Maternal nutrition, breast milk micronutrients and infant growth in rural Gambia

Kamilla Gehrt Eriksen

Darwin College, University of Cambridge
MRC Elsie Widdowson Laboratory

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The World Health Organization recommends exclusive breastfeeding for the first six months of an infant's life. However, the evidence base to support the adequacy of breast milk with respect to infant micronutrient status, across the duration of exclusive breastfeeding, among women who enter pregnancy and lactation with a poor nutritional status is limited. The research presented in this thesis explores the relationship between maternal nutritional status, breast milk micronutrients and infant status in a rural sub-Saharan context.

Existing evidence for associations between maternal dietary intake and nutritional status and breast milk micronutrient composition were systematically reviewed. Most effected by maternal nutrition were breast milk water-soluble vitamin concentrations (except for folic acid), fat-soluble vitamin concentrations were less influenced, and mineral concentrations were generally unaffected (except for iodine and selenium).

Next, the impact of feeding practice on infant growth in rural Gambia was explored. In this population, where growth faltering across the first two years of life is endemic, exclusive breastfeeding to six months of age had limited benefit on infant growth.

Finally, the impact of maternal multiple micronutrient supplementation on breast milk iodine, thiamin, riboflavin, vitamin B_6 and B_{12} was explored. Supplementation during pregnancy positively influenced maternal status for all investigated micronutrients, and modestly increased breast milk iodine and riboflavin concentrations across the first six months of lactation. No effects on breast milk concentrations of thiamin, vitamin B_6 or B_{12}, and limited effect on infant postpartum status, were observed.

The research presented in this thesis suggests that concentrations of breast milk micronutrients may be insufficient in settings where maternal micronutrient status is poor, with likely consequences for infant health. This research supports the need for interventions to improve the nutritional status of pregnant and lactating women in resource-poor settings alongside the promotion of exclusive breastfeeding for optimal health outcomes for infants as well as their mothers.

Kamilla Gehrt Eriksen, September 2017

Preface

This dissertation is the result of my own work and includes nothing which is the outcome of work done in collaboration except as specified in the text. It is not, in whole or in part, substantially the same as any that I have submitted, or am concurrently submitting for a degree or diploma or other qualifications at the University of Cambridge or any other university or similar institution. It does not exceed the word limit of 60,000 words prescribed by the Degree Committee for the Faculty of Biology.

The data and biological material analysed in this thesis were collected as a part of the Early Nutrition and Immune Development (ENID) study, a randomised trial conducted between April 2010 and February 2015 in rural Gambia. My contribution to the work in this thesis over the past three years from October 2014 to September 2017 consisted of (i) systematically collating and reviewing all relevant literature for the systematic review (ii) cleaning infant dietary data, infant growth data and all the remaining variables, and learning how to analyse infant growth data using multilevel modelling (iii) selecting blood, urine and breast milk samples for analysis, including preparation of aliquots (iv) learning how to quantify micronutrient concentrations in blood, urine and breast milk, and lastly (v) statistical analysis and interpretation of all data.

The blood, urine and breast milk micronutrient quantifications presented in this thesis were analysed in laboratories in Zurich, Switzerland, Cambridge, the UK and Davis, California, the United States as a part of collaborations. All collaborators are well-known experts in their fields. I was responsible for conducting the infant thyroglobulin assay during a two months' stay at ETH Zurich, Switzerland. For the remaning quantifications, I briefly assisted the responsible laboratory personel.

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Abstracts and presentations from this thesis are listed in Appendix 4.

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I am thankful for the help Alpha Jallow provided me in the laboratory in Keneba and Fajara getting all breast milk and maternal and infant blood samples aliquoted and ready for shipment. To the entire team in the Laboratory of Human Nutrition at ETH Zurich in Switzerland, thank you for letting me stay with you for two memorable months. Thank you for your open arms, for inviting me to your retreat in the Swiss mountains, for introducing me to the Swiss culture and for your friendship. A special thanks to Sandra Hunziker-Weibel and Dr Maria Andersson for teaching me how to do thyroglobulin quantifications, and for providing me with valuable input to the iodine chapter of this thesis. Thank you to Drs Alex Brito, Setti Shahab-Ferdows, Daniela Hampel and Lindsay Allen from USDA Western Human Nutrition Research Centre, California, for teaching me all about B-vitamins and especially thanks to Lindsay for hosting me during my trip to California. Thank you to Stephen Young and Amanda Mckillion for their work in the Elsie Widdowson Laboratory in Cambridge, and for guiding me through all the, for me, difficult quantification steps. Thank you, Dr Ayse Zengin and Komal Bhatia for your advice and input, and Ursula Kehlet for being the best possible study partner.

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List of abbreviations

AI	Average intake
apo-HC	Apo-haptocorrin
BCRP	Breast cancer resistance protein
BMI	Body-mass-index
BMIC	Breast milk iodine concentration
CV	Coefficients of variation
DBS	Dried blood spot
DIT	Diiodotyrosine
DSS	Demographic Surveillance System
EAR	Estimated average requirement
EBF	Exclusively breastfeeding
EBF-6	Infants exclusively breastfed to six months
nEBF-6	Infants not exclusively breastfed to six months
EGRAC	Erythrocyte glutathione reductase activation coefficient
ELISA	Enzyme-linked immunosorbent assay
ENID	Early Nutrition and Immune Development
aETK	Erythrocyte thiamin transketolase activity coefficient
EWL	Elsie Widdowson Laboratory
FAD	Flavin adenine dinucleotide
FeFol	Iron-folic acid
FFQ	Food frequency questionnaire
FMN	Flavin mononucleotide
FP	Feeding practice
hCG	Human chorionic gonadotropin
Hgb	haemoglobin
HIC	High-income country
holoTc	Holotranscobalamin
HPLC	High performance liquid chromatography
HPLC-FLD	High-performance liquid-chromatography-fluorescence detection
ICP-MS	Inductively coupled plasma mass spectrometry

IOM	Institute of Medicine
IQR	Interquartile range
IUGR	Intrauterine growth restriction
LAZ	Length-for-age z-score
LCMS/MS	Liquid chromatography-tandem mass spectrometry
LMIC	Low- and middle-income country
LNS	Lipid-based nutrient supplement
MIT	Monoiodotyrosine
MLM	Multilevel models
MMA	Methylmalonic acid
MMN	Multiple micronutrients
MRC	Medical Research Council
NIS	Sodium-iodide symporter
4-PA	4-pyrodoxic acid
PE	Protein-energy
PL	Pyridoxal
PLP	Pyridoxal-5'-phosphate
PM	Pyridoxamine
PMP	Pyridoxamine-5'-phosphate
PN	Pyridoxine
PNP	Pyrodixine-5'-phosphate
RDA	Recommended daily allowance
RCT	Randomised controlled trial
RSD	Residual standard deviation
SAM	Severe acute malnutrition
T_3	Triiodothyronine
T_4	Thyroxine
Tg	Thyroglobulin
tHcy	Total homocysteine
TMAH	Tetramethylammonium hydroxide
TMP	Thiamin monophosphate

TPO	Thyroid peroxidate
TPP	Thiamin pyrophosphate
TSH	Thyroid stimulating hormone
TTP	Thiamin triphosphate
UIC	Urinary iodine concentration
UIE	Urinary iodine excretion
UPLC-MS/MS	Ultra-performance liquid-chromatography tandem mass spectrometry
WAZ	Weight-for-age z-score
WHNRC	Western Human Nutrition Research Centre
WHO	World Health Organization
WLZ	Weight-for-length z-score

Chapter 1

Introduction and background

Every third person in the world is affected by malnutrition, either in the form of overweight, obesity, underweight or micronutrient deficiencies (1). Evidence on the severity and long-lasting consequences of malnutrition is compelling. Around 45% of all deaths in children under the age of five are linked to undernutrition and micronutrient deficiencies (2), and decreased cognitive function, poor growth and economic consequences are the negative impact of poor quality diets (1, 3). Malnutrition constitutes a major public health problem, mainly caused by poor quality diets and low dietary diversity (2).

Nutrition plays a vital role in determining both maternal and child health outcomes, and the effects of malnutrition can extend to the next generation. Maternal short stature and low body-mass-index (BMI) in pregnancy are associated with foetal growth restriction (2) and with childhood stunting (height-for-age z-score below -2 SD of the WHO Growth Standards) (4, 5). Infants who are growth restricted in utero are at risk of shorter height during adulthood (6), increasing the risk of an intergenerational cycle of stunting (3). According to the most recent estimates, 155 million children under the age of five years were stunted in 2016 (7), with reduced economic productivity as a detrimental outcome (8). It has been estimated that 20% of stunting prevalence originates from foetal growth restriction (9).

Around two billion people suffer from at least one micronutrient deficiency (1), and women of reproductive age in resource-poor areas are particularly at risk (2). Maternal micronutrient needs are increased during pregnancy due to the demand of the developing foetus (10). Existing deficiencies are often exacerbated during pregnancy and can lead to adverse outcomes for both the mother and her unborn child, such as growth deficits, foetal structural defects and some pregnancy disorders (11).

Impaired prenatal nutrition has also been linked to offspring risk of chronic disease in later life (12-14). Nutritional deprivation during early development triggers permanent changes in metabolism, increasing the risk of chronic diseases if rapid weight gain occurs after the age of two years (3). This is known as the developmental origins of health and disease hypothesis, linking poor intrauterine environment and foetal growth restriction to a higher risk of hypertension, type II diabetes and cardiovascular disease in adulthood (13, 14).

To address malnutrition and prevent adverse outcomes for mothers and children, implementation of evidence-based interventions at all stages of the life cycle are needed at scale (15). The first thousand days, from conception to the child's second birthday, has been determined the most important window of opportunity for preventing long lasting growth and development deficit (2, 8). It is a vulnerable period with rapid growth velocity and brain development, high susceptibility to programming effects along with high nutritional requirements (8). Furthermore, the pre-conceptional period is an important period to address to ensure nutrition adequacy during the first thousand days (8).

Evidence from three large meta-analyses of randomised controlled trials (RCT) showed that maternal supplementation during pregnancy with iron-folic acid (16), multiple micronutrients (10), and protein-energy (17) improved birth weight outcomes and decreased the risk of small-for-gestational age infants. The World Health Organization (WHO) currently recommends prenatal iron and folic acid supplementation as part of routine antenatal care to decrease maternal iron deficiency anaemia, low birth weight and to prevent neural tube defects (16, 18-20). Some researchers have argued for changing this recommendation to include multiple micronutrients (10). A recent meta-analysis of 17 RCTs concluded that multiple micronutrient supplementation during pregnancy compared to giving iron with or without folic acid decreased low birth weights, infants born small-for-gestational age and the rate of still births (10).

In terms of long-term effects of prenatal multiple micronutrients, a meta-analysis of RCTs reported that a multiple micronutrient supplementation during pregnancy did not impact growth, body composition or cardiovascular risk markers of children younger than nine years of age (21). Evidence of the beneficial effect of maternal supplementation on infant micronutrient status is limited. One RCT reported improved infant vitamin B_{12} status, but not vitamin A or zinc, at six months of age following maternal food and multiple micronutrient supplementation during pregnancy and the first 3 months of lactation (22). No other trials of multiple micronutrient supplementation during pregnancy reported infant postpartum micronutrient status.

After delivery, the focus of the recommended interventions is generally shifted from the mother towards the infant. During infancy, the WHO recommends optimal breastfeeding practices (see Sections 1.1. and 1.2) along with timely introduction of safe, adequate and nutrient-dense complementary foods from six months to two years of age (23). In some settings additional vitamin A, iron, vitamin D and iodine supplements for infants are recommended to sustain nutrient requirements for healthy development (15).

The mothers' postpartum nutritional status has not received much attention as a critical and vulnerable period in the prevention of maternal and child undernutrition. The WHO recommends iron supplementation, alone or in combination with folic acid, during the first 6-12 weeks postpartum, but only in populations with high prevalence of gestational anaemia (24). Bhutta et al (2013) (15) recommend several evidence-based interventions to address maternal and child undernutrition, but none of these interventions specifically address maternal nutritional status during lactation. A recent systematic literature review investigating the effect of multiple micronutrient supplementation during lactation on maternal and offspring outcomes concluded that it was impossible to quantify the effectiveness of maternal postnatal multiple micronutrient supplementation (25). The review included only two studies, both of which were of poor quality, highlighting that maternal postpartum nutrition is somewhat neglected in the scientific literature.

During lactation the energy and nutrient demands are greater than during pregnancy (26, 27). In the first few months of life healthy growing, exclusively breastfed infants double their birth weight, and the nutritional demand for lactating women is increased to support the growth of her offspring as well as her own metabolism (28). Lactating women who are not adequately nourished risk depleting their micronutrient stores, which can negatively influence the nutritional status of the breastfeeding child (29). Arguably, maternal pre- and postnatal nutritional status should be studied as a continuum, with emphasis on how it affects breast milk micronutrient composition and infant nutritional status, especially during the period of exclusive breastfeeding (EBF) (29).

In an attempt to address this research gap, this thesis focuses on breast milk micronutrients with the aim of exploring maternal nutrition during both pregnancy and lactation, breast milk composition, infant growth and nutritional status in a population in rural Gambia, where food availability and nutritional status are poor.

The following section introduces the relevance of breastfeeding and infant growth in settings where growth faltering is common, and highlights why the lactation period is an important period

to study, before focusing on lactation physiology, human milk composition and how maternal nutrition influences breast milk composition.

1.1 Breastfeeding

The WHO recommends EBF[1] until six months of age, with continued breastfeeding up to two years of age or beyond, along with nutritionally adequate, safe and appropriate complementary foods from six months of age (23).

The short-term benefits of breastfeeding have been well documented in both high-, (HIC) low- and middle-income countries (LMIC) (31-33). Observational studies have shown that breastfeeding has clear short-term advantages for child survival through reduction of morbidity and mortality from infectious diseases (31, 33, 34). One recent meta-analysis reported markedly reduced infant mortality, especially due to infectious diseases, with breastfeeding even into the second year of life (31). The benefit of early initiation of breastfeeding was recently reported in a meta-analysis, where delayed initiation of breastfeeding (2-23 hours after birth) increased the risk of neonatal mortality with 33% compared to initiation within the first hour after birth (33). Optimal breastfeeding practices have further been associated with a lower risk of childhood gastrointestinal infections, respiratory infection, sudden infant death syndrome and otitis media (31, 32). In addition to nutritional components, breast milk also contains a wealth of bioactive constituents that have beneficial non-nutritional functions for the infant; these include among others, a positive influence on the infant's immune system and gut microbiome (35, 36).

Breastfeeding also has long-term beneficial influences, with a wealth of observational evidence suggesting a protective effect on the incidence of non-communicable diseases, notably cardiovascular disease, diabetes (32, 37) and obesity (37-39). A recent observational study from Brazil found that breastfeeding is associated with improved performance in intelligence tests 30 years later (40). The evidence on longer-term outcomes is however more controversial than the shorter-term influences of breastfeeding. Studies have shown small effect sizes, and findings related to protective effects on obesity, raised blood pressure, other cardio metabolic risk factors (41-44) and cognitive performance (32) have been contradictory. The majority of these conflicting results (41-44), are based on the promotion of breastfeeding intervention trial

[1] EBF is defined by WHO as breastfeeding with no supplemental liquids or solid foods other than medications or vitamins (30)

(PROBIT), an RCT where more than 17,000 women were randomised to breastfeeding counselling in Belarus (45). Duration and exclusivity of breastfeeding were higher in the intervention group. However, the PROBIT study essentially compares "some" breastfeeding with "more" breastfeeding, and a difference in outcomes might have been observed comparing the "more" breastfeeding with an only formula fed control group.

Mothers also benefit positively from breastfeeding. Breastfeeding has been linked to a lower risk of breast cancer and possible also against ovarian cancer and type II diabetes in nursing mothers (31). A recent meta-analysis also found that breastfeeding are associated with lactational amenorrhea, increasing birth spacing (46), whereas evidence on the association between breastfeeding and postpartum weight loss is weak (46).

The evidence on breastfeeding's beneficial impact for infants and mothers is based largely on observational studies and in growing numbers on breastfeeding promotion intervention studies. It is unethical to randomise infants to various modes of feeding, which is why observational studies of breastfeeding practices and experimental designs of breastfeeding promotion offer the best available evidence of the causal relationships between breastfeeding and health outcomes. However, observational studies are prone to bias linked to confounding and reverse causality. The main confounders linked to breastfeeding are socioeconomic status and education. In HIC breastfeeding are associated with higher socio-economic status, and in LMICs this relationship is reversed (31). With reverse causality, the temporal sequence of the early signs of infection or growth faltering and cessation of breastfeeding may not be appreciated; infection or growth faltering may be responsible for the cessation of breastfeeding, rather than the other way round (47).

In experimental studies of breastfeeding promotion, noncompliance and similarities in the distribution of duration of breastfeeding can make causal inference problematic (47).

1.2 Exclusive breastfeeding

The WHO changed their recommendation of EBF from the first 4-6 months of life to six months after an Expert Consultation meeting in 2001 (48). The main evidence cited in support of the revision was a systematic review looking at infant health benefits (49) and a review evaluating the nutrient adequacy of EBF during the first six months of life (50).

The systematic review by Kramer and Kakuma (2012) (49) concluded: "The available evidence demonstrates no apparent risks in recommending, as a general policy, exclusively breastfeeding for the first six months of life in both developing and developed-country settings". This recommendation was based on comparing EBF for six months versus EBF for 3–4 months with continued mixed breastfeeding[2] until at least six months, in terms of growth, development, morbidity and survival of healthy, term infants. In total, 23 studies were summarized in the review, and 21 of these were observational studies that varied in both quality and geographic region. The last two studies were small RCTs conducted in Honduras, which did not classify as high methodological quality studies. Eleven of the 23 studies identified were conducted in LMICs. The authors concluded that in a LMIC setting, the most important advantage of EBF to six months of age was related to a decrease in infectious disease morbidity and mortality, especially due to gastrointestinal infections. No difference in infant allergic disease, growth, obesity or cognition was found between EBF to six versus EBF for 3-4 months (49).

Most of the scientific evidence included in this review was from observational studies, with well-recognised sources of potential bias. The quality assessment conducted in the Kramer and Kakuma (2012) (49) review showed that definitions of EBF varied considerably across studies. Few adhered to the WHO definition of EBF, and the majority defined EBF to also include the provision of water, teas or juices and a small amount of infant formula, which is by WHO definitions predominantly breastfeeding (30).

As a part of the 2001 Expert Consultation, the WHO evaluated the nutrient adequacy of EBF in meeting infants' nutrient requirements during the first six months of life (50). This was assessed by comparing infant nutrient intakes from breast milk with infant nutrient requirements, mostly evaluated by measuring infant growth.

The evaluation concluded that the total energy and protein concentration of breast milk was adequate to sustain infant needs in the first six months of life. In terms of micronutrients the evaluation was reduced to investigate only a few micronutrients; calcium, iron, zinc and vitamins A, D and B_6. The authors only identified vitamins A and B_6 as the micronutrients of concern, because maternal deficiencies of these two vitamins could lead to sub-optimal concentrations in breast milk. Iron status in exclusive breastfed children was also concluded as potentially problematic, as a study included in the review had demonstrated poorer iron status in infants who

[2] Mixed feeding is introduction of complementary liquid or solid foods with continued breastfeeding

were exclusive breastfed for six months versus four months in Honduras (51). Breast milk is a poor source of iron, and the estimated infant iron requirements cannot be met by breast milk alone at any stage of infancy. Nevertheless the authors concluded that in well-nourished populations the iron endowment infants are born with can sustain the need in early infancy, with iron from complementary foods needed after six months (50).

The WHO Expert Consultation recognised the lack of available data, and concluded that apart from a possible negative effect of EBF on infant iron status in poorly nourished populations, "the available evidence is grossly inadequate to assess risk of deficiency in other micronutrients" (48). Concerning the duration of EBF they also noted that: "the sample sizes were insufficient, however, to rule out an increased risk of growth faltering in some infants who are exclusively breastfed for 6 months, especially in populations with severe maternal malnutrition and a high prevalence of intrauterine growth retardation" (48). The Expert Consultation stated that investigating breast milk composition in poorly-nourished mothers and the adequacy to meet infant nutrient requirements is a highly-prioritised research area (48).

1.3 Infant growth

Regardless of the WHO recommendation, EBF practices differ widely between countries. In LMICs only 37% of infants younger than six months are exclusively breastfed (31). If looking at the prevalence of EBF to six months in LMICs, this is much lower, however due to lack of data no regional or global estimates exists.

These low percentages of EBF practices are likely linked to a longstanding debate about the optimal duration of EBF. One of the reasons for this debate is that growth faltering is commonly observed before six months of age in resource-poor settings, even in countries with high prevalence of breastfeeding (52-56). A secondary data analysis of national surveys from India reported that 21% of exclusive breastfed infants were stunted at six months of age, although there was no difference in stunting prevalence between exclusive breastfed and non-exclusive breastfed children, and the EBF definitions was based on a 24-hour recall (55).

When compared to the WHO International Growth Standards, infants from resource-poor settings are smaller at birth, show catch-up growth during the first few months of life, and then enter a period of reduced growth velocity marked by profound growth faltering up until 24 months of postnatal life (53, 54). Piwoz et al (2012) (57) stated that one of the main priorities in the work

of addressing poor growth is to understand why breastfed infants in resource-poor areas growth falter in early infancy (other determinants of poor growth are described at the end of this section).

There is limited evidence on how EBF *to* six months impacts infant growth in resource-poor populations. A recent meta-analysis of 35 RCTs investigated the effect of breastfeeding promotion interventions on infant and childhood growth (58). However, most studies included in this meta-analysis were conducted in affluent countries or urban areas in middle-income countries. In these populations, childhood overweight and obesity is a greater problem than growth faltering, and non-(exclusively) breastfed children are given infant formula (e.g. (41, 59)). The growth pattern of formula fed infants differs from that of infants who are breastfed, which is why the 2006 WHO Growth Standards are based on data from exclusively breastfed infants in the WHO Multicentre Growth Reference Study to represent healthy infant growth (60). Formula fed infants gain weight and length more rapidly than breastfed infants, starting around 2-3 months of age, and when they reach their first year of life they have a considerably larger body size (weight, length, BMI, skinfold) (61, 62). This accelerated postnatal growth has been linked to higher obesity rates in later life (63, 64). It is has been hypothesised that the higher protein content of infant formula (65), and possibly also a higher total energy intake in formula fed infants (66) drives larger body size. When investigating how EBF to six months influence infant growth in settings where growth faltering is common, it is important not to compare exclusive breastfed infants' growth against that of formula fed infants.

On examining the evidence from low-income countries in their meta-analysis where non-(exclusively) breastfed children were either given no or poor-quality infant formula, Guigliani et al (2015) (58) concluded that infant weight-for-age (WAZ) was significantly, although modestly, lower in the intervention group receiving breastfeeding counselling (z-score mean difference: -0.11). The effect was also reported for weight-for-length (WLZ) (z-score difference: -0.11), though no effect was seen on length/height-for age (LAZ) in low-income countries. This review assessed the impact of breastfeeding intervention on growth rather than the effect of breastfeeding *per se*, and so the results from the meta-analysis suggest that a longer duration of EBF in low-income countries has a modestly negative impact on infant WAZ and WLZ. This does not, however, tell us how EBF *to* six months influences growth, as most of the studies from low-income countries reported a low prevalence of EBF at six months of age.

Few studies have been able to investigate how EBF *to* six months influence growth in a resource-poor setting. Two RCTs have investigated the impact of EBF to six months on infant growth in

a setting where no infant formula was given (67, 68). These were both conducted in Honduras, and showed no difference in weight or length at six months of age between infants who were exclusively breastfed to six months versus to those to four months (with continued breastfeeding and solid foods). Both studies were also included in the Kramer & Kakuma (2012) (49) review that shaped the WHO recommendation of EBF to six month. These studies have however been criticised for having a low sample size (49) (n=119 and n=97 respectively), and only one of these trials followed the infants to one year of age (67), making it difficult to investigate any longer term influences. Two observational studies have also found the same overall results of no effect of EBF to six months on infant growth (69, 70). However, these observational studies were either cross-sectional or longitudinal studies that analysed serial measurements as cross-sectional data, reducing the power of longitudinal data (71).

The early growth faltering experienced in resource-poor areas has most commonly been attributed to several combined environmental influences such as, early introduction of low quality complementary foods, low dietary diversity, poverty, diarrheal diseases and other infections and poor hygiene and sanitation standards (8, 54, 72). A review of epidemiological studies has convincingly showed that suboptimal infant feeding practices, recurrent infections and micronutrient deficiencies are important determinants of stunting (2, 73). However, even with this knowledge, the most effective interventions to reverse growth faltering remain unclear (57, 74). No intervention study has normalised infant growth in settings where growth faltering is common (3). It has been argued that infant growth failure is further linked to more distant influences, such as economic growth, which increases access to health care, education and urbanisation (75). Intergenerational factors may also play a role, with maternal short stature being associated with offspring stunting (4, 5), and paternal in utero energy and nutrient restriction being associated with offspring postnatal growth (76).

A potential contributing factor to linear growth failure that has not received much attention is the possible inadequacies in breast milk micronutrient concentrations of mothers who enter pregnancy and lactation with a poor nutritional status (77). It is widely recognised that the response to deficiency of several nutrients, the so called Type II nutrients, is characterised by reduced growth or body weight without any reduction in tissue concentration (78). These includes for instance protein, essential amino acids, zinc and phosphorus. A Type I nutrient response, on the other hand, is characterised by a reduction in tissue concentration and body stores followed by clinical signs of deficiency. Type I nutrients include most micronutrients, for instance iron, iodine, selenium, thiamin, riboflavin and vitamin B_{12} (78).

Because few micronutrients are classified as Type II nutrients, it is often assumed that poor breast milk micronutrient concentration does not play a major causal role in the widespread growth faltering experienced in many LMIC settings (77). This has however not been ruled out by intervention studies or trials; few studies exists on how breast milk micronutrient concentrations is associated with infant growth in settings where infant growth faltering is common and where the mother is also at risk of micronutrient deficiencies. One observational study on breast milk micronutrient concentrations of Kenyan women examined vitamin B_{12}, a Type I nutrient, and found that it was not associated with infant growth at six months of age (79). Further, the low concentration of Type II nutrients such as zinc in breast milk, and the limited impact that maternal zinc status and dietary intake is believed to have on breast milk concentration, have led to a general lack of concern about the potential role that deficiencies in breast milk micronutrients play in contributing to infant growth failure (77). Overall, there is a notable lack of studies looking at the effects of breast milk micronutrient concentration on infant growth.

Irrespective of any impact on growth, the role of breast milk micronutrient concentration on infant nutritional status during the period of EBF is important for other infant health outcomes, and especially in resource-poor areas where lactating mothers are at risk of micronutrient deficiencies themselves (29). The data available, though limited, point to poor micronutrient status in breastfed infants at six months of age in resource-poor settings (22, 80-82). For example in Honduras 67% of breastfed infants had low vitamin B_{12} status, and 28% had low vitamin A status at six months of age (82). Low micronutrient status in infancy has typically been attributed to poor maternal status during pregnancy, low birth weight and/or preterm delivery resulting in poor infant stores at birth or low quality complementary foods, whereas breast milk micronutrient quality has been largely overlooked (77).

1.4 Lactation physiology and breast milk composition

Human milk is produced in the mammary gland, located in the breast, which consists of a series of ducts of epithelial origin branching through a connective tissue stroma and ends in clusters of grape-like alveoli, where milk secretion and storage take place. The alveoli are arranged into lobules, each of which drains into a ductal system which carries the milk product to the outside (83)(Figure 1).

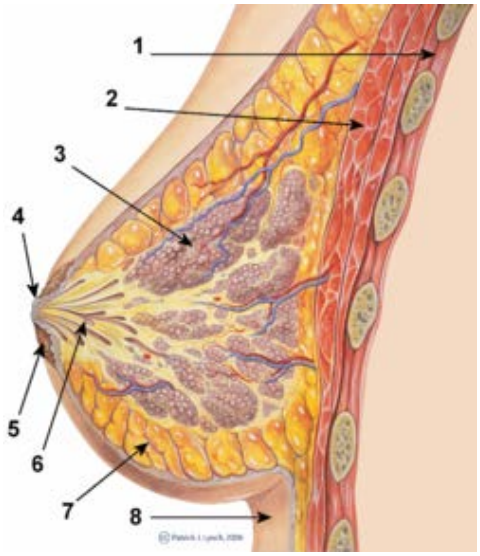

Figure 1. Cross-section of the mammary gland

1. Chest wall 2. Muscles 3. Lobules (groups of alveoli) 4.Nipple 5. Areola 6. Milk ducts 7. Fat 8. Skin. Source of picture: Patrick J. Lynch, medical illustrator.

The complex nature of breast milk composition also reflects the secretion and transport processes that take place within the mammary gland. Milk nutrients are secreted by epithelial cells in the alveoli of the mammary gland by numerous and complex systems, and are transported from the blood to the milk via transcellular pathways (84). There are two principle pathways by which solutes can move across an epithelium, the paracellular and the transcellular pathway (Figure 2). In the first pathway (pathway V in Figure 2) solutes are transported across the epithelium by passing through a tight junction. This pathway is only open during pregnancy, involution and in inflammatory states such as mastitis (85). In contrast the transcellular pathway involves the transport of solutes through the cell. The pathway responsible for the largest volume of milk is the excocytic pathway (pathway I in Figure 2). It is responsible for the exocytotic secretion of milk proteins, lactose, calcium and other components of the aqueous fraction of milk. It synthesizes milk proteins in the rough endoplasmic reticulum, then transfers these to the Golgi apparatus, where they are packaged into secretory vesicles for secretion. In the lipid pathway (pathway II) milk lipids are secreted by involvement of triglycerides that coalesce into larger fat droplets and move towards the apical membrane and are secreted as milk fat globules (83-85). Pathway III is the membrane transport pathway where distinct ions and small molecules such as glucose and amino acids are transported to the milk. This is done by specific transporters located

at the basal plasma membrane, Golgi apparatus, secretory vesicles and apical plasma membrane. Transcytosis is the last transcellular pathway (pathway IV) which is where the basal membrane takes up proteins, such as immunoglobulins, by endocytosis. The solutes are transported across the cell to the apical membrane where it is secreted into the milk space.

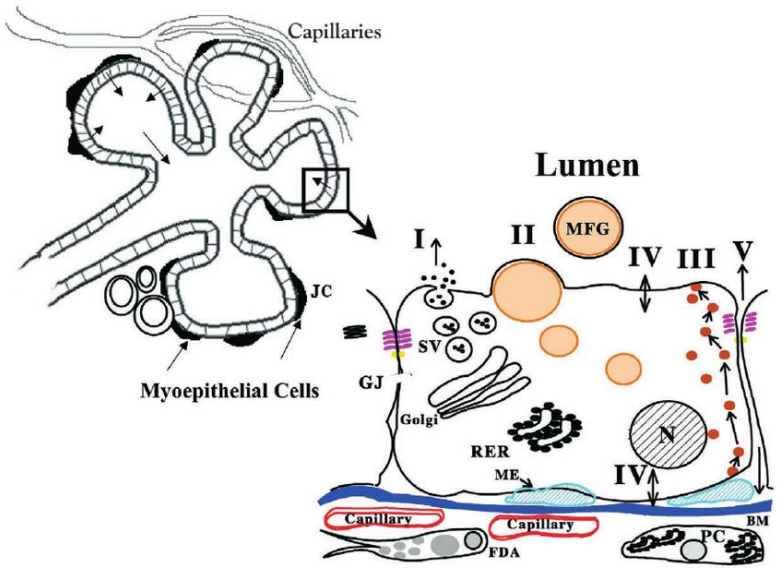

Figure 2. Pathways for milk secretion

Pathway I: Exocrine pathway. Pathway II: Lipid pathway. Pathway III: Membrane transport pathway. Pathway IV: Transcytosis pathway. Pathway V: Paracellular pathway. Abbreviations: RER – rough endoplasmic reticulum. MFG – milk fat globule. GJ – Gap junction. D – desmosome. BM – basement membrane. FDA – fat-depleted adipocyte. PC – plasma cell. From Neville (1995) (83), and McManaman and Neville (2003) (85).

Breast milk is produced during lactogenesis, which is the onset of milk secretion and includes all changes in the mammary epithelium necessary to go to full lactation after birth. Lactogenesis can be divided into two phases, where the onset of colostrum production in the final trimester of pregnancy is the first phase, and thereafter the mammary gland is sufficiently differentiated to secrete milk. Phase two of lactogenesis (secretory activation) is the onset of milk secretion after parturition following the initiation of lactation (86).

1.4.1 Milk composition

Human milk composition is dynamic, extremely complex and highly variable (36). Breast milk consist of more than 200 recognized elements, including water, macronutrients, minerals, vitamins, trace elements and non-nutritional bioactive components, such as hormones, antibodies and human milk oligosaccharides. Its composition has evolved over millions of years adapting to the requirements of infants (36).

Milk composition changes radically according to stage of lactation, especially during the first few days after birth when secretion changes from colostrum to milk. After a few weeks the composition stabilises and further changes in composition occur over a longer time frame (87). Human milk can roughly be divided into three types that differ in composition according to stage of lactation; colostrum, transitional, and mature milk. Colostrum is low in volume and is secreted in the first days postpartum; transitional milk is characterised by a gradual increase in volume and fat concentration and typically occurs from day five to two weeks postpartum. By four to six weeks postpartum the milk is considered fully mature (88). Even in mature milk, composition continues to change over the course of lactation.

Protein concentrations gradually decline in breast milk according to stage of lactation, whereas fat and carbohydrate concentration slightly increases from colostrum to mature milk (89, 90). For the majority of the water-soluble vitamins, concentrations are lower in early milk (1-5 days) compared to mature milk (91), except for vitamin B_{12} where the opposite has been reported (92). Fat-soluble vitamin concentrations are high in colostrum and drastically decline during the first week of lactation (93, 94). High concentration of fat-soluble vitamins in early lactation has been attributed to their storage in the mammary gland prior to delivery and released in response to endocrine changes associated with delivery and lactogenesis (95). Mineral concentrations are generally higher in colostrum than in mature milk, except for calcium where no changes have been observed according to stage of lactation (94).

Considerable variation in milk production and composition is also seen across population and between mothers (96, 97). For instance, milk protein and carbohydrate concentrations are relatively constant between individual women, whereas fat, and also the energy content of milk, varies widely between individuals and populations (50). Milk composition varies according to the time of day, within-feeding, and according to intervals between feedings (98, 99). The first milk expressed during a feed (foremilk), has a lower content of fat compared to the last milk

consumed during a feed (hindmilk) (98, 100). This change in composition is mostly seen for fat and fat-soluble components, where fat changes as much as five-fold within a single feed. Even within mature milk alone fat composition can change drastically over 24 hours. One study found an estimated variation coefficient in fat concentration in mature milk of 47% (101).

Breast milk volume is likely to also influence milk composition. Milk volume is primarily linked to infant demand (102), along with infant weight and total nursing time (103). Exclusively breastfed infants receive a higher milk volume than partially breastfed infants, which is especially apparent during the period when complementary foods replace breast milk (104). The variability in breast milk volume consumed by exclusively breastfed infants in the first six months of life is of particular importance, as milk transfer varies significantly from one mother-infant pair to another. It varies approximately from 450 to 1300 ml/day, averaging approximately 800 ml/day in healthy women across the first six months of EBF (99).

Evidence have shown that in women producing low milk volumes, the quantity of a nutrient increases to compensate (87, 105). A study investigating this in relation to macronutrient composition found lower concentrations of protein and fat with higher volumes of milk produced, whereas lactose increased with increasing volume (106). Literature on the influence of volumetric differences on micronutrient concentration is however lacking. A review reported breast milk calcium concentration to be independent of breast milk volume (107) and a recent study in Switzerland found the same results for milk iron concentration (Noever et al unpublished, personal communication). No studies have investigated how vitamin concentrations are influenced by volume. Breast milk volume/infant intake was previously measured by test-weighing the infant before and after breastfeeding for at least 24 hours (104), whereas now the dose-to-mother deuterium oxide method is considered the gold standard (108).

1.5 Maternal nutritional influences

Maternal nutritional status or dietary intake is the most important environmental influence on breast milk nutritional composition (97). Mixed evidence has been found on other environmental influences, such as maternal age, parity and gestational age (94, 109-111).

The influence of maternal nutritional status and dietary intake on macronutrient composition has been studied by several researchers (106, 112-115), and especially studies with a focus on breast milk fatty acid composition have been conducted (116-119). These studies have found that breast

milk protein concentration is generally not influenced by maternal diet (106, 113), neither is lactose, the main carbohydrate found in breast milk (106, 114). However, one intervention study in rural Gambia reported a small increase in breast milk protein and a small decrease in lactose concentration with a high energy dietary supplement (fortified biscuit) given during lactation (115). Although this study used the same participants as retrospective controls, and the change observed could therefore be due to stage of lactation rather than the supplement.

In relation to fat, findings have been contradictory. The total milk fat concentration seems to be unaffected (26, 97) whereas the fatty acid profile of breast milk appears responsive to maternal intake (116-119). In an RCT with 24 breastfeeding mothers assigned to receive either a daily docosahexaenoic acid (DHA) supplement or a placebo, authors found that postpartum DHA supplementation increased maternal plasma phospholipid and breast milk DHA (117).

The influence of maternal undernutrition on lactation performance and breast milk energy has also been studied (115, 120). Older studies have found that undernourished women only produced half the volume of milk produced by well-nourished women from more affluent countries. However, subsequent work using improved methods showed no distinction in the production of breast milk volume between undernourished and well-nourished women (97). A study combining data from 1060 women at three months postpartum found that maternal nutritional status, indicated as body mass index (BMI), was not associated with breast milk volume or breast milk energy content, not even in thin mothers (BMI <18.5 kg/m^2) (120) (Figure 3). Thin mothers living in resource-poor areas produced milk with energy levels comparable to those in milk produced by normal weight mothers. This was supported by the findings of an intervention study where 130 rural Gambian women were given a nutritional supplements during lactation (participants acted as retrospective controls), increasing energy intake from 1569 to 2291 kcal/day (115). The authors found no effect of the supplement on breast milk volume or total energy at any stage of lactation or in any season of the year.

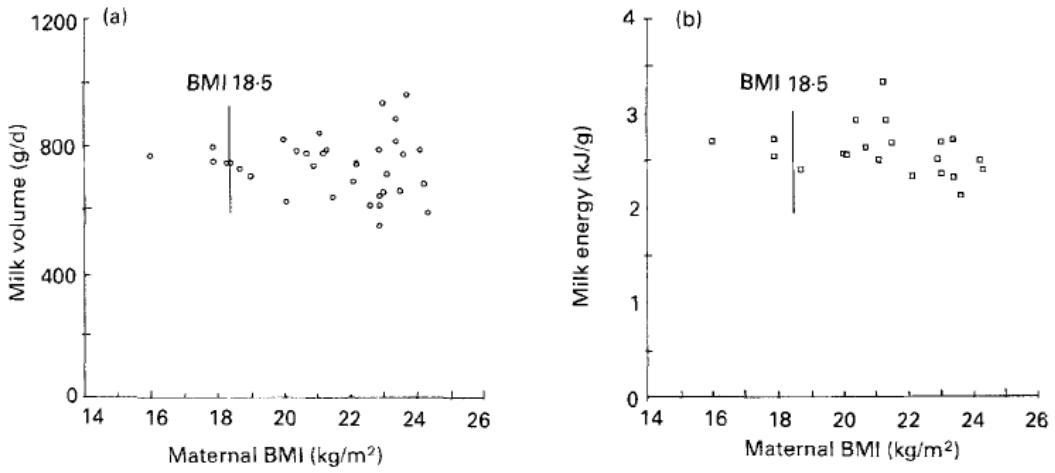

Figure 3. Relationship between maternal BMI and (a) milk volume and (b) energy content.

Data from 1060 women at three months postpartum from 35 separate studies. Values are means. From Prentice, Goldberg and Prentice (1994) (120).

The influence of maternal nutritional status on breast milk micronutrient composition is less well understood. A recent systematic review by Bravi et al (2016) (112) investigated how maternal dietary intake influences macro- and micronutrient concentrations in breast milk, but this review was restricted to studies investigating the influence of maternal dietary intake, excluding those investigating the effect of maternal nutritional status and micronutrient supplements. Furthermore, the review only included studies of well-nourished mothers, excluding studies conducted in poorly-nourished populations. Allen (1994) (121) also reported on how maternal nutritional status and dietary intake influence breast milk micronutrient composition, but this study was not based on a systematic approach and is now more than two decades old.

Other available studies or narrative reviews on breast milk micronutrient composition have either focused on a single breast milk micronutrient, or have used non-systematic literature review approaches without a specific objective of investigating maternal nutritional influences (77, 107, 121-127). Most of these available studies are clustered around specific micronutrients, leaving some micronutrients almost completely unexplored.

This introduction highlights the research gap that exists on maternal nutrition, breast milk micronutrients and infant growth in the first six months of life when infants are relying on breast milk as their only source of nutrients. The scientific literature to support the adequacy of breast milk micronutrient composition among women with a poor nutritional status is limited.

1.6 Aims and objectives (I)

There is limited evidence on the influence of maternal nutritional status and dietary intake on breast milk micronutrient composition in resource-poor areas where the mother is at risk of micronutrient deficiency. Data are lacking on how EBF to six months of age, in a resource-poor setting where infants experience early growth faltering, is associated with infant growth. The scientific literature on maternal nutrition, breast milk micronutrient composition and infant nutritional status during the period of EBF in a resource-poor setting, is lacking.

The overall *aim* of this thesis is to:

> Investigate maternal nutrition, breast milk micronutrient concentrations and infant growth and nutritional status during the period of exclusive breastfeeding in a resource-poor population.

The first two research *objectives* of this thesis are to:

1. Systematically define the existing evidence-base of maternal nutritional influences (nutritional status, dietary and supplement intake) on breast milk micronutrient (vitamins and minerals) concentrations across the first six months of lactation *(Chapter 2)*

2. Identify how exclusive breastfeeding practices during the first six months of life is associated with growth of rural Gambian infants up to two years of age (*Chapter 5*)

The results of the systematic literature review (*Chapter 2*) will guide the remaining objectives of this thesis, which are listed in *Chapter 3* (Section 3.4, pages 115-116).

Chapter 2

Systematic literature review

This Chapter presents a systematic literature review aiming to assess the influence of maternal nutritional status and intake (food or supplements) on breast milk micronutrient concentrations, and includes studies from all settings (high-, middle- and low income countries). The following micronutrients are included: vitamin A, vitamin D, vitamin E, vitamin K, vitamin C, thiamin, riboflavin, vitamin B_6, folic acid, vitamin B_{12}, calcium, copper, iodine, iron, selenium and zinc. This review is used to highlight the selection of micronutrients that will be focused on in the remainder of this thesis.

2.1 Methods

2.1.1 Search strategy

A comprehensive and systematic literature search was performed in PubMed/MEDLINE including articles published until March 1st 2017. The Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA) standard reporting guidelines were used. The following Mesh search terms were used: lactation, breastfeeding breast milk, human milk, vitamin B_1, thiamin, vitamin B_2, riboflavin, vitamin B_6, vitamin B_9, folate, folic acid, vitamin B_{12}, cobalamin, vitamin A, retinol, vitamin D, vitamin E, vitamin K, vitamin C, ascorbic acid, iodine, iron, calcium, zinc, selenium, copper and micronutrient. In addition to the studies found through PubMed, reference lists of the identified studies were checked and relevant studies were included.

2.1.2 Inclusion and exclusion criteria

All types of human studies were included for this review; randomised controlled trials (RCTs), interventions (no randomisation) and observational studies with no restriction on year of publication. Studies were included if (i) maternal nutritional status or intake was measured (either by biomarkers, dietary intake, supplementation or food fortification) during either pregnancy or lactation, (ii) breast milk micronutrient concentrations were measured and reported as means or medians, and (iii) the relationship between maternal nutritional status or intake and breast milk micronutrient concentrations in the first six months of life were investigated.

Studies investigating adolescents (<18 years), HIV positive women or pre-term deliveries were excluded, because of the likely difference in the concentration of some micronutrients in breast milk in these specific subgroups (110, 128-130). Studies that only investigated breast milk micronutrient concentrations beyond the first six months of lactation and studies providing breast milk concentrations in a figure without reporting the actual concentrations within the text were also excluded. Case studies and literature reviews were excluded along with studies with no full text access. For vitamin A, studies that did not report on breast milk retinol concentrations in relation to milk fat were excluded (n=17). Vitamin A is present almost exclusively in the lipid fraction of breast milk as retinyl esters, mainly retinyl palmitate (131, 132). Breast milk vitamin A concentration is strongly associated with milk fat, and retinol adjusted for fat is a more accurate reflection of total breast milk vitamin A concentrations. It has been suggested to express fat-soluble vitamins per grams of milk fat to reduce sampling inaccuracies (93, 131). This criterion was not applied to the other fat-soluble vitamins (D, E and K), as this would have led to exclusion of almost all the relevant studies.

2.1.3 Data extraction

All studies were read and information was extracted on study design, geographical area, sample size, micronutrient supplementation status, breast milk sampling protocols (including number of samples collected, collection method, stage of lactation when samples were collected, laboratory method and exclusively breastfeeding status if reported), maternal nutritional assessment (including method used to assess status or intake), and main results (including breast milk micronutrient concentrations and estimates of the relationship between maternal nutritional status or intake and micronutrient concentrations in breast milk).

2.2 Results

2.2.1 Description of included studies

Figure 4 details the publication selection flow. The initial search yielded 4505 publications, of which 4126 were excluded after title and abstract evaluation. Another 245 were excluded after considering the full text, leaving 135 eligible publications. Six further studies were identified by cross-referencing included publications. In total, 141 publications were included in this systematic review. The main characteristics and results of the included publications are summarised in Table 1 (vitamins) and Table 2 (minerals). Among the 141 included studies, 25 studies investigated several breast milk micronutrient components and were included more than once in Tables 1 and 2 and in the review below.

The studies included were published between 1960 and 2017, with 50% (70/141) published before 2000. Sixty studies were conducted in LMICs, 78 in HIC and 3 studies included participants from both HICs and LMICs. Twenty-nine of the studies were RCTs, 25 were interventions with no randomisation and often no placebo group, 77 were observational studies, and the remaining 10 studies were classified as pre-post intervention studies. Instead of using a control or placebo group, these ten studies compared breast milk micronutrient concentrations in breast milk samples collected pre-supplementation and post-supplementation. A fundamental problem with this study design is that it cannot be determined if a change in breast milk micronutrient concentration is due to supplementation or if it reflects a physiological increase or decrease in milk micronutrient concentrations with stage of lactation.

Analytical methods used among the included studies were highly variable. In addition to different methods being used between micronutrients, the methods also varied within each micronutrient. For instance, nine different analytical methods were used to quantify breast milk iodine concentration (Table 2). This was also the case for vitamin B_{12}, for which studies used various pre-treatments to extract breast milk vitamin B_{12} from its binding protein, apo-haptocorrin (apoHC), and various assays for vitamin B_{12} concentration (Table 1). Furthermore, a total of 11 studies did not describe the analytical method used.

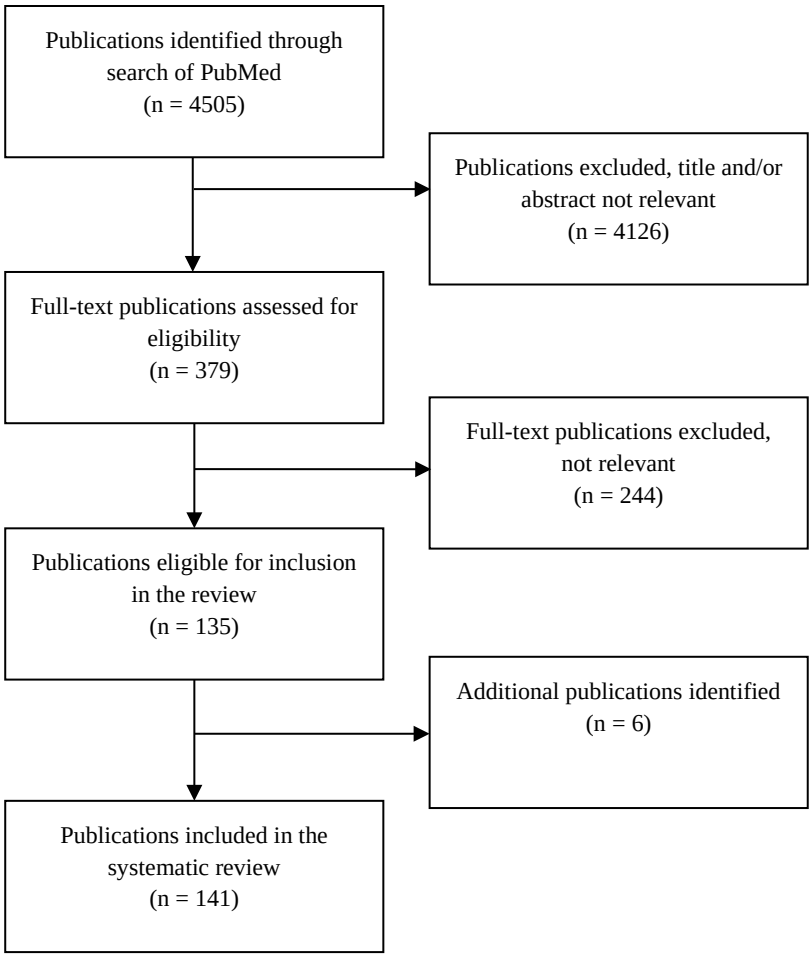

Figure 4. Publication selection flow

2.2.2 Maternal nutrition

Of the total 141 studies, 41 investigated more than one of the three exposure variables; maternal supplementation, dietary intake or status. The 63 RCTs, intervention and pre-post intervention studies included in this review provided the micronutrients in the form of either a single dose (tablet form or oil) or as a daily tablet (n=57), lipid-based nutritional supplement (n=1), as fortified foods (not including fortified oil) (n=4), or as a mixture of diet and supplementation (n=1). Among the 78 included observational studies, 34 investigated maternal nutritional status only by either blood or urine, and 29 studies investigated maternal dietary intake as the only exposure variable measured by either FFQs, 24-hour recalls or dietary records or diaries. Four observational studies investigated self-reported supplement use, and the remaining 11 observational studies investigated both maternal status and dietary intake.

Only 13 studies investigated maternal supplementation, intake or status during pregnancy, 14 investigated supplementation, intake or status during both pregnancy and lactation and 111 focused on supplementation, dietary intake or status on or around the day of breast milk sample collection. In three of the studies, it was not clear when the blood, urine or maternal dietary intake data was collected.

2.2.3 Breast milk collection

From the total 141 studies, 97 (69%) collected breast milk longitudinally (serial samples), and breast milk micronutrient concentrations were measured in colostrum to mature milk up to 12 months postpartum.

Several different breast milk expression methods were used across the included studies. In some, the milk was obtained by manual hand expression (n=58), in others the sample was collected with a manual breast pump (n=17) or electronic pump (n=20), and in many studies it was not specified how breast milk was expressed (n=46).

Protocols for the collection of breast milk varied widely across studies. Some studies obtained a sample from a full breast expression (n=21) (resulting in the collection of both foremilk and hindmilk), others collected a sample just before and after a feed (n=18) (foremilk and hindmilk), after a feed (n=2) (hindmilk only), and others collected a mid-feed milk sample (n=19) (hindmilk only). Several studies did not standardise the collection method around a feed, but collected the sample between feeds (n=30) (foremilk only) often standardising the collection to a specific time

of day. The majority of the studies did not specify how the breast milk sample was collected (n=51). Seven studies reported collection breast milk from both breasts, 33 from one breast only and 101 studies did not specify if the sample was from one or both breasts. Quite a large number of studies did not standardise breast milk sample collection according to stage of lactation (n=32), meaning that concentrations from across the lactation period were either pooled or used for comparison.

Only a small number of studies included data on breast milk volume, either expressed as a daily volume production or as daily infant intake. Similarly, only a few studies (n=42) reported if the infants were exclusively, predominantly or partially breastfed at the time of milk collection.

2.2.4 Main results

The relationship between maternal intake, nutritional status and breast milk micronutrient composition are presented below, ordered by type of micronutrient, with vitamins first followed by minerals. For each micronutrient, results are ordered as follows: The relationship between (i) maternal supplementation and breast milk micronutrient concentration (ii) maternal dietary intake and breast milk micronutrient concentration and (iii) maternal micronutrient status and breast milk micronutrient concentration.

Vitamin A

A total of 16 studies focused on vitamin A; nine RCTs, three intervention and four observational studies.

Four RCTs supplemented mothers with single mega-doses of 200,000-400,000 IU of vitamin A within one week after delivery, and showed an increase in breast milk retinol/g fat concentration (133-136). The effect was sustained for 3-4 months postpartum, however it disappeared around 6-9 months (133, 134, 136). Two of these RCTs, Rice et al (1999) (136) and Ayah et al (2007) (133), also measured maternal serum retinol and both studies found no effect of supplementation on serum retinol concentrations. This suggests that when supplementing the lactating mother, vitamin A is directed to the mammary gland rather than the liver, and that serum retinol is stable over a wide range of vitamin A intakes. These four RCTs were conducted in Kenya, Ghana/India/Peru, Brazil and Bangladesh; the Bangladeshi population particularly had a high prevalence of vitamin A deficiency during the study period.

One RCT and one intervention study, supplementing mothers during pregnancy or lactation with a weekly or daily vitamin A supplement (4800 RE weekly, 650 µg daily) reported an increase in breast milk retinol/g fat in breast milk (137, 138): 0.053 µmol/g vs 0.044 µmol/g ($p<0.05$) in mature milk from supplemented vs placebo women in Indonesia (137) and 29.5 µg/g vs 22.9 µg/g ($p<0.01$) in supplemented vs. unsupplemented women from The Gambia (138). Both studies failed to find any effect of vitamin A supplementation on maternal plasma and serum.

An RCT from Bangladesh found vitamin A supplementation of 0.25 mg retinyl acetat (given twice a day, six days a week) during lactation to increase breast milk retinyl equivalents/g fat in mature milk: 29 nmol/g fat vs. 20 nmol/g fat in supplemented vs. unsupplemented women (139). A recent RCT from Ghana supplemented mothers during both pregnancy and lactation with either a daily multiple micronutrient (MMN) supplement or a lipid-based nutrient supplement (LNS) both containing 800 µg vitamin A (111). The authors did not find a significant difference in breast milk retinol/g fat at six months postpartum in the supplemented group compared to the control group (MMN: 55.4 nmol/g fat, LNS: 54.7 nmol/g fat, control: 59.1 nmol/g fat).

Supplementation with β-carotene, during either pregnancy or lactation, did not increase breast milk retinol/g fat concentrations (140-143), and two of these studies were RCTs conducted in populations with a high prevalence of vitamin A deficiency (140, 143).

One study conducted in Cameroon demonstrated a positive association between maternal dietary vitamin A intake and breast milk retinol/g fat concentration ($r^2=0.13$, $p<0.001$) by FFQ and 24-hour recall (144), while other studies did not report any association (145, 146). For instance a dietary intake study in Brazil did not find an association between maternal dietary vitamin A intake (by FFQ) and concentration in colostrum (146).

Four studies investigated maternal vitamin A status (measured as serum or plasma retinol) and the association with breast milk retinol/g fat (137, 138, 146, 147). Two studies found a positive association (137, 147), however the remaining studies did not.

Vitamin B_1 (thiamin)

Eleven studies on breast milk thiamin concentration were included in this review, one RCT, four interventions, two pre-post intervention and four observational studies.

The only included RCT was conducted in Cambodia, in a population at risk of thiamin deficiency (148). The authors found that the consumption of thiamin fortified fish sauce (8 g/l) during pregnancy and lactation significantly increased total thiamin concentration in mature milk: 177 μg/l vs. 144 μg/l in women consuming fortified fish sauce (highest dose) vs. placebo. In line with this data, two pre-post intervention studies reported that thiamin supplementation (1.36 mg/day and 100 mg/day) during lactation increased breast milk thiamin in populations with poor thiamin status (115, 149).

A pre-post intervention study in a Thai-Myanmar population found that maternal intake of thiamin fortified flour (estimated intake of thiamin: 0.34 mg/day) increased breast milk free thiamin, however not breast milk total thiamin concentration (147). This study did not have a control group, but compared breast milk thiamin concentration after fortification with pre-fortification concentration in two different groups of women.

One intervention study conducted in a refugee camp near the Thai-Myanmar border supplemented participants who showed signs of thiamin deficiency with 100 mg of thiamin a day, but only during pregnancy (150). Women who did not show physical signs of deficiency did not receive any supplements. At three months postpartum, there was no difference in breast milk thiamin concentration between supplemented (128 μg/l) and unsupplemented women (117 μg/l) (150). In two intervention studies, in well-nourished women from the United States, breast milk thiamin concentration did not respond rapidly to maternal supplementation during lactation (151, 152). This contrasting observation to the findings from less well-nourished settings could suggest a preferential transport of thiamin into breast milk if maternal status is poor.

Maternal dietary thiamin intake during both pregnancy and lactation was positively associated with breast milk thiamin concentration in poorly- and well-nourished populations (153-155).

Maternal thiamin status was associated with breast milk total thiamin in Cambodia (149), and in a population living in a refugee camp at the Thai-Myanmar border (r^2=0.37, $p<0.05$) (156).

Vitamin B_2 (riboflavin)

A total of seven studies on riboflavin were included in this review; three interventions, one pre-post intervention and three observational studies.

One of the interventions and one of the pre-post intervention studies were conducted in rural Gambia, where there is a high rate of riboflavin deficiency (115, 157). Both studies found a positive effect of riboflavin supplementation (2 mg/day and 1.22 mg/day) during lactation in breast milk riboflavin concentrations. In the study published by Bates et al (1982) (157) the concentrations were 0.23 μg/ml vs. 0.14 μg/ml in the supplemented vs. placebo group ($p<0.03$) at approximately 40 days after supplementation commenced. In the pre-post intervention study published in 1983 by Prentice et al (1983) (115), a concentration of 0.21 μg/ml pre-supplementation vs. 0.28 μg/ml post supplementation ($p<0.001$) was observed.

An intervention conducted in a replete population in the United States also reported riboflavin supplementation (2 mg/day) during pregnancy and lactation to have a positive effect on riboflavin concentrations: 710 μg/l vs. 485 μg/l ($p<0.01$) in supplemented vs. unsupplemented women at six weeks postpartum (151). In contrast, another American study did not find any effect of supplementation (2 mg/day): 274 μg/l in supplemented vs. 243 μg/l in unsupplemented women at six months postpartum (152). Both of these studies had low sample sizes (n=11 and n=12).

Three observational studies investigated the relationship between maternal dietary riboflavin intake and breast milk riboflavin concentrations. Two studies, one conducted in India (where maternal riboflavin status was poor) and one in Spain, found a significant association (155, 158), whereas the last study, which was conducted in Russia, did not (154).

Only one study reported data on maternal riboflavin status, and found a positive effect between maternal status during third trimester of pregnancy and breast milk riboflavin in mature milk (158).

Vitamin B_6

A total of 11 studies on vitamin B_6 were identified for inclusion in this review; one RCT, five interventions, one pre-post intervention and four observational studies.

The only RCT, randomised participants to a supplement during lactation of either 0.5 mg pyridoxine or 4.0 mg pyridoxine as part of a daily multiple micronutrient supplement (159). There was a significant difference in total breast milk vitamin B_6 concentrations between the two groups, at 24 weeks postpartum; 1317 nmol/l and 2666 nmol/l ($p<0.01$) for the 0.5 and 4.0 mg/day supplement groups, respectively.

Four interventions and one observational study (160-164) from the United States found a positive effect of vitamin B_6 supplementation during lactation on breast milk vitamin B_6 concentration. One of these studies (160) also reported a positive effect of prenatal vitamin B_6 supplementation (this was an observational analysis, as the women self-reported their supplement use). However, Chang et al (1990) (160), Styslinger et al (1985) (162) and Hamaker et al (1990) (164) did not include an unsupplemented group, but showed that breast milk vitamin B_6 concentration paralleled the level of supplementation.

One intervention and one pre-post intervention study did not observe an effect of vitamin B_6 supplementation (115, 152). Thomas et al (1980) (152) found no effect of supplementation during lactation (4 mg/day) on breast milk vitamin B_6 concentration at six months postpartum: 235 μg/l vs. 212 μg/l in the supplemented vs. unsupplemented American women, however this study had a low sample size (n=12). The pre-post intervention study, conducted in rural Gambia, found that a daily fortified biscuit (containing 0.71 mg of vitamin B_6) given during lactation did not increase breast milk vitamin B_6 concentrations (115). This study did not use a placebo or a control group; milk vitamin B_6 concentrations after supplementation were compared with pre-supplement concentrations.

Several studies reported a positive association between maternal dietary vitamin B_6 intake and breast milk vitamin B_6 concentrations (165) (161, 162, 166). A study conducted in the United States (161), reported that maternal dietary vitamin B_6 intake measured by 4-day dietary records was associated with breast milk vitamin B_6 concentration at 5-7 days postpartum ($r=0.84$, $p<0.01$), and at 43-45 days ($r=0.76$, $p<0.05$) in unsupplemented women.

Three studies reported a positive association between maternal serum/plasma vitamin B_6 and breast milk vitamin B_6 concentrations (160, 161, 165). For instance, Chang et al (1990) (160) from the United States showed that maternal plasma pyridoxal phosphate (PLP) was associated with breast milk vitamin B_6 at one month ($r=0.49$, $p<0.01$), four months ($r=0.57$, $p<0.01$) and six months postpartum ($r=0.63$, $p<0.01$).

Vitamin B_9 (folate)

Eleven studies on breast milk folate were identified for inclusion in this review; two RCTs, three interventions, four pre-post intervention and three observational studies.

Two RCTs from Canada (167) and the United States (168) showed no effect of maternal folic acid supplementation during lactation (400 μg/day and 1 mg/day, respectively) on breast milk total folate concentration: 159 nmol/l vs. 183 nmol/l in supplemented vs. placebo women in Canada at 16 weeks postpartum (167) and 182 nmol/l vs. 187 nmol/l in supplemented vs. placebo women from the United States at six months postpartum (168).

