either while working or in public. The results showed that mothers in the inclusion groups were more likely to disagree with the statements that do not favour breastfeed in public or at work. Previous studies have also reported that being a working mother and/or feeling uncomfortable to breastfeed in public were factors that contributed to non-exclusive breastfeeding [211, 244-246, 250]. These factors could have been related to the perception of the exclusion group mothers who were unable to exclusively breastfeed their infants. In the context of this study, perhaps some mothers would also feel uncomfortable with the idea of an outsider (a researcher) coming to the house for the breastfeeding study.

4.8. Conclusion

In general, the mothers who enrolled in the present study were better educated and had a higher motivation to breastfeed than the general population in Malaysia. This is most likely related to the eligibility criteria (or selection criteria) as suggested by the positive association between maternal education levels and attitudes toward breastfeeding. Therefore, I should later consider the socio-demographic factors of the current study population when generalising the main outcomes of this study to the whole population of Malaysian at large. Nevertheless, since the present study is a randomised controlled trial, the primary outcomes were focused solely on the comparison of results between randomised groups. On the other hand, the inclusion and exclusion groups were similar in socio-demographic background and attitude towards breastfeeding except that the inclusion group mothers were significantly younger and more likely to have older siblings, which might result in them getting more breastfeeding support from family members. In the next chapter, the results of phase II (post-randomisation) are presented, comparing the randomised groups: control (n=31) and intervention (n=33). The main results for the whole population (n=64) are presented in Chapter 6.

Summary points:

- The study population had similar socio-demographic characteristics to the general population of mothers in the study region apart from the fact that they were more educated.
- The study population was also highly motivated to breastfeeding: all mothers planned to exclusively breastfeeding their infants for at least 5 months and were confident about achieving their target.
- The inclusion and exclusion groups had similar maternal characteristics and demographic background, except that the inclusion group mothers were significantly younger.
- More husbands were the main primary maternity care person in the inclusion group compared to the exclusion group, and thus they would more likely to support the mothers to breastfeed.
- Both inclusion and exclusion groups had similar perceptions towards breastfeeding, indicating a positive attitude towards breastfeeding.
- However, the exclusion group mothers were more likely to have less favourable opinions towards breastfeeding in public.

CHAPTER 5

5. PRIMARY HYPOTHESES AND OTHER RANDOMISED CONTROLLED TRIAL OUTCOMES

5.1. Introduction

This chapter presents outcomes of the randomised controlled trial (RCT). The outcome results can be categorised into 3 main components:

I) Baseline results prior to the intervention (HV1): maternal descriptive characteristics, breastfeeding perceptions and early postnatal experience.

II) Primary RCT hypothesis outcomes: i) maternal stress and anxiety; ii) breast milk cortisol concentrations; iii) infant behaviour measured using a 3-day diary; iv) infant growth (weight, BMI and body composition) and v) physiological changes in maternal salivary cortisol, breast milk cortisol concentrations and breast milk macronutrient levels before and after a breastfeeding session during the home visits.

III) Secondary RCT outcomes: i) milk hormones (ghrelin and leptin); ii) maternal depression; iii) infant appetite; iv) infant temperament

Although there are 3 outcome components, the discussion section of this chapter is mainly focused on the results relevant to the primary hypotheses. In the next chapter, I present the observational cohort study outcomes for the whole study population, together with a further discussion about milk hormones and infant behaviour and their relationship with growth.

5.2. Study population and follow-up visits

As indicated in Chapter 3 (Section 3.6), the planned sample size was 80-100 mothers to allow for drop-outs, and a total of 88 primiparous pregnant mothers were recruited. However, after mothers gave birth, a second screening was performed and only 64 exclusively breastfeeding mothers and their full term infants were eligible to be randomised (section 3.5). This still exceeded the target sample size of 56 mother-infant dyads, which was calculated to allow detection of a 0.76 SD difference in milk volume between groups at 80% power with a

significance level of α=0.05. During recruitment, once mothers were enrolled in the study, they were asked about their breastfeeding perceptions using the IIFAS questionnaire (section 3.13). They were randomised into control (n=31) and intervention (n=33) groups prior to the first home visit. Mothers were blinded to the randomisation process in order to prevent the mothers in the control group from seeking or using some form of relaxation therapy. The recruitment, screening and randomisation process were described in detail in Chapter 3.

Figure 5.1 Research flow chart of the MOM Study

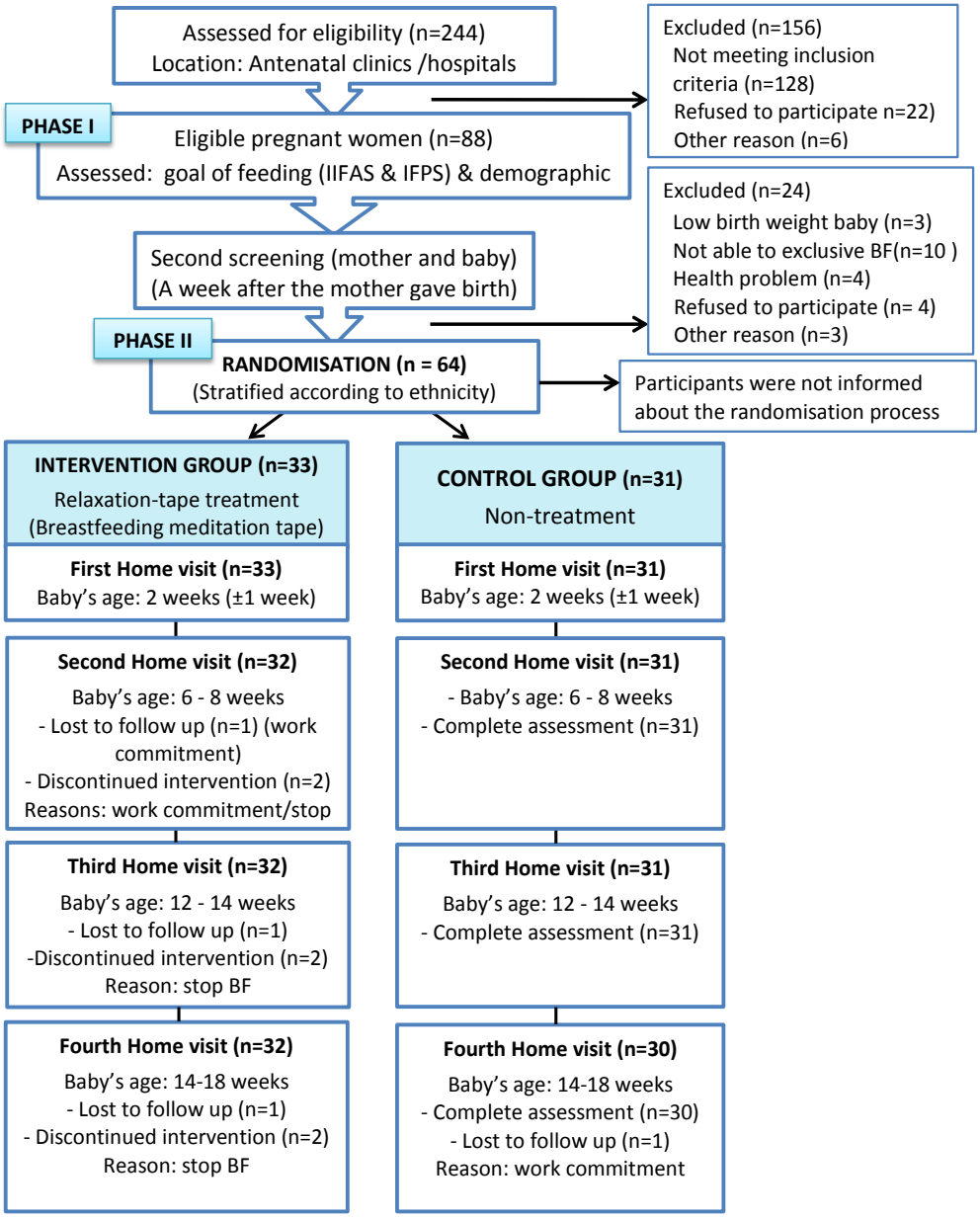


Figure 5.1 provides an overview of the research procedures including the number of home visits (HV). HV1 was conducted with all mother-infant dyads (n=64) when the infants were 2 weeks (±1 week) old. HV2 was conducted with 63 dyads when the infants were aged between 6 and 8 weeks. One HV was missed because a mother returned to work earlier than she expected and therefore could not give a commitment for this HV, but she was followed-up at HV3. HV3 was conducted with 63 mother-infant dyads when the infants were aged between 12 and 14 weeks and the last home visit (HV4) was conducted 2-3 weeks post HV3 with 62 mothers. Missed HV sessions were due to the mother's work commitments.

5.3. Research methods

5.3.1. Data collection

The data collection procedures and detailed information about questionnaires and measurements were described in Chapter 3. The assessments of breastfeeding and the anthropometric measurements of the infants were performed by the same researcher (myself) throughout all home visits. During the HV, mothers were asked about infant appetite (at HV1-3) and infant temperament (HV4 only). Breast milk and maternal saliva samples were collected before and after a breastfeeding session during the HV as described in section 3.17. Mothers in the intervention group were given a relaxation therapy audio-recording to listen to while breastfeeding during HV1-3 (described in 3.9). They were also asked to listen to the therapy whilst breastfeeding on a daily basis for at least two weeks after each HV and were asked to record the frequency of listening to the therapy on a calendar provided for this purpose. All mothers were given questionnaires about their psychological state (PSS, BAI and EPDS – detail in 3.15) for them to answer after the HV (HV1-3) at their convenience. Mothers were also asked to record their infant's behaviour in a 3-day diary after HV1 and 2. Breast milk and saliva samples were stored at -80°C until analysis. All assays for milk hormones (cortisol, leptin and ghrelin) were performed by my collaborators in Slovenia, from the Faculty of Health Sciences, University of Primorska; Dr Ana Petelin & Dr Zara Praznikar. They have previously conducted various analyses of hormones in biological samples including human milk.

5.3.2. Analysis of cortisol

Milk and saliva samples were thawed at room temperature for duplicate analyses (500 µL for breast milk and saliva). Samples were first vortexed and centrifuged at 2500 x g for 20 minutes at 4^{o}C and then the fat layer was removed with a spatula and liquid was assayed for cortisol concentration using commercially available ELISA kits (RE52611-IBL International, Germany). The sensitivity limit of this assay is 0.01 µg/dL and the upper range is 3 µg/dL. The intra-assay and inter-assay variation was around 5 and 10% respectively. For cortisol analysis the assay kits for saliva were used for both measurements (breast milk samples and saliva samples). Additional optimization of the protocol was performed for breast milk samples by using breast milk samples with known concentration of cortisol.

5.3.3. Analysis of milk leptin

Milk samples were thawed for 2 hours in a refrigerator for duplicate analyses. Leptin analysis is best performed using skim milk [141], thus prior to analysis, samples were centrifuged at 2500 x g for 20 minutes at 4^{o}C and the fat layer was discarded. The resulting skim milk was used to measure leptin concentration using a commercially available human leptin ELISA kit (BioVendor, Czech Republic). The sensitivity limit of this assay is 0.1 ng/mL and the upper range is 50 ng/mL. The intra-assay and inter-assay variation were both around 10%. However, leptin results <0.1 ng/mL (e.g 0.05 or 0.001), were not changed and they were still included for statistical analysis. Because the commercially available kit is optimized for serum/plasma concentrations of leptin, additional optimization of the protocol was performed by using breast milk samples with a known concentration of leptin.

5.3.4. Sampling and analysis of milk ghrelin

Within 1-2 hours after collection of breast milk samples during the HV, one set of milk samples (2ml) was acidified by adding 1 N HCL (10% of volume, pH 3-4), as recommended [236]. This is to stabilise the labile side chain of active ghrelin and to prevent its rapid deacylation. The samples were then stored at -80^{o}C until analysis. **Assay:** Prior to analysis, samples were thawed for 2 hours in a refrigerator for duplicate analyses. The samples were then vortexed continuously to ensure uniformity and centrifuged at 3000 g for 10 minutes at 4^{o}C. Samples (20 mcg) were assayed using commercially available ELISA kits EZGRT-89K (EMD Millipore, USA) for total ghrelin concentration and ELISA kits EZGRT-88K (EMD Millipore, USA) for active ghrelin concentration. The lower and upper ranges of the total and active ghrelin assays are

50 to 5000 pg/mL, and 5 to 2000 pg/mL respectively, with an intra- and inter-assay variation of 8 and 10% respectively for both total and active ghrelin. Because the commercially available kit is optimized for serum/plasma concentrations of ghrelin, additional optimization of the protocol was performed by using breast milk samples with a known concentration of active and total ghrelin.

5.3.5. Analysis of breast milk macronutrient content

Frozen milk samples (2.5-3ml) were thawed and warmed in a water bath at 37-40°C and were then homogenised (1.5-2 seconds per 1.5-2.0 ml) using the MIRIS Sonicator (MIRIS AB, Apsala, Sweden). Samples were measured in duplicate using the MIRIS Human Milk Analyser (HMA) based on mid-infrared transmission spectroscopy, set on the calibration mode for homogenised human milk according to the manufacturer's guideline. A calibration check was performed prior to analysis using the MIRIS check solution provided by the manufacturer. The machine was also cleaned using a MIRIS clean solution prior to and post analysis. Both calibration and cleaning processes were performed by injecting 3-5 ml check solution and 10-15ml clean solution respectively prior to analysis and after every 10 samples.

The HMA provides results for fat, carbohydrate (both lactose and oligosaccharide as a total carbohydrate content), protein (true protein) without non-protein nitrogen and crude protein (including non-protein nitrogen) and total milk energy (kcal/100ml). The measurement ranges for each of the macronutrients are as follows: fat 0-8 g/100ml, crude protein 0-3 g/100ml and carbohydrate 4-8 g/100ml. The HMA uses four different wavebands to measure fat, carbohydrate and protein content specifically according to the functional groups for each macronutrient in breast milk through waveband filters [251].

According to the manufacturer's information, the total energy in the milk is based on the following equation: Energy kcal/100ml= (9.25 Kcal/g x fat g/100ml) + (4.40 Kcal/ g x protein g/100ml) + (3.95 Kcal/g x carbohydrate g/100ml). The mean of the duplicate analyses was calculated and used for later statistical analysis. The intra- and inter-subject variation for milk energy was 0.025% and 4.8% respectively.

5.3.6. Calculation of total body water (TBW)

TBW was estimated based on the measurement of deuterium enrichment by isotope ratio mass spectrometry (IRMS) at ICH, UCL. The deuterium (stable isotope) dose administration and sample collection was described in 3.17 and 3.18 (Chapter 3). Once isotope is administered, it will equilibrate uniformly throughout the infant body water pool, which is known as the dilution space. The isotope analysis of urine samples provided data to calculate the dilution space (N) using the back-extrapolation method (Formula 1). Since the dilution space is assumed to overestimate the TBW, it was divided by 1.044 (Formula 2) [252, 253]. Based on the assumption that 79% of fat free mass (FFM) is water (in infants), TBW was divided by 0.79. Finally, the difference between FFM and body weight was then calculated to estimate fat mass (FM). Fat-Free-Mass- and Fat-Mass-Index (FMI and FFMI) were also calculated. FFM, FM, FFMI and FMI were then compared between groups.

Calculation / Formula:

1) Dilution space (N) = AT/a (Ed-Et/ Es-Ep)

A is the dose given to infants, **T** is the volume of tap water in which the dose is diluted, **a** is the portion of dose diluted, **E** is the isotope enrichment of: **d**=dose; **t**=tap water; **s**=post dose and **p**=pre-dose.

2) TBW = Dilution space (N) / hydrogen space (1.04)

3) FFM (kg) = TBW (kg) / 0.79

4) FM (kg) = Body weight (kg) – FFM kg)

5.3.7. Statistical Analysis

5.3.7.1. General

The statistical package IBM SPSS (version 23) was used for data analysis with the significance level set at $p<0.05$. Results involving nominal or ordinal data were presented as frequencies or percentages, followed by univariate analysis (Chi-square or Fisher Exact test where appropriate) in order to compare results between groups. Outcome measurements involving continuous data were checked first for normality by using Q-Q Plots and histograms. For normally distributed data, the mean ± standard deviation (SD) was presented along with the statistical result (T-test or ANOVA) for group comparisons. Alternatively, the median ± interquartile range (IQR) was presented along with the non-parametric test (Mann-Whitney or Kruskal-Wallis test) result. For all primary outcomes variables, intention-to-treat analyses were performed using univariate analyses to compare the mean differences between groups

at each time point (HV) and also the changes between time points (mean change from baseline (HV1) to HV2 and/or HV3). Repeated measures analysis of variance was performed where necessary to compare differences between groups across time points (HV1, HV2 and HV3). Associations between variables were also assessed using Pearson Product or Spearman Rank correlation where appropriate. To test for dose response effects, Spearman correlation was used to examine associations between the frequency of listening to the therapy and primary outcomes.

Overall, for each outcome, the main analysis chosen is summarised in Table 5.1, together with supporting analyses. To ascertain the long-term effects of the intervention, assuming no imbalance in outcomes between randomised groups was found at baseline (HV1) , the values at the endpoint were compared between groups (either at HV2 for hormones/infant behaviours or HV3 for other outcomes). The outcomes at other time points (at HV2 or HV4) or changes between time points (e.g. from HV1 to 3) were also compared between groups in additional analyses to support the main outcome results. To ascertain the short-term or acute effects of the intervention, the changes in outcomes (maternal psychological state or breast milk composition) from foremilk to hindmilk at HV1 were considered as the main analysis. The changes in these outcomes over the test feed at later time points (HV2 and/or HV3) were considered as supporting outcomes because the mothers had been exposed to the intervention starting after the measurement of baseline (foremilk, pre-feed) variables at HV1.

Anthropometric data were converted to standard deviation (Z-score) score (SDS) for infant weight, height, head circumference and BMI using WHO 2007 standard data (LMS growth add-in for Microsoft Excel) and SDS were used in all analyses. Infant growth (weight gain) was calculated using 2 methods: i) the LMS 'Weight gain to SDS' based on the WHO 2007 growth standard data (ie. external data); ii) simple linear regression to calculate the 'conditional weight gain' based on the study population data (ie. internal data). Both methods take into account the baseline weight (HV1) in calculating the change from baseline to an endpoint.

Table 5.1 The outcome measures for the RCT and the statistical analyses for each outcome

Randomised trial hypotheses		Outcome measures		Statistical analyses	
		Baseline	Outcomes		
a) Primary RCT outcomes:					
Primary hypotheses: *The use of relaxation tape therapy during breastfeeding starting at 2-week postpartum will result in:*				**Final main analyses**	**Supporting statistical analyses for additional outcomes :**
i	reduced maternal stress and anxiety	*Long term effects of the intervention on:*			
		maternal stress and anxiety scores at HV1	maternal stress and anxiety scores at HV2 and HV3	T-test for scores at HV3	- T-test for scores at HV2 - GLM repeated measure: change in scores across HV points*groups
		Short term / acute effects of the intervention (within a feed):			
		maternal mood/ emotions (mini-BF test) prior to BF at HV1	maternal mood/emotions post-BF session at HV1-3	T-test for the changes in scores during a feed at HV1	- T-test for the changes in scores (within a feed) at HV2 and HV3 - GLM repeated measure: change in scores across HV points*groups)
ii	lower milk cortisol levels	*Long term effects of the intervention on:*			
		breast milk cortisol in foremilk at HV1	breast milk cortisol in fore- and hindmilk at HV2	T-test for the foremilk cortisol at HV2	- T-test for hindmilk cortisol at HV2
		Short term / acute effects of the intervention (within a feed):			
		breast milk cortisol in foremilk at HV1	the changes in cortisol levels within a feed at HV1 and hindmilk cortisol at HV1	T-test for the changes in cortisol within a feed at HV1	- T-test for hindmilk cortisol at HV1 - T-test for the changes in cortisol within a feed at HV2
iii	Increased in breast milk energy (higher calories)	*Long term effects of the intervention on:*			
		total energy in fore milk HV1 (total calories)	total energy in fore- and hindmilk at HV2 and HV3.	T-test for the foremilk total energy at HV3	- T-test for the foremilk energy at HV2 and hind milk energy at HV2 & 3 - Pooled data for breast milk (t-test)
		Short term / acute effects of the intervention (within a feed):			
		total energy in fore milk HV1 (total calories)	the changes in milk energy within a feed at HV1-3 and hindmilk energy at HV1	T-test for the changes in milk energy within a feed HV1	- T-test for hindmilk energy at HV1 - T-test for the changes in milk energy levels at HV2 and HV3

Randomised trial hypotheses		Outcome measures		Main statistical analyses	Supporting statistical analyses
		Baseline	Outcomes		
iv	favourable effects on infant behaviour	Duration of infant feeding, sleeping, crying and awake at HV1	Duration of infant feeding, sleeping, crying and awake at HV2	T-test for the duration of each behaviour at HV2	T-test for the changes in duration for each behaviour (HV1 to 2)
vi	more optimal infant growth	infant weight & BMI HV1	- Infant weight & BMI at HV1-3 - Infant weight and BMI gain (HV1 to 3)	T-test for weight and BMI at HV3	- T-test for weight & BMI gain from HV1-3 - T-test for weight and BMI at HV2 & HV4 - GLM repeated measure: changes in weight/BMI across HV points*groups
		-	- Infant fat mass, fat-free-mass - Infant fat mass index (FMI) & fat-free-mass index (FFMI)	T-test at for FM, FFM, FMI and FFMI at HV4	-
b) Secondary RCT outcomes: Comparison between randomised groups					
i	Maternal depression	depression scores at HV1	depression scores at HV3	T-test for scores at HV3	- T-test for scores at HV2
ii	Milk leptin and ghrelin (hormones)	*Long term effects of the intervention on:*			
		breast milk hormone levels in fore milk at HV1	breast milk hormone levels in fore- and hindmilk at HV2	T-test for the foremilk leptin/ghrelin at HV2	- T-test for hindmilk leptin/ghrelin at HV2
		Short term / acute effects of the intervention (within a feed):			
		breast milk hormone levels in fore milk at HV1	the changes in milk hormones within a feed at HV1-3 and hindmilk hormone levels at HV1	T-test for the changes in hormone levels at HV2	- T-test for hormone levels in hind milk at HV1 - T-test for the changes in hormone levels within a feed at HV2
iii	Infant temperament	-	Temperament scores at HV4	T-test for scores at HV4	
iv	Infant appetite	appetite scores at HV1	appetite scores at HV3	T-test for scores at HV3	- T-test for scores at HV2
v	Other macronutrient components (for long term effects).	fat, protein and CHO in foremilk at HV1	foremilk fat, protein and CHO at HV2 & 3 and hindmilk levels at HV1 to 3	T-test for the foremilk levels at HV3	- T-test for the foremilk levels at HV2 - T-test for hindmilk levels at HV1 to 3 - T-test for the changes in levels within a feed at HV1-3 and also pooled data

* T-test = independent t-test to compare between groups; CHO: carbohydrate

5.3.7.2. Biochemical data

Cortisol, leptin and ghrelin concentrations in breast milk and salivary cortisol were analysed for samples collected at HV1 and 2 only, whereas the macronutrient composition of breast milk was determined in samples collected at all visits (HV1-3). Since data were also collected before and after a feed at each HV, the changes in breast milk composition (or salivary cortisol) from fore to hind milk were calculated. The main objective was to summarise the short-term effect of the relaxation therapy on maternal salivary cortisol and breast milk composition (hormones and macronutrient concentrations) during a breastfeeding session. This is based on the hypothesis that mothers who listened to the therapy during breastfeeding would be more relaxed and hence produce breast milk with lower cortisol concentrations and higher fat levels in hind milk (or show a greater reduction in cortisol concentrations and/or higher increment of milk fat from fore to hind milk). Data that were not normally distributed were transformed to natural logarithms (ln) prior to analysis, and hence the geometric mean (GM), standard deviation (GM x log SD) and sympercent (s%) are presented to show the percentage mean differences between groups [254]. Pearson's Product correlation was also performed to examine the association between saliva cortisol and breast milk cortisol in order to investigate whether the cortisol from maternal plasma is transferred into breast milk.

5.4. Results I : Baseline data prior to HV1

5.4.1. Maternal descriptive characteristics and BF perceptions during pregnancy

Table 5.2 presents the descriptive characteristics of mothers by randomised group, including their breastfeeding goals. There were no significant differences between groups for any variable. Both control and relaxation groups had a similar perception towards breastfeeding with IIFAS mean scores of 67.6±6.7SD and 66.4±6.3 respectively (p=0.46, CI:-1.9, 4.4). The agreement with the breastfeeding statements was also not significantly different between groups (p=0.28), with a median percentage score of 76 (IQR:40) and 80 (IQR:20) for control and relaxation groups respectively. There were no significant differences between groups in breastfeeding duration goals (X^2=1.78, p=0.78) or in their confidence levels for attaining these goals (X^2=5.77, p=0.22). Breastfeeding duration goals were not significantly associated with confidence levels (p=0.16).

Table 5.2 Descriptive characteristics of participants and their breastfeeding duration goals

Descriptive characteristics :		Groups					Stat. test	
		Control		Relaxation			X^2	P-value
		n	%	n	%	n (%)		
Mother's ethnicity	Malay	30	96.8	30	90.9	60 (94)	0.34	0.49
	Others	0	3.2	4	9.1	4 (6)		
Age groups	20-25	10	32.3	11	33.3	21 (33)	5.41	0.08
	26-30	21	67.7	17	51.5	38 (59)		
	31-34	0	0	5	15.2	5 (8)		
Marital status	Married	31	100	33	100	64(100)		-
Highest educational qualification	School	5	16.1	5	15.2	10 (16)	3.00	0.59
	Cert./Diploma	3	9.7	5	15.2	8 (13)		
	Bach. degree	21	67.7	18	54.5	39 (61)		
	Postgraduate	2	6.5	5	15.2	7 (11)		
Household income	1500-3000	8	25.8	11	33.3	19 (30)	1.38	0.89
	3001-5000	9	29.0	7	21.2	16 (25)		
	5001-8000	10	32.3	9	27.3	19 (30)		
	8001-10000	2	6.5	4	12.1	6 (9)		
	>10000	2	6.5	2	6.1	4 (6)		
Baby's gender	female	20	64.5	19	57.6	39 (61)	0.32	0.62
	male	11	35.5	14	42.4	25 (39)		
Birth order	Median (IQR)	3 (2)		3(3)				
Breastfeeding plan:		Control		Relaxation			1.96	0.90
	Category:	n	%	n	%	n (%)		
Breastfeeding duration goals	2-6 months	2	6.5	1	3.0	3 (4.7)		
	7-12 months	3	9.7	3	9.1	6 (9.4)		
	13-18 months	1	3.2	2	6.1	3 (4.7)		
	19-24 months	24	77.4	27	81.8	5 (79.7)		
	25-36 months	1	3.2	0	0	1 (1.6)		
Confidence levels (1-5:Not to strongly confident)	1	6	19.4	2	6.1	8 (12.5)	5.57	0.24
	2	5	16.1	2	6.1	7 (10.9)		
	3	5	16.1	5	15.2	10 (15.6)		
	4	11	35.5	15	45.5	26 (40.6)		
	5	4	12.9	9	27.3	13 (20.3)		

5.4.2. Early postnatal experience

Table 5.3 shows that mothers in both groups received similar maternity support during labour and had similar birth experiences, with no significant differences between groups for any variable (all p-value>0.05 by Chi-square test). Overall, 50% of the mothers in the study were attended by an obstetrician during labour. The majority of the mothers underwent vaginal delivery (75%), were accompanied by their husband (78.1%) in the labour room and spent 1-2 nights (71.9%) in hospital post-delivery. About a third of the mothers (32%) received information about breastfeeding during their antenatal class and 55% indicated that mass-media (internet and printed materials) was one of the sources for breastfeeding information.

Table 5.3. Maternity support and services in hospital during labour

Description	Control		Relaxation		Total		X^2	P-value
	n	%	n	%	n	(%)		
Birth attendant							0.09	0.96
Specialist	16	51.6	16	48.5	32	(50.0)		
Medical officer	11	35.5	12	36.4	23	(35.9)		
Midwife/Nurse	4	12.9	5	15.2	9	(14.1)		
Additional support (at labour)							1.94	0.6
Husband	25	80.6	25	75.8	50	(78.1)		
Birth support person	0	0	2	6.1	2	(3.1)		
None	6	19.4	6	18.2	12	(18.8)		
Mode of delivery				0.0			6.78	0.07
Vaginal, not induced	13	41.9	20	60.6	33	(51.6)		
Vaginal, induced	10	32.3	5	15.2	15	(23.4)		
Planned caesarean	3	9.7	0	0	3	(4.7)		
Unplanned caesarean	5	16.1	8	24.2	13	(20.3)		
Medication during labour							0.02	0.89
Anaesthesia	8	25.8	9	27.3	17	(26.6)		
Spinal/Epidural	13	41.9	11	33.3	24	(37.5)		
Nitrous oxide gas	6	19.4	9	27.3	15	(23.4)		
Other pain medication	7	22.6	6	18.2	13	(20.3)		
None medication	6	19.4	9	27.3	15	(23.4)		
Hospital stay after birth							3.93	0.29
1 night	11	35.5	15	45.5	26	(40.6)		
2 nights	8	25.8	12	36.4	20	(31.3)		
3 nights	10	32.3	4	12.1	14	(21.9)		
4-7 nights	2	6.5	2	6.1	4	(6.3)		
Source of BF information								
Antenatal class	10	32.3	11	32.4	20.5	(32.0)	0.71	0.447
Mass-media	19	61.3	16	47.1	35	(54.7)	1.06	0.327
No BF info at all	4	12.9	2	5.9	6	(9.4)	0.88	0.419

*BF = breastfeeding

There were also no significant differences in early postnatal experience between groups ($p>0.05$) as shown in Table 5.4. Overall, the majority of the mother-infant dyads (72%) experienced skin-to-skin contact directly after birth, mostly lasting for less than 20 minutes. 72% of the mothers were able to breastfeed their infant directly after birth while others experienced their first breastfeeding later, ranging from less than 30 minutes to 48 hours post-delivery.

Table 5.4. Early postnatal experience

Early postnatal experience	Control		Relaxation		Total	X^2	P-value
	n	%	n	%	n (%)		
How soon did skin-to-skin contact occur after delivery?						5.8	0.12
Directly after birth	20	64.5	26	78.8	46 (71.9)		
About 15-30 mins after birth	5	16.1	1	3.0	6 (9.4)		
More than 30 mins after birth	2	6.5	0	0	2 (3.1)		
More than 1 hour after birth	4	12.9	6	18.2	10 (15.6)		
For how long was the skin-to-skin contact after birth?						3.7	0.16
None	4	12.9	1	3.0	5 (7.8)		
Less than 20 mins	23	74.2	23	69.7	46 (71.9)		
More than 20 mins	4	12.9	9	27.3	13 (20.3)		
How soon was the first breastfeeding?						5.5	0.6
Directly after birth	7	22.6	12	36.4	19 (29.7)		
Less than 30 mins	2	6.5	3	9.1	5 (7.8)		
Within 30-60 mins	5	16.1	5	15.2	10 (15.6)		
Within 1-2 hours	6	19.4	7	21.2	13 (20.3)		
Within 3-6 hours	7	22.6	3	9.1	10 (15.6)		
Within 7-12 hours	1	3.2	2	6.1	3 (4.7)		
Within 13-24 hours	1	3.2	1	3.0	2 (3.1)		
After 2 days	2	6.5	0	0	2 (3.1)		
Co-sleeping with baby at home							
Bed-sharing	25	80.6	28	84.8	53 (84.1)	0.6	0.75
Baby-cot (in the same room)	6	19.4	4	12.1	10 (15.9)		

5.5. Results II : Primary outcomes

In this section, I present first the effects of the relaxation therapy on maternal psychological state (stress and anxiety) and infant outcomes (growth and behaviour). I also include the maternal depression and emotion (mini-breastfeeding) results since, although they are not part of the primary hypothesis, these variables are related to maternal psychological state. Next, I present the effects of the therapy on breast milk composition (cortisol and macronutrient concentrations) within a feed at different time points.

5.5.1. Maternal psychological state

5.5.1.1. Maternal stress, anxiety and depression across time points (HV1-3)

At baseline (HV1), maternal stress scores (PSS) were not significantly different between groups (p=0.42), but the relaxation group mothers had a significantly lower stress score at both HV2 (p=0.01) and HV3 (p=0.03) (Table 5.5). The relaxation group mothers also had significantly greater reduction in stress score across time (HV1-3) using GLM repeated measures test (F (1,58)=5.22,p=0.026). Maternal anxiety scores (BAI) of the control group mothers were significantly higher at baseline (p=0.02), but were not significantly different than those in the relaxation group at later visits (HV2 & HV3). There were no significant differences in maternal depression scores (EPDS) between groups at any home visit, although the relaxation group mothers showed a non-significantly greater reduction in the score across time points. Nevertheless, maternal stress, anxiety and depression scores were significantly associated at HV2 and HV3 (shown by moderate to strong correlations in Table 5.6). 13% mothers in the control and 16% in the relaxation groups had an EPDS score above the clinical cut-off point of 13 at HV1, and this reduced to 2% and 1% mothers in the control and relaxation groups respectively at HV3. These results were not significantly different between groups (p>0.05).

Table 5.5. Results of maternal psychological test scores (PSS, BAI & EPDS)

Groups :		Control	Relaxation			
	n	Mean (SD)	Mean (SD)	p-value	Mean diff	C.I
Stress - PSS						
Hv1	63	17.28 (5.6)	16.27 (4.3)	0.42	1.01	-1.5, 3.5
Hv2	62	16.06 (5.9)	12.55 (4.4)	0.011	3.51	0.8, 6.2
Hv3	61	15.10 (6.1)	11.97 (4.9)	0.029	3.13	0.3, 5.9
Anxiety - BAI						
Hv1	63	15.23 (8.9)	10.48 (71.2)	0.022	4.75	0.7, 8.8
Hv2‡	62	10.0 (14)‡	6.0 (9)‡	0.13		
Hv3‡	61	9.0 (12)‡	6.0 (10)‡	0.24		
Depression - EPDS						
Hv1	63	9.55 (4.3)	8.94 (4.1)	0.57	0.61	-1.5, 2.7
Hv2	62	9.22 (4.6)	7.38 (3.5)	0.08	1.84	-0.2, 3.9
HV3	61	7.33 (4.4)	6.0 (3.5)	0.19	1.33	-0.7, 3.3

‡ Mann-Whitney test: Results as Median (IQR)

Table 5.6 Correlations between scores for maternal stress (PSS), anxiety (BAI) and depression (EPDS).

		BAI HV2	BAI HV3	EPDS HV2	EPDS HV3
PSS HV2	R-value	0.58**	0.35*	0.59**	0.52**
	p-value	<0.001	0.006	<0.001	<0.001
	N	61	60	62	61
PSS HV3	R-value	0.52**	0.50**	0.61**	0.67**
	p-value	<0.001	<0.001	<0.001	<0.001
	N	59	61	60	61
BAI HV2	R-value			0.73**	0.62**
	p-value	-	-	<0.001	<0.001
	N			61	60
BAI HV3	R-value			0.57**	0.65**
	p-value	-	-	<0.001	<0.001
	N			60	61

* moderate correlation ** strong correlation

5.5.1.2. Mini-breastfeeding test (change within a feed at HV1-3)

The Mini-breastfeeding questionnaire data showed no significant differences between groups in the changes in maternal mood and emotions (e.g. stressed, anxious, happy, calm, relaxed, etc) or baby's mood (Question 8-9) within a feeding session at all HV (all p-values>0.05) (Table 5.7). The internal consistency reliability coefficients (Cronbach's α) for the Mini-Bf test were also low - ranging from an average of 0.1 to 0.4 from HV1 to HV3.

Overall, the mothers in the intervention group seemed to have a higher reduction of scores for 'stress' and' anxious', and higher increase in 'relax' and 'happiness' within a test-feed only at HV1 (Table 5.6), but the results were not significant (all p>0.05). Additional analyses were also performed using GLM repeated measures and the results showed no significant differences in any variable across time points between groups (mood/emotion change across HV points * groups; all p>0.05). Thus, there was no trend towards reduction in maternal distress or increase in positive maternal or infant emotions or mood across HV points in either group.

Table 5.7 Mini-breastfeeding test results at HV1-3

MINI-BF QUESTIONS		CONTROL			RELAXATION			T-TEST
NO	HV1	N	Mean	SD	N	Mean	SD	P-value
1	Stress	31	-2.79	19.0	33	-7.19	22.7	0.41
2	Anxious	31	-2.26	17.5	33	-5.26	21.9	0.55
3	Alert	31	4.91	28.0	33	-3.23	28.5	0.25
4	Relax	31	8.71	28.4	33	11.6	19.5	0.63
5	Happy	31	1.60	22.2	33	7.11	12.7	0.22
6	Tired	31	-4.99	28.0	33	-7.12	22.4	0.74
7	Sleepy	31	-5.79	30.7	33	0.22	17.6	0.34
8	Baby - Calm	31	21.5	28.4	33	22.6	30.2	0.89
9	Baby - Happy	31	17.9	27.3	33	17.7	26.6	0.97
	HV2	N	Mean	SD	N	Mean	SD	P-value
1	Stress	30	0.15	21.2	31	-2.10	22.5	0.69
2	Anxious	30	-5.96	24.2	31	-2.61	24.6	0.59
3	Alert	30	6.08	30.3	31	-1.27	21.3	0.28
4	Relax	30	13.8	24.0	31	3.47	24.2	0.10
5	Happy	30	11.7	21.5	31	5.1	15.6	0.17
6	Tired	30	1.3	26.3	31	1.88	35.5	0.94
7	Sleepy	30	7.53	23.4	31	1.35	27.6	0.35
8	Baby - Calm	30	21.1	29.6	31	13.9	24.4	0.30
9	Baby - Happy	30	23.7	30.2	31	8.61	23.7	0.03
	HV3	N	Mean	SD	N	Mean	SD	P-value
1	Stress	30	-3.42	15.9	31	-2.48	8.3	0.77
2	Anxious	30	-5.71	17.9	31	-1.43	6.1	0.21
3	Alert	30	3.07	20.8	31	2.63	18.9	0.93
4	Relax	30	6.75	21.9	31	9.05	18.7	0.66
5	Happy	30	3.97	17.6	31	5.59	16.9	0.72
6	Tired	30	-5.98	18.2	31	-3.26	23.3	0.61
7	Sleepy	30	3.66	21.1	31	0.19	18.9	0.50
8	Baby - Calm	30	13.1	31.8	31	10.4	15.6	0.67
9	Baby - Happy	30	8.32	30.4	31	9.83	15.3	0.81

* SD = standard deviation

5.5.2. Infant anthropometric measurements and growth

5.5.2.1. Weight, length and head circumference

The mean population absolute values for infant weight, length, head circumference and BMI at HV1 were 3.48 kg ±0.4, 52.2 cm ±1.9, 35.8 cm ±1.3 and 12.7 kg/m^2 ±1.1 respectively. Table 5.8 shows the mean Z-score (SD-for-age) for infant weight, length, head circumference and BMI from birth to HV4. As indicated previously, SDS data are presented and discussed in this chapter.

Standardised values for infant weight, length, head circumference and BMI were not significantly different between groups at baseline (at birth and HV1) with all p-values >0.05. The mean changes in weight, length, and head circumference from birth to HV1 (SDS-gain values) were also not significantly different between groups.

The relaxation group infants had significantly higher weight and BMI than the control group infants at HV2 to HV4 (all p-values <0.01–Table 5.7). Weight gain and BMI gain from HV1 to HV3 were also significantly higher in the intervention than the control group ($p<0.05$). The conditional weight gain results, calculated using the internal data, were also similar to the results of the weight gain calculated WHO growth standard data using the LMS 'Weight gain to SDS' function (all $p<0.05$).

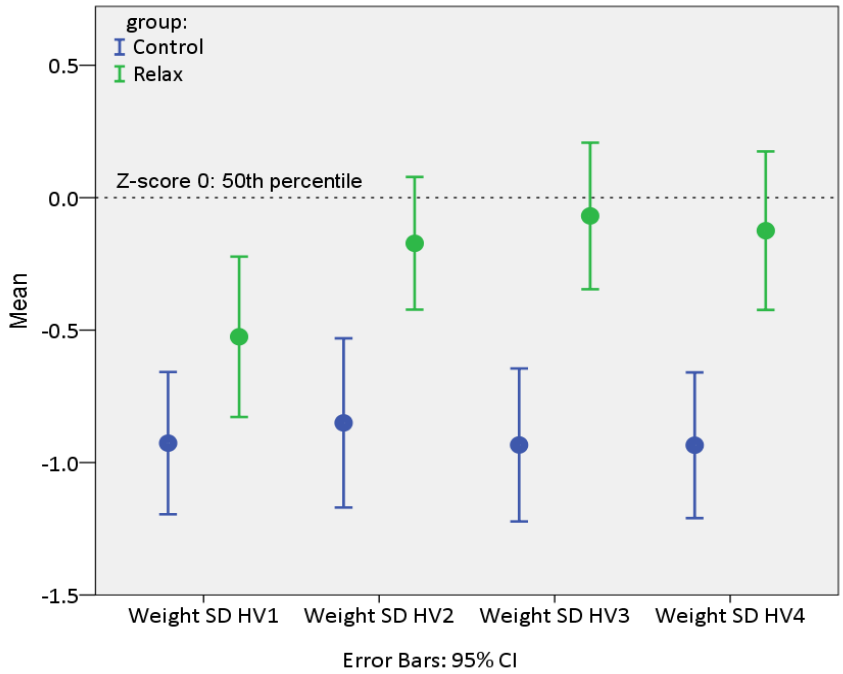
Overall, GLM repeated measures analyses showed that the relaxation group infants had significantly higher weight and BMI gain across all time points (HV1-3); (Weight SDS: $F(1,60)=12.1$, $p=0.001$) and (BMI SDS: $F(1,60)=15.7$, $p<0.001$). However, the mean weight SDS for both groups was still below 0 (below the 50^{th} centile) as shown in Figure 5.2.

However, there were no significant differences between groups for length and head circumference at any home visit (HV1-HV4). There was also no significant different in mother's weight at HV1 between groups (mean difference: 0.78, $p=0.78$, CI: -4.2, 5.54), with a mean population value of 61.5 kg ±9.7.

Table 5.8. SDS-scores for infant weight, length, head circumference and BMI from baseline to HV4, and FM and FFM at HV4

GROUPS:	CONTROL			RELAXATION			T-test			
	n	Mean	SD	n	Mean	SD	p-value	Mean diff.	C.I	
Weight SDS										
At birth	31	-0.62	0.7	33	-0.36	0.9	0.19	-0.26	-0.65	0.14
HV1	31	-0.92	0.7	33	-0.56	0.8	0.06	-0.36	-0.75	0.02
HV2	31	-0.82	0.9	32	-0.19	0.7	0.002	-0.63	-1.02	-0.24
HV3	31	-0.90	0.8	32	-0.12	0.8	<0.001	-0.78	-1.18	-0.39
HV4	30	-0.93	0.7	32	-0.17	0.8	<0.001	-0.76	-1.17	-0.36
Weight gain HV1-3	31	-0.32	0.9	32	0.44	1.0	0.002	-0.76	-1.22	-0.30
Conditional gain 1-3	31	-0.39	0.9	32	0.38	0.9	0.001	-0.77	-1.24	-0.31
Weight gain HV1-4	30	-0.40	0.85	32	0.29	1.0	0.005	-0.70	-1.18	-0.22
Conditional gain 1-4	30	-0.37	0.86	32	0.35	1.0	0.003	-0.72	-1.20	-0.25
Length SDS										
At birth	31	0.04	1.37	33	0.34	1.42	0.39	-0.30	-1.00	0.40
HV1	31	-0.29	.92	33	-0.06	.90	0.30	-0.24	-0.70	0.22
HV2	31	-0.06	1.1	32	0.29	1.1	0.21	-0.35	-0.90	0.20
HV3	31	0.33	1.1	32	0.47	0.9	0.59	-0.14	-0.66	0.37
HV4	24	0.17	0.8	26	0.61	0.9	0.07	-0.44	-0.92	0.03
Length gain HV1-3	31	0.63	1.0	32	0.54	0.7	0.70	0.08	-0.35	0.51
Head circumference (HC) SDS										
At birth	28	-1.05	1.27	30	-0.97	1.34	0.83	-0.08	-0.77	0.61
HV1	31	-0.27	1.01	33	0.01	0.91	0.24	-0.29	-0.77	0.19
HV2	31	-0.22	1.0	32	-0.07	1.0	0.55	-0.15	-0.65	0.35
HV3	31	-0.51	0.9	32	-0.18	0.9	0.15	-0.34	-0.79	0.12
HV4	18	0.07	0.4	18	0.01	0.6	0.75	0.06	-0.30	0.41
HC gain HV1-3	31	-0.24	0.5	32	-0.19	0.5	0.68	-0.05	-0.28	0.18
BMI SDS										
At birth	31	-0.97	1.09	33	-0.82	1.50	0.66	-0.15	-0.81	0.51
HV1	31	-1.11	.80	33	-0.76	.89	0.10	-0.35	-0.77	0.07
HV2	31	-1.11	0.9	32	-0.49	0.8	0.006	-0.62	-1.05	-0.18
HV3	31	-1.48	0.8	32	-0.52	0.9	<0.001	-0.96	-1.41	-0.51
HV4	24	-1.50	0.6	26	-0.69	1.0	0.001	-0.80	-1.28	-0.33
BMI gain HV1-3	31	-0.37	0.9	32	0.22	1.1	0.022	-0.59	-1.10	-0.09
Body composition at 14-18 weeks										
FM (kg)	12	1.05	0.5	17	1.4	0.6	0.13	-0.33	-0.76	0.10
FFM (kg)	12	4.7	0.8	17	5.2	0.7	0.10	-0.47	-1.0	0.10
FMI (kg/m^2)	12	2.6	1.3	17	3.5	1.5	0.13	-0.83	-1.9	0.27
FFMI (kg/m^2)	12	11.8	1.7	17	12.9	1.4	0.09	-1.04	-2.3	0.17

Figure 5.2 The mean weight SDS for both groups across time (HV1-4)



5.5.2.2. Infant body composition

The fat mass (FM) and fat-mass-index (FMI) were also not significantly different between groups (p>0.05) (Table 5.8). However, there was a non-significant trend of higher fat-free-mass (FFM) and fat-free-mass-index (FFMI) in intervention group infants than those in the control group (FFM: 5.2±0.7 vs 4.7±0.8, p=0.10; and FFMI: 12.9±1.4 vs 11.8±1.7, p=0.09).

5.5.3. Infant behaviour (3-day diary)

Mothers recorded their infant's behaviours for 72 hours in a 3-day diary post-HV1 and -HV2. Thus, the average duration for sleeping, awake and content, fussy and crying, and feeding over 24 hours were calculated. 'Awake and content' is described here as 'awake' only, while the duration for 'fussy and crying' over 72 hours were presented here as 'distress'. The compliance with completion of the diary was only 78%. There were no significant differences in maternal characteristics or socio-demographic background between those who did and did not completed the diary, within each randomised groups (p>0.05). However, the completion rate was significantly higher in the relaxation group then the control group (90% v 65%, p=0.011).

Figure 5.3 below shows the mean time spent in each behaviour over 24 hours at both HV1 and HV2. At baseline, no significant differences were found between groups for time spent sleeping (p=0.45, CI:-49, 109), feeding (p=0.21, CI: -19, 86), awake (p=0.08, CI:-125, 8) or distressed (p=0.83, CI:-46, 37). Sleeping duration was negatively associated with feeding (n=46, r=-0.57, p<0.001) and awake (n=46, r=-0.59, p<0.001) duration at HV1. There was also a negative association between time spent awake (and content) and distressed at HV1 (n=46, r=-0.38 p=0.008).

However, at HV2, the intervention group infants had significantly longer sleep duration than the control group (mean difference=82 minutes, p=0.017, C.I=-148.6, -15.6). On average, the intervention group infants spent about 14.3±1.6 hours sleeping, whereas control group infants spent approximately 12.9±1.6 hours sleeping over 24 hours at 6-8 weeks of age (HV2). However, the duration of other individual infant behaviours was not significantly different between groups; feeding (p=0.07, CI: -3.8, 98.8), awake (p=0.142, CI:-19.5, 130.8) and distressed (p=0.38, CI:-69.2, 27), although there was a trend for longer awake duration in the control group and shorter feeding duration in the intervention group.

There was also no significant difference in the change of duration for any behaviour from HV1 to HV2 between groups (p>0.05). However, non-significant trends were apparent: for example, the control group had a greater reduction in sleeping duration (849 to 733 minutes; p=0.1) and a greater increase in awake time duration (247 to 416 minutes; p=0.09) from HV1 to HV2 than the intervention group. Conversely, the intervention group had an increase in sleeping duration (819 to 856 minutes) and a greater reduction in feeding duration (243 to 169 minutes) from HV1 to HV2 (Figure 5.3 and 5.4). Reflecting these trends, the sleeping duration at HV2 was negatively associated with awake duration (n=37, r=-0.59, p<0.001), and the change in sleeping duration from HV1 to HV2 was also negatively associated with the change in awake duration from HV1 to HV2 (n=33, r=-0.61, p<0.001).

Figure 5.3 Mean time spent sleeping, feeding, awake and distressed over 24 hours:

Mean values over 3 days

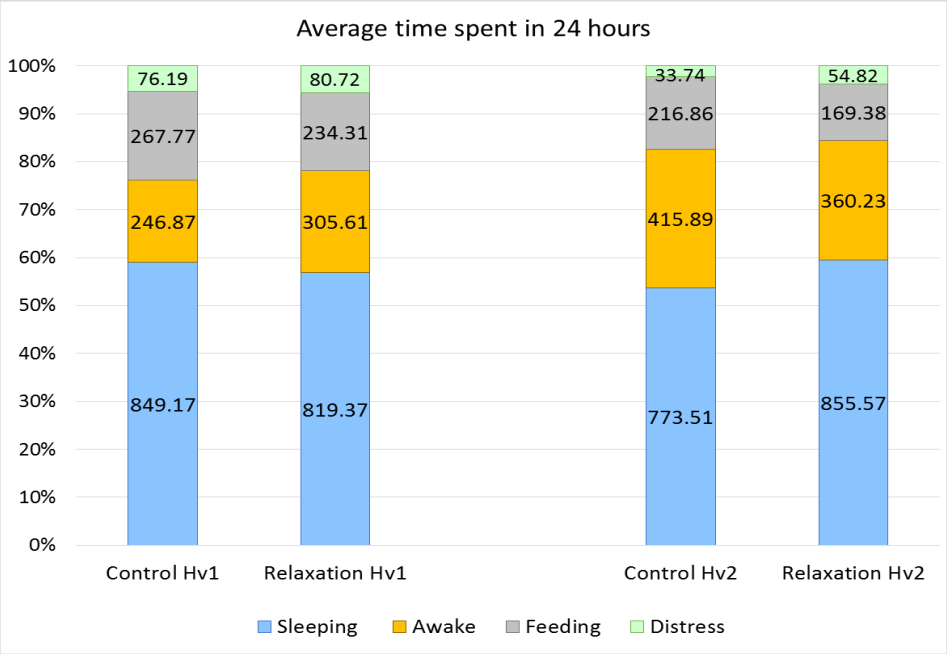
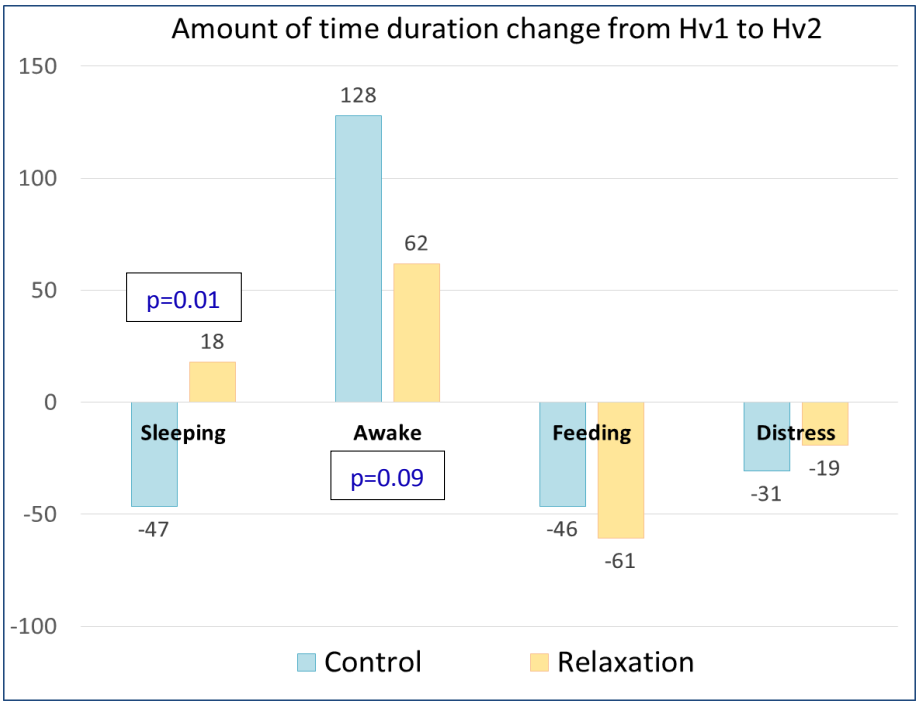


Figure 5.4. Change in duration for each behaviour from HV1 to HV2, according to randomised groups



5.5.4. Changes in milk composition within a feed

5.5.4.1. Cortisol

All cortisol data were transformed to natural logarithms (ln) prior to analysis. At HV1, there was no significant difference in fore milk cortisol between groups, but the relaxation group mothers had significantly lower cortisol concentrations in hind milk at HV1, with an average of 44.5 s% less than in the control group (C.I: 12.9 s%, 76.1 s%). Thus, the relaxation group had a significantly greater reduction (34%) in cortisol concentration within a feed at HV1 than the control group. However, there were no significant differences in milk cortisol at HV2, nor in maternal saliva cortisol at HV1 or 2 between groups. Overall, both saliva and milk cortisol concentrations decreased within a feed with lower levels of cortisol in the relaxation group at both visits. Full results are shown in Table 5.9.