An intervention study reported no effect of folic acid supplementation as there was no increase in breast milk folate concentration in women supplemented with 0.8 mg/day folic acid during lactation; 55 μg/l vs. 50 μg/l in supplemented vs. unsupplemented American women (152). In contrast, Sneed et al (1981) (161) reported a positive effect of 0.8 mg/day folic acid supplementation during lactation on breast milk folate compared to a placebo group in the United States. However, participants in this study were not randomised to a supplement group.

Maternal dietary folate intake, measured by 4-day dietary records was not associated with breast milk folate concentration in American women ($r=0.25$ $p>0.05$) (161). Similarly, maternal meat intake was also not associated with breast milk folate in Indian women (169).

Three observational studies did not find evidence for an association between maternal serum folate and breast milk folate concentration (161, 170, 171). This suggests that breast milk folate is tightly regulated and minimally influenced by maternal folate status or intake.

Four pre-post intervention studies, with no placebo or control groups, which compared milk folate concentrations after supplementation with pre-supplement concentrations were included (115, 172-174). One of these studies, conducted in Mexico, found an increase in breast milk total folate (172), whereas the remaining three studies conducted in the United States, Japan and The Gambia did not (115, 173, 174). However, West et al (2012) (173) did find an increase in breast milk folic acid concentration with folic acid supplementation (750 μg/day) and natural food folate (400 μ/day).

Vitamin B_{12}

A total of 15 studies investigated how maternal vitamin B_{12} intake or status is associated with breast milk B_{12} concentrations, two RCTs, two interventions, two pre-post intervention and eight observational studies.

Six studies investigated the influence of vitamin B_{12} tablet supplementation during pregnancy or lactation on breast milk vitamin B_{12} concentration (115, 152, 161, 163, 175, 176). Two of these studies were recent RCTs conducted in India (175) and Bangladesh (176) in populations with poor maternal vitamin B_{12} status. In India, vitamin B_{12} supplementation (50 μg/day) during pregnancy (given from <14 weeks gestation) and through to six weeks postpartum, increased breast milk vitamin B_{12} concentration: 136 pmol/l vs. 87 pmol/l ($p<0.001$) in supplemented vs. placebo women at six weeks postpartum (175). Supplementation also increased breast milk vitamin B_{12} concentration at three and six months postpartum, however this effect was not significant. In Bangladesh, vitamin B_{12} supplementation (250 μg/day) during pregnancy and lactation had an effect on vitamin B_{12} concentration in colostrum (778 pmol/l vs. 320 pmol/l in supplemented vs. placebo group, $p=0.001$) and in mature milk (235 pmol/l vs. 170 in supplemented vs. placebo group, $p=0.03$) (176). This increase in breast milk vitamin B_{12} with supplementation was supported by two intervention studies from the United States supplementing women with 8 μg/day of oral vitamin B_{12} during lactation (161, 163). Another intervention study conducted in the United States found contrasting results, reporting no evidence of an increase in breast milk vitamin B_{12} at six months postpartum when supplementing women with 8 μg/day during lactation (152).

The two included pre-post intervention studies (115, 177) did not detect a difference in breast milk vitamin B_{12} concentration. In The Gambia, a daily fortified biscuit (containing 1.19 μg of vitamin B_{12}) did not increase breast milk vitamin B_{12} concentration (115), and in the United States, 10 weeks of supplementing lactating women with a vitamin B_{12} rich diet and supplement (a total of ~8.6 μg/day) did not change breast milk concentration (177). Both of these studies did not use a placebo or a control group; but compared breast milk vitamin B_{12} concentrations after supplementation with pre-supplement concentrations.

In general, the included observational studies did not find any evidence for an association between maternal dietary vitamin B_{12} intakes and breast milk vitamin B_{12} concentrations (79, 161, 169, 178, 179). A study in Kenya found no evidence for dietary vitamin B_{12} intake which was measured by a quantitative weighing method and dietary recall, to have an influence on breast milk vitamin B_{12} at 0-1 month postpartum ($r^2=0.27$, $p=0.08$). However the study found evidence for an association at 1-4 months ($r^2=0.20$, $p<0.01$), and at 4-6 months postpartum ($r^2=0.26$, $p<0.001$) (79).

Mixed results were reported on the association between maternal vitamin B_{12} status and breast milk vitamin B_{12} concentration. A recent Danish observational study found an association between maternal plasma vitamin B_{12} and breast milk vitamin B_{12} at four months postpartum ($r=0.58$, $p=0.002$), however not at two weeks or nine months postpartum (180). Three other studies reported evidence for an association between maternal serum/plasma vitamin B_{12} and breast milk vitamin B_{12} in a Canadian (181) and American population (177, 182), whereas four other observational studies from Guatemala (178), Cambodia (181), Brazil (171) and the United States (161) did not.

Vitamin C

A total of 12 studies investigated how maternal vitamin C intake or status was associated with breast milk ascorbic acid concentration. Seven of these studies were intervention studies and five of them were observational studies.

Three of the intervention studies were conducted in poorly nourished populations, and two of them in the 1980's in rural Gambia (183-185). All three studies found a positive impact of maternal ascorbic acid supplementation (ranging from 35-1000 mg/day) on breast milk ascorbic acid concentrations. For instance in The Gambia, 90 mg of ascorbic acid given during lactation, in the form of a daily tea drink, had an effect on breast milk vitamin C; 3.40 mg/dl vs. 5.51 mg/dl in supplemented vs. unsupplemented women (184). The remaining four intervention studies were conducted in well-nourished populations (the United States and Australia), and ascorbic acid supplementation had less impact on breast milk concentration in these women compared to the effect observed in poorly-nourished populations (152, 161, 163, 186). However, it should be noted that that all four studies date back to the 1960's and had small sample sizes.

Three observational studies conducted in well-nourished populations reported mixed results on the influence of dietary ascorbic acid intake on breast milk concentrations (154, 187, 188). A study from Finland reported dietary vitamin C, measured by 7-day food consumption record, to be associated with breast milk vitamin C ($r=0.39$ to 0.46, $p<0.01$) (188), however the remaining studies conducted in well-nourished populations generally did not (154, 187). Three observational studies conducted in poorly nourished populations all reported a positive correlation of dietary ascorbic acid intake with breast milk ascorbic acid (155, 185, 189).

Only one study investigated maternal status and the relationship with breast milk vitamin C. This study was conducted in Finland and reported that maternal plasma vitamin C was associated with breast milk vitamin C (r=0.60, p=0.001), even after adjusting for milk volume (188).

Vitamin D

A total of 10 studies reported on how maternal vitamin D status or intake influence breast milk vitamin D; three RCTs, two interventions and five observational studies.

Two of the included RCTs and the two included intervention studies generally supported an increase in breast milk vitamin D and 25(OH)D concentration with daily maternal vitamin D supplementation of 1000 to 6400 IU during lactation (190-193). One of these studies (conducted in Finland) only found an increase in breast milk 25(OH)D concentration and not total vitamin D (190). Conversely, a study from Japan showed no increase in breast milk 25(OH)D concentration with supplementation (193). Of these four studies, only one was a RCT, however with a small sample size (n=19) (192).

The final RCT that was included reported a daily vitamin D supplement of 5000 IU or a single dose of 150,000 IU to increase breast milk cholecalciferol during the first month of lactation (194).

Only one study investigated maternal dietary vitamin D (measured by a three day food diary) and the relationship with breast milk vitamin D (195). This study was conducted in the United States reported and an association with total breast milk vitamin D concentration (r=0.57, p=0.005), but not with breast milk 25(OH)D (r=0.25, p=0.2).

The evidence for an association between circulating serum/plasma 25(OH)D and breast milk 25(OH)D was varied. Two older studies found no association during lactation (193, 196), however a recent Danish study found longitudinal associations at 2 weeks, 4 and 9 months postpartum (r=0.51 to r=0.74, p<0.01) (197), as did another study at one week postpartum (r=0.62, p<0.05) (198). Jan Mohammed et al (2014) (199) found maternal serum 25(OH)D in the second trimester to be associated with breast milk 25(OH)D in the first days after delivery, however this association was not observed at 2, 3, 6 or 12 months postpartum. A positive association between maternal serum cholecalciferol and breast milk cholecalciferol (r=0.38, p=0.02) was reported (194).

Vitamin D was present in low concentrations in breast milk compared to other micronutrients (Table 1). A seasonal variation in breast milk 25(OH)D concentrations was found, with higher concentrations during the summer months in both Finland (190) and in Denmark (197).

Vitamin E

There was limited evidence investigating maternal nutritional influences on breast milk vitamin E concentration, with a total of only seven studies identified, one RCT and six observational studies.

The only included RCT was conducted in Brazil, and found supplementation of both natural and synthetic vitamin E (400 IU RRR-α-tocopherol or 400 IU all-rac-α-tocopherol, respectively) to significantly increase colostrum α-tocopherol concentration 24 hours after supplementation; 2187 µg/dl vs. 2508 µg/dl vs. 1643 µg/dl in natural vs. synthetic supplementation vs. control group ($p<0.0001$) (200).

In general, the studies reviewed failed to find an association between maternal dietary vitamin and breast milk vitamin E concentrations (201-204). For instance, two studies that were both conducted in Poland reported no association between maternal dietary vitamin E intake (measured by a 3 day food diary) and breast milk vitamin E ($r=0.034$, $p=0.2$) (202, 203). In contrast, a single study from Spain observed a positive association between maternal dietary vitamin E intake during third trimester of pregnancy (measured by a 5 day dietary record and FFQ) and vitamin E concentration in transitional milk, but not in mature milk (205).

Two studies reported no association between maternal serum α-tocopherol and breast milk vitamin E concentration: $r=0.07$ ($p=0.4$) (200) and $r=-0.12$ ($p=0.22$) (206) in a Brazilian population.

Vitamin K

Few studies have investigated the impact of maternal nutritional influences on breast milk vitamin K concentration. In total, only four studies were included in this review; two RCTs and two observational studies.

The two included RCTs were conducted in the United States and the Netherlands, and included small sample sizes ($n=22$ and $n=32$, respectively). They found vitamin K supplementation during

lactation to increase breast milk vitamin K concentration; 82 ng/ml vs. 1.2 ng/ml at 12 weeks postpartum in supplemented vs. placebo women (given 5 mg/day) (207) and 140 nmol/l vs. 4.9 nmol/l in supplemented vs. placebo women at 16 weeks postpartum (given 4 mg/day) (208).

Two of the reviewed studies found no association between maternal dietary vitamin K intake (measured by FFQ and 3-day dietary recalls, respectively) and breast milk vitamin K concentrations (209, 210).

Only one study investigated the relationship between maternal plasma vitamin K and breast milk vitamin K concentration, and did not find any evidence for an association (208).

Calcium

A total of seven studies investigated maternal nutritional influences on breast milk calcium concentrations, three RCTs and four observational studies.

Two of the RCTs were conducted in rural Gambia supplementing participants with 1500 mg/day calcium during pregnancy (211) and 1000 mg/day during lactation (212). Breast milk calcium concentration was not affected by supplementation during pregnancy; 231 mg/l vs. 234 mg/l ($p>0.05$) in supplemented vs. placebo group at 13 weeks gestation (211), or by supplementation during lactation (212). Regardless of low habitual calcium intake in rural Gambia, neither of the two studies found an effect of calcium supplementation, suggesting that breast milk calcium concentration is unresponsive to maternal calcium status and intake. Another RCT conducted in the United States (213) supports this finding; no effect of calcium supplements of 1000 mg/day during lactation on breast milk calcium was found; 24 mg/dl vs. 24 mg/dl in supplemented vs. placebo women at six months postpartum.

Four observational studies from Spain (214), the United States (215, 216) and China (217) generally found no relationship between maternal dietary calcium intake and breast milk calcium concentrations. One study did find maternal dietary calcium intake in the third trimester of pregnancy (measured by a 5-day dietary record and FFQ) to be associated with breast milk calcium in mature milk, however not with calcium concentration in transitional milk (214).

One study reported no association between maternal serum calcium and breast milk calcium concentration in an American population (216).

Copper

A total of 11 studies investigated how maternal copper intake and status influence breast milk copper concentration: one intervention and 10 observational studies.

All studies found no association between maternal copper supplementation (218, 219), maternal dietary copper intake (216, 217, 220, 221), maternal serum copper (216, 222-225) and breast milk copper concentration. The single intervention study included was conducted in Italy, and supplemented women with 2 mg copper sulphate daily, reported breast milk copper concentration of 0.52 mg/l vs. 0.51 mg/l in supplemented vs. unsupplemented women at one month postpartum (219). The studies were from a variety of countries: Italy, Greece, Japan, Iran, India, Finland, the United States, Poland and China. All studies except for one (225) investigated at breast milk copper concentration in relation to either iron and/or zinc concentrations.

Iodine

A total of 22 studies from across Europe, Africa, Asia, the Middle East, and North America investigated how maternal iodine intake or status influences breast milk iodine concentration (BMIC). Three RCTs, two interventions, two pre-post interventions and 15 observational studies, with seven of the studies conducted in populations with insufficient iodine intake (reported maternal urinary iodine concentration (UIC) <150 μg/l during pregnancy or <100 μg/l during lactation) (226-232), and one study in a Korean population with exceptionally high iodine intake from seaweed (233).

The three included RCTs were conducted in Morocco (227), Denmark (232) and Belgium (231) in populations with insufficient iodine intake. In Morocco a strong effect of a single dose of 400 mg of iodine during lactation was reported; 61.4 μg/l vs. 33.2 μg/l ($p<0.001$) in supplemented vs. placebo women at three months postpartum (227). A similar effect was observed in Belgium, where women were given 100 μg/day of iodine during pregnancy, which increased iodine concentration in colostrum; 61 μg/l vs. 29 μg/l ($p<0.01$) in supplemented vs. placebo women (231). Pedersen et al (1993) (232) also found an increase in BMIC in Danish women with supplementation (200 μg/day) during pregnancy and lactation, however this increase did not reach statistical significance ($p=0.06$).

Two intervention studies also found a positive effect of iodine supplementation on BMIC. Chaouki et al (1994) (228) conducted their study in a iodine deficient population in Algeria, and

observed that iodised oil (0.5 ml iodised oil, approximately 240 mg of iodine) given pre-conception, during the first month of pregnancy or during the first three months of pregnancy had a positive effect on BMIC at one and six months postpartum compared to a control group ($p<0.001$). One included pre-post intervention study was conducted in a well-nourished population (in the United States), where women were given 600 µg of potassium iodine, equalling to 456 µg of iodine, and eight hours after ingestion BMIC increased from a baseline of 46 µg/l to 281 µg/l (234). Two other studies failed to find an effect of supplementation during lactation (116 ug/day potassium iodide and 150 µg/day iodine) (219, 235). The study by Kirk et al (2012) (235) was a pre-post intervention conducted in the United States, and reported no difference in BMIC in women supplemented with 150 µg of iodine.

Maternal intake of iodised salt (235, 236), self-administered iodine supplements (226, 237) and other foods high in iodine (233, 238, 239) significantly increased BMIC. However, the evidence was poor from studies investigating the correlation between maternal dietary iodine intake and BMIC, with three studies reporting no association (219, 235, 240). For instance, Hannan et al (2009) (240) reported that maternal dietary iodine intake (measured by 24-hour recall) was not associated with BMIC in early lactation ($r=0.28$, $p=0.2$) or in late lactation ($r=-0.09$, $p=0.7$) or combined ($r=0.22$, $p=0.2$). However, these three studies all had low sample sizes ($n=10$, $n=13$ and $n=31$ respectively), and a poor study design (all observational studies).

Studies investigating associations between maternal iodine status, measured as UIC, and BMIC found mixed results. Seven studies found a relationship (226, 227, 229, 239, 241-243), however three studies did not (244-246). Costeira et al (2009) (229) found an association between maternal UIC and BMIC at three months postpartum in an iodine deficient population in Portugal ($r=0.46$, $p<0.01$). Whereas, Leung et al (2009) (244) from the United States, with sufficient iodine intake, did not find an association between maternal iodine intake and iodine concentration in colostrum 60 hours after birth ($r^2=0.006$ $p=0.5$).

A large difference in BMIC was observed between populations with differing iodine status. For example, in an iodine deficient population from Morocco a median concentration in mature milk (~3 months postpartum) of 33.2 µg/l in unsupplemented mothers (227) was observed. In contrast, in Korea where maternal iodine intake was exceptionally high due to habitually high consumption of seaweed, mean BMIC was 892 µg/l at 4 weeks postpartum (233).

Iron

A total of 12 studies, two interventions, 1 pre-post intervention and 9 observational studies, were included in this review on breast milk iron.

Six of the studies were conducted in iron-replete populations and showed that iron supplementation (247), dietary iron intake (216, 217, 220, 221, 240) and maternal iron status (171, 216, 247) did not influence breast milk iron concentrations. The intervention study conducted by Zapata et al (1994) (247) found no effect of iron supplementation (40 mg/day) during lactation on Brazilian women's breast milk iron concentration; 12.5 μmol/l vs. 13.4 μmol/l in supplemented vs. control group at one month postpartum.

Studies involving women with low iron status found mixed results. The intervention study conducted by Zavaleta et al (1995) (248) supplemented anaemic Peruvian mothers (haemoglobin (Hgb) <110 g/l) with 100 mg/day of iron during lactation and found no difference in breast milk iron concentration; 0.4 μg/ml vs. 0.4 μg/ml in anaemic mothers (supplemented) vs. non-anaemic mothers (nonsupplemented). A pre-post intervention conducted in a Maela refugee camp in the Thai-Myanmar border found breast milk iron to increase after implementation of iron fortified flour in a group of women with a high prevalence of iron deficiency and anaemia (iron deficiency defined by serum ferritin <12 μg/l or soluble transferrin receptor >8.5 mg/l, anaemia defined as Hgb <120 g/l) (147). However this study did not have a control group, but compared breast milk iron concentration after fortification with pre-fortification concentration in two different groups of women.

Two studies conducted in Egypt and India both reported a significant association between maternal serum iron and breast milk iron (249, 250) and maternal haemoglobin (collected at delivery) and breast milk iron (250). Mildly and severely anaemic women were included in these two studies, and Kumar et al (2008) (249) reported that breast milk iron concentration was significantly reduced in severely anaemic women (Hgb≤60 g/l) compared to non-anaemic women (Hgb≥110 g/l) but not in those with mild-to-moderate anaemia (Hgb between 61-109 g/l). In line with this, Shashiraj et al (2006) (251) demonstrated an association between maternal haemoglobin and breast milk iron concentration in a group of anaemic women (Hgb ≥110 g/l) from India ($r=0.339$, $p=0.01$) one day after birth. This association was not seen in non-anaemic women in the same population ($r=-0.016$, $p>0.05$). Shashiraj et al (2006) (251) did not find any association between maternal serum iron and breast milk iron in anaemic or non-anaemic women.

Breast milk iron concentration was consistently low between populations compared to other micronutrients (Table 2).

Selenium

A total of 13 studies were included in this review on selenium, three interventions, one pre-post intervention and nine observational studies.

All three interventions and one pre-post intervention study found a significant, albeit weak, effect of selenium supplementation during pregnancy or lactation on breast milk selenium concentrations (252-255). For instance, Moore et al (2000) (253) supplemented Chinese women with a selenium-enriched yeast tablet (100 ug/day) during the last trimester of pregnancy and three months postpartum which increased breast milk selenium concentration; 16.2 ng/g vs. 8.4 ng/g (p=0.04) in supplemented vs. placebo women at one month postpartum.

One study conducted in Italy found maternal egg intake (measured by FFQ) during pregnancy to be positively associated with breast milk selenium (r=0.20, p=0.04) and maternal fish intake during lactation to be associated with breast milk selenium (r=0.21, p=0.04) (256). In contrast Hannan et al (2009) (240) found maternal dietary selenium intake (measured by 24-hour recall) to not be associated with breast milk selenium in early lactation (r=-0.10, p=0.7) or in late lactation (r=-0.39, p=0.3) or combined (r=-0.18, p=0.4), and the same overall conclusion of no association was reached by two other studies (217, 257).

The majority of the included studies found a significant association between maternal plasma or serum selenium and breast milk selenium (224, 252, 254, 255, 257-259), however other studies failed to find a significant association (258, 260, 261). All of the studies that did not find a significant association had small sample sizes (n=~20).

Zinc

A total of 24 studies were included on zinc in this review; four RCTs, two interventions and 18 observational studies.

The four RCTs generally found no effect of maternal zinc supplementation (100 mg/weekly, or 30, 25, 15 mg/daily) during pregnancy or lactation on breast milk zinc concentration (143, 159, 262, 263). Two of the RCTs were conducted in the United States (159, 263), one in Indonesia (143), and the most recent one in Iran (262). The latter found no effect of maternal zinc

supplementation of 100 mg/weekly during lactation on breast milk zinc at 1 month (226 μg/dl vs. 212 μg/dl in supplemented vs. placebo, p=0.3), 4 months (111 μg/dl vs. 103 μg/dl in supplemented vs. placebo, p=0.2) or at 5 months postpartum (118 μg/dl vs. 109 μg/dl in supplemented vs. placebo, p=0.3). However, the authors reported an effect at 2 months (182 μg/dl vs. 152 μg/dl in supplemented vs. placebo, p=0.02) and 3 months postpartum (159 μg/dl vs.129 μg/dl in supplemented vs. placebo, p=0.005).

Several observational studies generally did not find maternal zinc supplementation/fortification (147, 215, 218, 219), maternal dietary zinc intake (216, 217, 220, 221, 240, 264-266) or maternal serum/plasma zinc (171, 216, 222-224, 264-269) to be associated with breast milk zinc concentration. However three observational studies reported a positive association between maternal intake or status and breast milk zinc concentration (270-272).

Table 1. Studies on the relationship between maternal nutritional status or dietary intake and breast milk vitamin concentrations

First author, year	Study design (sample size) and setting	Micronutrient supplementation status	Milk samples	Maternal nutritional assessment	Main results	Relationship
Vitamin A						
Ayah 2007	Randomised, placebo controlled, double-blind trial (n=564) Kenya	Supplements were given 24 hours after delivery. Group 1: Mother received a single dose of 400,000 IU vitamin A and infant received 100,000 IU vitamin A as retinyl palmitate (n=142). Group 2: Mothers received a single dose of 400,000 IU vitamin A and infant received placebo (n=140). Group 3: Mother received placebo and infant received 100,000 IU vitamin A (n=143). Group 4: Mother received placebo and infant received placebo (n=139)	3 samples collected at 4, 14 and 26 weeks postpartum. A sample of 2-5 ml foremilk was obtained from each mother by manual expression, after at least 1 hour without nursing. HPLC was used for analysis	Supplement group	Mean breast milk retinol/g fat concentration, 4 weeks supplemented group (SG): 0.025 µmol/g fat, placebo group (PG): 0.019 µmol/g fat, 14 weeks, SG: 0.02 µmol/g fat, PG:0.019 µmol/g fat, week 26 SG: 0.020 µmol/g fat, PG: 0.019 µmol/g fat Mean (±SD) breast milk retinol, 4 weeks SG: 0.67 µmol/l, PG: 0.60 µmol/l, 14 weeks, SG: 0.52 µmol/l, PG:0.44 µmol/l, week 26 SG: 0.50 µmol/l, PG: 0.44 µmol/l (significantly different at all time-points) Mean serum retinol at 36 weeks gestation: 0.81µmol/l (no difference between groups). Week 14 SG: 1.05 µmol/l, PG: 1.01 µmol/l (no significantly different), week 26 SG: 0.96 µmol/l, PG:0.98 µmol/l (not significantly different, p=0.5) Maternal vitamin A dose of 400,000 IU increased breast milk retinol/g fat at 4 weeks postpartum (p=0.02), but not at 14 and 26 weeks postpartum (p=0.43, and p=0.31 respectively)	+ ÷
Bahl 2002	Randomised, double-blind, placebo controlled trial (n=631) Ghana, India and Peru	Supplements were given during lactation. Participants in the intervention group received a single dose of 60 mg vitamin A, as retinol palmitate, between 18–42 days postpartum, in the second group mothers received a placebo	2 samples were collected, 1 at enrolment (between 18-42 days postpartum), and 1 sample at 2, 6 or 9 months postpartum in randomly selected subgroups. 10 ml samples were collected in amber-coloured glass bottles from either one of the breasts using a breast pump. Breast milk samples were collected independently of the time since the previous feed, usually between 0900 and 1200 hours HPLC was used for analysis	Supplement group	Mean (±SD) breast milk retinol/g fat concentration, baseline, supplemented group (SG): 52.2±27.4 nmol/g fat, placebo group (PG): 51.6±26.4 nmol/g fat (no difference between groups), 2 months, SG: 49.8±24.6 nmol/g fat, PG: 42.7±22.1 nmol/g fat, 6 months SG: 42.9±21.6 nmol/g fat, PG: 41.8±25.8 nmol/g fat, 9 months SG: 43.6±22.4 nmol/g fat, PG: 45.2±27.9 nmol/g fat Maternal vitamin A supplementation increased breast milk retinol/g fat at 2 months postpartum (p<0.05), however not at 6 or 9 months postpartum (p>0.05)	+ ÷
Bezerra 2009	Randomised, controlled trial, no placebo group (n=143) Brazil	Supplements were given after delivery, group 1: retinyl palmitate single dose of 200,000 IU, group 2: a double dose of 200,000 IU 24 hours apart, and group 3: No supplementation. The vitamin	2 samples were collected at baseline (up to 16 hours after delivery) and 4 weeks postpartum. After overnight fast, breast milk was collected by manual expression of a single breast. The first ejection of milk was discarded to avoid fluctuations in retinol and fat content. The samples contained between 1 and 2 ml of	Supplement group	Mean (±SD) breast milk retinol/g fat concentration, baseline (colostrum), group 1: 48.9±38.0 µg/g fat, group 2: 49.2±41.2 µg/g fat, group 3:50.4±38.5 µg/g fat (no difference between groups), 4 months, group 1: 15.6±8.3 µg/g fat, group 2: 17.2±8.9 µg/g fat, group 3:12.7±6.7 µg/g fat Mean (±SD) breast milk retinol at baseline (colostrum), group 1: 3.22±1.81 µmol/l, group 2: 3.21±1.87 µmol/l, group 3: 3.31±1.40	+ ÷

Table 1. Studies on the relationship between maternal nutritional status or dietary intake and breast milk vitamin concentrations

First author, year	Study design (sample size) and setting	Micronutrient supplementation status	Milk samples	Maternal nutritional assessment	Main results	Relationship
		A capsules also contained vitamin E (40 mg)	colostrum milk, and the aliquots were collected in polypropylene tubes protected from the light and duly identified HPLC was used for analysis All infants were exclusively breastfed		µmol/l, 4 months, group 1: 1.78±1.00 µmol/l, group 2: 1.93±1.10 µmol/l, group 3: 1.28±0.61 µmol/l (significant difference between groups, p=0.01) Maternal vitamin A supplementation of 400,000 IU increased breast milk retinol/g fat at 4 weeks postpartum ($p<0.05$). Supplementation of 200,000 IU had no effect on breast milk retinol/g fat ($p>0.05$)	
Canfield 1997	Intervention, no randomisation or placebo group (n=12) USA	Supplements were given during lactation, group 1: 60 mg, group 2: 210 mg β-carotene	7 samples collected on -1, 0, 1, 2, 4, 7, and 9 days postpartum, with study baseline at 8 days postpartum. Milk samples were collected by electric breast pumps under subdued lighting. The complete contents of one breast were collected into sterile polypropylene containers or glass bottles HPLC was used for analysis	Supplement group	Mean (±SEM) breast milk retinol/g fat concentration, baseline (n=3), group 1: 1.1±0.3 µmol/g fat, group 2: 1.7±0.3 µmol/g fat (concentrations after supplementation not reported) Maternal β-carotene supplementation did not increase breast milk retinol/g fat (statistics not reported)	÷
Canfield 1998	Intervention, no randomisation or placebo group (n=5) USA	Supplements were given during lactation, 30 mg β-carotene for 28 days	13 samples collected over an 8 week period, days -1, 0, 1, 2, 4, 6 and once weekly for 7 weeks thereafter (stage of lactation not clear). The breast were completely emptied using an electronic pump, with the infant to breastfed from the breast 2-3 hour before collection. Participants used the same breast for collection of all samples. Subdued lighting and light-protected sterile polypropylene containers were used HPLC was used for analysis Infants were exclusively breastfed	Supplement group	Mean (±SE) breast milk retinol/g fat concentration, baseline (n=6): 0.034±0.003µmol/g fat (concentrations after supplementation not presented) Mean (±SE) breast milk retinol, baseline (n=3): 2.24±0.0003 µmol/l (concentrations after supplementation not presented) Maternal β-carotene supplementation did not increase breast milk retinol/g fat (statistics not reported)	÷
Canfield 2001	Randomised, placebo controlled trial (n=86)	Supplements were given during lactation. Group 1 (n=32): 90 mg β-carotene as red palm oil, group 2 (n=36): 90 mg purified β-carotene as tablets, group 3 (n=18):	2 samples collected, one sample at baseline (stage of lactation not clear) and one sample 10 days after supplementation. Participants	Supplement group	Mean (±SEM) breast milk retinol/g fat concentration, baseline, group 1: 0.04±0.01 µmol/g fat, group 2: 0.05±0.04 µmol/g fat, group 3: 0.06±0.04 µmol/g fat (no significant difference between groups). +10 days, group 1: 0.03±0.02 µmol/g fat, group 2: 0.04±0.02 µmol/g fat, group 3: 0.03±0.02 µmol/g fat	÷

Table 1. Studies on the relationship between maternal nutritional status or dietary intake and breast milk vitamin concentrations

First author, year	Study design (sample size) and setting	Micronutrient supplementation status	Milk samples	Maternal nutritional assessment	Main results	Relationship
	Honduras (deficient population)	placebo tablets. Participants returned on days 3, 5, 7 and 9 for an additional supplement	provided milk (5–10 ml) mid-morning by manual expression HPLC was used for analysis		Mean (±SEM) breast milk retinol baseline, group 1: 1.63±1.01 µmol/l, group 2: 1.28±0.59 µmol/l,, group 3: 1.95±0.87 µmol/l, (no significant difference between groups). +10 days, group 1: 1.10±0.42 µmol/l, group 2: 1.28±1.37 µmol/l,, group 3: 1.09±0.63 µmol/l, Maternal β-carotene supplementation did not increase breast milk retinol/g fat (statistics not reported)	
Dijkhuizen 2004	Randomised, double-blind, placebo controlled trial (n=170) Indonesia (deficient population)	Supplements were given daily during pregnancy until delivery (supplementation started <20 weeks gestation). Group 1: β-carotene (4.5 mg), group 2: zinc (30 mg), group 3: β-carotene (4.5mg) and zinc (30 mg), group 4: placebo. All groups were also given iron (30 mg) and folic acid (0.4 mg)	2 samples collected at 1 and 6 months postpartum. Breast milk was collected from the right breast 45–60 min after the last feeding from that breast. The breast was completely expressed with the use of a manual pump HPLC was used for analysis	Supplement group	Median (range) breast milk retinol/g fat concentration, 1 month, group 1: 32.7 (34.4, 73.5) nmol/g fat, placebo: 50.6 (44.1, 88.6) nmol/g fat. 6 months, group 1: 30.9 (19.7, 47.1) nmol/g fat, placebo: 27.9 (18.9, 36.6) nmol/g fat Maternal daily β-carotene supplementation (4.5 mg) during pregnancy did not increase breast milk retinol/g fat at 1 or 6 months postpartum (statistics not reported)	÷
Engle-Stone 2014	Observational (n=440) Cameroon	No supplements were given by investigators	1 sample collected (stage of lactation not clear, minimum age of infant was 1 month). Milk was collected from the breast from which the infant had not fed for a longer time. The mother first allowed her child to feed from the breast from which milk was to be collected. After exactly 30 seconds, the mother manually expressed 5–10 ml of milk from the same breast HPLC was used for analysis	FFQ and 24-hour recall (n=246) collected at the same time as breast milk sample Plasma (n=242) collected at the same time as the breast milk sample	Mean (95% CI) breast milk retinol/g fat concentration: 23.6 (22.2, 25.1) µg/g fat Mean (95% CI) breast milk retinol: 3.79 (3.53, 4.05) µmol/l, Dietary vitamin A intake: 346 µg RAEs/day Mean (95% CI) inflammation-adjusted pRBP 1.53 (1.47, 1.59) Maternal dietary vitamin A intake was associated with breast milk retinol/g fat (r^2=0.13, p<0.001) Maternal inflammation-adjusted pRBP was associated with breast milk retinol/g fat (r^2=0.09, p<0.0001)	+
Klevor 2016	Randomised, partially double-blind, controlled-trial Ghana	Supplements were given during pregnancy and lactation. Group 1: daily supply of an lipid-based nutritional supplement (LNS)	1 sample collected at 6 months postpartum. Casual ‘‘spot’’ breast milk samples were collected after feeding the infant for 1 min on that breast, by manual expression. Approximately 10 ml was collected from each participant	Supplement group	Mean (±SD) breast milk retinol/g fat concentration, group 1: 54.7±2.5 nmol/g fat, group 2: 55.4±2.5 nmol/g fat, group 3: 59.1±2.8 nmol/g fat Mean (±SD) breast milk retinol concentration, group 1: 2.5±0.1 µmol/l group 2: 2.5±0.1 µmol/l, group 3: 2.5±0.1 µmol/l	÷

Table 1. Studies on the relationship between maternal nutritional status or dietary intake and breast milk vitamin concentrations

First author, year	Study design (sample size) and setting	Micronutrient supplementation status	Milk samples	Maternal nutritional assessment	Main results	Relationship
		providing 800 µg RE of vitamin A from Before 20 weeks gestation to 6 months postpartum. Group 2: daily supply of a Multiple micronutrient (MMN) providing 800 µg RE of vitamin A from 20 weeks gestation to 6 months postpartum. Group 3: Control group given a daily supply of iron and folic acid only during pregnancy and a calcium placebo tablet during the first 6 months postpartum	HPLC was used for analysis		Maternal LNS or MMN supplementation during pregnancy and lactation did not increase breast milk retinol/g fat concentration (p=0.5)	
Muslimatun 2001	Randomised double-blind, community-based trial (n=170) Indonesia (deficient population)	Supplements were given during pregnancy. Participants were randomly assigned to two groups. They were supplemented once weekly from enrolment (16-20 weeks gestation) until delivery with two tablets each containing 60 mg iron as ferrous sulfate and 250 mg folic acid or with two tablets each containing 2400 retinol equivalents (RE) vitamin in addition to the same amount of ferrous sulfate and folic acid	2 samples collected at 4-7 days (n=73, transitional milk) and 3 months postpartum (n=85, mature milk). Between 08.00 and 11.00 am all milk from the right breast, which had not been used to feed the child during the previous hour, was collected using a breast milk pump. The breast milk was stored in dark brown glass bottles HPLC was used for analysis	Supplement group Serum collected at the same time as breast milk samples	Mean breast milk retinol/g fat concentration, transitional milk, supplemented group (SG): 0.113 µmol/g fat, placebo group (PG): 0.097 µmol/g fat, mature milk SG: 0.053 µmol/g fat, PG: 0.044 µmol/g fat Mean breast milk retinol, transitional milk, SG: 3.37 µmol/l, PG: 2.29 µmol/l, mature milk SG: 1.24 µmol/l, PG: 1.06 µmol/l (Mean serum retinol concentrations not reported, however there were no difference between groups) Maternal retinol supplementation during pregnancy increased breast milk retinol/g in mature milk ($p<0.05$) however not in transitional milk ($p>0.05$) Maternal serum retinol (in the placebo group) was associated with breast milk retinol/g fat in mature milk ($r=0.487$, $p<0.01$)	+ ÷

Table 1. Studies on the relationship between maternal nutritional status or dietary intake and breast milk vitamin concentrations

First author, year	Study design (sample size) and setting	Micronutrient supplementation status	Milk samples	Maternal nutritional assessment	Main results	Relationship
Olafsdottir 2001	Observational (n=77) Island	No supplements were given by investigators	1 samples collected between 2-4 months postpartum. 40 ml of milk from each participants in a sterile glass bottle either manually or by using simple sterile pumps. The first drops of milk were put aside before starting the collection HPLC was used for analysis	24-hour recall, specifically reporting intake of cod liver oil, collected at the same time as breast milk sample	Median breast milk retinol equivalent/fat concentration: 11.8 µg/g fat Median breast milk retinol equivalent 58.4 µg/100 ml Median dietary vitamin A intake: 889 µg/day in participants with a cod liver oil intake, and 4127 with no intake of cod liver oil (significantly different) Maternal intake of cod liver oil was not associated with breast milk retinol equivalent/g fat (statistics not reported)	÷
Rice 1999	Randomised double-blind, placebo controlled trial (n=220) Bangladesh (deficient population)	Supplements were given during lactation. Group 1: Participants received one dose of 200,000 IU (60,000 RE) retinyl palmitate + daily placebo tablets until 9 months postpartum. Group 2: 7.8 mg of beta-carotene daily. Group 3: daily placebo tablet	4 samples collected at 0.5 (baseline, casual collection, n=74) and 3 (n=69), 6 (n=70) and 9 (n=64) months postpartum (full collection). Samples were collected using two different techniques. For full collection, a trained field worker used a manual breast pump to express the entire contents of one breast which had not been used to feed an infant for ≥2 hours. Milk was collected from the left breast except when a breast infection was present or if milk production had stopped. Samples were collected between 10.30 and 21.15 hours. For casual milk collection, participants manually expressed 5 ml of milk into a glass collection jar without control over the time since last breastfeeding episode. A milk sample was collected from the breast which had not been used to feed the infant for the longer period of time. Casual milk samples were collected between 08.00–19.00 hour HPLC was used for analysis	Supplement group	Mean (±SD) breast milk retinol/g fat concentration, baseline, supplemented group, group 1 (SG): 0.37±0.19 µmol/g fat, placebo group, group 3 (PG): 0.34±0.18 µmol/g fat (no difference at baseline), 3 months, SG: 0.28±0.14 µmol/g fat, PG: 0.23±0.11 µmol/g fat, 6 months SG: 0.24±0.11 µmol/g fat, PG: 0.24±0.16 µmol/g fat, 9 months, SG: 0.24±0.13 µmol/g fat, PG: 0.21±0.10 µmol/g fat Mean (±SD) breast milk retinol baseline, SG: 1.71±1.34 µmol/l, PG: 1.51±1.08 µmol/l (no difference at baseline), 3 months, SG: 1.20±1.00 µmol/l, PG: 0.83±0.43 µmol/l (statistically different, $p<0.01$), 6 months SG: 0.85±0.53 µmol/l, PG: 0.87±0.61 µmol/l, 9 months, SG: 0.91±0.68 µmol/l, PG: 0.79±0.44 µmol/l Maternal supplementation of 200,000 IU increased breast milk retinol/g fat at 3 months postpartum ($p<0.05$), however not at 6 or 9 months ($p>0.05$)	+ ÷

Table 1. Studies on the relationship between maternal nutritional status or dietary intake and breast milk vitamin concentrations

First author, year	Study design (sample size) and setting	Micronutrient supplementation status	Milk samples	Maternal nutritional assessment	Main results	Relationship
da Silva Ribeiro 2010	Observational (n=86) Brazil	No supplements were given by investigators	1 sample collected up to 16 hours postpartum. The sample was collected by manual expression from a single full breast not suckled in the previous feeding. The first milk ejection was discarded to avoid fluctuations in retinol content HPLC was used for analysis	Serum, collected at the same time as breast milk sample FFQ, used to divide the participants into two groups according to predominant vitamin A source: Group A: >50% preformed vitamin A (n = 37). Group B: >50% pro-vitamin A carotenoid (n=49)	Mean (±SD) colostrum retinol/g fat concentration, group A: 40.0±28.7 µg/g fat, group B: 35.8±9.7 µg/g fat Mean (±SD) colostrum retinol, group A: 3.4±1.7 µmol/l, group B: 3.6±1.9 µmol/l (not significantly different) Mean (±SD) serum retinol, group a: 1.4±0.40 µmol/l, group B: 1.2±0.57 µmol/l (significantly different, p=0.03) Mean (±SD) dietary vitamin A, group A: 2071.0±1465.9 µg RAE/day, group B: 1051.6±920.4 µg RAE/day (significantly different, p<0.001) Maternal serum retinol was not associated with colostrum retinol/g fat (statistics not reported) Maternal dietary vitamin A intake was not associated with colostrum retinol/g fat (p=0.3)	÷
Stuetz 2012 (eur)	Pre-post intervention, no randomisation (n=86 before fortification, n=99 after fortification) Thai-Myanmar border (Maela Refugee camp)	Vitamin A fortified wheat flour (estimated daily intake: 235 µg/RE) was provided for all participants (the flour was also fortified with other micronutrients)	2 samples were collected, 1 before fortification of flour was introduced at 12 weeks postpartum and 1 sample after flour fortification was introduced at 12 weeks postpartum (two different groups of women). Milk samples were collected by manual expression into glass tubes wrapped in aluminium foil in order to protect against degradation HPLC was used for analysis	Fortification (pre-post intervention design, the study used pre-supplement concentrations as comparison group) Blood collected 12 weeks postpartum	Geometric mean breast milk retinol/g fat concentration, before fortification: 46.2 µmol/kg, after fortification: 43.2 µmol/kg Mean (±SD) serum retinol, before fortification: 159±0.40 µmol/l, after fortification: 1.69±0.47 µmol/l, (not significantly different, p=0.1) Maternal intake of vitamin A fortified flour did not increase breast milk retinol/fat (p=0.3) Maternal serum retinol was associated with breast milk retinol (β= 0.127, p<0.001)	÷ +
Turner 2013	Randomised controlled trial (n=135) Bangladesh (deficient population)	Supplements were given during lactation. Participants were stratified by their screening serum retinol concentrations (>0.875 mmol/l and <0.875 mmol/l) and randomly allocated to group 1: 100g orange-fleshed sweet potatoes (naturally rich in β-	2 samples collected, at baseline (between 2.6-8.9 months postpartum) and 3 weeks after when the study ended. Milk from a full breast was collected from the breast not fed on in the previous hour. Milk samples were collected via an electric breast pump until no milk was expressed from the breast HPLC was used for analysis	Supplement group	Mean (±SEM) breast milk retinyl equivalents/g fat concentration, baseline, supplemented group, group 3 (SG): 25±3.1 nmol/g fat, control group, group 4 (CG): 26±3.4 nmol/g fat (not significantly different), post-intervention, SG: 29±2.3 nmol/g fat, CG: 20±1.6 nmol/g fat Mean (±SEM) breast milk retinyl equivalents, baseline SG: 0.77±0.06 µmol/l, CG: 0.63±0.06 µmol/l (not significantly different), post-intervention, SG: 1.04±0.08 µmol/l, CG: 0.55±0.04 µmol/l	+

Table 1. Studies on the relationship between maternal nutritional status or dietary intake and breast milk vitamin concentrations

First author, year	Study design (sample size) and setting	Micronutrient supplementation status	Milk samples	Maternal nutritional assessment	Main results	Relationship
		carotene) and a cornoil capsule, group 2: 127 g canned tangerines (naturally rich in beta-cryptoxanthin) and a corn-oil capsule, group 3: low-dose vitamin A supplement (0.25 mg retinyl acetate) and 100g white-fleshed sweet potatoes (WFSP), group 4: 100g WFSPs and a corn-oil (control). The treatments were consumed twice a day, 6 days a week for 3 weeks			Maternal supplementation of 0.25 mg retinyl acetate increased breast milk retinyl equivalents/g fat ($p<0.05$)	
Villard 1987	Intervention, no randomisation or placebo group (n=55) The Gambia (deficient population)	Supplements were given during pregnancy and lactation, to women from Keneba, a 650 µg daily vitamin A dose, as a food supplement consisting of an energy-rich biscuit and a multi-vitamin fortified tea drink. Women from Manduar received no vitamin A supplementation	12 samples collected weekly between 3-15 weeks postpartum. A 5-10 ml sample was collected by manual expression in the morning, usually from the right breast. The sample was collected between feeds The breast milk vitamin A concentration was measured fluorimetrically	Supplement group Plasma collected at the same time as breast milk sample	Mean (±SEM) breast milk vitamin A/g fat concentration, supplemented group: 29.5±1.10 µg/g fat, unsupplemented group: 22.9±1.79 µg/g fat Mean (±SEM) plasma retinol, supplemented group: 52.2±1.69 µg/dl, unsupplemented group: 46.1±3.69 µg/dl (not significantly different) Maternal daily vitamin A supplementation of 650 µg during pregnancy and lactation increased breast milk vitamin A/g fat concentration ($p<0.01$) Maternal plasma retinol was not associated with breast milk vitamin A (statistics not reported)	+ ÷
Vitamin B1 (thiamin)						
Coats 2013	Pre-post Intervention. no randomisation (n=16) Cambodia	Supplements were given during lactation. All participants received oral thiamin hydrochloride, 100 mg for 5 days	2 samples collected at baseline (day 1) and 6 days after supplementation (not standardised to stage of lactation). Breast milk was expressed directly by mothers into a container immediately before oral thiamin administration Fluorescent thiochromes was used for analysis	Supplementation (Pre-post intervention design, the study used pre-supplement concentrations as comparison group)	Median (range) breast milk total thiamin concentration, baseline: 179.5 (85-359) nmol/l, day 6: 502.7 (360-808) nmol/l Median (range) total plasma thiamin concentration, baseline: 2.4 (0-6.9) nmol/l, 6 days: 18.6 (13.4, 25.3) nmol/l (significantly different) Maternal thiamin supplementation increased breast milk thiamin ($p<0.001$) Maternal total plasma thiamin was associated with breast milk total thiamin ($p<0.001$)	+

Table 1. Studies on the relationship between maternal nutritional status or dietary intake and breast milk vitamin concentrations

First author, year	Study design (sample size) and setting	Micronutrient supplementation status	Milk samples	Maternal nutritional assessment	Main results	Relationship
Deodhar 1960	Observational (n=60) India	No supplements were given by investigators	1 sample collected between two feeds at about 3 p.m. by voluntary expression. The sampling was done on three consecutive days and the average value for three samples was taken (stage of lactation was not clear) Laboratory method used not clear	Dietary intake of 1 day collected at the same time as the breast milk sample. The data was used to divide participants into 4 groups, according to their intake of thiamin, with the median and the two quartiles determined the distribution	Mean breast milk thiamin concentration, group 1: 11.51 mcg/100ml, group 2: 11.59 mcg/100ml, group 3: 13.16 mcg/100ml, group 4: 15.86 mcg/100ml Mean dietary thiamin intake, group 1: 0.21 mg/day, group 2: 0.36 mg/day, group 3: 0.58 mg/day, group 4: 1.23 mg/day Maternal dietary thiamin intake was associated with breast milk thiamin ($r=0.35$, $p<0.05$)	+
Kodentsova 2006	Observational (n=35, excluding preterm infants) Russia	Supplements were not given by investigators, however many of the participants consumed supplements, which was captured in a 24-hour recall. The participants were divided into two groups, group 1: no vitamin supply and group 2: adequate vitamin supply	1 sample collected between 3-10 days postpartum. The women were fasting when a single sample of breast milk was collected (breast milk collection method not clear) The thiochrome method was used for analysis	24-hour recall collected a few days after birth	Mean (±SD) breast milk thiamin concentration, group 1: 92±21 µg/l, group 2: 238±49 µg/l Maternal dietary thiamin intake (including supplement use) was associated with breast milk thiamin ($p<0.05$)	+
McGready 2001	Intervention, no randomisation or placebo group (n=50) Thai/Burmese border (Karen refugees)	Supplements were given through pregnancy. The participants that showed sign of thiamin deficiency during pregnancy were given a supplement of oral thiamin hydrochloride (100 mg/day) during pregnancy. The women having no clinical signs of deficiency did not receive thiamin supplementation	1 sample collected 3 months postpartum (n=16). 10 ml of breast milk were collected ≥1 hour after the previous feed. Once the infant was attached and sucking, the milk was expressed from the contralateral breast HPLC was used for analysis	Supplement group	Median (range) breast milk thiamin concentration, supplemented group: 128 (890, 155) µg/l, unsupplemented group: 117 (7.6, 34.2) µg/l Maternal thiamin supplementation during pregnancy did not increase breast milk thiamin at 3 months postpartum (statistics not reported)	÷
Nail 1980	Intervention, no randomisation or placebo group (n=11)	Supplements were given during pregnancy and lactation. All participants received supplements during pregnancy (4-36 weeks before	2 samples collected at 1 and 6 weeks postpartum. The supplemented participants expressed milk four times per day for 3 days, at 0, 4, 8, and 12 hours after ingestion of the vitamin supplement The unsupplemented	Supplement group	Mean breast milk thiamin concentration, 1 week, supplemented group: 133 µg/l, unsupplemented: 138 µg/l, 6 week, supplemented group: 238 µg/l, unsupplemented: 220 µg/l Maternal thiamin supplementation during pregnancy and lactation did not increase breast milk thiamin (statistics not reported)	÷

Table 1. Studies on the relationship between maternal nutritional status or dietary intake and breast milk vitamin concentrations

First author, year	Study design (sample size) and setting	Micronutrient supplementation status	Milk samples	Maternal nutritional assessment	Main results	Relationship
	USA	birth) and 11 participants were supplemented during lactation and 4 were not. The supplemented women were given a multivitamin and mineral supplementation containing 1.7 mg thiamin	participants obtained the sample immediately upon arising in the morning and at the same 4-hour intervals. Milk was with the aid of a breast pump Laboratory method used not clear			
Ortega 2004	Observational (n=51) Spain	No supplements were given by investigators	2 samples collected at 13-14 (transitional milk) and 40 days postpartum (mature milk) (breast milk collection method not clear) Laboratory method used not clear	Food record booklet for 5 days collected during third trimester of pregnancy, which was used to divide participants into two groups, group 1: thiamin intake < RI (n=13), group 2: thiamin intake ≥ RI (n=38)	Mean (±SD) breast milk thiamin concentration, transitional milk, group 1: 0.90 (±1.03) μmol/l, group 2: 0.88±0.57 μmol/l, mature milk, group 1: 0.25±0.07 μmol/l, group 2: 0.59±0.44 μmol/l Maternal dietary thiamin intake during third trimester of pregnancy was associated with breast milk thiamin in mature milk, however not in transitional milk ($p<0.05$)	+
Prentice 1983	Pre-post intervention, no randomisation (n=130) The Gambia	Supplements were given during lactation, 1.36 mg/day of thiamin to all participants as a fortified biscuit.	2 samples collected pre-supplementation (n=21) and post-supplementation (n=23). (stage of lactation not clear, however it was confined to the first 6 months of lactation). Milk was expressed from each breast before and after a mid-afternoon feed on two occasions (pre-supplement) and on two further occasions (post-supplementation). Milk was collected four times throughout the day, at regular intervals Laboratory method used not clear	Supplementation (pre-post intervention design, the study used pre-supplement concentrations as comparison group)	Mean (±SE) breast milk thiamin concentration, pre-supplement: 0.16±0.006 μg/ml, post-supplement: 0.22±0.006 μg/ml Maternal thiamin supplementation during lactation increased breast milk thiamin ($p<0.001$)	+
Stuetz 2012 (plos one)	Observational (n=636) Thai-Myanmar border (Maela refugee camp)	Supplements were given during lactation, 100 mg of thiamin a day to all participants	1 sample collected at 12 weeks postpartum. Milk samples (5–10 ml) were collected by manual expression HPLC was used for analysis	Whole blood collected at the same time as the breast milk sample	Median (IQR) breast milk total thiamin: 755.4 (730.4, 780.7) nmol/l Mean (±SD) thiamin diphosphate (TDP) concentration: 129.8±42.1 μg/l	+

Table 1. Studies on the relationship between maternal nutritional status or dietary intake and breast milk vitamin concentrations

First author, year	Study design (sample size) and setting	Micronutrient supplementation status	Milk samples	Maternal nutritional assessment	Main results	Relationship
					Maternal TDP was associated with breast milk total thiamin (r^2=0.37, p<0.05)	
Stuetz 2012 (eur)	Pre-post intervention, no randomisation (n=86, before fortification, n=99 after fortification) Thai-Myanmar border (Maela Refugee camp)	Thiamin fortified wheat flour (estimated daily intake: 0.34 mg of thiamin) was provided for all participants (the flour was also fortified with other micronutrients). All participants were furthermore supplemented with 92 mg/day of thiamin during pregnancy and lactation in both the before and after fortification groups. The investigators did not provide the flour, or the supplements.	2 samples were collected, 1 before fortification of flour was introduced at 12 weeks postpartum and 1 sample after flour fortification was introduced at 12 weeks postpartum (two different groups of women). Milk samples were collected by manual expression into glass tubes wrapped in aluminium foil in order to protect against degradation Breast milk thiamin was analysed using precolumn derivatization, reversed-phase liquid chromatography and fluorescence detection	Fortification (pre-post intervention design, the study used pre-fortified concentrations as comparison group, in two different groups of women) Whole blood collected 12 weeks postpartum	Mean (±SD) breast milk total thiamin concentration, before fortification: 256.6±129.9 µg/l, after fortification: 290.8±83.5 µg/l Mean (±SD) breast milk thiamin monophosphate (TMP) concentration, before fortification: 178.1±68.4 µg/l, after fortification: 176.8±49.5 µg/l Geometric mean breast milk free thiamin concentration, before fortification: 100.6 µg/l, after fortification: 129.4 µg/l Mean (±SD) whole blood thiamin diphosphate (TDP), before fortification: 67.4±21.9 µg/l, after fortification: 69.0±17.7 µg/l, (not significantly different, p=0.6) Maternal intake of thiamin fortified flour did not increase breast milk total thiamin (p=0.1), it did increase breast milk free thiamin (p=0.03)	+ ÷
Thomas 1980	Intervention, no randomisation or placebo group (n=12) USA	Supplements were given during lactation. Supplemented participants were given a multivitamin tablet (containing 1.7 mg/day of thiamin) and the remaining participants were not given any supplements	1 sample collected 6 months postpartum. Milk was expressed 4 times per day at 4-hour intervals for three consecutive days, and was expressed immediately before taking the supplement in the morning at 0 time and 4, 8, and 12 hour thereafter. Those participants not taking supplements expressed milk at corresponding times The thiochrome method was used for analysis	Supplement group	Mean (±SD) breast milk thiamin concentration, supplemented group 228±42 µg/l, not supplemented: 208±34 µg/l Maternal thiamin supplementation during lactation did not increase breast milk thiamin (statistics not reported)	÷
Whitfield 2016	Randomised double-blind, placebo controlled trial (n=90) Cambodia	Fortified fish sauce were given during pregnancy and lactation. Participants were randomised to 1 of 3 groups (n = 30) for ad libitum fish sauce consumption for 6 months: Group 1: placebo (no thiamin),	1 sample collected at the study end-line (not standardised according to stage of lactation). A battery-powered single breast pump was used. One full breast expression was collected from the breast that the participant self-identified as	Supplement group	Mean (95%CI) breast milk total thiamin concentration, group 1: 144 (123, 165) µg/l, group 2: 207 (186, 227) µg/l, group 3: 177 (156, 199) µg/l Mean (95% CI) Erythrocyte thiamin diphosphate (eTDP) concentrations, group 1: 193 (164, 222) nM, group 2: 282 (235, 310) nM, group 3: 254 (225, 284) nM (significantly different)	+

Table 1. Studies on the relationship between maternal nutritional status or dietary intake and breast milk vitamin concentrations

First author, year	Study design (sample size) and setting	Micronutrient supplementation status	Milk samples	Maternal nutritional assessment	Main results	Relationship
		group 2: low-concentration (2 g/l), group 3: high-concentration (8 g/l) fish sauce	being more "full" (the breast not most recently emptied) HPLC was used for analysis		Maternal intake of thiamin fortified fish sauce during pregnancy and lactation increased breast milk total thiamin (statistics not reported)	
Vitamin B2 (riboflavin)						
Bates 1982	Intervention, no randomisation (n=60) The Gambia	Supplements were given by investigators during lactation, either 2 mg/day of riboflavin, or a placebo (double-blinded) for 12 weeks	7 samples was collected across the 12 weeks long study. Foremilk samples were collected just before the supplement was given in the evening on every 3rd day during the first 6 week of the study. One sample was also collected at 12 weeks (stage of lactation not clear) Fluorimetric procedure was used for analysis	Supplement group	Mean (±SD) breast milk riboflavin concentration, baseline, supplemented group (SG): 0.61±0.07 µg/ml, placebo group (PG): 0.15±0.07 µg/ml (not significantly different). Days 1-9, SG: 0.18±0.07 µg/ml, PG: 0.14±0.06 µg/ml, days 10-18, SG: 0.23±0.10 µg/ml, PG: 0.16±0.08 µg/ml, days 19-27, SG: 0.22±0.04 µg/ml, PG: 0.15±0.06 µg/ml, days 28-36, SG: 0.22±0.05 µg/ml, PG: 0.16±0.09 µg/ml, days 37-42 µg/ml, SG: 0.23±0.08 µg/ml, PG: 0.14±0.11 µg/ml, day 84, SG: 0.22±0.08 µg/ml, PG: 0.12±0.05 µg/ml Maternal riboflavin supplementation during lactation increased breast milk riboflavin at all time-points ($p<0.03$)	+
Deodhar 1960	Observational (n=60) India	No supplements were given by investigators	1 sample collected between two feeds at about 3 p.m. by voluntary expression. The sampling was done on three consecutive days and the average value for three samples was taken (stage of lactation not clear) Laboratory method used not clear	Dietary intake of 1 day collected at the same time as breast milk sample. The data was used to divide participants into 4 groups, according to their intake of riboflavin, with the median and the two quartiles determining the distribution	Mean breast milk riboflavin concentration, group 1: 20.98 mcg/100ml, group 2: 24.36 mcg/100ml, group 3: 25.12 mcg/100ml, group 4: 0.41 mcg/100ml Mean dietary riboflavin intake, group 1: 0.15 mg/day, group 2: 0.23 mg/day, group 3: 0.28 mg/day, group 4: 0.41 mg/day Maternal dietary riboflavin was associated with breast milk riboflavin ($r=0.54$, $p<0.05$)	+
Kodentsova 2006	Observational (n=35, excluding preterm infants) Russia	No supplements were given by investigators, however many of the participants consumed vitamins, which was captured in a 24-hour recall. The participants were divided into two groups, group 1: no	1 sample collected between 3-10 days postpartum. The women were fasting when a single sample of breast milk was collected Breast milk riboflavin was estimated spectrophotometrically by the method of titration	24-hour dietary recall collected a few days after birth	Mean (±SD) breast milk riboflavin concentration, group 1: 266±40 µg/l, group 2: 330±41 µg/l Maternal dietary intake of riboflavin (including supplement use) was not associated with breast milk riboflavin ($p>0.05$)	÷

Table 1. Studies on the relationship between maternal nutritional status or dietary intake and breast milk vitamin concentrations

First author, year	Study design (sample size) and setting	Micronutrient supplementation status	Milk samples	Maternal nutritional assessment	Main results	Relationship
		vitamin supply and group 2: adequate vitamin supply				
Nail 1980	Intervention, no randomisation or placebo group (n=11) USA	Supplements were given during pregnancy and lactation. All participants received supplements during pregnancy (4-36 weeks before birth) and during lactation 11 participants received supplements during lactation, 4 did not. The supplemented women were given a multivitamin and mineral supplementation containing 2.00 mg of riboflavin	2 samples collected at 1 and 6 weeks postpartum. The supplemented participants expressed milk four times per day for 3 days, at 0, 4, 8, and 12 hours after ingestion of the vitamin supplement The unsupplemented participants obtained the sample immediately upon arising in the morning and at the same 4-hour intervals. Milk was with the aid of a breast pump Laboratory method used not clear	Supplement group	Mean breast milk riboflavin concentration, 1 week, supplemented group: 880 µg/l, unsupplemented: 367 µg/l, 6 week, supplemented group: 710 µg/l, unsupplemented: 485 µg/l Maternal riboflavin supplementation increased breast milk riboflavin ($p<0.01$)	+
Ortega 1999	Observational (n=57) Spain	No supplements were given by investigators	2 samples collected at 13-14 (transitional milk) and 40 days postpartum (mature milk). Milk samples were taken between 10 and 11 am by manual expression of a 5 ml sample from each breast at the beginning and end of a feed Fluorometry was used for analysis	Food record booklet for 5 days collected during third trimester of pregnancy, which was used to divide participants into two groups, group 1: riboflavin intake < RI (n=25), group 2: riboflavin intake ≥ RI (n=32) Blood (activation coefficient of erythrocyte glutathione reductase (α-EGR) collected during third trimester of pregnancy	Mean (±SD) breast milk riboflavin concentration, transitional milk, group 1: 574.9±258.7 nmol/l, group 2: 948.1±700.1 nmol/l, mature milk, group 1: 725.4±254.3 nmol/l, group 2: 993.8±436.6 nmol/l Mean (±SD) serum (α-EGR) concentration, group 1: 704±241.8 nmol/l, group 2: 996.4±302.9 nmol/l Maternal α-EGR during third trimester of pregnancy was associated with breast milk riboflavin in mature milk (statistics not reported) Maternal dietary riboflavin intake during third trimester of pregnancy was associated with breast milk riboflavin in transitional and mature milk ($p<0.05$)	+
Prentice 1983	Pre-post intervention, no	Supplements were given during lactation, 1.22 mg/day of riboflavin to all participants as a fortified biscuit.	2 samples collected pre-supplementation (n=21) and post-supplementation (n=23) (stage of lactation not clear, however it was confined to	Supplementation (pre-post intervention design, the study used	Mean (±SE) breast milk riboflavin concentration pre-supplement: 0.21±0.01 µg/ml, post-supplement: 0.28±0.01 µg/ml	+

Table 1. Studies on the relationship between maternal nutritional status or dietary intake and breast milk vitamin concentrations

First author, year	Study design (sample size) and setting	Micronutrient supplementation status	Milk samples	Maternal nutritional assessment	Main results	Relationship
	randomisation (n=130) The Gambia		the first 6 months of lactation). Milk was expressed from each breast before and after a mid-afternoon feed on two occasions during Sep 1979 (pre-supplement) and on two further occasions during Sep 1980 (post-supplementation). Milk was collected four times throughout the day, at regular intervals Laboratory method used not clear	pre-supplement concentrations as comparison group)	Maternal riboflavin supplementation during lactation increased breast milk riboflavin ($p<0.001$)	
Thomas 1980	Intervention, no randomisation or placebo group (n=12) USA	Supplements were given during lactation, 2.0 mg/day of riboflavin, and the remaining participants were not given any supplements	1 sample collected 6 months postpartum. Milk was expressed 4 times per day at 4-hour intervals for three consecutive days, and was expressed immediately before taking the supplement in the morning at 0 time and 4, 8, and 12 hour thereafter. Those participants not taking supplements expressed milk at corresponding times Laboratory method used not clear	Supplement group	Mean (±SD) breast milk riboflavin concentration, supplemented group 274±46 µg/l, unsupplemented: 243±35 µg/l Maternal riboflavin supplementation during lactation did not increase breast milk riboflavin (statistics not reported)	÷
Vitamin B_6						
Boylan 2002	Observational (n=25) USA	No supplements were given by investigators, however 20 of the women took supplements regularly	1 sample collected between 8-11 days postpartum (transitional milk). The breast milk was collected in the morning after a period of at least 2 hours in which the mother had not breastfed the infant. All milk was collected from one breast only into a sterile tube connected directly to an electric breast pump HPLC was used for analysis	24-hour recall, collected at the same time as breast milk sample, which was used to divide the participants into two groups, group 1 (n=12): dietary intake of B_6 below the median (median=2.90 mg/day) and group 2 (n=13): above the median	Median (min, max) breast milk pyridoxal concentration, group 1: 0.18 (0.12, 0.30) µmol/l, group 2: 0.65 90.33, 1.29) µmol/l Maternal dietary intake was associated with breast milk pyridoxal ($p<0.008$)	+
Chang 1990	Intervention, no randomisation or placebo group (n=47)	Supplements were given during lactation (until 6 months postpartum). Group 1: 2.5 mg/day of pyridoxine, group 2: 4.0 mg/day, group 3:	7 samples collected, one at 3-5 days post-partum (colostrum) and 6 samples each months during the first 6 months postpartum. Breast milk was expressed manually by the mother (10 ml), or	Supplement group Plasma pyridoxal phosphate (PLP)	Mean (±SEM) breast milk vitamin B_6 colostrum, group 1: 107±9 nmol/l, group 2: 191±11 nmol/l, group 4: 658±132 nmol/l (group 3 not reported)	+

Table 1. Studies on the relationship between maternal nutritional status or dietary intake and breast milk vitamin concentrations

First author, year	Study design (sample size) and setting	Micronutrient supplementation status	Milk samples	Maternal nutritional assessment	Main results	Relationship
	USA	7.5 mg/day, or group 4: 10 mg/day. No supplements were given by investigators during pregnancy, but most participants did take supplements themselves during pregnancy. For colostrum analysis, the participants were divided into groups according to B_6 supplement intake during pregnancy	with the use of a breast pump, each feeding for one 24-hour period Saccharomyces uvarum microbiological assay was used for analysis	collected at 1, 4 and 6 months postpartum	(B_6 concentrations in the remaining 6 months were reported in a figure) Mean (±SEM) plasma PLP, 1 month, group 1: 56±7 nmol/l, group 2: 99±6 nmol/l, group 3: 106±14 nmol/l, 4 months, group 1: 94±11 nmol/l, group 2: 111±9 nmol/l, group 3: 184±22 nmol/l, group 4: 173±7 nmol/l, 6 months, group 1: 93±7 nmol/l, group 2: 103±8 nmol/l, group 3: 155±9 nmol/l, group 4: 320±12 nmol/l Maternal prenatal supplementation increased breast milk B_6 at 3-5 days postpartum (statistics not reported) Maternal postpartum supplementation increased breast milk B_6 across the first six months postpartum (statistics not reported) Maternal plasma PLP was associated with breast milk B_6 at 1 month postpartum ($r=0.49$, $p<0.01$), and at 4 months postpartum ($r=0.57$, $p<0.01$), and at 6 months postpartum ($r=0.63$, $p<0.01$)	
Hamaker 1990	Observational (n=15) USA	Supplements were not given by investigators, but participants took supplements themselves during lactation, and were divided into two groups based on their supplement intake, group 1 (n=8): 2.5 mg pyridoxine, group 2 (n=9): 15.0 pyridoxine (routinely intake)	1 sample collected at 1 month postpartum. Between 5-10 ml of foremilk was collected at each infant feeding during one 24-hour period. Milk samples were expressed manually Saccharomyces uvarum microbiological assay was used for analysis	Supplement group	Mean (±SD) breast milk total vitamin B_6, after supplementation was consumed, group 1: 1297±608 nmol/l, group 2: 4098±698 nmol/l Maternal pyridoxine supplementation during lactation increased breast milk total B_6 ($p<0.05$)	+
Moser-Villon 1990	Randomised, double-blinded controlled trial (n=40) USA	Supplements were given during lactation, starting from delivery to 9 months postpartum. Participants were given a multiple micronutrient tablet daily, that only differed in amount of zinc and pyridoxine, 1) 0 mg zinc and 0.5 mg pyridoxine (n=10), 2) 0 zinc and 4.0 mg pyridoxine (n=10), 3) 25 mg zinc and 0.5 mg pyridoxine (n=10) 4) 25	6 samples collected at 1, 2, 4, 12, 24, 36 weeks postpartum. Samples was collected into polypropylene containers from the first breastfeed of the day, by manually expression. Half from the beginning and half from the end of the feeding Atomic-absorption spectrophotometry was used for analysis	Supplement group	Mean (±SEM) breast milk total vitamin B_6 concentration, 1 week, group 1: 495±88 nmol/l, group 2: 1095±119 nmol/l, 2 weeks group 1: 823±93 nmol/l, group 2: 1469±155 nmol/l, 4 weeks group 1: 1023±144 nmol/l, group 2: 2421±290 nmol/l, 12 weeks group 1: 1410±280 nmol/l, group 2: 2556±339 nmol/l, 24 weeks group 1: 1317±183 nmol/l, group 2: 2666±408 nmol/l, 36 weeks group 1: 1406±290 nmol/l, group 2: 3100±473 nmol/l Mean (±SEM) total plasma vitamin B_6 concentration at 1 week group 1: 53±5 nmol/l, group 2: 102±10 nmol/l, 2 weeks group 1: 55±3 nmol/l, group 2: 112±12 nmol/l, 4 weeks group 1: 76±6 nmol/l, group 2: 143±19 nmol/l, 12 weeks group 1: 89±9 nmol/l, group 2: 243±39 nmol/l, 24 weeks group 1: 99±10 nmol/l, group	+

Table 1. Studies on the relationship between maternal nutritional status or dietary intake and breast milk vitamin concentrations

First author, year	Study design (sample size) and setting	Micronutrient supplementation status	Milk samples	Maternal nutritional assessment	Main results	Relationship
		mg zinc and 4.0 mg pyridoxine (n=10)			2: 242±33 nmol/l, 36 weeks group 1: 86±10 nmol/l, group 2: 205±30 nmol/l (significantly different) Maternal vitamin B_6 supplementation of 4 mg/day during lactation increased breast milk vitamin B_6 compared to 0.5 mg/day ($p<0.01$)	
Prentice 1983	Pre-post intervention, no randomisation (n=130) The Gambia	Supplements were given during lactation, 0.71 mg/day of vitamin B_6 to all participants as a fortified biscuit.	2 samples collected pre-supplementation (n=21) and post-supplementation (n=21) (stage of lactation not clear, however it was confined to the first 6 months of lactation). Milk was expressed from each breast before and after a mid-afternoon feed on two occasions during Sep 1979 (pre-supplement) and on two further occasions during Sep 1980 (post-supplementation). Milk was collected four times throughout the day, at regular intervals Laboratory method used not clear	Supplementation (pre-post intervention design, the study used pre-supplement concentrations as comparison group)	Mean (±SE) breast milk vitamin B_6 pre-supplementation: 0.12±0.005 µg/ml, post-supplementation: 0.10±0.005 µg/ml Maternal vitamin B_6 supplementation during lactation did not increase breast milk vitamin B_6 ($p>0.05$)	÷
Sneed 1981	Intervention, no randomisation (n=16) USA	Supplements were given during lactation. Supplemented participants (n=9) were given a multivitamin tablet (containing 4 mg/day of vitamin B_6), and the remaining participants (n=7) were given a placebo	2 samples collected at 5-7 days and 43-45 days postpartum. Milk was expressed 4 times per day at 4-hour intervals, beginning with the first infant feeding of the day. The milk was expressed immediately before taking the supplement in the morning Microbiological assay was used for analysis	Supplement group Blood, collected at the same time as breast milk samples 4-day dietary records collected between 4-7 and 42-45 days postpartum	Mean (±SD) breast milk vitamin B_6 concentration, 5-7 days, supplemented group (SG): 248±60 µg/l, placebo group (PG): 123±34 µg/l. 43-45 days, SG: 240±57 µg/l, PG: 120±33 µg/l Mean (±SD) dietary vitamin B_6 intake, 5-7 days, SG: 5.33±0.29 mg/day, PG: 1.52±0.40 mg/day. 43-45 days, SG: 5.12±0.31 mg/day, PG: 1.41±0.56 mg/day Maternal vitamin B_6 supplementation increased breast milk vitamin B_6 ($p<0.01$) Maternal blood vitamin B_6 was associated with breast milk vitamin B_6 at 5-7 days postpartum ($r=-0.80$, $p<0.01$), and at 43-45 days ($r=-0.89$, $p<0.01$) in participants not supplemented Maternal dietary vitamin B_6 intake was associated with breast milk vitamin B_6 at 5-7 days postpartum ($r=0.84$, $p<0.01$), and at 43-45 days ($r=0.76$, $p<0.05$) in participants not supplemented	+
Styslinger 1985	Intervention, no randomisation or	Supplements were given during lactation. Group 1: no supplementation , group 2:	1 sample collected (stage of lactation not clear). 5 ml was collected following milk let down and before the infant nursed. Sample collection was	Supplement group	Mean (±SEM) breast milk vitamin B_6 concentration, group 1: 93±8 µg/l, group 2: 192±16 µg/l, group 3: 247±25 µg/l, group 4: 413±45 µg/l	+

Table 1. Studies on the relationship between maternal nutritional status or dietary intake and breast milk vitamin concentrations

First author, year	Study design (sample size) and setting	Micronutrient supplementation status	Milk samples	Maternal nutritional assessment	Main results	Relationship
	placebo group (n=24) USA	supplements of 2.5 mg pyridoxine, group 3: 10.0 mg pyridoxine , group 4: 20.0 mg pyridoxine per day for three consecutive days	initiated at 6 am on the first day of the study and continued to midnight of day three. Milk was expressed manually or by hand pump Saccharomyces uvarum microbiological assay was used for analysis Infants were exclusively breastfed	3 day diet records (not clear when collected)	Mean (±SEM) dietary vitamin B_6 intake (excluding supplement intake) was 1.8±0.2 mg/day for all four groups (no difference between groups) Maternal vitamin B_6 supplementation increased breast milk vitamin B_6 ($p<0.05$) Maternal dietary vitamin B_6 intake was associated with breast milk B_6 ($r=0.79$, $p<0.001$)	
Roepke 1979	Observational (n=106 during pregnancy and n=62 during lactation) USA	No supplements were given by investigators	2 samples collected at 3 and 14 days postpartum. Milk was manually expressed, and 5 ml was collected at an early morning feed Saccharomyces uvarum microbiological assay was used for analysis	24-hour diet recall and also a 3 day diet record, which was used to divide participants into two groups, group 1: B_6 intake ≤2.5 mg/day, group 2: B_6 intake >2.5 mg/day (data collected between 5 and 7 months of gestation) Serum collected at 5 months gestation	Mean (±SE) breast milk vitamin B_6 concentration 3 days, group 1 (n=9) : 8.1±1.5 µg/l, group 2 (n=42): 16.1±3.0 µg/l. Day 14, group 1 (n=9): 40.1±10.3 µg/l., group 2 (n=38): 57.4±7.2 µg/l Maternal dietary vitamin B_6 intake during pregnancy was associated with breast milk vitamin B_6 at 3 days postpartum ($p<0.02$), however not at 14 days postpartum Maternal serum vitamin B_6 at 5 months gestation was associated with breast milk vitamin B_6 at 14 days postpartum ($r=0.51$, $p<0.001$)	+ ÷
Thomas 1979	Intervention, no randomisation or placebo group (n=17) USA	Supplements were given during lactation. Supplemented participants (n=10) were given a multivitamin tablet (containing 4 mg/day of vitamin B_6), and the remaining participants (n=7) were not given any supplements	2 samples collected at 5-7 days and 43-45 days postpartum. Milk was expressed 4 times per day at 4-hour intervals. The milk was expressed immediately before taking the supplement in the morning at 0 time and 4, 8, and 12 hour thereafter. Those participants not taking supplements expressed milk at corresponding times Microbiological assay was used for analysis	Supplement group	Mean (±SE) breast milk vitamin B_6 concentration, 5-7 days, supplemented group (SG): 225±87 µg/l, not supplemented (NS): 128±59 µg/l. 43-45 days, SG: 237±57 µg/l, NS: 204±53 µg/l Maternal vitamin B_6 supplementation influenced breast milk vitamin B_6 in transitional milk ($p<0.05$), however not in mature milk ($p>0.05$)	+ ÷
Thomas 1980	Intervention, no randomisation or placebo group (n=12) USA	Supplements were given during lactation. Supplemented participants were given a multivitamin tablet (containing 4 mg/day of vitamin B_6) and the remaining participants	1 sample collected 6 months postpartum. Milk was expressed 4 times per day at 4-hour intervals for three consecutive days, and was expressed immediately before taking the supplement in the morning at 0 time and 4, 8, and 12 hour thereafter. Those participants not taking	Supplement group	Mean (±SD) breast milk vitamin B_6 concentration, supplemented group 235±49 µg/l, unsupplemented: 212±58 µg/l Maternal vitamin B_6 supplementation did not increase breast milk vitamin B_6 (statistics not reported)	÷

Table 1. Studies on the relationship between maternal nutritional status or dietary intake and breast milk vitamin concentrations

First author, year	Study design (sample size) and setting	Micronutrient supplementation status	Milk samples	Maternal nutritional assessment	Main results	Relationship
		were not given any supplements	supplements expressed milk at corresponding times Microbiological assay was used for analysis			
West 1976	Observational (n=19) USA	No supplements were given by investigators	Participants collected samples of foremilk before the first morning feed for 3 consecutive days during 1 week, and for 1 day on each of the following 2 week (stage of lactation not clear, between 3 weeks and 30 months) Saccharomyces carlsbergensis microbiological assay was used for analysis	Diet records for 3 consecutive days collected during the first week of milk collection, which was used to divide participants into three groups, group 1: B_6 intake <2.5 (n=6), group 2: B_6 intake between 2.5-5.0 (n=8), group 3: B_6 intake >5.0 (n=5) mg per day	Mean (±SD) breast milk vitamin B_6 concentration, group 1: 129±39 mg/day, group 2: 239±51 mg/day, group 3: 314±52 mg/day Maternal dietary vitamin B_6 intake was associated with breast milk vitamin B_6 ($p<0.01$)	+
Vitamin B9 (folate)						
Donangelo 1989	Observational (n=83) Brazil	No supplements were given by investigators	1 sample collected between 9.00 and 10.00 am by manual expression before the infant was due to be fed (5-10 ml) (stage of lactation was mixed, between 1-180 days) Radioisotope dilution assay was used for analysis Infants were exclusively breastfed	Serum collected at the same time as breast milk sample	Mean (±SE) breast milk folate concentration, 1-5 days postpartum (n=17): 23.0±4.1 nmol/l, 6-30 days (n=18): 61.8±6.9 nmol/l, 31-280 days (n=11): 107±17.3 nmol/l Mean (±SE) serum folate concentration, 1-5 days postpartum (n=9): 10.3±1.0 nmol/l, 6-30 days (n=18): 18.8±3.8 nmol/l, 31-280 days (n=10): 11.0±2.0 nmol/l Maternal serum folate was not associated with breast milk folate at any stage of lactation (statistics not reported)	÷
Houghton 2009	Randomised, placebo controlled trial (n=69) Canada	Supplements were given during lactation. Group 1: [6S]-5-methylTHF (416 µg/day). Group 2: folic acid (400 µg/day). Group 3: placebo Supplementation started 1 week postpartum and ended 16 week postpartum	3 samples collected at 4, 8 and 16 weeks postpartum. Complete breast expression (manually or by electric breast pump) was used to collect milk samples. Milk samples were collected between 13.00 and 14.50 HPLC was used for analysis	Supplement group Plasma collected at the same time as breast samples	Mean (±SD) breast milk folate concentration, 4 weeks, group 1: 189±52 nmol/l, group 2: 155±55 nmol/l, group 3: 193±62 nmol/l. 8 weeks, group 1: 175±43 nmol/l, group 2: 176±80 nmol/l, group 3: 207±76 nmol/l. 16 weeks, group 1: 182±102 nmol/l, group 2: 159±79 nmol/l, group 3: 183±57 nmol/l Mean (±SD) plasma folate concentration, 16 weeks, group 1: 104±55 nmol/l, group 2: 97±27 nmol/l, group 3: 48±24 nmol/l (significantly different)	÷

Table 1. Studies on the relationship between maternal nutritional status or dietary intake and breast milk vitamin concentrations

First author, year	Study design (sample size) and setting	Micronutrient supplementation status	Milk samples	Maternal nutritional assessment	Main results	Relationship
					Maternal folic acid supplementation did not increase breast milk folate (statistics not reported) Maternal plasma folate was not associated with breast milk folate (statistics not reported)	
Jathar 1970	Observational (n=47) India	No supplements were given by investigators	1 sample collected early in the morning before feeding the infant, by manual expression (stage of lactation not clear) Folate concentration was determined by the Lactobacillus casei microbiological assay	Dietary assessment, collected at the same time as breast milk sample, which divided the participants into three groups, group 1 (n=15): lacto-vegetarian, group 2 (n=16): non-vegetarian, occasional meat eaters, group 3 (n=17): non-vegetarian, frequent meat eaters.	Mean (±SE) breast milk folic acid activity group 1: 10.3±2.1 mμg/ml, group 2: 6.6±1.3 mμg/ml, group 3: 10.8±2.2 mμg/ml Maternal meat intake did not influence breast milk folate (statistics not reported)	÷
Khambalia 2006	Pre-post intervention, no randomisation (n=71) Mexico	Supplements were given during lactation. During the first clinic visit after childbirth (22±13 days postpartum), participants were randomised to group 1: receiving a multivitamin supplement containing 400 μg folic acid and 18 mg of elemental Fe. Group 2: receiving a multivitamin supplement containing 400 μg folic acid without Fe. Supplements were provided daily for 6 months	3 samples collected at 22 (baseline), 82 (mid-study) and 138 days (end-study) postpartum. Milk was collected by complete expression of 1 breast at least 2 hours after the previous feeding using an electric breast pump Folate concentration was determined by microbiological assay	Supplementation (Pre-post intervention design, the study used pre-supplement concentrations as comparison group)	Median (IQR) breast milk folate concentration, baseline: 102.5 (89.6, 129.2) nmol/l, mid-study: 154.9 (128.6, 178.2) nmol/l, end-study: 144.2 (122.1, 179.3) nmol/l Maternal folic acid supplementation during lactation increased breast milk folate ($p<0.001$)	+
Mackey 1999	Randomised double-blinded, placebo controlled trial (n=42)	Supplements were given during lactation. The participants received either a supplement with 0 or 1 mg folic acid/day.	2 samples collected at 3 months (baseline) and 6 months postpartum (end point). Complete breast expression was used to collect milk samples	Supplement group	Mean (±SEM) breast milk folate concentration, baseline, supplemented group (SG): 186±9.6 nmol/l, placebo group (PG): 224±11.6 nmol/l (significantly different at baseline). 6 months, SG: 181.9±10.6 nmol/l, PG: 187.0±11.9 nmol/l	÷

Table 1. Studies on the relationship between maternal nutritional status or dietary intake and breast milk vitamin concentrations

First author, year	Study design (sample size) and setting	Micronutrient supplementation status	Milk samples	Maternal nutritional assessment	Main results	Relationship
	USA	Supplementation started 3 months postpartum and ended 6 months postpartum	(15–30 ml) by manual means or with a mechanical pump Folate concentration was determined by the Lactobacillus casei microbiological assay At 3 months, all but 2 infants were exclusively breastfed; at 6 month, all but 8 infants were exclusively breast-fed	Plasma collected at the same time as breast milk samples	Mean (±SEM) plasma folate concentration, baseline, SG: 44.9±4.1 nmol/l, PG: 42.1±5.07 nmol/l. 6 months, SG: 46.7±6.5 nmol/l, PG: 36.8±4.2 nmol/l (not significantly different) Maternal folic acid supplementation during lactation did not increase breast milk folate (statistics not reported) Maternal plasma folate was associated with breast milk folate, however only in supplemented women (r=-0.52, p<0.01) at 6 months postpartum	
Prentice 1983	Pre-post intervention, no randomisation (n=130) The Gambia	Supplements were given during lactation, 5.7 µg/day of folic acid to all participants as a fortified biscuit.	2 samples collected pre-supplementation (n=21) and post-supplementation (n=22). (stage of lactation not clear, however it was confined to the first 6 months of lactation). Milk was expressed from each breast before and after a mid-afternoon feed on two occasions during Sep 1979 (pre-supplement) and on two further occasions during Sep 1980 (post-supplementation). Milk was collected four times throughout the day, at regular intervals Laboratory method used not clear	Supplementation (pre-post intervention design, the study used pre-supplement concentrations as comparison group)	Mean (±SE) breast milk folic acid pre-supplementation: 38.2±2.9 ng/ml, post-supplementation: 47.3±3.95 ng/ml Maternal folic acid supplementation during lactation did not increase breast milk folic acid (p>0.05)	÷
Tamura 1980	Pre-post intervention, no randomisation (n=16) Japan	Supplements were given during lactation to participants for 4 weeks (1 mg of folic acid/day)	2 samples collected (stage of collection unclear). Milk samples were collected between 2:00 to 3:00 PM, just before the infant was fed. Approximately 10 ml was collected by manual expression Folate concentration was determined by the Lactobacillus casei microbiological assay	Supplementation (Pre-post intervention design, the study used pre-supplement concentrations as comparison group) Plasma collected at the same time as breast milk samples	Mean (±SD) breast milk folate concentration, baseline: 130.2±45.9 ng/ml, post-supplementation: 136.6±41.2 ng/ml Mean (±SD) plasma milk folate concentration, baseline: 6.0±2.0 ng/ml, post-supplementation: 41.8±53.8 ng/ml (significantly different) Maternal folic acid supplementation (1 mg/day) during lactation did not increase breast milk folate (p>0.6) Maternal plasma folate was not associated with breast milk folate (statistics not reported)	÷
Smith 1983	Observational study (n=11)	Supplements were not given by investigators, however all participants reported taking a daily multivitamin and mineral supplement with 0.8-1 mg folic	2 samples collected at 6 and 12 weeks postpartum. Foremilk and hindmilk were collected by the participants manually or with a breast pump, at a morning, midday and evening	Serum collected at the same time as breast milk samples	Mean (±SD) breast milk total folate concentration: 78.9±44.7 ng/ml (concentrations from both study visits and from foremilk and hindmilk combined)	÷

Table 1. Studies on the relationship between maternal nutritional status or dietary intake and breast milk vitamin concentrations

First author, year	Study design (sample size) and setting	Micronutrient supplementation status	Milk samples	Maternal nutritional assessment	Main results	Relationship
	USA	acid during pregnancy and they continued taking the supplement for the duration of the study	feed. A total of 6 samples at each time-point was collected from each participant on three consecutive days Folate concentration was determined by the Lactobacillus casei microbiological assay Infants were exclusively breastfed	Red blood cell (RBC) folate collected at the same time as breast milk samples	(Maternal serum and RBC folate concentrations presented in a figure) Maternal serum/RBC folate was not associated with breast milk folate (statistics not reported)	
Sneed 1981	Intervention, no randomisation (n=16) USA	Supplements were given during lactation. Supplemented participants (n=9) were given a multivitamin tablet (containing 0.8 mg/day of folic acid) and the remaining participants (n=7) were given a placebo	2 samples collected at 5-7 days and 43-45 days postpartum. Milk was expressed 4 times per day at 4-hour intervals, beginning with the first infant feeding of the day. The milk was expressed immediately before taking the supplement in the morning Folate concentration was determined by the Lactobacillus casei microbiological assay	Supplement group Serum collected at the same time as breast milk samples 4-day dietary records collected between 4-7 and 42-45 days postpartum	Mean (±SD) breast milk folate concentration, 5-7 days, supplemented group (SG): 49.4±5.4 µg/l, placebo group (PG): 41.6±7.5 µg/l. 43-45 days, SG: 49.9±3.6 µg/l, PG: 42.8±5.1 µg/l Mean (±SD) serum folate, 5-7 days, SG: 10.04±2.52 ng/ml, PG: 6.75±3.41 ng/ml. 43-45 days, SG: 7.43±2.26 ng/ml, PG: 6.26±3.65 ng/ml (not significantly different) Mean (±SD) dietary folic acid intake, 5-7 days, SG: 1060±100 µg/day, PG: 290±100 µg/day, 43-45 days, SG: 1010±80 µg/day, PG: 340±200 µg/day, Maternal folic acid supplementation increased breast milk folate at both time-points ($p<0.01$) Maternal serum folate was not associated with breast milk folate at 5-7 days postpartum ($r=0.37$, $p>0.05$), or at 43-45 days ($r=0.03$, $p>0.05$) in participants not supplemented Maternal dietary folic acid intake was not associated with breast milk folate at 5-7 days postpartum ($r=0.25$, $p>0.05$), or at 43-45 days ($r=-0.14$, $p>0.05$) in participants not supplemented	+ ÷
Thomas 1980	Intervention, no randomisation or placebo group (n=12) USA	Supplements were given during lactation. Supplemented participants were given a multivitamin tablet (containing 0.8 mg/day of folic acid) and the remaining participants were not given any supplements	1 sample collected 6 months postpartum. Milk was expressed 4 times per day at 4-hour intervals for three consecutive days, and was expressed immediately before taking the supplement in the morning at 0 time and 4, 8, and 12 hour thereafter. Those participants not taking supplements expressed milk at corresponding times	Supplement group	Mean (±SD) breast milk folate concentration, supplemented group 54.8±7.0 µg/l, unsupplemented: 50.1±4.5 µg/l Maternal folic acid supplementation did not increase breast milk folate (statistics not reported)	÷

Table 1. Studies on the relationship between maternal nutritional status or dietary intake and breast milk vitamin concentrations

First author, year	Study design (sample size) and setting	Micronutrient supplementation status	Milk samples	Maternal nutritional assessment	Main results	Relationship
			Folate concentration was determined by the Lactobacillus casei microbiological assay			
West 2012	Pre-post intervention, no randomisation (n=28) USA	Supplements were given during lactation to all participants. All participants consumed a daily multivitamin supplement containing 750 µg folic acid, plus an intake of natural food folate (400 µg/day) for 10-12 weeks (from approximately 5- 15 weeks postpartum)	3 samples collected, at 5 (baseline) and 15 weeks (study end) postpartum. Breast milk samples were collected from a full expression of one breast 2 hour after the first feed of the day. Participants expressed the same breast throughout the study LC-MS/MS was used for analysis	Supplementation (pre-post intervention design, the study used pre-supplement concentrations as comparison group)	Mean (95% CI) breast milk total folate concentration, baseline: 56.2 (48.8, 64.2) ng/ml, 15 weeks: 61.8 (54.1, 70) ng/ml Mean (95% CI) breast milk folic acid concentration, baseline: 16.2 (11.8, 21.3) ng/ml 15 weeks: 24.1 (18.7, 30.3) ng/ml Maternal folic acid supplementation during lactation did not increase breast milk total folate (p=0.2) Maternal folic acid supplementation during lactation increased breast milk folic acid (p<0.003)	÷ +
Vitamin B_{12}						
Bae 2015	Pre-post intervention, no randomisation (n=28) USA	All participants, pregnant (n=26), lactation (n=28) and controls (non-pregnant, non-lactating) (n=21) consumed equivalent vitamin B_{12} amounts of ~ 8.6 µ/day for 10 weeks. The vitamin B_{12} was given as a meal (~6 µ/day) and as a supplement (2.6 µ/day). The effect of supplementation on breast milk concentration was only investigated in lactating women, not in pregnant women.	2 samples collected, at baseline 5 weeks postpartum and 10 weeks after, 15 weeks postpartum, only collected from the lactating women entering the study. Breast milk samples were collected from fasting women (10 hours), one full breast expression two hours after the first feed of the day Protein binding immunoassay was used for analysis Infants were exclusively breastfed	Supplementation (diet + supplement) (Pre-post intervention design, the study used pre-supplement concentrations as comparison group) Serum (B_{12} and holotranscobalamin (holoTC) collected at the same time-point as breast milk samples	Geometric mean (95% CI) breast milk vitamin B_{12} concentration at baseline (n=28): 318 (227, 447) pmol/l, 15 weeks (study end): 298 (213, 419) pmol/l Median (IQR) serum vitamin B_{12} concentration, baseline 463 (419, 511) pmol/l Median (IQR) serum holoTC concentration, baseline: 96 (82, 111) Maternal vitamin B_{12} supplementation (diet + supplement) during lactation did not increase breast milk vitamin B_{12} (p=0.5) Maternal serum vitamin B_{12} was associated with breast milk vitamin B_{12} at baseline (5 weeks postpartum) (r=0.48, p=0.01) Maternal serum holoTC was associated with breast milk vitamin B_{12} at baseline (r=0.42, p=0.03) Maternal serum vitamin B_{12} was not associated with breast milk B_{12} at study end (r=0.18, p=0.4) Maternal serum holoTC was associated with breast milk vitamin B_{12} at study-end (15 weeks postpartum) (r=0.40, p=0.04)	+ ÷
Casterline 1997	Observational (n=113)	No supplements were given by investigators	1 sample collected at 3 months postpartum. A milk sample was collected by complete expression of one breast, using and electric breast pump while the child was nursing from	Plasma collected at the same time as the breast milk sample	Mean (±SD) breast milk vitamin B_{12} concentration: 689.7±490.7 pmol/l	÷

Table 1. Studies on the relationship between maternal nutritional status or dietary intake and breast milk vitamin concentrations

First author, year	Study design (sample size) and setting	Micronutrient supplementation status	Milk samples	Maternal nutritional assessment	Main results	Relationship
	Guatemala		the opposite breast to stimulate a let-down reflex. Samples were collected from the opposite breast from the one used for the last feed and at least 1.5 hour after the last feed MAGIC vitamin B_{12}/folate radioassay was used for analysis Infants were exclusively breastfed	24-hour recalls on two separate occasions at 1 and 2 months postpartum	Mean (±SD) plasma vitamin B_{12} concentration: 252.2±101.7 pmol/l Mean (±SD) holotranscobalamin concentration: 70.3±61.9 pmol/l Mean (±SD) dietary vitamin B_{12} intake: 3.9±12.0 μg/day Maternal plasma vitamin B_{12} was not associated with breast milk vitamin B_{12} (r=0.18, p>0.05) Maternal holotranscobalamin was not associated with breast milk B_{12} (statistics not reported) Maternal dietary B_{12} intake was not associated with breast milk vitamin B_{12} (r=0.02, p>0.05)	
Chebaya 2017	Observational (n=129 Canadians, n=69 Cambodians) Canada and Cambodia	No supplements were given by investigators, however Canadian mothers consumed a daily vitamin B_{12}-containing multiple micronutrient supplement throughout pregnancy and lactation (12 μg/day); Cambodian mothers were unsupplemented	1 sample collected at 8 weeks postpartum in the Canadian study and between 3-27 weeks in the Cambodian study. Milk from one full breast expression was collected using an electric breast pump, more than two hours after the previous feeding Chemiluminescent enzyme immunoassay was used for analysis Infants were exclusively breastfed	Serum and plasma collected at the same time as the breast milk sample	Geometric mean breast milk vitamin B_{12} concentration, Canada (n=109): 452 pmol/l, Cambodia (n=59): 317 pmol/l Geometric mean serum B_{12}, Canada (n=124): 698 pmol/l Geometric mean plasma B_{12}, Cambodia (n=69): 620 pmol/l Maternal serum vitamin B_{12} was associated with breast milk vitamin B_{12} in Canadian mothers (β=0.498, p<0.001) Maternal plasma vitamin B_{12} was not associated with breast milk vitamin B_{12} in Cambodian mothers (β=0.105, p=0.4) (adjusted for stage of lactation)	+ ÷
Donangelo 1989	Observational (n=83) Brazil	No supplements were given by investigators	1 sample collected between 1-180 days postpartum, between 9.00 and 10.00 am by manual expression before the infant was due to be fed (5-10ml) Radioisotope dilution assay was used for analysis Infants were exclusively breastfed	Serum collected at the same time as breast milk sample	Mean (±SE) breast milk vitamin B_{12} concentration, 1-5 days postpartum (n=17): 0.82±0.26 nmol/l, 6-30 days (n=18): 1.18±0.39 nmol/l, 31-280 days (n=10): 0.67±0.15 nmol/l Mean (±SE) serum B_{12} concentration, 1-5 days postpartum (n=17): 0.23±0.04 nmol/l, 6-30 days (n=18): 0.35±0.05 nmol/l, 31-280 days (n=10): 0.32±0.04 nmol/l Maternal serum vitamin B_{12} was not associated with breast milk vitamin B_{12} at any stage of lactation (statistics not reported)	÷

Table 1. Studies on the relationship between maternal nutritional status or dietary intake and breast milk vitamin concentrations

First author, year	Study design (sample size) and setting	Micronutrient supplementation status	Milk samples	Maternal nutritional assessment	Main results	Relationship
Duggan 2014	Randomised, placebo controlled trial (n=366) India	Supplements were given during pregnancy and lactation. Participants were randomly assigned to receive daily oral dose of vitamin B_{12} (50 µg) or a placebo from enrolment (<14 weeks postpartum) through 6 weeks postpartum. All women received the standard care of 60 mg of iron and 500 µg of folic acid	3 samples collected at 6 weeks, 3 months and 6 months postpartum. Breast milk was collected without restriction regarding time since last feed. After cleaning the nipple and before the mothers nursed the infants, 10 ml of breast milk was expressed manually from one breast Competitive protein binding immunoassay was used for analysis	Supplement group	Median (IQR) breast milk vitamin B_{12} concentration, 6 weeks, supplemented group (SG) (n=68): 136 (93, 203) pmol/l, placebo group (n=73): 87 (44, 127) pmol/l. 3 months, SG (n=47): 97 (63, 146) pmol/l, PG (n=57): 68 (37, 102) pmol/l. 6 months, SG (n=37): 106 (65, 160) pmol/l, PG (n=44): 80 (51, 113) pmol/l. Median plasma vitamin B_{12}, second trimester, SG: 216 pmol/l, PG: 112 pmol/l (significantly different). Third trimester, SG: 184 pmol/l, PG: 105 pmol/l (significantly different) Maternal vitamin B_{12} supplementation during pregnancy and lactation (until 6 weeks postpartum) increased breast milk B_{12} at 6 weeks postpartum ($p<0.001$), however not at 3 ($p=0.7$) or 6 months postpartum ($p=0.8$)	+ ÷
Greibe 2013	Observational (n=25) Denmark	No supplements were given by investigators, however the majority of the participants supplemented their diet with a daily multivitamin pill containing 1.0–4.5 µg vitamin B_{12}	3 samples collected at 2 week (15 ± 7 days), 4 months (129 ± 12 days), and 9 months (280 ± 15 days) postpartum. At the day of each visit (or the night before), foremilk and hindmilk were manually collected by the mothers ELISA was used for analysis Infants were exclusively breastfed to after 4 months postpartum	Plasma collected at the same time as breast milk sample (n=60)	Median (range) breast milk vitamin B_{12} concentration, hindmilk, 2 weeks: 760 (210, 1880) pmol/l, 4 months: 290 (140, 690) pmol/l, 9 months: 440 (160, 1940) pmol/l Median (range) plasma holotranscobalamin (holoTC) 2 weeks: 140 (50-360) pmol/l, 4 months: 130 (60, 290) pmol/l, 9 months: 110 (50-240) pmol/l Median (range) plasma vitamin B_{12},, 2 weeks: 400 (170,790) pmol/l, 4 months: 390 (190, 750) pmol/l, 9 months: 420 (160,750) pmol/l Maternal plasma vitamin B_{12} was associated with breast milk vitamin B_{12} at 4 months postpartum ($r=0.58$, $p=0.002$), however not at 2 weeks or 9 months lactation (statistics not reported) Maternal plasma holoTC was associated with breast milk vitamin B_{12} at 2 weeks ($r=0.45$, $p=0.02$) and at 4 months postpartum ($r=0.57$, $p=0.03$)	+ ÷
Jathar 1970	Observational (n=47) India	No supplements were given by investigators	1 sample collected early in the morning before feeding the infant, by manual expression (stage of lactation not clear) Breast milk vitamin B_{12} was estimated microbiologically using Euglena gracilis var. bacillaris as the test organism	Dietary assessment, which divided the participants into three groups, group 1 (n=15): lacto-vegetarian, group 2 (n=16): non-vegetarian, occasional meat eaters, group 3	Mean (±SE) breast milk folic acid activity group 1: 91.3±17.9 µg/ml, group 2: 100.6±18.5 µg/ml, group 3: 103.2±20.8 µg/ml Maternal meat intake was not associated with breast milk B_{12} (statistics not reported)	÷

Table 1. Studies on the relationship between maternal nutritional status or dietary intake and breast milk vitamin concentrations

First author, year	Study design (sample size) and setting	Micronutrient supplementation status	Milk samples	Maternal nutritional assessment	Main results	Relationship
				(n=17): non-vegetarian, frequent meat eaters. Data collected at the same time as breast milk sample		
Neumann 2013	Observational (n=138) Kenya	No supplements given by investigators	3 samples collected between 0-1 months, 1-4 months and 4-6 months postpartum. Breast milk samples were collected during morning hours in the middle of nursing episodes by hand expression Competitive binding isotope dilution method	Quantitative weighing method and dietary recall collected monthly from the time of measurement	Mean (±SD) breast milk vitamin B_{12}, 0-1 months: 198.8±167.5 pg/ml, 1-4 months: 135.9±129.5, 4-6 months: 143.1±152.0 Mean (±SD) dietary vitamin B_{12} intake during lactation: 0.70±0.20 µg/dl Maternal dietary vitamin B_{12} intake was not associated with breast milk B_{12} at 0-1 months (r^2=0.27, p=0.08), however it was associated at 1-4 months (r^2=0.20, p<0.01), and at 4-6 months (r^2=0.26, p<0.001)	+ ÷
Prentice 1983	Pre-post intervention, no randomisation (n=130) The Gambia	Supplements were given during lactation, 1.19 µg/day of vitamin B_{12} to all participants as a fortified biscuit.	2 samples collected pre-supplementation (n=16) and post-supplementation (n=22). (stage of lactation not clear, however the study was confined to only include participants who was in the first 6 months of lactation). Milk was expressed from each breast before and after a mid-afternoon feed on two occasions during Sep 1979 (pre-supplement) and on two further occasions during Sep 1980 (post-supplementation). Milk was collected four times throughout the day, at regular intervals Laboratory method used not clear	Supplementation (Pre-post intervention design, the study used pre-supplement concentrations as comparison group)	Mean (±SE) breast milk vitamin B_{12} pre-supplementation: 0.16±0.04 ng/ml, post-supplementation: 0.12±0.02 ng/ml Maternal vitamin B_{12} supplementation during lactation did not increase breast milk B_{12} (p>0.05)	÷
Siddiqua 2015	Randomised, double-blinded, placebo controlled trial (n=68) Bangladesh	Supplements were given during pregnancy and lactation (starting between 11-14 weeks gestation and end point was 3 months postpartum) 250 µg/day of vitamin B_{12} or a placebo, and both groups received a daily dose of 60 mg of iron and 400 µg of folic acid	2 samples collected within 72 hours (colostrum) and 3 months postpartum. Manually expressed breast milk was collected from the participants, usually at the end of a breastfeeding session Immunoassay was used for analysis	Supplement group	Mean breast milk vitamin B_{12} concentration colostrum (72 hours), supplemented group (SG): 778 pmol/l, placebo group (PG): 320 pmol/l, 3 months, SG: 235 pmol/l, PG: 170 pmol/l Mean (±SD) plasma vitamin B_{12} concentration, baseline PG: 198.7±94.2 pmol/l, SG: 191.6±97.3 pmol/l, median 72 hours after birth, PG: 142 pmol/l, SG: 267 pmol/l, 3 months, PG: 242 pmol/l, SG: 416 pmol/l	+

Table 1. Studies on the relationship between maternal nutritional status or dietary intake and breast milk vitamin concentrations

First author, year	Study design (sample size) and setting	Micronutrient supplementation status	Milk samples	Maternal nutritional assessment	Main results	Relationship
					Maternal vitamin B_{12} supplementation during pregnancy and lactation increased colostrum B_{12} ($p<0.001$) and B_{12} concentration in mature milk ($p=0.03$)	
Sneed 1981	Intervention, no randomisation (n=16) USA	Supplements were given during lactation. Supplemented participants (n=9) were given a multivitamin tablet (containing 8 µg/day of vitamin B_{12}) and the remaining participants (n=7) were given a placebo	2 samples collected at 5-7 days and 43-45 days postpartum. Milk was expressed 4 times per day at 4-hour intervals, beginning with the first infant feeding of the day. The milk was expressed immediately before taking the supplement in the morning Radioimmunoassay was used for analysis	Supplement group Serum collected at the same time as breast milk samples 4-day dietary records collected between 4-7 and 42-45 days postpartum	Mean (±SD) breast milk vitamin B_{12} concentration, 5-7 days, supplemented group (SG): 0.91±0.25 µg/l, placebo group (PG): 0.70±0.19 µg/l. 43-45 days, SG: 0.79±0.24 µg/l, PG: 0.55±0.16 µg/l Mean (±SD) serum vitamin B_{12}, 5-7 days, SG: 682±148 pg/ml, PG: 567±59 pg/ml. 43-45 days, SG: 638±155 pg/ml, PG: 666±122 pg/ml (not significantly different) Mean (±SD) dietary vitamin B_{12} intake, 5-7 days, SG: 12.9±2.2 µg/day, PG: 7.0±3.1 µg/day. 43-45 days, SG: 11.8±1.0 µg/day, PG: 5.2±1.8 µg/day Maternal vitamin B_{12} supplementation increased breast milk vitamin B_{12} at both time-points ($p<0.01$) Maternal serum vitamin B_{12} was not associated with breast milk vitamin B_{12} at 5-7 days postpartum ($r=0.28$, $p>0.05$), or at 43-45 days ($r=-0.18$, $p>0.05$) in participants not supplemented Maternal dietary vitamin B_{12} intake was not associated with breast milk vitamin B_{12} at 5-7 days postpartum ($r=0.54$, $p>0.05$), or at 43-45 days ($r=0.41$, $p>0.05$) in participants not supplemented	+ ÷
Specker 1990	Observational (n=19) USA	No supplements were given by investigators	1 sample collected between 2.0 and 13.9 months postpartum. Samples were collected at the first daylight feeding and were expressed by hand or by pump from one breast. Participants were asked to express all of the milk Radioassay was used for analysis	Serum collected at the same time as breast milk sample	Mean (±SD) breast milk vitamin B_{12} concentration, vegetarians: 231±94 pmol/l, omnivorous: 378±75 pmol/l Maternal serum vitamin B_{12} was associated with breast milk B_{12} ($r=0.787$, $p<0.001$)	+
Thomas 1979	Intervention, no randomisation or placebo group (n=17)	Supplements were given during lactation. Supplemented participants (n=10) were given a multivitamin tablet (containing 8 µg/day of vitamin B_{12}), and the	2 samples collected at 5-7 days and 43-45 days postpartum. Milk was expressed 4 times per day at 4-hour intervals. The milk was expressed immediately before taking the supplement in the morning at 0 time and 4, 8, and 12 hour thereafter. Those participants not taking	Supplement group	Mean (±SE) breast milk vitamin B_{12} concentration, 5-7 days, supplemented group (SG): 1.65±0.63 µg/l, not supplemented (NS): 1.22±0.41 µg/l. 43-45 days, SG: 1.10±0.57 µg/l, NS: 0.61±0.17 µg/l	+ ÷

Table 1. Studies on the relationship between maternal nutritional status or dietary intake and breast milk vitamin concentrations

First author, year	Study design (sample size) and setting	Micronutrient supplementation status	Milk samples	Maternal nutritional assessment	Main results	Relationship
	USA	remaining participants (n=7) were not given any supplements	supplements expressed milk at corresponding times Radioimmunoassay was used for analysis		Maternal vitamin B_{12} supplementation increased breast milk vitamin B_{12} in mature milk ($p<0.05$), however not in transitional milk ($p>0.05$)	
Thomas 1980	Intervention, no randomisation or placebo group (n=12) USA	Supplements were given during lactation. Supplemented participants were given a multivitamin tablet (containing 8 µg/day of vitamin B_{12}) and the remaining participants were not given any supplements	1 sample collected 6 months postpartum. Milk was expressed 4 times per day at 4-hour intervals for three consecutive days, and was expressed immediately before taking the supplement in the morning at 0 time and 4, 8, and 12 hour thereafter. Those participants not taking supplements expressed milk at corresponding times Radioimmunoassay was used for analysis	Supplement group	Mean (±SD) breast milk vitamin B_{12} concentration, supplemented group 0.866±0.295 µg/l, not supplemented: 0.642±0.098 µg/l Maternal vitamin B_{12} supplementation did not increase breast milk vitamin B_{12} (statistics not reported)	÷
Williams 2016	Observational (n=286) Kenya	No supplements were given by investigators	1 sample collected at 6 months postpartum. Breast milk was collected while following an observed non-breastfeeding period of ≤ 90 min. After 1 minute of breastfeeding, study mothers hand-expressed 5 ml breast milk from the right breast. Collection was restricted to times between 09.00 and 12.00 Solid-phase competitive chemiluminescent enzyme immunoassay was used for analysis	7 day FFQ and 24-hour recall collected at the same time as the breast milk sample	Median (IQR) breast milk vitamin B_{12} concentration: 113 (61, 199) pmol/l Median (IQR) dietary vitamin B_{12} intake: 1.45 (0.31, 9.65) µg/day Maternal vitamin B_{12} intake was not associated with breast milk vitamin B_{12} (β=-0.01 95%CI= -0.03, 0.02) (adjusted for maternal age, stage of lactation)	÷
Vitamin C						
Bates 1982	Intervention, no randomisation or placebo group (n=168) The Gambia	Supplements were given during pregnancy and lactation to women from Keneba, a daily tea drink containing 35 mg of ascorbic acid. Women from Manduar received no daily fortified tea drink	1 sample collected (breast milk collection method not clear) Dinitrophenyl hydrazine-based assay was used for analysis	Supplement group	Mean (±SD) breast milk vitamin C concentration, supplemented group: 4.55±1.59 mg/dl, not supplemented group: 3.43±1.54 mg/dl Maternal supplementation of 35 mg/day during pregnancy and lactation increased breast milk vitamin C ($p<0.001$)	+

Table 1. Studies on the relationship between maternal nutritional status or dietary intake and breast milk vitamin concentrations

First author, year	Study design (sample size) and setting	Micronutrient supplementation status	Milk samples	Maternal nutritional assessment	Main results	Relationship
Bates 1983	Intervention, no randomisation (n=80) The Gambia	Supplements were given during lactation, a daily tea drink containing 35 mg of ascorbic acid. Additional vitamin C was added to the tea drink. Group 1: 0 mg, group 2: 30 mg, group 3: 60 mg, group 4: 90 mg	2 samples collected, one before supplementation started (baseline), and one 2.5 weeks after supplementation started (stage of lactation was between 3 and 20 months) (breast milk collection method not clear) Dinitrophenyl hydrazine-based assay was used for analysis	Supplement group	Mean (±SD) breast milk vitamin C concentration, baseline, group 1: 3.36±0.13 mg/dl, group 2: 3.95±0.19 mg/dl, group 3: 3.88±0.22 mg/dl, group 4: 3.70±0.18 mg/dl. 2.5 weeks, group 1: 3.40±0.12 mg/dl, group 2: 4.74±0.21 mg/dl, group 3: 5.31±0.19 mg/dl, group 4: 5.51±0.24 mg/dl Maternal vitamin C supplementation during lactation increased breast milk vitamin C ($p<0.05$)	+
Daneel-Otterbech 2005	Intervention, no randomisation or placebo group (n=) Zurich, Switzerland, and Abidjan, Côte d'Ivoire	Supplements were given during lactation. Study 1 (n=28): 1000 mg ascorbid acid/day for 10 days in European and African women. Study 2 (n=26): 1, 3 or 5 serving of orange juice per week (approximately 100 mg ascorbic acid/serving) (only in Africans) (n=13)	Study 1: 10 samples collected for 10 consecutive days. Study 2: Samples of human milk were collected weekly during the food supplementation (1 sample/week from the women receiving 1 serving of orange juice per week and 2 samples/week from the women served 3 or 5 servings of orange juice per week). Sampling method was not standardised, however they were collected between 07.00 and 12.00, before intake of ascorbic acid supplements or orange juice A simple analytic technique based on titration was used for analysis	Supplement group	Study 1: Mean (±SD) breast milk vitamin C concentration, baseline, European: 60±12 mg/kg, African: 19±16 mg/kg. 10 days, European: 70±16 mg/kg, African: 60±11 mg/kg Study 2: Mean (±SD) breast milk vitamin C concentration, baseline, 1 serving: 23±5.3 mg/kg, 3 servings: 16±6.0 mg/kg, 5 servings: 21±4.3 mg/kg. 6 weeks: 1 serving: 26±7.1 mg/kg, 3 servings: 32±6.9 mg/kg, 5 servings: 46±6.2 mg/kg Maternal ascorbic acid (1000 mg/day) supplementation increased breast milk vitamin C ($p<0.001$) Maternal intake of orange juice 3 or 5 servings a week increased breast milk vitamin C ($p<0.001$)	+
Deodhar 1960	Observational (n=60) India	No supplements were given by investigators	1 sample collected between two feeds at about 3 p.m. by voluntary expression. The sampling was done on three consecutive days and the average value for three samples was taken (stage of lactation was not clear) Laboratory method used not clear	Dietary intake of 1 da, which was used to divide participants into 4 groups, according to their intake of ascorbic acid, with the median and the two quartiles determined the distribution, collected at the same time as breast milk sample	Mean breast milk ascorbic acid, group 1: 2.44 mg/100ml, group 2: 2.70 mg/100ml, group 3: 3.23 mg/100ml, group 4: 4.46 mg/100ml Mean dietary ascorbic acid intake, group 1: 0.57 mg/day, group 2: 2.39 mg/day, group 3: 4.79 mg/day, group 4: 1.34 mg/day Maternal dietary ascorbic acid was associated with breast milk ascorbic acid ($r=0.65$, $p<0.05$)	+
Hankin 1966	Intervention, no randomisation (n=71)	Supplements were given during pregnancy and lactation. One group received a daily supplement of 100 mg	3 samples collected at 5 days, 6 and 12 weeks postpartum. The sample was collected around 2 pm on the day they visited the clinic.	Supplement group	Mean (±SE) breast milk vitamin C concentration, 5 days, supplemented group (SG): 6.6±0.26 mg/100ml, not supplemented group (NG): 5.3±0.22 mg/100ml. 6 weeks, SG: 6.4±0.45	+

Table 1. Studies on the relationship between maternal nutritional status or dietary intake and breast milk vitamin concentrations

First author, year	Study design (sample size) and setting	Micronutrient supplementation status	Milk samples	Maternal nutritional assessment	Main results	Relationship
	Australia	ascorbic acid starting from around 20 week gestation and continued to lactation ended. The second group received no supplement.	The dinitrophenylhydrazine method was used for analysis	Plasma collected at the same time-points as breast milk samples	mg/100ml, NG: 4.9±0.36 mg/100ml. 12 weeks, 5.7±0.53 mg/100ml, NG: 4.5±0.53 mg/100ml Mean (±SE) plasma ascorbic acid concentration, 5 days, SG: 0.75±0.08 mg/100ml, NG: 0.38±0.11 mg/100ml. 6 weeks, SG: 0.54±0.06 mg/100ml, NG: 0.20±0.06 mg/100ml, 12 weeks, SG: 0.57±0.08 mg/100ml, NG: 0.19±0.09 mg/100ml (significantly different) Maternal ascorbic acid (100 mg/day) supplementation during pregnancy and lactation increased breast milk vitamin C at 5 days and 6 weeks postpartum, however not at 12 weeks postpartum (statistics not reported) Maternal plasma ascorbic acid was associated with breast milk vitamin C at 5 days and 6 weeks postpartum (r=0.28, p<0.05 and r=0.67, p<0.01, respectively)	
Kodentsova 2006	Observational (n=35, excluding preterm infants) Russia	Supplements were not given by investigators, however many of the participants consumed vitamins, which was captured in a 24-hour recall. The participants were divided into two groups, group 1: no vitamin supply and group 2: adequate vitamin supply	1 sample collected between 3-10 days postpartum. The women were fasting when a single sample of breast milk was collected The method of visual titration was used for analysis	24-hour recall collected a few days after birth	Mean (±SD) breast milk vitamin C concentration, group 1: 32±10 mg/l, group 2: 85±20 mg/l Maternal dietary vitamin C intake was not associated with breast milk vitamin C (p>0.05)	÷
Ortega 1998	Observational (n=57) Spain	No supplements were given by investigators	2 samples collected at 13-14 (transitional milk) and 40 (mature milk) days postpartum. Milk samples were collected between 10 and 11 am by manual expression of a 5 ml sample from each breast at the beginning and end of feed. 5 ml of foremilk and 5 ml of hindmilk were pooled Spectrophotometry was used for analysis	5-day dietary record and FFQs during third trimester of pregnancy, which was used to divide the participants into two groups, group 1: vitamin C intake < RI (n=12) and group 2: ≥ RI (n=45)	Mean (±SD) transitional breast milk vitamin C concentration, group 1: 256±220 µmol/l, group 2: 434±288 µmol/l. Mature milk, group 1: 471±385 µmol/l, group 2: 433±338 µmol/l Maternal dietary vitamin C intake during third trimester of pregnancy was associated with transitional milk vitamin C (p<0.05), not with mature milk vitamin C (p>0.05)	+ ÷
Salmenpera 1984	Observational (n=200)	No supplements were given by investigators	5 samples collected at 3-4 days and 2, 4, 6, 9 and 12 months postpartum. (breast milk collection method not clear)	Plasma collected at the same time as breast milk samples	Mean (±SD) breast milk vitamin C concentration, colostrum: 6.18±0.99 mg/100ml, 2 months: 5.91±1.18 mg/100ml, 4 months: 4.97±1.06 mg/100ml, 6 months: 4.68±1.02 mg/100ml, 9 months: 4.46±0.56 mg/100ml, 12 months: 4.14±1.13 mg/100ml	+

Table 1. Studies on the relationship between maternal nutritional status or dietary intake and breast milk vitamin concentrations

First author, year	Study design (sample size) and setting	Micronutrient supplementation status	Milk samples	Maternal nutritional assessment	Main results	Relationship
	Finland		Microfluorometric assay was used for analysis Infants were exclusively breastfed	7-day food consumption record (n=47) at 1-2 and 4-5 months postpartum	Mean (±SD) plasma vitamin C concentration, 3-4 days: 1.03±0.34 mg/100ml, 2 months: 0.86±0.39 mg/100ml, 4 months: 0.96±0.46 mg/100ml, 6 months: 0.95±0.43, 9 mg/100ml months: 1.07±0.47 mg/100ml, 12 months: 1.08±0.47 mg/100ml Maternal plasma vitamin C was associated with breast milk vitamin C (r=0.60, p=0.001), also after adjusting for milk volume Maternal dietary vitamin C intake was associated with breast milk vitamin C (r=0.39 to 0.46, p<0.01)	
Sneed 1981	Intervention, no randomisation (n=16) USA	Supplements were given during lactation. Supplemented participants (n=9) were given a multivitamin tablet (containing 90 mg/day of ascorbic acid), and the remaining participants (n=7) were given a placebo	2 samples collected at 5-7 days and 43-45 days postpartum. Milk was expressed 4 times per day at 4-hour intervals, beginning with the first infant feeding of the day. The milk was expressed immediately before taking the supplement in the morning Breast milk ascorbic acid concentration was measured photometrically	Supplement group Plasma collected at the same time as breast milk samples 4-day dietary records collected between 4-7 and 42-45 days postpartum	Mean (±SD) breast milk vitamin C concentration, 5-7 days, supplemented group (SG): 64.6±19.9 mg/l, placebo group (PG): 53.1±17.1 mg/l. 43-45 days, SG: 72.4±19.6 mg/l, PG: 61.0±10.2 mg/l Mean (±SD) plasma ascorbic acid concentration, 5-7 days, SG: 1.01±0.30 mg/100ml, PG: 0.77±0.59 mg/l. 43-45 days, SG: 1.17±0.44 mg/l, PG: 0.87±0.26 mg/l (not significantly different) Mean (±SD) dietary ascorbic acid intake, 5-7 days, SG: 202±56 mg/day, PG: 83±55 mg/day. 43-45 days, SG: 193±60 mg/day, PG: 152±115 mg/day Maternal vitamin C supplementation did not increase breast milk vitamin C (statistics not reported) Maternal plasma ascorbic acid was associated with breast milk vitamin C (r=0.60, p<0.05) at 5-7 days postpartum, not at 43-45 days (r=-0.19, p>0.05) in participants not supplemented Maternal dietary vitamin C intake was associated with breast milk vitamin C at 5-7 days postpartum (r=0.66, p<0.05), however not at 43-45 days (r=0.31, p>0.05) in participants not supplemented	÷ +
Thomas 1979	Intervention, no randomisation or placebo group (n=17) USA	Supplements were given during lactation. Supplemented participants (n=10) were given a multivitamin tablet (containing 90 mg/day of ascorbic acid), and the remaining participants (n=7)	2 samples collected at 5-7 days and 43-45 days postpartum. Milk was expressed 4 times per day at 4-hour intervals, and was expressed immediately before taking the supplement in the morning at 0 time and 4, 8, and 12 hour thereafter. Those participants not taking	Supplement group	Mean (±SE) breast milk vitamin C concentration, 5-7 days, supplemented group (SG): 58.4±34 mg/l, not supplemented (NS): 73.3±36 mg/l. 43-45 days, SG: 87.2±50 mg/l, NS: 61.1±36 mg/l Maternal vitamin C supplementation did not increase breast milk vitamin C (statistics not reported)	÷

Table 1. Studies on the relationship between maternal nutritional status or dietary intake and breast milk vitamin concentrations

First author, year	Study design (sample size) and setting	Micronutrient supplementation status	Milk samples	Maternal nutritional assessment	Main results	Relationship
		were not given any supplements	supplements expressed milk at corresponding times Breast milk ascorbic acid concentration was measured photometrically			
Thomas 1980	Intervention, no randomisation or placebo group (n=12) USA	Supplements were given during lactation. Supplemented participants were given a multivitamin tablet (containing 90 mg/day of ascorbic acid), and the remaining participants were not given any supplement	1 sample collected 6 months postpartum. Milk was expressed 4 times per day at 4-hour intervals for three consecutive days, and was expressed immediately before taking the supplement in the morning at 0 time and 4, 8, and 12 hour thereafter. Those participants not taking supplements expressed milk at corresponding times Breast milk ascorbic acid concentration was measured photometrically	Supplement group	Mean (±SD) breast milk vitamin C concentration, supplemented group 38.4±12.3 mg/l, not supplemented: 35.2±12.0 mg/l Maternal vitamin C supplementation did not increase breast milk vitamin C (statistics not reported)	÷
Tawfeek 2002	Observational (n=200) Iraq	No supplements were given by investigators	1 sample collected at different stages of lactation (between 1-26 weeks postpartum). Midstream breast milk samples (2–5 ml) were obtained from each participant. All breast milk samples were collected between 10:00 and 12:00 hours. The samples were manually expressed by the participant Ascorbic acid was measured spectrophotometrically	24-hour recall collected at the same time as breast milk sample	Mean (±SD) breast milk vitamin C concentration, 2 weeks (n=30): 4.2±2.08 mg/100ml, 3-6 weeks (n=55): 3.8±1.9 mg/100ml, 7-14 weeks (n=41): 3.2±1.5 mg/100ml, >24 weeks (n=35): 2.5±0.8 mg/100ml Maternal dietary vitamin C intake was associated with breast milk vitamin C (r=0.61, p<0.01)	+
Vitamin D						
Ala-Houhala 1988	Intervention, no randomisation or placebo group (n=45) Finland	Supplements were given during lactation. Participants received either 2000 or 1000 IU (50 or 25 µg) of cholecalciferol per day as a single daily dose in 1000-lU (25-ag) tablets or they received no supplementation	2 samples collected, at 8 and 15 weeks postpartum and at different seasons (collections were made in February and May or in September and December) Experiment 1: The participants collected foremilk and hindmilk samples during the first feeding in the morning except in February, when only foremilk samples were collected during daily examination visits to the	Supplement group Serum collected at same time as breast milk samples	Median (range) breast milk vitamin D concentration, week 8 (Feb) 2000 IU: 403 (75, 1105) pmol/l, 1000 IU: 286 (148, 949) pmol/l, no supplement (NS): 333 (174, 762) pmol/l. Week 15 (April) 2000 IU: 390 (148, 1043) pmol/l, 1000 IU: 367 (112, 751) pmol/l, NS: 359 (140, 988) pmol/l Median (range) breast milk 25-(OH)D, week 8 (Feb) 2000 IU: 993 (223,2295) pmol/l, 1000 IU: 583 (238, 2163) pmol/l, NS: 393 (140, 1193) pmol/l. Week 15 (April) 2000 IU: 843 (448, 5700)	+ ÷

Table 1. Studies on the relationship between maternal nutritional status or dietary intake and breast milk vitamin concentrations