Table 5.9. Comparison of maternal saliva cortisol and breast milk cortisol (µg/dL) between groups

Groups :	Control			Relaxation					
	n	Mean[+]	SD	N	Mean[+]	SD	P value	Mean diff. (s%)	C.I (s%)
Milk Cortisol HV1 (µg/dL)									
Fore	31	0.170	0.1	32	0.140	0.09	0.22	19.7	-12.4, 51.8
Hind	31	0.167	0.1	32	0.107	0.07	0.007	44.5	12.9, 76.1
Change (Hind-Fore)	31	-0.003	<0.1	32	-0.033	0.02	0.024	-33.9	-63.4, -4.5
Milk Cortisol HV2									
Fore	29	0.116	0.09	31	0.152	0.13	0.21	-26.8	-69.2, 15.7
Hind	30	0.096	0.07	31	0.099	0.06	0.86	-3.2	-39.7, 33.3
Change (Hind-Fore)	28	-0.020	0.01	31	-0.053	0.04	0.48	-12.8	-48.9, 23.4
Saliva Cortisol HV1									
Pre BF [+]	31	0.062	0.05	32	0.048	0.04	0.21	26.4	-14.9, 67.8
Post BF	31	0.041	0.03	32	0.039	0.02	0.72	6.4	-28.9, 41.7
Diff. (Post-Pre)	31	-0.021	0.02	32	-0.009	<0.1	0.23	20.0	-13.3, 53.4
Saliva Cortisol HV2									
Pre BF	30	0.062	0.04	31	0.044	0.04	0.10	33.5	-6.6, 73.7
Post BF	29	0.044	0.03	31	0.036	0.03	0.37	18.6	-22.9, 60.1
Diff. (Post-Pre)	29	-0.018	0.01	31	-0.008	<0.1	0.33	17.2	-17.8, 52.2

[+]Geometric mean; s% : sympercent; BF=breastfeeding

Although no significant difference in saliva cortisol was found between groups, the maternal saliva cortisol was significantly associated with breast milk cortisol concentrations at that feed; this was seen at both HV1 and 2 (Table 5.10).

Table 5.10.Correlations between saliva cortisol and breast milk cortisol

		Saliva Pre-BF HV1	Saliva Post-BF HV1	Saliva Pre-BF HV2	Saliva Post-BF HV2
Fore Milk HV1	R-value	0.476**	0.339**	0.133	
	P-value	0.000	0.007	0.311	
	n	62	62	60	
Hind Milk HV1	R-value	0.401**	0.418**		0.168
	P-value	0.001	0.001		0.202
	n	62	62		59
Fore Milk HV2	R-value	-0.029		0.568**	0.403**
	P-value	0.830		<0.001	0.002
	n	59		59	58
Hind Milk HV2	R-value		0.062	0.462**	0.410**
	P-value		0.636	<0.001	0.001
	n		60	60	60

**. Correlation is significant at the 0.01 level (2-tailed); BF=breastfeeding

5.5.4.2. Macronutrient composition of breast milk

The overall results for breast milk macronutrient concentrations (fat, carbohydrate and protein) including milk energy are shown in Table 5.11 to 5.13 and Figure 5.5 to 5.7. Macronutrient content (fat, protein and carbohydrate) and total energy of fore milk at HV1 (as a baseline) were not significantly different between groups.

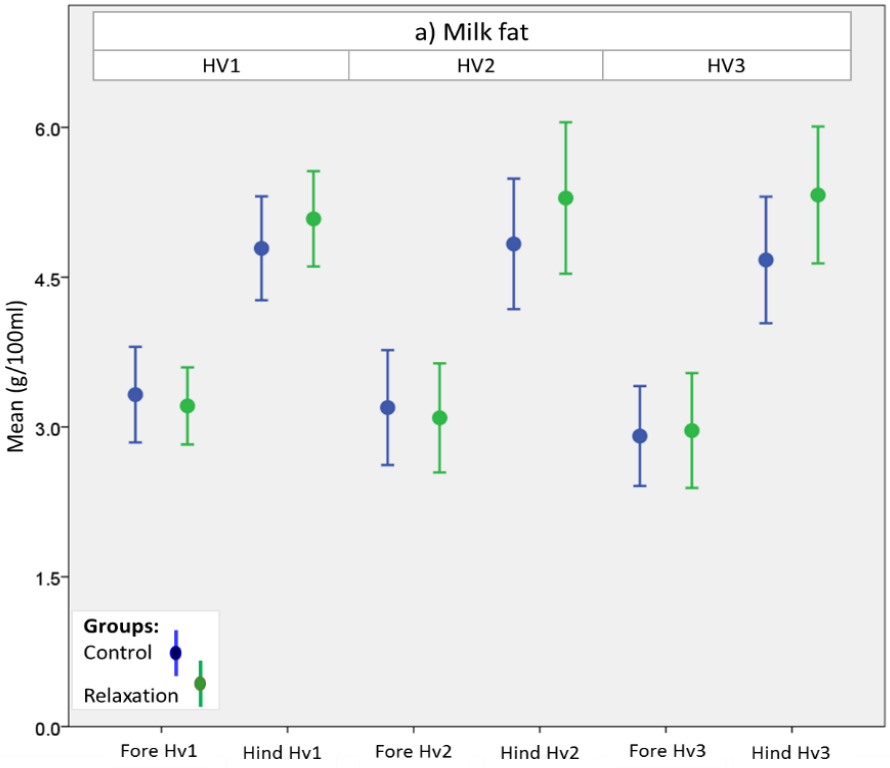
The milk fat content for the study population increased within a feed (increasing from 1.39 to 2.51 g/100ml), but no significant differences were found between groups at any HV. There was also no significant difference in milk fat content of hind milk at any HV. Nevertheless, there were non-significant trends: i) the relaxation group mothers overall had non-significant higher fat content of hind milk than the control group at all HV (from 5.17 at HV1 to 5.58 g/100ml at HV3 vs 4.73 at HV1 to 4.80 g/100ml at HV3); and ii) a non-significant greater increase in fat levels within a feed at all HV (ranging from 1.84 (HV1) to 2.51 (HV3) g/100ml vs 1.39 (HV1) to 1.73 (HV3) g/100ml), given that the milk fat content of fore milk (baseline) was similar to or slightly lower than that of the control group (Table 5.10).

Table 5.11 Milk fat of fore and hind milk from HV1 to HV3 by groups

Variables	Control			Relax.			p-	Mean diff.		
Milk fat (g/100ml)	n	mean	SD	n	mean	SD	value	(MD)	C.I	
Fore HV1	31	3.29	1.2	33	3.37	1.2	0.79	-0.08	-0.69	0.53
Hind HV1	29	4.73	1.3	31	5.17	1.3	0.20	-0.44	-1.12	0.24
Hind-Fore Hv1 (diff.)	29	1.39	1.8	31	1.84	1.2	0.28	-0.44	-1.25	0.36
Fore HV2	31	3.33	1.5	31	3.14	1.4	0.62	0.19	-0.55	0.92
Hind HV2	29	4.85	1.6	29	5.36	1.9	0.29	-0.50	-1.44	0.44
Hind-Fore Hv2 (diff.)	29	1.62	1.5	29	2.18	1.7	0.19	-0.56	-1.41	0.29
Fore HV3	30	3.07	1.4	30	3.07	1.5	1.00	0.00	-0.75	0.74
Hind HV3	30	4.80	1.6	30	5.58	2.0	0.10	-0.78	-1.72	0.16
Hind-Fore Hv3 (diff.)	30	1.73	2.1	30	2.51	2.5	0.19	-0.78	-1.96	0.41

C.I : confidence interval; Relax. : relaxation group; diff. : difference;

Figure 5.5 Milk fat of fore and hind milk from HV1 to HV3 by groups (Error Bars: 95% CI)



Unlike fat, the milk protein content was more stable and did not change much within a feed at any HV, with a change of only 0.01 to 0.03 g/100ml from fore to hind milk (Table 5.12; Figure 5.6). The overall protein content was not significantly different in either fore- or hind milk between groups at most HV apart from hind milk at HV1 where the control group had an average of 1.12 g/100ml higher protein content in hind milk (p=0.02).

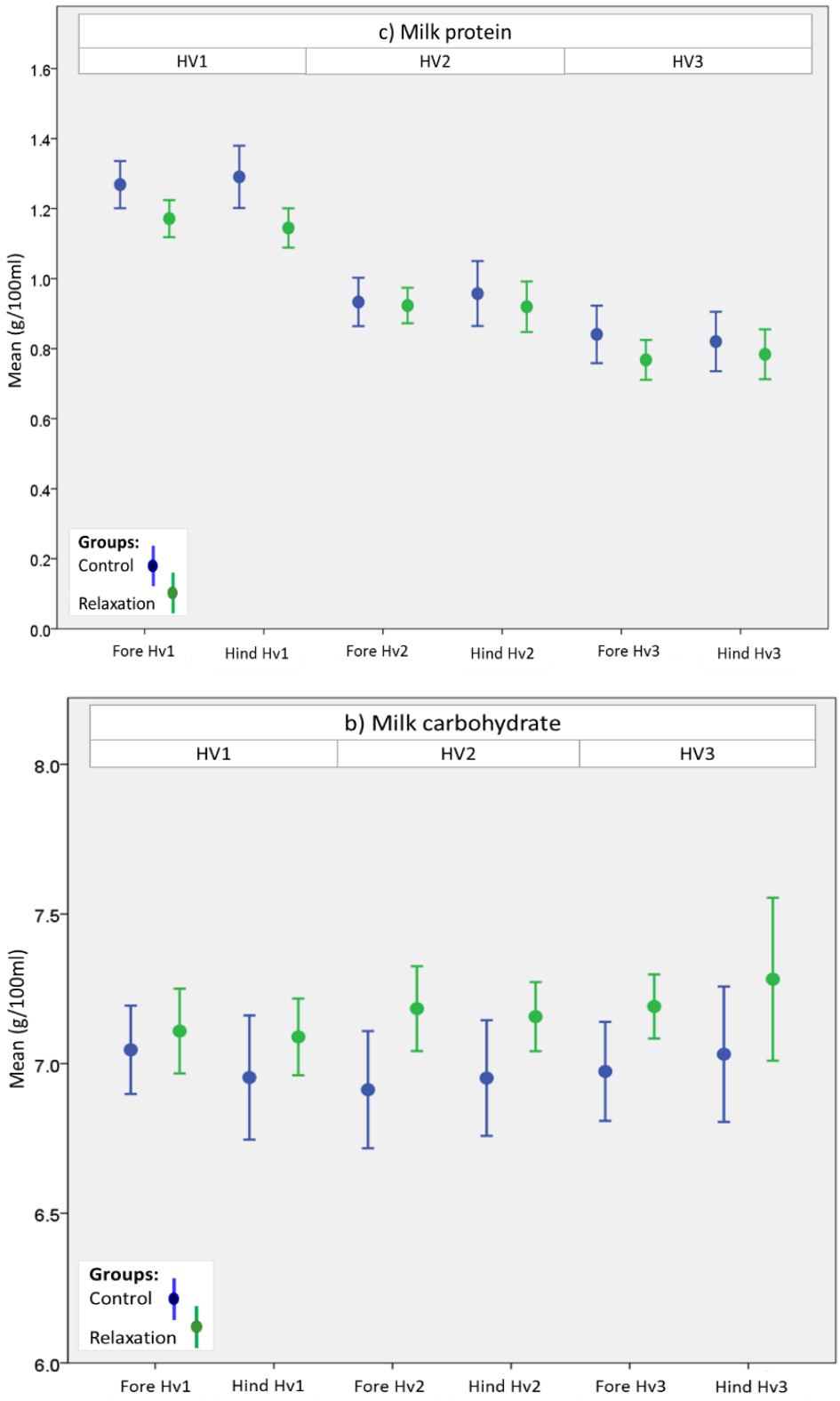
At HV1, the carbohydrate content in both fore and hind milk was not significantly different between groups, but at HV3, the carbohydrate content of fore milk was significantly higher in the relaxation group than in the control group (p=0.03). Similar to protein, the overall changes in carbohydrate content within a feed were small (0.01-0.09 g/100ml), however, there was a different long-term change in trend between groups; the carbohydrate content in fore milk of the relaxation group mothers increased across time points (from HV1 to HV3) whereas the control group showed an opposite trend – decreasing across time points. The GLM repeated measures test also suggested that there was a significant difference in the change of carbohydrate content of fore milk across time points between groups (F (1,59)=5.5, p=0.02).

Table 5.12. Milk protein and carbohydrate of fore and hind milk from HV1 to HV3 by groups

Variables	Control			Relax.			Mean diff.			
Milk protein (g/100ml)	n	mean	SD	n	mean	SD	p-value	(MD)	C.I	
Fore HV1	31	1.28	0.18	33	1.24	0.32	0.55	0.04	-0.1	0.17
Hind HV1	29	1.30	0.23	31	1.17	0.17	0.02	0.12	0.02	0.23
Hind-Fore Hv1 (diff.)	29	0.02	0.14	31	-0.02	0.11	0.28	0.03	-0.03	0.10
Fore HV2	31	0.96	0.20	31	0.94	0.16	0.56	0.03	-0.07	0.12
Hind HV2	29	0.98	0.24	29	0.95	0.25	0.71	0.02	-0.11	0.15
Hind-Fore Hv2 (diff.)	29	0.02	0.11	29	0.01	0.18	0.73	0.01	-0.06	0.09
Fore HV3	30	0.90	0.45	30	0.77	0.14	0.15	0.13	-0.04	0.30
Hind HV3	30	0.87	0.43	30	0.80	0.19	0.44	0.07	-0.10	0.24
Hind-Fore Hv3 (diff.)	30	-0.03	0.12	30	0.03	0.18	0.13	-0.06	-0.14	0.02
Milk CHO (g/100ml)		Control			Relax.		p-value	MD	C.I	
Fore HV1	31	7.08	0.37	33	7.02	0.46	0.58	0.06	-0.15	0.27
Hind HV1	29	6.96	0.51	31	7.04	0.35	0.48	-0.08	-0.31	0.15
Hind-Fore Hv1 (diff.)	29	-0.09	0.42	31	-0.01	0.40	0.44	-0.08	-0.29	0.13
Fore HV2	31	6.92	0.47	31	7.14	0.41	0.05	-0.22	-0.45	0.00
Hind HV2	29	6.94	0.48	29	7.10	0.44	0.20	-0.16	-0.40	0.09
Hind-Fore Hv2 (diff.)	29	0.03	0.38	29	-0.05	0.34	0.43	0.08	-0.11	0.27
Fore HV3	30	6.90	0.66	30	7.19	0.27	0.03	-0.29	-0.55	-0.03
Hind HV3	30	6.96	0.73	30	7.24	0.70	0.13	-0.28	-0.65	0.09
Hind-Fore Hv3 (diff.)	30	0.06	0.40	30	0.05	0.73	0.97	0.01	-0.30	0.31

C.I : confidence interval; Relax. : relaxation group; diff. : difference; CHO: carbohydrate

Figure 5.6 Milk protein and carbohydrate of fore and hind milk from HV1 to HV3 by groups (Error Bars: 95% CI)



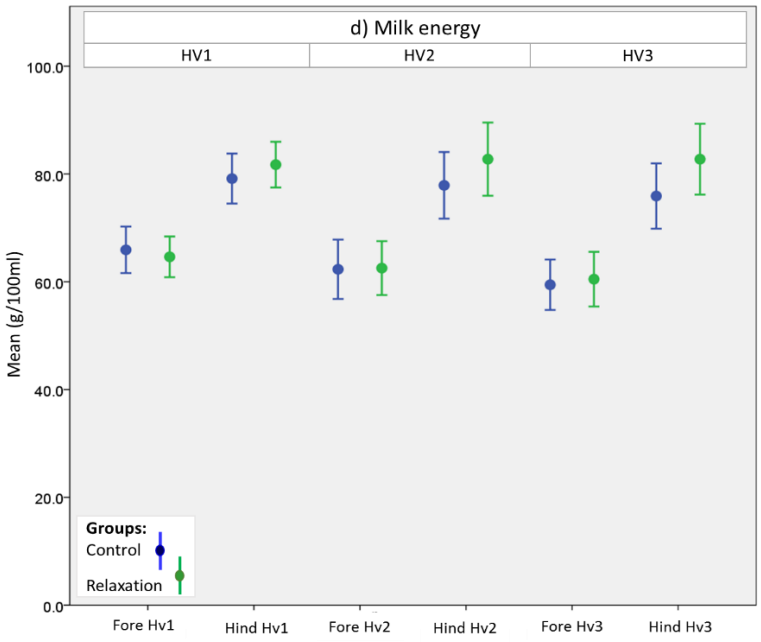
There were no significant differences in milk energy content in either fore- or hind milk at any HV between groups (Table 5.13; Figure 5.7). However, similar to the non-significant trend for fat content, the relaxation group mothers had overall non-significantly higher energy content in hind milk and also a non-significant greater increase in energy within a feed at all HV (16.8 (HV1) to 23.6 (HV3) vs 12.6 (HV1) to 16.1 (HV3) kcal/100 ml).

Table 5.13 Milk energy (kcal/100ml) of fore and hind milk from HV1 to HV3 by groups

Variables	n	mean	SD	n	mean	SD	Mean diff.			
Milk energy (kcal/100ml)		Control			Relax.		p-value	MD	C.I	
Fore HV1	31	65.8	11.1	33	66.1	11.6	0.91	-0.33	-6.0	5.4
Hind HV1	29	78.6	11.9	31	82.5	11.5	0.21	-3.81	-9.9	2.2
Hind-Fore Hv1 (diff.)	29	12.6	16.6	31	16.8	11.1	0.24	-4.29	-11.5	3.0
Fore HV2	31	63.7	14.2	31	62.9	12.9	0.82	0.77	-6.1	7.7
Hind HV2	29	78.1	15.3	29	83.3	17.4	0.24	-5.14	-13.7	3.5
Hind-Fore Hv2 (diff.)	29	15.3	13.5	29	20.0	16.2	0.24	-4.62	-12.5	3.2
Fore HV3	30	60.9	12.9	30	61.4	13.1	0.87	-0.55	-7.3	6.2
Hind HV3	30	77.0	15.5	30	85.0	18.8	0.08	-8.02	-16.9	0.9
Hind-Fore Hv3 (diff.)	30	16.1	19.6	30	23.6	23.3	0.18	-7.47	-18.6	3.6

C.I : confidence interval; Relax. : relaxation group; diff. : difference;

Figure 5.7 Milk energy of fore and hind milk from HV1 to HV3 by groups (Error Bars: 95% CI)



Pooled data: I also calculated the average of the macronutrient and energy content in foremilk and hindmilk, the average change within a feed (difference from fore to hind) at all HV and also the average for samples collected at all HV, as pooled data (Table 5.14). The relaxation group had significantly higher hindmilk fat and energy content than the control group (mean differences: 0.63 g/100 ml for fat and 6.2 kcal/100 ml for energy, p=0.03 and 0.02 respectively). Similarly, the average changes in fat and energy content within a feed for all HV pooled were significantly higher in the relaxation group than in the control group (mean differences of 0.66 g/100ml of fat and 6.1 kcal/100 ml of energy, both p=0.04: Table 5.11). The average foremilk carbohydrate (HV2 and HV3) was significantly higher in the relaxation group than the control group (p=0.007). In summary, the relaxation group mothers showed i) a trend towards having greater increases in milk energy and fat content within a feed across time points, with greater changes at later visits and; ii) higher foremilk carbohydrate and greater overall pooled milk carbohydrate.

Table 5.14 Pooled data: Milk composition of fore and hind milk by groups

Variables	Control			Relax.			Mean diff.			
Milk fat (g/100ml)	n	mean	SD	n	mean	SD	p-value	(MD)		C.I
Mean Fore (Hv2-3) [t]	30	3.19	1.19	29	3.05	1.21	0.65	0.14	-0.48	0.77
Mean hind (Hv1-3) [t]	31	4.79	1.01	31	5.42	1.26	**0.03**	-0.63	-1.21	-0.06
Mean diff. (HV1-3) [t]	31	1.53	1.16	31	2.18	1.30	**0.04**	-0.66	-1.28	-0.03
Milk carbohydrate (g/100ml)	Control			Relax.						
Mean Fore (Hv2-3) [t]	30	6.91	0.45	29	7.18	0.26	**0.007**	-0.27	-0.46	-0.07
Mean hind (Hv1-3) [t]	31	6.95	0.50	31	7.10	0.40	0.19	-0.15	-0.38	0.08
Mean diff. (HV1-3) [t]	27	0.00	0.28	28	0.01	0.31	0.86	-0.01	-0.17	0.15
Milk protein (g/100ml)	Control			Relax.						
Mean Fore (Hv2-3) [t]	30	0.93	0.28	29	0.85	0.11	0.18	0.08	-0.04	0.18
Mean hind (Hv1-3) [t]	31	1.04	0.29	31	0.99	0.18	0.39	0.05	-0.07	0.18
Mean diff. (HV1-3) [t]	27	0.01	0.08	28	0.00	0.09	0.56	0.01	-0.03	0.06
Milk energy (kcal/100ml)	Control			Relax.						
Mean Fore (Hv2-3) [t]	30	62.24	11.3	29	61.67	10.8	0.84	0.57	-5.20	6.34
Mean hind (Hv1-3) [t]	31	77.9	9.5	31	84.1	11.2	**0.02**	-6.23	-11.5	-1.0
Mean diff. (HV1-3) [t]	31	14.1	10.6	31	20.2	12.0	**0.04**	-6.05	-11.8	-0.3

C.I : confidence interval; Relax. : relaxation group; diff. : difference;

5.5.5. Dose-response effects

The mean and median frequencies of listening to the therapy at different duration and time points are shown in Table 5.15 : i) from day 1 up to 4 weeks post-HV1 (4 weeks duration post HV1); ii) from day 1 up to 4 weeks post-HV2 (4 weeks duration post HV2); iii) from day 1 post HV1 up to day 1 HV3 (2-3 months duration post HV1); and iv) from day 1 post HV1 up to day 1 HV4 (3-4 months duration post HV1). All mothers were encouraged to listen to the therapy at least once a day for a minimum of 2 weeks after each HV. If they did not manage to listen every day, they were suggested to listen for at least every alternate day for at least 2 weeks after each HV. Overall, the compliance rate was good since the majority of the mothers listened on average 12-13 times post HV1 and HV2.

Table 5.15 Descriptive statistics for the frequency of listening to the therapy

Duration of listening to the therapy:	4 weeks post HV1	4 weeks post HV2	2-3 months post HV1	3-4 months post HV1
	Hv1 (4 weeks)	Hv2-Hv3	Hv1 to Hv3	Hv1 to Hv4
Mean	12.1	12.6	24.7	34.0
Median	8.0	6.0	15.5	26.0
Minimum	1	0	2	3
Maximum	52	63	115	136

Table 5.16 shows that the frequencies of listening to the therapy from HV1 to later time points were on average moderately to strongly correlated with four main primary outcomes: maternal stress score (PSS), infant anthropometric data (weight SD and BMI SD) and sleeping duration. No significant correlation was found for other outcomes ($p>0.05$). Overall, more frequent listening to the therapy was associated with lower maternal stress score, increased sleeping duration and greater infant growth (weight SD, weight SD gain and BMI SD). There were no significant associations between the frequency of listening to the therapy and breast milk composition (either cortisol or macronutrient levels) ($p>0.05$).

Table 5.16 Correlations between frequencies of listening to the therapy and primary outcomes

Duration of listening to the therapy:		4 weeks post HV1	4 weeks post HV2	2-3 months post HV1	3-4 months post HV1
PSS score		**Hv1 (4 weeks)**	**Hv2-Hv3**	**Hv1 to Hv3**	**Hv1 to Hv4**
PSS HV2 score (n=62)	r =	-0.36	-0.38	-0.36	
	p =	0.004	0.003	0.004	
PSS HV3 score (n=61)	r =	-0.32	-0.34	-0.34	-0.33
	p =	0.012	0.007	0.007	0.009
Weight SDs		**Hv1 (4 weeks)**	**Hv2-Hv3**	**Hv1 to Hv2**	**Hv1 to Hv4**
Weight SD HV2 (n=63)	r =	0.43	0.33	0.4	
	p =	<0.001	0.007	0.001	
Weight SD HV3 (n=63)	r =	0.48	0.41	0.45	0.47
	p =	<0.001	0.001	<0.001	<0.001
Weight SD HV4 (n=62)	r =	0.45	0.39	0.43	0.45
	p =	<0.001	0.002	0.001	<0.001
Weight SD gain Hv1-3 (n=63)	r =	0.37	0.32	0.35	
	p =	0.003	0.01	0.004	
Weight SD gain Hv1-4 (n=62)	r =	0.33	0.29	0.32	0.36
	p =	0.009	0.02	0.011	0.004
BMI SDs		**Hv1 (4 weeks)**	**Hv2-Hv3**	**Hv1 to Hv3**	**Hv1 to Hv4**
BMI SD HV2 (n=63)	r =	0.37	0.38	0.39	
	p =	0.003	0.002	0.002	
BMI SD HV3 (n=63)	r =	0.53	0.50	0.53	0.54
	p =	<0.001	<0.001	<0.001	<0.001
BMI SD HV4 (n=50)	r =	0.49	0.45	0.46	0.46
	p =	0.001	0.001	0.001	0.001
Sleeping behaviour		**Hv1 (4 weeks)**	**Hv2-Hv3**	**Hv1 to Hv3**	**Hv1 to Hv4**
Duration of sleeping at 6-8 week (n=37)	r =	0.28	0.39	0.35	
	p =	0.09	0.018	0.035	
Changes in sleeping duration (n=34) (Increase from HV1-2)	r =	0.29	0.43	0.40	
	p =	0.09	0.012	0.019	

*correlation test: Spearman's rank; r= correlation co-efficient; p: p-value

Table 5.17 below shows a summary of the primary outcome findings.

Table 5.17.Summary of the primary outcomes for the relaxation group compared to the control group

No	Primary outcomes:	Baseline	End-point	The effects of the intervention:
1	**Maternal psychological state:**			
	Stress (PSS score)	NS	Lower *	Significantly reduced postnatal stress at HV2 and HV3.
	Anxiety (BAI score)	Lower *	NS	
2	**Infant growth (SDS) :**			
	Weight & weight gain	NS	Higher*	Significantly increased infant weight and BMI at later ages (HV2-4)
	Length	NS	NS	
	Head circumference	NS	NS	
	BMI	NS	Higher*	
	FM% & FFM%		NS	
	FM		NS	
	FFM		Higher NS T	
3	**Infant behaviour (3-day diary):**			
	Sleeping	NS	Longer*	Significantly increased sleeping duration post-HV2.
	Awake	NS	Shorter NS T	
	Feeding	NS	NS	
	Distress	NS	NS	
4	**Physiological changes in cortisol:**			
		Pre-BF:	**Post-BF:**	Significantly reduced cortisol levels within a feeding at HV1 only.
	Milk HV1	NS	Lower*	
	Milk HV2	NS	NS	
	Saliva HV1	Ns	NS	
	Saliva HV2	NS	NS	
	NS T: Non significant trend; NS: No significant different; *Significant different p<0.05			

No	Primary outcomes:	BASELINE	END-POINT	THE EFFECTS OF THE INTERVENTION:
5	**Physiological changes in macronutrient content:**			
		Fore:	**Hind:**	i) **Fat:** NS trend: Increased fat levels within a feed but NS between groups.
	Milk fat HV1,HV2 & HV3	NS	Higher NS T	
	Milk protein HV1	NS	Lower*	ii) **Protein:** Lower* protein levels in hind milk at HV1 only, but no significant differences at HV2 & HV3.
	Milk protein HV2 & 3	NS	NS	
	Milk CHO HV1	NS	NS	iii) **Carbohydrate:** Levels in fore milk were significantly higher at HV3. Higher NS trend of CHO in fore milk was shown at HV2 (p=0.05).
	Milk CHO HV2	Higher NS T	NS	
	Milk CHO HV3	Higher*	NS	
6	**Physiological changes in milk energy:**	**Fore:**	**Hind:**	
	Milk energy HV1, HV2 & HV3	NS	NS	NS trend: overall higher energy content in hind milk at all HV but NS between groups.
	Pooled results: Average of energy increase within a feed at all HV (1-3)	-	Higher*	Pooled results of milk energy changes within a feed at HV1 to HV3: significantly higher than the control group.

NS T: Non significant trend; NS: Not significantly different;
CHO: carbohydrate; *Significantly different $p<0.05$

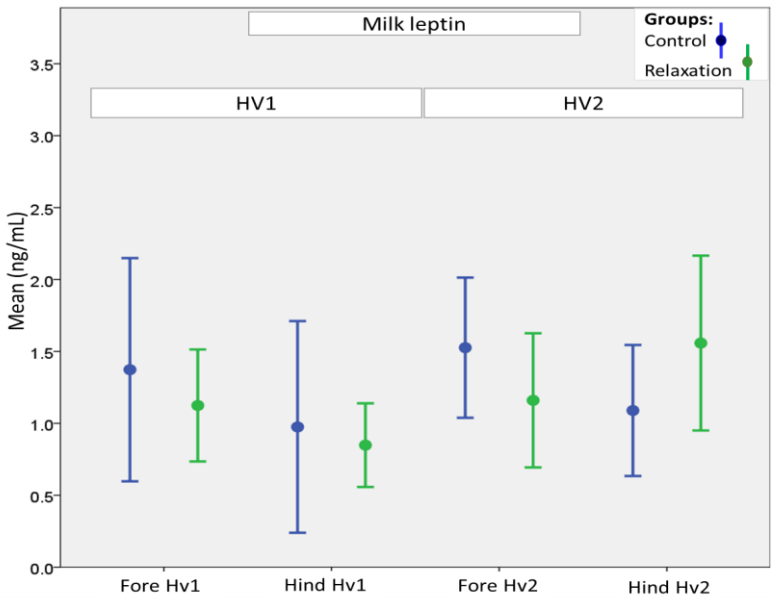
5.6. Results III : Secondary RCT outcomes

In this section, I present the effects of relaxation therapy on the changes in milk hormones (leptin and ghrelin) concentrations within a feed (fore and hind) and at two-time points (HV1 and HV2); and also the effects on infant appetite and temperament.

5.6.1. Breast milk leptin

All leptin data were transformed to natural logarithms prior to analysis and the results are shown in Table 5.18. At HV1, there was no significant difference in either fore- or hindmilk leptin between groups. At HV2, the control group had a non-significant trend towards higher foremilk leptin than the relaxation group (geometric mean: 1.12±1.1 v 0.59±0.8; p=0.051). However, the change in milk leptin concentration within a feed at HV2 was significantly different between groups: on average milk leptin concentration reduced within a feed in the control group, and increased within a feed in the relaxation group (p=0.02, CI:11.5 s%, 157.3 s%). In addition to the different direction of the change, the control group also had on average 84% greater change in leptin concentration within a feed than the control group: the mean value of foremilk leptin in the control group was double that in hindmilk, whereas in the relaxation group, the mean value of foremilk leptin was only 20% less than the value in hindmilk (Table 5.18). Figure 5.8 shows the absolute values of leptin concentrations in fore and hindmilk at HV1 and HV2.

Figure 5.8 Leptin concentrations (absolute values) in fore- and hindmilk at HV1 and HV2 by groups (Error Bars: 95% CI).



5.6.2. Breast milk ghrelin

Total ghrelin and active ghrelin data were transformed to natural logarithms prior to analysis. Table 5.18 shows the results for breast milk ghrelin (geometric mean values).

Table 5.18 Comparison of breast milk leptin and ghrelin between groups

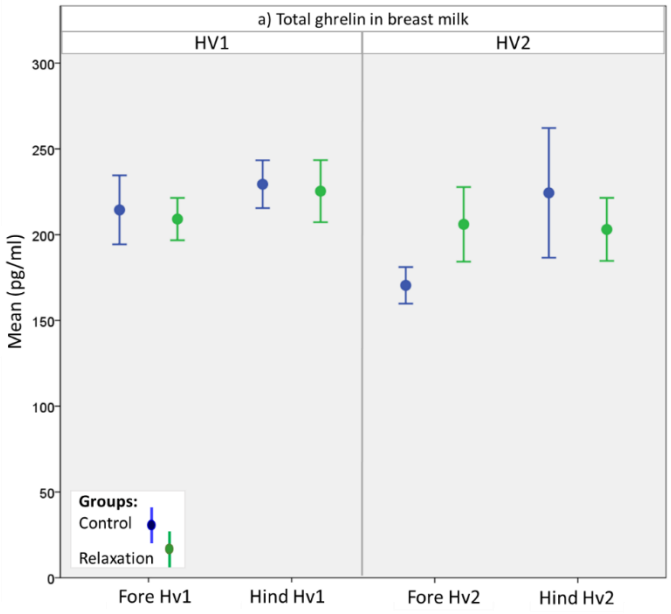
	n	Control Mean*	SD	n	Relaxation Mean*	SD	p-value	Mean diff: s%	C.I s%	
Leptin HV1										
Foremilk	32	0.50	0.8	32	0.55	0.9	0.807	-0.101	-0.9	0.7
Hindmilk	30	0.31	0.5	30	0.47	0.7	0.301	-0.421	-1.2	0.4
Change: Fore-Hind	30	0.19	0.2	30	0.08	0.1	0.227	0.412	-0.3	1.1
Leptin HV2										
Foremilk	30	1.12	1.1	29	0.59	0.8	0.051	0.640	0.0	1.3
Hindmilk	29	0.58	0.9	28	0.71	1.2	0.613	-0.214	-1.1	0.6
Change: Fore to Hind	29	0.54	0.8	28	-0.13	-0.15	0.024	0.844	0.1	1.6
Total ghrelin HV1										
Foremilk	31	208.7	40.7	31	205.12	27.6	0.688	0.017	-0.07	0.10
Hindmilk	31	224.9	34.8	29	220.81	39.7	0.674	0.018	-0.07	0.10
Change: Fore to Hind	30	-16.2	-3.5	29	-15.70	-2.5	0.93	-0.004	-0.10	0.09
Total ghrelin HV2										
Foremilk	30	168.2	27.4	29	198.38	45.0	0.002	-0.165	-0.27	-0.06
Hindmilk	30	210.5	69.7	28	198.68	43.1	0.439	0.058	-0.09	0.21
Change: Fore to Hind	30	-42.3	-15.6	28	-0.28	-0.08	0.01	-0.230	-0.40	-0.06
Active ghrelin HV1										
Foremilk	31	20.8	8.4	31	21.14	11.1	0.902	-0.015	-0.25	0.22
Hindmilk	31	30.5	13.5	30	25.39	9.7	0.09	0.182	-0.03	0.39
Change: Fore to Hind	31	-9.6	-4.9	30	-4.25	-2.22	0.131	-0.204	-0.47	0.06
Active ghrelin HV2										
Foremilk	30	12.2	11.3	29	16.47	10.5	0.149	-0.305	-0.72	0.11
Hindmilk	30	21.8	20.1	28	29.61	17.0	0.14	-0.304	-0.71	0.10
Change: Fore to Hind	30	-9.7	-7.0	28	-13.14	-7.26	0.972	-0.006	-0.34	0.34

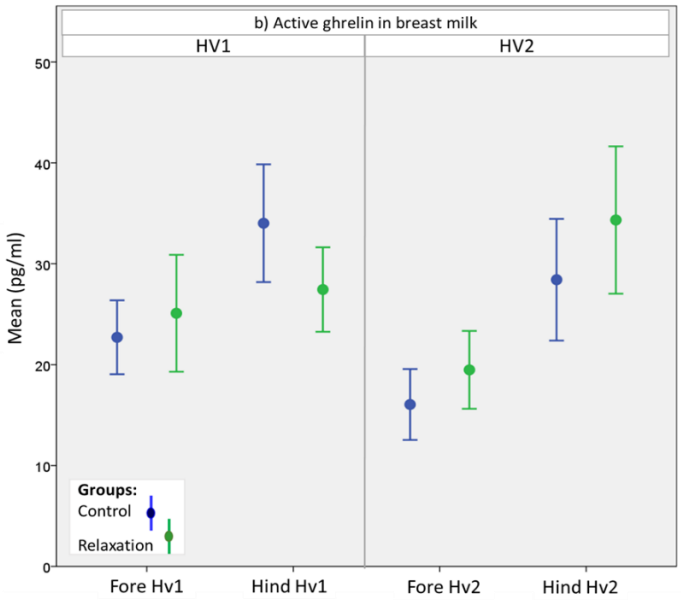
Mean*= geometric mean, S%=sympercent; p-value for t-test

The total ghrelin in both fore and hind-milk at HV1 was not significantly different between groups. However, at HV2, the foremilk total ghrelin was significantly different between groups (p=0.002): the control group had significantly lower total ghrelin in foremilk, with an average of 16.5 s% less than the relaxation group (geometric mean: 169 v 198 pg/ml, p=0.002, CI: -26.7 s%, -6.3 s%). Within a feed at HV2, total ghrelin concentrations in the control group

increased significantly (p=0.007) from fore- to hindmilk, but the relaxation group showed no major change within a feed (Table 5.18). With regards to the active ghrelin in breast milk, there were no significant differences in fore- and hindmilk active ghrelin concentrations between groups at either HV1 or HV2. Figures 5.8 and 5.9 show absolute values for total ghrelin and active ghrelin concentrations at HV1 and HV2 by groups.

Figure 5.9: a) Total ghrelin and b) active ghrelin concentrations (absolute values) in fore- and hindmilk at HV1 and HV2 by groups (all error bars: 95% CI).





5.6.3. Infant appetite (BEBQ)

The Baby-Eating-Behaviour questionnaire (BEBQ) results (Table 5.19) show that there were no significant differences in any appetite traits between groups at the different HV (all p>0.05). The repeated measures ANOVA also showed no significant differences between groups across home visits for any appetite trait (all p>0.05).

Table 5.19 Mean score of the appetite traits and t-test results

	Control			Relax						
Variables	n	mean	SD	n	mean	SD	p-value	Mean diff.	C.I	
HV1										
Enjoyment of food (EF)	31	4.38	0.4	33	4.26	0.4	0.229	0.12	-0.1	0.3
Food responsiveness (FR)	31	3.12	0.7	33	3.18	0.7	0.772	-0.05	-0.4	0.3
Slowness in eating (SE)	31	2.86	0.8	33	3.12	0.6	0.156	-0.26	-0.6	0.1
Satiety responsiveness (SR)	31	2.39	0.5	33	2.63	0.5	0.076	-0.24	-0.5	0.03
General appetite (GE)	31	4.23	0.8	33	4.12	0.9	0.617	0.11	-0.3	0.5
HV2										
Enjoyment of food (EF)	31	4.25	0.5	32	4.35	0.3	0.317	-0.1	-0.3	0.1
Food responsiveness (FR)	31	3.21	0.8	32	3.28	0.7	0.751	-0.06	-0.4	0.3
Slowness in eating (SE)	31	2.93	0.7	32	2.85	0.6	0.662	0.08	-0.3	0.4
Satiety responsiveness (SR)	31	2.54	0.4	32	2.59	0.5	0.649	-0.06	-0.3	0.2
General appetite (GE)	31	4.13	0.8	32	4.25	0.8	0.553	-0.12	-0.5	0.3
HV3										
Enjoyment of food (EF)	31	4.32	0.4	32	4.3	0.5	0.826	0.03	-0.2	0.3
Food responsiveness (FR)	31	3.14	0.7	32	3.05	0.7	0.616	0.09	-0.3	0.5
Slowness in eating (SE)	31	2.77	0.6	32	2.76	0.7	0.924	0.02	-0.3	0.4
Satiety responsiveness (SR)	31	2.68	0.5	32	2.6	0.5	0.573	0.07	-0.2	0.3
General appetite (GE)	31	3.94	0.9	32	4.0	0.9	0.673	-0.96	-0.55	0.36

5.6.4. Infant temperament (RIBQ)

Infant temperament characterised by three dimensions was not significantly different between groups (surgency p-value: 0.53, CI: -0.5, 0.3; negative affect p-value: 0.66, CI: -0.4, 0.6; and effortful control p-value: 0.78, CI:-0.3, 0.4).

Table 5.20 Summary of the RCT results on the effects of the intervention on secondary outcomes

		Baseline	End-point	Summary (comparison between groups)
1	Physiological changes in leptin:			
	Fore & hind milk	Fore:	Hind:	i) No significant diff. between groups at HV1, but at HV2, there was a NS trend : the relaxation group had lower foremilk leptin
	Milk HV1	NS	NS	
	Milk HV2	(R) Lower but NS trend	NS	
	Change within a feed :		Change:	ii) The change of leptin concentrations within a feed was significantly different between groups at HV2 only: Control: reduced within a feed Relaxation: increased within a feed
	Change fore-hind HV1	-	NS	
	Change fore-hind HV2	-	Opposite trend*	
2	Physiological changes in total ghrelin:			
	Fore & hind milk:	Fore:	Hind:	i) No significant diff. between groups at HV1, but at HV2, the relaxation group had significantly higher foremilk total ghrelin than the control group
	Milk HV1	NS	NS	
	Milk HV2	(R) Higher*	NS	
	Change within a feed :		Change:	ii) The change of total ghrelin concentrations within a feed was significantly different between groups at HV2 only: Control: increased within a feed Relaxation: slightly reduced within a feed
	Change fore-hind HV1	-	NS	
	Change fore-hind HV2	-	Opposite trend*	
3	Physiological changes in active ghrelin:			
	Fore & hind milk:	Fore:	Hind:	i) No significant diff. between groups at HV1 and HV2
	Milk HV1	NS	NS	
	Milk HV2	NS	NS	
	Change within a feed :		Change:	ii) The changes of active ghrelin concentrations within a feed were not significantly different between groups at HV1 and HV2
	Change fore-hind HV1	-	NS	
	Change fore-hind HV2	-	NS	
4	Infant appetite	HV1	HV3	
	All appetite traits HV1	NS	NS	No significant diff. between groups for appetite traits at any HV
	All appetite traits HV1	NS	NS	
	All appetite traits HV1	NS	NS	
5	Infant temperament	HV1	HV4	
	Surgency	-	NS	No significant diff. between groups for any temperament trait at HV4
	Negative affect	-	NS	
	Effortful control	-	NS	

NS : Not significant, *significant result at p<0.05; (R): Relaxation group; diff: different; BF=breastfeeding

Table 5.21 The outcome measures and the main results for the trial analyses

Randomised trial hypotheses		Outcome measures		Statistical analyses and results (intervention vs control)	
		Baseline	Outcomes		
a) Primary RCT outcomes:					
Primary hypotheses: *The use of relaxation tape therapy during breastfeeding starting at 2-week postpartum will result in:*				Final main analyses	Final results (main analyses only) :
i	reduced maternal stress and anxiety	*Long term effects of the intervention on:*			
		maternal stress and anxiety scores at HV1	maternal stress and anxiety scores at HV2 and HV3	T-test for scores at HV3	Stress: 11.97 v 15.10, p = 0.029, C.I: 0.3, 5.9 Anxiety: 6 v 9, p = 0.24
		Short term / acute effects of the intervention (within a feed):			
		maternal mood/ emotions (mini-BF test) prior to BF at HV1	maternal mood/emotions post-BF session at HV1-3	T-test for the changes in scores during a feed at HV1	Question 1-9 : all p > 0.05
ii	lower milk cortisol levels	*Long term effects of the intervention on:*			
		breast milk cortisol in foremilk at HV1	breast milk cortisol in fore- and hindmilk at HV2	T-test for the foremilk cortisol at HV2	0.15 v 0.12, p = 0.21, C.I: -69.2, 15.7
		Short term / acute effects of the intervention (within a feed):			
		breast milk cortisol in foremilk at HV1	the changes in cortisol levels within a feed at HV1 and hindmilk cortisol at HV1	T-test for the changes in cortisol within a feed at HV1	-0.033 v -0.003, p = 0.024, C.I: -63.4, -4.5
iii	Increased in breast milk energy (higher calories)	*Long term effects of the intervention on:*			
		total energy in fore milk HV1 (total calories)	total energy in fore- and hindmilk at HV2 and HV3.	T-test for the foremilk total energy at HV3	61.4 v 60.9, p = 0.87, C.I: -7.2, 6.3
		Short term / acute effects of the intervention (within a feed):			
		total energy in fore milk HV1 (total calories)	the changes in milk energy within a feed at HV1-3 and hindmilk energy at HV1	T-test for the changes in milk energy within a feed HV1	16.8 v 12.6, p = 0.24, C.I : -11.5, 3.0

Randomised trial hypotheses		Outcome measures		Main statistical analyses	Supporting statistical analyses
		Baseline	Outcomes		
iv	favourable effects on infant behaviour	Duration of infant feeding, sleeping, crying/distress and awake at HV1	Duration of infant feeding, sleeping, crying/distress and awake at HV2	T-test for the duration of each behaviour at HV2	Feeding, crying/distress, awake : all p>0.05 Sleeping : 855.6 v 733.6, p= 0.017
vi	more optimal infant growth	infant weight & BMI HV1	- Infant weight & BMI at HV1-3 - Infant weight and BMI gain (HV1 to 3)	T-test for weight SD and BMI SD at HV3	Weight SD: -0.12 v -0.9, p<0.001, -1.2, -0.4 BMI SD: -0.52 v -1.48, p<0.001, -1.41, -0.51
		-	- Infant fat mass, fat-free-mass - Infant fat mass index (FMI) & fat-free-mass index (FFMI)	T-test at for FM, FFM, FMI and FFMI at HV4	FM: 1.4 v 1.05, p=0.13, -0.76, 0.1 FFM: 5.2 v 4.7, p=0.10, -1.0, 0.1 FMI: 3.5 v 2.6, p=0.13, -1.9, 0.27 FFMI: 12.9 v 11.8, p=0.09, -2.3, 0.17
b) Secondary RCT outcomes: Comparison between randomised groups					
i	Maternal depression	depression scores at HV1	depression scores at HV3	T-test for scores at HV3	6.0 v 7.3, p=0.19, C.I:-0.7, 3.3
ii	Milk leptin and ghrelin (hormones)	*Long term effects of the intervention on:*			
		breast milk hormone levels in fore milk at HV1	breast milk hormone levels in fore- and hindmilk at HV2	T-test for the foremilk leptin/ghrelin at HV2	Leptin: 0.59 v 1.12, p=0.05, C.I: 0, 1.3 Ghrelin: 198 v 168, p=0.002, -0.27, -0.06
		Short term / acute effects of the intervention (within a feed):			
		breast milk hormone levels in fore milk at HV1	the changes in milk hormones within a feed at HV1-3 and hindmilk hormone levels at HV1	T-test for the changes in hormone levels at HV2	Leptin: 0.08 v 0.19, p=0.23, C.I: -0.3, 1.1 Ghrelin: -4.3 v -9.6, p=0.13, C.I: -0.47, 0.06
iii	Infant temperament	-	Temperament scores at HV4	T-test for scores at HV4	All temperament traits p>0.05
iv	Infant appetite	appetite scores at HV1	appetite scores at HV3	T-test for scores at HV3	All appetite traits p>0.05
v	Other macronutrient components (for long term effects).	fat, protein and CHO in foremilk at HV1	foremilk fat, protein and CHO at HV2 & 3 and hindmilk levels at HV1 to 3	T-test for the foremilk levels at HV3	Foremilk fat and protein at HV3: p>0.05 Foremilk CHO: 7.2 v 6.9, p=0.03, C.I: -0.55, -0.03

* T-test = independent t-test to compare between groups; CHO: carbohydrate; Ghrelin = total ghrelin

5.7. Discussion

In this section, firstly, I summarise the main findings and follow up with a discussion of each primary outcome. The possible mechanisms or pathways for the effects of the intervention are also discussed. At the end of this chapter, the discussion focusses on overall strengths and limitations of the study.

5.7.1. Summary of the results

Part I: At baseline, there were no significant differences in maternal socio-demographic factors, breastfeeding perception or birth experience between randomised groups. The overall scores of the attitudes and perceptions towards infant feeding in both groups indicate a positive attitude towards breastfeeding in all mothers in the study population. The majority of the mothers in both groups also seemed to be highly confident of achieving their long duration of breastfeeding goals. As indicated in the previous chapter, this is consistent with a study in Kuala Lumpur, Malaysia, showing that mothers who intended to breastfeed tend to have higher IIFAS score [246].

Part II: The primary outcome results are summarised in Table 5.20 and 5.21. Considering the primary hypothesis, listening to relaxation therapy showed significant effects in terms of i) reduced maternal stress at HV2 and HV3; ii) reduced milk cortisol concentrations over a feed at HV1; iv) increased sleeping duration in infants at HV2; and v) increased infant weight SDS and BMI SDS from HV2 to HV4. There were also consistent though non-significant trends in breast milk macronutrient content: a trend towards higher hindmilk fat (HV1-HV3) and foremilk carbohydrate (HV2-HV3), and also a greater increase in milk energy over a feed at HV1 to HV3 in the intervention group. The pooled results for milk energy change within a feed at HV1-3 also showed a significantly higher value in the intervention group. Interestingly, there were also consistent significant associations between the frequency of listening to the therapy from HV1 to later time points and several primary outcomes suggesting a 'dose-response'. Greater frequency of listening to the therapy was associated with a lower in maternal stress score, an increase in infant sleeping duration and higher infant weight and BMI SD at later time points.

Part III: The other RCT outcomes (secondary RCT outcomes) are summarised in Tables 5.19. The findings showed inconsistent results in terms of the short- and long-term effects of the relaxation therapy intervention during breastfeeding on breast milk leptin and ghrelin. The relaxation therapy had no apparent short-term effect (that is, no change within a feed) on breast milk hormones at HV1 but a longer-term effect was suggested by the significant difference in total foremilk ghrelin concentrations at HV2. Differences in the change in milk hormone concentrations within a feed (from fore- to hindmilk) were only shown at HV2, with opposite directions of the changes in leptin and total ghrelin concentrations within a feed between groups. Thus, leptin concentrations in the control group reduced within a feed at HV2 and vice-versa for the relaxation group. Conversely, the total ghrelin concentrations in the control group increased within a feed at HV2, but no major changes were found in the relaxation group (a small reduction within a feed). The relaxation therapy did not show any effects on infant appetite at any home visit or temperament recorded at HV4.

Taken together, these results suggest that listening to relaxation therapy positively influenced maternal psychological state, making the mother less stressed or more relaxed, with consequent effects on infant behaviour (longer sleeping duration at HV2) and growth (higher weight and BMI, and greater weight gain). The effects on infant outcomes could possibly be mediated by changes in milk composition within a feed or over time, although the observed trends did not reach statistical significance. These findings demonstrate psychological and physiological effects of the intervention on both mothers and infants during the study period, which support the overall primary hypothesis. The results were more convincing given the observed dose-response effects of listening to the therapy with the reduction in maternal stress score, increase in sleeping duration and higher infant growth (weight SD, weight SD BMI SD). Further results of the whole study population's breast milk hormone, infant appetite and temperament are presented and discussed in the next chapter; the observational cohort outcomes (Chapter 6).

5.7.2. Maternal psychological state

The intervention group mothers had significantly lower stress scores at later visits (HV2 and HV3), showing that the relaxation therapy is effective in reducing maternal stress. More convincingly, there was also a dose response effect showing by negative correlations between frequencies of listening to the therapy with maternal stress scores at HV2 and HV3. Similarly, previous studies using relaxation therapy among breastfeeding mothers also reported a significant decreased in maternal psychological distress [171, 188] and increased maternal mindfulness [188] during the postpartum period. However, the studies had several limitations. Firstly, both studies had a very small sample size (n=26-30) [171, 188], hence the precision and level of confidence in the results may be questioned. Secondly, the study by Ak et al., [171] was not a RCT and it measured the effectiveness of the intervention over a very short period of 4 days, with measurement of maternal stress on day 1 and day 4. Finally, the other study reported a reduction in maternal stress and anxiety and increase in mindfulness, but their intervention involved different meditation programs during several sessions over an 8-week period, and the control group was aware of the relaxation therapy (meditation program) offered to the intervention group mothers. This raises two main issues: i) there is a possibility that the mothers in the control group could seek and use a similar relaxation tool during the study period; ii) they did not identify which program contributed the most or was most effective in reducing maternal distress or increasing mindfulness during the postpartum period. A detailed critical appraisal of these studies is provided in the systematic review in Chapter 2.

Although there were no significant differences in maternal anxiety and depression at later visits in my study, the intervention group mothers had a non-significant trend towards lower scores for anxiety and depression at both HV2 and HV3, suggesting the potential for a positive effect of the intervention on these outcomes. The lack of significant effects of the intervention on measures of anxiety and depression could have a number of explanations. Firstly, this study had a relatively small sample size and thus may have been underpowered for some outcomes. As described in section 3.6, the sample size calculation was based on data on maternal stress from a trial in mothers with preterm infants. The healthy mothers with full-term infants enrolled in my study were likely to have a lower risk of psychological distress in general. Secondly, judging from the results of the questionnaires on breastfeeding perception, breastfeeding goals and confidence in achieving these goals (Table 5.1), all of the mothers in

my study were highly motivated to breast-feed and positive about doing so; this also suggests they were a relatively low risk group for psychological distress during the post-partum period. On the other hand, it is possible that the intervention might be effective in helping mothers to relax and therefore significantly reducing maternal stress, but not effective in reducing maternal anxiety and depression to any great degree. Studies indicate that anxiety and depression often co-exist, either as affective states or clinical disorders [255, 256], and treatments are usually focused on treating the depressive symptoms [256]. In my study, the main aim was to reduce stress levels or encourage the mother to relax, rather than treating clinical symptoms. My results also suggest that anxiety was more strongly correlated with depression than with stress, especially at HV2 ($r=0.73$, $p<0.001$). This is probably due to some overlap in the areas assessed by the questionnaires used to measure depression and anxiety, especially those relating to negative behavioural/emotional symptoms (e.g feeling anxious, panicking and losing control or tendency self-harm), whereas the stress questionnaire focussed mostly on the emotional state in general [257]. All the questionnaires used in this study to measure psychological state have established reliability (except the Mini-breastfeeding test questionnaire), and have been well validated worldwide including among Malaysian populations, so they can be considered to be robust instruments.

Although the relaxation therapy has been shown to effectively reduce maternal stress, it is not clear which component of the therapy – for example, the content or the tone of voice used (or both) in the guided-imagery recording – was responsible for this effect. The recording was also available in two languages – Malay and English - and the mothers could choose to listen to whichever version they preferred whilst breastfeeding. The majority of the mothers in the study population listened to both versions, which may reflect the similarity of the intonation and tone in the English and Malay versions. Moreover, as English is commonly used in Malaysia, [258], it is to be expected that some mothers would prefer to listen more frequently to the English version than the Malay version. Future studies should consider getting feedback from the mothers regarding these aspects of the intervention therapy in order to improve the relaxation tool and tailor it for different populations.

The Mini-breastfeeding questionnaire data showed no significant differences between groups in the changes in maternal mood and emotions within a feed at any HV. There are two possible explanations: i) the intervention did not have a significant short-term impact on maternal mood and emotions; or ii) the questionnaire used was not a valid tool for measuring the acute

changes (short-term effect) of the intervention. As indicated in Chapter 3, this questionnaire was not validated or test for reliability, and it certainly cannot be considered to be as robust as the other questionnaires used in the study. Moreover, it was difficult to standardise the timing for the mothers to complete the questionnaire since this depended on the infant's mood or demand for a feed. Some infants were crying or distressed prior to breastfeeding, thus the mothers could not complete the questionnaire prior to the feed, but could only manage to do it during the beginning of the feeding session. Although some mothers were able to complete the questionnaire prior to breastfeeding (test-feed), some of them completed the task in a hurry, as the infant was crying whilst others could complete the questionnaire prior to feeding in a very calm or relaxed state since their infant was calm, even though they were expecting a feed. This contributed to the very high variability of the data.