First author, year	Study design (sample size) and setting	Micronutrient supplementation status	Milk samples	Maternal nutritional assessment	Main results	Relationship
			clinic. They were stored at -70 C until analysed. Experiment 2: Only hindmilk was collected. Laboratory method used not clear		pmol/l, 1000 IU: 773 (403, 2190) pmol/l, NS: 398 (110, 2353) pmol/l Maternal serum concentrations not reported Maternal oral vitamin D supplementation (either 2000 or 1000 IU) did not increase breast milk vitamin D concentration (statistics not reported) Maternal oral vitamin D supplementation (either 2000 or 1000 IU) increased breast milk 25-(OH)D concentrations in both February and in April (statistics not reported) Maternal serum 25-(OH)D was not associated with breast milk 25-(OH)D or vitamin D (statistics not reported)	
Hoogenboezem 1989	Observational (n=39) The Netherlands	No supplements were given by investigators	7 samples collected at 1, 2, 3, 4, 8, 13 and 21 weeks postpartum (breast milk collection method not clear) HPLC was used for analysis Infants were exclusively breastfed	Plasma collected at birth and the same time as breast milk samples	Mean breast milk 25-(OH)D concentration: 325 pmol/l (data combined) Mean plasma 25-(OH)D concentration at birth: 84±6 nmol/l Maternal plasma 25-(OH)D was associated with breast milk 25-(OH)D 1 week after delivery (r=0.62, p<0.05)	+
Hollis 2004	Randomised controlled trial, no placebo group (n=18) USA	Supplements were given daily during lactation. Group 1: 1600 IU vitamin D_2 and 400 IU vitamin D_3 or group 2: 3600 IU vitamin D_2 and 400 IU vitamin D_3 for a three month period.	4 samples collected at 1 (baseline), 2, 3 and 4 months postpartum (breast milk collection method not clear) HPLC and radioimmunoassay techniques was used for analysis Infants were exclusively breastfed	Supplement group	Mean (±SEM) breast milk vitamin D_2 concentration, group 1, baseline: <0.5, 3 months: 2.2±0.7 ng/ml (p<0.01), group 2, baseline: 0.6±0.1 ng/ml, 3 months:6.6±2.4 ng/ml Mean (±SEM) breast milk vitamin D_3, group 1, baseline: 1.0±0.4 ng/ml, 3 months: 1.2±1.2 ng/ml, group 2, baseline: 0.9±0.4 ng/ml, 3 months: 2.8±1.0 ng/ml Mean (±SEM) breast milk total 25-(OH)D, group 1, baseline: 27.6±3.3 ng/ml, 3 months: 36.1±2.3 ng/ml, group 2, baseline: 32.9±2.4 ng/ml, 3 months: 44.5±3.9 ng/ml Maternal vitamin D supplementation (1600 IU and 3600 IU) increased breast milk vitamin D_2 (p<0.01 and p<0.04, respectively) Maternal vitamin D supplementation (1600 IU and 3600 IU) did not increase breast milk vitamin D_3 (p<0.7 and p<0.06, respectively)	+ ÷

Table 1. Studies on the relationship between maternal nutritional status or dietary intake and breast milk vitamin concentrations

First author, year	Study design (sample size) and setting	Micronutrient supplementation status	Milk samples	Maternal nutritional assessment	Main results	Relationship
					Maternal vitamin D supplementation (1600IU and 3600 IU) increased breast milk total 25-(OH)D ($p<0.05$ and $p<0.04$, respectively)	
Cancela 1986	Observational (n=11) France	No supplements were given by investigators	3 samples collected at 3-5 (visit 1), 15-18 (visit 2) and 30-45 (visit 3) days postpartum, by manual expression (5-10 ml). The sample was collected at the end of the first morning fed (first visit) or at different times during the mornings (second and third visit) HPLC was used for analysis Infants were exclusively breastfed	Serum collected at 3-5 and 30-45 days postpartum	Mean (±SEM) breast milk vitamin D concentration, visit 1: 0.89±0.32 nmol/l, visit 2: 1.13±0.32 nmol/l, visit 3: 1.12±0.32 nmol/l Mean (±SEM) breast milk 25-(OH)D visit 1: 0.50±0.11 nmol/l, visit 2: 0.65±0.11 nmol/l, visit 3: 0.56±0.11 nmol/l Mean (±SEM) serum 25-(OH)D visit1: 22.0±2.61 µmol/l, visit3: 23.97±2.49 µmol/l Maternal serum 25-(OH)D was not associated with breast milk 25-(OH)D (statistics not reported)	÷
Mohamed 2014	Observational study (n=102) Malaysia	No supplements were given by investigators, but <30% of the participants were supplemented with multivitamins during pregnancy	4 samples collected, 1–14 days (delivery) (n=101) and 2 (n=90), 6 (n=69) and 12 (n=49) months postpartum. Breast milk samples were collected using an electric breast pump HPLC was used for analysis	Serum collected during second and third trimester of pregnancy	Mean breast milk 25-(OH)D concentration at delivery: 1.26 nmol/l, 2 months: 1.18 nmol/l, 6 months: 1.01 nmol/l, 12 months: 1.16 nmol/l Mean (±SD) serum 25-(OH)D concentration second trimester: 28.5±15.3 nmol/l, third trimester: 59.0±20.4 nmol/l Maternal serum 25-(OH)D in second trimester was associated with breast milk 25-(OH)D at delivery (1-14 days postpartum) ($\beta=0.002$, $p=0.03$), however not at 2, 3, 6 or 12 months postpartum (adjusted for maternal age and prenatal multivitamin supplement)	+ ÷
Oberhelman 2013	Randomised controlled trial, no placebo group (n=40) USA	Supplements were given during lactation. Participants were administrated cholecalciferol either 150,000 IU at once (n=20) or 5000 IU/day for 28 days (n=20)	6 samples collected on day 0, 1, 3, 7, 14, and 28. (Stage of lactation not clear, mean age of infants 13 weeks). Participants collected the sample by breast pump or self-expression. Isotope dilution liquid chromatography tandem mass spectrometry was used for analysis Infants were exclusively breastfed	Supplement groups Serum collected at the same time as breast milk samples	Mean breast milk cholecalciferol concentration, daily dose, baseline: <7 ng/ml, day 1: <0.7 ng/ml, day 3: 8.0±3.7 ng/ml, day 7: 7.2±4.8 ng/ml, day 14: 8.6±5.4 ng/ml, day 28: 7.7±3.7 ng/ml, single dose, baseline: <0.7, day 1: 39.7±16.2 ng/ml, day 3: 24.6±8.9 ng/ml, day 7: 11.2±4.7 ng/ml, day 14: <0.7 ng/ml, day 28: <0.7 ng/ml Breast milk 25(OH)D was undetectable in all samples. Mean (±SD) serum cholecalciferol daily dose, baseline: 2.6±1,4 ng/ml, day 1: 10.6±3.8 ng/ml (significantly different), single dose, baseline: 4.7±6.0 ng/ml, day 1:160.0±38.8 ng/ml (significantly different)	+

Table 1. Studies on the relationship between maternal nutritional status or dietary intake and breast milk vitamin concentrations

First author, year	Study design (sample size) and setting	Micronutrient supplementation status	Milk samples	Maternal nutritional assessment	Main results	Relationship
					Mean (±SD) serum 25-(OH)D, daily dose, baseline:29.0±8.3 ng/ml, day 1: 30.7±9.7 ng/ml (not significantly different), single dose, baseline: 29.3±7.5 ng/ml, day 1: 43.3±9.7 ng/ml (significantly different) Maternal daily vitamin D supplementation (5000 IU) increased breast milk cholecalciferol at day 3, 14 and 28 ($p<0.05$) Maternal single dose vitamin D supplementation (150,000 UI) increased breast milk cholecalciferol at day 1, 3 and 7 ($p<0.05$) Maternal serum cholecalciferol was associated with breast milk cholecalciferol at baseline ($r=0.38$, $p=0.02$)	
Specker 1985	Observational (n=25) USA	No supplements were given by investigators	1 sample collected (stage of lactation not clear). The sample was collected on the first feeding of the day from one breast, 3 ml were collected at the beginning, middle and end of the feeding Competitive ligand-binding assay was used for analysis Infants were exclusively breastfed	3 day food diary collected at the same time as breast milk sample	Mean breast milk total vitamin D concentration: 315 pg/ml Mean breast milk total 25-(OH)D: 188 pg/ml Mean vitamin D intake: 457 IU/day Maternal dietary vitamin D intake was associated with breast milk total vitamin D ($r=0.57$, $p=0.005$) Maternal dietary vitamin D intake was not associated with breast milk 25-(OH)D ($r=0.25$, $p=0.2$)	+ ÷
Streym 2016	Observational (n=107) Denmark	No supplements were given by investigators	3 samples collected at 2 weeks, 4 and 9 months postpartum. Milk samples were collected at the day of each visit (or the night before), foremilk (milk before feeding the child) and hindmilk (milk after feeding the child) were collected manually liquid chromatography–tandem mass spectrometry was used for analysis	Plasma collected at the same time as breast milk samples	Median breast milk vitamin D concentration, 2 weeks, foremilk:0.5 nmol/l, hindmilk: 0.7 nmol/l, 4 months foremilk: 0.7 nmol/l, hindmilk:1.4 nmol/l, 9 months foremilk: 0.9 nmol/l, hindmilk: 1.0 nmol/l Median breast milk 25-(OH)D (n=106) 2 weeks foremilk: 0.9 nmol/l, hindmilk: 1.3 nmol/l, 4 months foremilk: 0.8 nmol/l, hindmilk: 1.4 nmol/l, 9 months foremilk: 0.9 nmol/l, hindmilk: 1.2 nmol/l Mean (±SD) plasma 25-(OH)D 2 weeks: 73.2±30.6 Streym, 4 months: 64.9±19.8 Streym, 9 months: 50.7±19.0 Streym Maternal plasma 25-(OH)D was associated with breast milk 25-(OH)D at all three time-points ($r=0.51$ to 0.74, $p<0.01$)	+

Table 1. Studies on the relationship between maternal nutritional status or dietary intake and breast milk vitamin concentrations

First author, year	Study design (sample size) and setting	Micronutrient supplementation status	Milk samples	Maternal nutritional assessment	Main results	Relationship
Takeuchi 1989	Intervention, no randomisation or placebo group (n=50) Japan	Supplements were given during lactation from 1 weeks to 4 weeks postpartum. Participants received a daily oral dose of vitamin D_2 (1200 IU/day), and another group of women served as control group (not given any D_2)	2 samples collected at 1 (baseline) and 4 weeks postpartum. The milk was collected manually and consisted of an entire expression in the morning before feeding HPLC was used for analysis	Supplement group Plasma collected at the same time as breast milk samples	Mean (±SE) breast milk vitamin D_2 concentration, baseline: 11±5 ng/l, not supplemented group (NS): no detection, supplemented group (SG):125±14 ng/l Mean (±SE) breast milk vitamin D_3, baseline: 117±9 ng/l, NS: 99±26 ng/l, SG: 122±13 ng/l Mean (±SE) breast milk 25-(OH)D_3, baseline: 309±28 ng/l, NS: 348±174 ng/l, SG: 263±33 ng/l Mean (±SE) plasma vitamin D_2, baseline: 0.3±0.1 ng/ml NS: no detection, SG:1,2±0.2 ng/ml Mean (±SE) plasma vitamin D_3, baseline: 1.1±0.2 ng/ml, NS: 0.3±0.2 ng/ml, SG: 1.0±0.2 ng/ml Mean (±SE) plasma 25-(OH)D_3, baseline: 16.9±0.8 ng/ml, NS: 17.3±2.0 ng/ml, SG: 19.0±1.2 ng/ml Maternal vitamin D_2 supplementation during lactation increased breast milk vitamin D (statistics not reported) Maternal vitamin D_2 supplementation during lactation did not increase breast milk 25-(OH)D (statistics not reported) Maternal plasma vitamin D_2 and D_3 was associated with breast milk vitamin D_2 and D_3 (statistics not reported) Maternal plasma 25-(OH)D_3 was not associated with breast milk 25-(OH)D_3	+ ÷
Wagner 2006	Randomised, double-blind, placebo controlled pilot trial (n=19) USA	Supplements were given during pregnancy and lactation. Group 1: 400 IU vitamin D_3/day (0 IU vitamin D_3, placebo and 1 prenatal vitamin containing 400 IU vitamin D_3), or Group 2: 6400 IU vitamin D_3/day (6000 IU vitamin D_3 and 1 prenatal vitamin containing 400 IU vitamin D_3)	6 samples collected at 1 (baseline), 2, 3, 4, 5, 6 and 7 months postpartum (breast milk collection method not clear) HPLC was used for analysis Infants were exclusively or fully breastfed	Supplement group	Mean breast milk vitamin D concentration, group 1, baseline: 59.6 IU/l, group 2: 82.4 IU/l, 2 months group 1: 71.2 IU/l, group 2: 387 IU/l, 3 months, group 1: 78.6 IU/l, group 2: 370.5 IU/l, 4 months, group 1: 45.7 IU/l, group 2: 374.4 IU/l, 5 months, group 1: 68.3 IU/l, group 2: 555.2 IU/l, 6 months group 1: 69.9 IU/l, group 2: 624.5 IU/l, 7 months, group 1: 76.3 IU/l, group 2: 873.5 IU/l Maternal supplementation of 6400 IU vitamin D_3/day during pregnancy and lactation increased breast milk vitamin D (statistics not reported)	+

Table 1. Studies on the relationship between maternal nutritional status or dietary intake and breast milk vitamin concentrations

First author, year	Study design (sample size) and setting	Micronutrient supplementation status	Milk samples	Maternal nutritional assessment	Main results	Relationship
Vitamin E						
Antonakou 2011	Observational (n=126) Greece	No supplements were given by investigators	3 samples collected at 1(n=64), 3 (n=39) and 6 (n=23) months postpartum. A total of 30 ml of foremilk was collected from one breast by an electric breast pump. Home visits were made during morning hours, and participants were instructed not to have breastfed their infants for at least two hours prior to using the breast pump HPLC was used for analysis Infants were exclusively breastfed	3 day dietary records collected at the same time as breast milk samples	Mean (±SD) breast milk vitamin E concentration (sum of α-(β+γ)- and δ-tocopherol) at 1 months: 8.9±3.6 µmol/l, 3 months: 8.7±4.6 µmol/l, 6 months: 9.5±5.6 µmol/l Mean (±SD) dietary vitamin E intake, 1 months: 7.2 ± 3.7 mg/day, 3 months: 6.8 ± 3.5 mg/day, 6 months: 10.9 ± 5.2 mg/day Maternal dietary vitamin E intake was not associated with breast milk vitamin E at 1, 3 or 6 months of lactation (r=0.002, p<0.7)	÷
Clemente 2015	Randomised double-blinded controlled trial (n=109) Brazil	Supplementation was given during lactation. The study consisted of group 1: control group (n=36), group 2: receiving an acetate capsule with natural vitamin E (RRR-α-TOH) (n=40), and group 3: receiving an acetate capsule with synthetic vitamin E (all-rac-α-TOH) (n=33). The capsule contained either 400 IU of RRR-α-TOH or 400 IU of all-rac-α-TOH	2 samples collected at baseline and 24-hours after supplementation (colostrum). Milk samples were collected from participants after an overnight fast (12 hours postpartum). Colostrum was obtained by manual expression at the end of breastfeeding. The foremilk was discarded, and 2 ml colostrum was collected HPLC was used for analysis	Supplement group Serum collected at the same time as breast milk samples	Mean (±SD) breast milk α-tocopherol concentration, baseline, group 1: 1665.2±160.2 µg/dl, group 2: 1387.1±176.5 µg/dl, group 3: 1802±208.1 µg/dl (not significantly different). After supplementation, group 1: 1642.5±181.9 µg/dl, group 2: 2187.2±284.6 µg/dl, group 3: 2508±303.7 µg/dl Mean (±SD) serum α-tocopherol concentration, baseline, group 1: 1016 ± 52 µg/dl, group 2: 1236 ± 51 µg/dl, group 3: 1083 ± 61 µg/dl (not significantly different) (after supplementation concentrations not reported) Maternal supplementation of 400 IU of RRR-α-TOH or 400 IU of all-rac-α-TOH increased α-tocopherol in colostrum, with RRR-α-TOH being most efficient (p<0.0001) Maternal serum α- tocopherol was not associated with colostrum α-tocopherol (r=0.07, p=0.4)	+ ÷
de Lira 2013	Observational (n=102) Brazil	No vitamin E supplements were given by investigators (vitamin A supplements were given)	1 sample collected during the first three days postpartum. The breast milk sample was collected after night-time fasting on the first day post-partum. Colostrum was collected for three consecutive days. It was collected by manual expression of a single breast that had not been previously suckled and the first ejection was discarded to avoid fluctuations in fat content	Serum collected on the first and second day postpartum	Mean (±SD) breast milk α-tocopherol concentration: 26.1±8.0 µmol/l Maternal serum α-tocopherol was not associated with colostrum α-tocopherol (r=-0.12, p=0.22)	÷

Table 1. Studies on the relationship between maternal nutritional status or dietary intake and breast milk vitamin concentrations

First author, year	Study design (sample size) and setting	Micronutrient supplementation status	Milk samples	Maternal nutritional assessment	Main results	Relationship
			HPLC was used for analysis			
Jiang 2016	Observational (n=102) China	No supplements were given by investigators	3 samples collected at 1 (colostrum, n=34), 14 (transitional milk, n=34) and 42 (mature milk, n=34) days postpartum. 35 ml was collected before breastfeeding, between 10 a.m. and 11 a.m. HPLC was used for analysis	24-hour recall collected at the same time as breast milk samples	Mean (±SD) breast milk α-tocopherol concentration, colostrum: 612.6±412.3 µg/100g, transitional: 248.5±218.5 µg/100g, mature: 177.1±109.0 µg/100g Maternal dietary vitamin E intake was not associated with breast milk vitamin E (p>0.05)	÷
Martysiak-Zurowska, 2013	Observational (n=48) Poland	No supplements were given by investigators	4 samples collected at 2 (colostrum, n=17), 14 (n=30), 30 (n=27) and 90 (n= 19) days postpartum. The breast was fully expressed with help from an electric pump 2 hours after the first morning feed (between 5 and 7 am.) HPLC was used for analysis	3 day food diary collected at the same time as breast milk samples	Mean (±SD) breast milk vitamin E concentration, day 2: 10.13±1.5 mg/l, day 14: 4.59±0.93 mg/l, day 30: 3.00±0.85 mg/l, day 90: 2.13±0.67 mg/l Mean (±SD) breast milk vitamin E/g total fat concentration, day 2: 357.4±52.8 µg/g total fat, day 14: 138.0±28.0 µg/g total fat, day 30: 83.8±23.7 µg/g total fat, day 90: 56.6±17.9 µg/g total fat Mean daily intake of vitamin E: 14.9±8.3 mg Maternal dietary vitamin E intake was not associated with breast milk vitamin E (r=0.034, p=0.2)	÷
Ortega 1999	Observational (n=57) Spain	No supplements were given by investigators, however 3.5% of participants took vitamin E during pregnancy	2 samples collected at 13-14 days (transitional) and 40 days (mature) postpartum. Milk samples were collected between 10 and 11 am by manual expression of a 5 ml sample from each breast at the beginning and end of feed. 5 ml of foremilk and 5 ml of hindmilk were pooled HPLC was used for analysis	5 day dietary record and FFQs during third trimester of pregnancy, which was used to divide the participants into two groups, group 1: vitamin E intake < 75% of RI (n=39) and group 2: ≥ 75% RI (n=18)	Mean (±SD) transitional breast milk vitamin E concentration, group 1: 3.80±1.32 µmol/l, group 2: 5.01±1.81 µmol/l. Mature milk, group 1: 2.20±0.72 µmol/l, group 2: 2.27±0.77 µmol/l Maternal dietary vitamin E intake during third trimester was associated with transitional milk vitamin E (p<0.05), not mature milk (p>0.05)	+ ÷
Szlagatys-Sidorkiewicz 2012	Observational (n=49) Poland	No supplements were given by investigators, however vitamin supplementation was reported by 63.18% and 52% of women whose milk samples were collected on the 3rd and 30th–	2 samples collected at 3 days (colostrum) and between the 30th and 32nd days (mature milk) postpartum. The breast was fully expressed with help from an electric pump 2 hours after the first morning feed (between 5 and 7 a.m.)	3 day food diary collected at the same time as breast milk samples	Median (IQR) breast milk vitamin E concentration, colostrum: 8.86 (5.22, 12.0) mg/l, mature milk: 1.10 (0.74, 3.94) mg/l Maternal dietary vitamin E intake was not associated with breast milk vitamin E (r=0.034, p=0.2)	÷

Table 1. Studies on the relationship between maternal nutritional status or dietary intake and breast milk vitamin concentrations

First author, year	Study design (sample size) and setting	Micronutrient supplementation status	Milk samples	Maternal nutritional assessment	Main results	Relationship
		33nd day postpartum, respectively	HPLC was used for analysis			
Vitamin K						
Canfield 1991	Observational (n=60) USA	No supplements were given by investigators	4 samples collected at 30-81 hours (colostrum), 1, 3 and 6 months postpartum. Colostrum was collected between 0800-1200 am, 2.5-3.5 hours after the last nursing. Mature milk samples were collected using an electric breast pump, with the infant nursing on the alternate breast. Mothers were instructed to pump for 15 minutes or until milk flow was markedly reduced HPLC was used for analysis At 1 and 3 months sample collection infants were exclusively breastfeed	FFQ (not clear when data was collected)	Mean(±SD) breast milk vitamin K concentration, colostrum (n=15): 7.52±5.90 nmol/l, 1 month (n=15): 6.98 ± 6.36 nmol/l, 3 months (n=15): 5.14 ± 4.52 nmol/l, 6 months (n=15): 5.76 ± 4.48 nmol/l Maternal dietary vitamin K intake was not associated with breast milk vitamin K (statistics not reported)	÷
Greer 1991	Observational (n=23) USA	No supplements were given by investigators	3 samples collected at 6, 12 and 26 weeks postpartum. 10 ml were collected from a complete expression of the contents of one breast by means of an electric breast pump Fluorescence spectrophotometry was used for analysis	3 day dietary recalls collected at the same time as breast milk samples	Mean (±SD) breast milk vitamin K concentration, 6 weeks: 0.86±0.52 ng/ml, 12 weeks: 1.14±0.72 ng/ml, 26 weeks: 0.87±0.50 ng/ml Mean (±SD) dietary vitamin K intake, 6 weeks: 302±361 µg/day, 12 weeks: 296±169 µg/day, 26 weeks: 436±667 µg/day Maternal dietary vitamin K intake was not associated with breast milk vitamin K (statistics not reported)	÷
Greer 1997	Randomised, double-blind, placebo controlled trial (n=22) USA	Supplements were given during lactation, supplemented group: 5 mg/day vitamin K (n=11) and a placebo group (n=11)	4 samples collected with 3 days of delivery (baseline) and 2, 6 and 12 weeks postpartum. Samples were collected 18-24 hours after maternal ingestion of vitamin K or the placebo. A complete expression of a single breast was collected with an electric pump Fluorescence spectrophotometry was used for analysis	Supplement group	Mean(±SD) breast milk vitamin K concentration, baseline supplemented group (SG): 0.69±0.39 ng/ml, placebo group (PG): 1.10±0.75 ng/ml (not significantly different), 2 weeks, SG: 76.53±26.98 ng/ml, PG: 1.17±0.70 ng/ml, 6 weeks, SG: 75.27±46.23 ng/ml, PG: 1.14±0.46 ng/ml, 12 weeks, SG: 82.10±40.10 ng/ml, PG: 1.17±0.40 ng/ml Mean (±SD) plasma vitamin K concentration, baseline, SG: 0.28±0.09 ng/ml, PG: 0.28±0.14 ng/ml (not significantly different), 2 weeks SG: 11.04±4.66 ng/ml, PG: 0.30±0.19 ng/ml, 6 weeks, SG: 15.14±17.81 ng/ml, PG: 0.32±0.11 ng/ml, 12 weeks ,	+

Table 1. Studies on the relationship between maternal nutritional status or dietary intake and breast milk vitamin concentrations

First author, year	Study design (sample size) and setting	Micronutrient supplementation status	Milk samples	Maternal nutritional assessment	Main results	Relationship
			Infants were exclusively breastfed		SG: 10.71±10.76 ng/ml, PG: 0.41±0.23 ng/ml (significantly different at all-time-points) Maternal daily vitamin K supplementation of 5 mg/day increased breast milk vitamin K at all time-points ($p<0.0001$)	
Thijssen 2002	Randomised placebo controlled trial (n=32) The Netherlands	Supplements were given on day 4 postpartum and continued until day 16 group 1: 0.0, group 2: 0.8, group 3: 2.0 and group 4: 4.0 mg vitamin K/day	4 samples collected at 4 (baseline), 8, 16 and 19 days postpartum, before 11.00 am from the breast that had not been used for the previous feed. The first 10 ml of the collected milk were discarded and the second 5–10ml were taken as a sample Fluorescence detection following HPLC separation was used for analysis	Supplement group Plasma collected at baseline and 16 days postpartum	Mean (±SD) breast milk vitamin K concentration, baseline, group 1: 5.51±2.47 nmol/l, group 2: 6.84±2.29 nmol/l, group 3: 5.15±2.48 nmol/l, group 4: 6.21±1.86 nmol/l (not significantly different). Day 8, group 1: 4.37±3.03 nmol/l, group 2: 23.33±14.72 nmol/l, group 3: 41.34±36.18 nmol/l, group 4: 88.72±43.40 nmol/l. Day 16, group 1: 4.87±1.43 nmol/l, group 2: 24.51±10.14 nmol/l, group 3: 60.64±31.60 nmol/l, group 4: 139.64±45.83 nmol/l. Day 19, group 1: 4.80±2.96 nmol/l, group 2: 12.35±12.51 nmol/l, group 3: 12.07±4.64 nmol/l, group 4: 44.88±39.83 nmol/l Mean (±SD) plasma vitamin K concentration, baseline, group 1: 2.56±1.72 mmol/l, group 2: 2.04±2.03 mmol/l, group 3: 3.10±2.21 mmol/l, group 4: 2.63±1.76 mmol/l (not significantly different). Day 16, group 1: 3.15±1.98 mmol/l, group 2: 9.37±6.75 mmol/l, group 3: 15.28±8.08 mmol/l, group 4: 31.64±8.16 mmol/l (significantly different) Maternal vitamin K supplementation (0.8, 2.0 and 4.0 mg/day) significantly increased breast milk vitamin K ($p<0.001$) Maternal plasma vitamin K was not associated with breast milk vitamin K (statistics not reported)	+ ÷

Table 2. Studies on the relationship between maternal nutritional status or dietary intake and breast milk mineral concentrations

First author, year	Study design (sample size) and setting	Micronutrient supplementation status	Milk samples	Maternal nutritional assessment	Main results	Relationship
Calcium						
Kirksey 1979	Observational (n=52) USA	No supplements were given by investigators, however 64% took calcium containing supplements	2 samples collected at 3 and 14 days postpartum (n=21). And for a sub-group 3 additional samples at 1-3 months (n=6), 5-7 months (n=8) and 1 year (n=5). Five to 10 ml of milk were obtained after milk let down at the first morning feeding. Samples were collected by manual expression into plastic vials Atomic absorption spectrophotometry was used for analysis	3 day dietary record preceding the day of milk sample collection, capturing supplement use	Mean (±SD) breast milk calcium concentration, day 3: 236±50 ppm, day 14: 221±45 ppm, 1-3 months: 190±30 ppm, 5-7 months: 335±35 ppm, 1 year: 176±28 ppm (milk concentrations were not reported according to supplement group) Mean (±SD) calcium intake day 14 (including supplement use): 320±60 mg, 1-3 months: 318±59 mg, 5-7 months: 329±58 mg, 1 year: 250±71mg Maternal calcium supplementation use, did not increase breast milk calcium (statistics not reported)	÷
Kalkwarf 1997	Randomised, placebo controlled trial (n=87) USA	Supplements were given during lactation. Calcium or a placebo supplement. The calcium supplement provided 1 g of calcium/day	3 samples collected at 0.5 (n=17), 3 (n=20) and 6 (n=32) months postpartum. (breast milk sampling method not clear) Atomic-absorption spectroscopy was used for analysis	Supplement group	Mean (±SD) breast milk calcium concentration: 0.5 months, supplemented group (SG): 30±3 mg/dl, placebo group (PG): 28±4 mg/dl, 3 months, SG: 27±5 mg/dl, PG: 28±4 mg/dl, 6 months, SG: 24±4 mg/dl, PG: 24±4 mg/dl Maternal calcium supplementation did not increase breast milk calcium at 0.5, 3 or 6 months postpartum (statistics not reported)	÷
Jarjou 2006	Randomised, double-blind, placebo controlled supplementation study (n=125) The Gambia	Supplements were given during pregnancy (started at 20 weeks gestation, ended at delivery). The calcium supplement provided 1500 mg of calcium/day. Placebo group were given 0 mg of calcium/day	3 samples collected at 2, 13 and 52 weeks postpartum. Samples were collected by manual expression of 1–2 ml samples directly into low-calcium tubes A validated semi-automated micro-method was used for analysis At 3 months of age 30% of infants were exclusively breastfed.	Supplement group	Mean (±SD) breast milk calcium concentration, week 2, supplemented group (SG): 250±54 mg/l, placebo group (PG): 248±47 mg/l. Week 13, SG: 231±34 mg/l, PG: 234±24 mg/l. Week 52, SG: 188±34 mg/l, PG:183±24 mg/l Geometric mean urinary calcium concentration, baseline, 20 weeks gestation: 67 mg/day, 36 weeks gestation, SG: 89.1 mg/day, PG: 49.6 mg/day (significantly different) Maternal calcium supplementation during pregnancy did not increase breast milk calcium at 2, 13 or 52 weeks postpartum (statistics not reported)	÷
Ortega 1998	Observational (n=57)	No supplements were given by investigators	3 samples collected 13, 14 (transitional milk) and 40 days postpartum (mature milk). Milk samples were collected between 10 and 11 a.m. by manual	5 days dietary record and FFQs during third trimester of pregnancy, which was used to divide	Mean (±SD) breast milk calcium concentration, transitional milk, group 1: 6.44±2.20 mmol/l, group 2: 6.37±1.96 mmol/l, mature milk, group 1: 5.95±1.56 mmol/l, group 2: 6.82±1.31 mmol/l	÷ +

Table 2. Studies on the relationship between maternal nutritional status or dietary intake and breast milk mineral concentrations

First author, year	Study design (sample size) and setting	Micronutrient supplementation status	Milk samples	Maternal nutritional assessment	Main results	Relationship
	Spain		expression of a 5 ml sample from each breast at the beginning and end of feeds	the participants into two groups, group 1: Calcium intake <1100 mg/day (n=40), group 2: ≥1100 mg/day (n=17)	Maternal dietary calcium intake in third trimester of pregnancy was not associated with breast milk calcium in transitional milk ($p>0.05$), however it was associated with calcium concentration in mature milk ($p<0.05$).	
Prentice 1995	Randomised placebo controlled supplementation study (n=60) The Gambia	Supplements were given through lactation, starting at 10 days postpartum for 12 months. Calcium (1000 mg) or placebo, taken 5 days a week	9 samples collected at 6, 13, 19, 26, 39, 52, 65, 78 weeks postpartum. 1-2 ml was expressed manually from each breast separately into unused, disposable polystyrene tubes. No specific sampling protocol with respect to time of day or stage of feed was adopted A semiautomated spectrophotometnic method was used for analysis	Supplement group	Mean (±SD) breast milk calcium concentration, 6 weeks (groups combined): 5.60±0.78 mmol/l, 13 weeks: 5.23±0.68 mmol/l, 19 weeks: 5.00±0.68 mmol/l, 26 weeks: 4.68±0.50 mmol/l, 39 weeks: 4.43±0.63 mmol/l, 52 weeks: 3.98±0.60 mmol/l, 65 weeks: 3.93±0.65 mmol/l, 78 weeks: 3.73±0.55 mmol/l (not significantly different) Mean (±SD) urinary calcium concentration, 1.5 weeks (baseline): SG 1.31±1.09 mmol/day, PG: 1.13±0.92 mmol/day. 13 weeks SG: 1.82±1.41 mmol/day, PG: 0.62±0.58 mmol/day (significantly different), 52 weeks SG: 2.07±1.39 mmol/day, PG:0.90±0.72 mmol/day (significantly different), 78 weeks SG: 1.55±1.43 mmol/day, PG: 1.16±1.20 mmol/day Maternal calcium supplementation did not increase breast milk calcium at any time-point (statistics not reported)	÷
Vaughan 1979	Observational (n=38) USA	No supplements were given by investigators	Monthly milk samples between 1-31 months postpartum. Subjects remained in the study on an average of 4 consecutive months. 150-200 ml of milk was hand expressed into acid-washed glass. Subjects were instructed to collect the milk over a period of 3 to 5 days, at morning, afternoon, and evening feedings and at random intervals within the feeding Atomic absorption spectrophotometer was used for analysis	Serum collected during the same 3- to 5-day period of milk collection for the particular month (n=24) 3 day dietary records. Intakes were recorded during the same 3- to 5-day period of milk collection (n=11)	Mean (±SEM) breast milk calcium concentration, 1-3 months: 257±29 μg/ml (n=28), 4-6 months: 236±25 μg/ml (n=39) Mean serum calcium concentration 4-6 months: 9.9 mg/100ml Mean dietary calcium intake 4-6 months: 1402 mg/day Maternal serum calcium was not associated with breast milk calcium (statistics not reported) Maternal dietary calcium intake was not associated with breast milk calcium (statistics not reported)	÷
Zhao 2014	Observational (n=90)	No supplements were given by investigators	1 sample collected between 5−240 days postpartum. All were instructed to empty one breast during 6 to 7 a.m. At 9 to 11 a.m., the full milk of one breast (which was emptied before) was	FFQs and 24-hour dietary recall collected during lactation	Mean (±SD) breast milk calcium concentration: 5-11 days: 303.3±52.4 mg/kg, 12-30 days: 293.6±46.7 mg/kg, 31-60 days: 309.6±43.1 mg/kg, 61-120 days: 287.4±40.0 mg/kg and 121-240 days: 267.4±43.8 mg/kg	÷

Table 2. Studies on the relationship between maternal nutritional status or dietary intake and breast milk mineral concentrations

First author, year	Study design (sample size) and setting	Micronutrient supplementation status	Milk samples	Maternal nutritional assessment	Main results	Relationship
	China (Beijing, Guangzhou and Suzhou)		collected using an electric breast pump. Fore and hind milk were gently mixed Inductively coupled plasma mass spectrometry was used for analysis		Maternal dietary calcium intake was not associated with breast milk calcium (r=0.016, p>0.05)	
Copper						
Chierici 1999	Intervention, no randomisation or placebo group (n=32) Italy	Supplements were given during lactation, containing 2 mg copper sulphate per day (and 116 ug potassium iodide and 20 mg zinc sulphate)	3 samples were collected at 3, 30 and 90 days postpartum (n=11). Milk was collected using a breast pump after the subjects had cleaned the nipple and areola with deionized water. 10 ml milk sample was pumped before the baby nursed Inorganic mass spectrometer was used for analysis	Supplement group	Mean (±SD) breast milk copper concentration, day 3, not supplemented group (NS): 0.53±0.12 mg/l, supplemented group (SG): 0.52±0.20 mg/l, day 30 group NS: 0.51±0.12 mg/l,, SG: 0.52±0.16 mg/l,, day 90 NS: 0.39±0.10 mg/l,, SG: 0.42±0.11 mg/l Maternal copper supplementation during lactation did not increase breast milk copper concentration (statistics not shown)	÷
Feeley 1983	Observational (n=102) Greece	No supplements were given by investigators, but 38% of participants took copper supplements of 2 mg/day during pregnancy and lactation	3 samples were collected between 4-7 days (early transitional milk), 10-14 days (transitional mil), 30-45 days postpartum (mature milk). 30 ml was collected, with one-third of the sample after let down and before feeding (foremilk); one third half-way through the feeding, and one-third after feeding (hindmilk) Laboratory method used not clear	Maternal supplementation information (self-reported) was collected at the same time as breast milk sample collections	Mean (±SEM) breast milk copper concentration, early transitional milk: 104.1±5.4, ug/100g, transitional milk: 93.9±3.6 ug/100g, mature milk: 84.7±3.8 ug/100g Maternal copper supplementation during pregnancy and lactation was not associated with breast milk copper (statistics not reported)	÷
Higashi 1982	Observational (n=65) Japan	No supplements were given by investigators	5 samples were collected at the first lactation (colostrum), one week (transitional milk), one month, 3 months and 5 months postpartum (mature milk). Milk were collected at morning by manual milking before the baby was due to be fed. App.10 ml were obtained directly into clean polyethylene bottles, after breasts were cleaned twice with deionized water	Serum collected 3 months postpartum (n=44)	Mean (±SD) breast milk copper concentration, colostrum: 0.45±0.23 mg/l, 1 week: 0.45±0.15 mg/l, 1 month: 0.44±0.10 mg/l, 3 months: 0.29±0.09 mg/l, 5 months: 0.22±0.08 mg/l Mean (±SD) serum copper: 1.29±0.11 mg/l Maternal serum copper was not associated with breast milk copper (statistics not reported)	÷

Table 2. Studies on the relationship between maternal nutritional status or dietary intake and breast milk mineral concentrations

First author, year	Study design (sample size) and setting	Micronutrient supplementation status	Milk samples	Maternal nutritional assessment	Main results	Relationship
			Atomic absorption spectrophotometry was used for analysis			
Mahdavi 2010	Observational (n=182) Iran	No supplements were given by investigators	1 sample collected between 90-120 days postpartum. Breast milk (10–15 ml) samples were collected before nursing the baby in the morning Laboratory method used not clear Infants were exclusively breastfed	24-hour recalls for 3 days (one weekend day included) collected at same time as breast milk sample collection	Mean (±SD) breast milk copper concentration: 0.53±0.3 mg/l Mean (±SD) dietary copper intake 1.16±0.7 mg/day Maternal dietary copper intake was not associated with breast milk copper (β=0.8, p=0.17)	÷
Rajalakshmi 1980	Observational (n=412 from urban areas, n=208 from rural areas) India	No supplements were given by investigators	1 sample collected between delivery and 13 months postpartum. 24 participants delivered more than one sample. Samples were collected by manual expression just before the baby was due to feed. 10 ml were obtained. Varian-Techtron-100 atomic absorption flame spectrophotometer was used for analysis	Serum collected between 1 and 6 months postpartum (n=152)	Mean (±SEM) breast milk copper concentration colostrum (n=76): 0.46± 0.019 µg/ml, transitional (n=31) (6-10 days): 0.50±0.028 µg/ml, 11 days to 1 month (n=28): 0.45±0.028 µg/ml, 1-3 months (n=77): 0.29±0.01 µg/ml, 4-6 months (n=89): 0.21±0.01 µg/ml, 7-12 months (n=88): 0.17±0.009 ug/ml, 13 months and above (n=23): 0.16±0.014 µg/ml Mean (±SEM) serum copper between 1-6 months: 1.73±0.123 µg/100ml Maternal serum copper was not associated with breast milk copper between 1-6 months postpartum (β=0.19, p>0.05)	÷
Salmenpera 1986	Intervention, (but only observational results presented) (n=200) Finland	Supplements were given during lactation, starting after delivery, group 1) no supplementation, 2) low, 2 mg copper/day, 3) high, 4 mg copper/day	9 samples collected at 4-5 days (baseline), 2, 4, 6, 7.5, 9, 10, 11, and 12 months postpartum. The milk samples were collected at home during a 24-hour period. The milk was manually expressed pooling 10 ml of milk from the beginning (foremilk) and end (hindmilk) of each feed Atomic absorption spectrophotometry was used for analysis 167 infants were exclusively breastfed for 2 months 140 for 4 months, 116 for 6 months, 36 for 9 months, and 7 for 12 months. Infants not exclusively breastfed were excluded	Serum collected at 4 months postpartum	Median (±SEM) breast milk copper concentration, colostrum: 0.34± 0.01 mg/l, 9 months: 0.12± 0.01 (copper concentrations at the remaining time-points were illustrated in a figure) Maternal serum copper was not associated with breast milk copper at 4 months postpartum (statistics not reported)	÷

Table 2. Studies on the relationship between maternal nutritional status or dietary intake and breast milk mineral concentrations

First author, year	Study design (sample size) and setting	Micronutrient supplementation status	Milk samples	Maternal nutritional assessment	Main results	Relationship
Vaughan 1979	Observational (n=38) USA	No supplements were given by investigators	Monthly milk samples were collected between 1-31 months postpartum. Participants remained in the study on an average of 4 consecutive months. 150-200 ml of milk was hand expressed into acid-washed glass. Subjects were instructed to collect the milk over a period of 3 to 5 days, at morning, afternoon, and evening feedings and at random intervals within the feeding Atomic absorption spectrophotometer was used for analysis	Serum collected during the same 3- to 5-day period of milk collection for the particular month (n=24) Three day dietary records. Intakes were recorded during the same 3- to 5-day period of milk collection (n=11)	Mean (±SEM) breast milk copper concentration, 1-3 months: 0.43±0.05 μg/ml (n=28), 4-6 months: 0.33±0.03 μg/ml (n=39) Mean serum copper concentration, 1-3 months: 1.5 μg/ml, 4-6 months: 1.9 μg/ml Mean dietary copper intake 4-6 months: 3.64 mg/day Maternal serum copper was not associated with breast milk copper (statistics not reported) Maternal dietary copper intake was not associated with breast milk copper (statistics not reported)	÷
Vuori 1980	Observational (n=27) Finland	No supplements were given by investigators	Not clear how many breast milk samples were collected and when. Milk aliquots of 8 ml were obtained at the beginning and at the end of each feed during a period of 24-hours and pooled to one sample Ash solutions by the flame atomic absorption spectrophotometric method was used for analysis	Two 7 day food records. The first record from the first survey period between 6-8 weeks and the second record from the second survey period between 17 to 22 weeks postpartum (n=15).	Mean (±SD) breast milk copper concentration, 6-8 weeks: 0.36±0.07 mg/l and 17-22 week 0.21±0.07 mg/l Mean (±SD) dietary copper intake: 1.88±0.62 mg/day in first survey period and 1.73±0.55 mg/day in second survey period Maternal dietary copper intake was not associated with breast milk copper (statistics not reported)	÷
Wasowicz 2001	Observational (n=131) Poland	No supplements were given by investigators	3 samples collected at 0-4 days (colostrum, n=43), 5-9 days (transitional milk, n=46)) and 10-30 days postpartum (mature milk, n=41). Milk samples (5–7 ml) were hand expressed after participants cleaned the nipple and areola with deionized water Inductively coupled plasma–atomic electron spectrometry was used for analysis	Plasma collected at 0-4 days (n=43), 5-9 days (n=46) and 10-30 days postpartum (n=41)	Mean (±SD) breast milk copper concentration, colostrum: 0.45±0.11 mg/l, transitional milk: 0.39±0.091 mg/l, mature milk: 0.27±0.09 mg/l Mean (±SD) plasma copper, 0-4 days: 1.70±0.55 mg/l, 5-9 days: 1.38± 0.43 mg/l, 10-30 days: 1.03±0.30 mg/l Maternal plasma copper was not associated with breast milk copper at any time (statistics not reported)	÷
Zhao 2014	Observational (n=90) China (Beijing, Guangzhou and Suzhou)	No supplements were given by investigators	1 sample collected between 5−240 days postpartum. All were instructed to empty one breast during 6 to 7 a.m. At 9 to 11 a.m., the full milk of one breast (which was emptied before) was collected using an electric breast pump. Fore and hind milk were gently mixed.	FFQs and one cycle of 24-hour dietary recall during lactation	Mean (±SD) breast milk copper concentration: 5-11 days: 0.56±0.15 mg/kg, 12-30 days: 0.50±0.16 mg/kg, 31-60 days: 0.35±0.09 mg/kg, 61-120 days: 0.31±0.07 mg/kg, 121-240 days: 0.29±0.16 mg/kg Maternal dietary copper intake was not associated with breast milk copper when adjusting for stage of lactation (r=-0.071, p>0.05)	÷

Table 2. Studies on the relationship between maternal nutritional status or dietary intake and breast milk mineral concentrations

First author, year	Study design (sample size) and setting	Micronutrient supplementation status	Milk samples	Maternal nutritional assessment	Main results	Relationship
			Inductively coupled plasma mass spectrometry was used for analysis			
Iodine						
Andersen 2014	Observational (n=127) Denmark	No supplements were given by investigators, however 83% of participants were iodine supplement users during pregnancy, and 47% took an iodine-containing supplement during lactation	1 sample collected 31 days postpartum. Breast milk were collected non-fasting. Information on timing of sampling in relation to breastfeeding of the child, timing of last iodine supplement intake prior to sampling, and whether milk was collected from one or both breast was collected Cerium/arsenite method was used for analysis	Supplement use during lactation (self-reported) Spot urine collected 31 days postpartum	Median (IQR) breast milk iodine concentration: 83 (61–125) µg/l (combined groups), iodine supplemented; 112 (80–154) µg/l, unsupplemented: 72 (47–87) µg/l Median UIC (IQR): 72 (46–107) µg/l, and in supplemented: 83 (63–127) µg/l and unsupplemented: 65 (40–91) µg/l (significantly different) Maternal iodine supplementation during lactation increased breast milk iodine ($p<0.001$) Maternal urinary iodine was associated with breast milk iodine ($r=0.28$, $p=0.015$)	+
Bazrafshan 2005	Observational (n=100) Iran	No supplements were given by investigators	1 sample collected between 30-180 days postpartum. 5-10 ml was collected (breast milk collection method not clear) Sandell–Kolthoff method was used for analysis	Spot urine collected between 30-180 days postpartum.	Median (range) breast milk iodine concentration: 93.5 (17–696) ug/l Median (range) urinary iodine concentration (UIC): 259 (35–519) ug/l Maternal UIC was associated with breast milk iodine ($r=0.44$, $p<0.0001$)	+
Bouhouch 2014	Randomised, double-blind, placebo controlled trial (n=239) Morocco (deficient population)	Supplements were given by investigators during lactation. Participants were block randomised to receive either: one dose of 400 mg iodine to the mother and placebo to the infant (indirect infant supplementation), or one dose of about 100 mg iodine to the infant and placebo to the	4 samples collected at baseline (≤ 8 weeks postpartum), 3, 6 and 9 months postpartum. 5 ml of breast milk was collected by manual expression Inductively coupled plasma mass spectrometry was used for analysis	Supplement group Spot urine collected at baseline, 3, 6 and 9 months postpartum	Median (IQR) breast milk iodine concentration at baseline, supplemented group (SG): 40.8 (26.1, 86.4) µg/l, placebo group (PG): 42.8 (25.4, 70.9) µg/l (not significantly different), 3 months SG: 61.4 (35.8, 94.8) µg/l, PG: 33.2 (18.4, 53.1) µg/l, 6 months SG: 49.1 (31.3, 70.9), PG: 35.7 (19.1, 52,2) µg/l, 9 months: SG: 39.4 (23.5, 66.7) µg/l, PG: 26.2 (17.7, 42.7) µg/l Median (IQR) urinary iodine concentration (UIC), baseline, SG: 37 (22-72) µg/l, PG: 30 (18-61) µg/l (not significantly different), 3 months, SG: 58 (36-59) ug/l, PG: 34 (23-60) µg/l. At 6 months SG: 67 (39, 114), µg/l PG: 44 (25, 75) µg/l, 9	+

Table 2. Studies on the relationship between maternal nutritional status or dietary intake and breast milk mineral concentrations

First author, year	Study design (sample size) and setting	Micronutrient supplementation status	Milk samples	Maternal nutritional assessment	Main results	Relationship
		mother (direct infant supplementation)	Exclusive breastfeeding at baseline: 95.8%, 3 months: 75.0%, 6 months: 66.3% and 9 months: 70.3%		months: SG: 58 (34, 135) µg/l, PG: 39 (24, 62) µg/l (significantly different) Median (IQR) TSH at baseline SG: 0.6 (0.4, 0.8) mU/l, PG: 0.6 (0.5, 0.9 mU/l), 3 months SG: 0.6 (0.4, 0.8) mU/l, PG: 0.7 (0.6, 0.9) mU/l, 6 months SG: 0.6 (0.5, 0.8) mU/l, PG: 0.6 (0.5, 0.9) mU/l, 9 months SG: 0.5 (0.4, 0.7) mU/l, PG: 0.7 (0.5,0.9) mU/l (not significantly different) Median (IQR) T_4 baseline SG: 88.5 (72.0, 111.5) nmol/l, PG: 100.0 (81.0, 123.3) nmol/l, 3 months SG: 79.1 (69.0, 94.4) nmol/l, PG: 81.0 (67.2, 99.0) nmol/l, 6 months SG: 92.0 (75.0, 107.0) nmol/l, PG: 88.8 (73.8, 110.0) nmol/l, 9 months SG: 92.8 (77.2, 114.5) nmol/l, PG: 89.8 (80.5, 110.0) nmol/l (not significantly different) Maternal iodine supplementation during lactation increased breast milk iodine ($p<0.0001$) Maternal UIC was associated with breast milk iodine at all time-points (ß=0.675–0.739, p<0·0001).	
Chaouki 1994	Intervention, no randomisation, or placebo group (n=1536) Algeria (deficient population)	Supplements were given during pregnancy, 0.5 ml iodised oil (lipiodol, 240 mg of iodine, given orally). Group A (n=213) participants were given iodised oil 1-3 months before conception, group B (n=190) iodised oil was given during first month of pregnancy. Group C (n=151), iodised oil given during the first 3 months of pregnancy. Group D (n=982) no treatment given	2 samples collected at 1 and 6 months postpartum (breast milk collection method not clear) Laboratory method used not clear	Supplement group	Mean (±SEM) breast milk iodine concentration, 1 months, group A: 520±11.8 nmol/l, group B: 559±10.8 nmol/l, group C: 551±13.4 nmol/l, group D: 307±3.2 nmol/l. 6 months, group A: 307±2.1 nmol/l, group B: 346±2.8 nmol/l, group C: 386±1.8 nmol/l, group D: 260±1.2 nmol/l Mean (±SEM) urinary iodine concentration (UIC) before supplementation, group A: 149±0.4 nmol/l, group B: 157±1.6 nmol/l, group C: 133±3.1 nmol/l, group D: 141±0.7 nmol/l. At delivery, group A: 748±2.1 nmol/l, group B: 803±3.9 nmol/l, group C: 780±3.1 nmol/l, group D: 141±0.4 nmol/l (significant difference in UIC before and after supplementation for group A, B and C), 6 months, group A: 338±2.1 nmol/l, group B: 378±1.0 nmol/l, group C: 394±2.5 nmol/l, group D: 149±0.7 nmol/l Mean (±SEM) TSH before supplementation, group A: 4.0±0.06 mU/l, group B: 3.8±0.05 mU/l, group C: 3.6±0.06 mU/l, group D: 3.9±0.01 mU/l. At delivery, group A: 2.1±0.001 mU/l, group B: 2.1±0.000 mU/l, group C: 1.9±0.001 mU/l, group D: 4.1±0.001 mU/l (significant difference in TSH before and after supplementation for group A, B and C). 6 months postpartum,	+

Table 2. Studies on the relationship between maternal nutritional status or dietary intake and breast milk mineral concentrations

First author, year	Study design (sample size) and setting	Micronutrient supplementation status	Milk samples	Maternal nutritional assessment	Main results	Relationship
					group A: 2.1±0.00 mU/l, group B: 2.1±0.01 mU/l, group C: 2.0±0.00 mU/l, group D: 4.0±0.00 mU/l Mean (±SEM) free T_4 before supplementation, group A: 10.3±0.09 pmol/l, group B: 11.2±0.07 pmol/l, group C: 11.8±0.13 pmol/l, group D: 11.6±0.06 pmol/l. At delivery, group A: 14.1±0.08 pmol/l, group B: 15.2±0.13 pmol/l, group C: 16.2±0.12 pmol/l, group D: 11.2±0.00 pmol/l (significant difference in T_4 before and after supplementation for group A, B and C). 6 months postpartum, group A: 12.9±0.04 pmol/l, group B: 14.1±0.10 pmol/l, group C: 15.0±0.08 pmol/l, group D: 11.0±0.05 pmol/l Maternal iodine supplementation increased breast milk iodine. Breast milk iodine was higher in groups A, B and C compared to group D at 1 month ($p<0.001$) and at 6 months postpartum (statistics not shown)	
Chierici 1999	Intervention, no randomisation or placebo group (n=32) Italy	Supplements were given during lactation containing 116 ug potassium iodide per day (and 20 mg zinc sulphate and 2 mg copper sulphate)	3 samples collected at 3, 30 and 90 days postpartum (n=10). Milk was collected using a breast pump after the subjects had cleaned the nipple and areola with deionized water. 10 ml milk sample was pumped before the baby nursed Inorganic mass spectrometer was used for analysis	Supplement group	Mean (±SD) breast milk iodine concentration day 3 not supplemented group (NG): 0.27±0.14 mg/l, supplemented group (SG): 0.32±0.55 mg/l, day 30 NG: 0.15±0.09 mg/l, SG: 0.13±0.08 mg/l, day 90 NS: 0.11±0.04 mg/l, SG: 0.08±0.05 mg/l Maternal iodine supplementation during lactation did not increase breast milk iodine (statistics not shown)	÷
Costeira 2009	Observational (n=140) Portugal (iodine deficient population)	No supplements were given by investigators	2 samples collected at 3 days and 3 months postpartum. Breast milk samples were collected in the morning and, whenever possible, in the fasting state, in a screw-capped plastic bottle, with no added preservatives A modification of the chloric acid digestion method was used for analysis	Urine samples collected at 3 days and 3 months postpartum	Median (IQR) breast milk iodine concentration, day 3: 95 (68, 143) ug/l, 3 months: 70 (50, 102) ug/l Median (IQR) urinary iodine concentration (UIC) 3 days (n=88): 35 (15,98) ug/l, 3 months (n=105): 50 (28, 84) ug/l Maternal UIC was associated with breast milk iodine at 3 days postpartum ($r=0.44$, $p<0.01$) and at 3 months postpartum ($r=0.46$, $p<0.01$)	+
Dold 2017	Observational (n=866) China, Croatia, Philippines (iodine sufficient)	No supplements were given by investigators	1 sample collected at 3 months postpartum. A 10-ml foremilk sample was obtained by manual expression into a plastic container Multicollector inductively coupled plasma mass spectrometry (MC-ICP- MS) was used for analysis	Spot urine collected at the same time as breast milk sample	Median (IQR) breast milk iodine concentration, pooled from China, Croatia and Philippines (iodine sufficient): 171 (123, 335) µg/kg Maternal UIC was associated with breast milk iodine at 3 months postpartum ($r=0.11$, $p<0.001$)	+

Table 2. Studies on the relationship between maternal nutritional status or dietary intake and breast milk mineral concentrations

First author, year	Study design (sample size) and setting	Micronutrient supplementation status	Milk samples	Maternal nutritional assessment	Main results	Relationship
	and Morocco (iodine deficient)		All infants were exclusively breastfed			
Glinoer 1995	Randomised double-bind, placebo controlled trial (n=180) Belgium (iodine deficient population)	Supplements were given during pregnancy. Group A: daily placebo. Group B: 131 µg potassium iodide (KI) a day, corresponding to 100 µg iodide a day. Group C: a combination of 131 µg KI and 100 L-T_4 a day. Supplements were given from the day of enrolment until delivery	1 sample collected between 2-6 days postpartum. (Not clear how breast milk was collected or how iodine concentration was analysed)	Supplement group	Mean (±SEM) breast milk iodine concentration, group A: 29±2 µg/l, group B: 61±10 µg/l, group C: 45±5 µg/l Mean urinary iodine concentration at baseline: 36 µg/l Maternal iodine supplementation during pregnancy increased breast milk iodine ($p<0.001$).	+
Gushurst 1984	Observational (n=37) USA	No supplements were given by investigators	2 samples collected between 14 days and 3.5 years postpartum (max interval between collections was 1 month). Before nursing their infants, mothers manually expressed the milk into a 75-ml plastic vial Iodide-specific electrode was used for analysis	Dietary questionnaire collected at the time of the first breast milk collection to assess iodised salt intake	Median (range) breast milk iodide concentration 14 days: 108 (29-450) µg/l, 3.5 years: 85 (32, 731) µg/l Mean (±SD) breast milk iodide concentration in women with no iodised salt intake (n=6): 113±64 µg/l, in women with low iodised salt intake (n=19): 143±105 µg/l, in women with a high iodised salt intake (n=11): 270±146 µg/l Maternal intake of iodised salt was associated with breast milk iodine ($p<0.01$) Maternal dietary iodine intake was associated with breast milk iodine ($p<0.01$)	+
Hannan 2009	Observational (n=31) USA	No supplements were given by investigators	2 samples collected between 30-45 days postpartum (early lactation) (n=31), and between 75-90 days postpartum (late lactation) (n=17). Approximately 20–30 ml of milk was collected either by manual expression into pre-washed	24-hour recall at two time-points in early and late lactation	Mean (±SD) breast milk iodine concentration in early lactation: 47.8±17.1 µg/l and late lactation: 42.3±8.71 µg/l. Mean (±SD) dietary iodine intake, early lactating: 75.7±73.5 µg/day, late lactation 51.5±52.7 µg/day	÷

Table 2. Studies on the relationship between maternal nutritional status or dietary intake and breast milk mineral concentrations

First author, year	Study design (sample size) and setting	Micronutrient supplementation status	Milk samples	Maternal nutritional assessment	Main results	Relationship
			plastic vials, or via a pre-washed trace mineral-free breast pump and container Flame Atomic Absorption Spectrometry and Neutron Activation Analysis was used for analysis		Maternal dietary iodine intake was not associated with breast milk iodine in early lactation (r=0.28, p=0.2) or in late lactation (r=-0.09, p=0.7) or combined (r=0.22, p=0.2)	
Kirk 2012	Pre-post ntervention, no randomisation (n=13) USA	All participants were given 150 µg/day of iodine during lactation	4 samples were collected by each subject between 1 to 8 months postpartum, at different times of day for three days in each of three regimens: (a) prior to and without iodine supplementation, (b) 150 µg/day iodine supplement taken in the evening and (c) 150 µg/day iodine supplement taken in the morning. Collection periods were spaced by at least 24-hours without supplementation. Subjects were asked to alternate between foremilk and hindmilk collection. Inductively coupled plasma mass spectrometry was used for analysis Infants were exclusively breastfed	Supplementation (Pre-post intervention design, the study used pre-supplement concentrations as comparison group) Self-reported iodised salt intake	Mean (±SD) breast milk iodine concentration before supplementation 53.0±35.7 µg/l, after supplementation PM: 56.5±34.5, AM: 56.5±50.4 µg/l Mean (±SD) breast milk iodine concentration for users of iodised salt: 71.3±26.3 µg/l, non-users: 37.9±14.9 µg/l Mean (±SD) total urinary iodine excretion, before supplementation 208±146 µg, after supplementation, PM: 261±173 µg, AM: 245±138 ug Maternal iodine supplementation did not increase breast milk iodine (p>0.05) Maternal iodised salt intake increased breast milk iodine (p<0.03)	÷ +
Leung 2009	Observational (n=97) USA	No supplements were given by investigators	1 sample collected within the first 60 hours postpartum. 2 ml colostrum by hand expression (n=61) Spectrophotometric was used for analysis	Spot urine collected at same time as breast milk collection (n=97)	Median (range) iodine concentration in colostrum: 51.4 (21.3, 304.2) µmol/l Median (range) urinary iodine concentration (UIC): 82.2 (10.3, 417.1) µmol/l Maternal UIC was not associated with breast milk iodine (r^2=0.006 p=0.5)	÷
Leung 2012	Pre-post intervention, no randomisation (n=16) USA	All participants were given a supplement of 600 µg of potassium iodine, equalling to 456 µg of iodine during lactation	9 samples collected at baseline (before ingestion of supplement) and then hourly for 8 hours. (Not clear at what stage of lactation samples were collected) (breast milk collection method not clear)	Supplementation (Pre-post intervention design, the study used pre-supplement concentrations as comparison group)	Median (IQR) breast milk iodine concentration, baseline: 45.5 (34.5–169.0) µg/l, after supplementation: 280.5 (71.5–338.0) µg/l Maternal iodine supplementation increased breast milk iodine (p<0.01)	+

Table 2. Studies on the relationship between maternal nutritional status or dietary intake and breast milk mineral concentrations

First author, year	Study design (sample size) and setting	Micronutrient supplementation status	Milk samples	Maternal nutritional assessment	Main results	Relationship
Mekrungcharas2014	Observational (n=100) Thailand	No supplements were given by investigators, however 18% and 7% of the participants used iodine supplements during pregnancy and lactation, respectively. 90% of the participants used iodised salt while 76% use iodised fish sauce	1 sample collected between birth and approximately 71 days postpartum. Approximately 10 ml of breast milk was collected (breast milk collection method not clear) The spectrophotometer kinetic assay method was used for analysis 85% of infants were exclusively breastfed	FFQ collected at the same time as breast milk collection	Median (IQR) breast milk iodine concentration: 129.7 (81.0, 205.7) mcg/l Maternal weekly egg intake (iodised) was associated with breast milk iodine (p=0.01)	+
Moon 1999	Observational (n=50) Korea	No supplements were given by investigators	2 samples collected between 2-5 days and 4 weeks postpartum. Milk samples were obtained by manual expression. Sampling was done at a mid-morning feed from both breasts Neutron activation analysis was used for analysis	24-hour recalls (high iodine intake from seaweed) collected at the same time as breast milk samples	Mean (±SD) breast milk iodine concentration, 2-5 days: 2170.0±1694.9 ug/l, 4 weeks 891.5±1036.7 ug/l Mean (±SD) dietary iodine intake 2-5 days: 2744.0±888.4 µg/day, 4 weeks: 1295.3±946.2 µg/day Maternal dietary iodine intake was associated with breast milk iodine in early lactation (r=0.81, p<0.0001) and in later lactation (r=0.82, p<0.0001)	+
Nohr 1994	Observational (n=152) Denmark (iodine deficient population)	No supplements were given by investigators, but 36% of participants took iodine containing supplements during pregnancy and lactation (150 µg per tablet)	1 sample collected 5 days postpartum (breast milk collection method not clear) The CE/AS method was used for analysis	Supplement intake during pregnancy and lactation (self-reported)	Median (IQR) breast milk concentration, unsupplemented: 33.6 (19.1, 56.6) µg/l, supplemented group: 57.0 (33.0, 113.5) µg/l Maternal iodine supplementation during pregnancy and lactation increased breast milk iodine (p<0.001)	+
Ordookhani 2007	Observational (n=48) Iran	No supplements were given by investigators	1 sample collected between 7-30 days postpartum. 5-10 ml of breast milk was collected (breast milk collection method not clear) The Sandell–Kolthoff method was used for analysis Infants were exclusively breastfed	Urine collected at the same time as breast milk collection	Median (range) breast milk iodine concentration: 148 (45–750) µg/l Median urinary iodine concentration (UIC): 107 (20–710) µg/l Median (range) TSH: 1.1 (0.3, 3.3) mIU/l Median (range) T_4: 10 (5.6, 14) µg/dl Median (range) T_3: 249 (144, 1212) ng/dl Maternal UIC was associated with breast milk iodine (r=0.434, p=0.004)	+

Table 2. Studies on the relationship between maternal nutritional status or dietary intake and breast milk mineral concentrations

First author, year	Study design (sample size) and setting	Micronutrient supplementation status	Milk samples	Maternal nutritional assessment	Main results	Relationship
Pearce 2007	Observational (n=57) USA	No supplements were given by investigators	1 sample collected between 10-250 days postpartum. Approximately 10 ml were collected at the start of a feed using either hand expression or a breast pump. In a subset (n=30) breast milk was collected at the start of a feed and sequentially throughout a single feed Spectrophotometric was used for analysis	Spot urine collected at the same time as breast milk collection	Median (range) iodine breast milk concentration: 155 (2.7–1968) µg/l Median (range) urinary iodine concentration (UIC): 114 (25–920) µg/l Maternal UIC was not associated with breast milk iodine (r^2=0.06; p=0.08). Maternal urinary iodine per creatinine was associated with breast milk iodine (r^2=0.27; p=0.0001).	÷ +
Pedersen 1993	Randomised controlled trial, no placebo group (n=54) Denmark (iodine deficient population)	Supplements were given during pregnancy and lactation. Group A (n=26): control group, Group B (n=28): 200 µg iodine a day starting from weeks 17-18 of pregnancy until 12 months postpartum	1 sample collected 5 days postpartum (breast milk collection method not clear) The Ceri/Arsenium method was used for analysis	Supplement group	Median breast milk iodine concentration, group A: 28 µg/l group B: 41 µg/l Median urinary iodine concentration (UIC) at baseline, group A: 51 µg/l, group B: 55 µg/l Maternal supplementation during pregnancy and lactation did not increase breast milk iodine (p=0.06)	÷
Sukkhojaiwaratkul 2014	Observational (n=87) Thailand	No supplements were given by investigators, however 34 participants took iodine supplements during lactation	1 sample collected at 2 months postpartum (n=57) (breast milk collection method not clear) The Sandell–Kolthoff method was used for analysis	Supplement intake (self-reported)	Median (range) breast milk iodine concentration: 90.8 (0, 311.5) µg/l, supplemented group: 108.6 (8.8, 311.5) µg/l, unsupplemented group: 69.5 (0, 172.4) µg/l Median (range) urinary iodine concentration (UIC): 138.0 (26.8, 735.8) µg/l, supplemented group: 198.8 (31.4, 735.8) µg/l, unsupplemented group: 119.8 µg/l (26.7, 620.8) µg/l (not significantly different) Maternal iodine supplementation during lactation increased breast milk iodine (p=0.032)	+
Trabzuni 1998	Observational (n=104) Saudi Arabia	No supplements were given by investigators	1 sample collected between 6-12 weeks postpartum (breast milk collection method not clear) The Sandell–Kolthoff method was used for analysis	Urine collected at the same time as breast milk sample (n=78) Dietary recall collected at the same time as breast milk sample	Mean (±SE) breast milk iodine concentration: 14.24 ±1.00 µg/dl Mean (±SE) urinary iodine concentration (UIC): 226.15 ±24.89 µg/gm creatinine Mean dietary intake of iodine: 368.28 µg/day.	+

Table 2. Studies on the relationship between maternal nutritional status or dietary intake and breast milk mineral concentrations

First author, year	Study design (sample size) and setting	Micronutrient supplementation status	Milk samples	Maternal nutritional assessment	Main results	Relationship
					Maternal UIC was associated with breast milk iodine (statistics not reported) Maternal dietary iodine intake (dairy) was associated with breast milk iodine ($p<0.05$)	
Wang 2009	Observational (n=49) China (Yongjin county, were iodised salt coverage rate is more than 90%)	No supplements were given by investigators	1 sample collected before 6 months postpartum (stage of lactation and breast milk collection method not clear) The Sandell–Kolthoff method was used for analysis	Urine collected at the same time as breast milk sample	Median breast milk iodine concentration: 240 ug/l Mean urinary iodine concentration (UIC): 126 µg/l Maternal UIC was not associated with breast milk iodine (statistics not reported)	÷
Iron						
Donangelo 1989	Observational (n=83) Brazil	No supplements were given by investigators	1 sample collected between 1-180 days between 9.00 and 10.00 am by manual expression before the infant was due to be fed (5-10 ml) Infants were exclusively breastfed Atomic absorption spectrophotometry was used for analysis	Serum collected at the same time as breast milk sample	Mean (±SE) breast milk iron concentration, 1-5 days postpartum (n=17): 1.23±0.18 µg/ml, 6-30 days (n=13): 0.84±0.17 µg/ml, 31-280 days (n=10): 0.42±0.05 µg/ml Mean (±SE) serum iron concentration, 1-5 days postpartum (n=11): 95.2±16.3 µg/dl, 6-30 days (n=11): 103±14.2 µg/dl, 31-280 days (n=7): 75.3±16.8 µg/dl Maternal serum iron was not associated with breast milk iron at any stage of lactation (statistics not reported)	÷
El-Farrash 2012	Observational (n=80, n=50 anaemic and n=30 non-anaemic) Egypt	No supplements were given by investigators	1 sample collected 15 days postpartum. Breast milk samples were collected by manual expression. Participants were instructed to first clean their breasts with a gauge piece soaked with plain water. After discarding an initial 4–5 ml of milk, an aliquot of 10 ml was collected in deionized glass jars and finally transferred to iron-free polyethylene tubes	Blood samples collected at delivery. The participants were divided into 3 groups for analysis: Group A haemoglobin 8.6–10.9 g/dl, mild anaemic, n=29), group B haemoglobin 8.5–7 g/dl to haemoglobin <7 g/dl, moderate-severe anaemia, n=21), group C	Mean (±SD) breast milk iron concentration in group C: 1.6±0.08 mg/L. Group A: 0.78±0.14. Group B: 0.29±0.12. There was a significant difference between group A and C ($p<0.001$) (Maternal serum iron and haemoglobin concentrations were not reported) Maternal serum iron was associated with breast milk iron ($r=0.54$, $p<0.01$) Maternal haemoglobin was associated with breast milk iron ($r=0.563$, $p<0.01$)	+

Table 2. Studies on the relationship between maternal nutritional status or dietary intake and breast milk mineral concentrations

First author, year	Study design (sample size) and setting	Micronutrient supplementation status	Milk samples	Maternal nutritional assessment	Main results	Relationship
			Atomic absorption spectrophotometry was used for analysis Infants were exclusively breastfed	haemoglobin ≥ 11 g/dl, normal, n=30)		
Hannan 2009	Observational (n=31) USA	No supplements were given by investigators	2 samples collected between 30-45 days postpartum (n=31) (early lactation), and between 75-90 days postpartum (n=17) (late lactation). Approximately 20–30 ml of milk was collected either by manual expression into pre-washed plastic vials, or via a pre-washed trace mineral-free breast pump and container Flame Atomic Absorption Spectrometry and Neutron Activation Analysis was used for analysis	24-hour recall collected at the same time as breast milk samples	Mean (±SD) iron concentration in early lactation: 0.5±1.0 mg/l and late lactation: 0.4±0.3 mg/l Mean (±SD) dietary iron intake, early lactating: 15.6±12.3 mg/day, late lactation: 10.8±5.8 mg/day Maternal dietary intake of iron was not associated with breast milk iron in early lactation (r=0.07, p=0.7) or in late lactation (r=-0.37, p=0.2) or combined (r=0.08, p=0.6)	÷
Kumar 2008	Observational (n=75, n=55 anaemic and n=20 non-anaemic) India	No supplements were given by investigators	2 samples collected at 3 days postpartum (early transitional milk) and 15 days postpartum (late transitional milk). Breast milk samples were collected by manual expression. Participants were instructed to first clean their breasts with a gauge piece soaked with plain water. After discarding an initial 4 to 5 ml of milk, an aliquot of 10 ml was collected in deionized glass jars and finally transferred to iron-free polyethylene tubes. Milk expression was done from 1 breast only, between 9:00 am to 12:00 pm, 1.5 to 2.0 hours after the last breastfeeding Atomic absorption spectrophotometry was used for analysis Infants were exclusively breastfed	Blood samples collected at delivery. Participants were divided into 4 groups for analysis: group 1: haemoglobin ≤60 g/l (n=21); group 2: haemoglobin between 61–85 g/l; (n =16); group 3: haemoglobin between 86–109 g/l (n=18); and group 4: haemoglobin ≥110 g/l; (n=20)	Mean (±SD) breast milk iron concentration, early transitional milk, group 1: 11.543±3.921 μmol/l, group 4:15.231±2.767 μmol/l, late transitional milk, group 1: 12.293±3.064 μmol/l, group 4: 14.901±2.412 μmol/l Mean (±SD) serum iron concentration group 1: 4.885±1.461 μmol/l l, group 4: 18.292±1.452 μmol/l (significantly different). Mean (±SD) haemoglobin group 1: 52±7 g/l, group 4: 121±5 g/l (significantly different). Breast milk iron concentration was reduced in severely anaemic mothers compared to non-anaemic mothers, but not in those with mild-to-moderate anaemia Maternal serum iron was associated with breast milk iron in early transitional milk (r=0.439; p<0.001), and late transitional milk (r=0.451; p<.001) Maternal ferritin was not associated with breast milk iron in early transitional milk (r=0.226, p>0.05) but in late transitional milk (r=0.251, p<0.05)	+
Mahdavi 2010	Observational (n=182) Iran	No supplements were given by investigators	1 sample collected between 90-120 days postpartum. Breast milk (10–15 ml) samples were collected into metal free tubes by self-expression before nursing the baby in the morning	24-hour recalls for three days (one weekend day included)	Mean (±SD) breast milk iron concentration: 0.85±0.2 mg/l for rural and urban women combined. Rural: 0.9±0.3 mg/l, urban: 0.81±0.2 mg/l	÷

Table 2. Studies on the relationship between maternal nutritional status or dietary intake and breast milk mineral concentrations

First author, year	Study design (sample size) and setting	Micronutrient supplementation status	Milk samples	Maternal nutritional assessment	Main results	Relationship
			Infants were exclusively breastfed		Mean (±SD) dietary iron intake was 11.8±8.2 mg/day for rural and urban combined. Rural: 11.1±8.1 mg/day, urban: 12.6±8.4 mg/day Maternal dietary intake of iron was not associated with breast milk iron (β=0.11, p=0.2)	
Shashiraj 2006	Observational (n=200, 100 anaemic and 100 non-anaemic) India	All participants were supplemented with iron and folic acid. Not clear if this was given by investigators	3 samples were collected, 1 day, 14 weeks and 6 months postpartum. Participants were requested to collect the breast milk by manual expression. After collecting around 10 ml of foremilk in a sterile acid-washed and rinsed iron-free container, the baby was breast-fed for about 15 min and thereafter an equal volume of hindmilk from the same breast was collected, and the samples were mixed Atomic absorption spectrometry was used for analysis	Blood collected on 1 day and 6 months postpartum	Mean breast milk iron concentration day 1: 0.89 mg/l in non-anaemic mothers and 0.86 mg/l in anaemic mothers. At 14 weeks 0.34 mg/l and 0.33 mg/l, respectively, at 6 months 0.27 mg/l and 0.26 mg/l, respectively Median (IQR) haemoglobin, 12.4 (11.4, 13.1) g/dl in non-anaemic, 1 day, and 9.7 (9.0, 10.5) g/dl in anaemic. At 6 months postpartum 13.1 (12.8, 13.3) g/dl and 12.2 (11.8, 12.8) g/dl, respectively Median (IQR) serum iron, 76.0 (76.0, 95.3) µg/dl in non-anaemic 1 day, and 64.0 (56.5, 70.0) µg/dl in anaemic. At 6 months postpartum 80.0 (72.0, 86.0) µg/dl and 80.0 (74.8, 84.5) µg/dl respectively Maternal serum iron of both groups (anaemic and non-anaemic) was not associated with breast milk iron on 1 day postpartum (r=-0.102, p>0.05 and r=0.178, p>0.05, respectively) and the same for 6 months (r=-0.141, p>0.05 and r=-0.048, p>0.05, respectively). Maternal haemoglobin was associated with breast milk iron in the anaemic group on 1 day postpartum (r=0.339, p=0.01), but not in the non-anaemic group (r=-0.016, p>0.05). No correlation at 6 months in the two groups was found (r=-0.012, p>0.05, r=0.199, p>0.05 respectively)	÷ +
Stuetz 2012 (eur)	Pre-post intervention, no randomisation (n=86 before fortification, n=99 after fortification)	Iron fortified wheat flour (estimated daily intake: 4.5 mg) was provided for all participants (the flour was also fortified with other micronutrients)	2 samples were collected, 1 before fortification of flour was introduced at 12 weeks postpartum and 1 sample after flour fortification was introduced at 12 weeks postpartum (two different groups of women). Milk samples were collected by manual expression into glass tubes wrapped in aluminium foil in order to protect against degradation	Fortification (Pre-post intervention design, the study used pre-supplement concentrations as comparison group)	Geometric mean breast milk iron concentration, before fortification: 0.242 mg/l, after fortification: 0.288 mg/l Geometric mean serum ferritin, before fortification: 41.8 ug/l, after fortification: 38.2 ug/l (not significantly different) Mean (±SD) haemoglobin (whole blood), before fortification: 124.5±11.0 g/l, after fortification: 124.4±9.5 g/l (not significantly different).	+

Table 2. Studies on the relationship between maternal nutritional status or dietary intake and breast milk mineral concentrations

First author, year	Study design (sample size) and setting	Micronutrient supplementation status	Milk samples	Maternal nutritional assessment	Main results	Relationship
	Thai-Myanmar border (Maela Refugee camp, high prevalence of iron deficiency and anaemia)		Inductively coupled plasma optical emission spectrometry (ICP-OES) after microwave-heated digestion was used for analysis	Blood collected 12 weeks postpartum	Geometric mean sTfR (soluble transferrin receptor) before fortification: 8.06 mg/l, after fortification:7.27 (significantly different) Maternal intake of fortified flour increased breast milk iron (p=0.04) Maternal sTfR was associated with breast milk iron (β -0.590, p<0.001)	
Vaughan 1979	Observational (n=38) USA	No supplements were given by investigators	Monthly milk samples were collected between 1-31 months postpartum. Subjects remained in the study on an average of 4 consecutive months. 150-200 ml of milk was hand expressed into acid-washed glass. Subjects were instructed to collect the milk over a period of 3 to 5 days, at morning, afternoon, and evening feedings and at random intervals within the feeding Atomic absorption spectrophotometer was used for analysis	Blood collected during the same 3 to 5-day period of milk collection for the particular month (n=24) 3 day dietary records. Intakes were recorded during the same 3 to 5-day period of milk collection (n=11)	Mean (±SEM) breast milk iron concentration 1-3 months: 0.49±0.05 (unit not clear) (n=28), 4-6 months: 0.43±0.04 (n=39) Mean serum iron concentration 4-6 months: 0.75 (unit not clear) Mean dietary iron intake 4-6 months: 39.3 mg/d Maternal serum iron was not associated with breast milk iron from the same stage of lactation (statistics not reported) Maternal dietary intake of iron was not associated with breast milk iron from the same stage of lactation (statistics not reported)	÷
Vuori 1980	Observational (n=27) Finland	No supplements were given by investigators	2 samples collected between 6-8 and 17-22 weeks postpartum. Milk aliquots of 8 ml were obtained at the beginning and at the end of each feed during a period of 24 hours and pooled to one sample Ash solutions by the flame atomic absorption spectrophotometric method was used for analysis	Two 7 day food records. The first record from the first survey period between 6-8 weeks and the second record from the second survey period between 17 to 22 weeks postpartum (n=15)	Mean (±SD) breast milk iron concentration, 6-8 weeks: 0.40± 0.10 mg/l, 17-22 weeks: 0.29±0.09 mg/l Mean (±SD) dietary iron intake, 6-8 weeks: 15.8±2.4 mg/day, 17-22 weeks: 13.4±2.6 mg/day Maternal dietary iron intake was not associated with breast milk iron (statistics not reported)	÷
Zapata 1995	Intervention, no randomisation or placebo group (n=28)	Iron supplements were given by investigators during lactation for 3 months starting at delivery, 40 mg FE/day. Participants had taken 30-60 mg FE/day during last trimester of pregnancy	3 samples collected at 1-2, 30-40, and 90-100 days postpartum. Milk samples of 10-15 ml were collected between 8:00 and 10:00 am by manual expression of both breasts into metal-free plastic tubes	Supplement group Plasma collected at 1-2 days and 90-100 days postpartum	Mean (±SD) breast milk iron concentration 1-2 days (baseline) supplemented group (SG):17.7±5.6 µmol/l, not supplemented group (NS): 18.3±5.0 µmol/l, 30-40 days SG: 12.5±5.2 µmol/l, NS:13.4±6.1 µmol/l, 90-100 days SG: 12.9±7.5 µmol/l, NS:10.0±3.4 µmol/l Mean (±SD) plasma iron concentration at 1-2 days (baseline) SG: 16.3±3.8 µmol/l, NS: 15.8±6.1 µmol/l, 90-100 µmol/l, SG:	÷

Table 2. Studies on the relationship between maternal nutritional status or dietary intake and breast milk mineral concentrations

First author, year	Study design (sample size) and setting	Micronutrient supplementation status	Milk samples	Maternal nutritional assessment	Main results	Relationship
	Brazil (low prevalence of iron deficiency)		Milk iron was analysed colorimetrically with sulfonated bathophenantroline by adaptation of methods used for serum iron Infants were exclusively breastfed		21.9±5.9 µmol/l, NS: 19.9±7.9 µmol/l. (No significant difference between supplement groups at any time-point) Maternal iron supplementation during lactation did not increase breast milk iron at any time-point (statistics not reported) Maternal plasma iron was not associated with breast milk iron (statistics not reported)	
Zavaleta 1995	Intervention, no randomisation or placebo group (n=29) Peru (high prevalence of iron deficiency)	Iron supplements were given to anaemic participants during lactation, starting from two days postpartum, 100 mg/day. Non-anaemic women did not receive any supplements	2 samples collected at 2 and 30 days postpartum. 10 ml of milk were collected at the second nursing of the day using a hand pump, with equal volumes of foremilk and hindmilk expressed and then mixed Atomic absorption spectrophotometry was used for analysis	Blood collected at study entry and participants were divided into two groups, group 1: anaemic (Hgb <110 g/l, n=19), group 2: non-anaemic Hgb ≥110 g/l, n=10)	Mean (±SD) breast milk iron concentration, 2 days (baseline), group 1: 0.9±0.2 µg/ml, group 2: 0.8±0.1 µg/ml, 30 days, group 1: 0.4±0.1 µg/ml, group 2: 0.4±0.1 µg/ml Mean (±SD) lactoferrin, 2 days, groups 1: 6.7±3.4 µg/l, group 2: 5.3±1.1 µg/l, 30 days, group 1: 3.7±0.9 µg/l, group 2: 4.4±1.1 µg/l Mean haemoglobin in group 1: 92 g/l before treatment and 105 g/l one months after (significantly different) Maternal iron supplementation of anaemic mothers did not increase breast milk iron or breast milk lactoferrin (statistics not reported) Maternal haemoglobin was not associated with breast milk iron or breast milk lactoferrin in any of the two groups (statistics not reported)	÷
Zhao 2014	Observational (n=90) China (Beijing, Guangzhou and Suzhou, urban areas)	No supplements were given by investigators	1 sample collected between 5−240 days postpartum. All participants were instructed to empty one breast between 6 to 7 a.m. At 9 to 11 a.m., the full milk of one breast (which was emptied before) was collected using an electric breast pump. Foremilk and hindmilk were gently mixed Inductively coupled plasma mass spectrometry was used for analysis	FFQs and one cycle of 24-hour dietary recall collected at the same time as the breast milk sample	Mean (±SD) breast milk iron concentration: 5-11 days 0.90±0.3 mg/kg, 12-30 days 1.0±0.7 mg/kg, 31-60 days 1.0±1.0 mg/kg, 61-120 days 0.9± 0.9 mg/kg, 121-124 days: 1.1± 1.1 mg/kg Maternal dietary iron intake was not associated with breast milk iron (r=0.089, p=0.06)	÷
Selenium						

Table 2. Studies on the relationship between maternal nutritional status or dietary intake and breast milk mineral concentrations

First author, year	Study design (sample size) and setting	Micronutrient supplementation status	Milk samples	Maternal nutritional assessment	Main results	Relationship
Cumming 1992	Observational (n=20) Australia	No supplements were given by investigators	1 sample collected between 6-12 weeks postpartum. Approximately 10 ml of breast milk were manually expressed at the beginning and end of a mid-morning feed, from the first breast offered at that feed. Milk samples were collected in acid washed containers Gamma-spectrometer was used for analysis	Serum and whole blood collected between 6-12 weeks postpartum	Mean (±SD) breast milk selenium concentration, foremilk (n=19): 10.8±3.5 ng/g, hindmilk (n=13): 13.9±3.5 ng/g, combined 11.9±3.5 ng/g Mean (±SD) maternal serum selenium 81±15 ng/g Mean (±SD) maternal blood selenium 101±19 ng/g Maternal serum selenium was not associated with breast milk selenium (p=0.9). Maternal blood selenium was not associated with breast milk selenium (p=0.2)	÷
Hannan 2009	Observational (n=31) USA	No supplements were given by investigators	2 samples collected between 30-45 days postpartum (n=31), and between 75-90 days postpartum (n=17). Approximately 20–30 ml of milk was collected either by manual expression into pre-washed plastic vials, or via a pre-washed trace mineral-free breast pump and container Flame Atomic Absorption Spectrometry and Neutron Activation Analysis was used for analysis	24-hour recall collected at the same time as breast milk samples	Mean (±SD) breast milk selenium concentration in early lactation: 15.9±4.1 mg/l, late lactation: 15.7±5.3 mg/l Mean (±SD) dietary selenium intake in early lactating: 38.8±26.3 ug/day, late lactation: 35.8±22.3 ug/day Maternal dietary selenium intake was not associated with breast milk selenium in early lactation (r=-0.10, p=0.7) or in late lactation (r=-0.39, p=0.3) or combined (r=-0.18, p=0.4)	÷
Higashi 1983	Observational (n=22) Japan	No supplements were given by investigators	5 samples collected: colostrum, 1 week (transitional milk), 1 month, 3 months and 5 months postpartum (mature milk) (n=10). 2 samples collected from group of participants 1 month or 3 months postpartum (n=12). Samples were collected in the morning by manual expression before the baby was due to feed. Approximately 10 ml was obtained directly into clean polyethylene bottles, after the breasts were cleaned twice with deionized water Diaminonaphthalene fluorimetric method of Watkinson was used for analysis	Serum collected at 3 months postpartum (n=16)	Median (range) breast milk selenium concentration, colostrum: 80 (35-152) ng/ml, 1 week: 29 (15-79 ng/ml), 1 month: 18 (9-39) ng/ml, 3 months: 17 (6-28) ng/ml, 5 months: 18 (9-33) ng/ml Mean (±SD) maternal serum selenium: 148±47 ng/ml Maternal serum selenium was not associated with breast milk selenium at 3 months postpartum (statistics not reported)	÷
Kumpulainen 1985	Intervention, no randomisation or	Selenium supplements were given during lactation, either group 1: no supplement, group	4 samples collected at 4-5 days (baseline), 2, 4 and 6 months postpartum. Milk samples were collected	Supplement group	Geometric mean (±SD) breast milk selenium concentration 4-5 days (baseline) (all groups combined): 11±2 ug/l. Group 1, 6 months: 7.2±2.5 ug/l	+

Table 2. Studies on the relationship between maternal nutritional status or dietary intake and breast milk mineral concentrations

First author, year	Study design (sample size) and setting	Micronutrient supplementation status	Milk samples	Maternal nutritional assessment	Main results	Relationship
	placebo group (n=200) Finland	2: 100 ug of selenite, group 3: 100 ug of yeast-selenium. Supplementation started 5-6 days postpartum	by the mothers by pooling 10 ml of milk from the beginning and end of each feed Electrothermal atomic absorption spectrometry was used for analysis 167 infants were exclusively breastfed for 2 months 140 for 4 months, 116 for 6 months. Infants not exclusively breastfed were excluded	Blood collected at 3-4 days (baseline), 2, 4 and 6 months postpartum	(the remaining milk concentrations were illustrated in a figure) Mean (±SD) serum selenium baseline, group 1: 59±13 μg/l, group 2: 58±13 μg/l, group 3: 53±11 μg/l. 4 months, group 1: 29 μg/l, group 2: 51 μg/l, group 3: 86 μg/l Maternal selenium supplementation increased breast milk selenium (statistics not reported) Maternal serum selenium was associated with total breast milk selenium excretion ($r=0.84$, $p<0.0001$)	
Levander 1987	Observational (n=23) USA	No supplements were given by investigators	3 samples collected at 1, 3 and 6 months postpartum (breast milk collection method not clear) Fluorometry of the diaminonaphthalene complex was used for analysis	Duplicate-plate food and drink composites and dietary records were collected daily for 3 days at 37 weeks gestation and at 1, 3, and 6 months postpartum Plasma collected at 37 weeks gestation, and at 1, 3, and 6 months postpartum	Mean (±SEM) breast milk selenium concentration, 1 month: 20±1 μg/l, 3 months: 15±1 μg/l, 6 months: 15±1 μg/l Mean (±SEM) dietary selenium intake at 37 weeks gestation: 97±4 μg/day, 1 month: 84±4 μg/day, 3 months: 84±4 μg/day, 6 months: 87±4 μg/day Mean (±SEM) plasma selenium at 37 weeks gestation: 112±5 μg/l, 1 month: 136±5 μg/l, 3 months: 137±5 μg/l, 6 months: 138±5 μg/l Maternal dietary selenium intake during lactation was not associated with breast milk selenium (statistics not reported) Maternal plasma selenium during lactation was associated with breast milk selenium ($r=0.38$, $p<0.03$)	÷ +
Mannan 1987	Observational (n=10) USA	No supplements were given by investigators	4 samples collected at 4, 8, 12 and 16 weeks postpartum. Milk samples were collected with mechanical pump between 07.00 and 11.00 hours, one of foremilk and the other of hindmilk at a single nursing period Laboratory method used not clear	Plasma collected at 4 and 8 weeks postpartum	Mean breast milk selenium concentration: 16.8 μg/l (pooled data). Mean (±SE) plasma selenium: 97±6.0 μg/l (data pooled) Maternal plasma selenium was associated with breast milk selenium ($r=0.61$, $p=0.01$)	+
Micetic-Turk 2000	Observational (n=20) Slovenia	No supplements were given by investigators	1 sample collected on the second or third day postpartum. Around 6 ml was collected (breast milk collection method not clear) Flow injection hydride generation atomic absorption spectrometry was used for analysis	Serum collected at birth	Mean (±SD) breast milk selenium concentration (n=18): 29±10 μg/l Mean (±SD) serum selenium (n=20): 62±15 μg/l Maternal serum selenium at birth was not associated with breast milk selenium in colostrum ($r^2=0.0174$, $p>0.05$)	÷

Table 2. Studies on the relationship between maternal nutritional status or dietary intake and breast milk mineral concentrations

First author, year	Study design (sample size) and setting	Micronutrient supplementation status	Milk samples	Maternal nutritional assessment	Main results	Relationship
Moore 2000	Intervention, no randomisation (n=21) China (rural area with low selenium intake)	Supplements were given by during last trimester of pregnancy and 3 months postpartum Group 1 (n=10): yeast (placebo), group 2 (n=11): a selenium-enriched yeast tablet providing 100 ug/day of selenium	6 samples collected at birth, 1, 2, 3, 4 and 12 weeks postpartum. The participants cleaned their breasts with 75% ethanol and expressed the milk by hand into a sterile, 15 ml container. The participants expressed approximately 10 ml of foremilk, from one breast, at the beginning of a feeding A semi-automated fluorimetric method was used for analysis	Supplement group	Mean (±SD) breast milk selenium concentration at birth, group 1: 7.4±1.3 ng/g, group 2: 16.7±1.3 ng/g. Week 1, group 1: not reported, group 2: 17.9±1.3 ng/g. Week 2, group 1: not reported, group 2: 19.8±1.3 ng/g. Week 3, group 1: 11.7±1.3 ng/g, group 2: 16.9±1.3 ng/g. Week 4, group 1: 8.4±.1.3 ng/g, group 2: 16.2±1.3 ng/g. Week 12, group 1: not reported, group 2: 9.9±1.3 ng/g Maternal selenium supplementation during pregnancy and lactation increased breast milk selenium (p=0.04)	+
Trafikowska 1996	Pre-post intervention, no randomisation (n=16) Poland	Supplements were given during lactation to all participants. Participants were given 200 ug/day of selenium starting from 3-4 weeks postpartum and continued to 3 months postpartum	4 samples collected at baseline (3-4 weeks postpartum) and after 1, 2 and 3 months of supplementation (breast milk collection method not clear) Fluorometric method of Watkinson was used for analysis	Supplementation (pre-post intervention design, the study used pre-supplement concentrations as comparison group) Blood collected at baseline and after 1, 2 and 3 months of supplementation	Mean (±SD) breast milk selenium concentration 3-4 weeks (baseline): 9.20±2.66 ng/ml, 1 month: 15.9±2.85 ng/ml, 2 months: 15.0±4.18 ng/ml, 3 months: 14.4±6.54 ng/ml Mean (±SD) plasma selenium concentration at 3-4 weeks (baseline): 54.0±13.2 ng/ml, 1 month: 101±23.6 ng/ml, 2 months: 127±20.2 ng/ml, 3 months: 116±19.7 ng/ml Mean (±SD) whole blood selenium concentration at 3-4 weeks (baseline): 77.6±14.4 ng/ml, 1 month: 129±36.9 ng/ml, 2 months: 148±33.3 ng/ml, 3 months: 147±35.0 ng/ml Maternal selenium supplementation increased breast milk selenium (p<0.001) Maternal whole blood and plasma selenium was associated with breast milk selenium concentration (r=0.591,p<0.0001 and r=0.612, p<0.0001, respectively)	+
Trafikowska 1998	Intervention, no randomisation (n=67) Poland	Supplements were given by investigators during lactation. Group 1 (n=24): 200 Ilg selenium per day in the form of yeast-rich-Se. Group 2 (n=30): 200 Ilg selenite-selenium per day mixed with baker's yeast. Group 3 (n=13): plain brewer's yeast with no added Se (placebo)	4 samples collected at baseline (3-5 weeks postpartum) and after 1, 2 and 3 months of selenium supplementation. Milk samples were collected by manual expression prior to the first morning feeding of the blood sampling day. About 10 ml milk was collected in polyethylene bottles after the breast had been washed with deionized water Fluorometric method of Watkinson was used for analysis Infants were exclusively breastfed	Supplement group Plasma and whole blood collected at baseline and after 1, 2 and 3 months of supplementation	Mean (±SD) breast milk selenium concentration baseline: 8.9 ± 2.81 µg/l (no difference between groups). Mean breast milk selenium, placebo group, 1 month: 8.1±2.6 µg/l, 3 months: 7.0±2.5 µg/l (Concentrations for the different groups after supplementation are presented in a figure, with no actual concentrations reported) Mean (±SD) whole blood selenium at baseline 76.6±15.2 µg/l and plasma selenium 53.2±14.2 µg/l (Concentrations for the different groups after supplementation are presented in a figure, with no actual concentrations reported)	+

Table 2. Studies on the relationship between maternal nutritional status or dietary intake and breast milk mineral concentrations

First author, year	Study design (sample size) and setting	Micronutrient supplementation status	Milk samples	Maternal nutritional assessment	Main results	Relationship
					Maternal selenium supplementation during lactation increased breast milk selenium concentration for group 1 ($p<0.0001$) and group 2 ($p<0.001$) Maternal whole blood selenium was associated with breast milk selenium in group 1 ($r=0.591$, $p=0.0001$) and in group 2 ($r=0.675$, $p=0.0001$). Maternal plasma selenium was associated with breast milk selenium in group 1 ($r=0.612$, $p=0.0001$) and in group 2 ($r=0.668$, $p=0.0001$)	
Valent 2011	Observational (n=100) Italy	No supplements were given by investigators	1 sample collected (not clear when it was collected, likely 3 months postpartum) Participants were allowed to collect their breast milk at any time, no indication was provided to them on when to express Hydride generation-atomic fluorescence spectrometry (HGAFS) system was used for analysis 82% of infants were exclusively breastfeed	FFQ at 3 months postpartum asking about habitual intake during pregnancy and lactation	Mean (±SD) breast milk selenium concentration: 12.1±3.0 ng/g Maternal egg intake during pregnancy was associated with breast milk selenium ($r=0.20$, $p=0.04$) Maternal fish intake during lactation was associated with breast milk selenium ($r=0.21$, $p=0.04$)	+
Wasowicz 2001	Observational (n=131) Poland	No supplements were given by investigators	3 samples collected: 0-4 days (colostrum, n=43), 5-9 days (transitional milk, n=46)) and 10-30 days (mature milk, n=41). Milk samples (5–7 mL) were hand expressed after subjects cleaned the nipple and areola with deionized water The fluorometric method of Watkinson was used for analysis	Plasma collected at 0-4 days (n=43), 5-9 days (n=46) and 10-30 days (n=41)	Mean (±SD) breast milk selenium concentration, colostrum: 22.8±10.1 µg/l, transitional milk: 11.3±3.8 µg/l, mature milk: 9.2±3.6 µg/l Mean (±SD) plasma selenium concentration: 0-4 days: 34.9±11.8 µg/l, 5-9 days: 44.6±12.7 µg/l, 10-30 days: 54.3±14.6 µg/l Maternal plasma selenium was associated with transitional milk selenium ($r=0.310$, $p<0.05$), and mature milk selenium ($r=0.382$, $p<0.05$). No association was found with colostrum (statistics not shown)	+ ÷
Zhao 2014	Observational (n=90)	No supplements were given by investigators	1 sample collected between 5−240 days postpartum. All were instructed to empty one breast during 6 to 7 a.m. At 9 to 11 a.m., the full	FFQs and one cycle of 24-hour dietary recall during lactation	Mean (±SD) breast milk selenium concentration: 5-11 days 21.0±9.1 mg/kg, 12-30 days 17.8±7.5 mg/kg, 31-60 days	÷

Table 2. Studies on the relationship between maternal nutritional status or dietary intake and breast milk mineral concentrations

First author, year	Study design (sample size) and setting	Micronutrient supplementation status	Milk samples	Maternal nutritional assessment	Main results	Relationship
	China (Beijing, Guangzhou and Suzhou, urban areas).		milk of one breast (which was emptied before) was collected using an electric breast pump. Foremilk and hindmilk were gently mixed. Inductively coupled plasma mass spectrometry was used for analysis		19.5±8.5 mg/kg and 61-120 days 15.1±7.5 mg/kg and 121-124 days: 14.3±7.2 mg/kg Maternal dietary selenium intake was not associated with breast milk selenium (r=0.055, p=0.26)	
Zinc						
Chierici 1999	Intervention, no randomisation or placebo group (n=32) Italy	Supplements were given by investigators during lactation, containing 20 mg zinc sulphate per day (and 2 mg copper sulphate and 116 μg potassium iodide)	3 samples were collected at 3, 30 and 90 days postpartum (n=11). Milk was collected using a breast pump after the participants had cleaned the nipple and areola with deionized water. 10 ml milk sample was pumped before the baby nursed Inorganic mass spectrometer was used for analysis	Supplement group	Mean (±SD) breast milk zinc concentration, day 3, not supplemented group (NS): 8.16±2.96 mg/l, supplemented group (SG): 5.89±2.65 mg/l, day 30 NG: 3.99±1.01 mg/l, SG: 3.36±1.40 mg/l, day 90 NS: 2.87±1.23 mg/l, SG: 2.63±1.23 mg/l Maternal zinc supplementation during lactation did not increase breast milk zinc (statistics not shown)	÷
Dijkhuizen 2001	Observational (n=155) Indonesia	No supplements were given by investigators	1 sample collected between 2.4-10.5 months postpartum. Breast milk was obtained from the right breast 45–60 min after the last feeding from that breast. The breast was completely expressed and all milk was collected Flame atomic absorption spectrophotometry was used for analysis	Plasma collected at the same time as breast milk sample	Median (IQR) breast milk zinc concentration: 30.3 (20.5, 47.2) μmol/l Mean (±SD) plasma zinc: 12.6±2.7 μmol/l Maternal plasma zinc was not associated with breast milk zinc (statistics not shown)	÷
Dijkhuizen 2004	Randomised, double-blind, placebo controlled trial (n=170) Indonesia	Supplements were given daily during pregnancy until delivery (supplementation started <20 weeks gestation). Group 1: β-carotene (4.5 mg), group 2: zinc (30 mg), group 3: β-carotene (4.5mg) and zinc (30 mg), group 4: placebo. All groups were also given iron (30 mg) and folic acid (0.4 mg)	2 samples collected at 1 and 6 months postpartum. Breast milk was collected from the right breast 45–60 min after the last feeding from that breast. The breast was completely expressed with the use of a manual pump	Supplement group Serum collected at 6 months postpartum	Median (range) breast milk zinc concentration, 1 month, group 2: 49.3 (31.3, 62.1) μmol/l, placebo: 42.1 (31.1, 51.7) μmol/l. 6 months, group 2: 15.3 (11.8, 27.2) μmol/l, placebo: 16.8 (11.2, 24.3) μmol/l Mean (±SD) serum zinc 6 months, group 2: 11.4±1.4, μmol/L placebo: 11.7±1.3 (no significant difference between groups) Maternal zinc supplementation (30 mg/day) during pregnancy did not increase breast milk zinc at 1 or 6 months postpartum (statistics not reported)	÷

Table 2. Studies on the relationship between maternal nutritional status or dietary intake and breast milk mineral concentrations

First author, year	Study design (sample size) and setting	Micronutrient supplementation status	Milk samples	Maternal nutritional assessment	Main results	Relationship
Donangelo 1989	Observational (n=83) Brazil	No supplements were given by investigators	1 sample collected between 9.00 and 10.00 am by manual expression before the infant was due to be fed (5-1 0ml) (stage of lactation was mixed, between 1-180 days) Atomic absorption spectrophotometry was used for analysis Infants were exclusively breastfed	Serum collected at the same time as breast milk sample	Mean (±SE) breast milk zinc concentration, 1-5 days postpartum (n=17): 5.94±0.55 µg/ml, 6-30 days (n=13): 2.84±0.37 µg/ml, 31-280 days (n=10): 1.65±0.38 µg/ml Mean (±SE) serum zinc concentration, 1-5 days postpartum (n=19): 0.54±0.02 µg/ml, 6-30 days (n=18): 0.79±0.04 µg/ml, 31-280 days (n=12): 0.76±0.05 µg/ml Maternal serum zinc was not associated with breast milk zinc at any stage of lactation (statistics not reported)	÷
Dumrongwongsiri 2015	Observational (n=176) Thailand	No supplementation were given by investigators	1 sample was collected (n=34) between 4-6 months postpartum. Milk collection was done during 2:00-4:00 p.m. on the visit day using an electric breast pump. Nipples and areolas were cleaned with deionized water before milk collection. The first 15-20 ml of from one breast was obtained Inductively coupled plasma mass spectrometry was used for analysis Infants were either breastfed (exclusively and predominantly) or mixed feed	Serum collected between 4-6 months postpartum (n=44)	Median (max, min) breast milk zinc concentration between 4 and 6 months: 1.57 (3.2,0.50) mg/l Maternal serum zinc was associated with breast milk zinc (r=0.56, p=0.016)	+
Feeley 1983	Observational (n=102) Greece	No supplements were given by investigators, but 40% of participants took zinc supplements of at least 15 mg/d during pregnancy and lactation	3 samples collected, early transitional milk (days 4 to 7 postpartum), transitional milk (days 10 to 14 postpartum), mature milk (days 30 to 45 postpartum). 30 ml was collected, with one-third of the sample after let down and before feeding (foremilk); one third half-way through the feeding, and one-third after feeding (hindmilk)	Maternal self-administered supplementation information	Mean (±SEM) breast milk zinc concentrations in early transitional milk: 0.52.±0.02 µg/100g, in transitional milk: 0.41±0.01 µg/100g, in mature milk: 0.29±0.01 µg/100g Maternal zinc supplementation (self-reported) did not increase breast milk zinc (statistics not reported)	÷
Fung 1997	Observational (n=13) USA	No supplements were given by investigators, but some of the participants consumed zinc containing supplements	1 sample was collected between 7-9 weeks postpartum (breast milk collection method not clear) Laboratory method used not clear	3 day weighed food records collected at the same time as breast milk sample	Mean breast milk zinc concentration: 26.3 µmol/24h Mean (±SEM) dietary zinc intake: 14.2±0.7 mg/day including zinc supplements Mean (±SEM) urinary zinc concentration: 1.18±1.39 µmol/24h Mean plasma zinc concentration were not reported	÷

Table 2. Studies on the relationship between maternal nutritional status or dietary intake and breast milk mineral concentrations

First author, year	Study design (sample size) and setting	Micronutrient supplementation status	Milk samples	Maternal nutritional assessment	Main results	Relationship
				Urine collected at the same time as breast milk sample Plasma collected at the same time as breast milk sample	Maternal plasma zinc was not associated with breast milk zinc (statistics not reported) Maternal urine zinc was not associated with breast milk zinc (statistics not shown) Maternal dietary zinc intake was not associated with breast milk zinc (statistics not shown)	
Gross 1998	Observational (n=91) Indonesia	No supplements were given by investigators	5 samples collected from each participant for 5 consecutive days between 1-5 months postpartum (n=91). Milk from one breast was taken by a manual pump in the morning between 09.00 and 11.00. The entire content of one breast was taken from the breast that was not last suckled Inductively coupled plasma atomic emission spectroscopy was used for analysis	Plasma collected between 1-5 months postpartum (n=86)	Median (min, max) breast milk zinc concentration: 2.7 (0.5, 12.8) mg/l Mean (±SD) plasma zinc concentration: 855±242 µg/l Maternal plasma zinc was not associated with breast milk zinc (r=0.10, p>0.05)	÷
Hannan 2009	Observational (n=31) USA	No supplements were given by investigators	2 samples collected between 30-45 days postpartum (n=31), and between 75-90 days postpartum (n=17). Approximately 20–30 ml of milk was collected either by manual expression into pre-washed plastic vials, or via a pre-washed trace mineral-free breast pump and container. Flame atomic absorption spectrometry and neutron activation analysis was used for analysis	24-hour recall at two time-points	Mean (±SD) breast milk zinc concentration in early lactation: 2.1±1.4 mg/l and late lactation: 2.0±1.7 mg/l Mean (±SD) dietary zinc intake, early lactating: 6.1±4.6 mg/day, late lactation: 5.0±3.1 mg/day Maternal dietary zinc intake was not associated with breast milk zinc in early lactation (r=-0.03, p=0.9) however it was in late lactation (r=-0.48, p<0.05).	÷ +
Higashi 1982	Observational (n=65) Japan	No supplements were given by investigators	5 samples collected; at the first lactation (colostrum), one week (transitional milk), one month, 3 months and 5 months postpartum (mature milk). Milk were collected at morning by manual milking before the baby was due to be fed. 10 ml were obtained directly into clean polyethylene bottles, after breasts were cleaned twice with deionized water	Serum collected 3 months postpartum (n=44)	Mean (±SD) breast milk zinc concentration: colostrum: 10.39±4.43 mg/l, at 1 week: 4.56±3.01 mg/l, 1 month: 2.66±1.03 mg/l, 3 months: 1.14±0.67 mg/l, 5 months: 1.05±0.46 mg/l Mean (±SD) zinc concentration in serum: 0.76±0.13 µg/l Maternal serum zinc was not associated with breast milk zinc (statistics not reported)	÷

Table 2. Studies on the relationship between maternal nutritional status or dietary intake and breast milk mineral concentrations

First author, year	Study design (sample size) and setting	Micronutrient supplementation status	Milk samples	Maternal nutritional assessment	Main results	Relationship
			Atomic absorption spectrophotometry was used for analysis			
Khosravi 2007	Randomised double-blind, placebo controlled trial (n=138) Iran	Supplements were given through lactation. The participants were given 100 mg of elemental zinc weekly (n=67) or a placebo (n=71) after 1 week postpartum	6 samples were collected at 1 week (baseline) and 1, 2, 3, 4, 5 months postpartum. 5-10 ml milk were hand expressed Atomic absorption spectrophotometer was used for analysis Infants were exclusively breastfed	Supplement group	Mean (±SD) breast milk zinc concentration, 1 week (baseline), supplemented group (SG) (n=68): 310±138 µg/dl, placebo group (PG) (n=71): 322±161 µg/dl (not statistical significant). 1 month SG (n=56): 226±84 µg/dl, PG (n=64): 212±90 µg/dl. 2 months SG (n=49): 182±79 µg/dl, PG (n=59): 152±69 µg/dl. 3 months, SG (n=48): 159±73 µg/dl, PG (n=58): 129±57 µg/dl. 4 months, SG (n=45): 111±54 µg/dl, PG (n=56): 103±66 µg/dl. 5 months, SG (n=43): 118±64 µg/dl, PG (n=56): 109±70 µg/dl Maternal zinc supplementation did not increase breast milk zinc concentration at 1, 4 or 5 months postpartum (p=0.3, p=0.2, p=0.3, p=0.3 respectively). It did increase breast milk zinc at 2 and 3 months postpartum (p=0.02, p=0.005 respectively)	÷ +
Kirksey 1979	Observational (n=52) USA	No supplements were given by investigators, however 64% took zinc containing supplements	2 samples collected at 3 and 14 days postpartum (n=21). And for a sub-group 3 additional samples at 1-3 months (n=6), 5-7 months (n=8) and 1 year (n=5). Five to 10 mL of milk were obtained after milk let down at the first morning feeding. Samples were collected by manual expression into plastic vials Atomic absorption spectrophotometry was used for analysis	Maternal self-administered supplementation information	Mean (±SD) breast milk zinc concentration, day 3: 4.61±1.06 ppm, day 14: 3.12±1.02 ppm, 1-3 months: 2.04±0.54 ppm, 5-7 months: 0.93±0.47 ppm, 1 year: 0.45±0.16 ppm (milk concentrations were not reported according to supplement group) Maternal zinc supplementation did not increase breast milk zinc (statistics not reported)	÷
Krebs 1985	Intervention, no randomisation or placebo group (n=53) USA	Supplements were given during lactation. 15 mg of zinc was given daily (n=14), or nothing (n=39). 43% of supplemented participants started zinc supplementation during first trimester of pregnancy, due to participation in a different study	Breast milk samples were collected starting from 1 months postpartum, at monthly intervals thereafter until lactation was stopped. Milk samples of 5 ml were collected by manual expression directly into zinc-free polypropylene containers Flame atomic absorption spectrophotometry was used for analysis	Supplement group	Mean (±SD) breast milk zinc concentration, 1 month supplemented group (SG) (n=14): 2.83±1.05 µg/ml, non-supplemented group (NG) (n=25): 2.65±0.81µg/ml, 9 months SG (n=4): 0.82±0.54 µg/ml, NG (n=8): 0.67±0.40 µg/ml (concentrations at the remaining time-points were presented in a figure and not readable) Maternal zinc supplementation was associated with a rate of decline in milk zinc concentration that was significantly less than that for the non-supplemented group (p=0.03)	+
Krebs 1995	Randomised, double-blind,	Supplements were given through lactation, starting 2	10 samples collected at 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9 months postpartum. 5-10 ml were hand expressed	Supplement group	Mean (±SD) breast milk zinc concentration, 0.5 months (data pooled, as no difference between the two groups): 59.4±15.1	÷

Table 2. Studies on the relationship between maternal nutritional status or dietary intake and breast milk mineral concentrations

First author, year	Study design (sample size) and setting	Micronutrient supplementation status	Milk samples	Maternal nutritional assessment	Main results	Relationship
	placebo controlled supplementation trial (n=71) USA	weeks after delivery. A subgroup of participants were enrolled in pregnancy (<20 weeks of gestation). Participants were randomised to, group 1:a tablet containing 15 mg of a day (n=40) or, group 2: a placebo group (n=31)	after subjects cleaned the nipple and areola with deionized water. For each of the first 29 participants, a mid-feeding sample was obtained with every feeding and from each breast during 3 days at 0.5, 3, 5 and 7 months. The remaining 42 participants collected three mid-feeding samples per day, with more or equal to 4 hours between samples, during 3 days. For the remaining visits, a single sample was obtained Flame atomic absorption spectrophotometry was used for analysis	Plasma collected at 0.5, 3, 5 and 7 months postpartum (n=71) 3 day dietary records at 0.5, 3, 5 and 7 months postpartum (n=71)	µmol/l, 1 month: 46.8±17.1 µmol/l, 2 months: 31.2±12.7 µmol/l, 3 months: 22.6±9.9 µmol/l, 4 months: 22.0±10.1 µmol/l, 5 months: 17.7±9.5 µmol/l, 6 months: 16.7±10.2 µmol/l, 7 months: 13.0±7.8 µmol/l, 8 months: 13.3±8.9 µmol/l, 9 months: 11.9±7.8 µmol/l Mean (±SD) plasma zinc, 0.5 months supplemented group (SG): 12.2±1.5 µmol/l, placebo group (PG): 12.2±1.0 µmol/l, 3 months SG: 13.6±1.7 µmol/l, PG: 13.2±2.1 µmol/l, 5 months SG: 13.5±1.3 µmol/l, PG: 12.8±1.4 µmol/l, 7 months SG: 13.6±1.9 µmol/l, PG: 13.3±1.8 µmol/l (significantly different) Mean (±SD) dietary zinc intake was overall across the study period, SG: 25.7±3.9 mg/day (including the supplement), PG: 13.0±3.4 mg/day Maternal zinc supplementation during pregnancy or lactation did not increase breast milk zinc (p>0.05) Maternal plasma zinc was not associated with breast milk zinc (statistics not reported) Maternal dietary zinc intake was not associated with breast milk zinc (statistics not reported)	
Mahdavi 2010	Observational (n=182) Iran	No supplements were given by investigators	1 sample collected between 90-120 days postpartum. Breast milk (10–15 ml) samples were collected into metal free tubes by self-expression before nursing the baby in the morning Laboratory method used not clear Infants were exclusively breastfed	24-hour recall method for 3 days (one weekend day included)	Mean (±SD) breast milk zinc concentration: 1.93±0.5 mg/l Mean (±SD) dietary zinc intake was 6.1±2.5 mg/day Maternal dietary intake of zinc was not associated with breast milk zinc (β=0.12, p=0.1)	÷
Moser 1983	Observational (n=23) USA	No supplements were given by investigators	3 samples collected at 1, 2, and 6 months postpartum. 30 ml of breast milk was collected in the morning as a part of the first morning feeding after 6 am and contained approximately half foremilk and half hindmilk. Samples were hand expressed into polypropylene containers Atomic absorption spectrophotometry was used for analysis	Chemical analysis of 3-day duplicate plate food composites at 1, 3 and 6 months postpartum Plasma collected at 1, 3, and 6 months postpartum	Mean (±SEM) breast milk zinc concentration, 1 month: 2.6±0.2 µg/ml, 2 months: 1.3±0.1 µg/ml, 6 months: 1.1±0.1 µg/ml Mean (±SEM) plasma zinc, 1 month: 79.1±1.7 µg/100ml, 3 months: 87.6±2.2 µg/100ml, 6 months: 84.4±2.4 µg/100ml Mean (±SEM) dietary zinc intake, 1 month: 9.4±0.5 mg/day, 3 months: 12.8±1.8 mg/day, 6 months: 9.6±0.7 mg/day. Maternal serum zinc was not associated with breast milk zinc at any time-point (statistics not reported) Maternal dietary zinc	÷

Table 2. Studies on the relationship between maternal nutritional status or dietary intake and breast milk mineral concentrations

First author, year	Study design (sample size) and setting	Micronutrient supplementation status	Milk samples	Maternal nutritional assessment	Main results	Relationship
					intake was not associated with breast milk zinc at any time-point (statistics not reported)	
Moser-Villon 1990	Randomised, double-blinded controlled trial, no placebo group (n=40) USA	Supplements were given by investigators starting from delivery to 9 months postpartum. Participants were given a multiple micronutrient tablet daily, that only differed in amount of zinc and B_6, group 1: 0 mg zinc and 0.5 mg B_6 (n=10), group 2: 0 zinc and 4.0 mg B_6 (n=10), group 3: 25 mg zinc and 0.5 mg B_6 (n=10), group 4: 25 mg zinc and 4.0 mg B_6 (n=10)	6 samples collected at 1, 2, 4, 12, 24, 36 weeks months postpartum. Samples was collected into polypropylene containers from the first breastfeed of the day, by manually expression. Half from the beginning and half from the end of the feeding Atomic absorption spectrophotometry was used for analysis	Supplement group Plasma collected at 1 and 2 weeks and 1, 3, 6 and 9 months postpartum	Mean (±SEM) breast milk zinc concentration, 1 week group 1: 70.6±7.3 µmol/l, group 3: 66.7±9.4 µmol/l, 2 weeks group 1: 55.5±5.1 µmol/l, group 3: 54.5±5.7 µmol/l, 4 weeks group 1: 41.8±2.7 µmol/l, group 3: 40.2±4.7 µmol/l, 12 weeks group 1: 26.3±1.9 µmol/l, group 3: 19.4±3.0 µmol/l, 24 weeks group 1: 21.0±3.4 µmol/l, group 3: 12.6±2.5 µmol/l, 36 weeks group 1: 11.3±1.2 µmol/l, group 3: 9.8±0.6 µmol/l Mean (±SEM) plasma zinc concentration, 1 week group 1: 11.8±0.5 µmol/l, group 3: 12.3±0.4 µmol/l, 2 weeks group 1: 12.1±0.5 µmol/l, group 3: 13.3±0.4 µmol/l, 4 weeks group 1: 12.3±0.5 µmol/l, group 3: 13.5±0.4 µmol/l, 12 weeks group 1: 14.4±0.5 µmol/l, group 3: 14.1±0.4 µmol/l, 24 weeks group 1: 12.2±0.8 µmol/l, group 3: 13.1±1.1 µmol/l, 36 weeks group 1: 14.7±0.9 µmol/l, group 3: 14.0±1.1 µmol/l (not significantly different) Maternal zinc supplementation during lactation did not increase breast milk zinc (statistics not reported)	÷
Ortega 1997	Observational (n=57) Spain	No supplements were given by investigators	2 samples collected at day 13-14 (transitional milk) and 40 days (mature milk) postpartum. Samples were collected between 10 and 11 am by manual expression of a 5 ml sample from each breast at the beginning and end of a feed Atomic absorption spectroscopy was used for analysis	5 days dietary record and FFQs collected during third trimester of pregnancy, which was used to divide the participants into two groups 1) zinc intake <50% of RI, 2) zinc intake ≥50% of RI Serum collected during third trimester of pregnancy	Mean (±SD) breast milk zinc concentration, transitional milk, group 1: 46.7±7.3 µmol/l, group 2: 51.0±9.2 µmol/l, mature milk, group 1: 28.7±6.2 µmol/l, group 2: 33.1±8.0 µmol/l Mean (±SD) serum zinc third trimester of pregnancy group 1: 12.0±1.5 µmol/l, group 2: 13.3±2.6 µmol/l Mean (±SD) dietary zinc intake group 1: 8.3±1.0 mg/day, group 2: 12.3±1.9 mg/day (significantly different) Maternal serum zinc during third trimester of pregnancy was associated with breast milk zinc in transitional ($r=0.5275$, $p<0.05$) and mature milk ($r=0.6075$, $p<0.05$) Maternal dietary zinc intake during third trimester of pregnancy was not associated with transitional milk zinc ($p>0.05$), but with mature milk zinc ($p<0.05$)	+ ÷

Table 2. Studies on the relationship between maternal nutritional status or dietary intake and breast milk mineral concentrations

First author, year	Study design (sample size) and setting	Micronutrient supplementation status	Milk samples	Maternal nutritional assessment	Main results	Relationship
Rajalakshmi 1980	Observational (n=412 from urban areas, n=208 from rural areas) India	No supplements were given by investigators	1 sample collected between delivery and 13 months. Some participants delivered more than one sample. Samples were collected by manual expression just before the baby was due to feed. 10 ml were obtained and delivered into polyethylene centrifuge tubes Varian-Techtron-100 atomic absorption flame spectrophotometer was used for analysis	Serum collected between 1 and 6 months postpartum (n=152)	Mean (±SEM) breast milk zinc concentration, colostrum (n=76) 5.32±0.312 µg/ml, transitional (n=31) (6-10 days): 4.72±0.275 µg/ml, 11 days to 1 month (n=28): 3.33±0.266 µg/ml, 1-3 months (n=77): 2.00±0.101 µg/ml, 4-6 months (n=89): 1.33±0.055 µg/ml, 7-12 months (n=88): 1.12±0.052 µg/ml, 13 months and above (n=23): 1.16±0.116 µg/ml Mean (±SEM) serum zinc, 1-6 months: 118.1±3.990 µg/100ml Maternal serum zinc was not associated with breast milk zinc between 1 to 6 months postpartum (β=-0.12, p>0.05)	÷
Samuel 2014	Observational (n=50) India	No supplements were given by investigators	3 samples were collected at 1, 3 and 6 months postpartum. Participants provided three mid-feeding samples of 5 ml, one each in the morning, afternoon and evening. Milk samples were collected in acid washed plastic bottles by hand expression after the nipple and areola were cleaned with deionised water Flame atomic absorption spectrophotometry was used for analysis	Serum collected at 3 and 6 months postpartum Interviewer-administered 24-hour recall collected at 1, 3 and 6 months postpartum	Median (IQR) breast milk zinc concentration, 1 month: 2.50 (2.01, 3.26) mg/l, 3 months: 1.37 (0.89, 1.79) mg/l, 6 months: 1.17 (0.80, 1.60) mg/l Mean (±SD) serum zinc at 3 months: 83.5±26.5 µg/l, 6 months: 92.2±25.1 µg/l Mean (±SD) dietary zinc intake, 1 month: 7.6±2.9 mg/day, 3 months: 7.7±2.3 mg/day, 6 months:7.8±2.8 mg/day Maternal serum zinc was not associated with breast milk zinc (statistics not reported) Maternal dietary zinc intake was not associated with breast milk zinc (statistics not reported)	÷
Severi 2013	Observational (n=151) Uruguay	No supplements were given by investigators	1 sample collected at 4 months postpartum. After washing the breast with de-ionized water, milk samples of 5 ml were collected at 5 minutes of feeding by manual expression directly into zinc free polypropylene containers Flame atomic absorption spectrophotometer was used for analysis	Plasma collected in early pregnancy (<14 weeks gestation)) and at 4 months postpartum (n=123) (not clear if pregnancy or lactation plasma data was used to assess association with breast milk zinc)	Median breast milk zinc concentration: 1.20 mg/l Mean (±SD) plasma zinc in early pregnancy: 85.2±13.6 µg/dl, 4 months: 84.6±12.2 µg/dl Maternal plasma zinc was not associated with breast milk zinc (r=-0.02, p>0.05)	÷
Stuetz 2012 (eur)	Pre-post intervention, no randomisation	Zinc fortified wheat flour (estimated daily intake: 2.7 mg) was provided for all	2 samples were collected 1 before fortification of flour was introduced at 12 weeks postpartum and 1 sample after flour fortification was introduced (two	Fortification (Pre-post intervention design, the study used pre-	Geometric mean breast milk zinc concentration breast milk, before fortification: 1.78 mg/l, after fortification: 1.79 mg/l	÷

Table 2. Studies on the relationship between maternal nutritional status or dietary intake and breast milk mineral concentrations

First author, year	Study design (sample size) and setting	Micronutrient supplementation status	Milk samples	Maternal nutritional assessment	Main results	Relationship
	(n=86 before fortification, n=99 after fortification) Thai-Myanmar border (Maela Refugee camp)	participants (the flour was also fortified with other micronutrients)	different groups of women). Milk samples were collected by manual expression into glass tubes wrapped in aluminium foil in order to protect against degradation Inductively coupled plasma mass spectrometry was used for analysis	supplement concentrations as comparison group)	Maternal intake of zinc fortified flour did not influence breast milk zinc ($p>0.05$).	
Vaughan 1979	Observational (n=38) USA	No supplements were given by investigators	Monthly milk samples between 1-31 months postpartum. Participants remained in the study on an average of 4 consecutive months. 150-200 ml of milk was hand expressed into acid-washed glass. Participants were instructed to collect the milk over a period of 3 to 5 days, at morning, afternoon, and evening feedings and at random intervals within the feeding Atomic absorption spectrophotometer was used for analysis	Serum sample collected during the same 3 to 5-day period of milk collection for the particular month (n=24) 3 day dietary records. Intakes were recorded during the same 3 to 5-day period of milk collection (n=11)	Mean (±SEM) breast milk zinc concentration, 1-3 months: 1.60±0.23 (unit not clear) (n=28), 4-6 months: 1.05±0.15 (unit not clear) (n=39) Mean serum zinc concentration, 1-3 months: 1.7 μg/ml, 4-6 months: 2.3 μg/ml Mean dietary iron intake 4-6 months: 12.7 mg/day Maternal serum zinc was not associated with breast milk zinc from the same stage of lactation (statistics not reported) Maternal dietary intake of zinc was not associated with breast milk zinc from the same stage of lactation (statistics not reported)	÷
Vuori 1980	Observational (n=27) Finland	No supplements were given by investigators	Not clear how many breast milk samples was collected and when. Milk aliquots of 8 ml were obtained at the beginning and at the end of each feed during a period of 24-hours and pooled to one sample Ash solutions by the flame atomic absorption spectrophotometric method was used for analysis	Two 7 day food records. The first record from the first survey period between 6-8 weeks and the second record from the second survey period between 17 to 22 weeks postpartum (n=15).	Mean (±SD) breast milk zinc concentration, 6-8 weeks: 1.89±0.74 mg/l, 17-22 week: 0.72±0.44 mg/l Mean (±SD) dietary zinc intake: 13.7±2.7 mg/day in first survey period and 12.8±2.8 mg/day in second survey period Maternal dietary zinc intake was not associated with breast milk zinc (statistics not reported)	÷
Wasowicz 2001	Observational (n=131) Poland	No supplements were given by investigators	3 samples collected at 0-4 days (colostrum, n=43), 5-9 days (transitional milk, n=46) and 10-30 days (mature milk, n=41). Milk samples (5–7 ml) were hand expressed after subjects cleaned the nipple and areola with deionized water Inductively coupled plasma–atomic electron spectrometry was used for analysis	Plasma collected at 0-4 days (n=43), 5-9 days (n=46) and 10-30 days (n=41)	Mean (±SD) breast milk zinc concentration: colostrum 8.2±2.8 mg/l, transitional milk: 3.7±1.8 mg/l, mature milk: 1.4±0.7 mg/l Mean (±SD) plasma zinc concentration, 0-4 days: 0.51±0.13 mg/l, 5-9 days: 0.62±0.23 mg/l, 10-30 days: 0.76±0.20 mg/l Maternal plasma zinc was associated with breast milk zinc in colostrum ($r=0.506$, $p<0.001$). No association was found with transitional or mature milk (statistics not reported)	+ ÷

Table 2. Studies on the relationship between maternal nutritional status or dietary intake and breast milk mineral concentrations

First author, year	Study design (sample size) and setting	Micronutrient supplementation status	Milk samples	Maternal nutritional assessment	Main results	Relationship
Zhao 2014	Observational (n=90) China (Beijing, Guangzhou and Suzhou, urban areas).	No supplements were given by investigators	1 sample collected between 5−240 days postpartum. All were instructed to empty one breast during 6 to 7 a.m. At 9 to 11 a.m., the full milk of one breast (which was emptied before) was collected using an electric breast pump. Fore and hind milk were gently mixed. Inductively coupled plasma mass spectrometry was used for analysis	FFQs and one cycle of 24-hour dietary recall during lactation	Mean (±SD) breast milk zinc concentration, 5-11 days: 3.9±1.5 mg/kg, 12-30 days: 2.8±1.2 mg/kg, 31-60 days: 2.0±0.7 mg/kg, 61-120 days: 1.5±0.6 mg/kg, 121-124 days: 1.3±0.5 mg/kg Maternal dietary intake of zinc was not associated with breast milk zinc after adjusting for stage of lactation (r=-0.063, p=0.2)	÷

2.3 Discussion

This systematic literature review included 141 studies, all providing an insight to the influence of maternal micronutrient supplementation, food fortification, dietary intake and nutritional status on breast milk micronutrient composition in women living in contrasting settings.