5.7.3. Infant behaviour

Besides the benefits to the mothers, the intervention also showed favourable effects on infant behaviour in terms of significantly longer sleeping duration among infants in the intervention group. Infants in the intervention group spent on average 82 minutes longer per day sleeping at HV2. More interestingly, time spent sleeping in the control group reduced from HV1 to HV2, whereas the intervention group infants showed the opposite effect with an increase in time spent in sleeping from HV1 to HV2. Conversely, the change in time spent awake from HV1 to HV2 in the control group was higher (doubled) than in the intervention group, although the difference between groups was not significant. Overall, the control group infants spent more time awake and less time sleeping at HV2, whereas the opposite behaviour was shown in the intervention group. More interestingly, there was also a dose response effect showing that the more frequent the mothers listened to the relaxation therapy, the longer their infants sleep during the early age (6-8 weeks).

It is intriguing to explore the potential mechanisms behind the relative effects of the intervention on these infant behaviours. Such mechanisms could operate through psychological or physiological mother-infant signalling. Firstly, mothers who were less stressed might have had longer and better quality time to physically bond with their infants (e.g. skin-to-skin or comforting their infant); this could possibly stimulate or facilitate infant sleep. Experimental studies [259, 260], including randomised trials [261, 262] found that kangaroo care (skin-to-skin) promotes better self-regulation in the sleep-wake cycle in infants, characterised by a longer quiet sleep state. There is also a possibility that mothers who were

more relaxed might sleep longer themselves than mothers who were more stressed, and this could also have affected the sleeping duration of the infant, given that the all mothers in the study were co-sleeping with their infants. An observational study of mothers of preterm infants also found that listening to a guide-imaginary recording given as relaxation therapy was associated with reduction in stress score and improvement of maternal sleep quality [263]. This is supported by other experimental studies among adults, which have reported that relaxation techniques, either guide-imaginary recordings [264-266] or music relaxation [266-268], improve sleep quality. However, none of these studies was performed among healthy women nor mothers during the postpartum period. I did not record maternal sleep patterns or time spent carrying/comforting the infant in my study, but these would be relevant outcomes for consideration in future research.

A second explanation could be physiological effects of maternal stress on breast milk composition or/and breast milk intake: i) mothers who were less stressed and more relaxed may have produced milk with a higher energy content, or milk with altered content of other bioactive factors such as the hormones, which may have consequently affected infant behaviour (e.g sleeping behaviour), and hence later growth; ii) mothers who were less stressed and more relaxed may have ejected milk more easily or frequently, which may consequently have affected nutrient intake in infants, via the more efficient release of fat-rich hind milk at the end of the feed. Unfortunately, at this stage, the isotope samples that were collected to estimate breast milk intake are yet to be measured. Further discussion on the association of milk hormones and infant behaviours and their influence on infant growth are discussed in the next chapter. Other than milk hormones measured in the present study, the maternal psychological state could also affect other bioactive factors in breast milk, such as melatonin [77, 78] or beta-endorphin [76, 269, 270], all of which have been suggested to influence maternal mood or infant behaviour.

In terms of the results for other aspects of infant behaviour, the time spent feeding or showing distress did not differ much between groups, with both groups demonstrating a reduction in the amount of time spent in both behaviours from HV1 to HV2. This could be because sleeping and awake were the main behaviours at this age making it easier to detect differences in these behaviours. Consistent with the present study results on sleeping duration, previous studies have shown that in early infancy, infants spent most of their time sleeping: about 60-65% of each 24-hour period (approximately 14.5 to 16 hours) at 2 to 8 weeks of age [271, 272].

There are some limitations that should be considered regarding the findings from the 3-day infant behaviour diary. Firstly, infant behaviours were recorded for only 2-3 days on each occasion in this study, although the diary was originally developed to be used to record infant behaviours over a 7-day period [224]. This is to allow for day-to-day variation within individuals. However, considering many other follow-up tasks that mothers were asked to perform after the home visit sessions (e.g. completing other questionnaires and collecting biological samples), it would have been difficult for them to complete the diary over a longer period, and I considered that asking them to do so was likely to reduce the amount and quality of the data obtained. There is a possibility that the overall infant behaviour results could be less precise or representative than if a longer behaviour recording could have been completed. Nevertheless, this limitation would be expected to apply to all infants and the results still permit comparison of the randomised groups, by comparing the differences in infant sleeping and awake duration.

Secondly, although the diary was shortened to 2-3 days, the compliance with completion of the diary from HV1 to HV2 was not high in contrast to other studies that have reported good compliance (around 80-90%) [224, 225, 273]. This could be due to the large number of different tasks that mothers were asked to perform over the study period. The low compliance reduced the sample size further and may have introduced bias, so the results may not be representative of the whole study population. This is especially the case since this parental report diary is open to parental manipulation. For example, it is possible that the non-compliant mothers were not able to complete the diary because of 'problematic' behaviour of their infants (for example, long periods awake or distressed). Alternatively, mothers who completed the diary may have been more motivated and observant of their infant's behaviours, or more educated and conscious about the importance of research. In this study, the completion rate for the diary was also significantly higher among mothers in the intervention group. Since mothers in the intervention group were not blinded towards the relaxation therapy, there is a possibility they might have felt they should be more conscious towards their infant's behaviour. It is also possible that the intervention group mothers managed to complete the diary more reliably because the infant was sleeping longer. Moreover, they were also asked to record the frequency of listening to the therapy, and hence were more aware of the tasks given to them as a follow-up to the home visit. However, although the completion rate was not even between groups, no significant differences in

infant behaviours were shown between groups at baseline. In addition, there were no significant differences in maternal characteristics or socio-demographic background between compliant and non-compliant within each randomised groups, suggesting that the available data on infant behaviour can still be considered representative of the whole study population. Thus, the results at later visit (HV2) could still be considered convincing in showing differences between groups.

5.7.4. Infant growth

The evidence on infant growth showed an effect of the intervention on infant weight SDS and BMI SDS. The results are remarkable and consistent in showing that infants in the intervention group had significantly higher weight SDS and BMI SDS at all later HV (HV2-4), and also significantly higher gain in weight SDS from baseline to study endpoint. It is not yet clear whether faster infant growth in the intervention group should be considered more optimal than the control group. This is especially the case since evidence has suggested that rapid weight gain during infancy may be associated with increased risk of obesity and other cardiovascular risk factors in later life [274, 275].

The majority of infants had acceptable weight-for-age and BMI-for-age SDS score (within -2 and +2 SD) throughout the study period. No marked increment (more than 1 band on the growth chart or ±0.67 SD between measurements [275]) of infant weight was found between home visits during the study period. In addition, the average weight and BMI SDS scores of the intervention groups at all HV were still within the normal limits of the WHO Growth chart, and slightly below the 50^{th} percentile (Z-score of 0 value), showing a close match to the optimal growth of breastfed infants according to the WHO growth standards [276]. Therefore, referring to the growth pattern of infants in the intervention group, I would not say that the intervention group infants showed 'rapid weight gain'.

More interestingly, the weight SDS in the control group slightly decreased from HV1 to HV4 whereas the intervention group increased gradually. This seems similar to the observational studies that have used the WHO growth standards in exclusively breast-fed infants, in which the infants often show downward centile crossing in the first 3-4 months [277-280]. It has been suggested that this may be because the WHO reference dataset consisted of mother-infant dyads living in 'ideal' circumstances (i.e a highly selected sample population) [281] and that infants living in the real world, especially among populations with middle to low socio-

economic status, may have difficulty attaining this ideal growth pattern [278-280]. It is possible that the relaxation intervention allowed the breastfed infants in my study to come closer to the 'ideal' growth pattern according to the WHO growth standards.

Another parameter to consider in defining 'optimal growth' is infant body composition. In the present study, the relaxation group had a non-significant trend towards higher fat free mass and fat free mass index (kg) than the control group. This suggests that the intervention group infants have a relatively non-significant greater increase in lean tissue than the control group. However, the body composition result was based on a limited number of isotope samples. Due to several methodological issues with the samples (described later in this chapter), not all data were plausible (only 29 samples) and this reduced the number available for the calculation of total body water, limiting the statistical power to detect differences. Nevertheless, this result is consistent with the significantly higher weight in the intervention group infants.

A longitudinal study [34] reported that fat mass increases gradually in the first three months of life regardless of infant feeding method and stabilises starting at 4 to 6 months of age, with high variation in fat mass, supporting a critical window for the development of adiposity in early infancy [34]. More interestingly, a meta-analysis reported that breast-fed infants have higher fat-mass than formula-fed infants in the first-four months of life [282], despite the frequent observation that breastfed infants have a reduced risk of obesity in later life [5, 6]. Since the anthropometric assessments of infants were performed in the first four months in the present study, it is unclear what would represent 'optimal growth' or 'optimal body composition' during this critical period given that all infants have normal weight gain according to WHO growth standards. Overall, I conclude that the infant growth results support the hypothesis that listening to the relaxation therapy would have favourable effects on infant growth shown by higher weight gain and BMI, although further research, including follow-up of the infants from this study, is required to confirm this. This result is more convincing since there were also consistent dose-response effects showing positive associations between the frequencies of listening to the therapy and infant weight, BMI and weight gain at different time points. The potential underlying mechanisms that could have contributed to these findings were explored using multivariate analysis and are discussed in the next chapter.

5.7.5. Maternal cortisol

This trial also investigated the effects of the intervention on the changes in breast milk hormone concentrations and macronutrient levels within a feed from fore to hind milk. Significant differences in breast milk cortisol between groups were only found at HV1 (cortisol in hind milk and the change in concentration within a feed at HV1). At HV2, there was a non-significant difference in the change in cortisol within a feed, with a trend towards a higher reduction in cortisol concentration in the intervention group. This suggests that the intervention was possibly more effective in reducing breast milk cortisol concentrations within a feed when the intervention group mothers were first exposed to the relaxation therapy recording at HV1 than after they had being exposed to the intervention over the period between HV1 and HV2. A recent study using a music therapy among mothers of premature infants reported a significant reduction in maternal saliva cortisol at the final therapy session [171]. However, the study had a small sample size (n=30) and the study design was not a randomised trial. They compared maternal psychological state before and after the study period for all mothers, without having a control group, hence, causality cannot be determined.

The inconsistent results between HV1 and HV2 could also partly reflect practical issues with the timing of data collection. Many HV2 were performed in the afternoon although the plan was to carry them out in the morning only. This was due to the limited time available for data collection since priority for conducting a home visit in the morning was given to HV1 (baseline) and HV3 (endpoint). Studies among postpartum mothers have reported that maternal cortisol concentration is usually highest in the morning and decreases gradually throughout the day [283-285]. It is possible that the diurnal pattern of cortisol in the mother's plasma may also affect the concentrations in breast milk; consistent with Patacchioli et al. [92], I found significant positive associations between maternal salivary cortisol and breast milk cortisol. Thus, the timing of data collection might have contributed to the inconsistent results of breast milk cortisol between HV1 and HV2.

Another factor to consider is that, due to the limited availability of specific assay kits for measuring breast milk cortisol, analyses were performed using assay kits specific for saliva samples. Although adjustments were made during analysis to correct for the use of human milk samples, this could still have contributed to the high variability of the resulting data. Therefore, improvement in assay kits for measuring breast milk cortisol is recommended for future research.

5.7.5.1. Breast milk macronutrient content

The effects of listening to the relaxation recording on changes in fat, carbohydrate and protein levels within a feed were less clear. Overall, the increase in fat within a feed was non-significantly higher in the intervention group than the control group at all HV. Consistently, there were non-significant trends towards higher hindmilk fat and a greater increase in fat levels within a feed among mothers in the intervention group at all HV. When I calculated the average of hind milk fat levels and the average of the changes in fat levels within a feed over all HV as pooled results, I found significantly higher values in the intervention group (Table 5.10). This suggests that repeated listening to the relaxation therapy may have contributed to a significantly higher breast milk fat level, which would be consistent with more efficient release of hindmilk. This is supported by the finding from a RCT [169] demonstrating that mothers in the intervention group that listened to the verbal-protocol of a relaxation therapy tape produced significantly higher fat in breast milk than those in the control group. Another RCT demonstrated a similar non-significant trend towards higher breast milk fat among mothers in the relaxation therapy group [170]. Both of these studies used the creamatocrit method to calculate the milk fat content, whilst I used MIRIS milk analyser. However, this would not be likely to influence the results since the fat content data was compared between randomised groups, not between study populations. Perhaps of greater importance, the milk collection procedure was not standardised in these two RCTs (e.g time of the day, fore/hind/mid-feed and stage of lactation), and this was discussed in detail in the systematic review (Chapter 2).

Not surprisingly, findings for breast milk energy were similar to the effects of the intervention on breast milk fat, since fat makes the greatest contribution to the energy content of breast milk. In the current study, the average milk fat content (mean of fore + hind milk) at HV3 was 3.9 g/100ml for the control and 4.3 g/100ml for the intervention group. Assuming a breast milk fat absorption of 85% and a milk intake of 150 ml/kg/day, the 3.9 and 4.3 g fat/100ml content in breast milk translate to caloric intake of approximately 46 kcal/kg/day and 51 kcal/kg/day for control and intervention group infants respectively. Thus, if the calorie intake either stabilises or gradually increases over time, infants in the intervention group may have received higher breast milk energy than infants in the control group. This cumulative increment in calorie intake over time could have also contributed to the greater infant weight gain and BMI. Thus, mothers who were more relaxed as a result of listening to the relaxation therapy could have had more efficient milk ejection, which produced higher hind milk that

contained high milk fat. If this continued over a long period, infants in the intervention group could have ingested higher milk energy over a long period of lactation, which is consistent with the finding of a significantly higher pooled milk fat during the study period.

The relaxation therapy had no significant effect on the changes in carbohydrate and protein levels within a feed at any HV. However, the foremilk carbohydrate levels of the relaxation group increased significantly over time, whereas the opposite trend was shown in the control group. The average of all foremilk carbohydrate and all milk carbohydrate (pooled milk data) were also significantly higher in the intervention group. This suggests a possible long-term effect of listening to the therapy on carbohydrate levels in breast milk. Overall, the effects of the intervention could potentially be mediated through higher milk energy, resulting from both milk carbohydrate and/or fat levels. There is a possibility that mothers who were relaxed and less stressed produced a larger volume of milk that contained higher milk carbohydrate which could have contributed to a higher nutrient intake. Consistent with that, both of the previous RCTs which have investigated the effectiveness of relaxation therapy reported that mothers in the intervention group produced significantly larger milk volumes than those in the control group. However, milk carbohydrate was not measured, hence it could not be related to milk volume [169, 170].

5.7.6. Summary discussion for results of other RCT outcomes (results III)

Significant effects of the intervention on the changes in breast milk leptin and ghrelin concentrations within a feed were found at HV2, but not at HV1. Both foremilk leptin and ghrelin were also significantly different between groups at HV2: the foremilk leptin was significantly lower whilst the foremilk ghrelin was significantly higher in the intervention group than the control group. Thus, there is a possibility of a long-term rather than an immediate effect of the intervention on the breast milk hormones concentrations.

Nevertheless, the inconsistent results of breast milk hormones might be explained by several factors. Firstly, as previously mentioned, although it was planned to conduct all home visits in the morning, due to time restrictions, several HV2 had to be performed in the early afternoon (following another subject's HV1 or HV3 session was done in the morning). Sample collection at a different time of the day may have increased the variability of the results, particularly for breast milk composition. Secondly, although the majority of HV were performed in the morning, a few mothers reported that they did not have breakfast prior to the home visit

session although they had previously been advised to do so. This may have influenced leptin and/or ghrelin levels in maternal plasma, and also possibly in breast milk [103, 108]. Finally, assay kits that were used for breast milk leptin and ghrelin analysis were not designed for human milk samples, but for human plasma, which might also have contributed to inaccuracy and /or variability in the results.

There were no significant differences in infant appetite and temperament between groups. It is possible that infant appetite or temperament are innate traits, in which case they would not be expected to be affected by the intervention or by breast milk composition. However, since the instruments used to assess these traits rely on maternal perceptions and descriptions of their infant, I considered that that the intervention could potentially influence the results. Thus, the results could also be interpreted as indicating either that the intervention does not influence the expression of these innate appetite and temperament traits, or that these traits were not responsive to the maternal signals that were influenced by the intervention during the study period, either through breast milk composition (or maternal behaviour).

5.7.7. Strengths and limitations of the study

5.7.7.1. Novelty of the research

Based on the systematic review presented in Chapter 2, this intervention study is the first randomised controlled trial investigating the effects of relaxation therapy on both mother and infant outcomes. Furthermore, this study also combined both psychological and physiological mother-infant aspects, including incorporating an anthropological perspective of the mother-infant relationship during the breastfeeding period, whereas many studies focus on each aspect separately. Many previous studies on infant nutrition in early life are also observational, hence they can only show associations, because the findings might also be explained by various confounding factors [38, 80].

Many factors have been found to influence maternal psychological state and breastfeeding outcomes during the postpartum period. As reported by recent studies [286-288], including a meta-analysis [289], socio-demographic background, labour experiences, social support and prenatal distress are contributing factors to postpartum distress. All of these factors are inter-related, and it is important to consider them as potential confounding factors. The use of an experimental design for my study was chosen to minimise the potential for confounding by

these factors. However, given the relatively small sample size, information on these factors was collected to allow adjustment if they differed between randomised groups at baseline, although in fact no such differences were identified. In addition, to reduce any potential bias in practices and attitudes towards breastfeeding and caring for a new-born baby, my study only involved primiparous mothers, so that these experiences were new to all mothers. A review [153] reported that fatigue and tiredness among new mothers, and infant crying in the first-three months, could increase the risk of postpartum depression. However, since all studies in this review were observational, it is difficult to determine which is the cause and which is the effect (maternal distress or infant crying) since, once again, the factors are inter-related. I aimed to clarify cause and effect by manipulating maternal psychological state using an experimental approach.

5.7.7.2. Sample size

I investigated the effects of the intervention on maternal psychological state, breast milk composition and infant growth and behaviour. Although not all results were statistically significant at all HV points, the findings collectively suggest positive effects of the therapy on both mothers and infants during the study period. Nevertheless, one of the main limitations of this study is the small sample size. Furthermore, no adjustment of sample size for multiple outcomes was performed. The p-value cut-off point for statistical analysis was also not adjusted for multiplicity since separate univariate analyses were done and results of maternal and infant outcomes were independent of each other. The possibility of a type 1 error should, however, be considered when interpreting the findings.

5.7.7.3. Breast milk macronutrient analysis

In terms of breast milk composition, although fat content in breast milk is highly variable compared to lactose and protein content, the HMA measurements have been reported to be more reliable for fat [251, 290-293] compared to protein and lactose [251, 291]. Nevertheless, many recent studies have validated the HMA against conventional laboratory assays [251, 290-293] or other infrared analysers [292], and found that the HMA generally produces good precision and accurate results. Moreover, since the study is a RCT, as long as any inaccuracy is the same across the range, it should pose less of a problem since the aim was to compare the results between groups rather than consider the absolute values.

5.7.7.4. Lack of blinding

It is also acknowledged that the intervention was not blinded to either the mothers or the researcher due to the nature of the therapy tool (guided-imagery protocol). It was impossible to blind the mothers since they were asked to listen to the therapy. However, in theory it would be possible to blind the researchers if more staff were available to conduct the home visits and analyse the data; for example, one researcher could conduct the mini-breastfeeding test and collect data on the use of the intervention (which could not be blinded since the mother needed to listen to the tape) whilst another could collect the rest of the data and take the anthropometric measurements, blind to the intervention. Due to restricted resources, and to maintain consistency in infant growth measurements, I conducted all the home visits and obtained all the measurements myself; and I also analysed the data. The best that I could do to reduce bias among mothers was to blind the randomisation process so that those in the control group were not aware of the existence of the intervention therapy whilst those in the intervention group assumed everyone received the therapy. All mothers were also informed that the main aim of the study was to investigate mother-infant factors that influence breastfeeding, and they were not told that it was also investigating the effects of maternal psychological state on infant outcomes. Mothers that received the intervention therapy were also told that the purpose of listening to the therapy was to test whether it can help the mother to be relaxed during breastfeeding and that by doing so this may or may not have beneficial effects on breastfeeding outcomes.

5.7.7.5. Isotope data

Finally, there were several methodological issues with the isotope data for body composition results. Firstly, the majority of the infants had high isotope enrichment in their pre-dose urine; this was due to persistence of the isotope in the mothers and also in expressed breast milk, since mothers were dosed with isotope at HV3, 2-3 weeks prior to HV4. This seemed to be a particular problem among infants that were regularly fed expressed breast milk containing high isotope enrichment because it had been expressed a few hours after mothers were dosed with isotope during HV3. It caused problems in detecting the lower level of isotope enrichment in pre-urine samples using isotope ratio mass-spectrometry since the low levels were expected to be around 0, whereas in many infants they were >100 delta unit.

Secondly, expressed breast milk mixed with isotope was used due to mother's request, in order to dose the infants, and because the milk contained some leftover isotope enrichment, this resulted in very high levels of isotope in the dose solution (>1000 delta unit). The highest standard that was available in the lab for the analysis was only 1000 units, and thus, there is a possibility that some results were less reliable due to the lack of an appropriate standard. These problems were unfortunately not predicted since they had not occurred in our previous studies using the same protocol, most probably because previous studies had mostly used plain water to dose the breast-fed infants since the mothers were not routinely expressing milk at the time [234].

Thirdly, there were a few implausible results: instead of having a reduction in isotope levels over time, there were some samples in which the 24-hour post dose isotope enrichment values were higher than the 5 hour post-dose samples. These data were excluded from the analysis. The explanation for this problem is not clear but it may relate to the intake of expressed breast milk that contained isotopes during the first hours post-dose. Again, this issue had not been predicted from previous studies using the same protocol because in those populations the mothers were not systematically expressing breast milk and feeding it to their infants at the time of the measurements, whereas many of the mothers in this study were returning to work around the time of the isotope study and had therefore started to use expressed milk.

Clearly there is a need in future studies using this protocol to make sure that mothers do not give their infant expressed milk enriched with isotopes, and that this is also not used for dosing the infant. Finally, there were some issues related to the recording of data: several mothers did not record the time of sample collection accurately, and a few did not record the time at all. This caused a problem in calculating the dilution space, and hence the total body water could not be estimated. The combination of these issues resulted in a reduced sample size for the calculation of total fat and fat-free mass in infants.

5.8. Conclusion

In conclusion, this trial showed significant effects of the relaxation therapy intervention which were most convincing for maternal stress and infant weight and BMI, with consistent effects at different time-points. The intervention therapy also had significant effects on infant behaviour with increased infant sleeping duration at HV2, and on milk composition, with greater reduction in milk cortisol concentrations within a feed at HV1, and higher foremilk carbohydrate levels at HV3. Adding to the validity of the observed intervention effects, there were also dose response effects between the frequency of listening to the therapy and maternal stress score, sleeping duration and infant weight and BMI. The pooled results from HV1 to HV3 also suggested possible long-term or cumulative effects of the intervention in increasing fat and carbohydrate levels and total energy in breast milk. Taken together, the results support the primary hypothesis: listening to the relaxation therapy resulted in reduced maternal stress, and altered breast milk composition with consequent effects on infant sleeping behaviour and growth. There is a possibility that the effects of the relaxation therapy on infant outcomes could be mediated through physiological signalling by alterations in breast milk composition (change in concentrations within a feed and over the study period), or behavioural signalling through the influence on infant sleeping duration. Several limitations of the study must be considered when interpreting the results, especially the small sample size. It is nevertheless intriguing to consider the potential signalling mechanisms underlying the effects of the intervention on infant growth.

In the next chapter, I present the results of the secondary outcomes (breast milk leptin and ghrelin, and infant appetite and temperament) for the whole MOMS population. The results of associations between secondary variables are presented and discussed in order to answer the secondary hypothesis of my study. The potential mechanisms of the effect of the intervention or influence of other factors that were independent of the intervention on infant growth measurements are also discussed.

Summary points:

- Listening to relaxation therapy showed significant effects in

i. reducing maternal stress at HV2 and HV3
ii. reducing milk cortisol concentrations within a feed at HV1 and thus producing significantly lower cortisol concentrations in hindmilk at HV1
iii. increasing foremilk carbohydrate levels across HV points and thus producing significantly higher foremilk carbohydrate at HV3
iv. producing significantly higher pooled milk fat and carbohydrate
v. increasing infant sleeping duration at HV2
vi. increasing infant weight and BMI from HV2 to HV4.

- The overall results suggest that listening to relaxation therapy had positively manipulated the maternal psychological state and altered breast milk composition, and also produced consequent effects on infant behaviour (longer sleeping duration) and growth (higher weight gain and BMI).
- These findings demonstrate the psychological and physiological effects of the intervention on both mothers and infants during the study period, which support the primary hypothesis.
- The physiological effects could be mediated through breast milk (by the changing of milk composition within a feed or between HV points),
- The main limitation of the study is the small sample size, which could have reduced power to detect effects, and the fact that it was non-blinded.

CHAPTER 6

6. OBSERVATIONAL COHORT STUDY OUTCOMES

6.1. Introduction

The aim of this chapter is to address the hypotheses using observational data from the whole study population, by investigating the relationship between infant behaviours (appetite and temperament), breast milk composition and infant growth. The observational cohort study hypotheses were:

I. Infant temperament, appetite and breast milk composition are associated with infant growth, and these associations also differ by gender.

II. Non-nutrient factors in breast milk (specifically hormonal constituents; ghrelin and leptin) are associated with infant appetite and behaviour and hence infant growth.

The observational cohort outcome measures were:

i. non-nutrient factors in breast milk – leptin and ghrelin

ii. infant temperament measured using the Rothbart's questionnaire (RIBQ)

iii. infant appetite assessed using the Baby Eating Behaviour Questionnaire (BEBQ)

As I have highlighted in previous chapters (Chapter 2 and 5), the analysis of observational data of this type is not straightforward given the existence of complex inter-relationships between variables. In addition, I have a potentially large number of both predictor and outcome variables available which introduces the potential for spurious findings due to multiple statistical testing. I therefore carefully considered the best analytical approach to use and focussed where possible and appropriate on using summary variables. The pros and cons of different approaches and justification for the chosen approach are discussed in this chapter.

6.2. Study population and follow-up visits

As indicated previously, the study population involves the 64 mother-infants dyads that were randomised into control (n=31) and intervention (n=33) groups. The recruitment process and follow-up visit procedures were described previously in Chapters 3-5.

6.3. Research methods

6.3.1. Data collection and analyses

The data collection procedure and detailed information about questionnaires and measurements were described in Chapter 3. Infant appetite was measured using the BEBQ questionnaire at HV1-3, whereas infant temperament was only measured at the final home visit (HV4) when the infants were aged 14-18 weeks. Both questionnaires were completed by the mothers during the HV session. Fore and hind milk samples were collected during HV1 to HV3 but, given a more complete set of milk samples were collected at HV1 and HV2, analyses were performed on samples collected at these time points only. Milk hormones (leptin and ghrelin) were analysed by a lactation research laboratory team from the Faculty of Health Sciences, University of Primorska, Slovenia.

6.3.2. Analysis of milk hormones

A detailed description of the analysis of milk leptin and ghrelin was presented in Chapter 5.

6.3.3. Questionnaire data

Infant appetite - BEBQ

The Baby-Eating-Behaviour questionnaire (BEBQ) involves a scale score from 1 (never) to 5 (always) for 18 items -designed to measure four main appetite traits; enjoyment of food (4 items); food responsiveness (6 items); slowness in eating (4 items); and satiety responsiveness (3 items)- including a single item to rate general appetite (GA). This is a parental report questionnaire, in which the mothers were asked to rate or evaluate these appetite traits of their infants: i) Enjoyment of Food (**EF**): the infant's liking for milk or how much the infant enjoys the feeding time in general; ii) Food Responsiveness (**FR**): infant responsiveness to maternal cues for feeding, as well as his/her demandingness for feeding; Slowness in Eating (**SE**): the pace of feeding or how slowly/quickly an infant feeds; and Satiety Responsiveness (**SR**): a measure of the extent to which the infant gets full easily, or satiety level during a feed or between feeds. Mothers also rated the overall appetite of their infant on a single question asking about whether they considered that their infant has a big appetite (**GA**) level [223, 294].

Infant temperament – RIBQ

The Rothbart's Infant-Behavior-Questionnaire-Revised (RIBQ) data involves a scale score from 1 (never) to 7 (always) for 37 items, which are designed to measure three main behaviour traits: surgency (13 items); negative affectivity (12 items) and effortful control (12 items). **Surgency** can be characterised by these scales: impulsivity (a tendency to give an immediate response without giving any thought to the action) [295]; intensity pleasure ('pleasure or enjoyment related to high stimulus intensity, rate, complexity, novelty and incongruity')[226]p.72, such as playing a 'peek-a-boo' game; activity levels (gross motor activity or movement) [226]; positive anticipation ('positive excitement and rapid approach toward pleasurable activities')[226]p.67 such as showing excitement after received a new toy; and smiling and laughter [226]. **Negative affectivity** includes domains describing emotionally less stable infants such as [73], irritable behaviours or negative emotions (e.g sadness, discomfort, frustration, anger and fear) [226]. Infants who score highly on this domain are typically described as having a challenging or difficult temperament [296]. **Effortful control** can be defined as having the ability to self-regulate and/or self-control emotions characterised by these scales: Low intensity pleasure ('pleasure or enjoyment related to low stimulus intensity, rate, complexity, novelty and incongruity')[226]p.72, such as playing quietly with a favourite toy; Inhibitory and attentional control (ability to suppress interference and sustain attention)[226]; and perceptual sensitivity ('detection of slight, low intensity stimuli from the external environment') [226]p.72 such as ability to notice something different or new, such as a new fabric cloth [226]. In my study, infants who scored high in the negative affectivity domain (more than 3.5 out of 7) were considered to have a challenging or difficult temperament, whereas those who scored high in effortful control were considered to have an easy or less challenging infant temperament.

A mean score and standard deviation were determined for each trait from both BEBQ and RIBQ. Higher mean scores indicate greater reported expression towards the behaviour or trait.

6.3.4. Use of primary outcome data from the RCT

For exploratory purposes, these data were included in the analyses:

i. Infant growth: Infant weight and BMI (see below).
ii. Infant behaviour: a) sleeping, awake and content, and feeding and distress (crying and colic) duration over 72 hours (3-day behaviour diary); b) breastfeeding duration of the 'test' feed during the HV (in minutes). (NB. Throughout the results, 'Feeding duration' refers to the value from the 3-day diary and 'breastfeeding duration' refers to the duration of the single observed feed during the HV).
iii. Milk macronutrient and cortisol concentrations: To correlate with infant behaviour and infant weight, BMI and weight gain.

6.4. Statistical analyses

6.4.1. General considerations

As previously mentioned, when planning the statistical analyses it was important to consider the large number of predictor and outcome variables available and the likelihood that they would be inter-related. To address these issues, I : (1) defined my hypotheses in advance and used these to structure the analyses; (2) considered the use of summary variables where this was possible and appropriate (for example, calculating the mean where the same variable was measured on more than one occasion). The use of summary variables has some clear advantages in minimising the number of statistical comparisons performed and also improving the clarity of presentation. However, I also considered the potential disadvantages of this approach. For example, it would not be sensible if there were clear changes in a variable over time and, from a biological perspective, there may be situations where an effect or association might genuinely be present at one time-point but not at another and this could be missed using a summary variable. Another argument against using summary variables could be that it removes the opportunity to look for consistency in associations which might help in assessing the reliability of findings. Since the balance of pros and cons differs depending on the variable, I considered them for each of the predictors and outcomes. This topic is further considered in the discussion.

Another issue relates to the temporal relationship between variables which is important in deciding whether one might predict another (since the predictor must logically precede the outcome). In the case of infant temperament, it is not possible to measure this before 12 weeks of age so strictly speaking, the infant temperament measured at 14 weeks in this study cannot predict any of the outcomes measured up to that point. However, it can be argued that temperament traits are at least partially heritable and therefore constant over time [297, 298]. On that basis, it could be considered reasonable to use the traits measured at 14 weeks as being stable indicators of personality which would have been present from birth, and therefore consider them as potential predictors of the growth outcomes.

6.4.2. Use of data for individual variables

Questionnaire data (temperament and appetite): Internal consistency estimates of reliability for each questionnaire were evaluated using Cronbach's α value. Appetite was recorded at 3 home visits. No significant differences were found for any appetite trait between visits (either from HV1 to HV2, HV2 to HV3 or HV1 to HV3 and using repeated measures ANOVA) except for slowness in eating (SE) between HV1 and HV3. Taken together, these results suggest that infant appetite score was stable over the study period, except for the slowness in eating (SE) trait, where the scores reduced over time. Hence, I calculated the mean score for each appetite trait as a summary variable, except for slowness in eating (SE), and used this in the analyses.

Breast milk hormones: Leptin and ghrelin in breast milk were analysed for samples collected at HV1 and 2. In addition to fore and hind milk concentrations, I calculated the mean milk leptin and ghrelin concentrations for each feed. Data for both leptin and total ghrelin were not normally distributed and thus were transformed to natural logarithms (ln) prior to statistical analysis. Hence, the geometric mean (GM), standard deviation (GM x log SD) and sympercent (s%) are presented to show the percentage mean differences between genders.

Weight and BMI: The data were converted to standard deviation score (SDS) using WHO 2006 standard data (LMS growth add-in for Microsoft Excel) to obtain the score for weight- and BMI-for-age by genders. The SDS data were used in the statistical analyses. Since weight and BMI were measured on 4 occasions, I considered whether to use one or two summary variables; for example, the change in SD from first to last visit, or the final measurement. However, I decided against this approach mainly because I felt it was important to examine

the consistency of the associations between milk hormones and later weight and BMI, especially given the potential for methodological errors in breast milk leptin and ghrelin analyses shown by high variability in the results. I considered that consistent results with anthropometric measurements at different time points could increase confidence in the overall reliability of the findings. On the other hand, several associations were only significant with infant weight or BMI at a particular time point. Thus, these associations were further tested using regression analysis where those growth measurements were used as a dependent variable in the regression model. Those variables that were significantly correlated with infant growth measurement at a particular time point were then added as a predictor. Other covariates were also added in the regression model to control for confounding factors.

Infant behaviour: The duration of sleeping, awake and content, feeding and distress (3-day diary data) from each home visit (HV1 and HV2) was used. The changes in time spent in each behaviour from HV1 to HV2 were calculated. The breastfeeding duration for each HV was used to correlate with relevant data collected at that visit (e.g. the correlation between breast milk leptin at HV1 and breastfeeding duration during HV1 and the correlation between breast milk leptin at HV2 and breastfeeding duration during HV2.

6.4.3. Analysis plan

I firstly examined descriptive data for the outcome measures including comparisons between genders and socio-demographic groups using independent t-test and ANOVA. Repeated measures ANOVA was used to examine changes in appetite trait scores from HV1 to HV3. Paired t-tests were performed to examine the changes in milk hormones within a feed (fore to hind) and across time points (HV1 to HV2).

Next, I examined univariate associations between variables using Pearson's Product correlation, with partial correlation to control for randomised group where this had been previously shown to influence one of the variables.

Finally, I used multivariate analyses (multiple linear regressions) to examine associations between infant temperament, appetite scores and milk hormones and infant growth outcomes, controlling for potential confounders such as socioeconomic status, maternal characteristics and infant gender. For regression analyses, variables that showed significant or non-significant trend results ($p<0.1$) in the univariate analyses were included in the

regression models using the backward elimination method. The models that are presented here are those which showed infant behaviour and/or milk hormone variables as significant predictor(s) of growth outcomes. The normal plot of residuals was examined to assess the fit of the regression model. Where appropriate, I tested for interactions between infant gender or randomised groups and the predictor variable using GLM univariate analysis.

Overall, the univariate associations are considered to be exploratory. Subsequent multivariate models separately considered adjustment for potential confounders (maternal socio economic status (as educational levels and maternal income), maternal BMI, and infant gender and potential mediators. Randomised group was considered as a predictor of growth outcomes in all models (given the effects observed in the RCT) and other factors (appetite traits, temperament traits, breast milk hormones and milk carbohydrate) were added to the models to test whether they were potential mediators of this effect, or whether they predicted growth outcomes independent of the effect of the intervention.

6.5. Results: Observational cohort outcomes

Descriptive data

6.5.1. Breast milk leptin

The average milk leptin of the study population was 0.54 ± 0.79 ng/ml at HV1 and 0.85 ± 1.0 ng/ml at HV2 (Table 6.1; mean increase 51 s% (p=0.046, CI: -10 s%, -1.0 s%). There were no significant differences in either fore- or hindmilk leptin, or mean milk leptin between genders at either time-point (all p-values>0.05).

Table 6.1 The study population group results for breast milk hormones

Milk hormones	n	Mean	Std. Deviation	Minimum	Maximum
Leptin HV1*	60	0.54	0.8	0.001	7.7
Leptin HV2*	57	0.85	1.0	0.01	19
Total ghrelin HV1	60	216	30.9	167	346
Total ghrelin HV2	58	196.5	39.6	134	396
Active ghrelin HV1	61	27	9.8	11	51
Active ghrelin HV2	58	24.4	12.0	1.5	52

*geometric mean; leptin unit ng/ml; ghrelin unit pg/ml

Table 6.2 shows the breast milk hormone concentrations in fore and hindmilk and also between HV1 and HV2, with paired t-test results. The mean leptin concentration significantly reduced over a feed at HV1 (p=0.03, C.I: 3 s%, 70 s%), with a reduction of 37 s% from fore- to hindmilk. However, at HV2, mean leptin was not significantly different between fore- and hindmilk (p=0.30). This may be due to the opposite direction in changes of leptin concentrations within a feed between the randomised groups (as presented in Chapter 5); thus the mean leptin values in hindmilk and foremilk for the whole study population were similar. Leptin in foremilk and hindmilk was strongly correlated at both visits (r=0.67, p<0.001 at HV1 and r=0.51, p<0.001 at HV2).

Table 6.2. The changes in mean hormone concentrations within a feed and between HV

Study population results:	**n**	**Mean**	**SD**	**Mean**	**SD**	**p-value^{Ŧ}**	**Mean diff: s%**	**C.I s%**	
Leptin (ng/ml) :		**Fore***		**Hind***					
Changes within a feed (HV1)	60	0.55	0.89	0.38	0.60	**0.033**	0.37	0.03	0.70
Changes within a feed (HV2)	57	0.78	0.98	0.64	1.01	0.302	0.20	-0.18	0.57
		HV1		**HV2**					
Changes from HV1 to HV2	57	0.51	0.75	0.85	1.0	**0.046**	-0.51	-1.00	-0.01
Total ghrelin (pg/ml) :		**Fore***		**Hind***					
Changes within a feed (HV1)	60	207.3	34.6	222.9	37.0	**0.004**	-0.07	-0.12	-0.02
Changes within a feed (HV2)	58	182.9	39.0	204.7	57.5	**0.016**	-0.11	-0.20	-0.02
		HV1		**HV2**					
Changes from HV1 to HV2	57	217.4	30.7	196.5	39.9	**0.003**	0.10	0.04	0.17
Active ghrelin (pg/ml) :		**Fore***		**Hind***					
Changes within a feed (HV1)	61	21.1	9.8	27.85	11.7	**<0.001**	-0.28	-0.41	-0.14
Changes within a feed (HV2)	58	14.1	11.5	25.30	19.8	**<0.001**	-0.58	-0.75	-0.41
		HV1		**HV2**					
Changes from HV1 to HV2	57	25.6	9.6	20.55	14.5	0.066	0.22	-0.02	0.45

*= geometric mean, S%=sympercent; p-value^{Ŧ} for paired t-test

6.5.2. Breast milk ghrelin

6.5.2.1. Total ghrelin

The average milk total ghrelin for the study population was 216.0 ± 30.9 pg/mL at HV1 and 196.5 ± 39 pg/mL at HV2 (Table 6.1) with a decrease of 10 s% between visits (p=0.003, C.I: 4 s%, 17 s%). Total ghrelin increased from fore- to hindmilk at both HV1 (p=0.004, CI: -12 s%, -2 s%) and HV2 (p=0.016, CI: -20 s%, -2 s%) (Table 6.2) but there were no significant differences in fore- or hindmilk total ghrelin concentrations between genders at either HV1 or HV2 (all p-values>0.05).

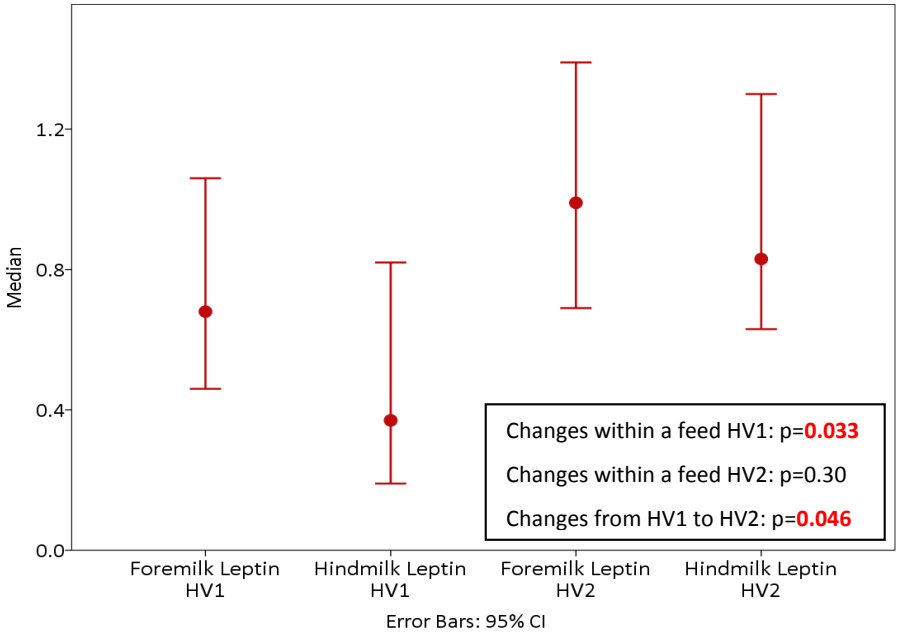
6.5.2.2. Active ghrelin

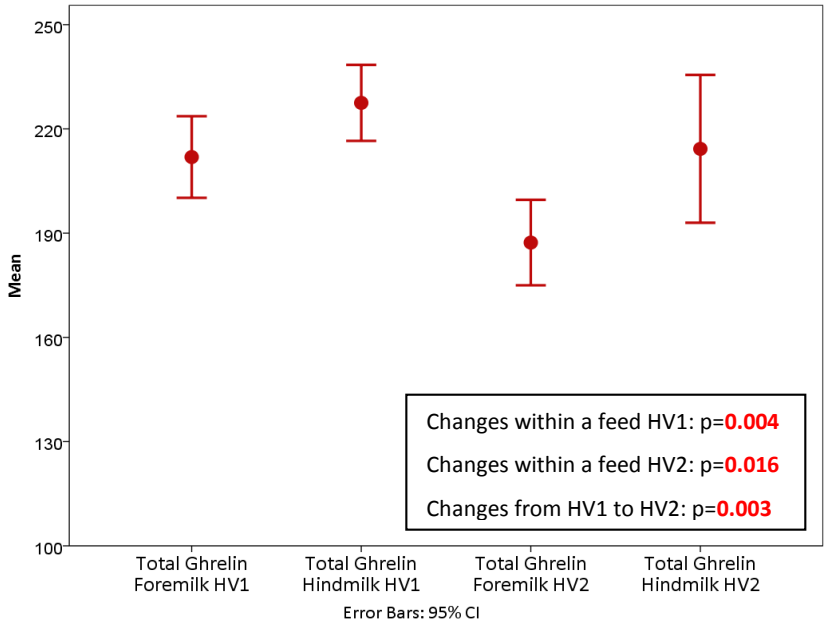
The mean milk active ghrelin for the study population was 27.0 ± 9.8 pg/mL at HV1 and 25.4 ± 12 pg/mL at HV2 (p=0.066; Table 6.1). The mean active ghrelin increased significantly from fore to hindmilk at both HV1 and HV2 (both p<0.001). At HV1, the mean study population active ghrelin concentration in hindmilk was on average 28 s% greater than the concentration in foremilk, with a difference of 58 s% at HV2. There were no significant differences in fore- and hindmilk active ghrelin concentrations between genders at either HV1 or HV2 (all p-values>0.05).

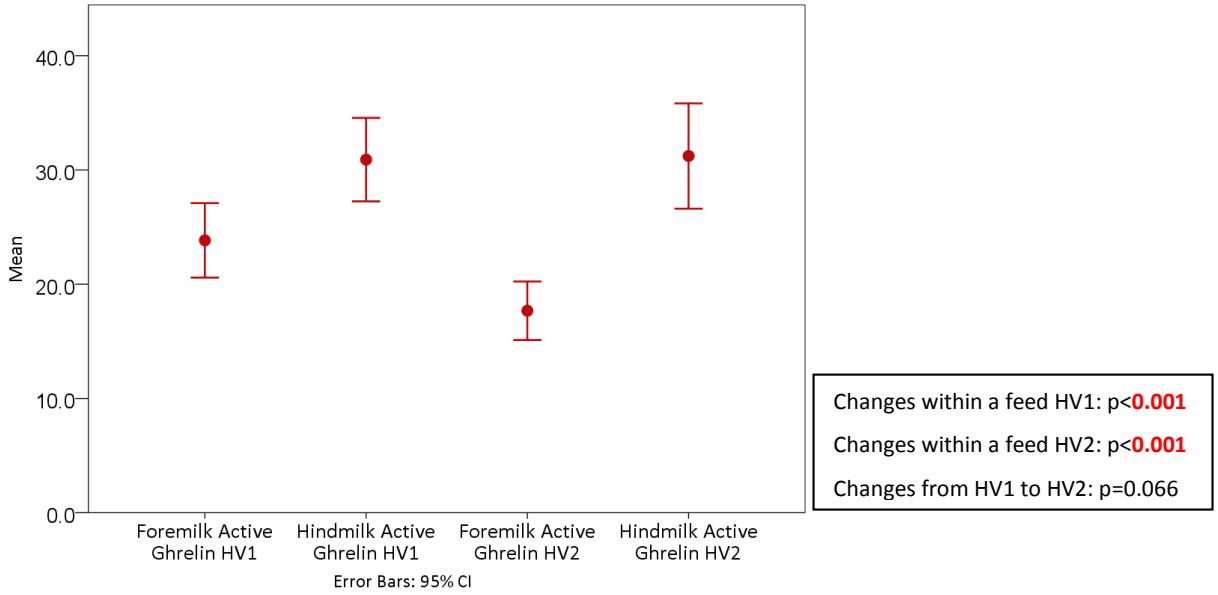
6.5.2.3. Associations between milk hormones

The mean total ghrelin at HV1 was significantly correlated with mean active ghrelin at HV1 (r=0.40, p=0.001), but at HV2, the correlation was not significant (r=0.25, p=0.06). No association was found between breast milk total ghrelin and leptin at HV1 or HV2. Figure 6.1 shows the population absolute value for median leptin, and mean population for total ghrelin and active ghrelin in fore- and hind milk at HV1 & HV2. The median value was used for leptin since the data were extremely skewed.

Figure 6.1 Median leptin (ng/mL), total ghrelin and active ghrelin (pg/mL) in fore- and hind milk at HV1 & HV2







6.5.3. Infant temperament (RIBQ)

In this study, the internal consistency reliability coefficients (Cronbach's α) for the Rothbart's Infant-Behaviour-Questionnaire-Revised (RIBQ) ranged from 0.79 to 0.85 for each question, with an average of 0.82. This indicates a high level of internal consistency of the questionnaire, consistent with previous studies [73, 226, 227]. The mean (+SD) population score for each dimension was 4.71 ±0.75 for surgency, 3.78 ±0.96 for negative affectivity and 5.53 ±0.64 for effortful control. Infant temperament characterised by these three dimensions was not significantly different between genders (all p-values >0.05) or socio-demographic groups at age 3-4 months.

6.5.4. Infant appetite (BEBQ)

The mean internal consistency reliability coefficients (Cronbach's α) for the Baby-Eating-Behaviour questionnaire (BEBQ) at HV1, HV2 and HV3 were 0.64, 0.66, 0.61 respectively. This indicates an acceptable internal reliability of the questionnaire, consistent with previous studies [223, 294, 299]. The BEBQ results show that there were no significant differences in any appetite traits between genders at all HV (Table 6.3), except for 'slowness in eating' (SE) at HV3: females scored significantly higher for slowness in eating than males suggesting slower feeding (2.91±0.56 v 2.54±0.78, p=0.03). When comparing the average score across all visits for each appetite trait, no gender differences were found (all p>0.05). Appetite traits were also not significantly different between socio-demographic, educational level and household income level groups.

Enjoyment of food (e.g. 'My baby enjoys feeding time', 'My baby loves milk'), food responsiveness (e.g. 'My baby frequently wants more milk than I provide', 'My baby is always demanding a feed'), and general appetite (e.g. '4. My baby has a big appetite') were positively skewed at all HV (values close to 5), whereas mean values for slowness in eating (e.g. 'My baby feeds slowly') and satiety responsiveness (e.g. 'My baby gets full up easily') were close to neutral (2.5). This suggests that the majority of the infants were perceived to enjoy feeding sessions, have a good response to breast milk or feeding cues and have a large appetite.

All appetite traits showed significant positive associations between visits (e.g Enjoyment of Food (EF): EF HV1 was positively associated with EF HV2 and EF HV3, and the same applied to all other traits). In addition, no significant differences were found in any of the appetite traits between home visits (either from HV1 to HV2, or HV1 to HV3 or HV2 to HV3) using paired t-test ($p>0.05$), except for slowness in eating between HV1 and HV3. This demonstrated the stability of all appetite traits across time points, except slowness in eating. The slowness in eating at HV1 (mean score=3.0) was significantly lower than at HV3 (mean score=2.8) but not HV2 (2.9), showing that older infants were perceived as feeding more quickly.

Table 6.3 Mean scores for appetite traits by genders

	Male			Female					
Variables	n	mean	SD	n	mean	SD	p-value[₮]	C.I	
HV1									
Enjoyment of food (EF)	25	4.39	0.40	39	4.27	0.40	0.24	-0.33	0.08
Food responsiveness (FR)	25	2.98	0.80	39	3.26	0.65	0.13	-0.08	0.64
Slowness in eating (SE)	25	2.94	0.76	39	3.03	0.71	0.62	-0.28	0.47
Satiety responsiveness (SR)	25	2.49	0.62	39	2.52	0.49	0.84	-0.25	0.31
General appetite (GA)	25	4.12	0.88	39	4.21	0.80	0.69	-0.34	0.51
HV2									
Enjoyment of food (EF)	25	4.42	0.38	38	4.22	0.40	0.06	-0.40	0.00
Food responsiveness (FR)	25	3.15	0.86	38	3.31	0.69	0.43	-0.24	0.55
Slowness in eating (SE)	25	2.78	0.70	38	2.96	0.66	0.31	-0.17	0.53
Satiety responsiveness (SR)	25	2.59	0.52	38	2.55	0.47	0.79	-0.29	0.22
General appetite (GA)	25	4.20	0.91	38	4.18	0.73	0.94	-0.43	0.40
HV3									
Enjoyment of food (EF)	25	4.32	0.62	38	4.30	0.32	0.88	-0.26	0.22
Food responsiveness (FR)	25	2.89	0.75	38	3.23	0.68	0.07	-0.02	0.71
Slowness in eating (SE)	25	2.54	0.78	38	2.91	0.56	0.03	0.04	0.71
Satiety responsiveness (SR)	25	2.56	0.57	38	2.69	0.45	0.31	-0.13	0.39
General appetite (GA)	25	4.00	0.91	38	3.97	0.88	0.91	-0.49	0.44

p-value[₮] for independent t-test

Two significant correlations were found among appetite traits: i) Mean satiety responsiveness was negatively associated with mean 'Enjoyment of Food' (n=62, r=-0.40, p=0.001). This suggests that infants perceived as having high satiety responsiveness were also perceived as less likely to enjoy feeding time. ii) Mean food responsiveness was positively associated with mean 'General Appetite' (n=62, r=0.48, p<0.001). This indicates that infants that were perceived as being highly responsive towards food were also thought to have a big appetite.

6.5.5. Associations between infant temperament, appetite and behaviour outcomes

Infant temperament was not associated with other infant behaviours (sleeping, awake, distress and feeding duration) (all p>0.05), but was correlated with infant appetite traits. Infant appetite was positively associated with the duration of the breastfeeding 'test feed' during the HV:

i) Infant temperament and appetite: Negative affectivity was inversely associated with the mean enjoyment of food trait (n=62, r=-0.27, p=0.03). This suggests that infants that were perceived as less likely to enjoy their feeding time in early life (2 and 6 weeks), were more likely to show difficult temperament at HV4: 3-4 months.

ii) Infant appetite and other infant behaviour: slowness in eating was significantly associated with breastfeeding duration during the test feed at the HV session: slowness in eating at HV1 was significantly positively correlated with breastfeeding 'test feed' duration during HV1 (n=63, r=0.43, p=0.001); and slowness in eating at HV3 was significantly correlated with breastfeeding duration at HV3 (n=60, r=0.32, p=0.014). There was a non-significant trend for a similar positive association at HV2: n=62, r=0.24, p=0.06. There were no significant associations among other appetite traits and behaviours.

6.5.6. Associations between infant temperament, appetite and behaviours and growth outcomes in univariate analyses:

Temperament: Effortful control was positively correlated with BMI SD at HV2 and weight SD gain from HV1 to HV2 and this remained statistically significant when adjusted for randomised group using partial correlation (Table 6.4).

Appetite: Mean food responsiveness score was positively associated with weight SD at HV4, weight SD gain from HV1-4 and infant BMI SD at HV3 (all p<0.05). In contrast, slowness in eating HV2 was negatively correlated with infant weight SD at HV2 to HV4 (all p<0.05). After controlling for randomised group in partial correlation analyses, the results remained statistically significant (Table 6.5). Overall, there were consistent trends towards positive associations between food responsiveness and infant growth, and negative associations between slowness in eating at HV2 and infant growth. There were no significant associations between other appetite traits and infant growth (p>0.05).