It is clear that all micronutrients in human breast milk are not equally influenced by maternal nutritional intake or status. Generally, milk water-soluble vitamin composition was influenced by maternal intake and status (except for folate). Less influenced was breast milk fat-soluble vitamin composition, likely due to buffering effects of maternal stores, and reasonably unaffected was breast milk mineral concentrations (except for iodine and selenium). This overall conclusion was also reached by Allen (1994) (121) who non-systematically reviewed the evidence available up until 1994.

For some of the micronutrients, the maternal nutritional influences had a different influence in well-nourished compared to poorly-nourished women. For instance, thiamin supplementation only influenced the breast milk thiamin concentration of women with a poor thiamin status, as opposed to well-nourished women. This suggests that as maternal thiamin intake increases the concentration in milk is increased. However for many vitamins, the concentration reaches a plateau when the mother is sufficient and does not respond to further supplementation (27).

A difference in the influence was also seen between maternal status and maternal dietary intake for some of the micronutrients. For instance, maternal iodine intake (iodine supplements and fortified salt) was overall a better predictor of BMIC than maternal iodine status measured as urinary iodine. This is likely due to the uptake of iodine by the mammary gland compensating for inadequate maternal iodine status by reducing maternal iodine reserves (273). An association between urinary iodine and breast milk concentration is therefore often inconsistent.

Additional data are however needed to make any causal conclusions. For many of the micronutrients (especially the water-soluble vitamins) few studies of high-quality design were available. For instance, of the studies exploring the B-vitamins, only 6 out of all 55 included studies were RCTs.

The comparability of the included studies was compromised by their dissimilar design and methods, in relation to the following: breast milk collection methods, the time of breast milk collection according to stage of lactation, the reference period of maternal status/diet/supplement

(on the day of breast milk collection, a week prior to collection or during pregnancy), the lack of breast milk volume collection and information on infant feeding practices, the difference in study design and statistical methods, and the lack of adjustment for confounding variables in the observational studies. Many studies had a small sample size, which may have led to inconsistent or null results even with the existence of real associations.

There were many different breast milk sampling methodologies in the included studies, ranging from a full breast expression to before or mid-fed sample. Comparing studies with different sample methodologies is especially problematic when measuring breast milk fat-soluble vitamins. Fat-soluble vitamin concentrations are strongly associated with milk fat, and are found in higher concentrations in hindmilk than foremilk (131, 190, 197). A difference in mineral or water-soluble vitamin composition between foremilk and hindmilk has generally not been observed (105, 107, 274). A few studies have found higher thiamin and riboflavin concentrations in hindmilk (274-276), however the differences were small. Inconsistent differences in the composition of breast milk from the right and left breast have been observed (277), although the likely reason for this difference is due to mastitis (105). The gold standard collection is collecting all breast milk expressed over 24 hours, with multiple collections across lactation from the same mother (88). Nevertheless, this is often not feasible, alternatively a full breast expression, standardised to time of day can be used (88). No studies from this review used the gold standard collection method, and only a few studies used the second best option.

Comparability was further compromised by inconsistencies among the laboratory methods used. Using the example of iodine, it was recently demonstrated that the calorimetric method based on the Sandell-Kolthoff reaction, used in five of the included studies measuring breast milk iodine (237, 239, 242, 243, 246) is unreliable (278, 279). ICP-MS is now considered the gold standard for breast milk iodine analysis (280), and this method was used in only three of the included iodine studies (227, 235, 241). This is also the case for vitamin B_{12}, where a new method was developed in 2009, successfully removing vitamin B_{12} in breast milk from apo-HC (281), estimating that previous quantifying methods would have either under- or over reported the amount of vitamin B_{12} in breast milk depending on the method used for pre-treatment and subsequent assay (281).

Almost all the intervention studies included in this review, provided maternal supplementation during lactation, and only a few studies investigated the effect of maternal nutrition during pregnancy on breast milk micronutrient composition.

2.3.1 Conclusion

In conclusion, this systematic review of the available literature highlights a weakness in the evidence for the relationship between maternal nutritional status, intake and breast milk micronutrient composition for the majority of micronutrients reviewed, and how difficult and problematic it is to compare results across studies. There is a need for more high-quality data where breast milk samples are collected longitudinally, and where standard methods for breast milk expression, collection, laboratory analysis methods and statistical methods, adjusting for important confounding variables, are used.

Chapter 3

Setting: The Gambia

Data and samples used in Chapters 5 to 8 of this thesis were collected from a randomised trial conducted in the rural West Kiang region of The Gambia. The Gambia is a small, low-income country in West Africa, with two million inhabitants. Approximately 40% of the population live in rural areas (282).

West Kiang is a rural district in the Lower River Region and the population consists of approximately 14,000 people spread across 36 villages (283) (Figure 5). Keneba is the largest village in the West Kiang region (283). In the 1940's a Medical Research Council (MRC) funded research programme was established in this area by Professor Sir Ian McGregor, with the explicit remit to investigate the relationship between parasitic infections and malnutrition (284). In 1974, a permanent, MRC-funded field station was established in Keneba, initiating a permanent research programme alongside the provision of free health care to the West Kiang population (284).

In West Kiang, the majority of the population is of Mandinka ethnicity (80%). The remaining ethnicities are Fula (16%), Jola (2.4%) and other (1.3%) (283). The predominantly religion is Muslim, and polygamy is widely practised (283). Life expectancy of West Kiang women was 73 years and 65 years for men in 2013 (283), which is higher than the average life expectancy for The Gambia; 67 years for women and 63 years for men (282). This difference is likely due to better access to a higher standard of health care (283).

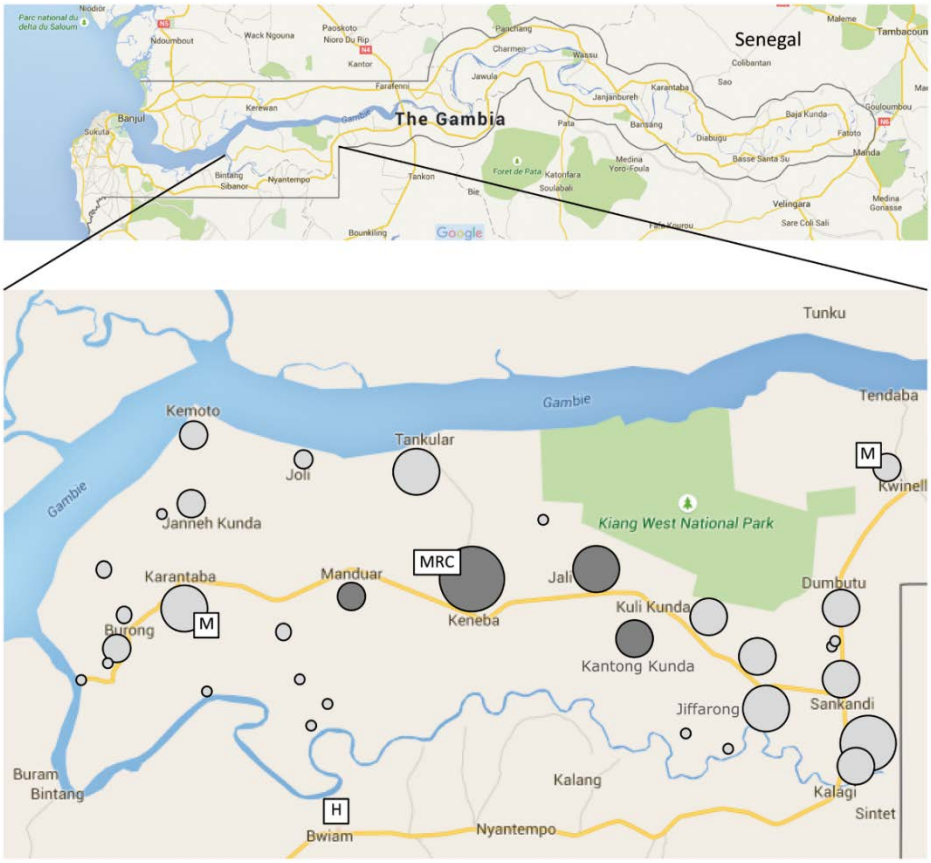

Figure 5. Location of MRC Keneba within West Kiang, The Gambia.

Village circle sizes are proportional to population, and dark grey villages are 'core' villages, which are the villages closest to the MRC. From Hennig et al (2015) (283).

The main livelihood in West Kiang is rural subsistence farming, and the country's economy relies predominantly on agriculture, making the population vulnerable to increasingly irregular rainfall and drought (285). Women are the main workforce in regards to agricultural labour and cooking (286), whereas men are mainly involved in groundnut cultivation, which traditionally is the main cash crop. The food consumed in West Kiang is mostly grown locally, and consists of a staple (rice, millets or maize) with a sauce made from a limited number of ingredients such as oil, ground nuts, green leaves, fish or vegetables (287). Meat intake is low in this population, with beef, goat or chicken being consumed only occasionally. Fish is consumed more regularly, due to the proximity to the river Gambia.

3.1 Seasonality

The environmental conditions in rural Gambia are characterised by a long, hot and dry season from November until May and a shorter wet season from June until October (288) (Figure 6). The staple crops are harvested between September and December, so food supplies are particular plentiful in November until December. However, because farming is dependent on the rains, there is a limited window for agriculture and the resulting crops are not enough to last for the entire year, and a 'hungry season' develops in June and lasts until October (288). An increase in infections are experienced during the wet season, including malaria, pneumonia and diarrhoea (289).

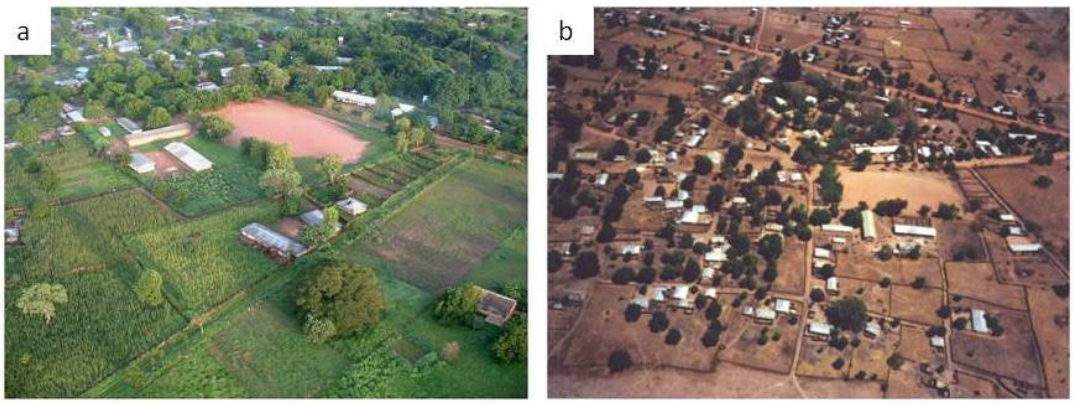

Figure 6. The Gambian rainy (a) and dry (b) season in Keneba, West Kiang.

Original photo source: Andrew Prentice.

This scarcity of food is further worsened by laborious seasonal farm work, where the land is cultivated during the hungry season. This results in an average weight loss of 3-6 kg in women in every hungry season (288). Even pregnant and lactating women participate in farm work, preparing the land prior to the hungry season, planting and weeding during the hungry season, and harvesting in the harvest season (290). The highly labour intensive work compounded by food shortage and increased exposure to infections, leads to a weight gain in pregnant women of 1400 g/month in the dry season and only 400 g/month in the hungry season (291). A difference in weight gain during pregnancy according to season has also been demonstrated in a more recent study (292).

Infants born in the hungry season are exposed to these nutritional stressors in mid-to-late foetal life, resulting in a lower birth weight compared with infants born in the harvest season (293). In this environment, birth during the annual hungry season is correlated with a higher mortality from infectious diseases in young adulthood (294). Seasonal effects have also been observed on prematurity and small-for-gestational age births (290).

3.2 Micronutrient status

Because of the low intake of fruit, vegetables, and animal source products, diets in West Kiang are low in micronutrients and the population has consequently a low status of several micronutrients.

3.2.1 Vitamins

Most of the work on vitamins in this population dates back to the 1980s. Moderate vitamin A deficiency has been recorded in pregnant and lactating women (295), along with riboflavin deficiency in infants and young children (81). Vitamin C status in this population is strongly determined by seasonal availability of mangoes, which are available during only May and June (183). Vitamin D status is adequate due to year long exposure to sunshine (296).

A more contemporary study by Dominguez-Salas et al (2013) (297) found that the mean dietary intake of folate, riboflavin, vitamin B_6 and choline in women of reproductive age over a 12 months period were significantly below the current international recommendations expressed as estimated average requirement (EAR). The intake of vitamin B_{12} was found to be 2.7 μg/day, just above the EAR of 2.0 μg/day. The low intake of several micronutrients has resulted in deficiencies being commonly experienced. Among these women, Dominguez-Salas et al (2013) (297) found that a significant percentage of the study population was below the range of adequacy for riboflavin (90%), choline (79%), vitamin B_{12} (19%) and folate (18%). For total vitamin B_6 the mean concentration was 39.7 nmol/l, however no suitable cut-off for adequacy was identified. Thiamin deficiency has historically not been an issue in this setting, however back in 1988 an outbreak of beri-beri occurred, which was resolved with distribution of thiamin supplements (298).

3.2.2 Minerals

Calcium has been of particular interest in this rural Gambian population, with the implementation of several studies over the years (e.g. (211, 212, 299)). Calcium intake and status are low in this population; however, a prenatal calcium supplementation did not benefit breast milk calcium concentrations, infant weight, growth or bone mineral status (211). Gambian women have likely adapted to a low dietary calcium intake (299). Iron physiology and deficiency has also been widely investigated in the West Kiang population (e.g (300-303)), especially in pregnant women who have high rates of anaemia.

Iodine status has rarely been investigated in rural Gambia. Initial results from a recent pilot study found iodine status to be low in this rural population. School children (n=204) had a median urinary iodine concentration of 50 μg/l (cut-off for insufficient intake is a median <100 μ/l) and lactation women (n=63) had a concentration of 39 μg/l (cut-off for insufficient iodine intake is a median <100 μ/l), indicating persistence of moderate iodine deficiency (R Wegmüller & M Andersson, unpublished data). National salt iodisation programmes have been implemented in The Gambia, however the coverage of adequate iodised salt is poor. A MICS survey from 2010 tested iodine levels in household salt, and found that only 22% of the households were using adequately iodised salt (304). This percentage is likely to be even lower in rural areas, as here it is estimated that 80% of the women locally source their salt by the river instead of purchasing the iodised salt available in the local shops (S Dalzell, unpublished data).

3.3 Infant and childhood growth

Rural Gambian infants are born small, show catch up growth in the early months, and after around three months of life they show growth faltering for the remainder of infancy and early childhood (54, 74). A recent retrospective cohort study, which used routine growth monitoring data collected from infant welfare clinics in Keneba, showed that between year 2000 and 2012, 30% of rural Gambian children were stunted, 11% wasted and 22% underweight at two years of age (74).

3.4 Aims and objectives (II)

The systematic literature review presented in Chapter 2, highlighted that human milk micronutrient composition is influenced by the mother's nutritional status and intake of especially water-soluble vitamins and iodine and selenium. Micronutrients most important for public health nutrition can therefore be divided into two groups; group I nutrients (thiamin, riboflavin, vitamin B_6, vitamin B_{12}, vitamin A, vitamin D, selenium, and iodine) are of most interest because the concentrations are considerably reduced by maternal depletion. In contrast, the concentration of group II nutrients (folate, calcium, iron, copper, and zinc) in breast milk is relatively unaffected by maternal nutritional status or dietary intake.

Chapters 6, 7 and 8 of this thesis focus on the following micronutrients: *thiamin*, *riboflavin*, *vitamin B_6*, *vitamin B_{12}*, and *iodine*. These micronutrients were selected because, as shown in Chapter 2, maternal nutritional status and intake influence the concentrations of these five micronutrients in breast milk. They were furthermore selected because as above presented evidence suggests that maternal status and dietary intake of these micronutrients are low in rural Gambia, increasing the risk of maternal and infant deficiency of particular these micronutrients.

From the systematic literature review it is also clear that few studies, and even less trials, exist on how maternal nutrition during pregnancy influence breast milk micronutrient composition. As presented in the introduction in Chapter 1, prenatal multiple micronutrients supplementation have a positive impact on birth outcomes, however whether this positive effect is carried over into the lactation period to benefit the mother, breast milk composition and the infant is unclear.

The remaining research *objectives* of this thesis are to:

1. Identify how multiple micronutrient supplementation during pregnancy influences maternal iodine status, breast milk iodine concentration and infant iodine status in rural Gambia (*Chapter 6*).

2. Identify how multiple micronutrient supplementation during pregnancy influences maternal vitamin B_{12} status, breast milk vitamin B_{12} concentration and infant vitamin B_{12} status in rural Gambia (*Chapter 7*).

3. Identify how multiple micronutrient supplementation during pregnancy influences maternal riboflavin and vitamin B_6 status and breast milk thiamin, riboflavin and vitamin B_6 concentrations in rural Gambia (*Chapter 8).*

Chapter 4

Methods and subjects

In Chapters 5 to 8, data and samples collected as part of the Early Nutrition and Immune Development (ENID) trial, were used. This Chapter provides an overview of the ENID trial, a detailed description of the exposure and outcome measures used in this thesis and details of the overall statistical methods used. Specific laboratory and statistical descriptions relevant to individual Chapters are not described here, but embedded within the relevant Chapters.

4.1 Study design and population

The randomised, partially blinded trial, ENID (ISRCTN49285450), was conducted in The Gambia between April 2010 and February 2015, with the main outcome to assess the effect of combined prenatal and infant nutritional supplementation on infant immune development (305). The analyses presented in this thesis use ENID data, however these analyses were not planned in the original study design.

Pregnant women were randomised to a nutritional supplement when they booked for antenatal care, which was before 20 weeks of gestation (baseline), with supplementation continuing until delivery. Their infants were further randomised from 6 to 12 months of age to a micronutrient supplement or placebo (305). In this thesis infant growth data from the ENID-Growth study was additionaly used, which was an extension to the main ENID trial, where the infant supplementation was continued up to 18 months of age, with infants followed to two years of age. In addition maternal biological samples (urine and postpartum blood) that were collected from ENID-Bone, an extension to the ENID trial on bone health were used.

The trial took place in the West Kiang region of The Gambia a rural, savannah region where the community relies on subsistence farming. The trial was based in all 36 villages registered in the West Kiang Demographic Surveillance System (DSS) (283). Using the DSS, all women of reproductive age (18-45 years) were invited to participate in the ENID trial. The exclusion criteria were women: who were currently pregnant (>20 weeks gestation), had a multiple pregnancy, currently enrolled in another study, severely anaemic (haemoglobin < 7 g/dl), HIV positive, or who had reached the onset of menopause (305). Each month starting from enrolment, a fieldworker interviewed participating women, and if menses was missed, a urine sample was collected for pregnancy testing. Women that were confirmed to be pregnant (and <20 weeks gestation, confirmed by ultrasound), were randomised into the trial and started supplementation the following week and continued until delivery (305).

The ENID, ENID-Growth and ENID-Bone studies were approved by the joint Gambia Government/MRC Unit The Gambia Ethics Committee (project numbers SCC1126v2, L2010.77 and L2009.66, respectively). Written informed consent was obtained from all women prior to enrolment into the trial. The trial observed Good Clinical Practice Standards and the current version of the Helsinki Declaration and was registered at ISRCTN49285450.

4.1.1 Intervention in pregnancy and infancy

Pregnant women were randomised and partially blinded to one of the following four intervention arms in early pregnancy (<20 weeks gestation) (305):

1. Iron-folic acid (FeFol), representing the usual standard of care during pregnancy as per Gambian Government Guidelines (iron 60 mg/day, folic acid 400 μ/day)
2. Multiple micronutrients (MMN), a combination of 15 micronutrients specifically designed and formulated by UNICEF, the World Health Organization (WHO) and United Nations University (1999) (306) for use during pregnancy in effectiveness trials in resource-poor settings. With the exception of iron and folic acid each tablet contained twice the recommended daily allowance (RDA) of each micronutrient. The decision to supplement at twice the RDA was based on a study from West Africa suggesting that 2xRDA were more effective with regard to birth outcomes (307). The FeFol and MMN supplements were formulated as tablets and manufactured by Scanpharm, Birkerød, Denmark.

3. Protein-energy and iron-folic acid (PE + FeFol), a lipid based nutritional supplement (LNS) providing the same amount of iron and folic acid as the FeFol arm but with the addition of energy, protein and lipids. The daily supplement contained 746 kilocalories.
4. Protein-energy and multiple micronutrients (PE+MMN), a micronutrient fortified LNS product providing the same level of micronutrients to the MMN arm (including iron and folic acid) in addition to energy, protein and lipid content. The two LNS products were manufactured by Valid Nutrition, Nairobi, Kenya. Table 3a details the nutrient composition of the four pre-natal supplement groups. Table 3b details the nutrient composition of the infant supplement.

Within the ENID study protocol, nurses, midwives, field assistants and community health workers were trained in optimal breastfeeding practices. However, no counselling to the participating women was implemented, beyond what is standard practice in this baby friendly community. ENID participants were encouraged to exclusively breastfeed, and at six months of age their infants were randomized to either an unfortified LNS supplement, or to the same LNS formulation fortified with multiple micronutrients (total energy of supplement: 108 kcal). Table 3b details the nutrient composition of the infant supplement. The ENID trial design is illustrated in Figure 7. The main focus of this thesis is on the antenatal arm of the ENID trial.

Table 3a. Nutritional composition of daily intake of pregnancy supplements. From Moore et al (2012) (305).

	Tablets		**LNS**	
	FeFol	**MMN**	**PE+FeFol**	**PE+MMN**
Iron (mg)	60	60	60	60
Folate (μg)	400	400	400	400
Vitamin A (RE μg)		1600	2.85	1600
Vitamin D (IU)		400	-	400
Vitamin E (mg)		20	4.2	20
Vitamin C (mg)		140	2.25	140
Vitamin B1 (mg)		2.8	0.3	2.8
Vitamin B2 (mg)		2.8	0.45	2.8
Niacin (mg)		36	1.35	36
Vitamin B_6 (mg)		2.8	0.15	2.8
Vitamin B_{12} (μg)		5.2	0.1	5.2
Zinc (mg)		30	3.3	30
Copper (mg)		4	1.05	4
Selenium (μg)		130	6.15	130
Iodine (μg)		300	2.6	300
Energy (kcal)			746	746
Protein (g)			20.8	20.8
Lipids (g)			52.6	52.6

FeFol, iron-folic acid; MMN, multiple micronutrients; PE, protein-energy.

Table 3b: Nutritional composition of daily intake of infant supplements. From Moore et al (2012) (305).

	Placebo	**MMN**
β-Carotene (μg RE)	1.84	400
Vitamin C (mg)	1.88	30
Folic acid (μg)	13.1	80
Thiamine (mg)	0.06	0.3
Riboflavin (mg)	0.04	0.4
Vitamin B3 (mg)	0.32	4
Pantothenic acid (mg)	0.08	1.8
Vitamin B6 (mg)	0.02	0.3
Vitamin B12 (μg)	0.06	0.5
Vitamin D (μg)	0.34	5
Vitamin E (mg)	0.12	2.7
Vitamin K (μg)	1.40	10
Iron (mg)	0.46	9
Zinc (mg)	0.24	4
Calcium (mg)	33.1	100
Potassium (mg)	91.76	152
Copper (mg)	0.02	0.2
Selenium (μg)	1.44	10
Iodine (μg)	1.40	90
Phosphorus (mg)	42.56	82
Magnesium (mg)	14.56	16
Manganese (mg)	0.08	0.08
Total energy (kcal)	108	108
Linoleic acid (g)	1.29	1.29
Linolenic acid (g)	0.29	0.29

MMN, multiple micronutrients. Data in shaded cells represent MMN content from base ingredients.

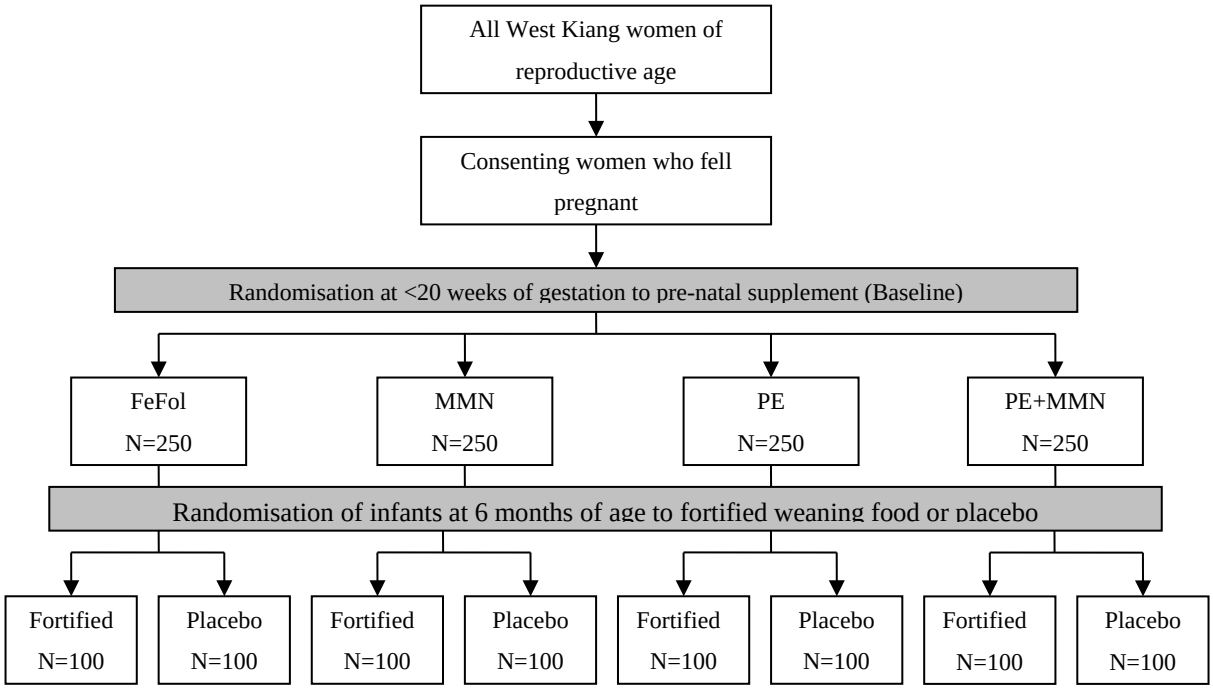

Figure 7. ENID trial design.

From Moore et al (2012) (305).

Antenatal supplements were given on a weekly basis by field workers. Compliance of tablets was assessed through a count on remaining tablets at the end of each week, and for the LNS-based supplement a score based on the amount of supplement left remaining in the jar was made (empty, half-empty, full). Women who received the tablet supplements had higher rates of compliance (FeFol and MMN, 95.7% and 93.1%, respectively) than women who received the LNS-based supplements (PE and PE+MMN, 81.7 % and 81.2%; p-value for difference between tablet and LNS groups $p<0.0001$) (Moore et al in preparation).

A total of 2,798 women consented to the study. Of these, 875 were eligible for supplementation. A total of 799 had live singleton births, with infants born between August 2010 and February 2015, and 723 mother-infant pairs completed the study till the one-year follow up. Loss to follow up was primarily due to migration, infant death or severe acute malnutrition. Severely malnourished infants were withdrawn from the trial and admitted to the MRC-led nutritional rehabilitation centre in Keneba.

4.2 Post hoc power calculation

In Chapters 6, 7 and 8, the analyses of blood (maternal and infant), urine (maternal) and breast milk only include participants from the tablet arm of ENID (intervention group 1 and 2), excluding participants randomised to the prenatal PE arm (intervention group 3 and 4). This was done due to the significant difference in compliance between the two study arms. Additionally, including only the tablet arm of the study distinguishes the effect of multiple micronutrients and thereby, excluding potential influences that PE supplementation may have on the outcome.

A post-hoc power calculation justified this decision to only include participants form the tablet arm of ENID. Using a population variance of 40 (based on previous studies' standard deviation of breast milk iodine concentration, see Chapter 2) a sample size of 200 in each of the tablet groups gives 96% power to detect a difference of 15 µg/l or more between FeFol and MMN arms, and 71% power to detect a difference of 10 µg/l.

4.3 Data collection

Figures 8 and 9 show the complete ENID and ENID-Growth data collection (pre)-pregnancy, at delivery and in infancy.

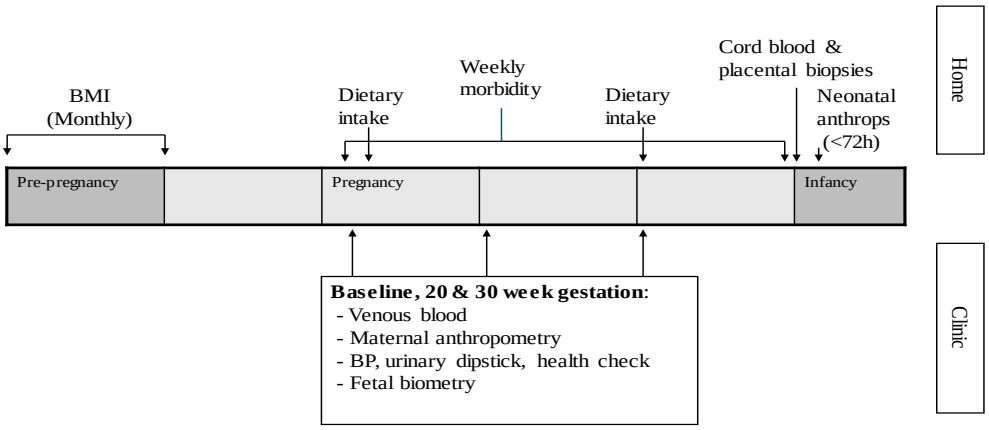

Figure 8. ENID pre-pregnancy, pregnancy and delivery protocol.

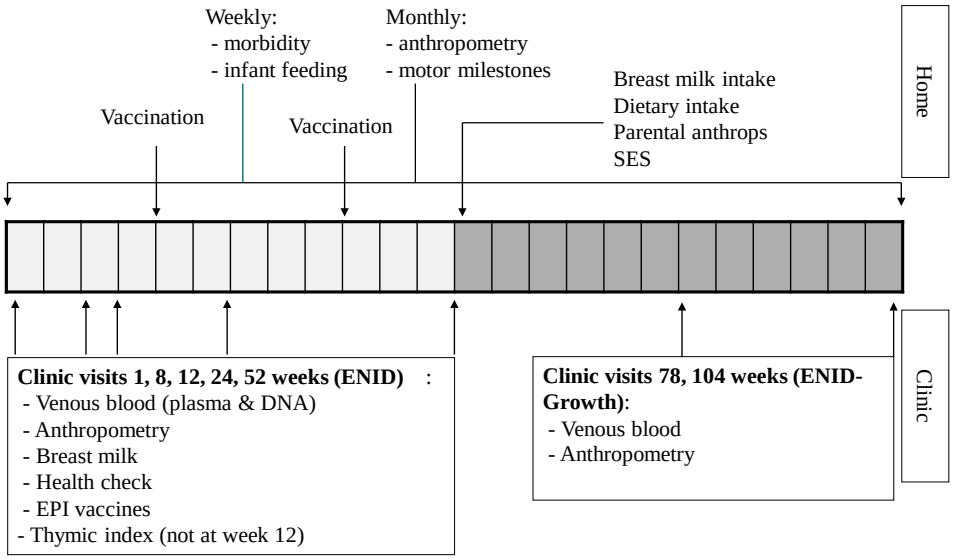

Figure 9. ENID and ENID-Growth infancy protocol.

4.3.1 Anthropometry

Infants had anthropometric measurements taken at birth (within 72 hours of delivery) and at scheduled visits to the MRC Keneba field station at 1, 8, 12, 24, 52, 78 and 104 weeks of age, with additional home visits at 16, 20, 32, 40, 65 and 91 weeks by trained field workers (Figures 8 and 9). An embedded sub-study[3] measured approximately 200 infants at the additional time-points of 4, 28, 36, 44 and 48 weeks of age, using the same procedures and anthropometric equipment as the main follow up. Data from all time-points were included in the analysis in Chapter 5.

The following anthropometric measurements were collected at all visits: weight and length, mid-upper-arm, thigh, head, abdominal-, and waist circumferences, knee-heel length and thigh, triceps, biceps, suprailiac and subscapular skinfold thicknesses. All field workers collecting the data were trained in infant anthropometric assessments. All weights and lengths were measured using electronic scales and length boards, which were precise to 10 g, and to 1 mm. Fixed length boards (Seca 417) were used for all clinic visits and field visits after the neonatal home visit, where a flexible length mat was used.

4.3.2 Infant feeding practice and morbidity data

From birth until the infant reached two years of age, trained field workers collected weekly infant feeding data by questionnaire. The infants were seen seven times at the MRC Keneba field station (weeks 1, 8, 12, 24, 52, 78 and 104 of infant age), and the remainder of data were collected at home visits (Figure 9). Structured questionnaires were used for data collection, and the flow of the questions is shown in Figure 10. The mother was asked to recall infant feeding practices in the previous seven days, i.e. if the infant was breastfed; if other foods or drinks had been introduced; and the frequency of these other foods and/or drinks. The drinks and foods included in the questionnaire and are available in Appendix 1 (Breastfeeding questionnaire). The field workers were trained in probing techniques, and were instructed to ensure that the mothers fully understood the questions asked. Infant feeding data are included in the analysis in Chapter 5.

[3] ENID-bioactive investigated the effects of maternal and grand-maternal birth season, and maternal breast milk bioactive factors on infant growth and intestinal inflammation. The data for this sub-study was collected in the field and not at the MRC Keneba field station.

1. Are you currently breastfeeding your infant? Yes ☐ No ☐

2. In the past 7 days, have you given your infant anything other than breast milk? Yes ☐ No ☐

If no, the questions are completed. If yes, please proceed to Question 3.

3. What other foods/drinks have been given, in the past 7 days

Drinks		Comments
Water	Once ☐ > Once ☐ Most days ☐ Never ☐	

Figure 10. Flow of infant feeding questionnaire in ENID.

Simultaneously with collecting infant feeding data, the mother was asked if the infant had experienced any diarrhoea, vomiting (not associated with eating), cough, rapid breathing, or fever in the past seven days. In addition, the mother was asked the number of days the infant had been sick, and whether the infants had been taken to a health facility and/or received any treatment (Appendix 1. Infant morbidity questionnaire). In the analyses in Chapter 5, infant diarrhoea was defined as having three or more loose stools a day and infant morbidity incidence rate was defined as combined episodes of either diarrhoea, vomiting, cough, rapid breathing, fever.

4.3.3 Other anthropometric and demographic variables

Maternal height and weight were collected at baseline when the mother was less than 20 weeks pregnant. Maternal body mass index (BMI) (kg/height^2) was calculated post data collection. Maternal parity was also collected at baseline and defined as the number of live children, the number of children who had died, and the number of stillbirths the enrolled mothers had prior to their current pregnancy. Gestational age at birth was calculated using the gestational age at baseline assessed by ultrasound.

In total, participants were from 28 villages in West Kiang, The Gambia. In the analyses in Chapters 5, 6, 7, and 8 the variable "village" was defined as participants from one of the four 'core' villages of West Kiang: Jali, Kantong Kunda, Keneba and Manduar, versus participants from one of the remaining 24 villages. This division was chosen as the core villages because they are situated close to the MRC Keneba field station, and therefore in closest proximity to the MRC Keneba clinic with known influence on health seeking behaviour (308). The mothers' educational level was defined as completed years of either English or Arabic schooling.

Seasonality was used as a variable in the analyses in Chapters 5, 6, 7 and 8. In this thesis, seasonality was defined as the time of year when infant anthropometric measurements or biomarkers were collected. As described in Chapter 3, seasonality in rural Gambia has a large influence on infant health. Seasonality was included in this thesis as either a binary variable; a wet (hungry) season (June to October) and a dry (harvest) season (November to May), or by using Fouriers terms (309), which allow the inclusion of the variation across all calendar months. Fourier series is a statistical model that allows the decomposition of any periodic function into a linear combination of simple oscillating functions (sines and cosines) parametrized by coefficients (the Fourier coefficients) (309). The first four sets of Fourier terms were used. Seasonality was fitted using Fourier terms in order to include the variation in the outcome variables across the calendar months, and not restrict it to the hungry or harvest season. Furthermore because the start of the two seasons in The Gambia is slightly different each year, as it depends on when the rain comes, using Fourier terms is a more accurate way of capturing differences between seasons.

4.3.4. Biomarkers

Several biomarkers (blood, breast milk and urine) from both mothers and infants and from several time-points are analysed in different chapters in this thesis. For convenience, Table 4 lists the biomarkers and the time-points used in Chapters 6, 7 and 8.

Table 4. Biomarkers and time-points used according to chapters.

	Chapter 6 (Iodine)	**Chapter 7 (B_{12})**	**Chapter 8 (B_1, B_2, B_6)**
Breast milk	Week 8,12, 24 postpartum	Week 8,12, 24 postpartum	Week 8,12, 24 postpartum
Maternal blood	Serum from baseline and 30 weeks' gestation	Plasma from baseline, 30 weeks' gestation and 12 weeks postpartum	Plasma from baseline, 30 weeks' gestation and 12 weeks postpartum
Maternal urine	Baseline, 30 weeks' gestation and 12 weeks postpartum		
Infant blood	Serum from cord, 12 and 24 weeks postpartum	Plasma from cord, 12 and 24 weeks postpartum	

Blood

A venous blood sample was collected from overnight fasting participants at baseline (enrolment, before the commencement of supplementation, <20 weeks gestation), 20 and 30 weeks' gestation (Figure 8). Furthermore a venous blood sample from fasting participants was collected at 12 weeks postpartum as part of ENID-Bone. Approximately 10 ml of blood was collected from the mother at each time-point by trained members of the Keneba nursing staff. The blood samples were collected in trace-free tubes and were immediately put on ice. The blood sample was centrifuged; plasma and serum aliquots were frozen at -80ºC. In this thesis, the focus is on the maternal blood samples collected at baseline, 30 weeks' gestation and 12 weeks postpartum, to see if the supplement had any effect during pregnancy, and how maternal status changed during lactation, when supplementation was no longer provided.

At birth, a sample of blood was obtained from the umbilical cord, and a venous blood sample was collected from each participating infant at 12 and 24 weeks postpartum. These samples are included in the analyses in Chapters 6, 7 and 8 to investigate infant status across the first six months postpartum. A cord blood sample (≤15 ml) was collected immediately after delivery, and if the mother delivered at home in her village, a residing field worker attended the delivery and collected the cord blood. The cord blood was immediately put on ice, or transported on ice to the MRC Keneba field station for processing. On arrival, cord blood was centrifuged and plasma and serum aliquots were frozen at -80ºC. At 12 weeks postpartum, 3 ml was collected and at 24 weeks 5 ml was collected by a trained member of Keneba nursing staff. All blood samples were centrifuged, aliquoted and stored at -80ºC at the MRC Keneba field station.

Urine

Maternal 24-hour urine samples were collected at baseline, 30 weeks' gestation and 12 weeks postpartum as part of ENID-Bone, and included in the analyses of Chapter 6. All urine excreted by participants over 24-hours was collected. A field worker visited the mother's home every four hours (during the day) to collect the urine samples; these were then transported on ice to the MRC Keneba field station where they were refrigerated. When in the mother's home the urine samples were similarly stored on ice. At the end of the 24-hours, all urine samples were pooled, and the total volume was noted. The samples were aliquoted and stored at -80ºC at the MRC Keneba field station. Infant urine was not collected as part of ENID or ENID-Bone.

Breast milk

Breast milk samples were collected from participants at 1, 8, 12, 24, 52, 78 and 104 weeks postpartum (Figure 9). The focus of this thesis is on samples collected at 8, 12 and 24 weeks postpartum to investigate longitudinal composition changes in mature milk during the first six months of lactation.

Participants provided a 5 ml breast milk sample from each breast, which was manually expressed between approximately 9 and 11 am at the MRC Keneba field station. The majority of the women were fasting when the sample was collected, as breakfast (provided at the MRC clinic) was served after the last sample collection. The breast milk sample was not collected during a feed or standardised according to the infant's last feed. Breast milk volume was not collected as part of ENID.

All breast milk samples used for analyses were aliquoted during late 2014 and early 2015. The samples (one from right breast and one from left breast) were first defrosted at room temperature, vortexed until thoroughly mixed and then samples from each breast were mixed and aliquoted into 2 ml tubes and stored at -80ºC. The breast milk samples used in the analyses in Chapters 6, 7 and 8 were pooled samples from the left and right breast.

4.4 Statistical analysis

Following data extraction from the main ENID database, data were cleaned, managed and analysed in STATA version 14 (310). Observations were deleted when infant and maternal ID were missing; outliers were removed. After cleaning all data, each data set on feeding data, infant growth and time-independent variables were all merged on infant or maternal ID into one STATA file.

Infant weight and length measurements were converted to weight-for-age z-score (WAZ), length-for-age z-score (LAZ) and weight-for-length z-score (WLZ) according to the WHO growth standard using the WHO Anthro program (Version 3.2.2 January 2011). If the difference between consecutive observations of the same infant was larger than three z-scores, the outlier was recorded as a missing value. In total 135 outliers of LAZ, 158 outliers of WLZ and 133 outliers of WAZ were dropped, together with the original length and weight values.

4.5 Baseline characteristics of ENID

Table 5 illustrates the baseline characteristics of ENID according to the tablet (FeFol and MMN) and PE arm (PE and PE+MMN), highlighting that there are no differences between the two supplement arms.

Table 5. Baseline characteristics of study population according to tablet and PE arm.

	Tablet (n=438)	PE (n=436)	All (n=875)
Maternal age (years) (n=1 missing)	29.5 (6.7)	29.6 (6.8)	29.6 (6.8)
Maternal weight (kg) (n=1 missing)	55.2 (9.4)	55.9 (10.0)	55.5 (9.7)
Maternal height (cm) (n=1 missing)	161.7 (6.0)	161.9 (5.7)	161.8 (5.9)
Maternal BMI (kg/m^2) (n=2 missing)	21.1 (3.5)	21.3 (3.4)	21.2 (3.5)
Gestational age at enrolment (weeks) (n=2 missing)	13.7 (3.4)	13.6 (3.2)	13.6 (3.3)
Parity, n (%)			
Primiparous	56 (12.8)	51 (11.7)	107 (12.2)
Multiparous (≥ 1 previous pregnancy)	382 (87.2)	386 (88.3)	768 (87.8)
Maternal education, n (%)			
No education	336 (76.7)	346 (79.2)	682 (77.9)
Low (1-7 years)	54 (12.3)	54 (12.4)	108 (12.3)
Medium (8-14 years)	48 (11.0)	37 (8.5)	85 (9.7)

Data are presented as mean (SD) unless otherwise stated. PE, protein-energy; n, sample size.

Chapter 5

Infant feeding practices and growth

This Chapter presents an original analysis investigating how exclusive breastfeeding (EBF) practices during the first six months of life are associated with longitudinal growth of rural Gambian infants from birth to two years of age. Parts of this Chapter are published (311).

5.1 Methods

The current analysis used data collected as a part of the ENID trial and the ENID-Growth study. Details on the ENID study design, data collection on anthropometry, infant feeding data and other demographic variables are all described in detail in Chapter 4 (page 117).

5.1.1 Infant anthropometry

Data availability on infant anthropometry according to time-points are detailed in Table 6. Weight and length measurements were converted to weight-for-age z-score (WAZ), length-for-age z-score (LAZ) and weight-for-length z-score (WLZ) according to the WHO growth standard using the WHO Anthro program (Version 3.2.2 January 2011). Z-scores were censored (potential outliers removed), based on WHO cut-offs: WAZ below -6 or above +5, LAZ below -6 or above +6 and WLZ below -5 or above +5 (312). The dataset consisted of more than 11,100 assessments of WAZ, LAZ and WLZ with a mean of 14.8 assessments per infant (range 2-19) over a mean of 23 months (range 0.2-25).

Table 6. Data availability on infant anthropometry according to time-point.

Weeks postpartum	Data availability
Birth	665
1	751
4	220
8	753
12	746
16	735
20	746
24	749
28	262
32	741
36	251
40	735
44	250
48	239
52	719
65	699
78	694
91	689
104	686

The main time-points were birth, 1, 8, 12, 16, 20, 24, 32, 40 and 52 weeks for ENID and 65-104 for ENID-Growth. Additional data were available from an embedded sub-study, making extra data available at 4, 28, 36, 44 and 48 weeks.

5.1.2 Exposure and confounding variables

Infant feeding practice was defined as exclusively breastfed to six months (EBF-6) (provision of breast milk only) versus not exclusively breastfed to six months (nEBF-6). The nEBF-6 infants were either predominantly breastfed (15%) (provision of breast milk and liquids only) or partially breastfed (85%) (provision of breast milk and solid foods) in the first six months.

The following variables were investigated as potential confounders or effect modifiers: maternal age, parity, weight, height, body mass index (BMI), educational level, and supplementation group during pregnancy, village, gestational age at birth and infant diarrhoea and morbidity incidence rate. Definitions of these variables are described in Chapter 4 (sections 4.3.2. and 4.3.3).

5.1.3 Statistical analysis

The crude association between infant feeding practice and continuous data were investigated using t-tests and for categorical data chi-squared tests were used.

In this analysis, individual age-related trajectories for each of weight, length, WAZ, LAZ and WLZ were modelled separately in multilevel models (MLM) with measurement occasion at level

one; individuals at level two, incorporating infant feeding practice associations with the sample-mean growth trajectories and adjustment for confounders and competing effects.

MLMs are known as hierarchical linear models or mixed effects models, and these models are appropriate to use in this analysis as they permit for the clustered structure of longitudinal data, with observations nested within individuals (313). The term 'mixed effects' is often used to describe MLMs, as they handle longitudinal data on a sample of individuals by simultaneously estimating random effects, which are allowed to vary between individuals, and fixed effects, which apply to all individuals. Fixed effects for instance describe the sample average growth curve and random effects are individual departures from the fixed effect (71).

The MLMs include components at two levels, a level-1 that describes how individuals change over time (called within-individual change) and a level-2 that describe how these changes (and the intercept) vary across individuals (called between-individual change), which makes MLMs superior to many other statistical methods (71). These two components are combined to form the composite MLM for change with random intercepts and random coefficients, and with the within-individual variation representing changes in the outcome over time. Another advantage of MLM, which makes it particular applicable for use in this current analysis of Gambian data, is that MLMs are not forced to consider time as a categorical variable, which is for instance the case with repeated measured ANOVA. Time-varying determinants, such as age at each visit, can be included in the models, meaning that MLM can handle variation in the timing of data collection (71). Another clear advantage of using MLM in this analysis of longitudinal data, is that missing data, which is missing due to for instance drop-outs, are handled by using information from other individuals to inform the shape of the curve. This occurs as a result of assuming that the fixed effects of the curve are similar across all subjects, and with the assumption that all missing data are missing at random (314).

Using MLM, regression equations can be derived at the individual level, which allows the estimation of between-individual differences and within-individual differences over time by modelling the variance (71). This makes MLM a powerful tool for modelling growth curves, as individual growth curve models only capture the within-individual differences and not between-individual differences (71). The within-individual difference is often referred to as 'level one residuals', and the between-individual as 'level two residuals'. Both levels of residuals should be normally distributed, which is an assumption of the model. The residual standard deviation (RSD) for level one residuals is further used to quantify the overall goodness of fit of the model (71).

The level one residuals capture measurement error, which is the deviation of observed measures from values predicted by the model. The aim is to fit a curve with an RSD similar to the measurement error of the variable analysed (315).

In this analysis, MLMs were built up by successfully adding parameters from a variance component model to a random intercept model, quadratic polynomial model, cubic polynomial model and lastly, for the length and weight measurements, the best fitted model, the Berkey Reed growth model (316). For the z-scores the shape of each trajectory was specified as a restricted cubic spline, with four knots (0.008, 0.389, 0.797 and 1.993 years). This model was chosen as it provided the lowest deviance compared to quadratic-, cubic- and fractional polynomial growth-curve models. Model fit was compared using likelihood-ratio tests, comparing the difference between the log likelihoods of two consecutive models. A p-value of <0.05 was used to denote statistical significance. Further, at each step the model with the lowest bayesian information criterion was selected, which is a measure of model fit. At each step of the model selection, diagnostic plots were generated to check model assumptions and identify outliers. The residuals were tested for normality and homogeneity of variance.

For all of the models included in this analysis (length, weight and z-scores), the constant and the (cubic spline) age terms were allowed to have random effect at level-2, allowing deviations from the intercept and gradient of the mean trajectory for each infant. Infant feeding practice was entered: (i) as a main effect, representing the association of infant feeding practice with the outcome at the intercept (i.e at birth); and (ii) as an interaction with the (spline) age terms, representing its association with the slope or rate of change in weight, length, WAZ, LAZ and WLZ. An unstructured variance-covariance matrix for the level-2 random effects was used.

Infant feeding data was coded as a binary variable; EBF-6 and nEBF-6. Seasonality of infant anthropometric measurements were included in the MLMs, using Fourier's term (309). The first four set of Fourier terms were used. In the weight and length models the seasonality function was further fitted as an interaction with age, as seasonality is likely to have a larger impact on growth in early life than when the infant reached two years of age. This was however not possible to do in the cubic spline models due to convergence. Infant morbidity incidence rate was added to the models as a time-dependent variable, and the rest of the potential confounding and competing effect variables as time-independent variables. The full model equation for Berkey Reed and the restricted cubic splines are detailed in Appendix 2 (model equations).

Overall model fits were improved by removing all length measurements at birth as the residuals were large, assumedly due to high measurement error. Birth measurements of length were often taken in the subject's home and the use of a flexible length mat likely introduced error. Data with large level-1 residuals (defined as >2 or <-2 z-score) were removed as they were considered as outliers and assumed to reflect large measurement error (WAZ n=8 data points were removed, LAZ n=28 and WLZ n=71). For weight and length no additional data points were removed as no outliers were detected.

The final MLMs provided a reasonable fit for the weight and length data with a RSD of 0.31 kg and 1.20 cm, respectively, which indicates the overall goodness of fit of the models. The final cubic spline models had RSD of 0.38, 0.49 and 0.58 z-score for WAZ, LAZ and WLZ, respectively. As indicated by the greater RSD for WLZ, the data did not fit the cubic spline model as well as the other z-score measurements. However as WLZ includes two sources of measurements errors (in weight and length), the RSD is inevitably larger for this model compared to WAZ and LAZ (71). The obtained RSDs for all models were acceptable and similar to the measurement error of the variable analysed.

The fully adjusted models were used to estimate between infant feeding-group differences at selected infant ages (0, 6, 12 and 24 months), which were presented with 95% confidence intervals. Furthermore trajectories were plotted according to infant feeding practice.

5.2 Results

5.2.1 Maternal and infant characteristics

A total of 756 mother and infant pairs were included in the analysis (Figure 11). At enrolment into the ENID trial (mean gestational age 13.7 weeks), the mothers had a mean (±SD) age of 30 years (±6.9) (Table 7). The population was lean: nineteen percent of the mothers were underweight (BMI <18.5) and only 11% were either overweight or obese (BMI ≥25). Educational levels were low, with 78% of women reporting to have received no formal education. The 756 infants included in this analysis were born with a mean birth weight of 3.02 kg (±0.4), 8% had low birth weights (<2.5 kg), and 22% were small for gestational age (Table 8). In the first two years of life the mean number of episodes of diarrhoea and other morbidity were 4.3 (±3.5) and 16.4 (±8.4), respectively.

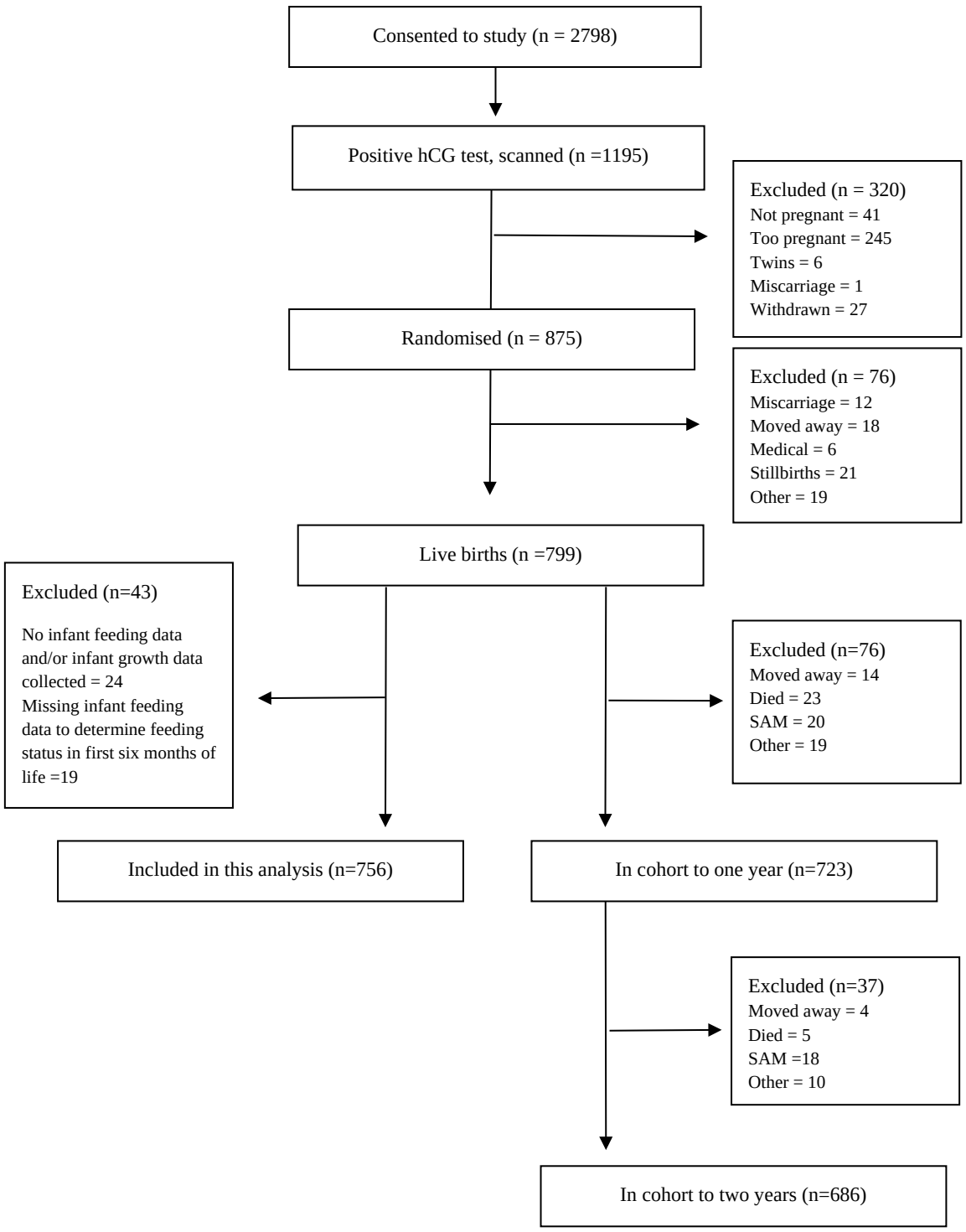

Figure 11. Flow diagram of included and excluded participants in ENID and in this analysis.

hCG, human chorionic gonadotropin; SAM; severe acute malnutrition.

Any excluded participants in ENID, either due to death, SAM, moving away or other reasons, had their data point prior to the exclusion included in the presented analyses.

Table 7. Maternal characteristics according to infant feeding practice.

Variable	n	Exclusively breastfed to six months	Not exclusively breastfed to six months	All
Maternal age (years)	755	29.9 (6.42)	30.5 (6.89)	30.3 (6.89)
Maternal weight (kg)	755	55.3 (9.42)	55.7 (9.59)	55.6 (9.53)
Maternal height (cm)	756	161.7 (5.71)	162.1 (5.89)	162.0 (5.84)
Maternal BMI	755	21.2 (3.58)	21.2 (3.33)	21.2 (3.41)
Parity categories, n (%)				
Primiparous	88	32 (13.5)	56 (10.8)	88 (11.6)
Multiparous	668	206 (86.6)	462 (89.2)	668 (88.4)
Maternal education categories [a], n (%)				
No education	591	192 (80.7)	399 (77.0)	591 (78.2)
Low (1-7 years)	94	25 (10.5)	69 (13.3)	94 (12.4)
Medium (8-14 years)	71	21 (8.8)	50 (9.7)	71 (9.4)
Maternal supplementation, n (%)				
FeFol	191	66 (27.7)	125 (24.1)	191 (25.3)
MMN	194	55 (23.1)	139 (26.8)	194 (25.4)
PE + FeFol	179	63 (26.5)	116 (22.4)	179 (23.7)
PE + MMN	192	54 (22.7)	138 (26.6)	192 (25.4)
Village [b], n (%)				
Core villages	194	43 (18.1)	151 (29.2)*	194 (25.7)
Outreach villages	562	195 (81.9)	367 (70.9)	562 (74.3)

Data are presented as mean (SD) unless otherwise stated. * Different from infants exclusively breastfed to age six months, $p \leq 0.05$.

[a] Maternal education was defined as completed years of either English or Arabic schooling

[b] Core villages are: Keneba, Jali, Kantong Kunda and Manduar situated close to the MRC Keneba. Outreach villages are the remaining 24 villages in West Kiang.

FeFol, iron-folic acid; MMN, multiple micronutrients; PE, protein-energy.

Table 8. Infant characteristics according to infant feeding practice.

Variable	n	Exclusively breastfed to six months	Not exclusively breastfed to six months	All
Birth weight (kg)	629	3.05 (0.41)	3.00 (0.39)	3.02 (0.40)
Birth weight categories, n (%)				
Low birth weight (<2.5 kg)	53	14 (7.2)	39 (9.0)	53 (8.4)
Normal birth weight (2.5-3.9 kg)	572	179 (91.8)	393 (90.6)	572 (90.9)
High birth weight (≥4.0 kg)	4	2 (1.0)	2 (0.5)	4 (0.6)
Birth length (cm) [a]	751	50.5 (2.06)	50.7 (2.08)	50.6 (2.08)
WAZ at birth	629	-0.55 (0.88)	-0.64 (0.87)	-0.62 (0.87)
LAZ at birth	751	-0.62 (0.99)	-0.50 (1.03)	-0.54 (1.01)
WLZ at birth	746	-0.55 (1.06)	-0.77 (1.07)*	-0.70 (1.07)
Gestational age at birth	750	40.0 (1.44)	40.2 (1.61)	40.2 (1.56)
Gestational age categories, n (%)				
<37 weeks	20	7 (3.0)	13 (2.5)	20 (2.7)
37-40 weeks	315	104 (44.4)	211 (40.8)	315 (42.0)
>40 weeks	415	123 (52.6)	292 (56.6)	415 (55.3)
Infant season of birth, n (%)				
Wet season (June/Oct)	290	90 (37.8)	200 (38.6)	290 (38.4)
Dry season (Nov/May)	466	148 (62.2)	318 (61.4)	466 (61.6)
Infant diarrhoea incidence rate (in the first two years of life)	756	4.50 (3.8)	4.21(3.3)	4.30 (3.5)
Infant morbidity incidence rate (in the first two years of life)	756	16.4 (8.7)	16.3 (8.3)	16.37 (8.4)

Data are presented as mean (SD) unless otherwise stated. * Different from infants exclusively breastfed to age six months, $p \leq 0.05$.

[a] Birth length used in this analysis is data collected at the second visit (infant age was between 0.2-1.3 months)

LAZ, length-for-age z-score; WAZ, weight-for-age z-score; WLZ, weight-for length z-score.

5.2.2 Infant feeding practices

Thirty-two percent of infants were exclusively breastfed at six months of age. Of the remainder, 9% of infants were given breast milk and liquids only and 59% were given breast milk accompanied by food before six months of age (Figure 12). The total mean age for introducing anything other than breast milk was 5.2 months, resulting in 67% of infants being exclusively breastfed to five months of age.

Among all infants, 1% were given water at one month of age, increasing to 62% by six months, and 1% were given semi-solid foods at one month of age increasing to 51% by six months. Other non-breast milk foods introduced to infants before six months of age included sugar water (in 2% of infants), tea (4%), cow's milk (3%), tinned milk (2%), powdered milk (5%), prepared weaning foods (4%) and solid foods (5%) although these feeds were given on few occasions. No infant formula was given at any time-point. All infants received some breast milk at one year of age and 49% of infants were still breastfed at two years of age.

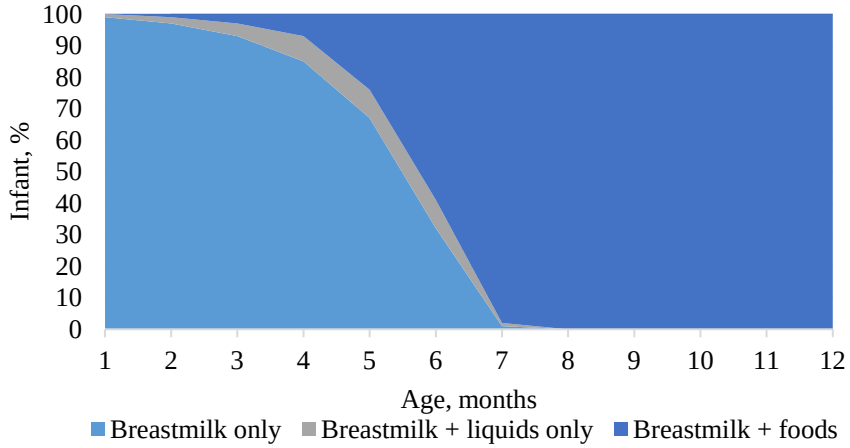

Figure 12. Infants' feeding practice by age.

5.2.3 Infant feeding practices and postnatal growth

Infants were born with a low mean WAZ, LAZ and WLZ of -0.62 (±0.9), -0.54 (±1.0), and -0.70 (±1.1), respectively (Table 8), and substantial growth faltering was indicated in the first two years of life (Figure 13). Rapid growth was observed in the first few weeks after birth, however growth faltering started at around 3.5 months of age (Figure 13). Mean weight and length z-scores declined to -1.34 (±0.9) WAZ, -1.31 LAZ (±1.0), -0.93 (±0.9) WLZ at two years of age, and by two years of age 26% of infants were stunted (LAZ <-2), 12% were wasted (WLZ<-2) and 23% were underweight (WAZ<-2).

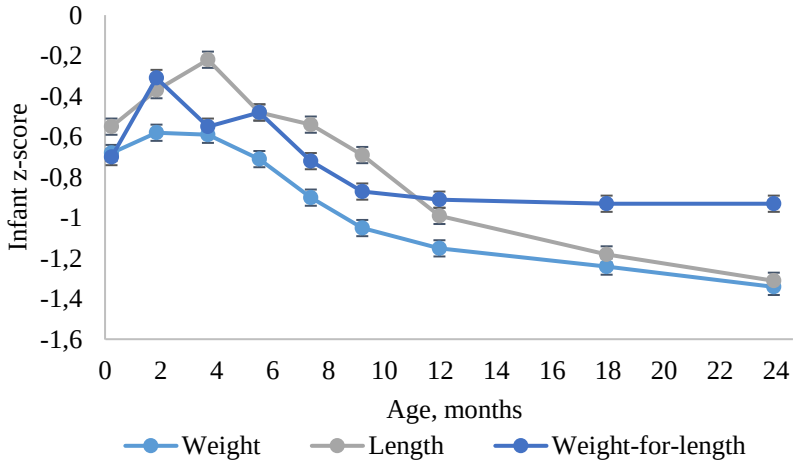

Figure 13. Infants' anthropometric measurements by age.

Values are means ±SD.

Infant weight and length

Modelling infant weight and length in the first two years according to infant feeding practice and adjusting for potential confounders and competing effects, showed limited evidence for a difference in growth between the EBF-6 and nEBF-6 infants (Figure 14). The shape of the estimated weight curve differed slightly by feeding status, with the weight curve of the EBF-6 group starting to diverge upwards from the nEBF-6 group at around six months of age, resulting in a slightly higher weight later in infancy for EBF-6 infants. EBF-6 infants tended to be on

average 143 grams heavier (95% CI: -0.024, 0.311, p=0.09) than nEBF-6 infants at two years of age, however the difference between the two groups did not reach statistical significance at any time point (Table 9).

Almost no difference was found in the estimated shape of the length curve for EBF-6 and nEBF-6 infants (Figure 14, Table 9). At birth, and at six months of age EBF-6 infants tended to be shorter than nEBF-6 infants. However, at one year of age this reversed, and by two years of age EBF-6 infants were on average 2.3 mm longer than nEBF-6 infants. However, this difference at two years of age did not reach statistical significance (95% CI: -0.230, 0.686, p=0.3).

No evidence was found for any interaction between infant feeding practice and the age terms, as infant feeding status was not associated with age-related increases (slopes) in any of the outcomes (Table 10). This indicates that the effect of infant feeding practice on infant weight or length did not vary with infant age.

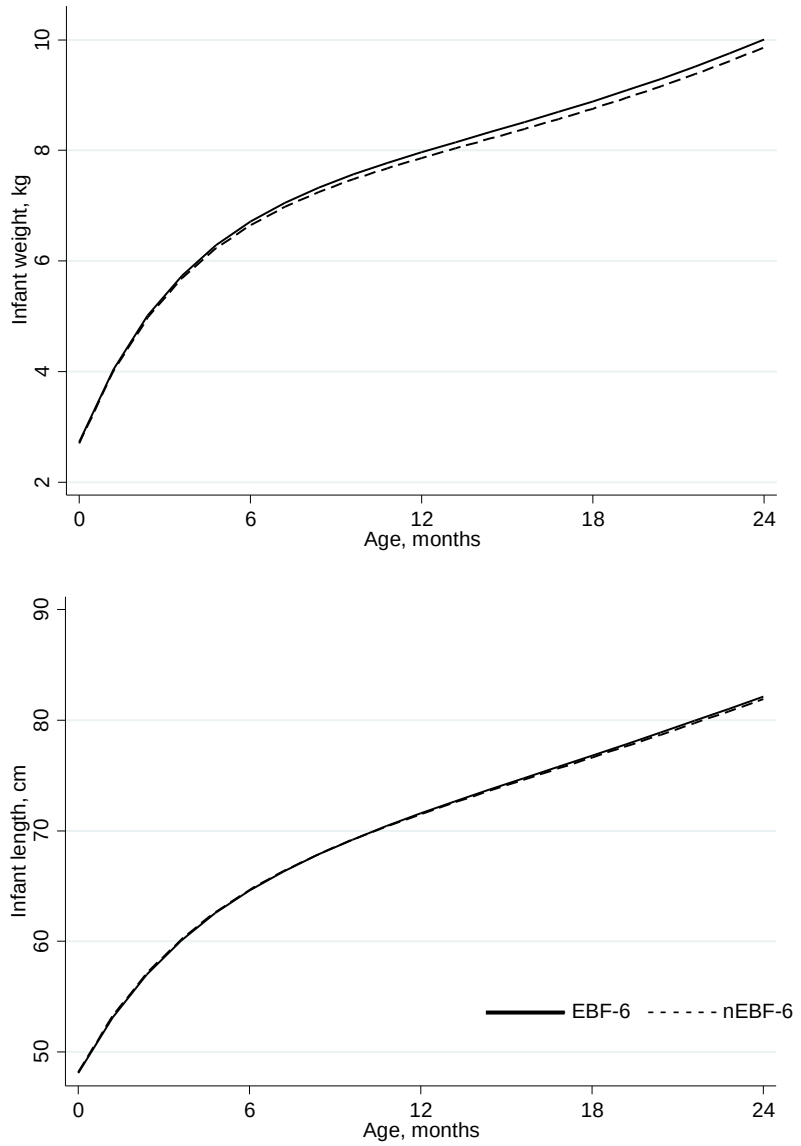

Figure 14. Weight and length by age trajectories between birth and two years of age according to infant feeding practice.

Trajectories are estimated from the multilevel models presented in Table 10 and are adjusted for sex (female: referent), village (Jali, Kantong Kunda, Keneba, Manduar) where applicable, morbidity (no incidence), gestational age at birth, parity (primiparous), maternal height and BMI. EBF-6, exclusively breastfed to six months; nEBF-6, not exclusively breastfed to six months.

Table 9. Differences in weight and length by infant feeding status at different ages.

	Weight (kg) [a]			Length (cm) [b]		
	Estimate [c]	95% CI	p-value	Estimate [c]	95% CI	p-value
At birth	0.050	-0.023, 0.123	0.2	-0.003	-0.328, 0.321	1.0
At 6 months	0.093	-0.029, 0.217	0.1	-0.022	-0.314, 0.270	0.9
At 1 year	0.136	-0.017, 0.290	0.08	0.131	-0.217, 0.479	0.5
At 2 years	0.143	-0.024, 0.311	0.09	0.228	-0.230, 0.686	0.3

[a] Adjusted for infant sex, village, infant morbidity, gestational age at birth, parity, maternal height and BMI.
[b] Adjusted for infant sex, infant morbidity, gestational age at birth, parity, maternal height and BMI.
[c] The estimates show the difference in weight and length between infants who were exclusively breastfed to age six months and infants who were not exclusively breastfed to age six months

Table 10. Fully adjusted multilevel model of serial weight and length for infants at birth, with exclusive breastfeeding to six months as exposure.

	Weight (kg)			Length (cm)		
	Estimate	95% CI	p-value	Estimate	95% CI	p-value
FIXED EFFECTS						
Constant	2.757	2.643-2.871	<0.001	48.284	47.864-48.704	<0.001
Age term [a]	11.815	11.206-12.424	<0.001	34.864	32.513-37.215	<0.001
lnage [a]	-43.546	-45.566- -41.526	<0.001	-118.029	-126.409- -109.649	<0.001
invage [a]	-47.028	-48.658- -45.399	<0.001	-140.346	-147.451- -133.240	<0.001
Sex						
Female (referent)	--	--	--	--	--	--
Male	0.100	0.033-0.167	0.003	0.477	0.176-0.778	0.002
Sex-by-age	1.706	0.941-2.471	<0.001	4.940	1.985-7.895	0.001
Sex-by-lnage	-7.334	-9.874- -4.794	<0.001	-22.978	-33.511- -12.445	<0.001
Sex-by-invage	-7.358	-9.411- -5.305	<0.001	-23.464	-32.395- -14.533	<0.001
Feeding practice (FP)						
Not exclusively breastfed to six months (referent)	--	--	--	--	--	--
Exclusively breastfed to six months	0.050	-0.022-0.123	0.2	-0.032	-0.338-0.321	0.9
FP-by-age	-0.414	-1.236-0.409	0.3	-1.834	-4.998-1.330	0.3
FP-by-lnage	1.469	-1.271-4.189	0.3	7.308	-3.979-18.594	0.2
FP-by-invage	1.023	-1.181-3.227	0.4	6.194	-3.380-15.768	0.2
Sin1 [b]	0.092	0.076-0.108	<0.001	0.052	-0.011-0.115	0.1
Cos1 [b]	-0.059	-0.075- -0.043	<0.001	-0.172	-0.236- -0109	<0.001
Sin2 [b]	0.004	-0.010-0.018	0.6	-0.104	-0.161- -0.046	<0.001
Cos2 [b]	0.023	0.009-0.037	0.002	0.045	0.012-0.102	0.1
Sin1-by-age	0.061	0.045-0.077	<0.001	0.031	-0.032-0.093	0.3
Cos1-by-age	0.018	0.001-0.034	0.03	-0.142	-0.205- -0.078	<0.001
Sin2-by-age	0.012	-0.003-0.027	0.1	-0.018	-0.077-0.041	0.6
Cos2-by-age	0.004	-0.011-0.019	0.6	0.024	-0.035-0.082	0.4
Maternal height [c]	0.012	0.007-0.017	<0.001	0.104	0.084-0.125	<0.001
Maternal BMI [c]	0.024	0.015-0.034	<0.001	0.029	-0.007-0.065	0.1
Gestational age [c]	0.079	0.059-0.100	<0.001	0.340	0.262-0.418	<0.001
Parity						
Primiparous (referent)	--	--	--	--	--	--
Multiparous	0.160	0.060-0.258	0.002	0.525	0.147-0.902	0.006
Village						
Core villages (referent)	--	--	--	--	--	--
Outreach villages	-0.075	-0.148- -0.002	0.05	--[d]	--	--
Infant morbidity						
No incidence in the past week (referent)	--	--	--	--	--	--
Incidenc in the past week	-0.017	-0.035-0.001	0.06	0.036	-0.031-0.104	0.3
RANDOM EFFECTS						
Variance (age)	4.471	3.943-5.068		9.990	7.704-12.954	
Variance (lnage)	16.554	14.658-18.697		38.894	30.572-49.482	

Variance (constant)	0.152	0.133-0.174	2.566	2.228-2.954
Covariance (age, lnage)	-8.403	-9.456- -7.351	-18.448	-23.299- -13.597
Covariance (constant, age)	-0.092	-0.168- -0.016	0.673	-0.073-1.419
Covariance (constant, lnage)	0.154	-0.009-0.300	-1.674	-3.113- -0.235
Var (residual)	0.097	0.095-0.100	1.430	1.387-1.475

Note: The third age term (invage) was not included in the random effect part of the model, because STATA could not make such a model converge. Core villages are: Keneba, Jali, Kantong Kunda and Manduar situated close to the MRC Keneba. Outreach villages are the remaining 24 villages in West Kiang.

[a] Age = age is centered to age at birth, lnage= ln(age+1), invage=(1/(age+1)) – 1

[b] sin1= sin*(1*2*π*toy), cos1= cos*(1*2*π *toy), sin2= sin*(2*2*π *toy), cos2= cos*(2*2*π *toy), toy= time of year (month) when anthropometric measurements were taken

[c] The variable was centred to the mean

[d] Village was not associated with length (p>0.05) at any time-point, and thus not included as a potential confounder.

Infant z-score

Modelling infant z-scores in the first two years of life according to infant feeding practice and adjusting for potential confounders and competing effects, showed limited evidence for a difference in growth between the EBF-6 and nEBF-6 groups of infants (Figure 15). Weak evidence suggested that the EBF-6 infants had a higher mean WAZ by 0.147 z-scores compared to nEBF-6 at six months of age (95% CI: -0.001, 0.293, p=0.05). For WLZ the observed difference between the groups was 0.189 (95% CI: 0.038, 0.341, p=0.01), whereas there was no difference in LAZ between EBF-6 and nEBF-6 infants at six months of age (Table 11).

Investigating the long-term influence of EBF to six months, weak evidence was found for a difference in mean WAZ between the two groups at one year of age, where EBF-6 infants were marginally heavier for their age (+0.183 WAZ; 95% CI: 0.011, 0.354, p=0.04) compared to nEBF-6 infants. A similar result was seen for WLZ, with a difference of 0.175 z-score (0.005, 0.345, p=0.04) between the two groups at one year of age. By two years of age, the difference in WAZ declined to 0.143 (-0.002, 0.283, p=0.05) and for WLZ to 0.097 (95% CI: -0.047, 0.242, p=0.2). For LAZ no difference was observed between EBF-6 and nEBF-6 infants at any time-point (Table 11).

No evidence was found for any interaction between infant feeding practice and the spline age terms, as infant feeding status was not associated with age-related increases (slopes) in any of the outcomes (Table 12). This indicates that the effect of infant feeding practice on infant z-scores did not vary with infant age. A greater incidence rate of morbidity episodes had a negative influence on infant WAZ and WLZ (Table 12).

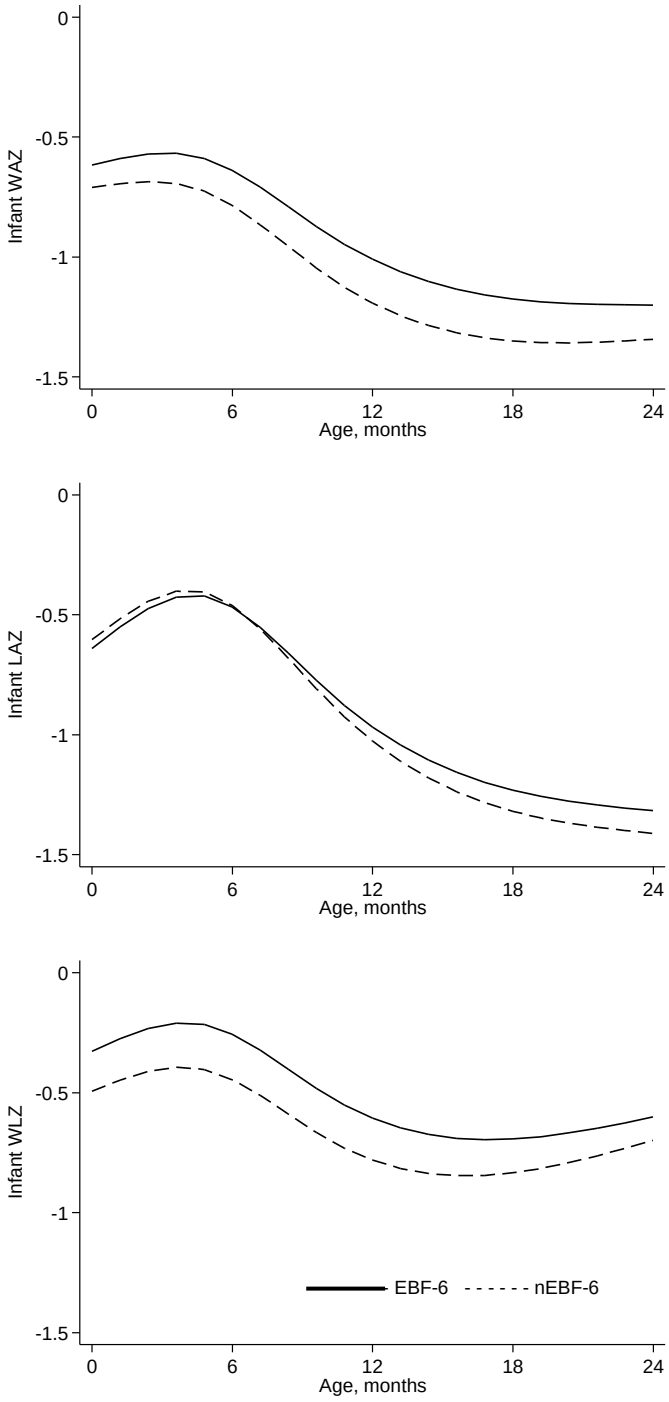

Figure 15. WAZ, LAZ, and WLZ trajectories between birth and 24 months of age according to infant feeding practice.

Trajectories are estimated from the multilevel models presented in Table 12 and were adjusted for sex (female: referent), gestational age at birth, infant morbidity (no incidence), maternal height, BMI, parity (primiparous), and village (Jali, Kantong Kunda, Keneba, or Manduar), when applicable. EBF-6, infants exclusively breastfed to six months; LAZ, length-for-age z-score; nEBF-6, infants not exclusively breastfed to six months; WAZ, weight-for-age z-score; WLZ, weight-for-length z-score.

Table 11. Differences in WAZ, LAZ, WLZ by infant feeding status at different ages.

	Weight-for-age z-score [a]			Length-for-age z-score [b]			Weight-for-length z-score [a]		
	Estimate [c]	95% CI	p-value	Estimate [c]	95% CI	p-value	Estimate [c]	95% CI	p-value
At birth	0.093	-0.040, 0.226	0.2	-0.037	-0.187, 0.114	0.6	0.166	-0.008, 0.340	0.06
At 6 months	0.147	-0.001, 0.293	0.05	-0.006	-0.137, 0.125	0.9	0.189	0.038, 0.341	0.01
At 1 year	0.183	0.011, 0.354	0.04	0.059	-0.085, 0.203	0.4	0.175	0.005, 0.345	0.04
At 2 years	0.143	-0.002, 0.283	0.05	0.095	-0.047, 0.237	0.2	0.097	-0.047, 0.242	0.2

[a] Adjusted for sex, village, infant morbidity, gestational age at birth, parity, maternal height and BMI.

[b] Adjusted for sex, infant morbidity, gestational age at birth, parity, maternal height and BMI

[c] The estimates show the difference in z-score between infants who were exclusively breastfed to six months and infants who were not exclusively breastfed to six months

Table 12. Fully-adjusted multilevel model of serial WAZ, LAZ and WLZ for infants at birth, testing exclusive breastfeeding to six months as exposure.

	Weight-for-age z-score			Length-for-age z-score			Weight-for-length z-score		
	Estimate	95% CI	p-value	Estimate	95% CI	p-value	Estimate	95% CI	p-value
FIXED EFFECTS									
Constant	-0.710	-0.904- -0.512	<0.001	-0.604	-0.797- -0.410	<0.001	-0.494	-0.716- -0.271	<0.001
Spline age term 1 (rs1) [a]	0.171	-0.028-0.369	0.09	0.900	0.656- 1.124	<0.001	0.466	0.173-0.759	0.002
Spline age term 2 (rs2) [a]	-5.456	-6.390- -4.522	<0.001	-10.279	-11.604- -8.955	<0.001	-6.284	-7.873- -4.696	<0.001
Spline age term 3 (rs3) [a]	12.302	10.338-14.266	<0.001	21.698	18.911-24.484	<0.001	14.140	10.808-17.472	<0.001
Sex									
Female (referent)	--	--	--	--	--	--	--	--	--
Male	-0.038	-0.160-0.084	0.5	-0.075	-0.214-0.064	0.3	0.055	-0.106-0.216	0.5
Sex-by-spline age term 1	-0.482	-0.731- -0.232	<0.001	-0.217	-0.512- -0.78	0.2	-0.579	-0.949- -0.209	0.002
Sex-by-spline age term 2	1.541	0.360-2.721	0.01	0.066	-1.602- 1.735	0.9	2.473	0.467-4.480	0.02
Sex-by-spline age term 3	-2.506	-4.988- -0.240	0.05	0.252	-3.258-3.763	0.9	-4.547	-8.755- -0.339	0.03
Feeding practice (FP)									
Not exclusively breastfed to six months (referent)	--	--	--	--	--	--	--	--	--
Exclusively breastfed to six months	0.093	-0.040-0.226	0.2	-0.037	-0.187-0.114	0.6	0.166	-0.008-0.340	0.06
FP-by-spline age term 1	0.107	-0.163-0.378	0.4	0.026	-0.290-0.342	0.9	0.069	-0.327-0.466	0.7
FP-by-spline age term 2	0.006	-1.261-1.272	1.0	0.607	-1.178-2.393	0.5	-0.374	-2.521-1.772	0.7
FP-by-spline age term 3	-0.339	-3.000-2.322	0.8	-1.427	-5.183-2.329	0.5	0.568	-3.932-5.069	0.8
Sin1 [b]	0.156	0.144-0.167	<0.001	0.024	0.010-0.039	0.001	0.193	0.176-0.211	<0.001
Cos1 [b]	-0.057	-0.068- -0.045	<0.001	-0.108	-0.122- -0.093	<0.001	0.011	-0.006-0.029	0.2
Sin2 [b]	0.013	0.003-0.024	0.02	-0.048	-0.061- -0.034	<0.001	0.057	0.041-0.074	<0.001
Cos2 [b]	0.033	0.023-0.044	<0.001	0.022	0.009-0.036	0.001	0.025	0.008-0.0413	0.003
Maternal height [c]	0.029	0.020-0.038	<0.001	0.055	0.046-0.065	<0.001	-0.005	-0.015-0.004	0.3
Maternal BMI [c]	0.043	0.027-0.058	<0.001	0.013	-0.003-0.030	0.1	-0.043	0.026-0.059	<0.001
Gestational age [c]	0.099	0.065-0.133	<0.001	0.113	0.078-0.149	<0.001	-0.001	-0.038-0.035	0.9
Parity									

Primiparous (referent)	--	--	--	--	--	--	--	--	--
Multiparous	0.232	0.068-0.396	0.006	0.199	0.025-0.372	0.03	-0.047	-0.226-0.131	0.6
Village									
Core villages (referent)	--	--	--	-- [d]	--	--	--	--	--
Outreach villages	-0.131	-0.252- -0.010	0.03	--	--	--	-0.100	-0.232-0.031	0.2
Infant morbidity	--	--	--	--	--	--	--	--	--
No incidence in the past week (referent)	--	--	--	--	--	--	--	--	--
Incidence in the past week	-0.017	-0.038 – 0.005	0.1	0.018	-0.009-0.046	0.2	-0.041	-0.075- -0.008	0.02
RANDOM EFFECTS									
Variance (rs1)	1.700	1.505-1.910		0.989	0.846-1.155		1.979	1.720-2.278	
Variance (rs2 term)	2.809	2.476-3.187		1.091	0.892-1.334		2.771	2.358-3.256	
Variance (constant)	0.647	0.579-0.723		0.729	0.646-0.822		0.941	0.835-1.061	
Covariance (rs1, rs2)	-2.076	-2.335- -1.816		-0.986	-1.164- -0.808		-2.231	-2.573- -1.888	
Covariance (rs1, constant)	-0.155	-0.244- -0.066		-0.298	-0.389- -0.206		-0.519	-0.659- -0.379	
Covariance (rs2, constant)	-0.024	-0.138-0.091		0.176	0.072-0.280		0.286	0.120- 0.453	
Var (residual)	0.149	0.145-0.153		0.242	0.235-0.250		0.343	0.332-0.354	

Note: The third spline age term (rs3) was not included in the random effect part of the model, because STATA could not make such a model converge. Core villages are: Keneba, Jali, Kantong Kunda and Manduar situated close to the MRC Keneba. Outreach villages are the remaining 24 villages in West Kiang.

BMI, body mass index; CI, confidence interval; FP, feeding practice; LAZ, length-for-age z-score; WAZ, weight-for-age z-score; WLZ, weigh-for-length z-score.

[a] rs1, rs2, rs3 = restricted cubic spline functions produced in STATA with the command: *mkspline rs = age cubic nknots(4) displayknots* (4 knots; 0.008, 0.389, 0.797 and 1.993 years).

[b] sin1= sin*(1*2*π*toy), cos1= cos*(1*2*π*toy), sin2= sin*(2*2*π*toy), cos2= cos*(2*2*π *toy). toy= time of year (month) when anthropometric measurements were taken

[c] The variable was centered to the mean.

Village was not associated with length-for-age z-score (p>0.05) at any time-point, and thus not included as a potential confounder

The difference in mean age at discontinuation of EBF between the EBF-6 and nEBF-6 groups was significant, however small (6.2 vs. 4.7 months). To explore differences between groups of infants who were exclusively breastfed for more distinct periods of time, a post-hoc MLM analysis with a different exposure variable was conducted; EBF to six months (±0.5 months) versus EBF to 3-4 months (±0.5 months) with continued mixed feeding (introduction of complementary liquids or solid foods). Using this new categorisation of infant feeding practice, the analysis showed no difference in LAZ between the two groups at any time-point. For WAZ and WLZ there was a difference between the two groups at six month of age, with infants who were exclusively breastfed to six months having a higher mean WAZ (+0.216 WAZ; 95%CI: 0.027, 0.404, p=0.03) and mean WLZ (+0.289 WLZ; 95% CI: 0.093, 0.485, p=0.004) than infants who were exclusively breastfed to 3-4 months (Table 13). However, these differences disappeared at one and two years of age, suggesting no longer-term benefit of EBF to six months on growth. Only 502 infants were included in this analysis, with 130 infants (26%) being exclusively breastfed to 3-4 months.

Table 13. Differences in WAZ, LAZ, WLZ by exclusive breastfeeding to six months (±0.5 months) and exclusive breastfeeding to 3-4 months (±0.5 months) (referent)

	Weight-for-age z-score [a]			Length-for-age z-score [b]			Weight-for-length z-score [a]		
	Estimate [c]	95% CI	p-value	Estimate	95% CI	p-value	Estimate	95% CI	p-value
At birth	0.071	-0.103, 0.246	0.4	0.030	-0.165, 0.224	0.8	0.082	-0.153, 0.317	0.5
At 6 months	0.216	0.027, 0.404	0.03	0.005	-0.163, 0.173	0.9	0.289	0.093, 0.485	0.004
At 12 months	0.120	-0.100, 0.339	0.3	0.101	-0.083, 0.286	0.3	0.099	-0.118, 0.317	0.4
At 24 months	0.048	-0.139, 0.236	0.6	0.034	-0.150, 0.218	0.7	0.034	-0.158, 0.227	0.7

[a] Adjusted for sex, village, infant morbidity, gestational age at birth, parity, maternal height and BMI.
[b] Adjusted for sex, infant morbidity, gestational age at birth, parity, maternal height and BMI.
[c] The estimates show the difference in z-scores between infants exclusively breastfed to 3-4 months (±0.5 months) and infants exclusively breastfed to six months (±0.5 months).

The unexpected difference in WLZ at birth according to feeding practice (Table 8 and 11, Figure 15) was further explored. It was investigated if this difference was explained by any environmental, maternal or infant characteristics, however this was not the case (Appendix 3). The possibility of reverse causality was therefore tested. Vail et al (2015) proposed a method for assessing reverse causality; including infants who were still exclusively breastfed at a given age and testing whether their mean weight, length and z-score change (between birth and a given age) were associated with subsequent age of discontinuation of EBF. This was applied here using the change in growth between (i) 2 weeks and 3 months of age and (ii) 2 weeks and 4 months of age. When applying this method, it was found that growth, either poor or good, in the first three or four months of life was not associated with when a mother stopped EBF (Table 14). Another method proposed by Kramer et al (2012) (47) in assessing reverse causality was also applied. This method did not look at the change in growth as Vail et al (2015) (317), but looked at the mean z-score at a given age and investigated how this was associated with a subsequent age at discontinuation of EBF. When applying this method in this analysis, it was found that a higher mean WLZ at three months of age was associated with a subsequent higher mean age at discontinuation of EBF (coefficient 0.064, 95% CI: 0.008, 0.120, p=0.03). No evidence for an association was found using WLZ at four months of age (coefficient 0.037, 95% CI: -0.008, 0.083, p=0.1) or if using other growth outcomes.

Table 14. Change in infant growth between (i) 2-12 weeks and (ii) 2-16 weeks of age and the association with subsequent age of discontinuation of exclusive breastfeeding

	Subsequent age of discontinuation of EBF					Subsequent age of discontinuation of EBF			
	n	Coefficient (SE)	95%CI	p-value		n	Coefficient (SE)	95% CI	p-value
Weight gain (2-12 weeks)	670	0.045 (0.06)	-0.06, 0.154	0.4	Weight gain (2-16 weeks)	595	0.002 (0.04)	-0.075, 0.078	1.0
Length gain (2-12 weeks)	673	-0.013 (0.02)	-0.051, 0.03	0.5	Length gain (2-16 weeks)	598	-0.005 (0.15)	-0.035, 0.024	0.7
WAZ change (2-12 weeks)	670	0.133 (0.04)	-0.070, 0.096	0.8	WAZ change (2-16 weeks)	595	-0.004 (0.03)	-0.063, 0.056	0.9
LAZ change (2-12 weeks)	673	-0.038 (0.04)	-0.125, 0.048	0.4	LAZ change (2-16 weeks)	598	0.0002 (0.03)	-0.064, 0.064	1.0
WLZ change (2-12 weeks)	666	0.021 (0.03)	-0.036, 0.078	0.5	WLZ change (2-16 weeks)	592	-0.012 (0.02)	-0.056, 0.032	0.6

CI, confidence interval; EBF, exclusive breastfeeding; LAZ, length-for-age z-score; n, sample size; SE, standard error; WAZ, weight-for-age z-score; WLZ, weight-for-length z-score.

5.3 Discussion

This Chapter analysed how EBF to six months was associated with infant growth from birth to two years of age, in a population where growth faltering is common and where food availability is limited. The WHO recommends EBF to six months; however, few studies have investigated the association between this practice and infant growth in a resource-poor setting where no infant formula is available.

In this analysis of rural Gambian mother-infant pairs, 32% of infants were exclusively breastfed to six months, and 67% to five months. This prevalence vastly exceeds estimates from other West African countries, for instance in Senegal only 19% of infants are exclusively breastfed to 4-5 months of age (318). Low prevalence of EBF is also found in many high-income countries, for instance in the UK only 1% are exclusively breastfed to six months (319). However, despite these impressive EBF practices recorded within this Gambian context substantial growth faltering in infants was observed across the first two years of life and, notably, this faltering started while most of the infants were still exclusively breastfed. Further high rates of stunting, wasting and underweight were found in this analysis, which is in line with previous findings in this rural Gambian setting (74), and similar to what has been found in other West African populations (320). The differences in weight and length between the infants who were exclusively breastfed to six months (EBF-6) versus infants not exclusively breastfed to six months (nEBF-6) were small. The observed differences in z-scores were small in magnitude (<0.2 SD at all time-points) and far lower than the z-score change indicative of crossing a major centile band (0.67 SD, (321).

To date, few published trials have investigated how following the WHO recommendation of EBF *to* six months benefits growth in a low-income setting. Only two controlled trials from Honduras (67, 68) were available, where mothers were randomised at four months postpartum to either continue EBF to six months or to feed solid foods from 4-6 months and continue breastfeeding. These data were re-analysed and combined by Kramer and Kakuma (2012) (49), who found that EBF to six months compared to EBF to four months did not improve infant weight or length between 4-6 months of age and subsequently (6-12 months). Monthly weight gain between 4-6 month was non-significantly higher among infants exclusively breastfed to six months compared to exclusive breastfed to 4 months (mean difference +20.78; 95% CI: -21.99, 63.54 g/month). For length, the gain between 4-6 months was almost identical between the two groups, with only

1.0 mm/month difference (95% CI: -0.40, 2.40 mm/month) (49). Similar results were found in observational studies (69, 70), which were also reanalysed by Kramer and Kakuma (2012) (49).

Kramer and Kakuma (2012) (49) further re-analysed and combined growth data from the two available trials from Honduras (67, 68) and found non-significant higher mean WAZ (+0.18, 95% CI: -0.06, 0.41), LAZ (+0.11, 95% CI: -0.11, 0.33) and WLZ (+0.09, 95% CI: -0.13, 0.31) in infants at six months of age who were exclusive breastfed to six months versus four months (with continued mixed feeding). The magnitude of the difference in z-scores were all small (<0.2 SD), which is in agreement with the findings in this analysis. The data presented in this current analysis adds to the existing evidence of limited benefit of EBF to six months of age on weight, length and z-scores in low-income settings. However, EBF to six months should still be encouraged, as following this practice benefits the infant in other aspects. For instance infants who are exclusively breastfed to six months are at lower risk of morbidity and mortality, especially due to gastrointestinal infections, which is of particular importance in this rural Gambian setting (49).

There are a number of possible reasons for the lack of a strong association between early infant feeding practice and infant growth in this rural Gambian population. Firstly, there was a low diversity in feeding behaviour observed in this population. The difference in feeding practice between the two groups were modest and the population had a high mean age of discontinuation of EBF (5.2 months). Secondly, drivers of growth faltering in this setting are potentially so powerful that EBF to six months is not sufficient to improve growth long-term. Several combined environmental influences such as a highly infectious disease environment, food insecurity, poor hygiene and sanitation standards and low quality of complementary foods could, in this setting, play a larger role in poor growth than EBF to six months. Further, a healthy gut microbiome has received considerable attention as potentially playing a role in healthy growth (57). These rural Gambian infants could possibly have a poor gut environment that is not optimal for healthy growth. Additionally, it is likely that mothers in this study population experienced growth faltering themselves in early life, increasing the risk of an intergenerational cycle of stunting (3).

To take into account the lack of heterogeneity between the two infant feeding groups (EBF-6 and nEBF-6) in terms of duration of EBF (6.2 months vs. 4.7 months), a post-hoc analysis of the data was initiated. It was found that infants exclusively breastfed to six months had a higher mean WAZ and WLZ at six months of age compared to infants who were exclusively breastfed to 3-4 months with continued mixed feeding. This difference had however disappeared by one year of age. The difference in z-scores at six months of age were higher than what was found in the

original analysis, but still small in magnitude (<0.3SD), and lower than the z-score change indicative of crossing a major centile band (0.67 SD, (321)). However, whilst this analysis indicated that infants exclusively breastfed to six months had improved growth at six months of age, changing the exposure variable meant that a large proportion of infants (36%) were excluded from the analysis, as they fell outside the new infant feeding categorisation. The results should therefore be interpreted with caution.

A surprising observation was the difference in WLZ at birth according to infant feeding practice. This observation could, firstly, reflect a consequence of some maternal, environmental or infant characteristic determining both feeding practice and infant size at birth. However, an analysis of predictors did not support this assumption. Secondly, this observation could reflect reverse causality, with mothers in this community choosing to continue EBF if their infant was growing well. Research from the UK has shown that rapid weight gain between birth and three months of age predicted subsequent earlier age of weaning (317). Kramer et al (2012) (47), using data from a Belarusian population, found contrary results; a low WAZ (<-1) at one month increased the risk of weaning by two months of age. In this analysis of rural Gambian infants, evidence that weight gain or loss in the first three or four months of life predicted discontinuation of EBF was not found, however a higher mean WLZ at three months of age predicted subsequent higher age at discontinuation of EBF. This could suggest that infant size influences EBF practice in this setting and not vice versa. This possibility that larger infants are more likely to remain exclusively breastfed reinforces the conclusion of this study that EBF to six months does not yield a growth benefit.

An important strength of this study was the comprehensive growth and feeding data collected prospectively. Infant feeding data was collected weekly, which made it possible to determine feeding practice accurately according to infant age. Weekly interviews of feeding practice are better than retrospective collected data, as the mother is only asked to remember what the infant consumed during the last week, and not to give an estimate of when EBF was no longer practiced. This comprehensive growth and feeding data further allowed the use of multilevel modelling to analyse the data longitudinally.

Further, several limitations of this analysis are acknowledged. Firstly, this is an observational study, with well-recognised sources of potential bias, such as confounding and reverse causality. Secondly, even though infant feeding practice was determined by weekly interviews, other studies have reported that only 70% of participating women accurately self-report breastfeeding

practices (322). To collect breastfeeding practises accurately the dose-to-mother deuterium oxide method would be needed.

5.3.1 Conclusion

In conclusion, these results suggest that EBF to six months has limited benefit to growth in rural Gambia; however following the WHO recommendation of EBF to six months is still advised in this setting due to other benefits of this practice.

Chapter 6

Iodine

This Chapter presents how an iodine-containing multiple micronutrient supplement given during pregnancy influences maternal status during pregnancy and lactation, breast milk composition and infant status in rural Gambia. The background begins with a review of iodine physiology, deficiency and iodine in human milk. This is followed by a description of assessment of iodine status, definitions of adequate status, intake requirements and epidemiological evidence for the importance of iodine. The focus of the background is on all three periods of pregnancy, lactation and infancy. The background is followed by a short introduction to the research area, methods, results and discussion.

6.1 Background

Iodine deficiency used to be a major global public health problem, however noteworthy progress has been made in the eradication of iodine deficiency disorders over the past two decades (323). In 1993, the World Health Organization (WHO) estimated that 110 countries were affected by goitre or other iodine deficiency disorders (324), and this number decreased to 15 in 2015 (325). The major contributing factor to this reduction has been the implementation of national salt iodisation programmes, with 75% of the global population having access to iodised salt in 2014 (326). Iodisation of salt is cost-effective, however, some countries experience low coverage of national programmes in rural areas. This is seen in The Gambia, as described in Chapter 3, where a large proportion of women in rural areas prefer to use locally sourced river salt. Despite the progress in national iodisation programmes, iodine deficiency remains a common micronutrient deficiency in all parts of the world (327).

Iodised salt used as table salt or as salt in food production is not the only source of iodine. Dairy products are another common source in some countries, due to iodine supplementation in animal feed. The iodine content in conventional cow's milk, for instance in the UK, is higher during winter months where cows are feed iodine fortified fodder (328). Salt-water fish and seafood have relatively high iodine content (329) because of their capability to concentrate iodine from seawater, however in many places fish and seafood are not consumed on a regular basis which makes the contribution of iodine from these sources low (330). Drinking water from certain aquifers or water disinfected with iodine can also be a rich source of iodine intake in some countries (331). Iodine is also found in soil, however the iodine content in crops is generally low and differs according to regions (332).

6.1.1 Iodine physiology

Iodine is mainly ingested as inorganic iodide (I-) and iodate (IO3-) and as organic iodine. Iodide is directly absorbed in the gastrointestinal track while iodate is reduced to iodide in the gut before absorption (333) (Figure 16). Organic iodine is digested before the released iodide is absorbed. More than 90% of ingested iodine is absorbed in the intestine, where it enters the plasma as iodide (333). From the plasma, iodide is transferred to the thyroid gland with losses mainly through the kidneys by excretion in urine; 90% of ingested iodine is excreted in urine in iodine sufficiency (333). Renal clearance is generally not influenced by iodine intake, whereas thyroid clearance is (334). Within the thyroid gland iodine is organified and used for synthesis of the thyroid hormones. A healthy adult has 15-20 mg of iodine, of which around 70-80% is stored in the thyroid gland, however in populations with chronic insufficient iodine intake, this storage might be as low as 20 µg (335).

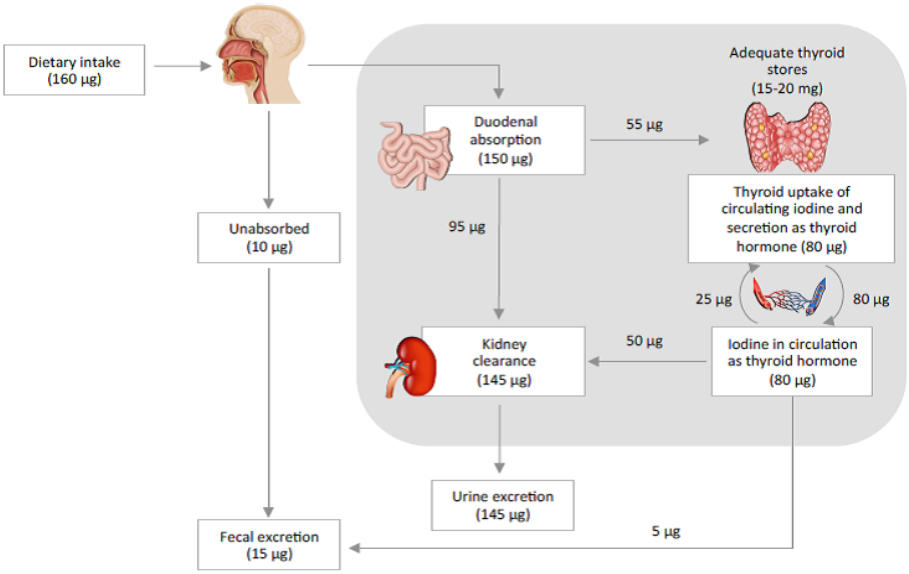

Figure 16. Iodine absorption and metabolism.

Adapted from Zimmermann (2012) (333), redrawn by S. Stinca (2016 personal communication, copied with permission).

The transport of iodide from plasma to the thyroid gland is active and mediated by a sodium-iodide symporter (NIS) (Figure 17). Iodide is transferred to the colloidal follicle lumen at the apical membrane, where it is oxidised and combined in thyroglobulin (Tg), which results in the formation of the hormone precursors monoiodotyrosine (MIT) and diiodotyrosine (DIT) (336). This process is catalysed by the thyroid peroxidate (TPO) enzyme which also catalyses the formation of triiodothyronine (T_3) and thyroxine (T_4). T_3 and T_4 are synthesised upon demand for thyroid hormone secretion that lead to internalisation of thyroglobulin into the follicular cell and digested in lysosomes (336). The thyroid stimulating hormone (TSH) is secreted by the anterior pituitary gland and regulates thyroid hormone synthesis and secretion (332).

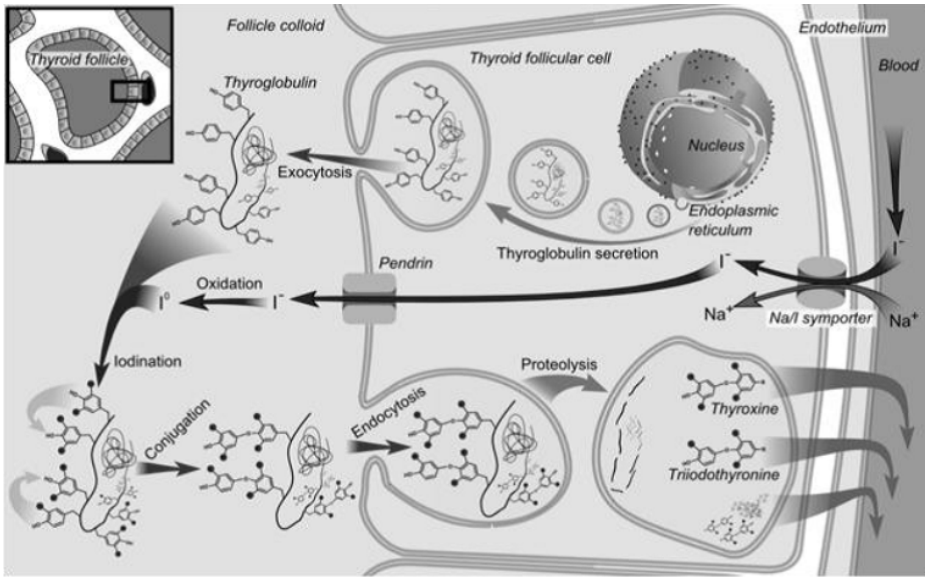

Figure 17. Uptake of iodide in the thyroid gland and synthesis of thyroid hormones.

From Andersen (2015) (337).

6.1.2 Iodine physiology during pregnancy and lactation

Iodide is transported to the placenta by NIS during pregnancy (338). In early pregnancy, the foetus is dependent on maternal thyroid hormones (339) transported across the placenta (340). From around mid-pregnancy, the foetal thyroid gland is increasingly capable of synthesising thyroid hormones, however still with a need for transport of iodide from the mother (341).

Uptake of iodine into the lactating mammary gland is also actively mediated by NIS (338). Lactation is associated with changes in maternal iodine metabolism, with iodine concentrated in the mammary gland for excretion in breast milk. Around 40-45% of ingested iodine is excreted in the milk, and consequently less is excreted in urine (342).

6.1.3 Iodine deficiency

Iodine is required for the biosynthesis of thyroid hormones (343), and iodine intake should not be below the threshold of what the thyroid needs in order to maintain normal function (335). Short-term low intakes of iodine can be buffered by intrathyroidal stores (up to 20 mg in iodine-replete populations) and increased fractional clearance of circulating iodine (335). In iodine-deficient populations, thyroidal iodine stores are generally low, making iodine turnover reliant

on dietary iodine intake (344). If the combined supply of absorbed dietary iodine and recycled endogenous iodine do not meet systemic need for synthesis of thyroid hormones, levels of T_3 and T_4 fall and TSH production increases (Figure 18). During deficiency, the increasing levels of TSH hyper-stimulate the thyroid, which in return increases Tg concentrations in the blood (328, 344). With continued stimulation to the thyroid and inadequate iodine intake, the thyroid can become enlarged (344).

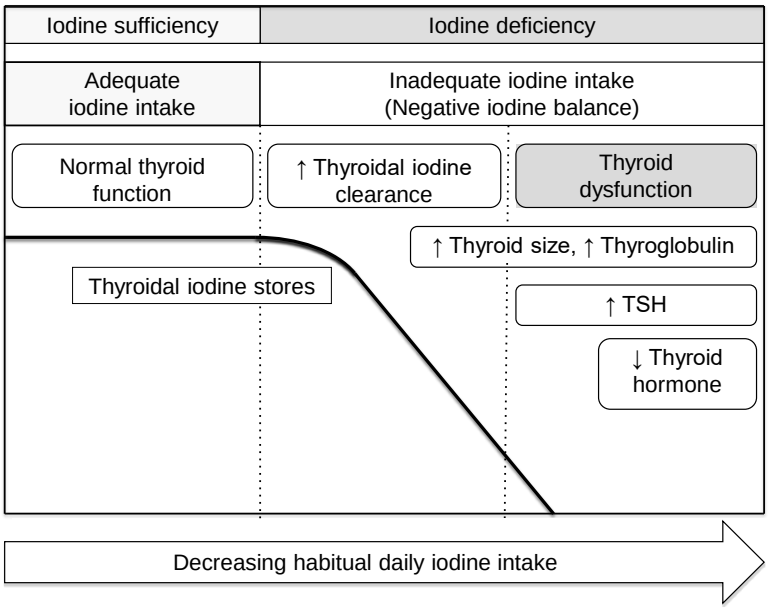

Figure 18. The physiological stages of iodine status.

The figure illustrates a model of human iodine and thyroid status at different stages (left to right) of iodine intake. The stages are separated by vertical dashed bars, first stage is sufficient intake, then low intake without thyroid dysfunction and lastly low intake with hypothyroidism (indicated by increased TSH and decreased T_4 and T_3). From Zimmermann and Andersson (2012) (344).

Insufficient iodine supply can result in adverse effects, which are termed iodine deficiency disorders (IDD) (334). Table 15 details the health consequences of iodine deficiency by population group. Consequences range from goitre to a range of outcomes arising from deficiency of the thyroid hormones, including growth impairment and neurodevelopmental damage (345).

Changes in thyroid hormones is likely caused by iodine deficiency but can also be due to for instance thyroid disease.

Table 15. Iodine deficiency disorders by population group. From Hetzel (1983) (345).

Population group	**Consequences of iodine deficiency**
All	Goitre Hypothyroidism (indicated by an increase in TSH and a decrease in T_4 and T_3)
Foetus	Abortion Stillbirth Congenital anomalies Perinatal mortality
Neonate	Endemic cretinism Infant mortality
Child and adolescent	Impaired mental function Delayed physical development Iodine-induced hyperthyroidism
Adult	Impaired mental function Iodine-induced hyperthyroidism

6.1.4 Iodine in human milk

In conditions of iodine adequacy the transport of iodide to human milk is effective and ensures an adequate supply of iodine to the breastfeeding newborn. Iodine in human milk is often found at concentrations that are 20-50 times higher than in plasma (346). Almost 80% of total iodine in mature milk is in the form of iodide and the remainder as organic iodine (347). T_3 and T_4 are present only in small quantities in human milk, constituting a small proportion of the total iodine in milk (348).

Iodine concentration is high in colostrum (219, 229), and reaches a plateau in mature milk (219, 227, 228, 233, 240). Researchers have found conflicting results regarding iodine concentration in foremilk versus hindmilk. Andersen et al (2014) (226) found a higher (however small) concentration of iodine in foremilk compared to hindmilk in a replete Danish population, whereas others report no difference between foremilk and hindmilk (245, 349). The lower breast milk iodine concentration (BMIC) in hindmilk is likely explained by the higher water content of foremilk (277). Dold et al (2016) (278) argue that the difference seen between foremilk and hindmilk is small, and is likely to be physiological irrelevant in iodine sufficiency. Considerable

diurnal and day-to-day variations in BMIC have been reported (350), however no consistent tendencies have been demonstrated by time of day or left or right breast (346).

BMIC is influenced by maternal iodine status and dietary intake as described in Chapter 2. Other determinants of breast iodine concentrations are maternal smoking (351), and caesarean section (217). Further, some studies have found a positive relationship between breast milk iodine and maternal age (238, 245). Gestational age at birth and parity are not associated with BMIC (127, 229).

6.1.5 Assessment of iodine status

The most common methods recommended to assess iodine status in humans are (i) iodine concentration in urine (ii) the concentration of thyroid function parameters; TSH, and Tg in serum samples and (iii) thyroid size (334, 352). These indicators complement each other with urinary iodine concentration (UIC) representing recent iodine intake (within days), Tg the intermediate response to inadequate or excessive iodine intake (weeks to months) and the changes in thyroid size reflecting long-term iodine intake and status (months to years) (334).

Urinary iodine concentration

UIC is a good marker of recent iodine intake. More than 90% of dietary iodine is absorbed and in healthy non-lactating adults, more than 90% of the absorbed iodine is excreted through urine (344). UIC is used to assess iodine status in populations; however it is not an optimal measure of individual status given urinary iodine substantially varies from day-to-day and within days due to differences in iodine intake (353). A 24-hour urine sample, expressed as a value of daily urinary iodine excretion (UIE) (µg/day), was initially considered as the reference standard for iodine status in populations (354). However, this biomarker is not practical in large studies or remote areas, and often has poor compliance (334, 354). Instead the use of a single urine spot sample is used (expressed as the median UIC, µg/l), and recommended by the WHO as the biomarker to use for assessment of iodine deficiency in populations (352).

Compared to UIC from a spot sample, UIC from 24-hour urine samples is not affected by within-day variation, and is more accurate and reproducible in assessing population iodine (355). UIC from 24-hour samples is correlated with spot UIC, however a noteworthy difference is observed in the distribution of the concentrations (355). Both biomarkers are however affected by day-to-

day variations in iodine intake, and 10 repeat samples are needed to reliably estimate individual iodine status and intake (353).

Urinary iodine can also be expressed in relation to urinary creatinine (µg iodine/g creatinine). However, urinary creatinine excretion varies with age, sex and body size, reducing its reliability as a denominator for the standardisation of iodine excretion (356, 357). Due to these limitations and the extra cost associated with measuring creatinine, expressing urinary iodine as µg/l, and not as µg/g creatinine, is now accepted (344, 352). If a large number of samples are analysed (50-100 as a minimum), the variation in hydration and iodine intake is generally evened out (344).

Serum thyroid stimulating hormone (TSH) and thyroid hormones (T_4 and T_3)

TSH is an insensitive indicator of iodine status, because even with iodine deficiency, TSH only increases slightly and often remains within the normal range (352). In general it is not recommend to routinely use TSH for monitoring iodine status in the adult population (327). It is however valuable to measure TSH, T_4 and T_3 to identify at-risk women, due to the impact of maternal thyroid hormone concentrations for normal foetal brain development (327). Thyroid hormone concentrations are poor indicators of iodine status, and it is not recommended to measure these for monitoring iodine status (358).

Serum thyroglobulin (Tg)

Tg is a thyroid-specific protein synthesised only in the thyroid and is the most abundant intrathyroidal protein (359). Serum Tg is elevated during iodine deficiency due to TSH hyperstimulation (344, 354). It is considered a robust biomarker of iodine nutrition in populations; it is more sensitive than TSH or thyroid hormones, and it reflects subtle changes in iodine nutrition over a period of weeks to months (360). Furthermore, assays measuring Tg using dried whole-blood spots (DBS) have been developed, making blood collection, storage and transport easier (361). Tg correlates well with UIC, thyroid size and thyroid hormones (360, 362-364) and data from several studies suggest that Tg in combination with UIC could be used as a sensitive indicator of iodine status in both children, adults and pregnant women (365, 366). DBS-Tg is recommended by the WHO for the monitoring of iodine status in school-aged children (352). However Tg measurement is technically challenging and inter-assay variability is high (367), and not enough studies have been conducted in populations other than school-aged children, for the WHO to recommend it as a monitoring biomarker.

6.1.6 Criteria for adequate iodine nutrition

Pregnancy

WHO recommends the use of UIC in spot samples as an indicator of iodine status in pregnant women, with a median UIC <150 μg/l defined as insufficient iodine intake (Table 16) (352). Zimmermann and Andersson (2012) (344) argue that even though the WHO indirectly has endorsed it, classifying *individuals* as having an insufficient iodine intake based on their UIC, is discouraged. Instead, they suggest to use the median UIC to interpret the iodine status of the population, and not calculate the prevalence of individuals below the median, due to the large day-to-day variations in iodine intake. Even in populations that are iodine-replete, studies have shown an individual day-to-day variation in UIC by 30% to 40% (353).

Table 16. Criteria for assessing iodine nutrition based on the median UIC (μg/l). From WHO, UNICEF, ICCD (2007) (352).

Median UIC (μg/l)	**Iodine intake**
Pregnant women	
<150	Insufficient
150-249	Adequate
250-499	Above requirements
≥500	Excessive
Lactating women and children <2 years	
<100	Insufficient
≥ 100	Adequate

UIC, urinary iodine concentration.

A newly proposed international reference range (2.5th and 97th percentile) of 0.3-43.5 μg/l for DBS-Tg in pregnant women has been suggested by Stinca et al (2017) (366). The authors based this reference range on a cross-sectional analysis of 3870 pregnant women from 11 countries. Based on the pooled median DBS-Tg of the reference population, they also suggested a median DBS-Tg below 10 μg/l to categorise iodine sufficiency in a population of pregnant women. The authors did not observe any difference in Tg across pregnancy in iodine-replete women, thus recommending a single Tg cut-off over the course of pregnancy. They did not develop a reference range for serum Tg, but reported that serum Tg correlated well with DBS-Tg. They further reported that over a range of iodine intakes (measured by UIC), DBS-Tg concentrations were u-

shaped. They measured Tg concentration with the use of a newly developed enzyme-linked immunosorbent assay (ELISA) (368).

Lactation

WHO recommends UIC from spot samples as an indicator to assess iodine nutrition in lactating women, with a median UIC <100 μg/l defining insufficient iodine intake (Table 16) (352). There is an ongoing discussion regarding the use of UIC to assess iodine nutrition in lactating women (241, 369). A recent study by Dold et al (2017) (241) showed that women, who were iodine sufficient and exclusively breastfeeding, had a preferential partitioning of iodine into breast milk, and consequently a lower urinary iodine excretion, when dietary iodine intake was low. This indicates that the women were adapting to a low iodine intake, by increasing uptake and secretion of circulating iodine in the blood by the mammary gland (370). This was only observed in women with adequate iodine status; in iodine-deficient women a constant proportion of iodine was excreted into breast milk (241). This supports the hypothesis that BMIC is a better indicator of iodine status than UIC in exclusively breastfeeding women, especially in those with an iodine intake in the medium/lower range (241).

Further work is required to confirm the findings of Dold et al (2017) (241). In addition breast milk is a complex matrix sample, and advanced quantification techniques are needed to measure it accurately (241), which lowers the feasibility of BMIC as the monitoring biomarker.

Infancy

Tg is widely used in the diagnosis of thyroid diseases, however few studies have used Tg as an epidemiological biomarker for iodine status in infancy (360, 362). Sobrero et al (2007) (371) found a large variation in Tg concentration in infancy depending on the quantification method used, implying large assay variability. The authors also reported large differences according to infant age, with higher serum Tg values in early infancy (3-15 days) compared to later infancy (16-180 days). Thus, for interpretation of serum Tg levels in infancy, age and quantification method should be considered and comparison between studies should be done with caution (367).

6.1.7 Iodine intake requirements during pregnancy, lactation and infancy

Pregnancy

Iodine requirement increases during pregnancy by approximately 50% (372). This is due to; (i) an increase in maternal T_4 production to maintain normal metabolism in the mother; (ii) a transfer of T_4 and iodine to the foetus; and (iii) an increase in renal iodide clearance. Several institutions have set a recommended intake for iodine during pregnancy. Table 17 presents the intakes recommended by The Food and Nutrition Board of the Institute of Medicine (IOM) of the U.S National Academy of Sciences and the WHO.

The IOM's dietary iodine intake estimations during pregnancy are based on studies that roughly estimated iodine requirement by correlating the effect of iodine supplementation with changes in thyroid volume during pregnancy (231, 232, 373). The WHO recommends a daily iodine intake of 250 μg/day for pregnant women, a value that is approximately 10% higher than the recommended dietary allowance (RDA) suggested by IOM (352).

Table 17. Recommended dietary iodine intake (μg/day) for pregnant and lactating women and infants by IOM (374) and the WHO (352).

Women of reproductive age		**Pregnancy**		**Lactation**		**Infancy**	
IOM	WHO	IOM	WHO	IOM	WHO	IOM	WHO
EAR: 95	-	EAR: 160	-	EAR: 209	-	AI: 110*	-
RDA:150	RNI: 150	RDA: 220	RNI 250	RDA: 290	RNI: 250	-	RNI: 90*

EAR, estimated average requirement (the EAR is the daily dietary intake level of a nutrient expected to satisfy the needs of 50% of the population group). RDA, recommended dietary allowance, RNI, recommended nutrient intake, AI, average intake (dietary intake believed to be adequate for everyone in the demographic group to maintain health, established where no sufficient data to establish EAR are available) IOM, Institute of Medicine, WHO, World Health Organization. * The IOM recommendation is for the first six months of infancy, and the WHO recommendation is for the first two years of life.

Lactation

The IOM's estimated average requirement (EAR) during lactation (209 μg/day), was established based on the average requirement of non-pregnant, non-lactating women (EAR of 95 μg/day) plus the average daily iodine excretion in breast milk (374). The average daily breast milk iodine excretion was estimated as 114 μg/day, which was the daily median intake of 0-6 month old

infants in a test-weighing study (375). The WHO recommends a daily iodine intake of 250 µg/day for lactating women, the same recommendation given during pregnancy (352).