Table 6.4 Correlations between infant temperament and growth

Effortful control with:	n	Pearson correlation	p-value	Partial Correlation*	p-value
Weight SD HV2	62	0.19	0.15	0.21	0.10
Weight SD HV3	63	0.09	0.49	0.11	0.39
BMI SD HV2	62	0.25	0.047	0.29	0.03
BMI SD HV3	63	0.11	0.4	0.14	0.29
Weight SD gain HV1-2	62	0.33	0.013	0.33	0.016
Weight SD gain HV1-3	63	0.07	0.59	0.09	0.49

*adjusted for randomised group

Table 6.5 Correlations between infant appetite and growth

Variables:	n	Pearson correlation	p-value	Partial Correlation*	p-value
Mean Food Responsiveness					
Weight SD HV2	62	0.05	0.73		
Weight SD HV3	63	0.18	0.15		
Weight SD HV4	61	0.27	**0.035**	0.31	**0.018**
BMI SD HV2	62	0.01	0.95		
BMI SD HV3	62	0.27	**0.036**	0.31	**0.017**
BMI SD HV4	50	0.23	0.11	0.31	**0.03**
Weight SD gain HV1-3	62	0.20	0.13		
Weight SD gain HV1-4	61	0.29	**0.023**	0.31	**0.015**
Slowness in Eating HV2					
Weight SD HV2	62	-0.39	**0.001**	-0.40	**0.001**
Weight SD HV3	62	-0.34	**0.007**	-0.36	**0.005**
Weight SD HV4	62	-0.30	**0.02**	-0.32	**0.014**
BMI SD HV2	63	-0.24	0.06		
BMI SD HV3	62	-0.24	0.06		
BMI SD HV4	50	-0.20	0.16		
Weight SD gain HV1-3	62	-0.17	0.18		
Weight SD gain HV1-4	61	-0.12	0.36		

*adjusted for randomised group (blank space in the table = no correlation (p>0.05))

Infant behaviour (3-day diary): In partial correlation analyses controlled for randomised group, awake duration at HV2 and changes in awake duration from HV1 to HV2 were inversely associated with infant growth variables. In contrast, sleeping duration at HV2 and changes in sleeping duration from HV1 to HV2 were positively associated with infant BMI SD (Table 6.6). Feeding and crying behaviours were not significantly associated with infant weight or BMI.

Table 6.6 Correlations between infant sleeping and awake duration and infant weight, BMI and weight gain

		Awake duration HV2	Change in awake duration HV1-2	Sleeping duration HV2*	Change in sleeping duration (HV1-2)*
Weight SD HV2	r =	-0.02	-0.34	0.08	0.17
	p =	0.89	0.05	0.65	0.35
	n =	37	33	30	30
Weight SD HV3	r =	-0.17	-0.47	0.07	0.05
	p =	0.32	0.006	0.722	0.807
	n =	37	33	30	30
Weight SD HV4	r =	-0.25	-0.50	0.10	0.06
	p =	0.13	0.003	0.58	0.75
	n =	37	33	30	30
BMI SD HV2	r =	-0.33	-0.39	0.51	0.42
	p =	0.046	0.025	0.003	0.016
	n =	37	33	30	30
BMI SD HV3	r =	-0.40	-0.53	0.47	0.26
	p =	0.014	0.002	0.007	0.15
	n =	37	33	30	30
BMI SD HV4	r =	-0.42	-0.32	0.31	0.06
	p =	0.019	0.10	0.12	0.78
	n =	31	28	25	25
Weight SD gain Hv1-4	r =	-0.38	-0.41	0.12	0.01
	p =	0.021	0.019	0.50	0.97
	n =	37	33	30	30
		Awake: overall negative associations with weight, BMI and weight gain		***Partial correlation for sleeping variable :** overall positive associations with BMI	

6.5.7. Associations between infant temperament, appetite and behaviours and growth outcomes in multivariate analyses:

Referring to the correlation results between infant behaviour traits and growth (Table 6.4 and 6.5), those temperament and appetite variables that showed significant correlations were combined in regression models to further investigate their relationship with infant growth outcomes. As indicated earlier, the backward elimination method was used for regression analysis. Potential confounders were considered (Table 6.7) and all were included in the model for regression analysis. Although all covariates were included in the model, here, I only

present the results for models where the appetite or temperament variables were significant predictors. The infant behaviour data (3-day diary) were not included in the model due to the smaller number of mothers who completed the diary, which would result in a smaller number of subjects in the models with reduced statistical power. Moreover, I would not be able to include more than 3 predictors in the model if infant behaviour variables were one of the predictors due to the small sample size (n=37).

In total, there were six variables (Table 6.7) that were included in all models for regression analysis. Slowness in eating (SE) at HV2 was not significantly different than slowness in eating HV1, and the two were significantly correlated (r=0.45, p=0.001), hence this trait was considered stable from HV1 to HV2 and slowness in eating HV2 was included in the model to predict the weight gain from HV1 to later time points (HV3-4). Socio-economic status was included because the appetite and temperament data were based on maternal perception (questionnaire data), and thus, maternal socio-demographic background might influence the reported data as suggested by previous studies. The randomised groups and baby's gender were included since they were predicted to influence infant growth, especially given that the primary outcome results showed significant differences in infant weight and BMI between randomised groups. The overall presented models (Table 6.8) were chosen based on: i) models where the appetite or temperament variables were significant predictors; ii) higher values of adjusted r-square. The normal plot of residuals was also examined.

Table 6.7 Outcomes and independent variables that were included in the regression analysis

<table>
<tr><td rowspan="2">Outcomes for model 1-9</td><td colspan="2">Variables included in all models :</td></tr>
<tr><td>Observational study variables of the present study as potential predictor</td><td>Covariates (considered as potential confounding factors/ mediators)</td></tr>
<tr><td rowspan="2">Weight SD HV2
Weight SD HV3
Weight SD HV4
Weight SD gain HV1-2
Weight SD gain HV1-3
Weight SD gain HV1-4
BMI SD HV2
BMI SD HV3
BMI SD HV4</td><td rowspan="2">i. Effortful control
ii. Slowness in Eating (SE) at HV2
iii. Mean food responsiveness (FR)
(all are continuous variables)</td><td>iv. Socio-economic status (SES) groups :
1=Low, 2=Middle, 3=High
v. Baby's gender :
1=Male, 0=Female</td></tr>
<tr><td>Potential predictor (primary outcome of the RCT): Randomised groups: 1=Relaxation, 0=Control</td></tr>
</table>

Table 6.8 Regression models for associations between infant behaviour and growth outcomes

Model / Outcome:	Variables:	Statistics				Adjusted R-Square
		B	p-value	CI: upper	CI: lower	
Model 1	(Constant)	-1.04	0.24	-2.76	0.69	
Weight SD HV2	Randomised group	0.65	<0.001	0.30	1.00	0.33
	Baby's gender	-0.41	0.031	-0.78	-0.04	
	Slowness in Eating	-0.49	<0.001	-0.74	-0.23	
	Effortful control	0.32	0.026	0.04	0.60	
Model 2	(Constant)	-0.21	0.72	-1.38	0.95	
Weight SD HV3	Randomised group	0.83	<0.001	0.48	1.18	0.38
	Baby's gender	-0.37	0.045	-0.74	-0.01	
	Slowness in Eating	-0.47	0.001	-0.73	-0.21	
	Mean FR (all HV)	0.26	0.074	-0.03	0.54	
Model 3	(Constant)	-0.73	0.21	-1.88	0.43	
Weight SD HV4	Randomised group	0.82	<0.001	0.47	1.17	0.40
	Baby's gender	-0.37	0.048	-0.73	0.00	
	Slowness in Eating	-0.45	0.001	-0.71	-0.18	
	Mean FR (all HV)	0.38	0.009	0.10	0.67	
Model 4	(Constant)	-0.92	0.51	-3.70	1.86	
Weight SD gain HV1-2	Randomised group	0.54	0.029	0.06	1.03	0.23
	Slowness in Eating	-0.36	0.044	-0.71	-0.01	
	Effortful control	0.50	0.013	0.11	0.89	
	SES group	-0.11	0.117	-0.26	0.03	
Model 5	(Constant)	-0.64	0.37	-2.05	0.78	
Weight SD gain HV1-3	Randomised group	0.77	0.001	0.33	1.22	0.20
	Slowness in Eating	-0.26	0.13	-0.59	0.07	
	Mean FR (all HV)	0.34	0.06	-0.02	0.70	
Model 6	(Constant)	-1.32	0.07	-2.75	0.12	
Weight SD gain HV1-4	Randomised group	0.72	0.002	0.27	1.18	0.21
	Slowness in Eating	-0.23	0.18	-0.57	0.11	
	Mean FR (all HV)	0.50	0.009	0.13	0.86	
Model 7	(Constant)	-1.73	0.14	-4.04	0.57	
BMI SD HV2	Randomised group	0.70	0.001	0.31	1.09	0.29
	Baby's gender	-0.51	0.018	-0.93	-0.09	
	Slowness in Eating	-0.35	0.018	-0.64	-0.06	
	Effortful control	0.43	0.009	0.11	0.75	
	SES group	-0.07	0.23	-0.19	0.05	
Model 8	(Constant)	-2.83	0.011	-4.99	-0.66	
BMI SD HV3	Randomised group	0.99	<0.001	0.59	1.39	0.37
	Slowness in Eating	-0.36	0.018	-0.66	-0.06	
	Mean FR (all HV)	0.47	0.006	0.14	0.79	
	Effortful control	0.17	0.29	-0.15	0.49	
Model 9	(Constant)	-1.36	0.07	-2.85	0.13	
BMI SD HV4	Randomised group	0.87	<0.001	0.44	1.30	0.35
	Baby's gender	-0.58	0.014	-1.03	-0.12	
	Slowness in Eating	-0.36	0.027	-0.67	-0.04	
	Mean FR (all HV)	0.35	0.06	-0.01	0.71	

Backward multiple regression analysis models with infant temperament and appetite trait variables as dependant variables, and SES groups, randomised groups and gender as potential confounding variables (detailed in Table 6.7). Baby's gender : 1=Male, 0=Female. All these variables were included in all models. Only significant predictors are shown (p<0.05). All models ANOVA p<0.005.

Summary results for models 1 to 3 : Weight SD HV1 – HV3

Based on the presented models in Table 6.8, slowness in eating was the strongest predictor of infant weight compared to other infant behaviour variables included in the models, after controlling for other potential confounding factors. Weight SD at HV2, HV3 and HV4 was predicted to decrease by 0.49, 0.47 and 0.45 SD respectively for every 1-point increase in slowness in eating score (all p≤0.001). In contrast, effortful control and mean food responsiveness score were associated with an increase in infant weight: weight SD at HV2 was predicted to increase by 0.32 SD for a 1-point increase in effortful control (p=0.026), whereas weight SD at HV3 was predicted to increase by 0.38 SD for a 1-point increase in food responsiveness score (p=0.009), after adjusting for the other covariates. There was also a non-significant trend showing that food responsiveness was a predictor of infant weight at HV2 (p=0.007). Model 1-3 accounts for 33%, 38% and 40% of the variability in Weight SD at HV2, HV3 and HV4 respectively.

Summary results for models 4-6 : Weight SD gain at different time points

Slowness in eating and Effortful control variables were a significant predictor of infant weight gain from HV1 to HV2, and food responsiveness was a significant predictor of infant weight gain from HV1 to HV4, and a non-significant trend predictor of weight gain from HV1 to HV3, after adjusting for the other covariates. Thus, Weight SD gain (HV1-2) was predicted to decrease by 0.36 and increase by 0.5 for every increase in slowness in eating (p=0.04) and effortful control (p=0.01) score respectively (Model 4). Weight SD gain (HV1-4) was predicted to increase by 0.5 SD for a 1-point increase in food responsiveness (p=0.009) (Model 6). Model 4 and 6 account for 23% and 21% of the variability in weight gain SD HV1-2 and HV1-4 respectively.

Summary results for models 7-9 : BMI SD HV2-4

Similar to model 1-3, slowness in eating was the strongest predictor of infant BMI compared to other infant behaviour variables included in the models, after controlling for other potential confounding factors. BMI SD at HV2, and BMI SD HV3 and HV4 were predicted to decrease by 0.35 (model 7) and 0.36 (model 8-9) respectively for every 1-point increase in slowness in eating score (all p<0.03). In contrast, BMI SD at HV2 was predicted to increase by 0.43 SD for a 1-point increase in effortful control (p=0.009), whereas BMI SD at HV3 was predicted to increase by 0.47 SD for a 1-point increase in food responsiveness score (p=0.006), after

adjusting for the other covariates. There was also a non-significant trend showing that food responsiveness was a predictor of infant BMI at HV4 (p=0.007). Models 7-9 account for 29%, 37% and 35% of the variability in BMI SD at HV2-4 respectively.

Overall, all of these results showed that the effects of the behaviour variables were independent of the effect of the intervention.

6.5.8. Associations between milk composition and infant outcomes (temperament, appetite, behaviour and growth) in univariate analyses

This section reports the associations between breast milk leptin and ghrelin and infant outcomes: temperament, appetite and also infant growth and behaviours.

There were no significant associations between breast milk hormones (cortisol, leptin and ghrelin) and infant temperament (all p>0.05). Breastfeeding duration (of the 'test' feed) at HV1 was not significantly correlated with breast milk hormones at HV1, and similarly, breastfeeding duration at HV2 was not significantly correlated with breast milk hormones at HV2. All other correlation results are summarised in Table 6.9.

6.5.8.1. Milk hormones and infant appetite and behaviours

***Leptin*:** Foremilk leptin at HV1 was significantly correlated with the slowness in eating score at HV1 and HV2, and also the average score for general appetite (GA) (r=0.4, p=0.001). In contrast to HV1, foremilk leptin at HV2 was negatively correlated with the average score of GA (r=-0.28, p=0.03). No other association was found between milk hormones and other infant appetite traits (p>0.05).

Hindmilk leptin at HV1 was negatively associated with feeding duration at HV2 (r=-0.41, p=0.013) and consistently, there was a non-significant trend for a negative correlation between hindmilk leptin at HV2 and feeding duration at HV2 (r=-0.28, p=0.09). The decrease in leptin concentration within a feed at HV1 was positively correlated with feeding duration at HV2 (r=0.41, p=0.013). Consistently, the decrease in leptin within a feed at HV2 was positively correlated with feeding duration at HV2. Milk leptin showed no significant correlation with other infant behaviours. In general, the results were consistent in suggesting that higher milk leptin concentrations were associated with shorter feeding duration.

***Ghrelin*:** Hindmilk total ghrelin at HV1 was positively correlated with feeding duration at HV2 (r=0.42, p=0.005) and negatively associated with the reduction in feeding duration from HV1-2 (r=-0.37, p=0.04). Foremilk total ghrelin at HV2 was also positively associated with sleeping duration at HV2 (r=0.36, p=0.03) and inversely associated with the change (increase) in awake duration between visits (r=-0.37, p=0.038). Consistently, the increase in total ghrelin within a feed at HV2 was positively associated with sleeping duration at HV2 (r=0.38, p=0.02) and changes in sleeping duration between visits (r=0.42, p=0.016). Similarly, the increase in active ghrelin within a feed at HV2 was negatively associated with awake duration at HV2 (r=-0.40, p=0.015) and the change (increase) in awake duration between visits (r=-0.40, p=0.02), and also positively correlated with the change (increase) in sleeping duration from HV1-2 (r=0.47, p=0.006). In general, the findings for total and active ghrelin were in the opposite direction to those for leptin, suggesting that higher ghrelin is associated with longer feeding duration, and also with longer sleeping duration.

6.5.8.2. Associations between milk hormones and infant growth in univariate analyses

Milk leptin was negatively associated with infant growth: foremilk leptin at HV2 was negatively correlated with infant weight SD at HV2 to HV4. There were also non-significant negative correlations between milk leptin HV1 and infant weight and BMI. In contrast, both total and active ghrelin showed positive associations with infant growth: foremilk total ghrelin at HV2 with infant weight SD at HV2-4; increase in total ghrelin within a feed at HV2 with weight SD at HV2-4, and weight SD gain at HV1-3 and HV1-4; and foremilk active ghrelin at HV2 with BMI SD at HV2-4 (summarised in Table 6.8). Milk cortisol at both HV1 and HV2 were not significantly correlated with any infant growth measurements.

6.5.8.3. Associations between milk macronutrients and infant weight and BMI in univariate analyses

Milk carbohydrate (either in fore or hind milk at HV2 and HV3, or pooled data) consistently showed significant positive associations with infant weight and BMI at HV2 to HV4 (range of r-value= 0.27-0.51; range of p-value: less than 0.001 to 0.03). Hindmilk protein at HV1 and HV2 were negatively correlated with infant weight gain and BMI (range of r-value = -0.26 to -0.30; range of p-value: 0.02 to 0.04) (Appendix 9). Milk fat (either in fore or hind milk at any HV, or pooled data) were not significantly correlated with infant weight or BMI at any HV.

Table 6.9 Summary of correlations between milk hormones and infant appetite, behaviour and growth

Hormones	Infant appetite (BEBQ)	Infant behaviours duration (3-Day Diary)	Infant growth
LEPTIN			
Foremilk hv1	SE HV1 (r=0.26, p=0.04) SE HV2 (r=0.31, p=0.016) Mean GA (r=0.32, p=0.01)		Weight SD HV2-4 = NS -ve correlation
Hindmilk hv1		Feed HV2 (r=-0.41, p=0.013)	Weight SD HV2-4 = NS -ve correlation
Changes within a feed hv1 (reduction)		Feed HV2 (r=0.41, p=0.013)	
Foremilk hv2	SE HV2 (r=0.14, p=0.28) Mean GA (r=-0.23, p=0.08) (all NS results)		Weight SD HV2 (r=-0.27, p=0.04) Weight SD HV3 (r=-0.31, p=0.015) Weight SD HV4 (r=-0.28, p=0.03) BMI SD HV2-4 = NS -ve correlation
Hindmilk hv2		Feed HV2 (r=-0.28, p=0.09): NS -ve correlation	Weight SD HV2-4 = NS -ve correlation
Changes within a feed hv2		Feed HV2 (r=0.57, p<0.001)	
TOTAL GHRELIN			
Foremilk hv1			Weight SD HV2-4 = NS +ve correlation
Hindmilk hv1		Feed HV2 (r=0.42, p=0.005) Reduce in feed HV1-2 (r=-0.37, p=0.04)	
Increase within a feed hv1			Weight SD HV2-4 = NS +ve correlation
Foremilk hv2		Sleep HV2 (r=0.36, p=0.03) Changes in awake (HV1-2) (r=-0.37, p=0.038)	Weight SD HV2 (r=0.31, p=0.017) Weight SD HV3 (r=0.35, p=0.007) Weight SD HV4 (r=0.37, p=0.005)
Hindmilk hv2			
Increase within a feed hv2		Sleep HV2 (r=0.38, p=0.02) Changes in sleep duration HV1-2 (r=0.42, p=0.016)	Weight SD HV2 (r=0.26, p=0.046) Weight SD HV3 (r=0.38, p=0.003) Weight SD HV4 (r=0.39, p=0.002) Weight SD gain HV1-3 (r=0.36, p=0.006) Weight SD gain HV1-4 (r=0.35, p=0.007) BMI SD HV3 (r=0.31, p=0.019)

Hormones	Infant appetite (BEBQ)	Infant behaviours duration (3-Day Diary)	Infant growth
ACTIVE GHRELIN			
Foremilk hv1			
Hindmilk hv1			
Increase within a feed hv1			
Foremilk hv2			BMI SD HV2 (r=0.26, p=0.04) BMI SD HV3 (r=0.25, p=0.06) – NS trend BMI SD HV4 (r=0.47, p=0.001)
Hindmilk hv2			
Increase within a feed hv2		Awake HV2 (r=-0.40, p=0.015) Changes in awake (HV1-2) (r=-0.40, p=0.02) Changes in sleep (HV1-2) (r=0.47, p=0.006)	

*Results that were not presented (empty columns in the table) showed inconsistent trends or no correlation (very low r-value or high p-value); All NS trend (p<0.1)

6.5.9. Associations between milk hormones and infant growth in multivariate analyses

Breast milk hormone variables that were significantly associated with infant growth in univariate analyses were included in regression models. The backward method was used to examine associations with infant weight and BMI outcomes after adjusting for confounders (socioeconomic status, infant's gender and randomised groups). Apart from milk hormones (foremilk, hindmilk and changes within a feed concentrations), covariates that were included were maternal BMI, infant gender and randomised group. The maternal BMI was included since it was reported to be associated with milk hormones, especially milk leptin [300]. The milk leptin and ghrelin (fore and hind) used in the models were the log (ln) value, and the active ghrelin and change in total ghrelin within a feed used was the absolute value. Here, I only present the results for models where milk leptin or ghrelin variables were significant predictors for infant weight and/or BMI (Table 6.10). Since randomised group was a strong predictor of infant growth measurements, I ran the regression analyses twice, with and without the randomised group variable. Therefore, the strongest predictor of infant growth other than randomised group could be determined. The backward elimination method was used for all regression analyses. Non-collinearity between milk hormone variables was also validated by the value of variance inflation factors below 5.

Based on the results of the univariate analyses, the following variables were included in regression analyses for each of the outcomes below:

Outcomes:	**Variables included in all models :**	
	Independent variables (Predictor)	Covariates variables (considered as potential confounding factors)
Weight SD HV2 **Weight SD HV3** **Weight SD HV4**	i. Foremilk leptin HV2 (Ln) ii. Foremilk ghrelin HV2 (Ln) iii. Change in milk total ghrelin within a feed at HV2 (T.Ghrelin change HV2)	iv. Maternal BMI at HV2 v. Baby's gender : 1=Male, 0=Female vi. Randomised groups*: 1=Relaxation group, 0=Control group
BMI SD HV2 **BMI SD HV3** **BMI SD HV4**	i. Foremilk leptin HV2 (Ln) ii. Foremilk active ghrelin HV2	iii. Maternal BMI at HV2 iv. Baby's gender : 1=Male, 0=Female v. Randomised groups*: 1=Relaxation group, 0=Control group

*Randomised groups (the primary outcome of the RCT) is considered as a potential predictor of the outcome

Table 6.10 Regression models examining associations between milk hormones and infant weight SD and BMI SD

Model / Outcome:	Variables:	Statistics				Adjusted R-Square
		B	p-value	CI: upper	CI: lower	
1) Weight SD HV2	*Without randomised group variable:* Sig. predictor = FM Total ghrelin HV2					
	With randomised group variable: Only randomised group is a sig. predictor					
2) Weight SD HV3	(Constant)	-0.73	0.00	-1.02	-0.43	
	T.Ghrelin change HV2	0.43	0.02	0.06	0.80	0.26
	Randomised group	0.62	<0.001	0.22	1.02	
3) Weight SD HV4	*Without randomised group variable:* Sig. predictor = FM Total ghrelin HV2					
	(Constant)	-0.78	0.00	-1.08	-0.48	
	T.Ghrelin change HV2	0.42	0.03	0.05	0.78	0.25
	Randomised group	0.60	0.00	0.20	1.01	
4) BMI SD HV2	*Without randomised group variable:* Sig. predictor = FM Active ghrelin HV2					
	With randomised group variable: Only randomised group is a sig. predictor					
5) BMI SD HV3	*Without randomised group variable:* Sig. predictor = FM Active ghrelin HV2					
	With randomised group variable: Only randomised group is a sig. predictor					
6) BMI SD HV4	*With randomised group variable:*					
	(Constant)	-1.85	0.00	-2.35	-1.35	
	FM Active ghrelin HV2	0.03	0.01	0.01	0.06	0.36
	Baby's gender	-0.52	0.01	-0.93	-0.11	
	Randomised group	0.53	0.02	0.10	0.96	

Backward multiple regression analysis models with milk hormone variables as dependant variables, and maternal BMI, randomised groups and gender as potential confounding variables. All models ANOVA p<0.001.Only significant predictors are shown (p<0.05). *Sig. = significant; T.Ghrelin= Total ghrelin

All models were tested for interactions between gender or randomised groups and milk hormones using GLM univariate analysis, and all interaction terms were non-significant (p>0.05). This indicates that the associations between milk hormones and infant weight or BMI were not modified by gender or randomised groups. No collinearity was found between milk hormone variables in all models.

Summary results for regression model Table 6.10

The increase in total ghrelin concentration within a feed was a significant positive predictor of Weight SD at HV3 and HV4, after adjusting for the other covariates. Thus, a 1pg/mL increase in the change in total ghrelin from fore to hindmilk at HV2 was associated with a 0.43 SD and 0.42 increase in weight at HV3 (p=0.032) and HV4 (p=0.03) respectively. These models account for 26% and 27% of the variability in weight at HV3 and HV4 respectively. Foremilk total ghrelin was a positive predictor for infant weight SD at HV2 only if the randomised group variable was excluded from the model.

For model 6, every 1 pg/mL increase in foremilk active ghrelin at HV2 is associated with a 0.03 SD increase in BMI at HV4 (p=0.01). This model accounts for 36% of the variability in BMI at HV4. These effects were independent of gender and randomised group; randomised group remained a significant predictor in all models. There were also no significant interactions between gender and milk hormones in these models. If the randomised group variable was excluded from the model, Foremilk active ghrelin was also a positive predictor for infant BMI SD at HV2 and HV3.

6.5.10. Combined regression models to show associations of infant appetite, temperament and milk hormones with infant growth in multivariate analyses

The appetite, temperament and milk hormone variables that were significant in the previously presented models were combined, to ascertain the strongest predictors of growth or weight and BMI at different time points. The backward method was used to examine associations with infant weight, BMI and weight gain. In addition to infant gender and randomised groups, the average milk carbohydrate variable was also included in the model as a potential confounder. This is because many milk carbohydrate variables (Table 5.10) showed significant differences by randomised groups, and these variables were also significantly associated with infant weight and BMI. The following predictors in Table 6.11 were included in all models:

Table 6.11 Predictors used in the combined regression models

Outcomes:	Independent variables (Predictor)	Covariates variables
Weight SD HV2	i) Foremilk leptin HV2 ii) Foremilk total ghrelin HV2 iii) Slowness in Eating HV2 (SE HV2) iv) Effortful Control	- Average milk carbohydrate: Milk carbo - Randomised groups: 1=Relaxation, 0=Control (considered as strong potential predictor(s) of the outcome)
Weight SD HV3 Weight SD HV4	i) Foremilk leptin HV2 ii) Change in milk total ghrelin HV2 iii) Slowness in Eating HV2 (SE HV2) iv) Mean Food Responsiveness (FR)	
Weight SD gain HV1-3 Weight SD gain HV1-4	i) Foremilk leptin HV2 ii) Foremilk total ghrelin HV2 iii) Slowness in Eating HV2 (SE HV2) iv) Mean Food Responsiveness (FR)	
BMI SD HV2	i) Foremilk leptin HV2 ii) Foremilk active ghrelin HV2 iii) Slowness in Eating HV2 (SE HV2) iv) Effortful Control	
BMI SD HV3 BMI SD HV4	i) Foremilk leptin HV2 ii) Foremilk active ghrelin HV2 iii) Slowness in Eating HV2 (SE HV2) iv) Mean Food Responsiveness (FR)	

Table 6.12 Final regression models that showed significant results for predictors

Model / Outcome:	Variables:	Statistics				Adjusted R-Square
		B	p-value	CI: upper	CI: lower	
Model 1: Weight SD HV2	(Constant)	-11.06	0.001	-17.21	-4.92	
	FM Total ghrelin HV2	1.12	0.01	0.28	1.96	0.35
	Slowness in Eating HV2	-0.42	0.007	-0.72	-0.12	
	Milk carbo	0.85	0.008	0.23	1.46	
Model 2: Weight SD HV3	(Constant)	-0.15	0.785	-1.28	0.97	
	T.Ghrelin change HV2	0.66	0.02	0.11	1.21	0.39
	Slowness in Eating HV2	-0.53	0.001	-0.82	-0.24	
	Mean FR (all HV)	0.32	0.03	0.03	0.60	
	Randomised group	0.62	0.002	0.24	0.99	
Model 3: Weight SD HV4	(Constant)	-0.64	0.24	-1.73	0.45	
	T.Ghrelin change HV2	0.66	0.016	0.13	1.20	0.43
	Slowness in Eating HV2	-0.52	0.001	-0.81	-0.23	
	Mean FR (all HV)	0.44	0.003	0.16	0.72	
	Randomised group	0.64	0.001	0.27	1.01	
Model 4: Weight SD gain HV1-3	(Constant)	-6.14	0.04	-12.13	-0.16	
	Mean FR (all HV)	0.33	0.08	-0.04	0.70	0.18
	Milk carbo	0.70	0.09	-0.11	1.50	
	Randomised group	0.61	0.016	0.12	1.10	
Model 5: Weight SD gain HV1-4	(Constant)	-1.68	0.007	-2.88	-0.47	0.18
	Mean FR (all HV)	0.41	0.029	0.05	0.78	
	Randomised group	0.70	0.005	0.22	1.18	
Model 6: BMI SD HV2	(Constant)	-1.91	0.10	-4.20	0.38	
	Slowness in Eating HV2	-0.38	0.035	-0.73	-0.03	0.22
	Effortful control	0.35	0.048	0.003	0.69	
	Randomised group	0.62	0.007	0.18	1.06	
Model 7: BMI SD HV3	(Constant)	-2.04	0.003	-3.37	-0.71	
	FM Act.ghrelin HV2	0.02	0.08	-0.003	0.04	0.37
	Slowness in Eating HV2	-0.46	0.008	-0.79	-0.12	
	Mean FR (all HV)	0.52	0.003	0.19	0.85	
	Randomised group	0.86	<0.001	0.44	1.27	
Model 8: BMI SD HV4	(Constant)	-2.25	0.002	-3.63	-0.88	
	FM Act.ghrelin HV2	0.03	0.02	0.01	0.06	0.32
	Slowness in Eating HV2	-0.32	0.07	-0.67	0.03	
	Mean FR (all HV)	0.38	0.036	0.03	0.74	
	Randomised group	0.59	0.014	0.13	1.05	

Backward multiple regression analysis models with infant behaviours traits and milk hormones as dependant variables, and randomised groups and milk carbohydrate as other covariates (Table 6.11). All models ANOVA $p<0.005$. FM=foremilk; Act.ghrelin = Active ghrelin; FR = Food responsiveness.

All final model results are presented in Table 6.12. Models that are not presented here showed that the infant behaviours and milk hormones were not significant predictors of infant growth at the later age, after adjusting for the randomised group and milk carbohydrate variables. If randomised group was not included in the model, the milk carbohydrate variable was shown to be a significant positive predictor for infant growth in model 1-2, 4, 6-8, or a non-significant positive predictor in model 3 and 5.

Summary results for models 1 to 3 : Weight SD HV1 – HV3

Slowness in eating (SE) was a consistent negative predictor of infant weight SD HV1-3. Weight SD at HV2, HV3 and HV4 were predicted to decrease by 0.42, 0.53 and 0.52 SD respectively for every 1-point increase in slowness in eating score (all $p \leq 0.007$). In contrast, Mean food responsiveness was associated with a 0.32 and 0.44 SD increase in weight at HV3 ($p=0.03$) and HV4 ($p=0.003$), after controlling for the other variables. Milk ghrelin was also a positive predictor for infant weight. Weight SD at HV2 was predicted to increase by 1.12 SD for every 1% increase in foremilk total ghrelin at HV2. Whereas, for every 1pg/mL increase in the changes in total ghrelin from fore to hindmilk at HV2 was associated with a 0.66 SD increase in weight at both at HV3 ($p=0.02$) and HV4 ($p=0.001$). Model 1-3 accounts for 35%, 39% and 43% of the variability in Weight SD at HV2, HV3 and HV4 respectively.

Summary results for models 4-5 : Weight SD gain

Mean food responsiveness (FR) was also associated with a 0.41 increase in weight SD gain from HV1 to HV4 ($p=0.029$). There was a non-significant trend for a similar positive association between Mean food responsiveness and increase in weight SD gain from HV1 to HV3. Both models 4 and 5 account for 18% of the variability in weight SD gain from HV1 to HV3 and HV1 to HV4.

Summary results for models 6-8 : BMI SD HV2-3

BMI SD at HV2 and HV3 were predicted to decrease by 0.38 and 0.46 SD respectively for every 1-point increase in slowness in eating score (all $p<0.05$). On the other hand, consistent with Weight SD HV3-4 associations in model 1-3, mean food responsiveness was associated with 0.52 and 0.38 SD increase in BMI SD at HV3 and HV4. Effortful control and Foremilk active ghrelin were also a significant positive predictors for infant BMI at HV2 and HV4 respectively (all $p<0.05$), after adjusting for the other variables including randomised group. Models 6-8 account for 22%, 37% and 32% of the variability in BMI SD at HV2, HV3 and HV4 respectively.

Table 6.13. Summary of the observational outcome results for the whole population group

	VARIABLES	RESULTS
1	**Milk leptin**	
	Change within a feed : Fore to hind milk Milk HV1 Milk HV2	i. At HV1, the mean leptin significantly reduced over a feed with a reduction of 37 s% from fore- to hindmilk. ii. At HV2, mean leptin was not significantly different between fore- and hindmilk (this could be due to opposite trends in the change of concentrations within a feed between randomised groups)
	Change between visits : HV1 to HV2	iii. Milk leptin increased significantly from HV1 to HV2 ($p<0.05$)
2	**Milk total ghrelin**	
	Change within a feed : Fore to hind milk Milk HV1 & HV2 Change between visits : HV1 - HV2	i. The mean total ghrelin increased significantly from fore- to hindmilk at both HV1 and HV2. ii. The mean total ghrelin concentrations at HV1 were significantly higher than the concentrations at HV2 ($p<0.05$).
3	**Milk active ghrelin**	
	Change within a feed : Fore to hind milk Milk HV1 & HV2 Change between visits : HV1 to HV2	i. The mean active ghrelin increased significantly from fore- to hindmilk at both HV1 and HV2 ii. The mean active ghrelin was not significantly different between visits (from HV1 to HV2)
4	**Test for gender differences**	
		i. The breast milk leptin and active and total ghrelin concentrations in fore and hindmilk were not significantly different between genders at any HV.
5	**Infant temperament**	
	Temperament traits : Surgency, negative affectivity and effortful control	i. Infant temperament traits were not significantly different between genders (all $p>0.05$) at age 3-4 months. ii. Effortful control was positively associated with weight and BMI at HV2 and weight gain from HV1 to HV2.

	VARIABLES	RESULTS
6	**Infant appetite**	
	Enjoyment of food (EF) Food responsiveness (FR) Slowness in eating (SE) Satiety responsiveness (SR) General appetite (GA)	i. There were no significant differences in any appetite traits between genders at any HV, except SE at HV3. At HV3, the SE appetite trait was significantly higher in female than male. ii. EF, FR and GA were positively skewed (values close to 5) whereas mean values for SE and SR were close to middle value (2.5). iii. No significant differences were found in appetite traits between visits (either from HV1 to HV2, or HV1 to HV3 or HV2 to HV3) except for SE between HV1 and HV3. iv. The SE at HV1 was significantly higher than at HV3 showing that infants fed faster as their age increased. v. Mean SR was negatively associated with mean EF, whereas mean FR was positively associated with mean GA. vi. Mean FR was positively associated with infant growth, whereas SE HV2 was negatively associated with infant growth.
7	**Other infant behaviours**	
		i. Awake duration at HV2 and changes in awake duration from HV1 to HV2 were inversely associated with infant growth. ii. Sleeping duration at HV2 and changes in sleeping duration from HV1 to HV2 were positively associated with infant BMI SD.
8	**Associations between variables (univariate associations)**	
	i) Infant temperament, appetite and growth	i. Negative affectivity was inversely associated with the mean EF, suggesting that infants that were perceived as less likely to enjoy their feeding time in early life were more likely to show 'difficult' behaviour at the later age.
	ii) Milk hormones and infant appetite	i. Foremilk leptin at HV1 was significantly positively correlated with the average score of SE. ii. The average GA was positively correlated with foremilk leptin at HV1 but negatively correlated with foremilk leptin at HV2
	iii) Milk hormones and infant behaviour	i. Feeding duration at HV2 was negatively associated with hindmilk leptin at HV1 and positively associated with the reduction of leptin within a feed at HV1 & HV2. ii. Foremilk total ghrelin at HV2 was positively associated with sleeping duration at HV2 and inversely associated with the change in awake time duration between visits. iii. The increase in total ghrelin within a feed at HV2 was positively associated with sleeping duration at HV2 and changes in sleeping duration between visits. iv. The increase in active ghrelin within a feed (HV2) was: i) negatively associated with awake duration (HV2) & the increase in awake duration between visits; ii) positively correlated with the changes in sleeping duration.
	iv) Milk hormones and infant growth	i. Milk leptin was negatively associated with infant growth. ii. Both total and active ghrelin showed positive associations with infant growth.

7	**Associations between variables (multivariate associations)**		
	Infant behaviour and growth	i.	Slowness in Eating (SE) HV2 was a significant negative predictor of weight SD HV2-HV4, BMI SD HV2-HV4 and Weight SD gain HV1-2.
		ii.	Effortful control was a significant positive predictor of weight SD HV2, BMI SD HV2 and Weight SD gain HV1-2, but not a significant independent predictor of growth outcomes beyond HV2.
		iii.	Food Responsiveness (FR) was a significant positive predictor of Weight SD gain HV1-4 and BMI HV3.
		iv.	These associations were independent of the intervention, which was also a significant positive predictor of growth outcomes.
		v.	All interaction term results for infant behaviour variables were not significant: the associations above were not modified by gender or by randomised group.
	Milk hormones and growth	i.	Increased in total ghrelin concentrations within a feed was a significant positive predictor of Weight SD at HV3 & HV4.
		ii.	Foremilk active ghrelin at HV2 was a significant positive predictor of BMI SD HV4.
		iii.	These associations were independent of the intervention, which was also a significant positive predictor of growth outcomes.
		iv.	All interaction term results for milk hormone variables were not significant suggesting the associations were not modified by gender or randomised group.
	FINAL COMBINED MODELS	i.	The positive predictors of infant weight at different HV were: Weight SD HV2: Foremilk total ghrelin HV2; Weight SD HV3-4: Mean FR and the changes of total ghrelin within a feed at HV2.
		ii.	The Slowness in eating appetite trait was a significant negative predictor of infant weight SD HV1-3
		iii.	Mean FR was a positive predictor of infant weight gain SD from HV1 to 4, and also a non-significant trend positive predictor of infant weight gain SD from HV1 to HV3.
		iv.	The significant positive predictors of infant BMI at different HV were: BMI SD HV2: Effortful Control; BMI SD HV3: Mean FR; and BMI SD HV4: Mean FR and Foremilk active ghrelin at HV2.
		v.	These associations were independent of the effect of the intervention. All interaction terms for randomised group results were not significant.

6.7. Discussion

This chapter addresses the observational cohort hypotheses and explores the associations between mother-infant factors and infant growth. A summary of the results is presented in Table 6.10. The observational cohort hypotheses were:

I. Infant temperament, appetite and breast milk composition are associated with infant growth, and these associations also differ by gender.

II. Non-nutrient factors in breast milk (specifically hormonal constituents; ghrelin and leptin) are associated with infant appetite and behaviour and hence infant growth.

I firstly summarise the main findings and then discuss each of the secondary outcomes, comparing the present study findings with previously published data. At the end of this chapter, the discussion is focused on the findings of the combined regression models together with the overall strengths and limitations of the study.

6.7.1. Main findings

Referring to the first part of the hypothesis my findings confirmed that infant temperament and appetite traits were associated with infant growth. Thus, food responsiveness was positively associated with infant weight gain whereas slowness in eating was negatively associated with infant weight, BMI and weight gain. Effortful control was also positively associated with infant growth outcomes. The associations remained significant when both behaviour traits were included in multivariate models, in which they were shown to predict infant growth at different time points. Foremilk leptin was negatively associated with infant weight, whereas foremilk total ghrelin and the changes in total ghrelin within a feed were positively associated with infant weight, and foremilk active ghrelin was associated with infant BMI. However, after controlling for gender and randomised group in the multivariate models, only the changes in total ghrelin within a feed and foremilk active ghrelin were independently associated with infant weight and BMI respectively. Without including the randomised group variable in the models, foremilk total ghrelin was shown to be a significant positive predictor of weight at HV2 and HV4, with a similar though non-significant trend for weight at HV3.

Finally, when infant behaviour and milk hormone variables were included in the combined models (Table 6.12), the results consistently showed that slowness in eating was a significant negative predictor of weight and BMI at almost all time points (HV2-HV4) while mean food responsiveness was consistently shown to be a positive predictor of weight and BMI at later

time points (HV3-HV4), including weight gain from HV1 to HV4. Effortful control was a positive predictor of infant BMI but only at HV2. In terms of milk hormones, only foremilk total and active ghrelin and the changes in milk ghrelin within a feed were shown to be significant positive predictors of weight or BMI at different time points. These associations were all independent of randomised group and average milk carbohydrate, with no modification of the effects by randomised group. Thus, consistent with observational cohort hypothesis I, infant temperament, appetite and milk hormones appear to influence infant growth measurements at around 6-14 weeks.

However, contrary to my hypothesis, these associations did not differ by gender. Temperament and appetite traits were not significantly different between genders except for 'slowness in eating' at HV3. Breast milk composition (both macronutrient content and milk hormones) was also not significantly different between genders at any HV, suggesting that there was no bias or differing strategy of maternal investment in terms of milk composition in male and female infants.

Considering the second part of the observational cohort hypothesis, only foremilk leptin at HV1 was significantly associated with infant appetite traits – namely slowness in eating - and both of these variables were independently associated with infant growth measurements. When both variables were then included in multivariate models to predict infant growth, only slowness in eating showed significant inverse associations with infant weight and BMI at 6- to 14-weeks of infant age.

Breast milk leptin and ghrelin at 6 weeks of age (HV2) were also shown to be associated with other infant behaviours, namely feeding, sleeping and awake duration at 6-8 weeks. Thus, milk leptin was inversely associated with later feeding duration, whereas ghrelin was positively associated with feeding and sleeping duration, and inversely associated with the increase in awake duration from HV1 to HV2. Overall, ghrelin was shown to be positively correlated with sleeping and negatively associated with awake duration in early infancy, and these behaviours were in turn correlated with infant growth measurements, which were positively correlated with sleeping duration and negatively correlated with awake duration.

6.7.2. Breast milk leptin

The mean breast milk leptin levels of this study population at HV1 (0.54±0.79 ng/ml) and HV2 (0.85±1.0 ng/ml) were within the range reported in previous studies (Table 2.2 in Chapter 2: ranging from 0.01-3.65 ng/ml). Nevertheless, as described in Chapter 2, reported leptin levels are highly variable, and the method used to analyse the samples, choice of assay kit (e.g. radioimmunoassay or ELISA), or the use of whole milk instead of skimmed milk, could also have contributed to the high variability of the results between studies. The milk leptin levels in the present study increased significantly from 2 to 6 weeks postpartum, similar to a recent study [301], but in contrast to many other studies at similar stages of lactation, which reported decreasing or stable leptin levels across lactation (Figure 2.7 in Chapter 2). However, the results of the present study only involved two-time points and the gap between visits was short (only 4 weeks), thus conclusions cannot be drawn about the direction of leptin levels across longer periods of lactation. As discussed previously (Chapter 5), since some breast milk samples were collected in the afternoon at HV2, later than the intended home visit time, this could have contributed to the greater variability in the data (the milk hormone SD values and concentration range were larger at HV2 - Table 6.1). In terms of the changes in leptin concentrations from fore to hind milk, the present study showed a reduction over a feed at 2 weeks (0.55 to 0.38 ng/ml) and a similar non-significant reduction trend at 6 weeks (0.78 to 0.64 ng/ml). Previous studies showed inconsistent non-significant trends at different stages of lactation: an increase at 1-4 weeks [101, 142], a decrease at >12 weeks [123, 142], and no change within a feed at 4-5 weeks [139] and 10-12 weeks [101, 140].

Leptin has been shown to influence long-term energy balance which in turn affects growth [103]. The suggested mechanism involves the activation of anorexigenic neuropeptides that enhance satiety signals, which then influence food intake and energy balance [302]. However, the function and mechanism of action of leptin in breast milk on infants are not yet clear. My findings demonstrated negative correlations between milk leptin and infant weight, consistent with many previous studies at similar or later ages [127-131]. In the present study, the associations were only apparent starting at HV2 (6-8 weeks of age), shown by inverse associations between foremilk leptin at 6 weeks and infant weight at 6, 12 and 14 weeks. Similar non-significant trends were also shown between hindmilk leptin at HV2 and both fore and hindmilk leptin at HV1 with infant weight at week 6-14. It has been suggested that inverse associations between milk leptin and infant growth may result in protection against later overweight, or reduced risk of overweight at later ages [128, 132]. However, in the

multivariate analyses, when I included other predictors in the regression models (e.g. milk ghrelin and randomised group), milk leptin was no longer a significant negative predictor of infant weight.

Nevertheless, it is also interesting to note that milk leptin during early lactation (at 2 weeks) was significantly correlated with infant appetite and feeding behaviours. Foremilk leptin at HV1 was significantly associated with the slowness in eating (SE) appetite trait at both HV1 and HV2. These associations suggest that higher leptin at the beginning of the feed is associated with slower feeding (at least according to maternal perception) at HV1 and/or HV2. On the other hand, hindmilk leptin at HV1 was negatively associated with feeding duration at HV2, and consistently, the reduction of leptin within a feed at HV1 was positively associated with feeding duration at HV2. Taken together, the results are consistent with an effect of breast milk leptin on feeding behaviour, with higher levels associated with a slower feeding pace (e.g. sucking slowly) and shorter feeding duration at a later age. These feeding duration results were the data recorded over 72 hours in the 3-day diary.

Interestingly, leptin at HV1 was not associated with the duration of breastfeeding in the observed feed during that home visit, and similarly, leptin at HV2 was not associated with the duration of breastfeeding during HV2. This suggests that breast milk leptin may show a longer-term rather than a short-term effect on infant feeding duration. Consistently, a recent study [140] did not find any association between milk leptin dose and the time interval between feeding, suggesting no evidence of a short-term effect of leptin on the time between feeds. However, their sample size was very small (n=19) and the leptin dose was calculated solely based on the milk intake measured by test-weighing. This method has a high possibility of systematic error due to insensible water loss in infants during measurement. Furthermore, the duration of nursing bouts might vary, which could increase the variability of the milk intake data. Overall, due to the limited evidence, I conclude that the short-term effect of milk leptin on infant outcomes is not clear and largely unexplored.

In terms of long-term effects of milk leptin on infant outcomes, I found inverse associations between milk leptin at 2 weeks and infant weight at later ages in (bivariate correlations, but no longer significant in multivariate analysis). It is possible that the influence of leptin could be mediated through effects on infant appetite and feeding behaviour such that these variables displace leptin in multivariate analyses. Specifically, a slow pace in feeding and

shorter feeding duration could be a potential mechanism for the influence of breast milk leptin on infant weight. On the other hand, potentially, milk leptin could have long-term effects on infant growth by reducing milk intake, acting in the same manner as plasma leptin in adults. In human adult studies, leptin has been suggested to play a role in the control of meal size by stimulating other satiety neuropeptides in the brain [103, 125, 126]. Thus, since some studies have reported that ingested milk leptin could remain biologically active in the infant's body [122, 123], high leptin in breast milk could then potentially result in lower milk intake, probably through shorter feeding duration or higher satiety. Milk intake data from the present study are not yet available, and leptin in infant plasma was not measured. Nevertheless, studies have reported that breast-fed infants had higher serum leptin than formula-fed infants in the first-fourth months [143, 303-305], and breast milk leptin was significantly correlated with infant serum leptin [123], suggesting the transfer of breast milk leptin into the infant's circulation. As indicated earlier, breast milk leptin has also been shown to negatively correlate with infant weight, suggesting an influence of leptin on later growth or development. However, the result was not consistent after controlling for other covariates (randomised groups, gender, milk ghrelin and infant behaviour) in the multivariate regression; milk leptin was no longer a significant negative predictor of infant weight. Future studies using standardised milk sampling protocols with a larger sample size should be conducted to further explore the relationship between milk leptin and infant growth. In addition to feeding behaviour, future studies should also consider the combination of milk intake and infant serum leptin measurements in order to increase understanding of the function of breast milk leptin and the effects on infant growth and development.

Other than feeding behaviour, infant appetite also showed associations with infant growth, and this is discussed further in subsection 6.4.5. However, feeding duration (measured over 72 hours) was not associated with infant growth.

6.7.3. Breast milk ghrelin

The mean breast milk total ghrelin levels of this study population at HV1 (216 ± 30 pg/ml) and HV2 (196 ± 39 pg/ml) were within the range reported by previous studies (Table 2.1 in Chapter 2: ranging from around 100 to 3000 pg/ml). Active ghrelin in breast milk is measured less frequently. The concentration range of the present study population (11-52 pg/ml) was close to that reported in two previous studies (range: 9-39 pg/ml) [101, 111], but much lower compared to two other studies (range: 500-1670 pg/ml) [110, 112]. Thus, there appear to be

two distinct ranges of active ghrelin values reported in the literature. This could be possibly due to sample handling since active ghrelin (acyl-ghrelin) could be deacylated within 3-4 hours by enzymes, predominantly butyrylcholinesterase [106]. However, little is known about the presence or concentration of this enzyme in human milk. To maintain the concentration of acyl-ghrelin, I acidified my milk samples within 30 minutes to 3 hours after the sample was collected, either during the home visit or after the session, depending on the availability of time. Ideally, I should have standardised the timing of the acidification process during or after a home visit, but this was not possible due to time constraints and lack of manpower during data collection (the home visits were mainly being conducted just by myself). Some studies might acidify or add a protease inhibitor straightaway after the samples were collected and hence be able to maintain a larger proportion of active ghrelin in the breast milk. Thus, the results for milk active ghrelin could plausibly be different between studies in which samples are acidified straightaway or later after 2-3 hours. Although many studies have reported on the acidification procedure before sample storage, detailed information about the timing of acidification was generally not included. Hence, this is something that should be standardised and reported in future studies.

The change in concentration of milk ghrelin across lactation also shows inconsistent results between studies: the present study showed a significant reduction in milk total ghrelin from week 2 to week 6 of lactation, and no significant changes in milk active ghrelin between time points, similar to a previous study [101], but other studies showed an opposite trend, with milk total [109, 110] and/or active ghrelin [112] increasing across lactation (at either 2 or 3 time points), and one study showed inconsistent trends of milk total ghrelin at 3 time points across lactation [301]. Thus, similar to leptin, reported milk ghrelin is highly variable between studies, which may largely reflect methodological and sampling procedure issues. As indicated previously, due to time constraints and limited resources, it was not possible to conduct all home visits during late morning as planned in the protocol; some HV2 were conducted in the afternoon. This may explain the observation that, as with leptin, the results for milk ghrelin at HV2 were more variable than at HV1. Moreover, due to the lack of available assay kits specifically for human breast milk, the assay kits that were used to measured milk ghrelin were designed for human plasma samples. Overall, many factors may have contributed to the variability of the milk hormones results, as listed in the Table 6.14. These factors should ideally be taken into consideration and standardised when designing and performing research on human milk hormones. On the other hand, the high variability in the milk hormone results

between studies or between time-points within my study could also potentially be due to real differences in the degree or strength of biological signalling between the mother and infant through breast milk hormones over time. In other words, the different results could reflect differences in the tension of the tug-of-war at later time points, since older infants might signal more strongly to the mother, whereas the mother might respond differently since more of her energy resources have already been used for breastfeeding. Thus it is possible that diversity of ecological and behavioural factors could also contribute to the observed high variability in the results.

Table 6.14 Factors to consider when designing and conducting research on human milk hormones

a) Sampling procedure			
i) Timing of data collection: - Morning - Afternoon - Pooled milk samples	ii) Food intake of the BF mothers: - Before meal - After meal - Fasting state	iii) Milk samples: - Foremilk - Hindmilk - Mid-flow milk - Total/mixed	iv) Sample handling: - Acidify or add protease inhibitor - Timing of acidification - Freeze/thaw process
b) Lab analysis procedure			
i) Analysis method: - Radioimmunoassay - ELISA - Others (e.g. multiplex immunoassay)	ii) Assay kits: - Specific for human milk? Adjustment needed if using assay kits for: serum/plasma/saliva	iii) Analysis for: - Whole milk - Skimmed milk - active form state (e.g total or active ghrelin)	iv) Reliability: - duplicates - intra- and inter-assay variation - sensitivity and range
c) Population groups or stage of lactation			
i) Mother's health : - Healthy population - Medical condition (e.g. diabetic or obese/overweight)	ii) Mothers of : - Full term infants - Pre-term infants - Low birth weight or macrosomic-baby	iii) Maternal status: - Primiparous - Multiparous - Multiple births (twins)	iv) Lactation status/stage: - Exclusive BF - Mixed feeding - Early stage: colostrum - Later stage: mature milk

*BF: breastfeeding

In contrast to milk leptin, ghrelin has been suggested to stimulate appetite through the activation of anorexigenic neuropeptides that influence food intake and thus body weight [103]. In adults, ghrelin has been reported to have immediate effects on food intake in response to hunger, shown by a sharp rise prior to a meal and a sudden drop post-meal, and was therefore considered to function as a hunger signal [108]. However, the trend of plasma ghrelin in infants pre- and post-feeding is not known, and would be difficult to measure for practical and ethical reasons. Interestingly, instead of being viewed only as a hunger hormone, some studies have also suggested that higher ghrelin concentrations could signal the brain to

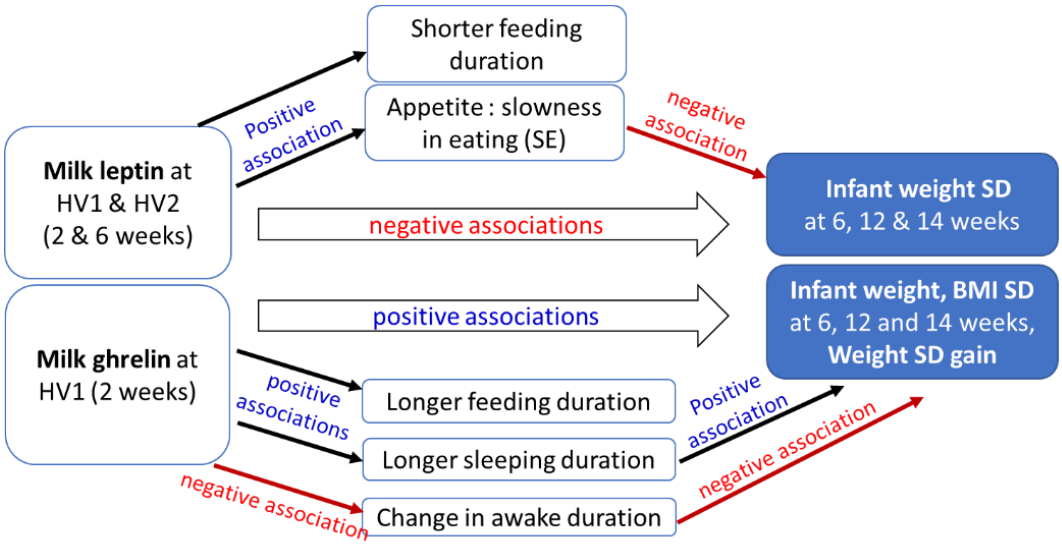
prepare for metabolism and energy storage [106, 306]. This was proposed due to the finding that active ghrelin in human plasma did not increase during food deprivation states or prolonged fasting [306, 307]. Ghrelin has also been reported to be produced in different parts of the human body, hence, it was suggested that it might function differently according to where it was derived or expressed in the human body, or the part of the brain where it was activated [106, 107]. Ghrelin has also been found to be produced by breast tissue [116, 118], and hence transferred into breast milk, yet the function of milk ghrelin in infants is still not well understood. Some studies have reported that ghrelin in breast milk could come from maternal plasma, due to an increase of plasma ghrelin after delivery, and the higher levels in maternal plasma than in breast milk [109].

Few studies have measured the change in ghrelin concentrations from fore to hind milk within a feed. Two studies reported a significant decrease in milk ghrelin within a feed at 1 and 3 months of lactation [101, 142], and suggested a potential role of milk ghrelin in infant appetite regulation and self-control in feeding. However, they did not find any associations with infant growth, and infant feeding behaviour or appetite were not investigated. In contrast to these findings, the present study demonstrated an increase in both total and active ghrelin within a feed at 2-3 and 6-8 weeks lactation. The present study also showed that the change in milk ghrelin within a feed was a positive predictor of infant weight, and that foremilk active ghrelin was a predictor of infant BMI, after controlling for possible confounding factors. If randomised group was not included in the model, foremilk total ghrelin was also a significant predictor for infant weight. These results consistently show that milk ghrelin was associated with an increase in infant weight at different time points. However, the results reported in the literature are not consistent: previous studies either found no correlation [142, 301] or reported an opposite result; with an inverse association between ghrelin and infant weight [114, 115]. The finding of inverse associations between milk ghrelin and infant growth measurements in previous studies has led to speculation that milk ghrelin may be involved in the regulation of infant body weight by stimulating more growth hormone production in infants with lower weight gain. However, the mechanism underlying a positive association of milk ghrelin with infant growth is less clear.

In addition to infant growth, milk ghrelin was also associated with infant behaviour in my study. The hindmilk total ghrelin at 2 weeks was positively associated with infant feeding duration at 6-8 weeks and inversely associated with the reduction in feeding duration from 2

to 6 weeks. However, similar to leptin, milk ghrelin at HV1 and HV2 were not correlated with the duration of breastfeeding of a single feed during the HV sessions. These findings suggest that both milk leptin and ghrelin may have longer-term rather than short-term effects on feeding duration. Taken together, leptin and ghrelin showed opposite correlations with later feeding duration: milk leptin at 2 and 6 weeks were associated with shorter feeding duration at 6-8 weeks, whereas ghrelin at 2 weeks was associated with longer feeding duration at 6-8 weeks. Consistently, similar effects were seen for the correlation between milk leptin and ghrelin and infant growth: leptin was inversely associated with infant weight and ghrelin was positively associated with infant weight and BMI. These associations are illustrated in Figure 6.2. Overall, these results support the suggested functions of milk leptin and ghrelin on infant feeding regulation, in terms of feeding duration and appetite, and the relationship to later weight or BMI or growth.

Figure 6.2 Associations between milk hormones and infant behaviour and growth



In addition to feeding duration, milk ghrelin was also positively associated with sleeping duration and negatively associated with awake duration (Table 6.9). To my knowledge, this is the first study to find correlations between milk ghrelin and infant sleeping and awake duration. Thus, there is a possibility that ghrelin might also facilitate sleep in the infant, and, since sleeping uses less energy, infants who sleep longer could conserve energy which can be used for growth. Fascinatingly, studies in adult humans, including an RCT [308], have also demonstrated a role of plasma ghrelin as a sleep-promoting factor [309-311], and it has been suggested to improve sleep quality [312]. Since it appears that the function of ghrelin may not

be restricted to metabolic regulation or energy homeostasis through regulation of food intake, as suggested in adult studies, it is plausible that ghrelin in breast milk could function through effects on sleeping behaviour instead of or in addition to effects on eating behaviour. Thus, this new finding suggests the need to broaden research on breast milk ghrelin to consider effects on infant behaviour and growth during early life, rather than focussing just on infant feeding or appetite regulation.

6.7.4. Associations between milk hormones and infant weight and BMI

In univariate analyses, milk hormones at HV1 did not show any association with infant growth but the milk hormones at HV2 were significantly associated with infant weight and BMI. Both total and active foremilk ghrelin at HV2 were shown to be significant positive predictors of infant weight or BMI only if the randomised group variable was not included in the regression models (Table 6.10) which suggests that the effect of the intervention on infant growth could partly be mediated via milk ghrelin. Conversely, the change in total ghrelin concentrations within a feed at HV2 was a significant predictor of growth outcomes with or without randomised group in the model (Table 6.10 and 6.12), which might suggest that this is not a mediator for the intervention effect on infant growth. Milk leptin was not a significant predictor of growth in any models, with or without including randomised group, after adjusting for other covariates (e.g. milk ghrelin and/or infant behaviour). Thus the evidence for an influence of milk leptin on infant growth was weaker and less consistent.

6.7.5. Infant temperament (RIBQ)

Temperament has been categorised into different component dimensions of personality to describe individual differences in reactivity and self-regulation [313, 314]. Although some temperament traits are partially heritable, they may also be influenced by the environment, maturation and life experiences [314]. In this context, heritability is defined by the proportion of variance in infant temperament traits attributable to genetic variance [315]; thus the behaviour trait is partially inherited from the parents.

Using the Rothbart's Infant-Behavior-Questionnaire-Revised (RIBQ), effortful control, negative affectivity and surgency were assessed as three main dimensions of infant temperament traits. The study population scored high in effortful control (5.5), rated in

between ‘more than half of the time’ (score of 5/7) to ‘almost always’ (score of 6/7), whereas negative affectivity (3.78) was close to the neutral score (3.5/7). Therefore, the infants in this study population seemed to have an easy or less challenging temperament at 3-4 months of age. Overall, they were perceived by their mothers to show relatively high self-regulation or controlled emotions during early life rather than demonstrating ‘challenging’ behaviour. The negative affectivity value of the study population was slightly higher than a sample population of breast-fed or mixed-fed infants in previous studies (2.4-3.0) [73, 226], but similar to another previous study (score of 3.5) [316], all in infants of a similar age. A study compared the temperament between infants with different methods of feeding and found that breast-fed and mixed-fed infants had a significantly lower score in effortful control and higher score in negative affectivity than formula-fed infants [73]. Hence, formula-fed infants were rated as having a less challenging temperament than breast-fed and mixed-fed infants, leading the authors to suggest the need for extra support to be considered for breastfeeding mothers to help prolong breastfeeding duration. However, this result could also suggest that mothers of breast-fed or mixed-fed infants were more aware of infant cues or signals, which could influence the overall temperament score.

On the other hand, the average population score for surgency in my study was rated as ‘more than half time’ in terms of frequency in demonstrating the trait, indicating moderate activity levels and a moderate degree of ‘extraversion’. These behaviours were not significantly different between genders, similar to other studies among infants at 3-4 months of age [73, 226]. There are limited studies investigating gender differences in infant temperament at this age since it is considered likely to be similar among boys and girls, as well as stable in early life. However, there is some evidence that gender differences develop at later ages during childhood [317, 318].

Although temperament in early life is usually considered to be fairly stable from birth to 18 months [317, 318], it could be associated with infant growth, especially during early life when growth is rapid. For example, a high activity level (high surgency trait) and distressed emotions or behaviour (negative affectivity traits) could affect the use of energy, and thus energy balance [319]. Distress behaviours such as crying and vocalisation have a high energy cost, and if this persists, it could contribute to higher total daily energy expenditure [320]. This is especially critical in the first 6 months when the energy cost for growth is markedly higher than at later ages [185]. On the other hand, infants with lower distress and a calmer

personality (higher effortful control) could save more energy for growth. This concept is supported by my findings since effortful control was positively associated with infant weight and BMI at 6-8 and 12-14 weeks, and weight gain from 6-8 to 12-14 weeks. However, surgency and negative affectivity were not correlated with infant growth, which is inconsistent with other studies that found positive correlations between these temperament traits and infant growth either at around 3 months or at later ages [297, 316, 321-323].

Apart from effects on energy balance, behaviour traits in early life could result in different strategies in parenting care or maternal investment, which could also eventually influence infant growth. In terms of biological factors (considering maternal investment in breast milk), the present study did not show any association between macronutrient content, total milk energy or milk hormones and infant temperament. These relationships are largely unexplored, either in humans or animals. Only one study has reported on these associations: higher milk energy was positively associated with surgency temperament (higher activity and greater confidence) among macaque rhesus infants in a stressful environment [96]. This study suggested that mothers may have the potential to shape infant temperament through alterations in milk composition, by transferring different hormones (including cortisol), macronutrients or total milk energy. However, the study had some limitations. The frequency or duration of feeding was not assessed and this could influence the fat concentrations in the milk or milk dilution, and hence the milk total energy. The growth of the infant macaques was also not measured, and therefore the later consequences for growth could not be investigated. Moreover, milk composition and infant behaviour development patterns in animals are considerably different than in humans since growth and maturation rates in humans are much slower. In my study, there was no significant correlation between milk cortisol and any temperament traits. Effortful control was found to be a positive predictor for infant growth measurements after controlling for other covariates such as milk composition (leptin, ghrelin and mean carbohydrate levels) and randomised group. This suggests that the associations between infant temperament and infant growth measurements in my study population were independent of milk composition or the intervention effect.

It is also possible that infant temperament could influence the parental feeding style or encourage more responsive feeding. This may be especially the case in first-time mothers who are mostly still learning to interpret their infant's cues. For example, higher vocalisation or irritable behaviour could lead to more frequent feeding, even though vocalisation may not

always indicate hunger. This could be one of the mechanisms for the findings of the positive association between challenging temperament and infant weight gain during infancy in many previous studies [316, 321-323] although the association was not found in the present study. Since vocalisation is metabolically expensive, infants may be more likely give true information or cues (honest signals) for hunger during early life. However, if this behaviour persists at later ages, they could well manipulate the parents, since the energy cost for growth reduces by more than half after 6 months of age [185]. Previous findings on the relationship between difficult temperament and infant growth relied solely on maternal report [316, 321-323], without measuring the actual physical activity or energy expenditure. Thus, the mechanism of the association between temperament and later growth is not clearly understood. However, maternal report could be regarded as an appropriate source of data as well if the aim is to measure maternal perception of infant appetite in order to understand how the mother perceives and responds to infant signalling during early life.

Previous studies also found a relationship between irritability and later child behaviour [319], or temperament traits with sleeping and fussiness patterns [321]. However, the present study did not find any association between infant temperament and appetite, or, between infant temperament and other behaviours (from the 3-day diary) during the study period. This may be due to the short length of follow-up and small sample size but it could also be related to the RIBQ tool.

There are several limitations of the usage of RIBQ as a tool to measure infant temperament. Firstly, infant temperament can only be measured starting at 3-4 months [226], and in the present study, the infant temperament was assessed at 14-16 weeks of age. There were several parts of the questionnaire that were not yet applicable for the infants (e.g. Q27: How often did your baby notice the sound of an aeroplane passing overhead?) and hence these questions were excluded from the analysis. This could have reduced to some extent the integrity of the temperament measure. Secondly, infant temperament measurement relied solely on maternal perception, which could possibly be influenced by maternal characteristics, mood or their own temperament or behaviour. Mother-infant bonding and daily interactions could also possibly influence the maternal report. For example, working mothers who were likely to have less time with their infants might have reported the infant temperament less accurately. A positive association has been reported between infant temperament rated by two different parties in a study population in Oregon, USA, showing moderate agreement

between primary and secondary caregivers [226]. However, the results might be different in other populations that have different lifestyles or environments (e.g long working hours or short maternity leave, and therefore heavy dependency on other caregivers with less interaction with the infant). Therefore, better measurements are needed including parent-infant interactions to increase the understanding of infant temperament and its relationship with other factors such as growth and development during infancy.

Thirdly, this tool did not identify or provide an objective cut-off score for difficult or challenging temperament, thus high scores towards 7 (always) in negative affectivity were assumed to represent difficult temperament in this study. Having a cut-off point to determine an abnormal level of challenging temperament might also be useful to detect which mothers potentially need extra support in prolonging breastfeeding. However, this would require the different scores to be validated against a relevant outcome to derive the appropriate cut-off (function as a diagnostic tool), such as breastfeeding duration. Thus, this would be a relevant topic for future research on breastfeeding.

6.7.6. Infant appetite (BEBQ)

The Baby-Eating-Behaviour questionnaire (BEBQ) results showed that the study population infants were generally perceived to enjoy feeding sessions, to be responsive to feeding cues, and to have a large appetite. Although breastfed infants are generally assumed to have higher satiety responsiveness which is suggested as a possible mechanism for protective effects of breastfeeding against obesity [324], this was not shown in my study population. The score on the satiety scale was not high (close to the median value), although almost all infants in the present study population were exclusively breastfed. This is consistent with a study which reported that breastfed infants have higher food responsiveness and lower sensitivity to satiety than formula fed infants [223].

In my study, since breastfeeding had mostly been established by 2-3 weeks of lactation, and the infants were well-fed at the time the BEBQ was first administered, so mothers were more likely to relate the enjoyment of food with the infant's liking for milk. This is shown by the negative association between enjoyment of feeding and satiety responsiveness in the study. Infants that had a high food responsiveness score also tended to be rated by their mothers as having a large appetite.

There were no gender differences in any of the infant appetite traits, apart from at HV3 when female infants had higher slowness in eating score than male. However, the average score for all visits (HV1-3) for slowness in eating was not significantly different between genders. Findings on gender differences in infant appetite in early life are inconsistent, and the evidence is limited [223]. In contrast to the present study, Mallan et al., [299] found higher slowness in eating score in male infants at about 4 months of age. On the other hand, Llewellyn et al., [223] found that female infants had higher sensitivity to satiety cues and lower food responsiveness score than males (mean age = 8 months). Their finding was consistent with those in older children assessed using the Child Eating Behaviour Questionnaire (CEBQ) [325]. However, other studies found no significant differences in appetite between genders among older children [326, 327]. Since growth is critical during early infancy, gender would possibly be less likely to be associated with infant appetite during this period.

Infant appetite was stable across HV between 2 to 12 weeks of age, except for the 'slowness in eating' appetite trait, which reduced across time points. A study in Australia showed similar results: infant fed faster from 2 to 12 weeks, and only became more sensitive to satiety at a later age (5 months) [328]. This is most probably due to well-established breastfeeding or efficient feeding among older infants, hence they were able to feed more quickly. Other studies have also reported that infant appetite in early infancy or childhood is associated with appetite at later age, demonstrating the stability of appetitive traits over time [223, 324, 329]. This also suggests that mothers tend to give a consistent report about their infant's appetite across time. However, there is a possibility that the parents may have become familiar with (or adapted to) their infant's feeding behaviour and appetite in early infancy, and hence tend to report similar appetite traits across time. As mentioned previously, the adaptation to infant behaviour (in this case, infant appetite) could well be higher among first-time parents who are still learning about their infant's behaviour and have no other children for comparison. Moreover, once the infant has started complementary feeding, infant appetite should be measured differently, or using a different tool, since feeding behaviour and appetite could change. Some studies have used the CEBQ to compare the same scales of infant appetite that are measured using the BEBQ [223, 294, 330]. In fact, the BEBQ is an adapted version of the CEBQ, and thus the appetite scale is considered to be comparable from infancy to childhood [223].

Since infant appetite seemed to be stable over time during the study period, the average score for each appetite trait (except slowness in eating) was used to examine associations with infant growth. Overall, infant growth in the present study was positively associated with food responsiveness and inversely associated with the slowness in eating during infancy. This is consistent with many other studies [299, 328, 330, 331]. It suggests that infants in the present study who were more responsive to breastfeeding tended to have greater weight gain from 2 to 18 weeks (HV1-4), and thus were also heavier at later ages. In contrast, infants who fed slowly at 6-8 weeks of age had lower weight and BMI at later ages. These two associations were shown to persist up to 9-15 months in other cohort studies [330, 332]. The UK cohort studies found that appetite is partially heritable. Hence, higher food responsiveness and/or large appetite during infancy could promote rapid weight gain at later ages, and this could increase the risk of overweight or obesity especially in an obesogenic environment [332, 333]. However, the majority of the infants in that study were formula-fed, and it should be acknowledged that the perception of breastfeeding mothers about their infants' appetite and feeding behaviours could be different. As with the Rothbart questionnaire, the reliance on parental report is one of the main limitations of the BEBQ, as it is subject to parental interpretation of their infant's eating behaviour, and may also be associated with maternal characteristics or socio-demographic background. However, in my study, the reported slowness in eating was positively associated with longer breastfeeding duration during each home visit suggesting that the maternal report was confirmed by an objective measurement. Moreover, as indicated previously, since I am measuring the mother-infant signalling, relying on the maternal report could be useful in assessing the perception and responsive of the mother toward her infant. In addition, infants that were perceived as having irritable behaviour or personality (high negative affectivity trait) were also perceived by mothers as showing less enjoyment of feeding times. This could be considered to show consistency in maternal reporting since there could be a link between irritable behaviour and dissatisfaction in feeding, which is one of the main ways in which the infant communicates early in life.

6.7.7. Associations of infant appetite with milk hormones and infant growth

With regard to the relationship of appetite with breast milk hormones, studies have suggested that breast-fed infants may have better appetite regulation and self-control of feeding due to the presence of bioactive factors in breast milk, particularly hormones such as leptin and ghrelin [101-103]. Breastfeeding has also been linked with a protective effect against obesity in later life, via its influence on appetite regulation and energy balance in early life [132].

Although many studies have reported associations between milk hormones and infant growth [129], this relationship is still poorly understood. As described above, my study results have shown that higher foremilk leptin at 2 weeks was associated with the infant's slowness in eating scores at 2 and 6 weeks of age (HV1 and HV2). Both of these variables were also shown to be negatively associated with infant weight at 6 weeks, 12-14 and 14-18 weeks. This is consistent with previous findings reporting inverse associations between breast milk leptin and infant growth (weight gain, BMI and body composition) in early infancy [127-130, 334]. It has generally been assumed that this involved effects of leptin on appetite behaviour or satiety [103], although as yet, no studies have reported relationships between milk leptin and satiety. In my study, no association was found between leptin and satiety responsiveness, but slowness in eating can be considered to indicate better appetite control [223]. Hence, it is possible that favourable effects of leptin on regulation of food intake may due to effects on satiety, but also on other aspects of appetite such as the pace of feeding. Moreover, slowness in eating was also reported to be moderately associated with satiety responsiveness in the Gemini twin cohort study among infants aged 4-20 months (n=2402) [223].

Conversely, ghrelin has been assumed to stimulate appetite and response to hunger [104, 105] and in adults, ghrelin is associated with appetite. There was also an assumption that breast milk ghrelin stimulates infant appetite, and hence influences infant weight. However, my study found no relationship between milk ghrelin and food responsiveness or general appetite. There are no other studies in infants that have investigated this. In my study, general appetite was positively correlated with foremilk leptin at HV1 rather than ghrelin, but the results were inconsistent in HV2 (negative correlation). It is possible that the inconsistent associations between leptin and appetite could relate to the many methodological issues with milk hormone sample analysis (described earlier) and also the limitations of the BEBQ questionnaire.

In multivariate analyses, slowness in eating was consistently shown to be a strong negative independent predictor of infant weight and BMI, after adjusting for randomised group and milk hormones. This could reflect the fact that this appetite trait appears to have a heritable component as reported by the Gemini twin cohort study team [333, 335]; they found that the heritability was high for slowness in eating (84%), followed by satiety responsiveness (72%), food responsiveness (59%) and enjoyment of food (53%) [335]. Thus, they suggested that the influence of genetics on infant growth could be mediated through early feeding behaviour,

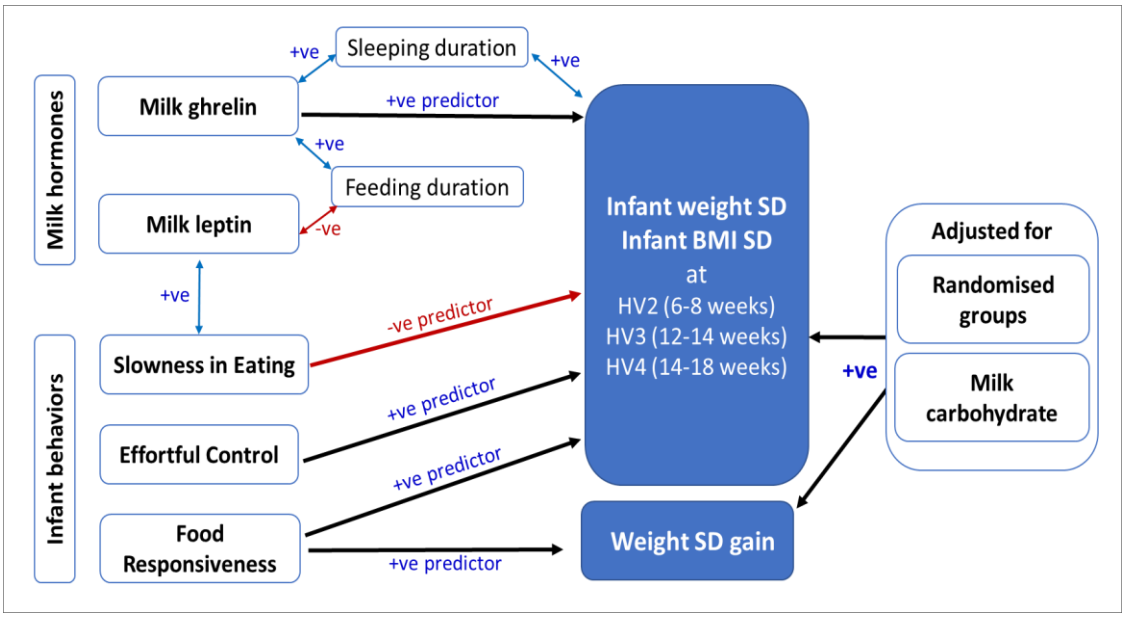
particularly speed or pace in eating and feeding rate. However, it is acknowledged that the majority of infants in the twin study are formula-fed, hence the infants were not likely to receive biological or hormonal signals from breast milk, which this could possibly associated with feeding behaviour. Nevertheless, consistent with my findings, a relationship between infant feeding style and growth during infancy was also reported in other studies demonstrated by positive associations between vigorous and faster suckling and infant adiposity [336, 337].

Overall, many studies have proposed that breast milk and infant plasma hormones, particularly leptin and ghrelin, influence infant appetite and growth during infancy, however, few studies have actually explored infant appetite. This is partly because there are a very limited number of available tools to objectively measure infant appetite. The BEBQ used in the study is an adapted version of the validated CEBQ but it has not itself been validated (indeed, this could be very difficult to do among young infants). Another limitation of the BEBQ is that there is no cut-off point for the scores to indicate the levels of each appetite trait. Thus, relationships with later outcomes such as overweight or obesity cannot be used for diagnostic purpose in the clinical setting. There is also no clear guidance on the interpretation of scores, particularly how or if scores for different appetite traits (e.g 'food responsiveness' and 'enjoyment of food') should be combined in order to determine whether the infant has low or high, good or bad appetite. Instead of describing the appetite behaviour for each trait, many studies use the BEBQ or CEBQ to ascertain associations between appetite traits and other factors, mainly growth. The BEBQ only asks a single question to determine the general appetite level for the infant, by asking the mother to rate the frequency of the infant of having a big appetite: the score ranges from 'Never' to 'Always' for the statement 'My baby has a big appetite'. The statement by itself could be considered biased, as it asks the mother to focus on a big appetite, rather than rating her baby's overall level of appetite. However, since appetite traits are generally expected to be consistent over time and also highly heritable, repeated measurements using the BEBQ at different ages would allow the investigator to assess consistency of maternal responses. This might help to identify parental manipulation. Thus, in my study, infant appetite was assessed at HV1-3 and scores for most appetite traits were considered to be stable.

6.7.8. Associations between infant behaviours, milk composition and infant growth outcomes; and consideration of these factors as potential mediators of the effects of the intervention

Although some studies have reported on the relationships between milk hormones and infant growth, the underlying mechanisms behind such associations were not explored. This is especially important considering there are so many inter-correlations between mother-infant factors that are involved in breastfeeding, breast milk and infant growth. Previous studies have generally investigated milk hormones and infant behaviour separately, in order to examine correlations with growth. One novel part of my study was exploring the relative associations of these different factors with infant growth outcomes in multivariate models, and attempting to investigate whether they might mediate the observed effect of the intervention. Milk carbohydrate was included in the combined models because it was consistently and significantly associated with infant weight and BMI at different time points, and was also shown to be significantly higher in the intervention group. Figure 6.3 provides a summary of the overall results for the combined models to show the associations of milk hormones and infant behaviours (as predictors) with infant growth measurements.

Figure 6.3 Overall associations between milk hormones and infant behaviours, and infant growth



In the final regression models, slowness in eating was the only significant negative predictor for infant weight and BMI, whilst other variables, namely milk ghrelin (especially the changes of ghrelin within a feed), effortful control and food responsiveness were positive predictors for infant growth measurements. These effects were all independent of the intervention and milk carbohydrate. However, milk leptin was no longer a significant predictor of infant weight in the multivariate analyses after adjusting for milk ghrelin, infant behaviour traits and/or randomised group. Interestingly, if the randomised group variable was not included in the analyses for the final combined models, milk carbohydrate was consistently shown to be a significant positive predictor of infant growth at almost all HV points. This is consistent with the univariate analyses showing that milk carbohydrate (either in fore- or hindmilk at HV2 or pooled data) was consistently associated with infant weight and BMI at HV2 to HV4. Moreover, in model 1 (Table 6.12), randomised group was no longer a significant predictor, but milk carbohydrate was shown to be positively associated with an increase in infant weight at HV2. However, in all other models, the randomised group variable was significantly associated with higher infant growth, whilst milk carbohydrate was excluded from the final regression models. These results suggest that the intervention effect on infant growth could be mediated by milk carbohydrate. Consistent with the present study, a recent study also reported that milk carbohydrate measured at 4-8 weeks of lactation was significantly associated with later infant weight and adiposity at 12 months, and weight and adiposity gains from 3 to 12 months of age [338]. They also found that hind milk fat was negatively associated with later infant weight and adiposity, whereas hind milk protein was positively associated with BMI [338]. However, I did not find significant associations between milk fat or protein and infant weight, BMI or weight gain, which could possibly be due to the sample size in my study (n=64) compared to the previous study (n=614) [338].

It is not possible from my results to determine which component of carbohydrate might be responsible for the observed association with infant growth since the method used (MIRIS analyser) measures total carbohydrate which includes both lactose and oligosaccharide. A recent study reported significant positive associations between human milk oligosaccharides (HMO) and infant growth and body composition [339]. They suggested that HMO could potentially improve breast milk nutrient absorption by the epithelial cell in the infant's digestive tract, or alternatively HMO could also affect neural development and influence infant appetite or feeding regulation [339]. However, more evidence is needed to understand the function of HMO and the mechanisms underlying these associations. On the other hand,

it is also possible that the lactose component of milk carbohydrate could influence infant growth, or that carbohydrate is a proxy for another factor which could mediate the effect of the intervention on infant growth. Lactose may influence liquid influx into breast milk, and this could have resulted in a lower fat percentage in breast milk, so that infants would possibly end up feeling less satiated during breastfeeding [338]. Subsequently, this could have caused infants to be fed a larger milk volume within a feed or to have more frequent feeding sessions within a day, with higher energy and nutrient intake throughout the day or over the lactation period. Therefore, it would be intriguing in future studies to focus on the relationships between milk composition, particularly milk carbohydrate, and ghrelin and infant growth.

In terms of the relationships between milk hormones and infant behaviours, there is a possibility that infant behaviour, particularly feeding and sleeping duration could be mediators for the effect of milk leptin and/or ghrelin on infant growth. To my knowledge, this study is the first to report associations of breast milk ghrelin with infant sleeping duration, consistent with the suggested role of ghrelin as a sleep-promoting factor in adults. However, infant sleeping and awake behaviour were not included in the regression models because of the reduced sample size due to the smaller number of mothers who completed the diary. It is possible that milk leptin could also influence infant feeding behaviour, through an increase in slowness in eating and shorter feeding duration, with subsequent effects on infant growth. However, this mechanism was not supported by my analyses since milk leptin was no longer a significant negative predictor of infant growth in the multivariate models. On the other hand, infant temperament and appetite traits were shown to be independent and consistent predictors of infant growth measurements. As indicated earlier, these traits are likely to be heritable, and hence their effects on infant growth may not be greatly influenced or modified by external factors. Thus, it is suggested for future studies to broaden research on the relationships between infant behaviour and milk hormones, particularly sleeping behaviour and milk ghrelin.

6.7.9. Strengths and limitations

6.7.9.1. Novelty of the research

To my knowledge, my study was the first to investigate the relationship of breast milk hormones and infant behaviours, and the consequences for infant growth. As indicated earlier, one of the strengths of this study was the novelty in linking milk hormones and infant behaviour in predicting infant growth during early infancy. Although mother-infant factors are inter-correlated, this study was to some extent able to summarise the link between factors to show the direction of these correlations in relation to infant growth. In addition to that, the finding of a relationship between milk ghrelin and sleeping and feeding behaviour could potentially broaden future research on this topic.

6.7.9.2. Prospective data

Although this chapter presents observational analyses of data from the MOM Study population, another strength is that all data were collected prospectively. All infants were followed up from birth up to 14-18 weeks. This is important considering that the infant appetite and behaviour data depend on parental report. There is a high risk of parental manipulation in studies using retrospective data to assess infant appetite and behaviour due to difficulty in recall or bias towards the infant's current behaviour. There is also a high possibility of reverse causality in studies examining associations between appetite traits and infant growth using retrospective data [294]. The use of prospective data may also provide greater confidence when determining the direction of the relationship between appetite or infant behaviour and growth.

6.7.9.3. Study limitations

There are several limitations of this study with regard to the secondary outcome results. Firstly, as indicated in the previous chapter, the sample size was small (n=64), hence reducing the power to detect significant effects. Secondly, the duration of the follow-up was short, only 14-18 weeks. This was largely due to the need to complete the study within the permitted time-frame for a PhD. Many previous studies followed infants up to at least 6 months to study the association between infant appetite and behaviour in early life and later growth. Early feeding behaviour may well influence later growth beyond the age of 3-4 months and later follow-up is important to understand the early programming of infant nutrition on health

outcomes in later life. Thirdly, in addition to small sample size, I was unable to standardise the timing of milk sample collection for all home visits and this might have contributed to the high variability in the milk hormone results. In addition, although the majority of HV were still performed in the morning, a few mothers reported that they did not have breakfast prior to the visit even though they had previously been advised to have a proper meal before the session. This may have influenced leptin and/or ghrelin levels in maternal plasma, and therefore possibly in breast milk since these hormones may be affected by food intake [103, 108]. Finally, assay kits that were used for breast milk leptin and ghrelin analysis were not designed for human milk samples, but for human plasma. All of these factors may have contributed to the variability of the data, especially the breast milk leptin results. This could have reduced the statistical power to identify or ascertain stronger correlation results between variables.

6.7.9.4. Statistical aspects

Since the present findings were based on observational analyses, there are a few statistical issues that need to be highlighted. Firstly, the sample size is small, but there are a large number of predictor and outcome variables which were expected to be inter-related in a complex way. Despite attempts to focus the analyses and use summary variables where possible, multiple testing was performed to investigate these relationships which would increase the probability of rejecting the true null hypothesis, or increase the chance of producing a false-positive result. As explained earlier in the chapter, I chose not to use summary variables for outcomes such as growth in order to assess the consistency of the results and to increase confidence in the overall reliability of the findings. However, I acknowledge that p-values were not adjusted for multiple testing since most variables were assumed to be independent of each other. Secondly, I used summary variables for measures which seemed to be well correlated over time and which are generally reported to be stable during early infancy (e.g. appetite traits). This is beneficial in terms of reducing the number of statistical comparisons performed, but may also have reduce the ability to detect genuine associations related to a measurement at a single time-point. To be cautious, I performed separate analyses for associations at individual time-points to assess for consistency of the summary results (data not presented) and found no contradictory results. Finally, there were a few variables that were measured at later time points of the study (e.g. temperament traits at HV4) but which were used to 'predict' infant outcome at an earlier time point (HV2-HV3). Logically, a predictor should be measured prior to the outcome. However, since temperament

is reported to be relatively stable from birth to later age during infancy, and also partially heritable, [317, 318, 340], the results for temperament measured at 3 months were assumed to be similar to what they would have been from birth.

The main aim of this study was to explore associations between mother-infant factors in early life and infant growth using observational analyses. Thus, the results cannot be considered to show causality but can show associations between factors and help to identify possible pathways or mechanisms for further investigation. Although there were several statistical and methodological limitations related to the present findings, the study also has a number of strengths, and the overall findings provide some insights and suggestions for future research on this topic.

6.8. Conclusion

Early life is a sensitive critical window for infant programming that may affect later growth and health outcomes. Therefore, further understanding of the relationships between infant behaviour (appetite and temperament) and milk composition and later growth warrant further investigation. Overall, the study findings supported my observational cohort hypotheses. Infant temperament, appetite and breast milk composition were found to influence infant growth, although the associations did not differ by gender, whilst milk ghrelin and leptin were found to influence infant appetite and behaviour and hence infant growth.

The findings of this study might not be generalisable to the whole Malaysian population, even breastfeeding mothers, since the study population involves predominantly Malay mothers with higher education level. This is most likely due to unintentional selection bias during recruitment since the study only involved exclusively breastfeeding mothers that were willing to cooperate and give their time to participate in the study. In addition to leptin and ghrelin, there are still many other bioactive factors including opiates in breast milk that may also influence infant behaviour. These bioactive factors may consequently affect infant growth and/or later health outcomes [80, 97]. Thus, further research with a larger sample size is needed to explore breast milk leptin and ghrelin, and also other biomarkers that may influence infant outcomes. Improvement in assay kits specialised for breast milk hormones (especially ghrelin) is also recommended for future studies. A larger study should also include follow up of the study population for other health outcomes.

CHAPTER 7

7. GENERAL DISCUSSION & CONCLUSION

7.1. Introduction

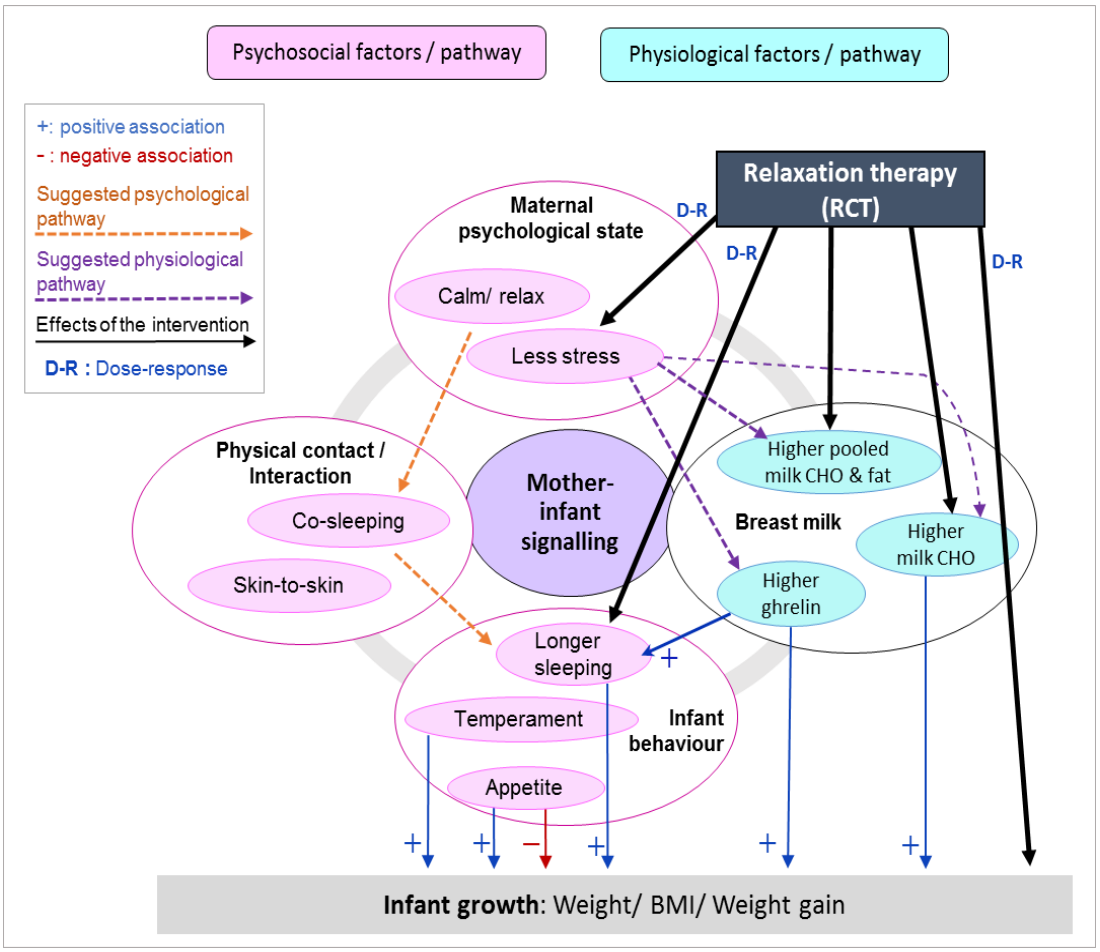
In this chapter, I combine the findings of both main results chapters to produce an overview of possible mother-infant signalling pathways or mechanisms that influence infant growth. I also discuss the anthropological perspective of the study findings, including the possible trade-offs and tug-of-war mechanisms that occur during lactation. Next, I describe the strengths of the study and its contribution to public health and anthropological theory. I also describe the general limitations of the study and suggest future directions for infant feeding research building on the study findings. Finally, I provide a conclusion for my research project.

In previous chapters I have discussed my findings in the context of previous studies. Although for some of the outcomes there were a number of previous publications, as indicated in the systematic review in Chapter 2, none of the previous studies that used relaxation therapy as an intervention during breastfeeding investigated the effects on infant outcomes. In term of maternal outcomes, only two studies included effects on breast milk volume and milk fat levels [169, 170]. There are also very few studies investigating the changing composition of breast milk within a feed and throughout lactation and fewer exploring the signalling between the mother and infant during breastfeeding. Hence, my ability to compare the findings from the RCT directly with existing data was somewhat limited. The suggested mechanisms or potential pathways of mother-infant signalling presented in this chapter take into account both published data and my own findings.

7.2. Summary of the findings – biological perspective

Supporting the primary hypothesis, the study findings reported in Chapter 5 have shown that the relaxation therapy was effective in reducing maternal stress during breastfeeding, favourably affecting breast milk composition and positively influencing infant behaviour and growth. The study findings of the whole population (Chapter 6) also supported the secondary hypotheses: infant temperament, appetite and breast milk composition were found to be associated with infant growth, although the associations did not differ by gender, whilst milk ghrelin and leptin were found to be associated with infant appetite and behaviour and hence infant growth. Figure 7.1 provides an overview of my findings – including the observed effects of the intervention and the observed associations from the observational cohort analyses - in the context of the findings from previous studies (discussed in Chapters 5 and 6) and proposed biological pathways of mother-infant signalling. The figure is an adapted version of the figure presented in Chapter 2 which summarised possible mother-infant signalling factors during lactation.

Figure 7.1 Overview of the observed effects of the intervention and the observed associations from the observational cohort analyses, including the suggested potential pathways



In summary, Figure 7.1 shows the effectiveness of the intervention in reducing maternal stress and the consequent effects on breast milk composition and infant sleeping behaviour, and also on growth. Infant temperament and appetite behaviours have independent effects on infant growth and were not affected by the intervention, which is most likely because these traits are heritable and largely stable. Overall, the influence of mother-infant factors on infant growth can be described (as I proposed in Chapter 6) by the suggested physiological and psychological signalling pathways.

My findings are consistent with two potential mechanisms for the physiological signalling pathway. Firstly, mothers who are less stressed or more relaxed may produce breast milk with higher carbohydrate and also higher pooled milk energy, leading to greater infant energy intake. This could occur as a result of more efficient milk ejection resulting in higher milk intake and/or higher intake of hind milk. Alternatively, more relaxed mothers might produce milk with different concentrations of bioactive factors as seen in my study for milk ghrelin, which might influence infant sleeping (or other) behaviours. The second, psychological signalling pathway, would operate via improved mother-infant interaction or bonding in more relaxed mothers, perhaps stimulating better sleeping quality in the mother-infant dyad; this pathway was not tested in my study. Both pathways could result in increased infant growth via increased nutrient intake and/or reduced energy expenditure as a result of a longer sleeping duration in the infant. This would be expected to be especially important during the first four to six months of infancy life when energy requirements for growth are greatest [185].

The figure is an attempt to provide an overview of my findings in the context of the findings from previous studies and proposed biological pathways and mechanisms of mother-infant signalling. However, it is important to consider the strength of the evidence from the different parts of my study with regard to causality. I can be more confident about a causal effect of the intervention in reducing maternal stress and increasing sleeping duration and infant weight and BMI since these data come from an experiment (RCT), with further support from the observed dose-response effects (presented in Chapter 6). However, the results from the secondary observational analyses can only demonstrate associations between variables, and thus should be interpreted with more caution. Moreover, it is also important to acknowledge that the pathways might work differently in other populations, due to human behavioural diversity [57]. For example, the environmental stress of a population might be different than

in the Malaysian population who took part in my study. This is especially true since the majority mothers in Malaysia practice the traditional confinement period which could reduce the potential for stress and anxiety. Thus, differences in culture or tradition, ecology or behavioural characteristics would be expected to contribute to the behavioural diversity within and/or between populations [57].

7.3. Summary of the findings – anthropological perspective

As described in Chapter 2, Trivers' theory predicts that the offspring is selected to demand more resources than the mother is selected to provide, and this mostly occurs unconsciously during the lactation period [62]. This conflict starts as early as fetal life, and the tension is expected to increase during postnatal life – specifically the period of parental care [62]. However, it is less clear whether the mother or the infant is more 'in control' in the tug-of-war during lactation. Based on my study findings, there are several potential mechanisms for the tug-of-war that may influence the maternal investment strategy during lactation. This is discussed further in 7.3.1.

Trivers also hypothesised that there is a potential for parental bias towards male offspring if environmental conditions are less stressful [206]. This is supported by the finding of previous studies on higher milk energy density among mothers of sons in humans [203] and in non-human mammals [200, 201, 341] and also higher milk intake among human male infants [191]. However, in less favourable condition, findings of animal studies have suggested that mothers might either invest more in daughters due to their faster maturation rate, enabling the next generation to start reproducing earlier [199, 342]. Differential investment between genders might be due to different biological factors, for example, different lean mass or energy requirements during infancy. My study did not find any differences between genders in breast milk composition, whether macronutrient content, total energy or milk hormones. This is consistent with some other human studies [203-205] that reported no significant difference in milk intake and/or composition between genders. These findings, however, could have been affected by several factors such as unstandardised methods of milk sampling or other confounding factors (e.g. parity and maternal body composition). I would conclude that the present study did not support the Trivers' theory regarding bias or a differing strategy of maternal investment in terms of milk composition in male and female infants during the lactation period. Nevertheless, I acknowledge that my sample size is small; specifically, the

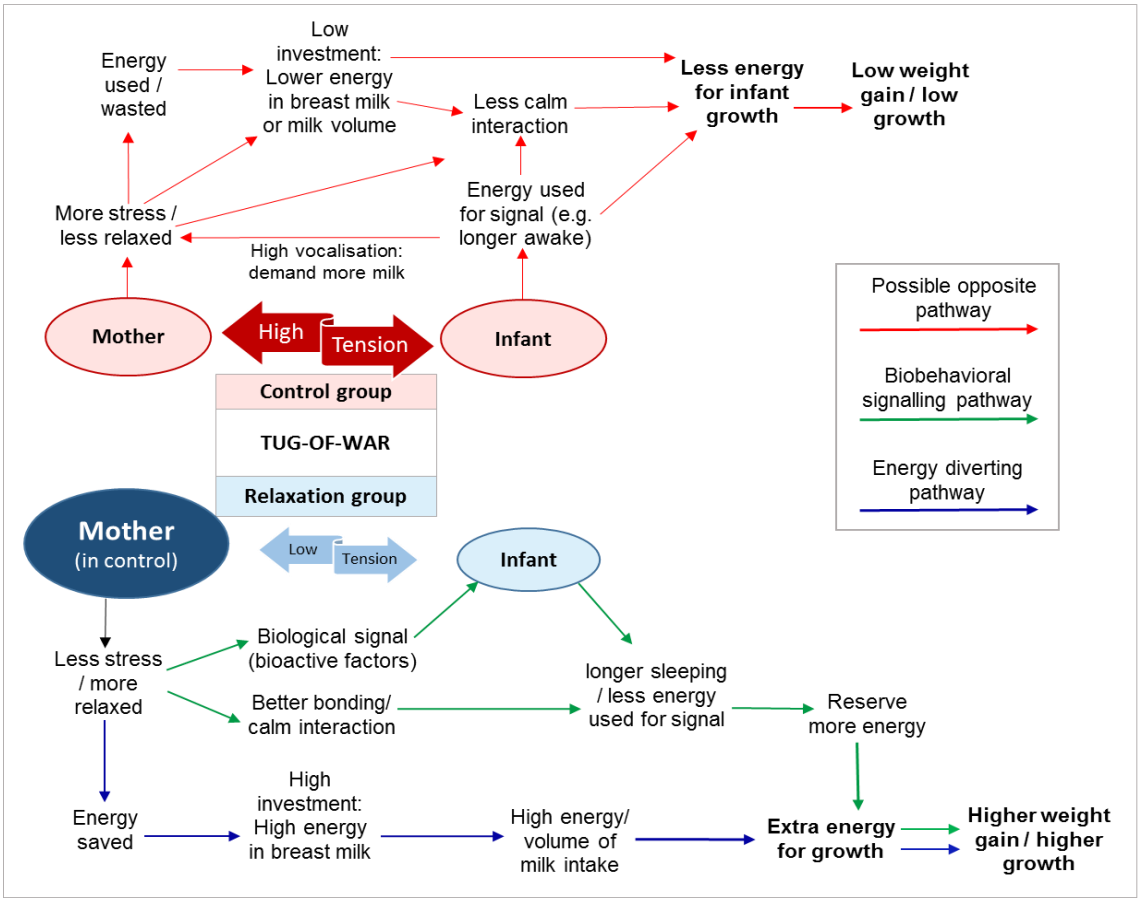
study was not powered for sub-group analyses and hence had low statistical power to detect differences in milk composition between genders.

7.3.1. Maternal investment strategy

Maternal energy capital might be more critical during early postnatal life which is a highly energetically demanding period for the mother as she recovers from delivery and at the same time needs to invest in her offspring, especially through breast milk [52, 343]. Since lactation is costly, postpartum distress may raise tension in the tug-of-war by affecting the maternal energy budget (as shown by the possible opposite pathway in Figure 7.2). As previously indicated in Chapter 2, psychological distress increases energy expenditure [175-179], so chronic stress may reduce energy allocation to breast milk. Hence, people with major depression use excess energy and need to trade-off resources between maintenance or immune function over growth and reproduction, depending on the type of depression [175, 176, 180]. Furthermore, emotional distress in mothers has been shown to inhibit the let-down reflex, leading to disruption of milk flow and reduced milk volume [163, 167, 168]. Thus, by having low milk energy or volume, this could also lead to high vocalisation or demand by the infants [64, 65]. My study findings show that manipulating maternal psychological state by giving relaxation therapy is effective in reducing maternal stress and possibly making the mothers more relaxed. Since stress uses energy, the relaxation therapy might have indirectly manipulated or affected the maternal energy budget during the lactation period. This could, in turn, have reduced the tension of the tug-of-war via several suggested mechanisms, as also illustrated in Figure 7.2.

Figure 7.2 provides an overview of the possible mechanisms for the tug-of-war between the mother and the infant during lactation. Here, I propose two possible pathways for the maternal investment strategy that could be affected by the intervention, which are: i) the 'Energy diverting pathway' and ii) the 'Biobehavioural signalling pathway'. The first pathway suggests that the maternal energy budget that was previously being used for stress during the postpartum period could be diverted and invested in breast milk. Hence a trade-off in energy occurs between maternal stress and breast milk energy. Based on my results, infants might receive the energy in two possible ways: i) higher carbohydrate content in breast milk which may result in ingestion of a higher milk volume; or ii) the intake of higher pooled carbohydrate and fat content in breast milk over a long period of lactation. Either way, infants in the intervention group could have received higher energy from breast milk via these mechanisms than those in the control group.

Figure 7.2 Suggested mechanisms and hypothetical pathways of the tug-of-war during lactation



Next, the biobehavioral signalling pathway suggests that the mother could also signal the infant to manipulate his sleeping behaviour so that he can reserve more energy for growth. For example, in the case of my study, it is possible that milk ghrelin was transferred to signal the infant to sleep longer, based on the positive association between ghrelin and sleeping duration. This concept is also supported by the higher foremilk ghrelin and longer sleeping duration found in the intervention group. There is also a possibility that the behaviour of the intervention group mothers who were less stressed or calmer, and perhaps experiencing better sleep quality, could have influenced infant sleeping behaviour. Therefore, infant energy that would have been used during awake periods could have been saved for growth by having longer sleeping duration. All of these mechanisms could have resulted in a trade-off between energy used during awake periods and growth via longer sleeping duration.

Previous studies have also suggested that mothers could send breast milk cortisol as a signal to the infant about their current environmental stress. As previously indicated in Chapter 3, it was hypothesised that the intervention tool would reduce milk cortisol and influence infant crying behaviour or temperament. However, this was not clearly demonstrated in my study

since there was no significant difference in crying duration and temperament between randomised groups, and the results of milk and saliva cortisol were inconsistent. There was also no significant association of milk cortisol with any infant behaviour (e.g. distress duration) or growth. Nevertheless, it would be intriguing to further explore the potential effects of bioactive factors in breast milk on infant growth and development.

Overall, my findings are consistent with these mechanisms, which propose that the mother may be the 'driver' of the tug-of-war during lactation. Thus, it is suggested that the mother is more in 'control' in provisioning energy for the infant via i) higher energy in breast milk or greater milk volume; and/or ii) manipulating the infant by sending behavioural or biological signal(s) in order for the infant to reserve more energy for growth. However, it is also possible that in my study, the mother may not necessarily be the one who is solely responsible in provisioning energy, or the 'driver' of the tug-of-war, since the findings could reflect the fact that it was the mother who was manipulated in the trial. It is possible that, if the infant had been manipulated, both mother and infant could have played important roles in provisioning energy within the environment constraints, generating parent-infant coadaptation. This has been demonstrated in previous studies, [64, 65, 344, 345], for example, where the baby plays a role in increasing milk volume by intense suckling, even if the mother is malnourished [64, 65], which could be important for infant survival and reproductive success. Nevertheless, by manipulating maternal psychological state, the mother-infant tug-of-war of the present study population was apparently pushed toward a positive energy balance, resulting in greater energy investment in milk production and higher infant weight gain in early life. In the long term, this could also help in improving BF success.

On the other hand, it is also interesting to consider the reverse situation, as suggested by the hypothetical opposite pathway in Figure 7.2. For example, if the mother is stressed, she might send signals to the infant that the environment is risky and he should sleep less or be more awake, so there would be less investment in growth (not to invest predominantly in growing as long as the survival could be maintained). Greater stress would also inhibit oxytocin production and reduce the flow of milk, especially hind-milk. Trade-offs like these are likely to occur depending on the life history strategy [16, 202]. This would be influenced by the hierarchy of priorities within the environmental constraint during early infancy life: survival, growth, and behavioural activity [202]. A previous study demonstrated a trade-off between growth and maintenance among baboon infants when maternal milk synthesis was

experimentally reduced via extreme maternal food restraint; the affected baboon infants suspended their growth during that stressful phase [345]. On the other hand, a trade-off between growth and behavioural activity was shown by a study demonstrating the role of milk cortisol in affecting infant activity and temperament in early life [202].

Applying evolutionary concepts in humans would increase the understanding of how the environment might affect lactating women and the development of the offspring. This is important since some environmental factors (e.g. stress) are modifiable and/or treatable (e.g. relaxation therapy), and hence breastfeeding rates or duration could be improved.

7.4. General strengths of the study

Overall, my study had a number of strengths; many of these have been mentioned previously but I provide an overview here:

- The use of a randomised study design allowed me to investigate causal effects of the relaxation therapy on maternal stress, breast milk composition and infant behaviour and growth.
- The follow-up rate during the study was good, and most mothers were fully compliant with the tasks given, except for the 3-day diary recording. The intervention tool is simple and cost-effective, which possibly made it easy for the mothers to comply and most of them found it useful.
- Novelty of the research topic: this was the first study to test the effectiveness of the relaxation therapy on both mother and infant outcomes, as previous studies generally studied these outcomes separately. It was also the first to combine both biological and anthropological perspectives.
- Non-randomised analyses using data from the whole study population was performed to study the direction or trends of associations between mother-infant factors and associations with infant growth. This enabled previously unexpected findings to emerge, such as the relation of higher milk ghrelin with longer infant sleeping duration, and the relation of higher milk carbohydrate with increased infant growth; which has led to new hypotheses.

- The combination of both primary and secondary outcomes for mother-infant factors during lactation enabled me to investigate the trends or direction of associations among complex inter-related factors that influence early infant growth. Thus, this study helped to link some of the many small pieces (mother-infant factors) into one big picture portraying the potential mechanisms or pathways that influence infant outcomes during early infant life.
- The study also considered the anthropological perspective of mother and infant signalling during the lactation period, by manipulating the maternal energy budget and investigating the effects on maternal investment (in breast milk) and, in the infant, by testing the parent-offspring conflict hypothesis that emerged from Trivers' theory.

7.5. Contribution of the study findings

7.5.1. Clinical and public health

Despite national and international initiatives designed to support BF, either at the individual or population level, as presented in Chapter 2, global BF rates, including those in Malaysia, are still low and below target levels. Most attempts to improve BF rates focus on providing additional support, but many aspects of the BF process are poorly understood. This study provides a better understanding of mother-infant factors during the lactation period, including the suggestion of potential mechanisms of biological and behavioural signalling that influence infant growth. Overall, the study attempted to summarise these complex mother-infant factors and provide insights which might be helpful in developing evidence-based interventions, programs or policies to support and promote breastfeeding.

My findings highlight the importance of minimising maternal stress, since the experimental relaxation intervention influenced infant behaviour, breast milk composition, and subsequently infant growth. This simple and cost-effective tool could be used in for all breastfeeding mothers, in order to reduce their stress levels. However, further research is required to confirm the findings, and future studies could consider revising the content of the therapy so that it could be used for all mothers during the postpartum period, regardless of breastfeeding status, with the aim of improving maternal psychological state in general. Although my study was not long enough to assess breastfeeding duration and exclusivity, the relaxation therapy might have the potential to help mothers achieve their breastfeeding goals at an individual level. It could also help to improve overall breastfeeding rates and exclusivity

at the population level. Thus, health organisations or bodies could consider using this simple tool to support breastfeeding mothers.

Since the relaxation group in the present study showed higher weight and BMI, greater weight gain, and a non-significant trend towards higher fat-free mass, it would be worth testing the therapy in clinical settings, for example, in mothers of preterm, low birth weight or growth challenged infants. The fact that the intervention was able to show an impact on infant growth even in healthy mother-infant dyads suggests that its use in settings where mothers are more stressed could have greater impact.

The study findings also provide new understanding on the influence of milk hormones on non-feeding behaviour, such as sleeping and awake duration. To my knowledge, it is the first study to show the potential of breast milk ghrelin in shaping infant behaviour during early infancy, and thus influencing infant growth. In addition, the suggested mother-infant signalling mechanisms or pathways based on my findings provide new directions for exploring maternal effects on infant behavioural plasticity and possibly on nutritional programming. The study has contributed to filling a research gap by combining both biological, psychological and anthropological aspects of mother-infant signalling during breastfeeding. In fact, it may also be the first to translate the biological perspectives of infant feeding to the anthropological perspectives, using a robust methodological study design.

7.5.2. Anthropological theory and research

Life history theory is the most important way that anthropologists can achieve an evolutionary perspective on the biology of living humans [48]. However, most life-history research has been done in animals and most of it is observational [346]. It is difficult using this approach to define cause and effect due to the complexity of the inter-relationships between many factors during lactation, as shown in the previous diagrams. In particular, trade-offs which are the most important insight of life history theory are difficult to detect in humans [346]. In terms of parent-offspring conflict, many factors will determine the energy budget available to a mother to invest for current or future offspring and this also varies between mothers [52, 56]. To my knowledge, previous life-history studies on human biology are all observational, and therefore only predictions can be made [192-194]. As previously indicated in Chapter 2, although anthropologists have occasionally used experimental approaches, this has generally been in the field of psychology [195], but limited in physiology.

My contribution to anthropological theory is therefore building on these previous studies by using an experimental design in humans. My project was the first human study to experimentally test Trivers' parent-offspring conflict theory [62]. I believe this makes my research ground-breaking, investigating the tug-of-war between mother and offspring during this critical period of human life. The most novel feature of my study is that I manipulated the maternal energy budget and then investigated the knock-on effects in the infant, testing several hypotheses that emerge from Trivers' theory. My experimental approach allowed me to maintain other factors constant while altering just the mother's psychological state during the lactation period. Thus, I was able to investigate the effect on both mother and infant, independent of other factors and was able to identify that the mother was more likely to be in control in the tug-of-war. However, I acknowledge that the results could not necessarily be generalised to other populations due to selection bias during recruitment.

The period during which infants show plasticity to nutrition coincides with the time they are nourished through maternal physiology [347]. This makes infants very sensitive to 'maternal capital', which may be expressed in social or somatic currencies [348]. As studies have shown that breastfeeding mothers rate their babies as more 'difficult' in temperament than do formula-feeding mothers [73], the 'tug-of-war' underlying lactation is one likely reason for this difference. Therefore, understanding who controls provisioning in the evolutionary conflict between the mother and infant is a key to understanding how parent-offspring coadaptation evolved in humans [344]. Using an evolutionary approach to study infant feeding can also broaden the understanding of parental effects on infant behavioural phenotype and growth [8, 9, 63]. My project also contributes to knowledge on the natural variation in milk composition that shapes infant development, giving new information about the trade-offs that occur between mothers and infants.

7.6. Limitations of the study

Many of the study limitations have been discussed at relevant points throughout the thesis, but here I provide an overview of the most important points:

- Statistical limitations: The study had a small sample size, which limited the ability to detect differences between randomised groups. This is especially the case for the breast milk hormones since the data were highly variable. The small sample size also limited my ability to perform structured equation modelling (SEM) for path analysis in order to further study the potential pathways and mechanisms that were discussed earlier. The required sample size, in order to perform SEM, is generally suggested to be more than 100 [349].
- Standardisation of milk sampling protocol: Due to manpower constraints, since the data collection was performed by one researcher (myself) during the limited time-frame of PhD, it was not possible to completely standardise the timing of data collection for milk sampling, so many HV2 were performed in the afternoon, instead of the intended morning time. This could have contributed to the high variability in the milk hormone results.
- It was not possible to blind the subjects or researcher to the intervention, apart from the milk hormone analyses which were performed by a lab with no access to information on the randomised group. Table 7.1 summarises the potential bias which could have been introduced by the non-blinded study design considering the different outcome measures, and how they could have affected the results. Precautions that were taken to reduce the possibility of bias are also included. Overall, the highest potential for bias was in the breast milk sampling process; it is possible that mothers could manipulate this to provide more or less milk, and I could also have sub-consciously altered the duration of the feed which could have influenced the amount of fat-rich hind-milk. Some studies have standardised the nursing time to a specific duration (e.g. 10-15 minutes) in order to collect hind milk samples. However it would be unphysiological to standardise the feeding time especially in early infancy, since the duration of nursing bouts naturally varies between infants and with the time of day.
- It would have been preferable to blind the statistical analyses. In practice, I was unable to do this because I needed to track and check original data from the folders from time to time, when there was a query, and this would have been difficult if the original identifiers had been removed from the database.

- Isotope samples: Due to methodological issues described in Chapter 5, not all results were suitable for inclusion in the analysis to calculate the infant total body water. Furthermore, due to time constraints and the availability of the mass spectrometer, it has not been possible to analyse the milk intake isotope data yet.
- Due to limited financial resources, the milk hormone samples collected at HV3 were not analysed yet.
- It was only possible to follow infants up to 4 months of age due to the time restrictions for a PhD. Thus, the sustainability of the observed effects beyond the study period, especially for growth outcomes, could not be investigated.
- Selection bias during recruitment: The study population were predominantly educated, Malay women. Therefore, the results cannot necessarily be generalised to the whole multi-racial community of the Malaysian population, or to other populations.

Table 7.1 Potential bias due to the non-blinded study design and strategies taken to limit bias

Main outcome		Potential bias of not blinding	Strategy to limit bias	Research tools
1	Maternal psychological state	Mothers: Probability to answer the questionnaires based on the perception that the relaxation therapy has made her less stressed, especially if the researcher is around. Researcher: Possibility to give extra BF support to the intervention group mothers so that they would perform better in BF or have reduced risk of BF problems.	Mothers: They were not asked to answer the questionnaire during HV when the researcher was present, but any time after the HV at their convenience. Researcher: All mothers were given standard guidelines and information on BF and infant care during the HV. They were given a list contacts of BF peer counsellors if they need any help with BF.	Questionnaire ▪ PSS ▪ BAI ▪ EPDS
2	Breast milk hormones	Researcher: Bias during the lab analysis	Researcher: Blind analysis – lab analysis was performed by a collaborator who did not have access to information on the study population	ELISA Kits
3	Breast milk macronutrients (fore and hind milk sampling)	Mothers: the mothers in the intervention group might possibly wait to unlatch the baby later when she finishes listening to the recording, hence resulting in more hind milk in the milk sample. Researcher: there is a possibility that the researcher might unconsciously ask the mothers in the control group to unlatch the baby earlier, and hence resulting in less hind milk in the milk sample.	Mothers: Told them that they can unlatch the baby whenever they wanted to, for example if the baby stopped suckling and fell asleep during BF. Researcher: Waited until the baby unlatched him/herself during the breastfeeding session.	MIRIS milk analyser

Main outcome / factor		Potential bias of not blinding	Strategy to limit bias	Research tools
4	Infant behaviour	Mothers: Intervention group mothers might be more observant and conscious of their infant's behaviour than the control group Researcher: Bias during data handling – since calculation of each behaviour duration requires measuring the shaded boxes length.	Mothers: All mothers were sent gentle reminders to complete the tasks given post-HV, this including to record the infant behaviour on the diary. Researcher: Data handling and entry was performed by a person who was not related to other parts of the research project.	3-day diary
5	Infant growth	Researcher: Bias during measurement, especially during height and head circumference measurement since the researcher could have rounded the measurement values differently for intervention and control groups.	Researcher: The researcher followed standard procedures for measurements.	Weight, length and head circumference
6	Statistical analysis	Researcher: Bias in performing the statistical analysis	Researcher: Recoding of IDs and blinding of the randomised groups should ideally have been done, but was not possible with the available personnel (see text).	SPSS or Excel

7.7. Future research directions

7.7.1. Biological research

Based on the findings of my study, I would like to propose suggestions for future research:

i. A larger RCT testing the usage of the relaxation therapy among different populations, especially those where breastfeeding is challenging but likely to have greater health benefits:
   - Mothers of preterm or low-birth-weight infants
   - Breastfeeding mothers of growth challenged infants
   - Breastfeeding mothers in other populations than Malaysia (need to consider changing the content and language of the guided-imagery therapy recording)
   - Different groups of mothers: multiparous, teenage mothers or multiple pregnancies (twin)

   Future trials should consider a follow-up to examine the long-term effects of the relaxation therapy on infant outcomes (behaviours and growth) and BF duration and exclusivity. Better standardisation of data collection should be applied: standardised timing of home visits and sample collection, especially the timing of milk acidification for ghrelin analysis. Blinding the researcher during data collection and sample analysis would be preferable.
ii. Follow up of the infants from the current study population at a later age to measure their behaviours (appetite and temperament), growth and body composition.
iii. Replication of the study in other populations with the addition of other health or developmental outcomes. Additional measurements of bioactive factors in breast milk such as oligosaccharides, melatonin or opiates should also be considered in future studies.
iv. Broaden research in the area of infant feeding by investigating the relationships between breast milk composition, focusing on ghrelin and carbohydrate, with feeding and other behaviours (e.g. sleeping or crying).
v. Investigate mother-infant signalling among formula feeding mothers using the same intervention tool and study design (RCT) with the aims: i) to reduce maternal stress during the early postpartum period; ii) to test the effect on mother-infant behaviours (bonding) and infant sleeping duration, with the hypothesis that relaxed mothers will have better mother-infant bonding and infant sleeping duration, which could influence infant weight and BMI during early infancy. This also offers the opportunity to solely investigate the behavioural pathway of the mother-infant signalling, since there is no breast milk involved.

7.7.2. Anthropological research

i. Future studies could use the same study design among teenage mothers or mothers with multiple pregnancies where the energy demands of both parties are higher, creating greater tension in the tug-of-war. Manipulating maternal psychological state by giving the relaxation therapy could have a greater impact in these populations, since it would have greater potential benefits for both parties. In addition, studying multiple pregnancies could also add another research perspective in terms of the tug-of-war: a conflict between siblings.

ii. Related to (i), it would also be interesting to follow up the mothers from the present study population by investigating mother-infant factors during the lactation period with their second children. This could also determine the differences in maternal investment and trade-off that occur between first and second children.

iii. The significant differences in breast milk composition and infant growth between groups in the present population showed that the maternal investment strategy might be different between groups. Previous studies have shown that higher maternal investment in early life could contribute to high 'metabolic capacity' in the offspring, or in other words, a greater capability of the human body to maintain homeostasis in later life [350, 351]. These previous studies were observational and involved retrospective data, hence there were many confounding factors and biases that could interfere with the results. Therefore, it would be interesting to extend follow-up of the offspring from this RCT to later life in order to study the human life-history strategy. This could be done by investigating the relation of maternal capital with metabolic capacity or load in the offspring during early life and the influence on later health and development, as proposed by Wells et al., [350]. Alternatively, it would be better to replicate the current study among high stressful or high energy demanding population (e.g. mothers of preterm or teenage lactating mothers) with a larger sample size to account for drop up over a long follow up.

iv. Extending the point above, follow-up of the infants should include measurement of their later growth (weight, height and body composition) and development or health (e.g. menarche age and blood pressure) to determine the effect of different levels of metabolic capacity in early infancy life on later life history strategy (e.g. early reproduction vs growth).

7.8. Conclusion

In this thesis, I have presented results from my research in which I used an experimental approach to investigate mother-infant signalling in early life. I have explored potential pathways for intervention effects, and discussed the findings from both a biological and anthropological perspective. The findings have scientific and practical relevance; they contribute to current understanding of the physiological, psychological and anthropological perspective of infant feeding, and also identify aspects that can be addressed to increase breastfeeding success. Thus, the practical relevance and potential applications of the results in terms of supporting breastfeeding mothers were also highlighted. Given the intervention tool is simple and practical, it could easily be used in future interventions aimed at increasing the rates and duration of BF and exclusive BF. To my knowledge, previous studies of human milk composition and infant growth have been predominantly presented through the biological perspective. However, this present study broadens that approach by incorporating behavioural and anthropological perspectives, including life-history theory. This contributes to knowledge on the natural variation in milk composition that shapes infant behaviour and development, providing new information about the trade-offs that occur between mothers and infants. Overall, by contributing data relevant to the mechanisms of biological and behavioural signalling during early life in humans, this project has increased understanding of maternal-infant factors during lactation and provided useful suggestions for the direction of future research.

REFERENCES:

1. Skibiel, A.L., et al., *The evolution of the nutrient composition of mammalian milks.* Journal of Animal Ecology, 2013. **82**(6): p. 1254-1264.
2. Oftedal, O., *The evolution of milk secretion and its ancient origins.* animal, 2012. **6**(Special Issue 03): p. 355-368.
3. Ip, S., et al., *Breastfeeding and maternal and infant health outcomes in developed countries.* Evid Rep Technol Assess (Full Rep), 2007(153): p. 1-186.
4. Van Rossum, C., F. Büchner, and J. Hoekstra, *Quantification of health effects of breastfeeding - Review of the literature and model simulation*, in *RIVM report*. 2005, National Institute for Public Health and the Environment.
5. Horta, B.L., et al., *Evidence on the long-term effects of breastfeeding*. 2007, World Health Organization: Geneva.
6. Victora, C.G., et al., *Breastfeeding in the 21st century: epidemiology, mechanisms, and lifelong effect.* The Lancet, 2016. **387**(10017): p. 475-490.
7. Rollins, N.C., et al., *Why invest, and what it will take to improve breastfeeding practices?* The Lancet, 2016. **387**(10017): p. 491-504.
8. Wells, J.C.K., *Parent-Offspring Conflict Theory, Signaling of Need, and Weight Gain in Early Life.* The Quarterly Review of Biology, 2003. **78**(2): p. 169-202.
9. Hinde, K., *Lactational Programming of Infant Behavioral Phenotype*, in *Building Babies*, K.B.H. Clancy, K. Hinde, and J.N. Rutherford, Editors. 2013, Springer New York. p. 187-207.
10. Lefèvre, C.M., J.A. Sharp, and K.R. Nicholas, *Evolution of Lactation: Ancient Origin and Extreme Adaptations of the Lactation System.* Annual Review of Genomics and Human Genetics, 2010. **11**(1): p. 219-238.
11. Capuco, A.V. and R.M. Akers, *The origin and evolution of lactation.* Journal of Biology, 2009. **8**(4): p. 1-4.
12. Hinde, K. and L.A. Milligan, *Primate milk: proximate mechanisms and ultimate perspectives.* Evol Anthropol, 2011. **20**(1): p. 9-23.
13. Oftedal, O., *Use of maternal reserves as a lactation strategy in large mammals.* Proceedings of the Nutrition Society, 2000. **59**(01): p. 99-106.
14. Maertens, L., F. Lebas, and Z. Szendro, *Rabbit milk: A review of quantity, quality and non-dietary affecting factors.* World Rabbit Science, 2010. **14**(4): p. 205-230.
15. Prentice, A.M. and A. Prentice, *Evolutionary and enviromental influences on human lactation.* Proceedings of the Nutrition Society, 1995. **54**(02): p. 391-400.
16. Milligan, L.A. and R.P. Bazinet, *Evolutionary modifications of human milk composition: evidence from long-chain polyunsaturated fatty acid composition of anthropoid milks.* J Hum Evol, 2008. **55**(6): p. 1086-95.
17. Brough, L., et al., *Iodine intake and status during pregnancy and lactation before and after government initiatives to improve iodine status, in Palmerston North, New Zealand.* Maternal and Child Nutrition, 2013. **In press**.
18. Bates, C.J., et al., *The effect of vitamin C supplementation on lactating women in Keneba, a West African rural community.* International journal for vitamin and nutrition research. Internationale Zeitschrift fur Vitamin- und Ernahrungsforschung. Journal international de vitaminologie et de nutrition, 1983. **53**(1): p. 68-76.
19. Hollis, B.W. and C.L. Wagner, *Vitamin D requirements during lactation: high-dose maternal supplementation as therapy to prevent hypovitaminosis D for both the mother and the nursing infant.* The American Journal of Clinical Nutrition, 2004. **80**(6): p. 1752S-1758S.

20. Wooding, F. and M. Peaker, *Comparative mammary fine structure.* UK, Zoological Society of London: Comparative aspects of lactation., 1977: p. 1-41.
21. Jones, E. and S.A. Spencer, *The physiology of lactation.* Paediatrics and Child Health, 2007. **17**(6): p. 244-248.
22. Neville, M.C. and J. Morton, *Physiology and Endocrine Changes Underlying Human Lactogenesis II.* The Journal of Nutrition, 2001. **131**(11): p. 3005S-3008S.
23. Kent, J.C., *How breastfeeding works.* J Midwifery Womens Health, 2007. **52**(6): p. 564-70.
24. Neville, M.C., et al., *Studies in human lactation: milk volume and nutrient composition during weaning and lactogenesis.* The American Journal of Clinical Nutrition, 1991. **54**(1): p. 81-92.
25. Pang, W. and P.E. Hartmann, *Initiation of Human Lactation: Secretory Differentiation and Secretory Activation.* Journal of Mammary Gland Biology and Neoplasia, 2007. **12**(4): p. 211-221.
26. Agostoni, C., et al., *Breast-feeding: A Commentary by the ESPGHAN Committee on Nutrition.* Journal of Pediatric Gastroenterology and Nutrition, 2009. **49**(1): p. 112-125 10.1097/MPG.0b013e31819f1e05.
27. Hytten, F.E., *Clinical and chemical studies in human lactation. VI. The functional capacity of the breast.* Br Med J, 1954. **1**(4867): p. 912-5.
28. Hytten, F.E., *Clinical and chemical studies in human lactation.* Br Med J, 1954. **1**(4855): p. 175-82.
29. World Health Organization. *Infant and young child nutrition*. in *Fifty-fourth World Health Assembly*. 2001. Geneva: World Health Organization.
30. Newton, E.R.M., *Breastmilk: The Gold Standard.* Clinical Obstetrics & Gynecology, 2004. **47**(3): p. 632-642.
31. Walker, A., *Breast Milk as the Gold Standard for Protective Nutrients.* The Journal of Pediatrics, 2010. **156**(2, Supplement): p. S3-S7.
32. Lucas, A., *Long-term programming effects of early nutrition - Implications for the preterm infant.* Journal of Perinatology, 2005. **25**(SUPPL. 2): p. S2-S6.
33. Lucas, A., *Programming by early nutrition: an experimental approach.* The Journal of Nutrition, 1998. **128**(2): p. 401S-406S.
34. Breij, L.M., et al., *Longitudinal fat mass and visceral fat during the first 6 months after birth in healthy infants: support for a critical window for adiposity in early life.* Pediatric Obesity, 2016: p. n/a-n/a.
35. Singhal, A., et al., *Nutrition in infancy and long-term risk of obesity: evidence from 2 randomized controlled trials.* The American Journal of Clinical Nutrition, 2010. **92**(5): p. 1133-1144.
36. Lucas, A., *Programming by early nutrition in man.* Ciba Found Symp, 1991. **156**: p. 38-50; discussion 50-5.
37. Singhal, A., *Early Nutrition and Long-Term Cardiovascular Health.* Nutrition Reviews, 2006. **64**(5): p. S44-S49.
38. Fewtrell, M., *The evidence for public health recommendations on infant feeding.* Early Human Development, 2011. **87**(11): p. 715-721.
39. Hoddinott, P., D. Tappin, and C. Wright, *Breast feeding.* BMJ, 2008. **336**(7649): p. 881-887.
40. Groër, M.W., *Differences Between Exclusive Breastfeeders, Formula-Feeders, and Controls: A Study of Stress, Mood, and Endocrine Variables.* Biological Research For Nursing, 2005. **7**(2): p. 106-117.
41. Garcia, C.R., et al., *Breast-feeding initiation time and neonatal mortality risk among newborns in South India.* J Perinatol, 2011. **31**(6): p. 397-403.

42. Chen, D.C., et al., *Stress during labor and delivery and early lactation performance.* The American Journal of Clinical Nutrition, 1998. **68**(2): p. 335-44.
43. Dennis, C.L., et al., *Traditional postpartum practices and rituals: a qualitative systematic review.* Womens Health (Lond Engl), 2007. **3**(4): p. 487-502.
44. Lauer, J.A., et al., *Deaths and years of life lost due to suboptimal breast-feeding among children in the developing world: a global ecological risk assessment.* Public Health Nutrition, 2006. **9**(06): p. 673-685.
45. Black, R.E., et al., *Maternal and child undernutrition: global and regional exposures and health consequences.* The Lancet, 2008. **371**(9608): p. 243-260.
46. UNICEF. *Childinfo : Monitoring the Situation of Children and Women*. Current status of breastfeeding 2012 [cited 2012 August]; Available from: http://www.childinfo.org/breastfeeding_status.html.
47. Fatimah, S., et al., *Breastfeeding in Malaysia: Results of the Third National Health and Morbidity Survey (NHMS III) 2006.* Malaysian Journal of Nutrition, 2010. **16**(2): p. 195-206.
48. Hill, K., *Life history theory and evolutionary anthropology.* Evolutionary Anthropology: Issues, News, and Reviews, 1993. **2**(3): p. 78-88.
49. Hill, K. and H. Kaplan, *Life History Traits in Humans: Theory and Empirical Studies.* Annual Review of Anthropology, 1999. **28**: p. 397-430.
50. Jasienska, G., *Reproduction and lifespan: Trade-offs, overall energy budgets, intergenerational costs, and costs neglected by research.* American Journal of Human Biology, 2009. **21**(4): p. 524-532.
51. Butte, N.F. and J.C. King, *Energy requirements during pregnancy and lactation.* Public Health Nutrition, 2005. **8**(7a): p. 1010-1027.
52. Dufour, D.L. and M.L. Sauther, *Comparative and evolutionary dimensions of the energetics of human pregnancy and lactation.* American Journal of Human Biology, 2002. **14**(5): p. 584-602.
53. Dewey, K.G., *ENERGY AND PROTEIN REQUIREMENTS DURING LACTATION.* Annual Review of Nutrition, 1997. **17**(1): p. 19-36.
54. Tracer, D.P., *Breastfeeding structure as a test of parental investment theory in Papua New Guinea.* American Journal of Human Biology, 2009. **21**(5): p. 635-642.
55. Tully, K.P. and H.L. Ball, *Trade-offs underlying maternal breastfeeding decisions: a conceptual model.* Maternal & Child Nutrition, 2013. **9**(1): p. 90-98.
56. Piperata, B.A., *Variation in maternal strategies during lactation: The role of the biosocial context.* American Journal of Human Biology, 2009. **21**(6): p. 817-827.
57. Brown, G.R., et al., *Evolutionary accounts of human behavioural diversity.* Philosophical Transactions of the Royal Society B: Biological Sciences, 2011. **366**(1563): p. 313-324.
58. Fadzil, F., K. Shamsuddin, and S.E. Wan Puteh, *Traditional Postpartum Practices Among Malaysian Mothers: A Review.* The Journal of Alternative and Complementary Medicine, 2015.
59. Chen, L.W., et al., *Dietary changes during pregnancy and the postpartum period in Singaporean Chinese, Malay and Indian women: the GUSTO birth cohort study.* Public Health Nutrition, 2013. **FirstView**: p. 1-9.
60. Sear, R. and M. Gibson, *Introduction to special issue on "Trade-Offs in Female Life Histories: Integrating Evolutionary Frameworks".* American Journal of Human Biology, 2009. **21**(4): p. 417-420.
61. Smith, C.C. and S.D. Fretwell, *The Optimal Balance between Size and Number of Offspring.* The American Naturalist, 1974. **108**(962): p. 499-506.
62. Trivers, R.L., *Parent-Offspring Conflict.* American Zoologist, 1974. **14**: p. 249–264

63. Haig, D., *Genetic Conflicts in Human Pregnancy.* The Quarterly Review of Biology, 1993. **68**(4): p. 495-532.
64. Delgado, H.L., et al., *Relationship of maternal and infant nutrition to infant growth.* Early Human Development, 1982. **6**(3): p. 273-286.
65. Loudon, A.S.I., A.S. McNeilly, and J.A. Milne, *Nutrition and lactational control of fertility in red deer.* Nature, 1983. **302**(5904): p. 145-147.
66. Haig, D., *Troubled sleep: Night waking, breastfeeding and parent–offspring conflict.* Evolution, Medicine, and Public Health, 2014. **2014**(1): p. 32-39.
67. Elias, M.F., et al., *Nursing Practices and Lactation Amenorrhoea.* Journal of Biosocial Science, 1986. **18**(01): p. 1-10.
68. Valeggia, C. and P.T. Ellison, *Lactational amenorrhoea in well-nourished Toba women of Formosa, Argentina.* J Biosoc Sci, 2004. **36**(5): p. 573-95.
69. Ellison, P.T., *Energetics and reproductive effort.* American Journal of Human Biology, 2003. **15**(3): p. 342-351.
70. Valeggia, C. and P.T. Ellison, *Interactions between metabolic and reproductive functions in the resumption of postpartum fecundity.* American Journal of Human Biology, 2009. **21**(4): p. 559-566.
71. Lucas, A., et al., *How much energy does the breast fed infant consume and expend?* Br Med J (Clin Res Ed), 1987. **295**(6590): p. 75-7.
72. Saarela, T., J. Kokkonen, and M. Koivisto, *Macronutrient and energy contents of human milk fractions during the first six months of lactation.* Acta Pædiatrica, 2005. **94**(9): p. 1176-1181.
73. de Lauzon-Guillain, B., et al., *Breastfeeding and Infant Temperament at Age Three Months.* PLoS ONE, 2012. **7**(1): p. e29326.
74. Savino, F. and S.A. Liguori, *Update on breast milk hormones: Leptin, ghrelin and adiponectin.* Clinical Nutrition, 2008. **27**(1): p. 42-47.
75. Neville, M.C., T.B. McFadden, and I. Forsyth, *Hormonal Regulation of Mammary Differentiation and Milk Secretion.* Journal of Mammary Gland Biology and Neoplasia, 2002. **7**(1): p. 49-66.
76. Jarmolowska, B., et al., *Changes of beta-casomorphin content in human milk during lactation.* Peptides, 2007. **28**(10): p. 1982-6.
77. Cohen Engler, A., et al., *Breastfeeding may improve nocturnal sleep and reduce infantile colic: Potential role of breast milk melatonin.* European Journal of Pediatrics, 2012. **171**(4): p. 729-732.
78. Illnerová, H., M. Buresová, and J. Presl, *Melatonin rhythm in human milk.* The Journal of Clinical Endocrinology & Metabolism, 1993. **77**(3): p. 838-841.
79. Lee, K.A., M.E. Zaffke, and G. Mcenany, *Parity and Sleep Patterns During and After Pregnancy.* Obstetrics & Gynecology, 2000. **95**(1): p. 14-18.
80. Neville, M.C., et al., *Lactation and neonatal nutrition: defining and refining the critical questions.* J Mammary Gland Biol Neoplasia, 2012. **17**(2): p. 167-88.
81. Khani, S. and J.A. Tayek, *Cortisol increases gluconeogenesis in humans: its role in the metabolic syndrome.* Clin. Sci., 2001. **101**(6): p. 739-747.
82. Meaney, M., et al., *Early environmental regulation of forebrain glucocorticoid receptor gene expression: implications for adrenocortical responses to stress.* Developmental neuroscience, 1996. **18**(1-2): p. 49.
83. Tu, M.T., S.J. Lupien, and C.-D. Walker, *Measuring stress responses in postpartum mothers: Perspectives from studies in human and animal populations.* Stress, 2005. **8**(1): p. 19-34.
84. Nguyen, D.-A. and M.C. Neville, *Tight Junction Regulation in the Mammary Gland.* Journal of Mammary Gland Biology and Neoplasia, 1998. **3**(3): p. 233-246.

85. Joffe, T.H., et al., *A new method for quantifying encephalization in the growing individual.* Journal of Theoretical Biology, 2003. **225**(3): p. 361-367.
86. Sullivan, E.C., et al., *Cortisol concentrations in the milk of rhesus monkey mothers are associated with confident temperament in sons, but not daughters.* Dev Psychobiol, 2011. **53**(1): p. 96-104.
87. Grey, K.R., et al., *Human milk cortisol is associated with infant temperament.* Psychoneuroendocrinology, 2013. **38**(7): p. 1178-1185.
88. Hart, S., et al., *Breast milk levels of cortisol and Secretory Immunoglobulin A (SIgA) differ with maternal mood and infant neuro-behavioral functioning.* Infant Behavior and Development, 2004. **27**(1): p. 101-106.
89. Groer, M.W., S. Humenick, and P. Hill, *Characterizations and psychoneuroimmunologic implications of secretory immunoglobulin A and cortisol in preterm and term breast milk.* Journal of Perinatal & Neonatal Nursing, 1994. **7**(4): p. 42-51.
90. Kulski, J.K. and P.E. Hartmann, *Changes in the concentration of cortisol in milk during different stages of human lactation.* Aust J Exp Biol Med, 1981. **59**(6): p. 769-778.
91. Kato, E.A., et al., *Evidence for the Direct Transfer of Corticosteroid-Binding Globulin from Plasma to Whey in the Guinea Pig.* Endocrinology, 1985. **117**(4): p. 1404-1408.
92. Patacchioli, F.R., et al., *Maternal plasma and milk free cortisol during the first 3 days of breast-feeding following spontaneous delivery or elective cesarean section.* Gynecol Obstet Invest, 1992. **34**(3): p. 159-63.
93. Pressley, L. and J.W. Funder, *Glucocorticoid and Mineralocorticoid Receptors in Gut Mucosa.* Endocrinology, 1975. **97**(3): p. 588-596.
94. Henning, S.J., P.L. Ballard, and N. Kretchmer, *A study of the cytoplasmic receptors for glucocorticoids in intestine of pre- and postweanling rats.* Journal of Biological Chemistry, 1975. **250**(6): p. 2073-2079.
95. Donovan, S.M., et al., *Noninvasive molecular fingerprinting of host–microbiome interactions in neonates.* FEBS Letters, 2014. **588**(22): p. 4112-4119.
96. Hinde, K. and J.P. Capitanio, *Lactational programming? Mother's milk energy predicts infant behavior and temperament in rhesus macaques (Macaca mulatta).* Am J Primatol, 2010. **72**(6): p. 522-9.
97. Bartol, F.F., et al., *Lactocrine signaling and developmental programming.* J Anim Sci, 2012. **2**: p. 696-705.
98. Fuller, P.J. and K. Verity, *Mineralocorticoid receptor gene expression in the gastrointestinal tract: Distribution and ontogeny.* Journal of Steroid Biochemistry, 1990. **36**(4): p. 263-267.
99. Glynn, L.M., et al., *Postnatal maternal cortisol levels predict temperament in healthy breastfed infants.* Early Hum Dev, 2007. **83**(10): p. 675-81.
100. van der Voorn, B., et al., *Determination of cortisol and cortisone in human mother's milk.* Clinica Chimica Acta, 2015. **444**: p. 154-5.
101. Karatas, Z., et al., *Breastmilk ghrelin, leptin, and fat levels changing foremilk to hindmilk: is that important for self-control of feeding?* European Journal of Pediatrics, 2011. **170**(10): p. 1273-1280.
102. Cummings, D.E., *Ghrelin and the short- and long-term regulation of appetite and body weight.* Physiology & Behavior, 2006. **89**(1): p. 71-84.
103. Klok, M.D., S. Jakobsdottir, and M.L. Drent, *The role of leptin and ghrelin in the regulation of food intake and body weight in humans: a review.* Obesity Reviews, 2007. **8**(1): p. 21-34.
104. Fields, D.A., C.R. Schneider, and G. Pavela, *A narrative review of the associations between six bioactive components in breast milk and infant adiposity.* Obesity, 2016. **24**(6): p. 1213-1221.

105. Perez-Tilve, D., et al., *Gut hormones ghrelin, PYY, and GLP-1 in the regulation of energy, balance, and metabolism.* Endocrine, 2006. **29**(1): p. 61-71.
106. Müller, T.D., et al., *Ghrelin.* Molecular Metabolism, 2015. **4**(6): p. 437-460.
107. Korbonits, M., et al., *Ghrelin—a hormone with multiple functions.* Frontiers in Neuroendocrinology, 2004. **25**(1): p. 27-68.
108. Nass, R., et al., *Evidence for Acyl-Ghrelin Modulation of Growth Hormone Release in the Fed State.* The Journal of Clinical Endocrinology & Metabolism, 2008. **93**(5): p. 1988-1994.
109. Aydin, S., et al., *Ghrelin is present in human colostrum, transitional and mature milk.* Peptides, 2006. **27**(4): p. 878-882.
110. Ilcol, Y.O. and B. Hizli, *Active and total ghrelin concentrations increase in breast milk during lactation.* Acta Pædiatrica, 2007. **96**(11): p. 1632-1639.
111. Aydin, S., *The presence of the peptides apelin, ghrelin and nesfatin-1 in the human breast milk, and the lowering of their levels in patients with gestational diabetes mellitus.* Peptides, 2010. **31**(12): p. 2236-40.
112. Cesur, G., et al., *The relationship between ghrelin and adiponectin levels in breast milk and infant serum and growth of infants during early postnatal life.* Journal of Physiological Sciences: JPS, 2012. **62**(3): p. 185-90.
113. Savino, F., et al., *Assay of ghrelin concentration in infant formulas and breast milk.* World Journal of Gastroenterology : WJG, 2011. **17**(15): p. 1971-1975.
114. Ng, P.C., et al., *Ghrelin in preterm and term newborns: relation to anthropometry, leptin and insulin.* Clinical Endocrinology, 2005. **63**(2): p. 217-22.
115. Savino, F., et al., *Serum ghrelin concentration and weight gain in healthy term infants in the first year of life.* Journal of Pediatric Gastroenterology & Nutrition, 2005. **41**(5): p. 653-9.
116. Kierson, J.A., et al., *Ghrelin and cholecystokinin in term and preterm human breast milk.* Acta Pædiatrica, 2006. **95**(8): p. 991-995.
117. Sato, T., et al., *Structure, regulation and function of ghrelin.* Journal of Biochemistry, 2012. **151**(2): p. 119-128.
118. Gnanapavan, S., et al., *The Tissue Distribution of the mRNA of Ghrelin and Subtypes of Its Receptor, GHS-R, in Humans.* The Journal of Clinical Endocrinology & Metabolism, 2002. **87**(6): p. 2988-2988.
119. Goldstein, J.L., et al., *Surviving Starvation: Essential Role of the Ghrelin-Growth Hormone Axis.* Cold Spring Harbor Symposia on Quantitative Biology, 2011. **76**: p. 121-127.
120. Barrenetxe, J., et al., *Distribution of the long leptin receptor isoform in brush border, basolateral membrane, and cytoplasm of enterocytes.* Gut, 2002. **50**(6): p. 797-802.
121. Sobhani, I., et al., *Leptin secretion and leptin receptor in the human stomach.* Gut, 2000. **47**(2): p. 178-183.
122. Bonnet, M., et al., *Mammary leptin synthesis, milk leptin and their putative physiological roles.* Reproduction Nutrition Development, 2002. **42**(5): p. 399-413.
123. Ucar, B., et al., *Breast milk leptin concentrations in initial and terminal milk samples: relationships to maternal and infant plasma leptin concentrations, adiposity, serum glucose, insulin, lipid and lipoprotein levels.* Journal of Pediatric Endocrinology, 2000. **13**(2): p. 149-56.
124. Sánchez, J., et al., *Leptin Orally Supplied to Neonate Rats Is Directly Uptaken by the Immature Stomach and May Regulate Short-Term Feeding.* Endocrinology, 2005. **146**(6): p. 2575-2582.
125. Attele, A.S., Z.Q. Shi, and C.-S. Yuan, *Leptin, gut, and food intake.* Biochemical Pharmacology, 2002. **63**(9): p. 1579-1583.

126. Picó, C., et al., *Gastric leptin: a putative role in the short-term regulation of food intake.* British Journal of Nutrition, 2003. **90**(04): p. 735-741.
127. Brunner, S., et al., *Breast milk leptin and adiponectin in relation to infant body composition up to 2 years.* Pediatric Obesity, 2015. **10**(1): p. 67-73.
128. Miralles, O., et al., *A physiological role of breast milk leptin in body weight control in developing infants.* Obesity, 2006. **14**(8): p. 1371-7.
129. Doneray, H., Z. Orbak, and L. Yildiz, *The relationship between breast milk leptin and neonatal weight gain.* Acta Paediatrica, 2009. **98**(4): p. 643-7.
130. Fields, D.A. and E.W. Demerath, *Relationship of insulin, glucose, leptin, IL-6 and TNF-α in human breast-milk with infant growth and body composition.* Pediatric Obesity, 2012. **7**(4): p. 304-312.
131. Schuster, S., et al., *Leptin in maternal serum and breast milk: association with infants' body weight gain in a longitudinal study over 6 months of lactation.* Pediatric Research, 2011. **70**(6): p. 633-7.
132. Palou, A., J. Sanchez, and C. Pico, *Nutrient-gene interactions in early life programming: leptin in breast milk prevents obesity later on in life.* Advances in Experimental Medicine & Biology, 2009. **646**: p. 95-104.
133. Bouret, S.G., *Early Life Origins of Obesity: Role of Hypothalamic Programming.* Journal of Pediatric Gastroenterology and Nutrition, 2009. **48**: p. S31-S38 10.1097/MPG.0b013e3181977375.
134. Smith-Kirwin, S.M., et al., *Leptin Expression in Human Mammary Epithelial Cells and Breast Milk.* The Journal of Clinical Endocrinology & Metabolism, 1998. **83**(5): p. 1810-1810.
135. Uysal, F.K., et al., *Breast milk leptin: its relationship to maternal and infant adiposity.* Clin Nutr, 2002. **21**(2): p. 157-60.
136. Houseknecht, K.L., et al., *Leptin Is Present in Human Milk and Is Related to Maternal Plasma Leptin Concentration and Adiposity.* Biochemical and Biophysical Research Communications, 1997. **240**(3): p. 742-747.
137. Ilcol, Y., Z.B. Hizli, and T. Ozkan, *Leptin concentration in breast milk and its relationship to duration of lactation and hormonal status.* International Breastfeeding Journal, 2006. **1**(1): p. 21.
138. Savino, F., et al., *Mother and Infant Body Mass Index, Breast Milk Leptin and Their Serum Leptin Values.* Nutrients, 2016. **8**(6): p. 383.
139. Schueler, J., et al., *Presence and dynamics of leptin, GLP-1, and PYY in human breast milk at early postpartum.* Obesity, 2013. **21**(7): p. 1451-8.
140. Cannon, A.M., et al., *The Effects of Leptin on Breastfeeding Behaviour.* International Journal of Environmental Research & Public Health [Electronic Resource], 2015. **12**(10): p. 12340-55.
141. Eilers, E., et al., *Leptin determination in colostrum and early human milk from mothers of preterm and term infants.* Early Human Development, 2011. **87**(6): p. 415-419.
142. Andreas, N.J., et al., *Impact of maternal BMI and sampling strategy on the concentration of leptin, insulin, ghrelin and resistin in breast milk across a single feed: a longitudinal cohort study.* BMJ Open, 2016. **6**(7).
143. Savino, F., et al., *Resistin and leptin in breast milk and infants in early life.* Early Human Development, 2012. **88**(10): p. 779-82.
144. Bronsky, J., et al., *Adiponectin, AFABP, and Leptin in Human Breast Milk During 12 Months of Lactation.* Journal of Pediatric Gastroenterology and Nutrition, 2011. **52**(4): p. 474-477 10.1097/MPG.0b013e3182062fcc.
145. Savino, F., et al., *Evaluation of leptin in breast milk, lactating mothers and their infants.* European Journal of Clinical Nutrition, 2010. **64**(9): p. 972-7.

146. Weyermann, M., et al., *Adiponectin and leptin in maternal serum, cord blood, and breast milk.* Clinical Chemistry, 2006. **52**(11): p. 2095-102.
147. Bielicki, J., R. Huch, and U. von Mandach, *Time-course of leptin levels in term and preterm human milk.* European Journal of Endocrinology, 2004. **151**(2): p. 271-6.
148. Brown, A., J. Rance, and P. Bennett, *Understanding the relationship between breastfeeding and postnatal depression: the role of pain and physical difficulties.* Journal of Advanced Nursing, 2016. **72**(2): p. 273-282.
149. O'Hara, M.W. and A.M. Swain, *Rates and risk of postpartum depression—a meta-analysis.* International Review of Psychiatry, 1996. **8**(1): p. 37-54.
150. Leahy-Warren, P., G. McCarthy, and P. Corcoran, *First-time mothers: social support, maternal parental self-efficacy and postnatal depression.* Journal of Clinical Nursing, 2012. **21**(3-4): p. 388-397.
151. Watkins, S., et al., *Early Breastfeeding Experiences and Postpartum Depression.* Obstetrics and Gynecology, 2011. **118**(2): p. 214-221.
152. Kendall-Tackett, K., *A new paradigm for depression in new mothers: the central role of inflammation and how breastfeeding and anti-inflammatory treatments protect maternal mental health.* International Breastfeeding Journal, 2007. **2**(1): p. 1-14.
153. Kurth, E., et al., *Crying babies, tired mothers: What do we know? A systematic review.* Midwifery, 2011. **27**(2): p. 187-194.
154. Moehler, E., et al., *Maternal depressive symptoms in the postnatal period are associated with long-term impairment of mother–child bonding.* Archives of Women's Mental Health, 2006. **9**(5): p. 273-278.
155. Gonidakis, F., et al., *A 6-month study of postpartum depression and related factors in Athens Greece.* Comprehensive Psychiatry, 2008. **49**(3): p. 275-282.
156. Field, T., *Postpartum depression effects on early interactions, parenting, and safety practices: A review.* Infant Behavior and Development, 2010. **33**(1): p. 1-6.
157. Myers, S., O. Burger, and S.E. Johns, *Postnatal depression and reproductive success in modern, low-fertility contexts.* Evolution, Medicine, and Public Health, 2016. **2016**(1): p. 71-84.
158. Hart, S.L., S.C. Jackson, and L.M. Boylan, *Compromised Weight Gain, Milk Intake, and Feeding Behavior in Breastfed Newborns of Depressive Mothers.* Journal of Pediatric Psychology, 2011. **36**(8): p. 942-950.
159. Shakespeare, J., F. Blake, and J. Garcia, *Breast-feeding difficulties experienced by women taking part in a qualitative interview study of postnatal depression.* Midwifery, 2004. **20**(3): p. 251-260.
160. Adedinsewo, D.A., et al., *Maternal Anxiety and Breastfeeding: Findings from the MAVAN (Maternal Adversity, Vulnerability and Neurodevelopment) Study.* Journal of Human Lactation, 2013.
161. Zanardo, V., et al., *Impact of Anxiety in the Puerperium on Breast-feeding Outcomes: Role of Parity.* Journal of Pediatric Gastroenterology and Nutrition, 2009. **49**(5): p. 631-634.
162. Taveras, E.M., et al., *Clinician Support and Psychosocial Risk Factors Associated With Breastfeeding Discontinuation.* Pediatrics, 2003. **112**(1): p. 108-115.
163. Ueda, T., et al., *Influence of psychological stress on suckling-induced pulsatile oxytocin release.* Obstet Gynecol, 1994. **84**(2): p. 259-62.
164. World Health Organization, *Infant and young child feeding: Model Chapter for textbooks for medical students and allied health professionals*, in *The physiological basis of breastfeeding*. 2009, WHO Press: Geneva.
165. Newton, M. and N.R. Newton, *The let-down reflex in human lactation.* The Journal of Pediatrics, 1948. **33**(6): p. 698-704.

166. Lau, C., *Effects of Stress on Lactation.* Pediatric Clinics of North America, 2001. **48**(1): p. 221-234.
167. Dewey, K.G., *Maternal and Fetal Stress Are Associated with Impaired Lactogenesis in Humans.* The Journal of Nutrition, 2001. **131**(11): p. 3012S-3015S.
168. Stuebe, A., K. Grewen, and S. Meltzer-Brody, *Association between maternal mood and oxytocin response to breastfeeding.* Journal of women's health, 2013. **22**(4): p. 352.
169. Keith, D.R., B.S. Weaver, and R.L. Vogel, *The Effect of Music-Based Listening Interventions on the Volume, Fat Content, and Caloric Content of Breast Milk–Produced by Mothers of Premature and Critically Ill Infants.* Advances in Neonatal Care, 2012. **12**(2): p. 112-119.
170. Feher, S.D.K., et al., *Increasing Breast Milk Production for Premature Infants With a Relaxation/Imagery Audiotape.* Pediatrics, 1989. **83**(1): p. 57-60.
171. Ak, J., et al., *Impact of Music Therapy on Breast Milk Secretion in Mothers of Premature Newborns.* Journal of Clinical and Diagnostic Research : JCDR, 2015. **9**(4): p. CC04-CC06.
172. Stuebe, A.M., et al., *Failed lactation and perinatal depression: common problems with shared neuroendocrine mechanisms?* Journal of Women's Health, 2012. **21**(3): p. 264-272.
173. Jolley, S.N., et al., *Dysregulation of the Hypothalamic-Pituitary-Adrenal Axis in Postpartum Depression.* Biological Research For Nursing, 2007. **8**(3): p. 210-222.
174. Rogers, S.L., et al., *Cortisol metabolism, postnatal depression and weight changes in the first 12 months postpartum.* Clinical Endocrinology, 2016: p. n/a-n/a.
175. Andrews, P.W., et al., *Is serotonin an upper or a downer? The evolution of the serotonergic system and its role in depression and the antidepressant response.* Neuroscience & Biobehavioral Reviews, 2015. **51**(0): p. 164-188.
176. Segerstrom, S.C., *Stress, Energy, and Immunity: An Ecological View.* Current Directions in Psychological Science, 2007. **16**(6): p. 326-330.
177. Moles, A., et al., *Psychosocial stress affects energy balance in mice: Modulation by social status.* Psychoneuroendocrinology, 2006. **31**(5): p. 623-633.
178. Michel, C., et al., *Chronic stress reduces body fat content in both obesity-prone and obesity-resistant strains of mice.* Hormones and Behavior, 2005. **48**(2): p. 172-179.
179. Meerlo, P., G.J.F. Overkamp, and J.M. Koolhaas, *Behavioural and physiological consequences of a single social defeat in Roman high- and low-avoidance rats.* Psychoneuroendocrinology, 1997. **22**(3): p. 155-168.
180. Lupien, S.J., et al., *Cortisol levels during human aging predict hippocampal atrophy and memory deficits.* Nat Neurosci, 1998. **1**(1): p. 69-73.
181. Lawson, D.W. and R. Mace, *Sibling configuration and childhood growth in contemporary British families.* International Journal of Epidemiology, 2008. **37**(6): p. 1408-1421.
182. Rao, M., et al., *Reduced heat loss following sucrose ingestion in premature and normal human newborns.* Early Human Development, 1997. **48**(1–2): p. 109-116.
183. Számadó, S., *The cost of honesty and the fallacy of the handicap principle.* Animal Behaviour, 2011. **81**(1): p. 3-10.
184. Mock, D.W., M.B. Dugas, and S.A. Strickler, *Honest begging: expanding from Signal of Need.* Behavioral Ecology, 2011. **22**(5): p. 909-917.
185. Wells, J.C.K. and P.S.W. Davies, *Estimation of the energy cost of physical activity in infancy.* Archives of Disease in Childhood, 1998. **78**(2): p. 131-136.
186. Rees, B.L., *Effect of Relaxation with Guided Imagery on Anxiety, Depression, and Self-Esteem in Primiparas.* Journal of Holistic Nursing, 1995. **13**(3): p. 255-267.
187. Liberati, A., et al., *The PRISMA Statement for Reporting Systematic Reviews and Meta-Analyses of Studies That Evaluate Health Care Interventions: Explanation and*

ElaborationPRISMA: Explanation and Elaboration. Annals of Internal Medicine, 2009. **151**(4): p. W-65.
188. Perez-Blasco, J., P. Viguer, and M. Rodrigo, *Effects of a mindfulness-based intervention on psychological distress, well-being, and maternal self-efficacy in breast-feeding mothers: results of a pilot study.* Archives of Women's Mental Health, 2013. **16**(3): p. 227-236.
189. O'Connor, M.E., et al. *Relaxation training and breast milk secretory IgA*. Archives of pediatrics & adolescent medicine, 1998. **152**, 1065-70.
190. Miller, E.M., et al., *Field and laboratory methods in human milk research.* American Journal of Human Biology, 2013. **25**(1): p. 1-11.
191. da Costa, T.H.M., et al., *How Much Human Milk Do Infants Consume? Data from 12 Countries Using a Standardized Stable Isotope Methodology.* The Journal of Nutrition, 2010. **140**(12): p. 2227-2232.
192. Kuzawa, C.W., et al., *Rapid weight gain after birth predicts life history and reproductive strategy in Filipino males.* Proceedings of the National Academy of Sciences, 2010. **107**(39): p. 16800-16805.
193. Nettle, D., *Dying young and living fast: variation in life history across English neighborhoods.* Behavioral Ecology, 2010. **21**(2): p. 387-395.
194. Sear, R., R. Mace, and I.A. McGregor, *Maternal grandmothers improve nutritional status and survival of children in rural Gambia.* Proceedings of the Royal Society of London B: Biological Sciences, 2000. **267**(1453): p. 1641-1647.
195. Henrich, J., et al., *"Economic man" in cross-cultural perspective: Behavioral experiments in 15 small-scale societies.* Behavioral and Brain Sciences, 2005. **28**(06): p. 795-815.
196. Feng, Z., et al., *Glucocorticoid and progesterone inhibit involution and programmed cell death in the mouse mammary gland.* The Journal of Cell Biology, 1995. **131**(4): p. 1095-1103.
197. Berg, M.N., A.M. Dharmarajan, and B.J. Waddell, *Glucocorticoids and Progesterone Prevent Apoptosis in the Lactating Rat Mammary Gland.* Endocrinology, 2002. **143**(1): p. 222-227.
198. Catalani, A., et al., *Maternal corticosterone effects on hypothalamus–pituitary–adrenal axis regulation and behavior of the offspring in rodents.* Neuroscience & Biobehavioral Reviews, 2011. **35**(7): p. 1502-1517.
199. Hinde, K., et al., *Holsteins Favor Heifers, Not Bulls: Biased Milk Production Programmed during Pregnancy as a Function of Fetal Sex.* PLoS ONE, 2014. **9**(2): p. e86169.
200. Hinde, K., *First-time macaque mothers bias milk composition in favor of sons.* Curr Biol, 2007. **17**(22): p. R958-9.
201. Hinde, K., *Richer milk for sons but more milk for daughters: Sex-biased investment during lactation varies with maternal life history in rhesus macaques.* Am J Hum Biol, 2009. **21**(4): p. 512-9.
202. Hinde, K., et al., *Cortisol in mother's milk across lactation reflects maternal life history and predicts infant temperament.* Behavioral Ecology, 2015. **26**(1): p. 269-281.
203. Powe, C.E., C.D. Knott, and N. Conklin-Brittain, *Infant sex predicts breast milk energy content.* Am J Hum Biol, 2010. **22**(1): p. 50-4.
204. Fujita, M., et al., *In poor families, mothers' milk is richer for daughters than sons: A test of Trivers–Willard hypothesis in agropastoral settlements in Northern Kenya.* American Journal of Physical Anthropology, 2012. **149**(1): p. 52-59.
205. Quinn, E.A., *No evidence for sex biases in milk macronutrients, energy, or breastfeeding frequency in a sample of filipino mothers.* American Journal of Physical Anthropology, 2013. **152**(2): p. 209-216.

206. Trivers, R.L. and D.E. Willard, *Natural Selection of Parental Ability to Vary the Sex Ratio of Offspring.* Science, 1973. **179**(4068): p. 90-92.
207. Department of Statistics, *Population Distribution and Basic Demographic Characteristic Report 2010 (Updated: 05/08/2011)*. 2011, Malaysia Federal Government Administrative Centre,: Putrajaya.
208. Malaysia Planning Division, *Health Indicators 2014 : Indicators for monitoring and evaluation of strategy health for all*, Malaysia Ministry of Health, Editor. 2015, Health Informatic Centre: Putrajaya.
209. Radzniwan, A., et al., *Breast feeding practice and knowledge among mothers attending an urban Malaysian maternal and child health clinic.* Medicine & Health, 2009. **4**(1): p. 1-7.
210. Tan, A.E., et al., *Knowledge, Attitudes and Sources of Information on Breastfeeding among Pregnant Mothers.* Med & Health, 2008. **3**(1): p. 30-7.
211. Adlina, S., et al., *Knowledge, attitude and practice of breastfeeding among mothers in the pre-baby friendly hospital initiative implementation at seven private hospitals in Malaysia.* Malaysian Journal of Public Health Medicine, 2006. **6**(1): p. 58-63.
212. Van Belle, G., *Sample size*, in *Statistical rules of thumb*, G. Van Belle, Editor. 2008, Wiley: New Jersey US. p. 27-52.
213. Geefhuysen, C.J., et al., *Malaysian Antenatal Risk Coding and the Outcome of Pregnancy.* Journal of Obstetrics and Gynaecology Research, 1998. **24**(1): p. 13-20.
214. Othman, R., N.I.A. Razak, and N. Ishak, *Traditional Malay midwifery practices of point massage (bertungku) and vaginal heat (bertangas) treatment for postnatal treatment among Malay culture at east coast of Malaysia.* p. 253.
215. Menelli, S., *Breastfeeding Meditation*. 2004, White Heart Publishing.
216. Shukri, N.H., et al. *Maternal perceptions of a Malay version of a breastfeeding relaxation therapy recording: A Pilot Study*. in *20th Malaysian Dietitians Association Scientific Conference*. 2014. Kuala Lumpur: Malaysian Dietitians Association
217. Mukhtar, F. and N.S. Zulkefly, *The Beck Anxiety Inventory for Malays (BAI-Malay): A Preliminary Study on Psychometric Properties.* Malaysian Journal of Medicine and Health Sciences, 2011. **7**(1): p. 73-79.
218. Mahmud, W.M., A. Awang, and M.N. Mohamed, *Revalidation of the Malay Version of the Edinburgh Postnatal Depression Scale (EPDS) Among Malay Postpartum Women Attending the Bakar Bata Health Center in Alor Setar, Kedah, North West Of Peninsular Malaysia.* Malays J Med Sci, 2003. **10**(2): p. 71-5.
219. Mazlan, N.H. and A. Ahmad, *A Validity Study of Malay-translated Version of Perceived Stress Scale.* Malaysian Journal of Forensic Sciences (MJOFS): Mission Statement, 2012. **3**(1): p. 52-57.
220. Mora, A.d.l., et al., *The Iowa Infant Feeding Attitude Scale: Analysis of Reliability and Validity.* Journal of Applied Social Psychology, 1999. **29**(11): p. 2362-2380.
221. Centers for Disease Control and Prevention. *Infant Feeding Practices Study II : The Questionnaire*. 2009 [cited 2012 july, 2nd]; Available from: http://www.cdc.gov/ifps/questionnaires.htm.
222. Nielsen, S.B., et al., *Adequacy of Milk Intake During Exclusive Breastfeeding: A Longitudinal Study.* Pediatrics, 2011. **128**(4): p. e907-e914.
223. Llewellyn, C.H., et al., *Development and factor structure of the Baby Eating Behaviour Questionnaire in the Gemini birth cohort.* Appetite, 2011. **57**(2): p. 388-96.
224. Barr, R.G., et al., *Parental diary of infant cry and fuss behaviour.* Archives of Disease in Childhood, 1988. **63**(4): p. 380-387.
225. St James-Roberts, I., J. Hurry, and J. Bowyer, *Objective confirmation of crying durations in infants referred for excessive crying.* Archives of Disease in Childhood, 1993. **68**(1): p. 82-84.

226. Gartstein, M.A. and M.K. Rothbart, *Studying infant temperament via the Revised Infant Behavior Questionnaire.* Infant Behavior and Development, 2003. **26**(1): p. 64-86.
227. Parade, S.H. and E.M. Leerkes, *The reliability and validity of the Infant Behavior Questionnaire-Revised.* Infant Behav Dev, 2008. **31**(4): p. 637-46.
228. Cohen, S., T. Kamarck, and R. Mermelstein, *A global measure of perceived stress.* J Health Soc Behav, 1983. **24**(4): p. 385-96.
229. Cohen, S. and D. Janicki-Deverts, *Who's Stressed? Distributions of Psychological Stress in the United States in Probability Samples from 1983, 2006, and 20091.* Journal of Applied Social Psychology, 2012. **42**(6): p. 1320-1334.
230. Fydrich, T., D. Dowdall, and D.L. Chambless, *Reliability and validity of the beck anxiety inventory.* Journal of Anxiety Disorders, 1992. **6**(1): p. 55-61.
231. Cox, J.L., J.M. Holden, and R. Sagovsky, *Detection of postnatal depression. Development of the 10-item Edinburgh Postnatal Depression Scale.* The British Journal of Psychiatry, 1987. **150**(6): p. 782-6.
232. Eberhard-Gran, M., et al., *Review of validation studies of the Edinburgh Postnatal Depression Scale.* Acta Psychiatrica Scandinavica, 2001. **104**(4): p. 243-249.
233. Abrams, S.A. and W.W. Wong, *Stable isotopes in human nutrition: laboratory methods and research applications*. 2003, UK: Cabi.
234. Nielsen, S.B., et al., *Administering labelled water to exclusively breast-fed infants in studies involving stable isotope dilution techniques.* Isotopes Environ Health Stud, 2011. **47**(1): p. 18-25.
235. International Atomic Energy Agency (IAEA), *Stable Isotope Technique to Assess Intake of Human Milk in Breastfed Infants*. 2010, Vienna, Austria: International Atomic Energy Agency.
236. Hosoda, H., et al., *Optimum collection and storage conditions for ghrelin measurements: octanoyl modification of ghrelin is rapidly hydrolyzed to desacyl ghrelin in blood samples.* Clin Chem, 2004. **50**(6): p. 1077-80.
237. Miller, E.M., et al., *Field and laboratory methods in human milk research.* Am J Hum Biol, 2012.
238. Amran, N., *Labour Force Survey Report*. 2015, Malaysia Department of Statistics: Putrajaya.
239. Hasan, A.R., *Household Income and Basic Amenities Survey Report*. 2013, Malaysia Department of Statistics: Putrajaya. p. 20.
240. Baba, Z., *Laporan Tahunan 2011 (Annual Report 2011)*. 2012, Selangor Department of Health: Shah Alam. p. 45.
241. Azidah, A.K., et al., *Postnatal depression and socio-cultural practices among postnatal mothers in Kota Bahru, Kelantan, Malaysia.* Med J Malaysia, 2006. **61**(1): p. 76-83.
242. Ali, A. and P. Howden-Chapman, *Maternity Services and the Role of the Traditional Birth Attendant, Bidan Kampung, in Rural Malaysia.* Journal of Public Health Management and Practice, 2007. **13**(3): p. 278-286.
243. Rostam, K., *Perubahan penduduk Wilayah Metropolitan Lanjutan Klang- Langat, Malaysia, 1957-2000 (Regional population change in Klang-Valley, Malaysia, 1957-2000).* Akademika 2010. **79**(Mei-Ogos): p. 1-18.
244. Tan, K., *Knowledge, attitude and practice on breastfeeding in Klang, Malaysia.* The International Medical Journal of Malaysia, 2009. **8**(1).
245. Tan, K., *Factors associated with exclusive breastfeeding among infants under six months of age in peninsular malaysia.* International Breastfeeding Journal, 2011. **6**: p. 2-2.
246. Ishak, S., et al., *Knowledge and Beliefs About Breastfeeding Are Not Determinants for Successful Breastfeeding.* Breastfeeding Medicine, 2014. **9**(6): p. 308-312.

247. Sear, R., *Beyond the nuclear family: an evolutionary perspective on parenting.* Current Opinion in Psychology, 2016. **7**: p. 98-103.
248. Snopkowski, K. and R. Sear, *Grandparental help in Indonesia is directed preferentially towards needier descendants: A potential confounder when exploring grandparental influences on child health.* Social Science & Medicine, 2015. **128**: p. 105-114.
249. Meehan, C.L., C. Helfrecht, and R.J. Quinlan, *Cooperative breeding and Aka children's nutritional status: Is flexibility key?* American Journal of Physical Anthropology, 2014. **153**(4): p. 513-525.
250. Tan, K., *Factors Associated with Non-exclusive Breastfeeding among 4-Week Post-partum Mothers in Klang District, Peninsular Malaysia* Malaysian Journal of Nutrition, 2009. **15**(1): p. 11-18.
251. Silvestre, D., et al., *Comparison of mid-infrared transmission spectroscopy with biochemical methods for the determination of macronutrients in human milk.* Maternal & Child Nutrition, 2014. **10**(3): p. 373-382.
252. Davies, P.S. and J.C. Wells, *Calculation of total body water in infancy.* European journal of clinical nutrition, 1994. **48**(7): p. 490-495.
253. Wells, J.C.K., et al., *Prediction of total body water in infants and children.* Archives of Disease in Childhood, 2005. **90**(9): p. 965-971.
254. Cole, T.J., *Sympercents: symmetric percentage differences on the 100 loge scale simplify the presentation of log transformed data.* Statistics in Medicine, 2000. **19**(22): p. 3109-3125.
255. Ross, L.E., et al., *Measurement issues in postpartum depression part 1: Anxiety as a feature of postpartum depression.* Arch Women Ment Health, 2003. **6**.
256. Miller, R.L., J.F. Pallant, and L.M. Negri, *Anxiety and stress in the postpartum: Is there more to postnatal distress than depression?* BMC Psychiatry, 2006. **6**(1): p. 1-11.
257. Lee, E.-H., *Review of the Psychometric Evidence of the Perceived Stress Scale.* Asian Nursing Research, 2012. **6**(4): p. 121-127.
258. Wong, I.F.H., *English in Malaysia*, in *English for Cross-Cultural Communication*, L.E. Smith, Editor. 1981, Palgrave Macmillan UK: London. p. 94-107.
259. Feldman, R., et al., *Skin-to-skin contact (kangaroo care) promotes self-regulation in premature infants: Sleep-wake cyclicity, arousal modulation, and sustained exploration.* Developmental Psychology, 2002. **38**(2): p. 194-207.
260. Feldman, R. and A.I. Eidelman, *Skin-to-skin contact (Kangaroo Care) accelerates autonomic and neurobehavioural maturation in preterm infants.* Developmental Medicine & Child Neurology, 2003. **45**(04): p. 274-281.
261. Ferber, S.G. and I.R. Makhoul, *The Effect of Skin-to-Skin Contact (Kangaroo Care) Shortly After Birth on the Neurobehavioral Responses of the Term Newborn: A Randomized, Controlled Trial.* Pediatrics, 2004. **113**(4): p. 858-865.
262. Ludington-Hoe, S.M., et al., *Neurophysiologic Assessment of Neonatal Sleep Organization: Preliminary Results of a Randomized, Controlled Trial of Skin Contact With Preterm Infants.* Pediatrics, 2006. **117**(5): p. e909-e923.
263. Schaffer, L., et al., *Guided Imagery: An Innovative Approach to Improving Maternal Sleep Quality.* The Journal of Perinatal & Neonatal Nursing, 2013. **27**(2): p. 151-159.
264. Akgün Şahin, Z. and N. Dayapoğlu, *Effect of progressive relaxation exercises on fatigue and sleep quality in patients with chronic obstructive lung disease (COPD).* Complementary Therapies in Clinical Practice, 2015. **21**(4): p. 277-281.
265. Innes, K.E., et al., *Effects of Meditation versus Music Listening on Perceived Stress, Mood, Sleep, and Quality of Life in Adults with Early Memory Loss: A Pilot Randomized Controlled Trial.* J Alzheimers Dis, 2016. **52**(4): p. 1277-98.

266. Blanaru, M., et al., *The effects of music relaxation and muscle relaxation techniques on sleep quality and emotional measures among individuals with posttraumatic stress disorder.* Mental Illness, 2012. **4**(2): p. e13.
267. Jespersen, K.V. and P. Vuust, *The Effect of Relaxation Music Listening on Sleep Quality in Traumatized Refugees: A Pilot Study.* J Music Ther, 2012. **49**(2): p. 205-29.
268. Ziv, N., et al., *The effect of music relaxation versus progressive muscular relaxation on insomnia in older people and their relationship to personality traits.* J Music Ther, 2008. **45**(3): p. 360-80.
269. McKinney, C., et al., *The Effect of Selected Classical Music and Spontaneous Imagery on Plasma β-Endorphin.* Journal of Behavioral Medicine, 1997. **20**(1): p. 85-99.
270. Zanardo, V., et al., *Beta Endorphin Concentrations in Human Milk.* Journal of Pediatric Gastroenterology and Nutrition, 2001. **33**(2): p. 160-164.
271. Parmelee, A.H., W.H. Wenner, and H.R. Schulz, *Infant sleep patterns: From birth to 16 weeks of age.* The Journal of Pediatrics, 1964. **65**(4): p. 576-582.
272. Wooding, A.R., J. Boyd, and D.C. Geddis, *Sleep patterns of New Zealand infants during the first 12 months of life.* Journal of Paediatrics and Child Health, 1990. **26**(2): p. 85-88.
273. Lam, J., et al., *Electronic and paper diary recording of infant and caregiver behaviors.* J Dev Behav Pediatr, 2010. **31**(9): p. 685-93.
274. Ong, K.K. and R.J.F. Loos, *Rapid infancy weight gain and subsequent obesity: Systematic reviews and hopeful suggestions.* Acta Pædiatrica, 2006. **95**(8): p. 904-908.
275. Monteiro, P.O.A. and C.G. Victora, *Rapid growth in infancy and childhood and obesity in later life – a systematic review.* Obesity Reviews, 2005. **6**(2): p. 143-154.
276. WHO Multicentre Growth Reference Study Group, *WHO Child Growth Standards based on length/height, weight and age.* Acta Paediatr Suppl, 2006. **450**: p. 76-85.
277. Júlíusson, P.B., et al., *Growth of Belgian and Norwegian children compared to the WHO growth standards: prevalence below −2 and above +2 SD and the effect of breastfeeding.* Archives of Disease in Childhood, 2011. **96**(10): p. 916-921.
278. Roelants, M., R. Hauspie, and K. Hoppenbrouwers, *Breastfeeding, growth and growth standards: Performance of the WHO growth standards for monitoring growth of Belgian children.* Annals of Human Biology, 2010. **37**(1): p. 2-9.
279. Julia, M., *Adoption of the WHO Child Growth Standards to Classify Indonesian Children under 2 Years of Age According to Nutrition Status: Stronger Indication for Nutritional Intervention.* Food and Nutrition Bulletin, 2009. **30**(3): p. 254-259.
280. de Onis, M., et al., *Comparison of the World Health Organization (WHO) Child Growth Standards and the National Center for Health Statistics/WHO international growth reference: implications for child health programmes.* Public Health Nutrition, 2006. **9**(07): p. 942-947.
281. WHO Multicentre Growth Reference Study Group, *Enrolment and baseline characteristics in the WHO Multicentre Growth Reference Study.* Acta Paediatr Suppl, 2006. **450**: p. 7-15.
282. Gale, C., et al., *Effect of breastfeeding compared with formula feeding on infant body composition: a systematic review and meta-analysis.* The American Journal of Clinical Nutrition, 2012.
283. Tu, M.T., S.J. Lupien, and C.-D. Walker, *Diurnal salivary cortisol levels in postpartum mothers as a function of infant feeding choice and parity.* Psychoneuroendocrinology, 2006. **31**(7): p. 812-824.
284. Gonzalez, A., et al., *The relation between early life adversity, cortisol awakening response and diurnal salivary cortisol levels in postpartum women.* Psychoneuroendocrinology, 2009. **34**(1): p. 76-86.

285. Taylor, A., et al., *Diurnal pattern of cortisol output in postnatal depression.* Psychoneuroendocrinology, 2009. **34**(8): p. 1184-1188.
286. Andersson, E. and I. Hildingsson, *Mother's postnatal stress: an investigation of links to various factors during pregnancy and post-partum.* Scandinavian Journal of Caring Sciences, 2015: p. 1-8.
287. Clout, D. and R. Brown, *Sociodemographic, pregnancy, obstetric, and postnatal predictors of postpartum stress, anxiety and depression in new mothers.* Journal of Affective Disorders, 2015. **188**: p. 60-67.
288. Ngai, F.-W. and S.-F. Ngu, *Predictors of maternal and paternal depressive symptoms at postpartum.* Journal of Psychosomatic Research, 2015. **78**(2): p. 156-161.
289. Grekin, R. and M.W. O'Hara, *Prevalence and risk factors of postpartum posttraumatic stress disorder: A meta-analysis.* Clinical Psychology Review, 2014. **34**(5): p. 389-401.
290. Billard, H., et al., *Calibration Adjustment of the Mid-infrared Analyzer for an Accurate Determination of the Macronutrient Composition of Human Milk.* Journal of Human Lactation, 2016. **32**(3): p. NP19-NP27.
291. Casadio, Y.S., et al., *Evaluation of a Mid-Infrared Analyzer for the Determination of the Macronutrient Composition of Human Milk.* Journal of Human Lactation, 2010. **26**(4): p. 376-383.
292. Fusch, G., et al., *Rapid measurement of macronutrients in breast milk: How reliable are infrared milk analyzers?* Clinical Nutrition, 2015. **34**(3): p. 465-476.
293. Menjo, A., et al., *Bedside analysis of human milk for adjustable nutrition strategy.* Acta Pædiatrica, 2009. **98**(2): p. 380-384.
294. French, S.A., et al., *Eating behavior dimensions. Associations with energy intake and body weight. A review.* Appetite, 2012. **59**(2): p. 541-549.
295. Putnam, S.P., L.K. Ellis, and M.K. Rothbart, *The structure of temperament from infancy through adolescence.* Advances in research on temperament, 2001: p. 165-182.
296. Thomas, A., S. Chess, and S.J. Korn, *The Reality of Difficult Temperament.* Merrill-Palmer Quarterly, 1982. **28**(1): p. 1-20.
297. Burton, P., et al., *Association between infant correlates of impulsivity - surgency (extraversion) - and early infant growth.* Appetite, 2011. **57**(2): p. 504-509.
298. Losonczy-Marshall, M., *Stability in temperament and emotional expression in 1- to 3-year-old children.* Social Behavior and Personality, 2014. **42**(9): p. 1421-1430.
299. Mallan, K.M., L.A. Daniels, and S.J. de Jersey, *Confirmatory factor analysis of the Baby Eating Behaviour Questionnaire and associations with infant weight, gender and feeding mode in an Australian sample.* Appetite, 2014. **82**: p. 43-49.
300. Andreas, N.J., et al., *Effect of Maternal Body Mass Index on Hormones in Breast Milk: A Systematic Review.* PLoS ONE, 2014. **9**(12): p. e115043.
301. Kon, I.Y., et al., *The study of breast milk IGF-1, leptin, ghrelin and adiponectin levels as possible reasons of high weight gain in breast-fed infants.* Annals of Nutrition & Metabolism, 2014. **65**(4): p. 317-23.
302. Farooqi, I.S., et al., *Leptin Regulates Striatal Regions and Human Eating Behavior.* Science, 2007. **317**(5843): p. 1355-1355.
303. Savino, F., et al., *Leptin levels in breast-fed and formula-fed infants.* Acta Pædiatrica, 2002. **91**(9): p. 897-902.
304. Savino, F., et al., *Breast-fed Infants have Higher Leptin Values than Formula-fed Infants in the First Four Months of Life*, in *Journal of Pediatric Endocrinology and Metabolism*. 2004. p. 1527.
305. Savino, F., et al., *Ghrelin, leptin and IGF-I levels in breast-fed and formula-fed infants in the first years of life.* Acta Pædiatrica, 2005. **94**(5): p. 531-537.
306. Kirchner, H., et al., *GOAT links dietary lipids with the endocrine control of energy balance.* Nat Med, 2009. **15**(7): p. 741-745.

307. Liu, J., et al., *Novel Ghrelin Assays Provide Evidence for Independent Regulation of Ghrelin Acylation and Secretion in Healthy Young Men.* The Journal of Clinical Endocrinology & Metabolism, 2008. **93**(5): p. 1980-1987.
308. Weikel, J.C., et al., *Ghrelin promotes slow-wave sleep in humans.* American Journal of Physiology - Endocrinology and Metabolism, 2003. **284**(2): p. E407-E415.
309. Kotronoulas, G., A. Stamatakis, and F. Stylianopoulou, *Hormones, hormonal agents, and neuropeptides involved in the neuroendocrine regulation of sleep in humans.* Hormones (Athens), 2009. **8**(4): p. 232-48.
310. Schuessler, P., et al., *Nocturnal ghrelin levels – relationship to sleep EEG, the levels of growth hormone, ACTH and cortisol – and gender differences.* Journal of Sleep Research, 2005. **14**(4): p. 329-336.
311. Frieboes, R.M., et al., *Growth Hormone-Releasing Peptide-6 Stimulates Sleep, Growth Hormone, ACTH and Cortisol Release in Normal Man.* Neuroendocrinology, 1995. **61**(5): p. 584-589.
312. Copinschi, G., et al., *Prolonged oral treatment with MK-677, a novel growth hormone secretagogue, improves sleep quality in man.* Neuroendocrinology, 1997. **66**(4): p. 278-86.
313. Rothbart, M.K., *Temperament, Development, and Personality.* Current Directions in Psychological Science, 2007. **16**(4): p. 207-212.
314. Rothbart, M.K. and S.A. Ahadi, *Temperament and the Development of Personality.* Journal of Abnormal Psychology 1994. **103**(1): p. 55-66.
315. Wells, J.C.K. and J.T. Stock, *Re-examining heritability: genetics, life history and plasticity.* Trends in Endocrinology & Metabolism, 2011. **22**(10): p. 421-428.
316. Slining, M., et al., *Infant temperament contributes to early infant growth: A prospective cohort of African American infants.* International Journal of Behavioral Nutrition and Physical Activity, 2009. **6**(1): p. 51.
317. Else-Quest, N.M., et al., *Gender differences in temperament: A meta-analysis.* Psychological Bulletin, 2006. **132**(1): p. 33-72.
318. Rothbart, M.K., *Measurement of temperament in infancy.* Child development, 1981: p. 569-578.
319. Wells, J.C.K., et al., *Investigation of the relationship between infant temperament and later body composition.* International journal of obesity, 1997. **21**(5): p. 400-406.
320. Wells, J.C.K. and P.S. Davies, *Relationship between behavior and energy expenditure in 12-week-old infants.* American Journal of Human Biology, 1996. **8**(4): p. 465-472.
321. Darlington, A.-S.E. and C.M. Wright, *The Influence of Temperament on Weight Gain in Early Infancy.* Journal of Developmental & Behavioral Pediatrics, 2006. **27**(4): p. 329-335.
322. Carey, W.B., *Temperament and Increased Weight Gain in Infants.* Journal of Developmental & Behavioral Pediatrics, 1985. **6**(3): p. 128-131.
323. Niegel, S., E. Ystrom, and M.E. Vollrath, *Is Difficult Temperament Related to Overweight and Rapid Early Weight Gain in Infants? A Prospective Cohort Study.* Journal of Developmental & Behavioral Pediatrics, 2007. **28**(6): p. 462-466.
324. Carnell, S. and J. Wardle, *Appetitive traits and child obesity: measurement, origins and implications for intervention.* Proceedings of the Nutrition Society, 2008. **67**(4): p. 343-355.
325. Carnell, S. and J. Wardle, *Appetite and adiposity in children: evidence for a behavioral susceptibility theory of obesity.* The American Journal of Clinical Nutrition, 2008. **88**(1): p. 22-29.
326. Sleddens, E.F., S.P. Kremers, and C. Thijs, *The Children's Eating Behaviour Questionnaire: factorial validity and association with Body Mass Index in Dutch*

children aged 6–7. International Journal of Behavioral Nutrition and Physical Activity, 2008. **5**(1): p. 1-9.
327. Wardle, J., et al., *Development of the Children's Eating Behaviour Questionnaire.* Journal of Child Psychology and Psychiatry, 2001. **42**(7): p. 963-970.
328. Shepard, D.N. and P.C. Chandler-Laney, *Prospective associations of eating behaviors with weight gain in infants.* Obesity, 2015. **23**(9): p. 1881-1885.
329. Parkinson, K.N., et al., *Do maternal ratings of appetite in infants predict later Child Eating Behaviour Questionnaire scores and body mass index?* Appetite, 2010. **54**(1): p. 186-190.
330. Quah, P.L., et al., *Prospective associations of appetitive traits at 3 and 12 months of age with body mass index and weight gain in the first 2 years of life.* BMC Pediatrics, 2015. **15**(1): p. 1-10.
331. van Jaarsveld, C.M., et al., *Appetite and growth: A longitudinal sibling analysis.* JAMA Pediatrics, 2014. **168**(4): p. 345-350.
332. van Jaarsveld, C.M., et al., *Prospective associations between appetitive traits and weight gain in infancy.* The American Journal of Clinical Nutrition, 2011. **94**(6): p. 1562-1567.
333. Llewellyn, C.H., et al., *Inherited behavioral susceptibility to adiposity in infancy: a multivariate genetic analysis of appetite and weight in the Gemini birth cohort.* Am J Clin Nutr, 2012. **95**(3): p. 633-9.
334. Schuster, S., et al., *Leptin in Maternal Serum and Breast Milk: Association With Infants' Body Weight Gain in a Longitudinal Study Over 6 Months of Lactation.* Pediatr Res, 2011. **70**(6): p. 633-637.
335. Llewellyn, C.H., et al., *Nature and nurture in infant appetite: analysis of the Gemini twin birth cohort.* The American Journal of Clinical Nutrition, 2010. **91**(5): p. 1172-1179.
336. Agras, W.S., et al., *Influence of early feeding style on adiposity at 6 years of age.* The Journal of Pediatrics, 1990. **116**(5): p. 805-809.
337. Stunkard, A.J., et al., *Predictors of body size in the first 2 yr of life: a high-risk study of human obesity.* Int J Obes Relat Metab Disord, 2004. **28**(4): p. 503-513.
338. Prentice, P., et al., *Breast milk nutrient content and infancy growth.* Acta Paediatrica, 2016. **105**(6): p. 641-647.
339. Alderete, T.L., et al., *Associations between human milk oligosaccharides and infant body composition in the first 6 mo of life.* The American Journal of Clinical Nutrition, 2015. **102**(6): p. 1381-1388.
340. Carranza Carnicero, J.A., et al., *A longitudinal study of temperament in infancy: stability and convergence of measures.* European Journal of Personality, 2000. **14**(1): p. 21-37.
341. Robert, K.A. and S. Braun, *Milk Composition during Lactation Suggests a Mechanism for Male Biased Allocation of Maternal Resources in the Tammar Wallaby Macropus eugenii.* PLoS ONE, 2012. **7**(11): p. e51099.
342. Hinde, K., et al., *Daughter dearest: Sex-biased calcium in mother's milk among rhesus macaques.* American Journal of Physical Anthropology, 2013. **151**(1): p. 144-150.
343. Rogowitz, G., *Trade-offs in Energy Allocation During Lactation.* American Zoologist, 1996. **36**(2): p. 197-204.
344. Hinde, C.A., R.A. Johnstone, and R.M. Kilner, *Parent-Offspring Conflict and Coadaptation.* Science, 2010. **327**(5971): p. 1373-1376.
345. Roberts, S.B., T.J. Cole, and W.A. Coward, *Lactational performance in relation to energy intake in the baboon.* The American Journal of Clinical Nutrition, 1985. **41**(6): p. 1270-6.

346. Zera, A.J. and L.G. Harshman, *The Physiology of Life History Trade-Offs in Animals.* Annual Review of Ecology and Systematics, 2001. **32**: p. 95-126.
347. Wells, J.C.K., *Adaptive variability in the duration of critical windows of plasticity: Implications for the programming of obesity.* Evolution, Medicine, and Public Health, 2014. **2014**(1): p. 109-121.
348. Wells, J.C.K., *Maternal capital and the metabolic ghetto: An evolutionary perspective on the transgenerational basis of health inequalities.* American Journal of Human Biology, 2010. **22**(1): p. 1-17.
349. Fan, X., B. Thompson, and L. Wang, *Effects of sample size, estimation methods, and model specification on structural equation modeling fit indexes.* Structural Equation Modeling: A Multidisciplinary Journal, 1999. **6**(1): p. 56-83.
350. Wells, J.C.K., et al., *Maternal investment, life-history strategy of the offspring and adult chronic disease risk in South Asian women in the UK.* Evolution, Medicine, and Public Health, 2016. **2016**(1): p. 133-145.
351. Proos, L.A., *Growth & development of Indian children adopted in Sweden.* Indian J Med Res, 2009. **130**(5): p. 646-650.

APPENDICES

1. Ethics Approval (UCL)
2. Ethics Approval (Ministry of Health Malaysia)
3. Abstract publications & conference presentations
4. MOM Study Consent form
5. MOM Study Info Sheet & Advertisement poster
6. MOM Study questionnaires (6.1 – 6.14)
7. Isotope procedures
8. SPSS result : Chi-Square test (Chapter 4)
9. SPSS result : Correlation between milk macronutrient and growth (Chapter 6)

UCL RESEARCH ETHICS COMMITTEE
GRADUATE SCHOOL OFFICE

Dr Mary Fewtrell
Childhood Nutrition Research Centre
Institute of Child Health
30 Guilford Street
UCL

16 December 2013

Dear Dr Fewtrell

Notification of Ethical Approval
Project ID: 4883/001: Mother-infant signalling during breastfeeding: a randomised controlled trial investigating the effects of a relaxation intervention in breastfeeding mothers on breast milk production, breast milk cortisol and infant behaviour and growth

I am pleased to confirm that your study has been approved by the UCL Research Ethics Committee for the duration of the project i.e. **until September 2015** to include the following changes to the actual study procedures from the original version of the protocol:

- Collection of infant urine as opposed to saliva.
- Removal of the doubly-labelled water measurement at 12wks and instead repeat the measurement of milk intake using the dose-to-mother technique with deuterium (already being administered at 2wks) and then dose the infant with deuterium 2wks later to measure his/her body composition.

Approval is subject to the following conditions:

1. You must seek Chair's approval for proposed amendments to the study for which this approval has been given. Ethical approval is specific to this project and must not be treated as applicable to research of a similar nature. Each research project is reviewed separately and if there are significant changes to the research protocol you should seek confirmation of continued ethical approval by completing the 'Amendment Approval Request Form'.

The form identified above can be accessed by logging on to the ethics website homepage: http://www.grad.ucl.ac.uk/ethics/ and clicking on the button marked 'Key Responsibilities of the Researcher Following Approval'.

2. It is your responsibility to report to the Committee any unanticipated problems or adverse events involving risks to participants or others. Both non-serious and serious adverse events must be reported.

   Reporting Non-Serious Adverse Events
   For non-serious adverse events you will need to inform Helen Dougal, Ethics Committee Administrator (ethics@ucl.ac.uk), within ten days of an adverse incident occurring and provide a full written report that should include any amendments to the participant information sheet and study protocol. The Chair or Vice-Chair of the Ethics Committee will confirm that the incident is non-serious and report to the Committee at the next meeting. The final view of the Committee will be communicated to you.

Reporting Serious Adverse Events
The Ethics Committee should be notified of all serious adverse events via the Ethics Committee Administrator immediately the incident occurs. Where the adverse incident is unexpected and serious, the Chair or Vice-Chair will decide whether the study should be terminated pending the opinion of an independent expert. The adverse event will be considered at the next Committee meeting and a decision will be made on the need to change the information leaflet and/or study protocol.

On completion of the study you must submit a brief report (a maximum of two sides of A4) of your findings/concluding comments to the Committee, which includes in particular issues relating to the ethical implications of the research.

With best wishes for the research.

Yours sincerely

[redacted]

Professor John Foreman
Chair of the UCL Research Ethics Committee

Cc:
Nurul Shukri & Professor Jonathan Wells, Applicants
Professor Atul Singhal

UCL Research Ethics Committee, c/o The Graduate School, North Cloisters, Wilkins Building
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ethics@ucl.ac.uk
www.ucl.ac.uk/gradschool

JAWATANKUASA ETIKA & PENYELIDIKAN PERUBATAN
(Medical Research & Ethics Committee)
KEMENTERIAN KESIHATAN MALAYSIA
d/a Institut Pengurusan Kesihatan
Jalan Rumah Sakit, Bangsar
59000 Kuala Lumpur

Tel. : 03 2282 9082/03 2282 9085
03 2287 4032/03 2282 0491
Faks : 03 2287 4030

Ref: ( ) dlm.KKM/NIHSEC/Jld 3.P13-957
Date : 2nd December 2013

NMRR-13-841-16720
MOTHER-INFANT SIGNALLING DURING BREASTFEEDING: A RANDOMISED TRIAL INVESTIGATING THE EFFECTS OF A RELAXATION INTERVENTION IN BREASTFEEDING MOTHERS ON BREAST MILK PRODUCTION, BREAST MILK CORTISOL AND INFANT BEHAVIOUR AND GROWTH.

Principal Investigator: Nurul Husna Mohd Shukri
Childhood Nutrition Research Centre
Institute of Child Health
University of College London (UCL)

Documents received and reviewed with reference to the above study:

1. Response letter to MREC dated 14th November 2013.
2. Revised Protocol version 2 dated 14th November 2013.
3. Patient information sheet (English) & Informed Consent Form (English) version 2 dated 14th November 2013.
4. Patient information sheet (Malay) & Informed Consent Form (Malay) version 2 dated 14th November 2013.
5. CV and IA-HOD-IA form of all investigators.

The Medical Research & Ethics Committee, Ministry of Health Malaysia operates in accordance to the International Conference of Harmonization Good Clinical Practice Guidelines.

Project Sites: Klinik Ibu dan Anak Cheras Baru, Klinik Kesihatan Bandar Baru Bangi, Klinik Kesihatan Ibu Dan Anak Kampung Pandan, Klinik Kesihatan Sungai Chua, Klinik Kesihatan Ampang, Klinik Kesihatan Bandar Seri Putra, Klinik Kesihatan Kajang

Comment: Please note that the approval is valid until 2nd December 2014. To renew the approval, a completed 'Continuing Review Form' (Appendix 1) has to be submitted to MREC at least 2 months before the expiry of the approval.

Decision by Medical Research & Ethics Committee:
(√) Approved
( ) Disapproved

Date of Decision: 2nd December 2013

[redacted]

(DATO' DR CHANG KIAN MENG)
Chairman
Medical Research & Ethics Committee
Ministry of Health

Academic contributions during my PhD study

Abstract publications :

- **Shukri, N. H. M.,** Wells, J., Mukhtar, F., & Fewtrell, M. (2016). A randomised trial to test the effectiveness of maternal relaxation therapy during breastfeeding: Effects on infant behaviour. *Journal of Pediatric Gastroenterology and Nutrition, 62*, 662.
- **Shukri, N. H.,** Wells, J., Mukhtar, F., Lee, M. S., & Fewtrell, M. (2016). Mother-infant signalling during breastfeeding: results of a randomised trial investigating the effects of relaxation therapy in breastfeeding mothers on maternal stress and infant growth & behaviour. Breastfeeding Medicine, 11(2), A16.

  http://online.liebertpub.com/doi/pdfplus/10.1089/bfm.2016.28999.abstracts?src=recsys
- **Shukri, N. H.,** Wells, J., Mukhtar, F., Lee, M. S., & Fewtrell, M. (2016). Knowledge and perception of breastfeeding among primiparous healthy pregnant women in Klang-valley, Malaysia. Breastfeeding Medicine, 11(2), A65.
- **Shukri, N. H.,** Wells, J., Mukhtar, F., Lee, M. S., & Fewtrell, M. (2015). Mother-infant signalling during breastfeeding: A randomised trial investigating the effects of a relaxation intervention in breastfeeding mothers on breast milk production, breast milk cortisol and infant behaviour and growth. *Maternal & Child Nutrition, 11 (S2)*, 110.
- **Shukri N.H.,** Fewtrell M., Wells J., Mukhtar F., & Lee, M.S. (2015). Mother-Infant signalling during breastfeeding: An ongoing investigation of physiological and psychological factors during breastfeeding to study causal relationship of maternal psychological state on breast milk production, cortisol levels, infant behaviour & growth. Malaysian Journal of Nutrition, 21(Supp), S47-S48 (Abstract).

Submitted for publications :

- **Shukri, N. H.,** Wells, J., Mukhtar, F., Lee, M. S., & Fewtrell, M. (2016). Study protocol: An investigation of mother-infant signalling during breastfeeding using a randomised trial to test the effectiveness of breastfeeding relaxation therapy on maternal psychological state, breast milk production and infant behaviour and growth, International Breastfeeding Journal (in review).

Oral presentations in conferences :

- **Nurul Husna MS**, Mokhtar F, Huang MSL, Wells J & Fewtrell M. A randomised trial to test the effectiveness of maternal relaxation therapy during breastfeeding: effects on infant behavior**.** 49th Annual Congress of ESPGHAN, 25-28 May 2016, Greece.
- **Nurul Husna MS**, Mokhtar F, Huang MSL, Wells J & Fewtrell M. A randomised trial to test parent-offspring conflict theory during the lactation period by manipulating maternal psychological state using relaxation therapy. 3rd International Conference on Nutrition & Growth, 17-19 March 2016, Vienna, Austria.
- **Nurul Husna MS**, Mokhtar F, Huang MSL, Wells J & Fewtrell M. Mother-infant signalling during breastfeeding: Results of a randomised trial investigating the effects of relaxation therapy in breastfeeding mothers on maternal stress and infant growth and behaviour. International Society for Research in Human Milk and Lactation (ISRHML), 3-7 March 2016, Cape Town, S.Africa
- **Nurul Husna MS**, Fewtrell M & Wells J. Mother-infant signalling during breastfeeding: A randomised trial investigating the effects of a relaxation intervention in breastfeeding mothers on breast milk production, breast milk cortisol and infant behaviour & growth, PhD Upgrade Seminar, Institute of Child Health UCL, 6 November 2013, London, UK.
- **Nurul Husna MS,** Fewtrell M & Wells J. Mother-Infant Signalling During Breastfeeding: Associations between Breast Milk Composition, Hormone Levels in Breast Milk and Infant Behaviour and Temperament: Background. ESPGHAN Summer School on Nutrition, 20 June-5 July 2013, Prague.

Poster presentations in conferences :

i) **Nurul Husna MS,** Mokhtar F, Huang MSL, Wells J & Fewtrell M. Knowledge and perception of breastfeeding among primiparous healthy pregnant women in Klang-Valley, Malaysia. International Society for Research in Human Milk and Lactation (ISRHML), 3-7 March 2016, Cape Town, South Africa.
ii) **Nurul Husna MS,** Wells J & Fewtrell M. The association of the psychological state of breastfeeding mothers with infant appetite and growth. 3rd International Conference on Nutrition & Growth, 17-19, 2016 March, Vienna, Austria.
iii) **Nurul Husna MS,** Mokhtar F, Huang MSL, Wells J & Fewtrell M. Mother-infant signalling during breastfeeding: An investigation of physiological and psychological factors during breastfeeding, including an ongoing randomised trial investigating the effects of a relaxation intervention in breastfeeding mothers on breast milk production, breast milk cortisol and infant behaviour & growth. Nutrition & Nurture in Infancy & Childhood Conference, 10-12 June 2015, UK.
iv) **Nurul Husna MS,** Mokhtar F, Huang MSL, Wells J & Fewtrell M. An ongoing investigation of physiological and psychological factors during breastfeeding to study causal relationship of maternal psychological state on breast milk production, cortisol levels, infant behaviour & growth. Asia Pacific Conference on Clinical Nutrition (APCCN) 2015, 26-29th January 2015, KL, Malaysia.
v) **Nurul Husna MS,** Mokhtar F, Huang MSL, Wells J & Fewtrell M. Maternal perceptions of a Malay version of a breastfeeding relaxation therapy recording: A Pilot Study. Poster presented at 20th Malaysian Dietitians Association Scientific Conference 2014, 20-21 June 2014, KL, Malaysia.
vi) **Nurul Husna MS** and Brough L (2012). Mandatory iodine fortification in bread: is it enough to eliminate iodine deficiency in New Zealand? Poster presented at 27th Scientific Conference of Nutrition Society of Malaysia, 24-25th May 2012, Kuala Lumpur, Malaysia.
vii) **Nurul Husna MS** and Brough L (2011). Dietary iodine intake and iodine nutritional knowledge of women of childbearing-age in Palmerston North, New Zealand. Poster presented at Malaysian Dietitians Association Scientific Conference & 17th AGM; Bridging Gaps in Nutrition Care for the Community, 20th-22nd July 2011, Kuching, Malaysia
viii) **Nurul Husna MS** and Brough L (2011). Iodine status of women of childbearing-age in Palmerston North, NZ after mandatory fortification of bread with iodised salt. Poster presented at 26th Scientific Conference of Nutrition Society of Malaysia, 24-25th March 2011, KL, Malaysia.
ix) **Nurul Husna MS** and Ramlah, R (2008). Breakfast habit among adolescent in Kulim, Kedah. Poster presented at 23rd Scientific Conference & AGM Nutrition Society of Malaysia, 25-26 March 2008, Kuala Lumpur, Malaysia.

Achievements and Awards

- The European Society for Paediatric Gastroenterology Hepatology and Nutrition (ESPGHAN) Young Investigator Award 2016 (500 EURO)
- The International Society for Research in Human Milk and Lactation (ISRHML) Trainee Travel Awards 2016 (1000 USD)
- UCL Institute of Child Health Travel Award Grant 2016 (500 GBP)
- Poster competition prize – UCL Institute of Child Health 2015

Supervising an MSc student (2016)

MSc student (MSc in Clinical and Public Health Nutrition), 2016 : Sarah Dib
Dissertation: The influence of hospital practices and family support on breastfeeding outcomes.

MOMS
Mother-Offspring Milk study

MRC Childhood Nutrition Research Centre
Institute of Child Health, 30 Guilford Street
London WC1N 1EH

Title of project:

Mother-infant signalling during breastfeeding: A study investigating breast milk production, breast milk composition, infant behaviour and growth.

Name of Investigators and Institutions:
Dr Mary Fewtrell, Prof. Jonathan Wells, UCL Institute of Child Health (ICH)
Dr Mary Huang Soo Lee, Universiti Putra Malaysia (UPM)
Nurul Husna Mohd Shukri, UCL ICH, UPM

Participant Information Sheet

We would like to invite you and your baby to take part in our research study. Before you decide whether you would like to take part, it is important for you to know why the research is being done and what it will involve. Please take time to read this information sheet carefully and discuss it with others if you wish. If there is anything that is not clear, or if you would like more information, please feel free to contact us. After you are properly satisfied that you understand this study, and that you wish to participate, we will ask you to sign the informed consent form.

What is the purpose of the study?

Breastfeeding involves communication between mother and baby. The baby can 'signal' his/her needs to the mother by his/her behaviour and the way in which s(he) feeds, and the mother can respond by producing different amounts of milk and altering the composition of the milk. We need to learn more about how this signalling works and understand what factors result in the most successful outcome for mother and baby. This study will investigate the relationships between the amount and composition of the breast milk a mother produces, including the hormones in breast milk such as cortisol, and the growth and behaviour of the baby. We hope that our findings will eventually be used to help mothers to breast-feed more successfully.

Breastfeeding provides short and longer-term health benefits to both infant and mother, and mothers are advised to exclusively breast-feed for the first 6 months. However, many mothers do not manage to breast-feed for as long as they would like, and breastfeeding rates in many countries are below target levels. Most attempts to improve breastfeeding rates focus on giving extra advice and support to mothers, yet there are a lot of things we do not fully understand about what makes breastfeeding successful. In order to give better advice and help to mothers it is important to study and understand better the relationships between the mother and baby during breastfeeding.

How is the study to be done?

We are inviting healthy women who are pregnant with their first baby and planning to exclusively breastfeed their baby for at least four months, to take part in this study. If you agree to take part in the study we will: (1) do a short interview with you before you deliver your baby (either at the antenatal clinic or at home (2) visit you at home when your baby is 2, 6 and 12 weeks of age. Each home visit will take about 2 hours. We will aim to visit during the morning and you will be able to choose a day which is convenient for you.

During the first interview we will:

1. Explain the study to you and answer all your questions. Then, we will ask you to sign a consent form.
2. Ask you some questions about your opinions on infant feeding and your plans for feeding your baby when (s)he is born.
3. Record your expected delivery date (EDD), and take your contact details so that we can contact you once your baby is born.
4. Give you a questionnaire to fill in during the week after your delivery, to give us information about the birth and your experience of feeding your baby in the first few days.

After you give birth we will contact you by phone (about 1 week after your EDD). If you still want to participate in the study, we will arrange for a home visit when your baby is 2 weeks old. (If you do not prefer to be contacted directly, we can contact your midwife to ask about your delivery).

What will be done during the first home visit?

During the first home visit we will do the following:

a) Ask you some questions to find out how you and your baby are getting on with breastfeeding, and about your baby's eating behaviour. We will also ask some information about your background, pregnancy and delivery.

b) Measure your baby's weight, length and head size.

c) Ask you to do a **'mini breastfeeding test'**:
This test will be done when your baby is ready for a feed so it fits in with your normal routine. Before you start feeding we will ask you to complete a short questionnaire about how you are feeling, and collect a saliva sample using a special swab which you can chew on for a few minutes. While you are doing this we will weigh your baby and collect a sample of his/her saliva using a special baby swab. We will measure the cortisol levels in the saliva which gives us an idea of how relaxed you and your baby are. We will ask you to express a small amount of breast milk before the feed; you can choose how to do this (by hand or using a breast pump – we can provide you with a hand pump if you don't have one). Then we will ask you to breastfeed your baby as normal, and we will record the length of the feed. We will leave you and your baby alone during the feed so as not to distract you. After the feed, we will ask you to repeat the measurements (that is, complete the questionnaire, collect a small breast milk and saliva sample). We will also measure your baby's weight again at the end of the feed so we can estimate how much milk (s)he has taken.

d) Measure the amount of milk your baby is taking each day. We can do this by giving you a drink containing heavy water molecules (deuterium) which acts as a marker so we can measure how much milk your baby takes from you. Deuterium is not harmful, and is naturally present in all humans. This method has been used safely in hundreds of mothers and babies. Firstly, we will give you a glass of plain water or juice containing the deuterium to drink. We will collect a sample of your baby's urine before you breastfeed your baby for the first time after the dosing. To do this, we will place cotton wool balls in the nappy to collect the urine. We will ask you to collect a saliva sample again at the 5 and 24 hours after the drink, and also anytime on day 4 and 14. We will also ask you to collect your baby's urine after 5 and 24 hours and on day 3, 4, 13 and 14. We will give you instructions on how to do this using cotton wool balls in the nappy. The samples can be kept in the fridge and will be collected by us.

e) We will leave a behaviour diary for you to fill in over a 3 day period to record your baby's behaviour. We will also ask you to fill in 3 questionnaires at your convenience which will measure different aspects of your mood and how you are feeling. The questionnaires are also available online and you can answer them online if you wish too.

At the end of the visit, we will arrange a date and time to visit you again when your baby is 6 weeks old for the second home visit.

2nd home visit when your baby is 6 weeks old

- We will do the same measurements and data collection on your second home visit, but without the deuterium measurement of breast milk intake. We will arrange a date and time to visit you when your baby is 12 weeks old for the 3rd and final home visit.

3rd home visit when your baby is 12 weeks old

- We will do the same measurements and data collection on your third home visit, but you will not be asked to complete the infant behaviour diary
- At this visit, we will ask you to complete a different infant behaviour questionnaire which is for babies from the age of 12 weeks.
- At this visit, we would like to measure your baby's body composition, milk intake and the energy content of the breast milk, using a doubly labelled water drink – this is like deuterium but also contains heavy oxygen molecules. We will collect a sample of your baby's urine, then give him/her the dose of water using a syringe. We will ask you to collect urine samples (after 5 and 24 hours and day 3, 4, 13 and 14) using the same method as you did after the first home visit.

Are there any risks?

We do not think there are any risks for you or your baby. All of the tests are painless and completely harmless. The isotope measurements have been done hundreds of times in very small babies and their mothers around the world.

What if something goes wrong?

The research project has been approved by the UCL Research Ethics Committee & Malaysia Medical Research Ethics Committee, which believes that it is of minimal risk to you and your baby. However, any research can carry unforeseen risks and we want you to be informed of your rights in the unlikely event that any harm should occur as a result of taking part in this project.

Who will have access to the research records?

Only the researchers will have access to the data collected during the study. The information and samples collected from you and your baby during the study will be identified only by a study number. Your personal information will only be used to keep in touch with you during the study and this will be kept securely in a locked cabinet for 10 years.

What will happen to our saliva, milk and urine samples?

The samples will be frozen until the study is finished and then analysed as described above. Any leftover milk sample will be frozen and, with your permission, could be used in future in an ethically approved research study. However, if you do not give your consent for us to store the milk sample, it will be destroyed.

Are there any benefits for me or my baby?

There are no direct benefits for you and your baby although you will have the opportunity to receive some additional support with breastfeeding, and we will be able to tell you how much breast milk your infant was receiving from the measurements at the 2 week and 12 week visits (the analysis will not be done until the end of the study). We will also provide you with a breast pump to express milk (or an alternative gift of you already have your own breast pump). It is hoped that this study will improve our understanding about how mothers and babies communicate during breastfeeding so that we can improve the advice we give to mothers in the future.

What will happen to the results of the research study?

The results of this research will be published in a medical journal and presented at scientific meetings. We will also send you a summary of the results at the end of the study.

Do I and my baby have to take part?

It is up to you to decide whether to take part or not. If you do decide to take part, you will be asked to sign a consent form. We will photocopy the consent form for you and keep one for our records. If you decide, now or at a later stage that you do not wish to participate in this study, that is entirely your right and will not in any way effect any present or future heath or medical care. If you withdraw, any data collected from you up to your withdrawal will still be used for the study after getting your permission first. If you refuse to take part or decide to withdraw, this will not affect your medical care.

UCL INSTITUTE OF CHILD HEALTH

MOMS
Mother-Offspring Milk study

UPM UNIVERSITI PUTRA MALAYSIA

Study Number:

Patient Identification Number for this trial:

PARTICIPANT CONSENT FORM

Title of Project: Mother-infant signalling during breastfeeding: A study investigating breast milk production, breast milk composition, infant behaviour and growth.

Name of Researcher: Nurul Husna M Shukri

Contact details: Phone: 0196768394 or Email: 2013moms@gmail.com or nurul.shukri.12@ucl.ac.uk

Thank you for your interest in taking part in this research.

Please initial box

1	I confirm that I have read and understand the information sheet dated **14 June 2013** (version 1.0) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily.	
2	I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected. If I withdraw from the study, I agree that the investigators can keep and use my data that has already been collected during the course of the study: **YES ( ) NO ( )**	
3	I understand that relevant sections of my or my child's data collected during the study may be looked at by responsible & authorised personnel from the study and regulatory authorities, where it is relevant to my taking part in this research. I give permission for these individuals to have access to our records.	
4	I understand that my and my child's samples are a gift, and agree that the samples can be stored anonymously and used by the custodian Dr Mary Fewtrell, UCL for future ethically approved research relating to infant feeding and as described in the attached information sheet.	
5	I agree to being contacted in the future about further study relating to infant feeding.	
6	I agree to my doctor or personal midwife being informed of my participation in the study.	
7	I agree to take part in the above study.	

Name of Participant	Date	Signature
Name of Person taking consent (if different from Investigator)	Date	Signature
Name of Investigator	Date	Signature

When completed: 1 copy for participant; 1 (original) for researcher file.

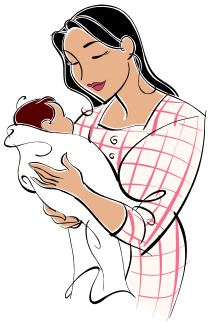
MOMS
Mother-Offspring Milk study

Would you like to take part in a study investigating:

➔ your breast milk volume and composition

➔ your baby's milk intake

➔ your baby's behaviour and growth?



- ➔ We are recruiting first time mothers aged **18 to 40 years old,** and their **babies** to participate in this study.
- ➔ We will do a short interview during the end of your pregnancy and then will visit you at your home when your baby is at **2, 6 and 12 weeks old**.
- ➔ We will ask you same questions about your thought and experiences about breastfeeding, and your **feelings** whilst you are breastfeeding.
- ➔ We will measure **how much milk you produce** and your milk composition
- ➔ We will also study your **baby's behaviour, milk intake and early growth.**
- ➔ We will provide you a **hand breast pump** & **a final report** about the study.

If you would like to take part or find out more about the study please contact:

Husna Shukri, UCL Institute of Child Health, & UPM.

Email : 2013moms@gmail.com

Tel: +60196768394

Version update: 16/08/2013

LIST OF QUESTIONNAIRES FOR MOM STUDY

No	Questionnaires / Forms	Language		Date/version	Stages
		ENG.	MALAY		
1	First Screen (Recruitment)	X		Ver 1 (01/08/13)	Recruitment
2	Demographic Q	X	X	Ver 1 (01/08/13)	Recruitment
3	Iowa Infant Feeding Attitude Scale (IIFAS)	X	X (v)	Ver 1 (01/08/13)	Recruitment
4	IIFAS Add	X	X	Ver 1 (01/08/13)	Recruitment
5	Neonatal Questionnaire - CDC	X	X	Ver 1 (01/08/13)	Recruitment / Home visit 1
6	Birth Screening Q	X		Ver 1 (01/08/13)	Screen 2 (After delivery)
7a	Infant Feeding Quest I (IFQ 1) – CDC	X		Ver 1 (01/08/13)	Home visit 1
7b	Infant Feeding Quest II (IFQ 2) – CDC/FF	X		Ver 1 (01/08/13)	Home visit 2
7c	Infant Feeding Quest III (IFQ 3) – CDC/FF	X		Ver 1 (01/08/13)	Home visit 3
8	Baby Eating Behaviour Q (BEBQ)	X	X	Ver 1 (01/08/13)	Home visit 1,2,3
9	Mini Breastfeeding test (Mini test)	X	X	Ver 1 (20/06/13)	Home visit 1,2,3
10	Baby behaviour/crying diary (BBD)	X	X	Ver 1 (01/08/13)	Home visit 1 & 2
11	Edinburgh Postnatal Depression Scale (EPDS)	X	X (v)	Ver 1 (01/08/13)	Home visit 1,2,3
12	Perceived Stress Scale (PSS)	X	X (v)	Ver 1 (01/08/13)	Home visit 1,2,3
13	Beck Anxiety Inventory (BAI)	X	X (v)	Ver 1 (01/08/13)	Home visit 1,2,3
14	Infant Behaviour Ques-Revised (IBQ-R)	X	X	Ver 1 (01/08/13)	Home visit 3

*** v :** validated

SCREENING QUESTIONNAIRE - Recruitment

Mother's initials : _____ Date: _____

No	Inclusion	State 'Yes'
1	First time pregnancy?	
2	Singleton?	
3	Free from serious illness / chronic disease? *(Category : Low risk - High risk?)*	
4	Planning to breastfeed exclusively (at least 4mo)	
5	Mother can communicate in English / Malay	
6	Mother is not planning to stay outside Selangor area during postpartum	
No	**Exclusion**	**State 'No'**
1	Does the mother on medication during pregnancy?	
2	Plan to give mixed feeding?	
3	Smoking ?	

Eligible to participate in the study? ----------> **YES ( )** **NO ( )**

Obtain consent form? ----------> YES ( ) NO ( )

Indicate EDD:

By calculation/ultrasound: ……..

Plan of place for delivery : ……

Plan of place to stay during postpartum : ……

Subject ID: _____ Date: _____

DEMOGRAPHIC QUESTIONNAIRE

1. D.O.B : [ ] / [ ] / [ ]

2. Age : [ ] years old

3. Ethnicity :

   Malay ( ) Chinese ( ) Indian ( ) Other Bumi ( ) Mixed : _____

4. Highest education level :

   No formal education ( )
   Primary school ( )
   Secondary school/ Pre-Uni (STPM/Matriculation) ( )
   Certificates/Diploma ( )
   Bachelor Degree / Advanced Diploma ( )
   Master degree / PhD /Doctorates ( )

5. Maternal occupation : …….. (please indicate)

   Employer (own/operate business, has employee) ( )
   Government sector ( )
   Private sector ( )
   Self employed ( )
   Unemployed / unpaid worker ( )

6. Paternal occupation : …….. (please indicate)

   Employer (own/operate business, has employee) ( )
   Government sector ( )
   Private sector ( )
   Self employed ( )
   Unemployed ( )

7. Household income :

   <RM1500 ( )
   RM 1500 – 3000 ( )
   RM3001 - 5000 ( )
   RM 5001 – 8000 ( )
   RM 8001 – 10 000 ( )
   > RM 10 000 ( )

8. Marital status :

Married ( )
Single ( )
Single, but living with partner ( )
Separated ( )
Widow ( )

9. Maternal birth order : _____

10. Age different from older sibling : _____

11. Age different from younger sibling : _____

12. Plan of place of delivery :

Public hospital( )
Private hospital( )
Home( )
Others( )

Name of the place: ……..

13. Who will be taking care of you during postpartum (tick 'X' that applies)

Husband ( )
Parents ( )
In-law's ( )
Sibling ( )
House maid ( )
Close relative/friend ( )
Self ( )

14. Who primarily will take care of you and your baby during postpartum?

Husband ( )
Parents ( )
In-law's ( )
Sibling ( )
House maid ( )
Close relative/friend ( )
Self ( )

15. Estimated of duration for confinement (if practice/apply) :

_____ days/ week(s)/ month(s)

16. How strong do you think you will practice confinement following to your cultural :

Very strong/strict ( )
Strong ( )
Medium ( )
Low ( )
None ( )

Subject ID: _____ Date: _____

The Iowa Infant Feeding Attitude Scale (IIFAS)

For each of the following statements, please indicate how much you agree or disagree by circling the number that most closely corresponds to your opinion. Choose any number from 1 to 5 :

(1 = Strongly disagree [SD], 2 = Disagree [D], 3 = Neutral [N], 4 = Agree [A], 5 = Strongly agree [SA]).

	Strongly disagree	Disagree	Neutral	Agree	Strongly agree
1. The nutritional benefits of breast milk last only until the baby is weaned from breast milk.	1	2	3	4	5
2. Formula-feeding is more convenient than breastfeeding.	1	2	3	4	5
3. Breastfeeding increases mother-infant bonding.	1	2	3	4	5
4. Breast milk is lacking in iron.	1	2	3	4	5
5. Formula-fed babies are more likely to be overfed than are breast-fed babies.	1	2	3	4	5
6. Formula-feeding is the better choice if a mother plans to work outside the home.	1	2	3	4	5
7. Mothers who formula-feed miss one of the great joys of motherhood.	1	2	3	4	5
8. Women should not breast-feed in public places such as restaurants.	1	2	3	4	5
9. Babies fed breast milk are healthier than babies who are fed formula.	1	2	3	4	5
10. Breast-fed babies are more likely to be overfed than formula-fed babies.	1	2	3	4	5
11. Fathers feel left out if a mother breast-feeds.	1	2	3	4	5
12. Breast milk is the ideal food for babies.	1	2	3	4	5
13. Breast milk is more easily digested than formula.	1	2	3	4	5
14. Formula milk is as healthy for an infant as breast milk.	1	2	3	4	5
15. Breastfeeding is more convenient than formula feeding.	1	2	3	4	5
16. Breast milk is less expensive than formula.	1	2	3	4	5
17. A mother who occasionally drinks alcohol should not breast-feed her baby.	1	2	3	4	5

A. Breast-feeding attitudes

1. Which of the following statements is closest to your opinion?
   The best way to feed a baby below 6 months old is:

   Breast-feeding ( )
   Formula feeding ( )
   A mix of both breast and formula feeding ( )
   Breast-feeding and formula feeding are equally good ways to feed a baby ( )

2. How strongly do you agree or disagree with the following statements?

STRONGLY AGREE…(1)	**SOMEWHAT AGREE…(2)**	**NEITHER AGREE NOR DISAGREE…(3)**	**SOMEWHAT DISAGREE…(4)**	**STRONGLY DISAGREE…(5)**	**NOT SURE..(6)**

   i. If a baby is breast-fed, he or she will be less likely to get ear infections....

      (1) ( ) **(2)** ( ) **(3)** ( ) **(4)** ( ) **(5)** ( ) Not sure -**(6)** ( )

   ii. If a baby is breast-fed he or she will be less likely to get a respiratory illness….

      (1) ( ) **(2)** ( ) **(3)** ( ) **(4)** ( ) **(5)** ( ) Not sure -**(6)** ( )

   iii. If a baby is breast-fed he or she will be less likely to get diarrhoea….

      (1) ( ) **(2)** ( ) **(3)** ( ) **(4)** ( ) **(5)** ( ) Not sure -**(6)** ( )

   iv. Babies should be exclusively breast-fed (fed only breast milk) for the first 4-6 months….

      (1) ( ) **(2)** ( ) **(3)** ( ) **(4)** ( ) **(5)** ( ) Not sure -**(6)** ( )

   v. If a child was breast-fed, he or she will be less likely to become obese….

      (1) ( ) **(2)** ( ) **(3)** ( ) **(4)** ( ) **(5)** ( ) Not sure -**(6)** ( )

B. Breast-feeding in future

3. How long do you plan to exclusively breastfeed your baby?

   _____ weeks/ months

4. How old do you think your baby will be when you first feed him or her formula or any other food besides breast milk?

   3 to 4 months ( )
   5 to 6 months ( )
   7 to 9 months ( )
   More than 9 months ( )

5. Do you plan to continue breast-feeding after you return to work?

   Yes ( )
   No ( )
   Do not plan to work after the baby's birth ( )

6. How old do you think your baby will be when you completely stop breastfeeding?

   _____ months/years

7. Using 1 to mean 'Not at all confident' to 5 as 'Very confident', how confident are you that you will be able to breastfeed until the baby is the age that you indicate above (Q17)?

   Not at all confident (1) (2) (3) (4) (5) Very confident

Subject ID: _____ Date: _____

NEONATAL QUESTIONNAIRE

1. What was your baby's weight and length at birth? ☐☐☐☐ g ☐☐☐ cm

2. When you were pregnant, did you attend any classes that discussed breastfeeding your baby?

   (Please "x" all that apply)

   Yes, a class on breastfeeding .....( )
   Yes, a child birth or baby care class that included breastfeeding .....( )
   No.....( )
   No, I just read from book/magazine, or get information from the Internet....( )

3. Which type of health professional was your birth attendant?

   An obstetrician ( )
   A family doctor, general practitioner, internist, or other physician ( )
   A midwife or nurse midwife ( )
   No health professional was present ( )

4. Other than the medical staff, who was with you during your labor? **(Please "x" all that apply)**

   The baby's father ( )
   Your mother/father/siblings ( )
   Close relatives or friends ( )
   A professional labor support person ( )
   No one other than medical staff ( )

5. How was your baby delivered?

   Vaginally and not induced ( )
   Vaginally and induced ( )
   A planned cesarean ( )
   An unplanned or emergency cesarean ( )

6. Which of the following medications did you have during labor or delivery?
   (Please "x" all that apply)

   General anesthesia (you were put to sleep) ( )
   A spinal or epidural ( )
   Nitrous oxide (gas breathed through a mask or mouthpiece while remaining conscious) ( )
   Other pain medication or don't know which pain medication ( )
   No pain medication ( )

7. How much weight did you gain during this pregnancy? _____kg (estimates or weighed?)

8. How many nights were you in the hospital or birth center after your baby was born?

   1 night ( )
   2 nights ( )
   3 nights ( )
   4 to 7 nights ( )
   More than 7 nights ( )

9. How **soon** your baby was placed direct on your body after birth (skin-to-skin contact)? :

   Directly after birth ( )
   15 to 30 minutes after birth ( )
   More than 30 mins after birth ( )
   More than 1 hour after birth ( )

10. Soon after birth, how **long** your baby was placed direct on your body (skin-to-skin contact)? :

    None ( )
    Less than 20 mins ( )
    More than 20 mins ( )

11. How soon after the delivery did you breastfeed or try to breastfeed your baby?

    Directly after birth (in the labour room) ( )
    Less than 30 minutes ( )
    Within 30 to 60 mins ( )
    Within 1 to 2 hours ( )
    Within 3 to 6 hours ( )
    Within 7 to 12 hours ( )
    Selepas 1 day (12 hours) ( )
    Within 13 to 24 hours ( )
    After 2 days ( )
    More than 2 days ( )

Subject ID: _____ Date: _____

BIRTH SCREENING QUESTIONNAIRE

Infant's Initial: _____ Baby's birthday : _____

Baby's gender : Boy ( ) Girl ( )

Mother's initials : _____

No	Inclusion	State 'Yes'
1	Singleton	
2	Birthweight : (2.5 – 4 kg?)	
3	Gestation >37 weeks (complete)	
4	Exclusive BF	
5	Mother can communicate in English / Malay	
6	Mother is not planning to stay outside Selangor area during postpartum	
No	**Exclusion**	**State 'No'**
1	Congenital malformation likely to affect growth	
2	Requirement for NICU other than for mild resp probs/ short term anti-biotics (more than 1 day)	
3	Mother has illness that prevented from BF	
4	Mixed feeding	

Eligible to participate in the study? ----------> **YES ( )** **NO ( )**

Randomisation : ----------> **Intervention ( )** **Control ( )**

Randomisation ID :

MOMS : Mother-Offspring-Milk Study

Subject ID: _____ Date: _____

INFANT FEEDING QUESTIONNAIRE I (IFQ I)

1. Was your baby given a pacifier by you, the medical staff, or anyone else while in the hospital or birth center?

   Yes ( ) No ( ) Don't know ( )

2. As best you know, what is the recommended number of months to exclusively breastfeed a baby, meaning the baby is only fed breast milk? _____ MONTHS

3. Did you receive a gift pack or diaper bag from the hospital or birth center? Include a gift pack from a child birth class if you took the class at the hospital or birth center.

   Yes ( ) No ( ) → (**go to question 5)**

4. Were any of the following included in the gift pack? If you received more than one gift pack from the hospital or birth center, answer for all that you received. **(please "x" all that apply)**

   Infant formula ( )
   Coupon for infant formula ( )
   Breastfeeding supplies (nursing pads, nipple cream, etc.) ( )

5. Did you receive a gift pack from any place besides the hospital or birth center, for example, from your doctor or a child birth class taken somewhere other than the hospital?

   Yes ( ) No ( )

6. While you were in the hospital for delivery baby, did anyone help you with breastfeeding (BF) by showing you how or talking to you about BF?

   Yes ( ) No ( ) → (**go to question 10)**

7. How many hours after the baby's birth did you *first* get help with breastfeeding?

   Less than 30 min ( )
   30 to 60 min ( )
   1 to 2 hours ( )
   3 to 6 hours ( )
   7 to 12 hours ( )
   1 day ( )
   13 to 24 hours ( )
   2 days ( )
   More than 2 days ( )
   Never ( )

8. Who helped you with breastfeeding? **(please "x" all that apply)**

   Doctor ( )
   Lactation consultant ( )
   Midwife ( )
   Nurse ( )
   Peer counselor ( )
   BF support group member ( )
   Family member(s) ( )
   Friend(s)/relative(s) ( )
   Someone else ( )……
   None ( )

9. While you were in the hospital or birth center, did your baby stay in your room day and night, except for doctor visits, bathing, or other treatments?

   Yes, all the time ( ) (**go to question 13)**
   Yes, some nights but not all ( )
   No ( )

10. Was your baby brought to you for feeding during the night? Yes ( ) No ( )

11. When your baby was not in your room, how did the staff decide when to feed the baby or to bring him or her to you for feeding? **(please "x" all that apply)**

Whenever he or she cried or seemed hungry ( )
Whenever you asked or went to get him or her ( )
On a schedule determined by the nurses or doctors ( )
Don't know ( )

12. During the first few days after your baby was born, did you feed him or her…

Whenever he/she cried or seemed hungry ( )
On a schedule or routine ( )
Sometimes on a schedule & sometimes when he/she cried or seemed hungry ( )

13. While you were in the hospital or birth center, was your baby fed water, formula, or sugar water at any time?

Water	Yes ( )	No ( )	Don't know ( )
Formula	Yes ( )	No ( )	Don't know ( )
Sugar water	Yes ( )	No ( )	Don't know ( )

14. How long did it take for your milk to come in?

1 day or less ( )
2 days ( )
3 days ( )
4 days ( )
More than 4 days ( )

15. Using 1 to mean "Disliked Very Much" and 5 to mean "Liked Very Much," how would you say you felt about breastfeeding during the <u>first week</u> you were breastfeeding?

<u>Disliked very much (1) (2) (3) (4) (5) liked very much</u>

16. Were you given information about any breastfeeding support groups or services before you went home from the hospital or birth center? Yes ( ) No ( )

17. When you left the hospital or birth center, how were you feeding your baby?

Breastfeeding only ( ) Formula feeding only ( ) Both breast and formula feeding ( )

18. Did you have any pain while breastfeeding at any time in the <u>first 2 weeks</u>?

Yes ( ) No ( ) → **(go to question 21)**

19. Using 0 to mean "No pain at all" and 10 to mean "The worst possible pain," how much pain, if any, were you in when you were breastfeeding during the following time periods?

Scale :	No pain ----------> Worst possible pain									
Pain level	1	2	3	4	5	6	7	8	9	10
1st day										
1st week										
2nd week										

20. Did you have any of the following problems breastfeeding your baby during your first 2 weeks of breastfeeding? **(please "x" all that apply)**

My baby had trouble sucking or latching on ( )
My baby choked ( )
My baby wouldn't wake up to nurse regularly enough ( )
My baby was not interested in nursing ( )
My baby got distracted ( )
My baby nursed too often ( )
It took too long for my milk to come in ( )
I had trouble getting the milk flow to start ( )
My baby didn't gain enough weight or lost too much weight ( )
I didn't have enough milk ( )
My nipples were sore, cracked, or bleeding ( )
My breasts were overfull (engorged) ( )
I had a yeast infection of the breast ( )
I had a clogged milk duct ( )
My breasts were infected or abscessed ( )
My breasts leaked too much ( )
I had no problems ( )
I had some other problem ( ) :

……..

21. Did you ask for help with these problems from a health professional (a doctor, midwife, or nurse), a lactation consultant, or a breastfeeding support group? Yes ( ) No ( )

22. Did you get any help with these problems from a health professional, a lactation consultant, or a breastfeeding support group? Yes ( ) No ( )

23. Did the help you received solve the problem(s) or make them better?

Not at all (1) (2) (3) (4) (5) Yes, very much

Other information :

24. Has your baby used a pacifier in the past 7 days? Yes ( ) No ( )

25. Has your baby had jaundice at any time since he or she was born? 3

Yes ( ) No ( ) → (**go to question 10)**

26. How was the jaundice treated? **(please "x"all that apply)**

I fed formula in addition to breastfeeding for a while ( )
I stopped breastfeeding for a while ( ) …
I stopped breastfeeding and did not begin breastfeeding again ( )
My baby was placed under a lamp (phototherapy) ( )…
My baby received an exchange transfusion ( )
My baby received some other treatment ( ) please indicate : ..... …
No treatment was given ( )

27. Since the time your baby was discharged from the hospital after the birth, has he or she been hospitalized for any reason or has your baby been taken to a hospital for any outpatient procedure or surgery? Yes ( ) → (**go to question 29)** No ( )

28. How many nights was your baby in the hospital for the most recent problem since discharge after the birth? (Write in 0 if your baby did not stay overnight.) _____ NIGHTS

Subject ID: _____ Date: _____

INFANT FEEDING QUESTIONNAIRE II (IFQ II)

A. Breast-feeding at present

1. Has your baby used a dummy in the past 7 days? Yes ( ) No ( )

2. During the past 7 days, how often was your baby put to bed with breast milk?

   At most bedtimes, including naps ( )
   At most night bedtimes, but not naps ( )
   At most naps, but not night bedtimes ( )
   Only occasionally at bedtimes, including nap ( )
   Never ( )

3. Does your baby usually feed from both breasts at each feeding?
   Yes ( ) No ( ) Baby is only fed pumped milk ( )→ **Go to Q7**

4. Does your baby usually let go of the breast him or herself?
   Yes, both breasts ( )
   Yes, first breast only ( )
   Yes, second breast only ( )
   No ( )

5. About how long does an average breast-feeding last?
   Less than 10 minutes ( )
   10 to 19 minutes ( )
   20 to 29 minutes ( )
   30 to 39 minutes ( )
   40 to 49 minutes ( )
   50 or more minutes ( )

6. In an average 24-hour period, what is the LONGEST time for you, the mother, between breast-feedings or pumping milk? Please count the time from the start of one breast-feeding or pumping session to the start of the next. Please think of time between feedings during both night and day to find the longest time. **(Write in the number of hours and minutes) :**

   _____ HOURS **and** _____ MINUTES

7. Since your baby was born, have you ever pumped or tried to pump milk? (Include expressing breast milk in any way as pumping milk.)

   Yes, but I did not get any milk ( ) Yes, and I got milk ( ) No ( ) → **Go to Q14**

8. How old was your baby the first time you pumped or tried to pump milk?

   _____ DAYS **or** _____ WEEK

9. How many times in the past 7 days was your baby fed pumped breast milk to drink? Include breast milk you expressed in any way as pumped milk _____ TIMES, **IF 0 → Go to Q14**

10. How often does your baby drink all of his or her cup or bottle of pumped milk?

    Never ( )
    Rarely ( )
    Sometimes ( )
    Most of the time ( )
    Always ( )

11. How often is your baby encouraged to finish a cup or bottle if he or she stops drinking before the pumped breast milk is all gone?

    Never ( )
    Rarely ( )
    Sometimes ( )
    Most of the time ( )
    Always ( )

12. How have you pumped or expressed milk since this baby was born? (**please "x" all that apply**)

Electric breast pump ( )
Battery operated pump ( )
By hand (without using a pump) ( )
Combination electric and battery operated breast pump ( )
Manual breast pump (no batteries, no cord to plug in) ( )

13. For what reasons have you pumped milk in the past 7 days? (**please "x" all that apply**)

To relieve engorgement..... ( )
Because my nipples were too sore to nurse..... ( )
To increase my milk supply ..... ( )
To get milk for someone else to feed to my baby..... ( )
To mix with cereal or other food..... ( )
To have an emergency supply of milk..... ( )
To donate to a baby other than my own..... ( )
For me to feed to my baby when I do not want to breast-feed or when my baby cannot breast-feed..... ( )
To keep my milk supply up when my baby could not nurse (such as while you were away from your baby or when your baby was too sick to nurse) ( )

B. Breast-feeding in future

14. How long do you plan to exclusively breastfeed your baby?

_____ weeks/ months

15. How old do you think your baby will be when you first feed him or her formula or any other food besides breast milk?

3 to 4 months ( )
5 to 6 months ( )
7 to 9 months ( )
More than 9 months ( )

16. Do you plan to continue breast-feeding after you return to work?

Yes ( )
No ( )
Do not plan to work after the baby's birth ( )

17. How long is your maternity leave : _____ days/ weeks/ months

18. How old do you think your baby will be when you completely stop breastfeeding?

_____ months/years

19. Using 1 to mean 'Not at all confident' to 5 as 'Very confident', how confident are you that you will be able to breastfeed until the baby is the age that you indicate above (Q17)?

Not at all confident (1) (2) (3) (4) (5) Very confident

C. Breast-feeding attitudes

20. Which of the following statements is closest to your opinion?
The best way to feed a baby below 6 months old is:

Breast-feeding ( )
Formula feeding ( )
A mix of both breast and formula feeding ( )
Breast-feeding and formula feeding are equally good ways to feed a baby ( )

21. How strongly do you agree or disagree with the following statements?

STRONGLY AGREE…(1)	**SOMEWHAT AGREE…(2)**	**NEITHER AGREE NOR DISAGREE…(3)**	**SOMEWHAT DISAGREE…(4)**	**STRONGLY DISAGREE…(5)**	**NOT SURE..(6)**

i. Infant formula is as good as breast milk….

(1) ( ) **(2)** ( ) **(3)** ( ) **(4)** ( ) **(5)** ( ) Not sure -**(6)** ( )

ii. If a baby is breast-fed, he or she will be less likely to get ear infections....

(1) ( ) **(2)** ( ) **(3)** ( ) **(4)** ( ) **(5)** ( ) Not sure -**(6)** ( )

iii. If a baby is breast-fed he or she will be less likely to get a respiratory illness….

(1) ( ) **(2)** ( ) **(3)** ( ) **(4)** ( ) **(5)** ( ) Not sure -**(6)** ( )

iv. If a baby is breast-fed he or she will be less likely to get diarrhoea….

(1) ( ) **(2)** ( ) **(3)** ( ) **(4)** ( ) **(5)** ( ) Not sure -**(6)** ( )

v. Babies should be exclusively breast-fed (fed only breast milk) at least for the first 4-6 months….

(1) ( ) **(2)** ( ) **(3)** ( ) **(4)** ( ) **(5)** ( ) Not sure -**(6)** ( )

vi. If a child was breast-fed, he or she will be less likely to become obese…

(1) ( ) **(2)** ( ) **(3)** ( ) **(4)** ( ) **(5)** ( ) Not sure -**(6)** ( )

22. Using **1 to mean 'Never' and 5 to mean 'Always'**, please choose the answer for each of the following statements that best describes how you feel about breastfeeding (BF) your baby:

	Never ---------- Always				
I feel that I can find out what I need to know about BF	**(1)**( )	**(2)**( )	**(3)**( )	**(4)**( )	**(5)**( )
I feel that BF takes too much time	**(1)**( )	**(2)**( )	**(3)**( )	**(4)**( )	**(5)**( )
I feel that my baby gets enough breast milk at each feeding	**(1)**( )	**(2)**( )	**(3)**( )	**(4)**( )	**(5)**( )
I feel that I can breastfeed my baby whether it hurts or not	**(1)**( )	**(2)**( )	**(3)**( )	**(4)**( )	**(5)**( )
I feel that my family supports my decision to breastfeed	**(1)**( )	**(2)**( )	**(3)**( )	**(4)**( )	**(5)**( )

23. Using 1 to mean "Very Uncomfortable" and 5 to mean "Very Comfortable," how comfortable would you be in the following situations?

	V. uncomfortable ---------- V. comfortable				
BF in the presence of women who are relatives	**(1)**( )	**(2)**( )	**(3)**( )	**(4)**( )	**(5)**( )
BF in the presence of men & women who are relatives	**(1)**( )	**(2)**( )	**(3)**( )	**(4)**( )	**(5)**( )
BF in the presence of close women friends	**(1)**( )	**(2)**( )	**(3)**( )	**(4)**( )	**(5)**( )
BF in the presence of men & women who are close friends	**(1)**( )	**(2)**( )	**(3)**( )	**(4)**( )	**(5)**( )
BF in the presence of men & women who are not close friends	**(1)**( )	**(2)**( )	**(3)**( )	**(4)**( )	**(5)**( )
BF your baby in the public	**(1)**( )	**(2)**( )	**(3)**( )	**(4)**( )	**(5)**( )

D. Sleeping arrangements

24. During the past 7 days, what was the longest time your baby usually slept at night without waking?

2 hours or less ( )
3 to 4 hours ( )
5 to 6 hours ( )
7 to 8 hours ( )
8 hours or more ( )

25. Where does your baby sleep at night?

Same bed with you ( )
In a cot beside your bed ( )
Baby's mattress beside your bed ( )
Different room than you ( ) → **Go to Q26**

26. What are your reasons for bringing your baby to bed with you? (**please "x" all that apply**)

It is commonly done in my family…… ( )
To bottle feed..... ( )
Sleeping with my baby helps the baby or me to sleep better..... ( )
To help with a blocked milk duct or other breast-feeding problem..... ( )
I think it is safer if my baby sleeps with me or us..... ( )
To be close or bond..... ( )
A doctor or nurse advised sleeping with my baby..... ( )
To comfort when fussy..... ( )
To breast-feed..... ( )
To comfort when sick..... ( )

27. What are your reasons for not bringing your baby to bed with you? (**please "x" all that apply**)

It is not commonly done in my family…… ( )
We wake each other up, or baby wakes me or others in the bed..... ( )
I think it is safer if my baby does not sleep with me or us..... ( )
I don't think the baby should sleep with me because my husband smoke, or I take sedative medicine or other reason..... ( )
A doctor or nurse advised not sleeping with my baby..... ( )
I think it will be too hard to get my baby to sleep in a crib when he or she is older..... ( )

E. Caring during postpartum

28. Who has has been taking care of you during postpartum (tick 'X' that applies)

Husband ( )
Parents ( )
In-law's ( )
Sibling ( )
Close relative/friend ( )
Self ( )

29. Who primarily take care of you and your baby during postpartum?

Husband ( )
Parents ( )
In-law's ( )
Sibling ( )
Close relative/friend ( )
Self ( )

30. Do you have a confinement period? Yes ( ) No ( ) → **Go to Q32**

Subject ID: _____ Date: _____

INFANT FEEDING QUESTIONNAIRE III (IFQ 3)

A. Breast-feeding at present

1. Has your baby used a dummy in the past 7 days? Yes ( ) No ( )

2. During the past 7 days, how often was your baby put to bed with breast milk?

   At most bedtimes, including naps ( )
   At most night bedtimes, but not naps ( )
   At most naps, but not night bedtimes ( )
   Only occasionally at bedtimes, including nap ( )
   Never ( )

3. Does your baby usually feed from both breasts at each feeding?

   Yes ( )
   No ( )
   Baby is only fed pumped milk ( )→ **Go to Q7**

4. Does your baby usually let go of the breast him or herself?

   Yes, both breasts ( )
   Yes, first breast only ( )
   Yes, second breast only ( )
   No ( )

5. About how long does an average breast-feeding last?

   Less than 10 minutes ( )
   10 to 19 minutes ( )
   20 to 29 minutes ( )
   30 to 39 minutes ( )
   40 to 49 minutes ( )
   50 or more minutes ( )

6. In an average 24-hour period, what is the LONGEST time for you, the mother, between breast-feedings or pumping milk? Please count the time from the start of one breast-feeding or pumping session to the start of the next. Please think of time between feedings during both night and day to find the longest time. **(Write in the number of hours and minutes) :**

   _____ HOURS **and** _____ MINUTES

7. Using **1 to mean 'Never' and 5 to mean 'Always'**, please choose the answer for each of the following statements that best describes how you feel about breastfeeding (BF) your baby:

	Never ---------- Always
I feel that I can find out what I need to know about BF	**(1)**( ) **(2)**( ) **(3)**( ) **(4)**( ) **(5)**( )
I feel that BF takes too much time	**(1)**( ) **(2)**( ) **(3)**( ) **(4)**( ) **(5)**( )
I feel that my baby gets enough breast milk at each feeding	**(1)**( ) **(2)**( ) **(3)**( ) **(4)**( ) **(5)**( )
I feel that I can breastfeed my baby whether it hurts or not	**(1)**( ) **(2)**( ) **(3)**( ) **(4)**( ) **(5)**( )
I feel that my family supports my decision to keep on breastfeeding my baby	**(1)**( ) **(2)**( ) **(3)**( ) **(4)**( ) **(5)**( )

B. Breast-feeding in future

8. How old do you think your baby will be when you first feed him or her formula or any other food besides breast milk?

3 to 4 months ( ) 7 to 9 months ( )
5 to 6 months ( ) More than 9 months ( )

9. Do you plan to continue breast-feeding after you return to work?

Yes ( ) Do not plan to work after the baby's birth ( )
No ( )

10. How old do you think your baby will be when you completely stop breastfeeding?

_____ months/years

11. Using 1 to mean 'Not at all confident' to 5 as 'Very confident', how confident are you that you will be able to breastdfeed until the baby is the age that you indicate above (Q17)?

Not at all confident (1) (2) (3) (4) (5) Very confident

C. Breast pumps & Expressing milk

12. Since your baby was born, have you ever pumped or tried to pump milk? (excluding the expressing breast milk during the study's home visit)

Yes, but I did not get any milk ( )
Yes, and I got milk ( )
No ( ) → **Go to section D, Q?**

13. How old was your baby the **first time** you pumped or tried to pump milk?

_____ DAYS **or** _____ WEEKS

14. Are you now pumping milk on a regular schedule? Yes ( ) No ( ) → **Go to Q16**

15. How old was your baby when you first began pumping milk on a **regular** schedule?

_____ DAYS **or** _____ WEEKS

16. How have you pumped or expressed milk since your baby was born? (**please "x" all that apply**) Please tick (/) in the second box on the one that you use most often.

Electric breast pump ( ) ( )
Battery operated pump ( ) ( )
By hand (without using a pump) ( ) ( )
Combination electric and battery operated breast pump ( ) ( )
Manual breast pump (no batteries, no cord to plug in) ( ) ( )

17. How did you learn to use the breast pump you use most often? (**please "x" all that apply**)

I read the printed directions that came with the pump ( )
I got instructions for the pump from the internet ( )
I watched a video about how to use the pump ( )
A lactation consultant, WIC staff, nurse, or doctor showed me how to use it ( )
A friend, relative, sales clerk, or other person showed me how to use it ( )
I figured it out without directions or being shown how ( )

18. For what reasons have you pumped milk in the past 7 days? (**please "x" all that apply**)

To relieve engorgement..... ( )
Because my nipples were too sore to nurse..... ( )
To increase my milk supply ..... ( )
To get milk for someone else to feed to my baby..... ( )
To mix with cereal or other food..... ( )
To have an emergency supply of milk..... ( )
To donate to a baby other than my own..... ( )
For me to feed to my baby when I do not want to breast-feed or when my baby cannot breast-feed..... ( )
To keep my milk supply up when my baby could not nurse (such as while you were away from your baby or when your baby was too sick to nurse) ( )

19. During the past 2 weeks, how many times did you pump milk? (Include expressing breast milk in any way as pumping milk.) _____ TIMES in past 2 weeks → (if 0→ **Go to section D, Q?)**

20. On average, in the past 2 weeks, how many ounces of milk did you pump each time?

1 ounce or less ( )
2 ounces ( )
3 to 4 ounces ( )
5 to 6 ounces ( )
7 to 8 ounces ( )
More than 8ounces ( )

21. How long was your milk usually stored in the refrigerator in the past 2 weeks?

1 day or less ( )
2 – 3 days ( )
4 - 5 days ( )
6 - 8 days ( )
More than 8 days ( )
I do not store my milk ( )

22. How many times in the past 7 days was your baby fed pumped breast milk to drink? Include breast milk you expressed in any way as pumped milk _____ TIMES, **IF 0 → Go to Q14**

23. How often does your baby drink all of his or her cup or bottle of pumped milk?

Never ( )
Rarely ( )
Sometimes ( )
Most of the time ( )
Always ( )

24. How often is your baby encouraged to finish a cup or bottle if he or she stops drinking before the pumped breast milk is all gone?

Never ( )
Rarely ( )
Sometimes ( )
Most of the time ( )
Always ( )

Subject ID: _____ Date: _____ Home visit: **1 / 2 / 3** (please circle)

BABY EATING BEHAVIOUR QUESTIONNAIRE (BEBQ)

These questions are about your baby's appetite over his/her first few months of life. We are specifically interested in the period during which your baby is fed milk only, i.e. no solid foods or pre-prepared baby food yet.

How would you describe your baby's feeding style at a typical daytime feed?

	Never	Rarely	Sometimes	Often	Always
1. My baby seems contented while feeding	☐	☐	☐	☐	☐
2. My baby frequently wants more milk than I provide	☐	☐	☐	☐	☐
3. My baby loves milk	☐	☐	☐	☐	☐
4. My baby has a big appetite	☐	☐	☐	☐	☐
5. My baby finishes feeding quickly	☐	☐	☐	☐	☐
6. My baby becomes distressed while feeding	☐	☐	☐	☐	☐
7. My baby gets full up easily	☐	☐	☐	☐	☐
8. If allowed to, my baby would take too much milk	☐	☐	☐	☐	☐
9. My baby takes more than 30 minutes to finish feeding	☐	☐	☐	☐	☐
10. My baby gets full before taking all the milk I think he/she should have	☐	☐	☐	☐	☐
11. My baby feeds slowly	☐	☐	☐	☐	☐
12. Even when my baby has just eaten well he/she is happy to feed again if offered	☐	☐	☐	☐	☐
13. My baby finds it difficult to manage a complete feed	☐	☐	☐	☐	☐
14. My baby is always demanding a feed	☐	☐	☐	☐	☐
15. My baby sucks more and more slowly during the course of a feed	☐	☐	☐	☐	☐
16. If given the chance, my baby would always be feeding	☐	☐	☐	☐	☐
17. My baby enjoys feeding time	☐	☐	☐	☐	☐
18. My baby can easily take a feed within 30 minutes of the last one	☐	☐	☐	☐	☐

MINI BREASTFEEDING TEST : Questionnaire on mood and feelings during breastfeeding (BF).

Subject ID : …….. Date : …… Home visit : **1 / 2 / 3** (please circle)

Time before BF : …… Time after BF : ……

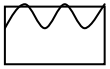
Please mark (|) on the scale for each question:

1) How stressed do you feel? *Not at all stressed* ●——— *Very stressed*
2) How anxious do you feel? *Not at all anxious* ●——— *Very anxious*
3) How alert do you feel? *Not at all alert* ●——— *Very alert*
4) How relaxed do you feel? *Not at all relaxed* ●——— *Very relaxed*
5) How happy do you feel? *Not at all happy* ●——— *Very happy*
6) How tired do you feel? *Not at all tired* ●——— *Very tired*
7) How sleepy do you feel? *Not at all sleepy* ●——— *Very sleepy*
8) How calm is your baby? *Not at all calm* ●——— *Very calm*
9) How happy is your baby? *Not at all happy* ●——— *Very happy*

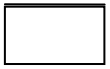
☺ THANK YOU ☺

MOM Study (3-Day Behaviour Diary) Study-ID_____

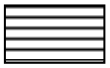
The record is filled in by shading on the 'time rulers' using the appropriate type of shading. An example is given here. Note that activities or behaviour don't have to last for 15 minutes to be filled in. The length of shading in tells us how long they lasted for. If you can be accurate to within about 5 minutes, that will be accurate enough.



Sleeping



Awake and content



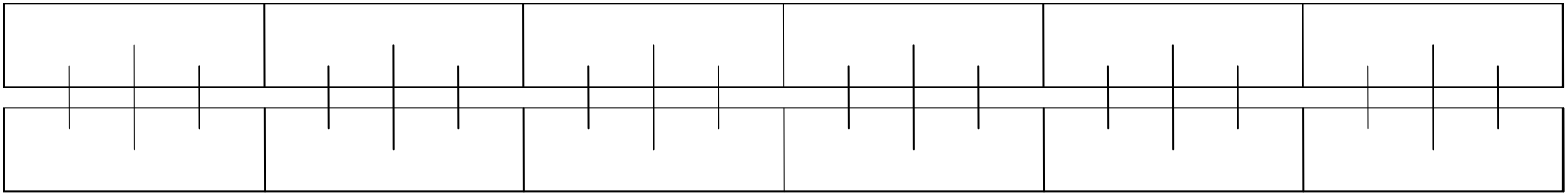
Colic: bouts of intense, unsoothable crying and other behaviour, possibly due to stomach or bowel pain

Fussy: your baby is unsettled and irritable,

Crying: periods of prolonged, distressed vocalisation

Feeding

.



12:0 noon 12:30 1:00 1:30 2:00 2:30 3:00 3:30 4:00 4:30 5:00 5:30 6:00

Symbols: H = held or carried; C = bath or nappy change; P = play

Edinburgh Postnatal Depression Scale (EPDS)

ID participant: ……

Date: ……. Home visit: **1 / 2 / 3** (please circle)

As you are pregnant or have recently had a baby, we would like to know how you are feeling. Please check the answer that comes closest to how you have felt **IN THE PAST 7 DAYS**, not just how you feel today.

Here is an example, already completed.

I have felt happy:
- ☐ Yes, all the time
- ☒ Yes, most of the time
- ☐ No, not very often
- ☐ No, not at all

This would mean: "I have felt happy most of the time" during the past week. Please complete the other questions in the same way.

In the past 7 days:

1. I have been able to laugh and see the funny side of things
   - ☐ As much as I always could
   - ☐ Not quite so much now
   - ☐ Definitely not so much now
   - ☐ Not at all

2. I have looked forward with enjoyment to things
   - ☐ As much as I ever did
   - ☐ Rather less than I used to
   - ☐ Definitely less than I used to
   - ☐ Hardly at all

*3. I have blamed myself unnecessarily when things went wrong
   - ☐ Yes, most of the time
   - ☐ Yes, some of the time
   - ☐ Not very often
   - ☐ No, never

4. I have been anxious or worried for no good reason
   - ☐ No, not at all
   - ☐ Hardly ever
   - ☐ Yes, sometimes
   - ☐ Yes, very often

*5 I have felt scared or panicky for no very good reason
   - ☐ Yes, quite a lot
   - ☐ Yes, sometimes
   - ☐ No, not much
   - ☐ No, not at all

*6. Things have been getting on top of me
   - ☐ Yes, most of the time I haven't been able to cope at all
   - ☐ Yes, sometimes I haven't been coping as well as usual
   - ☐ No, most of the time I have coped quite well
   - ☐ No, I have been coping as well as ever

*7 I have been so unhappy that I have had difficulty sleeping
   - ☐ Yes, most of the time
   - ☐ Yes, sometimes
   - ☐ Not very often
   - ☐ No, not at all

*8 I have felt sad or miserable
   - ☐ Yes, most of the time
   - ☐ Yes, quite often
   - ☐ Not very often
   - ☐ No, not at all

*9 I have been so unhappy that I have been crying
   - ☐ Yes, most of the time
   - ☐ Yes, quite often
   - ☐ Only occasionally
   - ☐ No, never

*10 The thought of harming myself has occurred to me
   - ☐ Yes, quite often
   - ☐ Sometimes
   - ☐ Hardly ever
   - ☐ Never

Perceived Stress Scale (PSS)

Subject ID: ……

Date : ……. Home visit : **1 / 2 / 3** (please circle)

The questions in this scale ask you about your feelings and thoughts during **THE LAST MONTH** (up till today). In each case, please indicate your response by circling the number in the corresponding space representing HOW OFTEN you felt or thought a certain way.

No.	Feelings / Thoughts	Never 0	Almost Never 1	Some-times 2	Fairly Often 3	Very Often 4
1	In the last month, how often have you been upset because of something that happened unexpectedly?	**0**	**1**	**2**	**3**	**4**
2	In the last month, how often have you felt that you were unable to control the important things in your life?	**0**	**1**	**2**	**3**	**4**
3	In the last month, how often have you felt nervous and "stressed"?	**0**	**1**	**2**	**3**	**4**
4	In the last month, how often have you felt confident about your ability to handle your personal problems?	**0**	**1**	**2**	**3**	**4**
5	In the last month, how often have you felt that things were going your way?	**0**	**1**	**2**	**3**	**4**
6	In the last month, how often have you found that you could not cope with all the things that you had to do?	**0**	**1**	**2**	**3**	**4**
7	In the last month, how often have you been able to control irritations in your life?	**0**	**1**	**2**	**3**	**4**
8	In the last month, how often have you felt that you were on top of things?	**0**	**1**	**2**	**3**	**4**
9	In the last month, how often have you been angered because of things that were outside your control?	**0**	**1**	**2**	**3**	**4**
10	In the last month, how often have you felt difficulties were piling up so high that you could not overcome them?	**0**	**1**	**2**	**3**	**4**

Beck Anxiety Inventory (BAI)

Subject ID: ……

Date : ……. Home visit : **1 / 2 / 3** (please circle)

Below is a list of common symptoms of anxiety. Please carefully read each item in the list. Indicate how much you have been bothered by that symptom during the **past month, including today**, by circling the number in the corresponding space in the column next to each symptom.

No.	**Symptoms of anxiety**	**Not At All** **0**	**Mildly** - but it didn't bother me much **1**	**Moderately** - it wasn't pleasant at times **2**	**Severely –** it bothered me a lot **3**
1	Numbness or tingling	**0**	**1**	**2**	**3**
2	Feeling hot	**0**	**1**	**2**	**3**
3	Wobbliness in legs	**0**	**1**	**2**	**3**
4	Unable to relax	**0**	**1**	**2**	**3**
5	Fear of worst happening	**0**	**1**	**2**	**3**
6	Dizzy or lightheaded	**0**	**1**	**2**	**3**
7	Heart pounding/racing	**0**	**1**	**2**	**3**
8	Unsteady	**0**	**1**	**2**	**3**
9	Terrified or afraid	**0**	**1**	**2**	**3**
10	Nervous	**0**	**1**	**2**	**3**
11	Feeling of choking	**0**	**1**	**2**	**3**
12	Hands trembling	**0**	**1**	**2**	**3**
13	Shaky / unsteady	**0**	**1**	**2**	**3**
14	Fear of losing control	**0**	**1**	**2**	**3**
15	Difficulty in breathing	**0**	**1**	**2**	**3**
16	Fear of dying	**0**	**1**	**2**	**3**
17	Scared	**0**	**1**	**2**	**3**
18	Indigestion	**0**	**1**	**2**	**3**
19	Faint / lightheaded	**0**	**1**	**2**	**3**
20	Face flushed	**0**	**1**	**2**	**3**
21	Hot/cold sweats	**0**	**1**	**2**	**3**

Rothbart's Infant Behaviour Questionnaire Revised (RIBQ-R)

1	2	3	4	5	6	7	NA
Never	**Very Rarely**	**Less Than Half the Time**	**About Half the Time**	**More Than Half the Time**	**Almost Always**	**Always**	**Does Not Apply**

1. When being dressed or undressed during the last week, how often did the baby squirm and/or try to roll away?

1 2 3 4 5 6 7 NA

2. When tossed around playfully how often did the baby laugh?

1 2 3 4 5 6 7 NA

3. When tired, how often did your baby show distress?

1 2 3 4 5 6 7 NA

4. When introduced to an unfamiliar adult, how often did the baby cling to a parent?

1 2 3 4 5 6 7 NA

5. How often during the last week did the baby enjoy being read to?

1 2 3 4 5 6 7 NA

6. How often during the last week did the baby play with one toy or object for 5-10 minutes?

1 2 3 4 5 6 7 NA

7. How often during the week did your baby move quickly toward new objects?

1 2 3 4 5 6 7 NA

8. When put into the bath water, how often did the baby laugh?

1 2 3 4 5 6 7 NA

9. When it was time for bed or a nap and your baby did not want to go, how often did s/he whimper or sob?

1 2 3 4 5 6 7 NA

10. After sleeping, how often did the baby cry if someone doesn't come within a few minutes?

Rothbart's Infant Behaviour Questionnaire Revised (RIBQ-R)

1	2	3	4	5	6	7	NA
Never	**Very Rarely**	**Less Than Half the Time**	**About Half the Time**	**More Than Half the Time**	**Almost Always**	**Always**	**Does Not Apply**

1 2 3 4 5 6 7 NA

11. In the last week, while being fed in your lap, how often did the baby seem eager to get away as soon as the feeding was over?

1 2 3 4 5 6 7 NA

12. When singing or talking to your baby, how often did s/he soothe immediately?

1 2 3 4 5 6 7 NA

13. When placed on his/her back, how often did the baby squirm and/or turn body?

1 2 3 4 5 6 7 NA

14. During a peekaboo game, how often did the baby laugh?

1 2 3 4 5 6 7 NA

15. How often does the infant look up from playing when the telephone rings?

1 2 3 4 5 6 7 NA

16. How often did the baby seem angry (crying and fussing) when you left her/him in the crib?

1 2 3 4 5 6 7 NA

17. How often during the last week did the baby startle at a sudden change in body position (e.g., when moved suddenly)?

1 2 3 4 5 6 7 NA

18. How often during the last week did the baby enjoy hearing the sound of words, as in nursery rhymes?

1 2 3 4 5 6 7 NA

19. How often during the last week did the baby look at pictures in books and/or magazines for 5 minutes or longer at a time?

1 2 3 4 5 6 7 NA

1	2	3	4	5	6	7	NA
Never	**Very Rarely**	**Less Than Half the Time**	**About Half the Time**	**More Than Half the Time**	**Almost Always**	**Always**	**Does Not Apply**

Rothbart's Infant Behaviour Questionnaire Revised (RIBQ-R)

20. When visiting a new place, how often did your baby get excited about exploring new surroundings?

1 2 3 4 5 6 7 NA

21. How often during the last week did the baby smile or laugh when given a toy?

1 2 3 4 5 6 7 NA

22. At the end of an exciting day, how often did your baby become tearful?

1 2 3 4 5 6 7 NA

23. How often during the last week did the baby protest being placed in a confining place (infant seat, play pen, car seat, etc.)?

1 2 3 4 5 6 7 NA

24. When being held, in the last week, did your baby seem to enjoy him/herself?

1 2 3 4 5 6 7 NA

25. When showing the baby something to look at, how often did s/he soothe immediately?

1 2 3 4 5 6 7 NA

26. When hair was washed, how often did the baby vocalize?

1 2 3 4 5 6 7 NA

27. How often did your baby notice the sound of an airplane passing overhead?

1 2 3 4 5 6 7 NA

28. When introduced to an unfamiliar adult, how often did the baby refuse to go to the unfamiliar person?

1 2 3 4 5 6 7 NA

29. When you were busy with another activity, and your baby was not able to get your attention, how often did s/he cry?

1	2	3	4	5	6	7	NA
Never	**Very Rarely**	**Less Than Half the Time**	**About Half the Time**	**More Than Half the Time**	**Almost Always**	**Always**	**Does Not Apply**

Rothbart's Infant Behaviour Questionnaire Revised (RIBQ-R)

1 2 3 4 5 6 7 NA

30. How often during the last week did the baby enjoy gentle rhythmic activities, such as rocking or swaying?

1 2 3 4 5 6 7 NA

31. How often during the last week did the baby stare at a mobile, crib bumper or picture for 5 minutes or longer?

1 2 3 4 5 6 7 NA

32. When the baby wanted something, how often did s/he become upset when s/he could not get what s/he wanted?

1 2 3 4 5 6 7 NA

33. When in the presence of several unfamiliar adults, how often did the baby cling to a parent?

1 2 3 4 5 6 7 NA

34. When rocked or hugged, in the last week, did your baby seem to enjoy him/herself?

1 2 3 4 5 6 7 NA

35. When patting or gently rubbing some part of the baby's body, how often did s/he soothe immediately?

1 2 3 4 5 6 7 NA

36. How often did your baby make talking sounds when riding in a car?

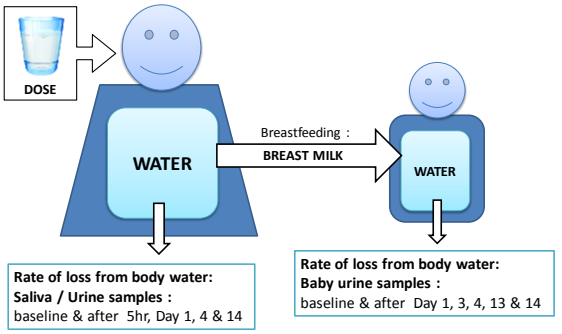
1 2 3 4 5 6 7 NA

37. When placed in an infant seat or car seat, how often did the baby squirm and turn body?

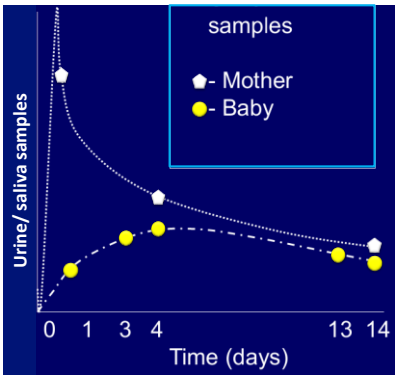
1 2 3 4 5 6 7 NA

Assessing milk intake by isotope method

Measuring milk transfer from mother to infant



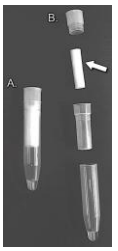
Average milk intake in 14 days :



Saliva sample - mother

Easy steps to collect saliva sample :

1. Remove the bottle cap
2. Take out the cotton swab
3. Roll the cotton inside your mouth (don't chewing it)
4. When it is wet, put it back inside the bottle
5. Put back the cap! Close tightly.



Dose preparation (lab)

(to the mother)

- Filter **10g deuterium** into a dose bottle
- Add 80-100 ml of plain water
- Close the lid & mix well
- **TAKE a dose sample of 2ml** (use clean pipette). Store in a freezer at -20C
- Put the dose bottle into a sealed bag and weigh. **RECORD the weight (prior dose)**

Dose administration (home visit)

(to the mother)

- **Weigh** mother & baby (Day 0)
- Collect **baseline** samples : Saliva (mother) & urine (baby)
- **Shake** the dose bottle → Give to the mother to drink
- **RECORD** the **TIME** & **DATE** of dosing
- Put the empty dose bottle into a sealed bag and weigh. **RECORD the weight (post dose)**

Dose preparation (lab)

(to the baby – body composition)

Filter **0.1 g/kg** body weight of deuterium into a sterile bottle (e.g 0.6 for 6 kg baby)

↓

Add **20-25 g** of plain water (or breast milk)

↓

Close the lid & **mix** well

↓

TAKE a dose sample of 2ml (use sterile pipette). Store in a freezer at -20C

↙ ↘

Put the dose bottle into a sealed bag with a syringe/bottle teat and weigh. **RECORD the weight (prior dose)**

Put some tissues in a sealed bag. **RECORD the weight & date.**

Dose administration (to the baby)

Measuring baby's body composition

Weigh the baby (Day 14)

↓

Collect **baseline** samples (pre-dose 14a): Baby urine

↓

Shake the dose bottle → Dose the baby by using syringe/bottle/teat & use labelled tissues to wipe any spillage

↓

RECORD the **TIME** & **DATE** of dosing

↓

Put the syringe/bottle/teat & used tissues into a sealed bag & weigh. **RECORD the weight (post dose)**

Baby urine sample collection

Baseline & Post dose **(Day 1, 3, 4, 13, 14)** & Body composition **(Post dose 5 hr & Day 1)**

Put 3-5 cotton wool balls/sheets in the nappy. Record the TIME

↓

After 30 minutes → check the cotton wool.

↙ ↘

If it is still dry :

put it back for 30 min & change '*this time*' to '*time dry*' → check again after 30 mins

If it is wet/damp :

Put the cotton wool in a syringe & squeeze the urine into the tube bottles →Record the **TIME WET**

Mother saliva sample collection

Baseline **(day 0)** & Post dose **(Day 1, 4, 14)**

DO NOT EAT/ DRINK/ CLEAN TEETH for 30 minutes before taking the sample

↓

Move the swab around your mouth until **WET.** DO not chew it.

↓

Place the wet cotton swab in the tube, put the lid firmly. **RECORD** time & date.

Appendix A: SPSS Output

Socio-demographic: Included vs Excluded groups.

1) Comparison of infant gender: Chi-Square test p-value of 0.275

Chi-Square Tests

	Value	df	Asymptotic Significance (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	1.190[a]	1	.275		
Continuity Correction[b]	.714	1	.398		
Likelihood Ratio	1.181	1	.277		
Fisher's Exact Test				.329	.199
Linear-by-Linear Association	1.176	1	.278		
N of Valid Cases	87				

a. 0 cells (0.0%) have expected count less than 5. The minimum expected count is 9.78.

b. Computed only for a 2x2 table

2) Comparison of maternal age: Equal variances not assumed p-value of 0.005

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Mother's age	Equal variances assumed	.135	.714	2.772	86	.007	1.802	.650	.510	3.094
	Equal variances not assumed			2.926	46.227	.005	1.802	.616	.563	3.042

3) Comparison across maternal age groups: Fisher's Exact test p-value of 0.008

Chi-Square Tests

	Value	df	Asymptotic Significance (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)	Point Probability
Pearson Chi-Square	8.123[a]	2	.017	.017		
Likelihood Ratio	10.064	2	.007	.009		
Fisher's Exact Test	9.098			.008		
Linear-by-Linear Association	7.343[b]	1	.007	.007	.005	.004
N of Valid Cases	88					

a. 1 cells (16.7%) have expected count less than 5. The minimum expected count is 2.45.

b. The standardized statistic is -2.710.

4) Comparison across educational levels: Fisher's Exact test p-value of 0.504

Chi-Square Tests

	Value	df	Asymptotic Significance (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)	Point Probability
Pearson Chi-Square	2.227[a]	3	.527	.550		
Likelihood Ratio	2.093	3	.553	.605		
Fisher's Exact Test	2.318			.504		
Linear-by-Linear Association	.051[b]	1	.822	.891	.461	.105
N of Valid Cases	88					

a. 3 cells (37.5%) have expected count less than 5. The minimum expected count is 2.73.

b. The standardized statistic is .225.

5) Comparison across household income categories: Fisher's Exact test p-value of 0.083

Chi-Square Tests

	Value	df	Asymptotic Significance (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)	Point Probability
Pearson Chi-Square	9.664[a]	5	.085	.076		
Likelihood Ratio	11.038	5	.051	.057		
Fisher's Exact Test	8.728			.083		
Linear-by-Linear Association	3.629[b]	1	.057	.064	.033	.014
N of Valid Cases	84					

a. 6 cells (50.0%) have expected count less than 5. The minimum expected count is .24.

b. The standardized statistic is 1.905.

6) Comparison of birth place: Fisher's Exact test p-value of 0.587

Chi-Square Tests

	Value	df	Asymptotic Significance (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)	Point Probability
Pearson Chi-Square	.001[a]	1	.981	1.000	.587	
Continuity Correction[b]	.000	1	1.000			
Likelihood Ratio	.001	1	.981	1.000	.587	
Fisher's Exact Test				1.000	.587	
Linear-by-Linear Association	.001[c]	1	.981	1.000	.587	.198
N of Valid Cases	86					

a. 0 cells (0.0%) have expected count less than 5. The minimum expected count is 8.95.

b. Computed only for a 2x2 table

c. The standardized statistic is -.023.

7) Comparison of main maternity care person: Fisher's Exact test p-value of 0.010

Chi-Square Tests

	Value	df	Asymptotic Significance (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)	Point Probability
Pearson Chi-Square	15.739[a]	5	.008	.005		
Likelihood Ratio	16.333	5	.006	.006		
Fisher's Exact Test	12.822			.010		
Linear-by-Linear Association	.679[b]	1	.410	.478	.230	.047
N of Valid Cases	86					

a. 8 cells (66.7%) have expected count less than 5. The minimum expected count is .51.

b. The standardized statistic is -.824.

Correlation results : Milk macronutrients and infant weight, BMI and weight gain

		Weight SD HV2	Weight SD HV3	Weight SD HV4	Weight SD gain HV1-3	Weight SD gain HV1-4	BMI SD HV3	BMI SD HV4
CHO Fore HV2	p-value	.270*	.200	.125	.095	.011	.071	.123
	r-value	.034	.122	.339	.468	.932	.584	.346
	n	62	61	60	61	60	62	61
CHO Hind HV2	p-value	.506**	.423**	.354**	.296*	.202	.306*	.220
	r-value	.000	.001	.007	.024	.132	.019	.098
	n	58	58	57	58	57	58	58
CHO Hind HV3	p-value	.327*	.257*	.179	.121	.038	.300*	.190
	r-value	.012	.048	.175	.356	.774	.021	.146
	n	59	60	59	60	59	59	60
Average CHO Fore	p-value	.310*	.208	.073	.000	-.137	.201	.159
	r-value	.017	.114	.586	.999	.303	.127	.229
	n	59	59	58	59	58	59	59
Average CHO Hind	p-value	.406**	.330**	.241	.174	.073	.289*	.231
	r-value	.001	.009	.061	.175	.576	.024	.071
	n	61	62	61	62	61	61	62
Average of CHO all HV	p-value	.448**	.354**	.259	.256	.131	.386**	.296*
	r-value	.001	.008	.059	.060	.345	.004	.028
	n	55	55	54	55	54	55	55
True protein Hind HV1	p-value	-.236	-.201	-.183	-.041	-.022	-.263*	-.289*
	r-value	.072	.123	.166	.758	.867	.044	.025
	n	59	60	59	60	59	59	60
True protein Hind HV2	p-value	-.184	-.196	-.213	-.299*	-.289*	-.220	-.191
	r-value	.166	.140	.111	.023	.029	.096	.152
	n	58	58	57	58	57	58	58

*CHO = Carbohydrate; Milk Fat = No significant correlation at all (all p>0.05)