Infancy

Infants are especially sensitive to iodine deficiency because they have the highest production of thyroid hormones per kilogram, and they are born with minimal thyroidal iodine stores (376). Exclusively breastfed infants rely on iodine from breast milk alone to cover their high rates of thyroid hormone production (334).

Infant iodine requirements are poorly defined. The average intake (AI) recommended by the IOM during the first six months of an infant's life (110 µg/day) is based on the median breast milk iodine concentration of 146 µg/l measured in a small (n=37), single study of American women (236) and an average breast milk intake of 0.781 l/day reported by a test-weighing study (375). In this American study, breast milk samples were collected between 14 days and 3.5 years postpartum in the 1980's, a period when iodine intake in the United States was considered excessive (236).

In contrast the WHO recommends a daily iodine intake of 90 µg for infants up to two years of age (352), based on a calculation that the iodine intake required to achieve positive iodine balance is at least 15 µg per kg/day in full term infants (323). However, assessment of iodine requirement and prevalence estimates of nutrient deficiencies in populations should ideally be based on an EAR (374). Recently, Dold et al (2016) (377) attempted to develop an EAR for iodine in infants. They reported an EAR of 72 µg/day for infants between 2-5 months of age. The EAR was established by a dose-response metabolic iodine balance study, where daily iodine intake, excretion and retention were measured over a range of iodine intakes in Swiss infants who were fed infant formula. The authors reported an iodine requirement of 70 µg/day in 2-5 months old infants, and by adding an allowance for accumulation of thyroidal stores they recommended an EAR of 72 µg/day. If using this EAR, and the estimate that infants consume approximately 0.781 l of breast milk a day, a BMIC of 92 µg/l is needed to meet an exclusively breastfed infant's daily iodine requirement (377). Further studies are required to support these findings before revised values can be accepted.

In countries that have successfully implemented national salt iodisation programmes, the iodine requirement of breastfed infants are predicted to be covered by breast milk; further supplementation of the infant is not recommended (352, 369). However, in areas without salt

iodisation programmes, the WHO recommends that infants (between 0-6 months of age) who are exclusively breastfed, should be supplemented indirectly through breast milk via maternal supplementation (352, 369). This recommendation was supported by a recent randomised controlled trial (RCT) comparing direct and indirect infant iodine supplementation and showed that indirect supplementation was associated with improved infant iodine status (227).

6.2 Iodine in the first 1000 days

Iodine is essential during all life stages, however it is especially vital during pregnancy and infancy, where the risk of deficiency for infants are high (376).

6.2.1 Pregnancy iodine status and health outcomes

Severe iodine deficiency[4] in pregnancy causes permanent brain damage with mental retardation and neurological abnormalities in the foetus (378). Severe iodine deficiency during pregnancy has further been linked to endemic cretinism in neonates, which is the extreme expression of the abnormalities and intellectual development caused by iodine deficiency (378, 379). The importance of adequate iodine intake was first shown in an intervention study in the 1960's in a severe iodine-deficient area in Papua New Guinea with endemic cretinism. This study showed that an injection of iodised oil given before conception or in early pregnancy reduced the incidence of cretinism and improved motor and cognitive functions in the offspring (380, 381). It has further been highlighted that neurological deficits caused by iodine deficiency in infants and young children are best prevented when iodine is given before or in early pregnancy (334, 382). The risk of infant mortality is further increased in infants born to women whose iodine deficiency is not corrected before or during pregnancy (334, 383).

Whether mild or moderate iodine deficiency during pregnancy[5] has the same effect on offspring outcomes is not as well established (327, 384). It has been speculated that mild-to-moderate iodine deficiency also has detrimental effects on brain development in the offspring (327). This is based on a number of trials that have found an improvement on maternal and offspring thyroid function with iodine supplementation in mild-to-moderate deficient women (231, 232, 385). However, none of these trials investigated the effect of iodine supplementation on offspring brain

[4] Severe iodine deficiency exists when more than 30% of children in the population or area have goitre, and the population has a median UIC<20 μg/l and pregnant women in the area give birth to cretins (327).

[5] Mild iodine deficiency during pregnancy is defined as a median UIC between 100-150 μg/l (327).

development or postnatal growth. An observational study from the UK by Bath et al (2013) (386) reported an UIC <150 μg/g Cr during pregnancy to be associated with an increased risk of suboptimal cognitive outcomes in the offspring at eight years of age (386).

These findings warrant additional RCTs in areas of mild-to-moderate iodine deficiency, however there is a debate about whether such trials are ethical (384). Trials in iodine poor areas are becoming increasingly difficult to justify because of the known harmful effect of iodine deficiency during pregnancy and infancy. One RCT has since been implemented, and preliminary results go against the speculations of a mild-to-moderate effect (Udomkesmalee et al in preparation). Pregnant women from India and Thailand were mildly iodine deficient with a median UIC just below 150 μg/l, when randomised to a prenatal iodine supplement of 200 μg/day, or a placebo during pregnancy. The authors found that iodine supplementation did not have any effect on offspring neuro development at 5-6 years of age (Udomkesmalee et al in preparation).

In iodine deficiency, the activity of the thyroid hormones is reduced which diminish the effect of growth hormone and circulating concentrations of insulin-like growth factor and its binding proteins (334, 335). The role of iodine in growth has been documented in goitre endemic areas (387). However, few trials have investigated neonatal and postnatal growth as an outcome measurement of iodine supplementation during pregnancy in women with mild-to-moderate iodine deficiency (384).

6.2.2 Infancy iodine status and health outcomes

Iodine deficiency during infancy can also irreversibly impair brain development and increase infant mortality (383, 388, 389). A large RCT implemented in Indonesia in an area of iodine deficiency found that infants treated with iodine supplements at six weeks of age had a 72% decrease in risk of infant death during the first two months of follow up (389). No RCTs investigating the effect of infant iodine supplementation on brain development in iodine-deficient areas were found.

6.3 Iodine status of mothers, breast milk and infants

Maternal iodine status and intake during lactation is of particular importance for exclusively breastfed infants who are relying on breast milk as their only source of iodine. As described in Chapter 2, several intervention studies have found a positive effect of maternal iodine

supplementation on BMIC during lactation, however few studies have investigated the effect of maternal supplementation exclusively during pregnancy. In fact of all available intervention studies, the majority have (i) not investigated the effect of supplementation exclusively during pregnancy (2 out of 7 studies have investigated the effect during pregnancy only (228, 231), (ii) not investigated the effect longitudinally across the first six months of lactation (3 out of 7 studies have investigated the longitudinal effect (219, 227, 228), and (iii) not investigated the effect simultaneously on breast milk iodine concentration and infant iodine status (3 out of 7 studies have investigated this (227, 231, 232). The picture is incomplete, and essentially no study has investigated the effect of supplementation during pregnancy on BMIC and infant iodine status across the first six months postpartum, highlighting the need for further research.

The existing literature that is available on iodine supplementation during pregnancy only, consists of one RCT (228), one intervention study (no randomisation) (231) and one observational study (230), where women self-reported their supplement use during pregnancy. In all three studies, a prenatal supplement had a positive effect on breast milk iodine concentrations, and some of the studies also showed a positive effect on infant neonatal iodine status (228, 231). None of the studies investigated longitudinal infant iodine status. These three studies were conducted in iodine-deficient populations in Algeria and in Belgium and Denmark, with the two latter studies conducted prior to implementation of salt iodisation in both countries. Furthermore, a RCT by Pedersen et al (1993) (232) supplemented Danish women with 200 µg/day of iodine, however during both pregnancy and lactation, which makes it difficult to conclude if the reported result of a borderline effect on median BMIC at 5 days postpartum (41 µg/l vs. 28 µg/l in supplemented vs. control women) was due to the iodine supplement received during pregnancy or during the first few days of lactation.

This Chapter presents an original analysis investigating; (i) the effect of an iodine containing multiple micronutrient supplementation during pregnancy on breast milk iodine concentration and infant thyroglobulin concentration during the first six months of life in rural Gambia; (ii) determinants of breast milk iodine concentration and (iii) estimated maternal and infant iodine intakes.

6.4 Methods

Data and samples for this analysis were collected as part of the ENID trial and the ENID-Bone extension to the ENID trial. Maternal urine (from a 24-hour urine collection collected at baseline,

30 weeks' gestation and 12 weeks postpartum), maternal serum during pregnancy (baseline and 30 weeks' gestation), breast milk (8, 12 and 24 weeks postpartum) and infant serum (delivery (cord blood), 12 and 24 weeks postpartum), were used from participating women and infants in the tablet arms of ENID (Iron-folic acid (FeFol) and multiple micronutrients (MMN)). Maternal UIC, serum Tg, BMIC and infant serum Tg were analysed. Participant recruitment, maternal supplementation, data and sample collection methodologies are all described in detail in Chapter 4 (page 117).

6.4.1 Sample analysis

Serum, urine and breast milk samples were aliquoted and frozen until analysis. Urine and breast milk samples were transported to MRC Elsie Widdowson Laboratory (EWL) (Cambridge UK), and serum samples were transported to the Human Nutrition Laboratory of ETH Zurich (Zurich, Switzerland) for analysis.

Urinary iodine concentration

Maternal UIC is presented as 24-hour UIC (µg/l) and not as 24-hour UIE (µg/day), as this increases comparability with other studies and with the WHO definition for insufficient iodine intake.

UIC was measured using Inductively Coupled Plasma Mass Spectrometry (ICP-MS), at MRC EWL using a routine in-house method based on published methods (390). Urine aliquots were defrosted and immediately prior to taking an aliquot for sample analysis, the sample was homogenised by using a vortex mixer. For each sample a 0.4 ml aliquot was pipetted into a 10 ml tube and diluted (1:10) with a solution of tetramethylammonium hydroxide (0.5%, TMAH), which included the internal standard tellurium (20 µg/l), and vortexed before analysis.

Urine (ClinChek Urine Control for Trace Elements, RECIPE Chemicals+ Instruments GmbH, Lot 432) was used for quality control. Table 18 details the inter-assay variability for urine quality controls. The certified acceptable range for level 1 and 2 is 90-150 and 373-622 µg/l respectively. The coefficients of variation (CV) obtained are of acceptable quality.

Table 18. Inter-assay variability of quality controls for urinary iodine.

	Urine (level 1)	**Urine (level 2)**
n	65	65
Mean (µg/l)	123	504
SD	3.8	15.5
CV (%)	3.1	3.1

SD, standard deviation; CV, coefficient of variation; n, sample size.

Breast milk iodine concentration

BMIC was measured by ICP-MS using a method specifically developed for BMIC measurement at MRC EWL. The samples were defrosted, inverted and placed in a rotary mixer for a minimum of 15 minutes, and immediately prior to aliquoting, the samples were further homogenised using a vortex mixer. For the sample preparation procedure 0.1 ml of each sample was pipetted into a 10 ml tube. Samples were diluted (1:50) with a solution of ultra-grade TMAH containing tellurium as the internal standard (0.5% TMAH, 20 µg/l tellurium). The samples were then analysed by ICP-MS along with external matrix-matched calibration standards (commercially sourced pooled breast milk, Sera Laboratories international LTD). Serum and whole blood (RECIPE Chemicals+, Instruments GmbH and Sero AS) were used as quality controls. Table 19 presents the inter-assay variability of the quality controls. The certified acceptable range for serum level 1 and 2 are 36-55 and 79-118 µg/l respectively, and 95-115 µg/l for whole blood. The CVs obtained are of acceptable quality.

Table 19. Inter-assay variability of quality controls for serum and whole blood iodine.

	Serum (level 1)	**Serum (level 2)**	**Whole blood**
n	78	78	76
Mean (µg/l)	50	109	102
SD	2.5	3.8	3.2
CV (%)	5.1	3.4	3.0

SD, standard deviation; CV, coefficient of variation; n, sample size.

Thyroglobulin

Tg was measured in maternal and infant serum using a newly developed sandwich serum-Tg ELISA at the Institute of Food, Nutrition and Health, ETH Zurich in Switzerland (368). For the

current analysis of the maternal serum samples, an identical protocol developed by Stinca et al (2016) (368) was used. For the infant samples however, the samples were diluted 1:20 (instead of 1:10 as per protocol). For both the maternal and infant serum analyses, the concentrations of the controls used to obtain a calibration curve were 5.48, 10, 20, 53.7 and 100 µg/l. Thyroglobulin Liquicheck™ Tumor Marker Control (BIO-Rad Laboratories AG, Cressier, Switzerland, LOT. 19990 and LOT. 19970) was used as the standard.

Two in-house serum samples (ID 1 and ID 3) and two in house serum samples (ID 1 and ID 2) were used for quality controls of the maternal and infant samples. Table 20 details the inter-assay variability of the quality controls. The CVs obtained are of acceptable quality.

Table 20. Inter-assay variability of quality controls for maternal and infant serum Tg.

	Serum maternal ID 1	**Serum maternal ID 3**	**Serum infant ID 1**	**Serum infant ID 2**
n	24	25	24	21
Mean (µg/l)	21.3	28.9	19.6	37.0
SD	3.2	3.4	3.5	4.5
CV (%)	15.0	11.7	18.0	12.1

SD, standard deviation; CV, coefficient of variation; n, sample size; Tg, thyroglobulin.

6.4.2 Statistical analysis

Baseline characteristics of the study population according to maternal supplement groups were assessed by t-tests (continuous outcome data) or ANOVA (categorical outcome data).

Individual linear mixed effects models for repeated measurements were used to assess the effect of maternal supplementation on maternal iodine status, BMIC and infant iodine status. For each continuous variable (maternal UIC, Tg, BMIC and infant Tg), an individual linear mixed effects model was derived with time (three visits (two for maternal Tg), coded as a categorical variable, with the baseline visit as the reference category) and maternal supplementation group (FeFol or MMN) as fixed effects. Potential predictors of BMIC were included in the models (based on results from section 6.5.2), but this made no substantive difference to the results (data not shown). The models were therefore reported without any covariates.

Between-individual variation was modelled using random effects. The residuals were tested for normality and homogeneity of variance using residual plots, and non-normally distributed data

were log-transformed and then reanalysed. Outliers were defined as data with residuals larger than 3 standard deviations (SD) from the mean in the mixed effect models and were excluded from the analysis (maternal UIC n=5 data points removed, maternal Tg n=3, BMIC n=5, infant Tg n=5).

Interactions between time and supplementation for each model were assessed by likelihood-ratio tests between two nested linear mixed effects models, one model with and one without the interaction terms. The overall supplementation effect for breast milk concentrations were assessed by a likelihood-ratio test comparing two nested mixed effects models, one with maternal supplement group (and its time interaction) and one without maternal supplement group (i.e. with fixed effects for time only). The likelihood-ratio test tests whether the model including maternal supplement group as a predictor gave a significantly better fit to the data than the one without.

For normally distributed data, the mean (SE) concentration at each time-point according to supplement group were derived from the mixed effects models that included an interaction term between time and supplement group (this was also how the baseline mean concentrations were derived). For non-normally distributed data, medians (interquartile range (IQR)) were derived from the raw data. The differences in concentrations between supplement groups at each time-point were derived from the mixed effects models. For log-transformed data the differences were reported as percentages, calculated by exponentiating coefficients from the log transformed model: $100(e^{\hat{\beta}} - 1)$, where $\hat{\beta}$ is the estimated difference in log plasma/breast milk concentration between groups. For normally distributed data the differences were reported in units (molar or mass units). P-values for change in iodine concentration between time-points were derived from the mixed effects models.

Determinants of BMIC at 12 weeks postpartum were examined using linear regression (continuous data) and *t*-tests or ANOVA (categorical data). Non-normally distributed data were log-transformed before analysis. Season of breast milk sample collection was categorised as a binary variable (dry season, November to May; wet season, June to October). Any crude associations between BMIC and exposure variables, identified with a p-value below or equal to 0.05 were taken forward for multiple linear regression, using step-wise regression. Each variable was added one by one to the multiple regression.

Estimated infant iodine intake was calculated based on the individual BMIC and assuming a mean breast milk intake of 0.782 l/day, which represents the estimated mean breast milk volume

consumed by Gambian infants at three months of age, assessed by test-weighing (391). This estimate is close to the average breast milk intake of 0.781 l/day reported by another test-weighing study from the United States (375), which is often used by other studies as the mean breast milk intake for infants between 0-6 months. Only infants who were exclusively breastfeed were included in this analysis.

Estimated maternal urinary iodine excretion was calculated based on individual UICs assuming a median urine excretion of 1.5 l/day (374). Estimated maternal daily iodine intake was calculated using total daily iodine excretion (through urine and breast milk) and assuming an average iodine bioavailability of 92% (374). The difference in estimated daily iodine intakes and excretions according to maternal supplement group were tested using the Mann-Whitney test.

6.5 Results

For this analysis, only women and children from the FeFol and MMN arms of ENID were included, representing a total of 381 mother and infant pairs (Figure 19).

At baseline, mean (SD) age of the participating women was 30 (6.8) years (Table 21). The mean gestational age at study enrolment was 13.8 (3.4) weeks, and a mean BMI of 21.1 (3.4) kg/m^2. Nineteen percent of the women were underweight (BMI<18.5 kg/m^2) and 10% were overweight (BMI $\geq$ 25 kg/m^2). A large percentage of the participating women had received no formal Arabic or English schooling (76%). The study population had a mean parity of 4.1 (2.7).

A large proportion of infants in this cohort were exclusively breastfed, with 97% of infants being exclusively breastfed to two months of age, and 93% and 31% to three and six months of age, respectively. The mean age of discontinuation of exclusive breastfeeding (EBF) was 5.2 (1.2) months. Age of discontinuation of EBF did not differ between supplement groups. As observed in Chapter 5, infants in this setting showed growth faltering from early infancy, with a high prevalence of stunting and wasting. In this study sample, 23% were stunted and 14% wasted at two years of age.

Maternal median (IQR) UIC at baseline was 51 (33, 82) µg/l, and did not differ between supplement groups (p=0.7, Table 22). At baseline, maternal median Tg was 22 (12, 39) µg/l, and did not differ between supplement groups (p=0.9, Table 22). At baseline, 24% (42/178) of women had elevated Tg (>43.5 µg/l) in the MMN group and 19% (33/170) in the FeFol group (p=0.3).

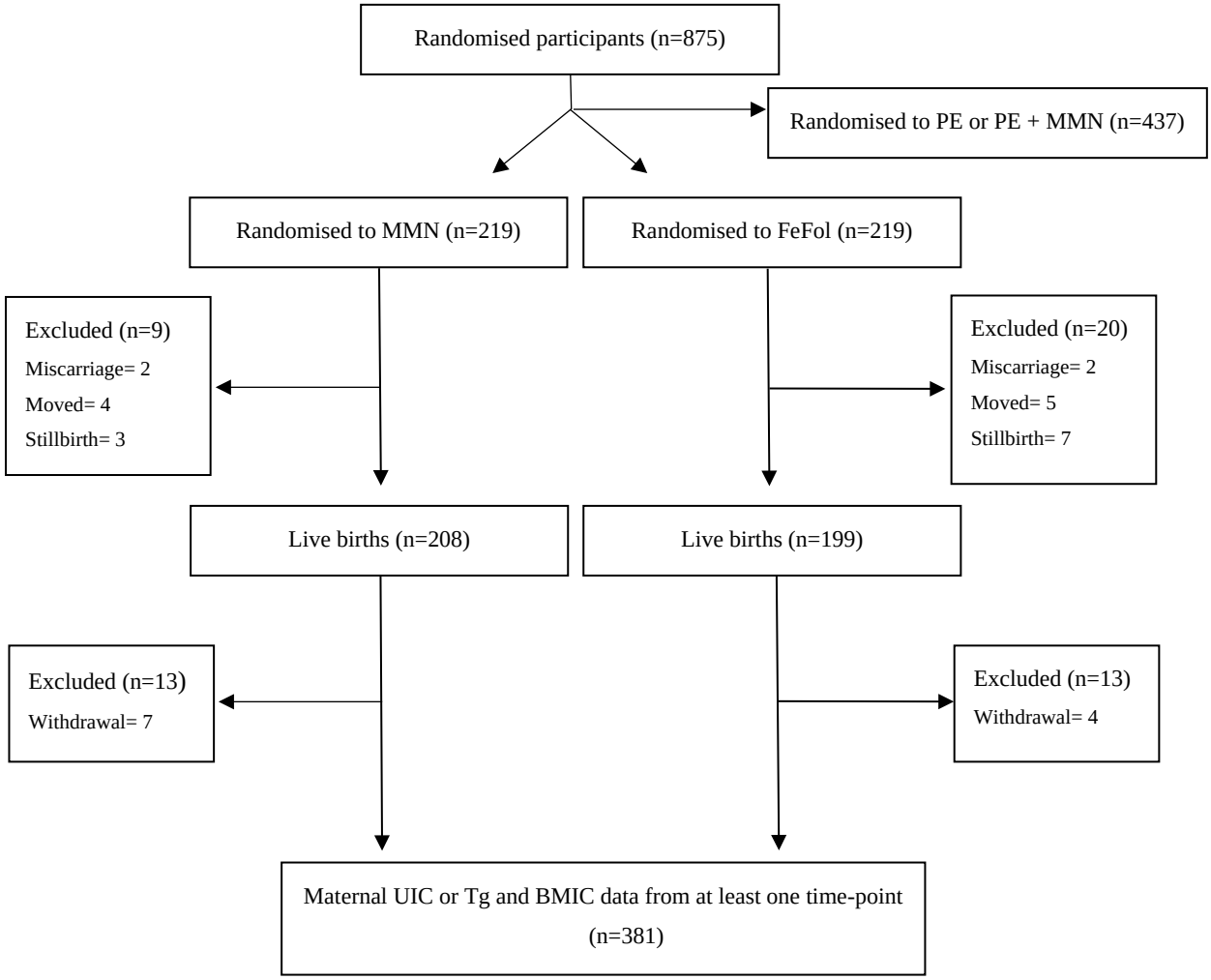

Figure 19. ENID trial profile and mother-infant pairs included in this analysis.

In Chapter 7 (vitamin B_{12}), total sample size is n=381, and in Chapter 8 (thiamin, riboflavin and vitamin B_6) total sample size is n=384 (13 excluded and 5 withdrawals from the MMN group and 13 excluded and 3 withdrawals from the FeFol group). FeFol, Iron-folic acid; MMN, multiple micronutrients, UIC, urinary iodine concentration; BMIC, breast milk iodine concentration; Tg, thyroglobulin; PE, protein-energy

Table 21. Baseline characteristics of study population according to intervention arm

	FeFol (n=186)	MMN (n=195)	All (n=381)
Maternal age (years)	30.3 (6.7)	29.1 (6.8)	29.7 (6.8)
Maternal weight (kg) (n=1 missing)	55.3 (8.8)	55.6 (9.9)	55.4 (9.4)
Maternal height (cm)	161.9 (6.2)	162.3 (5.6)	162.1 (5.9)
Maternal BMI (kg/m^2) (n=1 missing)	21.1 (3.1)	21.1 (3.7)	21.1 (3.4)
Gestational age at enrolment (weeks) (n=1 is missing)	13.7 (3.4)	13.8 (3.4)	13.8 (3.4)
Parity, n (%)			
Primiparous	19 (10.2)	28 (14.4)	47 (12.3)
Multiparous (≥ 1 previous pregnancy)	167 (89.8)	167 (85.6)	334 (87.7)
Maternal education [a], n (%)			
No education	149 (80.1)	142 (72.8)	291 (76.4)
Low (1-7 years)	24 (12.9)	25 (12.8)	49 (12.9)
Medium (8-14 years)	13 (7.0)	28 (14.4)	41 (10.8)
Village, n (%)			
Core villages [b]	47 (25.3)	55 (28.2)	102 (26.8)
Outreach villages	139 (74.7)	140 (71.8)	279 (73.2)
Maternal ethnicity, n (%)			
Mandinka	162 (87.1)	158 (81.0)	320 (84.0)
Other	24 (12.9)	37 (19.0)	61 (16.0)

Data are presented as mean (SD) unless otherwise stated. MMN, multiple micronutrient; FeFol, Iron-folic acid; n, sample size.

[a] Maternal education was defined as completed years of either English or Arabic schooling.

[b] Core villages are: Keneba, Jali, Kantong Kunda and Manduar situated close to the MRC Keneba. Outreach villages are the remaining 24 villages in West Kiang.

6.5.1 Supplement effect on iodine status and breast milk concentration

Maternal iodine status

Maternal MMN supplementation significantly improved maternal UIC compared with FeFol (p<0.001, Table 22, Figure 20). At 30 weeks' gestation median (IQR) UIC for the FeFol group was 41 (29, 76) μg/l and 90 (43, 177) μg/l for the MMN group. Between baseline and 30 weeks' gestation maternal UIC remained unchanged in the FeFol group (p=0.1), and significantly increased in the MMN group (p<0.001). Between 30 weeks' gestation and 12 weeks postpartum maternal UIC decreased in both supplement groups (p<0.001 for both groups) and the difference in UIC between the groups was not significant at 12 weeks postpartum (p=0.07). Median UIC at 12 weeks postpartum in the FeFol group was 33 (22, 51) μg/l and 39 (25, 64) μg/l in the MMN group.

The changes observed in maternal UIC in response to supplementation were also reflected in maternal serum Tg concentration. Maternal MMN supplementation during pregnancy significantly decreased maternal Tg compared with FeFol (p<0.001, Table 22, Figure 21). At 30 weeks' gestation median Tg for the FeFol group was 22 (13, 41) μg/l and 17 (9, 33) μg/l for the MMN group. Between baseline and 30 weeks' gestation, Tg increased in the FeFol group (p=0.03), and decreased in the MMN group (p<0.001). There was a significant difference in the prevalence of elevated Tg at 30 weeks' gestation between the two supplement groups, 13% (23/183) in the MMN group versus 21% (37/175) in the FeFol group (p=0.03).

Breast milk iodine concentration

No evidence was found for an overall time by supplement interaction (p=0.3, Table 22, Figure 22), however there was a significant difference in BMIC that was consistent over time, with a higher concentration in the MMN group (p=0.005). Median BMIC at 8 weeks postpartum was 57 (41, 82) μg/l and 51 (34, 73) μg/l for the MMN and FeFol groups, respectively. BMIC decreased between 8 to 24 weeks postpartum in both supplement groups (p<0.001). Median BMIC at 24 weeks postpartum was 51 (32, 74) μg/l and 39 (30, 57) μg/l for the MMN and FeFol groups, respectively (p=0.001).

Infant iodine status

Maternal MMN supplementation during pregnancy significantly decreased infant serum Tg compared with FeFol (evidence for an overall time by supplement interaction) ($p<0.001$, Table 22, Figure 23). Median infant cord serum Tg at birth was 100 (51, 140) μg/l in the MMN group and 127 (81, 192) μg/l in the FeFol group. A significant difference in infant serum cord Tg between the two groups was observed at birth ($p<0.001$). Median infant serum Tg concentration at 24 weeks was 69 (47, 90) μg/l and 66 (43, 92) μg/l for the MMN and FeFol groups, respectively. The difference in infant Tg observed at birth was attenuated at 12 and 24 weeks postpartum ($p=1.0$ and $p=0.6$ respectively). Infant serum Tg concentrations significantly decreased between birth and 24 weeks postpartum for both supplement groups ($p<0.001$).

Table 22. Maternal UIC, Tg, BMIC and infant Tg concentrations according to maternal supplement group, derived from individual mixed effects models

Mothers	Baseline	p-value *	30 weeks' gestation	p-value *	12 weeks postpartum	p-value *	p-value **
Urinary iodine concentration (µg/l)							
MMN	57 (31, 89)		90 (43, 177)		39 (25, 64)		
FeFol	48 (35, 80)		41 (29, 76)		33 (22, 51)		
Difference between supplement groups (%) [a]	3.2 (-12.2, 21.4)	0.7	95.9 (65.7, 131.7)	<0.001	17.1 (-1.2, 38.8)	0.07	<0.001
Serum thyroglobulin concentration (µg/l)							
MMN	21.3 (12.1, 41.6)		16.8 (8.6, 32.8)				
FeFol	21.6 (12.5, 38.0)		22.4 (12.9, 41.2)				
Difference between supplement groups (%) [a]	-0.8 (-18.5, 20.8)	0.9	-26.9 (-38.8, -11.0)	0.002			<0.001
Breast milk	8 weeks postpartum		12 weeks postpartum		24 weeks postpartum		
Breast milk iodine concentration (µg/l)							
MMN	57 (41, 82)		51 (35, 72)		51 (32,74)		
FeFol	51 (34, 73)		44 (33, 72)		39 (30, 57)		
Difference between supplement groups (%) [a]	17.0 (3.3, 32.7)	0.01	9.3 (-3.4, 23.6)	0.2	22.1 (8.0, 37.9)	0.001	0.3 (0.005)
Infants	Birth (cord blood)		12 weeks postpartum		24 weeks postpartum		
Serum thyroglobulin concentration (µg/l)							
MMN	100.0 (51.1, 140.1)		86.0 (58.7, 126.9)		66.1 (42.6, 91.9)		
FeFol	126.5 (80.8, 191.6)		87.8 (58.6, 124.4)		69.2 (46.7, 90.2)		
Difference between supplement groups (%) [a]	-27.7 (-37.5, -16.4)	<0.001	3.1 (-9.6, 17.6)	1.0	0.4 (-12.1, 14.8)	0.6	<0.001

Data presented on concentrations are medians (IQR) (non-normally distributed data), and are derived from raw data, not from the mixed effects models. The difference in concentrations between the two supplement groups are presented as the percentage (95% CI) difference between groups calculated by exponentiating coefficients from the log transformed model. All data were log-transformed

[a] FeFol group is the referent group

* This p-value tests the difference in concentration between supplement groups at the given time-point.

** This p-value tests the difference in concentration between the two supplement groups depending on time; in other words the p-value tests an overall time by supplementation interaction. For the breast milk analysis the p-value presented in brackets is the overall supplement effect independent of time.

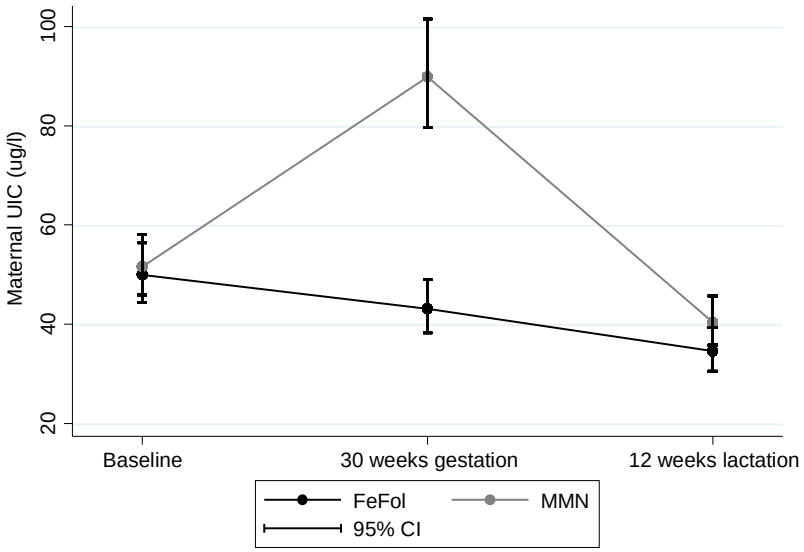

Figure 20. Longitudinal maternal urinary iodine concentration (UIC) (µg/l) (geometric means) according to supplement group at baseline, 30 weeks' gestation and 12 weeks postpartum.

Data were log-transformed and analysed using a mixed effects model. Values on y-axis are unlogged. The overall time by supplementation interaction p<0.001.

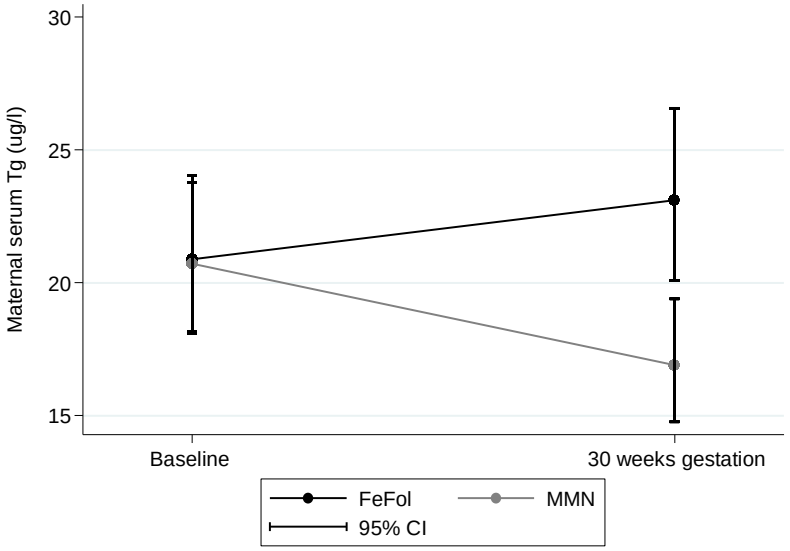

Figure 21. Longitudinal maternal thyroglobulin concentration (µg/l) (geometric means) according to supplement group at baseline and 30 weeks' gestation.

Data were log-transformed and analysed using a mixed effects model. Values on y-axis are unlogged. The overall time by supplementation interaction p<0.001.

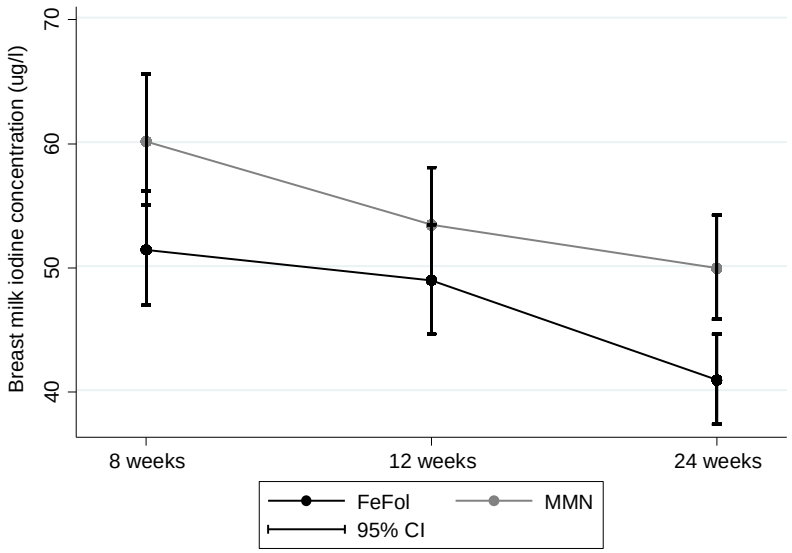

Figure 22. Longitudinal breast milk iodine concentration (µg/l) (geometric means) according to supplement group at 8, 12 and 24 weeks postpartum.

Data were log-transformed and analysed using a mixed effects model. Values on y-axis are unlogged. The overall time by supplementation interaction p=0.3. The overall time by supplementation effect p=0.005.

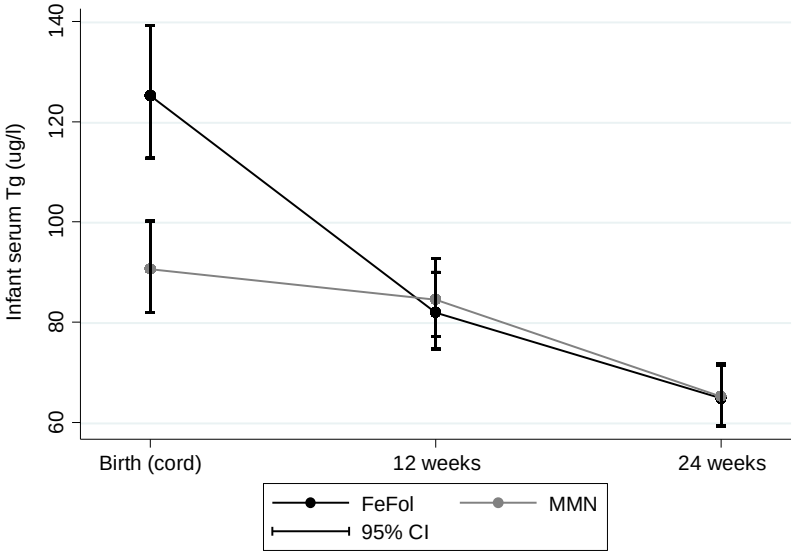

Figure 23. Longitudinal infant thyroglobulin concentration (µg/l) (geometric means) according to supplement group at birth, 12 and 24 weeks postpartum.

Data were log-transformed and analysed using a mixed effects model. Values on y-axis are unlogged. The overall time by supplementation interaction p<0.001.

6.5.2 Determinants of breast milk iodine concentration

Maternal BMIC was strongly associated with UIC at 12 weeks postpartum (crude coefficient (log-transformed): 0.217, 95%CI: 0.128, 0.306, p<0.001, r=0.290) (Figure 24). The same overall results were seen if only including mothers from the FeFol group (data not shown). BMIC at 12 weeks postpartum was further associated with seasonality (p=0.05) village (p=0.005), maternal ethnicity (p=0.008) and infant sex (p=0.01) (Table 23). After adjusting the association between maternal BMIC and maternal UIC at 12 weeks postpartum for potential confounders (seasonality, village, ethnicity, infant sex), the association remained (adjusted coefficient (log-transformed): 0.198, 95%CI: 0.106, 0.291, p<0.001, r=0.346) (Table 24).

Maternal BMIC at 12 weeks postpartum was also associated with maternal UIC in early pregnancy (baseline) (crude coefficient (log transformed): 0.154, 95% CI: 0.068, 0.240, p<0.001, r=0.210) (Figure 25). Data from both supplement groups were included in this analysis, as there were no differences in UIC or BMIC at 12 weeks postpartum between the two groups. The same overall results were seen if only including mothers from the FeFol group (data not shown). After adjusting the association between maternal BMIC and maternal UIC at baseline for potential confounders, the association remained (adjusted coefficient (log-transformed): 0.113, 95%CI: 0.025, 0.202, p=0.01, r=0.295) (Table 25).

Mothers who were exclusively breastfeeding their infant at six months postpartum did not have a different breast milk iodine concentration compared to mothers who were not exclusively breastfeeding (data not shown).

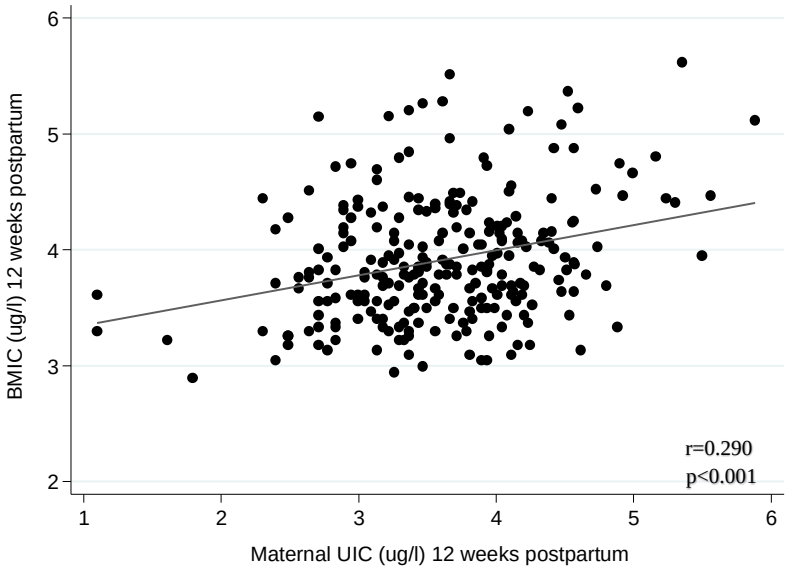

Figure 24. Association between maternal urinary iodine concentration (UIC) and breast milk iodine concentration (BMIC) at 12 weeks postpartum (n=255).

All values were log-transformed before analysis. The grey line is the linear regression fit.

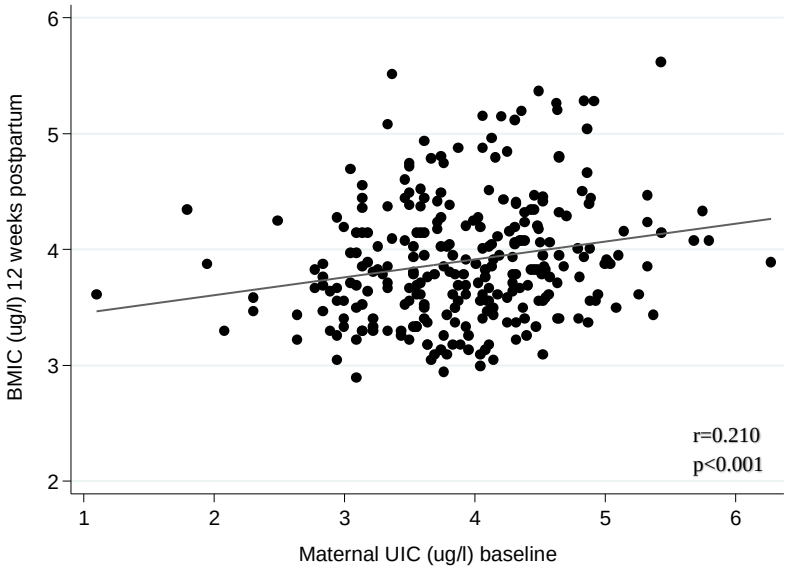

Figure 25. Association between maternal urinary iodine concentration (UIC) at baseline (early pregnancy) and breast milk iodine concentration (BMIC) at 12 weeks postpartum (n=275).

All values were log-transformed before analysis. The grey line is the linear regression fit.

Table 23. Determinants of breast milk iodine concentration (BMIC) at 12 weeks postpartum

	n	BMIC 12 weeks postpartum
Maternal age, years	323	-0.002 (0.005)
Maternal weight, kg	322	-0.003 (0.003)
Maternal height, cm	323	0.002 (0.005)
Maternal BMI (kg/m^2)	322	-0.25 (0.22)
Parity		
Primiparous	44	4.04 (0.09)
Multiparous (≥ 1 previous pregnancy)	279	3.93 (0.03)
Maternal education [a]		
No education	244	3.91 (0.58)
Low (1-7 years)	43	4.07 (0.55)
Medium (8-14 years)	36	4.02 (0.47)
Gestational age at birth (weeks)	319	0.002 (0.02)
Season of sample collection		
Dry season (Nov to May)	193	3.99 (0.04)
Wet season (June to Oct)	126	3.87 (0.05)*
Village		
Core villages [b]	80	4.10 (0.06)
Outreach villages	243	3.89 (0.04)*
Maternal ethnicity		
Mandinka	268	3.90 (0.03)
Other	55	4.13 (0.08)*
Infant sex		
Female	156	3.86 (0.04)
Male	167	4.02 (0.05)*
Infant age at 12 week visit	316	-0.007 (0.005)

Non-normal distributed data were log transformed. Continuous data are presented as logged beta coefficient (SE), categorical data as logged means (SD). Continuous data were analysed using linear regression and categorical data using *t*-tests or ANOVA. BMIC, breast milk iodine concentration; n, sample size.

* Evidence for a difference between outcome and exposure variable $p \leq 0.05$.

[a] Maternal education was defined as completed years of either English or Arabic schooling

[b] Core villages are: Keneba, Jali, Kantong Kunda and Manduar situated close to the MRC Keneba. Outreach villages are the remaining 24 villages in West Kiang.

Table 24. Adjusted regression of BMIC and maternal UIC at 12 weeks postpartum

	n	β-coefficient	SE	95% CI	p-value
Maternal UIC 12 weeks postpartum	251	0.198	0.047	0.106, 0.291	<0.001
Season (referent dry season (Nov to May))	251	-0.071	0.066	-0.202, -0.060	0.3
Village (referent core villages [a])	251	-0.143	0.076	-0.293, -0.006	0.06
Maternal ethnicity (referent Mandinka)	251	0.136	0.088	-0.037, 0.309	0.1
Infant sex (referent female)	251	0.122	0.065	-0.006, 0.250	0.06

The β-coefficient presented for each of the determinants are all adjusted for the remaining variables in the table. BMIC and maternal UIC were logged transformed

[a] Core villages are: Keneba, Jali, Kantong Kunda and Manduar situated close to the MRC Keneba.

BMIIC, breast milk iodine concentration; UIC, urinary iodine concentration; SE, standard error; CI, confidence interval; n, sample size

Table 25. Adjusted regression of BMIC at 12 weeks postpartum and maternal UIC at baseline (early pregnancy)

	n	β-coefficient	SE	95% CI	p-value
Maternal UIC baseline (early pregnancy)	271	0.113	0.045	0.025, 0.202	0.01
Season (referent dry season (Nov to May))	271	-0.073	0.064	-0.199, 0.054	0.3
Village (referent core villages [a])	271	-0.137	0.076	-0.287, 0.012	0.07
Maternal ethnicity (referent Mandinka)	271	0.201	0.085	0.034, 0.368	0.02
Infant sex (referent female)	271	0.111	0.063	-0.014, 0.236	0.08

The β-coefficient presented for each of the determinants are all adjusted for the remaining variables in the table. BMIC and maternal UIC were logged transformed.

[a] Core villages are: Keneba, Jali, Kantong Kunda and Manduar situated close to the MRC Keneba.

BMIC, breast milk iodine concentration; UIC, urinary iodine concentration; SE, standard error; CI, confidence interval; n, sample size

6.5.3 Estimated maternal and infant iodine intake

Estimated average daily iodine intake of mothers during pregnancy and lactation are presented in Table 26. The estimated maternal median iodine intake in early pregnancy (baseline) was 83 μg/day. Using the baseline estimates and the EAR of 160 μg/day for pregnant women developed by the IOM, 84% (274/327) had a daily iodine intake below the EAR in early pregnancy. Using maternal UIC and BMIC data at 12 weeks postpartum (both supplement groups combined), maternal estimated median iodine intake during lactation was 105 μg/day. Using the suggested EAR by IOM of 209 μg/day for lactating women, 90% (211/235) of the women had intakes below the EAR.

In exclusively breastfed infants, iodine intake is equal to excretion in breast milk. Table 26 shows the estimated average infant iodine intake (μg/day) at the three time-points. The estimated median intake of iodine for exclusively breastfed infants was 37 μg/day at 12 weeks postpartum. Eighty-seven percent (119/137) of the infants from the FeFol group had an estimated iodine intake below the EAR of 72 μg/day (developed by Dold et al (2016) (377)) at 12 weeks postpartum, and 85% (137/161) from the MMN group.

Table 26. Estimated average daily iodine intake for mothers and exclusively breastfed infants (μg/day) at varying time-points.

Maternal intake [a]	Baseline		30 weeks' gestation		12 weeks postpartum
FeFol, n=161	78.3 (57.1, 130.4)	FeFol, n=149	66.8 (47.3, 123.9)	FeFol, n=110	100.5 (70.8, 134.8)
MMN, n=166	92.9 (50.5, 145.1)	MMN, n=154	146.7 (70.1, 288.6)*	MMN, n=125	119.8 (77.0, 155.2)
Total, n=327 [c]	83.2 (53.8, 133.7)	Total, n=303	94.6 (57.1, 190.8)	Total, n=235	104.8 (74.5, 144.9)
Infant intake [b]	8 weeks postpartum		12 weeks postpartum		24 weeks postpartum
FeFol, n=144	39.5 (26.6, 55.5)	FeFol, n=137	34.4 (25.8, 55.5)	FeFol, n=89	29.7 (23.5, 40.7)
MMN, n=151	44.6 (32.8, 64.1)*	MMN, n=161	39.9 (27.4, 58.7)	MMN, n=91	39.1 (24.2, 56.4)*
Total, n=295	42.2 (28.9, 61.0)	Total, n=298	36.8 (26.6, 56.3)	Total, n=180	33.6 (23.9, 47.7)

Data are medians (IQR). *Difference in medians between supplement groups, $p \leq 0.05$. Analysed by Mann-Whitney test.

[a] Calculated for each individual as total estimated daily iodine excretion (through urine and breast milk) divided by 0.92, where 0.92 refers to 92% iodine bioavailability.

[b] Calculated as the concentration excreted in breast milk*0.782 l/day

[c] Total = data from both supplement groups.

MMN, multiple micronutrients; FeFol, iron-folic acid

6.6 Discussion

Supplementing pregnant Gambian women with a multiple micronutrient supplement, containing 300 µg/day of iodine, resulted in higher maternal UIC and lower maternal serum Tg during pregnancy, a lower infant cord Tg concentration at birth, and a modestly higher BMIC across the first six months of lactation. The positive effect of supplementation on maternal UIC during pregnancy was attenuated during lactation, and no effect of supplementation was observed on infant postnatal iodine status (Tg).

In this rural Gambian population, maternal supplementation starting in early pregnancy had a positive effect on maternal IUC and a positive effect on maternal thyroid activity, with a lower Tg concentration alongside a reduced prevalence of elevated Tg seen in the MMN group. In addition a significant increase in Tg was found in the FeFol group from baseline to 30 weeks' gestation, indicating a generalised increase in maternal thyroid activity over the course of pregnancy. This observation could indicate a physiological adaptation of the thyroid to low iodine intake by increasing plasma iodide clearance and turnover of thyroidal iodine stores. This observation further suggests that Tg is a more sensitive biomarker at the individual level, compared to UIC where a significant change between baseline and 30 weeks' gestation was not observed in the FeFol group.

The supplementation further had an effect on infant cord Tg concentration, with a significant lower Tg concentration in the MMN group. Regardless of a large difference in cord Tg concentration between the two groups, the effect was not sustained during infancy. No evidence for a difference in Tg between the two supplement groups was observed at 12 or 24 weeks postpartum. This is in agreement with other studies, finding that maternal iodine supplementation during pregnancy is reflective of foetal iodine status at birth (228, 231, 232). For instance an RCT supplementing deficient Danish women with a supplement of 200 µg/day of iodine during pregnancy (starting from 17-18 weeks gestation) reported a cord serum Tg decrease from 67 µg/l to 38 µg/l in the supplemented vs. control group (232). None of the available studies investigated any long-term effects on infant iodine status. This indicates that a prenatal supplement have a positive effect on infant iodine status at birth.

In addition, Tg concentration significantly declined during infancy in both groups in this population. This is in agreement with the findings from other studies. Several studies have reported a physiological rise in newborns' Tg from right after birth and up to 6 hours post birth,

and with a progressive decrease beginning 96 hours after birth until reaching a plateau in later infancy (371, 392-394). In fact a study found that Tg concentration did not reach adult concentration until eight years of age (393).

In this Gambian setting, the effect of supplementation during pregnancy was not sustained in the mother during lactation. No difference in maternal UIC was observed at 12 weeks postpartum. This is in agreement with an RCT finding that maternal UIC fell sharply a few days after delivery when supplementation ended in iodine-deficient Belgium women (231). In this rural Gambian population a difference in BMIC between the two supplement groups, independent of time was found, likely due to carry-over effect of the pregnancy supplementation. This suggest that any thyroidal storage obtained during pregnancy is transferred to the milk rather than excreted through maternal urine in this population.

The positive effect observed on BMIC was however modest, and as observed in the infant postnatal Tg concentrations, not sufficient to increase infant status. Only two existing intervention studies have investigated iodine supplementation during exclusively pregnancy and the effect on BMIC (228, 231). One RCT (231) and one intervention study (no randomisation or placebo group) (228), conducted in iodine-deficient populations (Algeria and Belgium, respectively), found a positive effect of an iodised oil supplementation (equivalent to 240 mg of iodine) (228) and a daily iodine tablet supplement (100 μg/day) (231) on BMIC at five days (231) and at one and six months postpartum (228). Chaouki et (al 1994) (228) supplemented deficient women either in (i) pre-pregnancy, (ii) during the first month of pregnancy or (iii) during the first trimester, which increased both maternal UIC at birth and BMIC compared to a control group (mean BMIC: (i) 307 nmol/l, (ii) 346 nmol/l, (iii) 386 nmol/l vs. 260 nmol/l at six months postpartum). Glinoer et al (1995) (231) supplemented deficient women in their RCT from the second trimester, which increased BMIC in early lactation to 61 μg/l in supplemented and 29 μg/l in placebo women. Glinoer et al (1995) did not investigate the longitudinal effect on BMIC. This indicates that supplementing women of low iodine status during pregnancy has an effect on breast milk iodine concentrations, however comparability between these two studies and the results presented here are low, as the type of supplement, iodine concentration of the supplement and timing of the supplementation were different in all three studies.

However, the results in this analysis suggest that supplementation during pregnancy alone with 2xRDA of iodine given as a multiple micronutrient is not sufficient in ensuring adequate iodine concentration in breast milk across the duration of EBF in a mild-to-moderate iodine-deficient

population as this Gambian population. Evidence suggest that maternal postnatal iodine supplementation is needed to increase both maternal status, breast milk iodine and infant status. An RCT among iodine deficient women in New Zealand (before iodisation of salt was implemented) found that an iodine supplement (150 μg/day) given to the mothers from birth to six months postpartum increased milk concentrations by 1.7 times compared to a placebo (395). Postnatal supplementation also improved both maternal and infant iodine status across lactation in this study. Similar results were observed in the RCT by Bouhouch et al (2014) (227). Here, deficient Moroccan women were supplemented with a single dose of 400 mg of iodine (≤8 weeks postpartum), which improved both maternal status and BMIC over the course of lactation.

In addition, median BMIC decreased longitudinally in both supplement groups in this rural Gambian population, potentially suggesting physiological changes in BMIC across lactation regardless of supplementation. This decline in BMIC according to stage of lactation, has also been reported in a recent systematic review on BMIC (396) and in several of the longitudinal studies included in Chapter 2. The observed decline could on the other hand imply that in the absence of adequate maternal iodine intakes, in an already iodine-deficient population, BMIC declines in mature milk across the course of lactation. As reported, in this rural Gambian population, maternal supply did not equal the demand in order to sustain breast milk iodine concentrations, which may explain the decrease in BMIC across lactation rather than physiological changes. Supporting this hypothesis is a recent RCT supplementing deficient women from birth to 24 weeks postpartum with either a placebo, 75 μg/day or 150 μg/day of iodine, finding that supplementation prevented a BMIC decline over the first six months of lactation compared to a placebo (395).

6.6.1 Iodine deficiency, intake and determinants of BMIC

The women in this study were considered iodine deficient in early pregnancy according to the WHO definition (<150 μg/l), with a median UIC at baseline of 51 μg/l. Similarly, the median maternal Tg was 22 μg/l at baseline, which is above the threshold of 10 μg/l suggesting iodine deficiency in pregnancy (366). This threshold was developed from DBS-Tg and not serum Tg, and although they correlate, interpretation should be made with caution (366). More than 80% of women in early pregnancy (baseline) were estimated to be below the IOM's EAR for iodine intake (160 μg/day).

A low UIC was observed in pregnancy regardless of supplementation group; the daily consumption of 300 µg/iodine did not increase UIC to 150 µg/l at 30 weeks' gestation. This is likely because the women in this study had depleted thyroidal iodine stores when entering pregnancy, and parts of the ingested iodine were used to build up their stores. This could imply that starting supplementation earlier than ~13 weeks gestation is preferable in order to allow enough time to re-build depleted iodine stores, or a higher dose of iodine is required at this level of deficiency to reach an acceptable UIC within a short period of time. This is supported by an RCT of iodine-deficient Belgium women who were either supplemented from second trimester until delivery with 100 µg/day of iodine or a placebo (231). In this study, maternal UIC was below 50 µg/l at baseline, and in the third trimester of pregnancy maternal UIC was below 100 µg/l in both supplement groups (reported in a figure, actual concentrations not reported). This indicates that also in this study a higher dose and/or an earlier start of supplementation would be needed to ensure adequate maternal status during pregnancy.

During lactation (12 weeks post-partum) mothers had a median UIC (FeFol group: 33 µg/l, MMN group: 39 µg/l) below the WHO cut-off for inadequate iodine intake (<100 µg/l). In addition, the median BMIC was between 39 and 57 µg/l for both supplement group across the first six months of lactation, which is considered low. For instance Bouhouch et al (2014) (227) reported a median UIC and BMIC of 34 µg/l and 33 µg/l, respectively at 3 months postpartum (in unsupplemented women, using ICP-MS to measure BMIC) in iodine-deficient Moroccan participants, close to the values reported here. Further, a recent study, also using ICP-MS to quantify concentrations, reported a pooled median BMIC of 171 ug/l between 2-26 weeks postpartum in replete populations (adequate intake of iodized salt) from Croatia, China and the Philippines (241), far from the concentrations reported here. Adding the calculations done by Dold et al (2017) (241) that a BMIC above 92 µg/l is needed to sustain exclusively breastfed infants' iodine need in the first months of lactation, BMIC is considered low in this population. However, to date no consensus exists on an optimal BMIC to define optimal iodine status for lactating women and infants. As seen in Chapter 2, large variations are seen in BMIC because of differences in maternal diet, iodine status, other environmental factors and differences in collection and analytical methods. More research is needed to define adequate BMIC in well-nourished women.

The interpretation of a low BMIC is supported by the low estimated maternal iodine intake during lactation (90% below the IOM's EAR, 209 µg/day), and low estimated intake of EBF infants, regardless of supplementation group (more than 80% below the EAR proposed by Dold et al (2016) (377), 72 µg/day). Nevertheless, day-to-day variation in iodine intake was not taken into

account in the calculation of these estimated iodine intakes, as the estimation was based on a single 24-hour median UIC for the mothers. Further, actual infant breast milk intake was not obtained in this analysis, and the daily infant iodine intake is based on an estimate.

Limited studies have used infant Tg as a biomarker for iodine status in infancy, and even fewer have measured Tg in cord blood. There are a few available studies that have reported Tg concentrations in cord blood (231, 232, 397), however comparability of the actual concentrations is low because of the use of different quantification methods. Tg measurement is technically challenging, and comparison between studies should be done with caution (367). Establishing reference values for infant Tg across infancy and according to analytical quantification method are needed before infant Tg concentrations can be interpreted.

In this analysis, maternal UIC during lactation correlated with BMIC at 12 weeks postpartum, which is in agreement with results from other studies (see Chapter 2). Maternal IUC in early pregnancy also correlated with BMIC at 12 weeks postpartum. BMIC was not associated with maternal age, gestational age at birth or parity, which is supported by most of the existing literature (127, 229). No association between BMIC and EBF was found in this analysis, which potentially indicates that BMIC is not determined by breast milk volume in this population. This is only plausible if exclusively breastfed children at six months of age had a higher breast milk intake compared to non-exclusively breastfed children, which may not be the case. More studies investigating the influence of breast milk volume on BMIC are needed.

6.6.2 Strengths and limitations

The strengths of this study are the randomised, double-blind design of the ENID trial conducted in a moderately deficient population of pregnant and lactating women, measuring the almost complete spectra of iodine status and thyroid function parameters along with BMIC. Few previous studies have been conducted in areas with moderate iodine deficiency, and even fewer studies have covered pregnancy, lactation and infancy and measured the range of biomarkers done in this study. This analysis exposed the need to focus on maternal iodine status during both periods of pregnancy and lactation, as both are equally important for maternal and infant iodine status. Further, this analysis identified the need of further research on definitions of iodine deficiency especially of Tg during infancy, and the need to define an adequate BMIC.

Advanced laboratory techniques were used to measure iodine status and BMIC and furthermore a 24-hour UIC sample was used to assess maternal status rather than a spot UIC. As already described, breast milk is a complex matrix hence the laboratory method used contributes to the reliability of the results obtained. In the current analysis, IC-PMS was used to measure BMIC, which is now considered the gold standard (280). EBF rates were high in this cohort, making it possible to estimate infant iodine intake without the need of analysing iodine intake from complementary foods.

The limitations of this study are that maternal or infant thyroid hormones were not investigated, which could have supported the interpretation of the severity of iodine deficiency. For instance if the mothers' TSH and T_4 were within the normal reference range, it may have explained why they were not visible goitres, and supported an interpretation of a mild-to-moderate iodine-deficient population. Thyroid hormone concentrations could have informed if the maternal thyroid, in this population, was able to adapt to meet the increased thyroid hormone requirements during pregnancy at mild-to-moderate iodine deficiency. Further, infant UIC was not measured, as infant urine was not collected as a part of ENID.

6.6.3 Conclusion

In conclusion, a daily multiple micronutrient supplementation (containing 300 µg/day iodine) given during pregnancy to mild-to-moderate iodine-deficient Gambian women, was not adequate to increase BMIC to a level that could sustain exclusively breastfed infants' iodine needs in the first six months of life. Efforts to increase access to iodine in this community is a high priority.

Chapter 7

Vitamin B_{12}

This Chapter presents how a vitamin B_{12}-containing multiple micronutrient supplement given during pregnancy influences maternal status during pregnancy and lactation, breast milk composition and infant status in rural Gambia. The background begins with a review of vitamin B_{12} physiology, deficiency and vitamin B_{12} in human milk. This is followed by a description of assessment of vitamin B_{12}, definitions of adequate status, intake requirements and epidemiological evidence for the importance of vitamin B_{12}. The focus of the background is on all three periods of pregnancy, lactation and infancy. The background is followed by a short introduction to the research area, the methods, results and discussion.

7.1 Background

Vitamin B_{12} deficiency is a public health problem. Clinical vitamin B_{12} deficiency, such as severe anaemia or neurological manifestations in the elderly, is rarely observed, however subclinical vitamin B_{12} deficiency, which is defined as a low or marginal B_{12} status, affects several population groups (398-400). Evidence from recent population-based studies suggests that low or marginal vitamin B_{12} status in either pregnancy or infancy is likely to have detrimental consequences for the infant (79, 401-403). Global prevalence data for subclinical vitamin B_{12} deficiency are not available, however in the United States, between 3-26% of the general population is affected, with a higher proportion in the elderly population. Higher prevalence is found in South America, Asia and Africa, where in some population groups more than 40% have a low vitamin B_{12} status, including pregnant women and young children (404).

Pregnant and lactating women, infants and the elderly are among the population groups at greatest risk of vitamin B_{12} deficiency. This is especially true in resource-poor settings where intakes of animal source foods are low; dietary vitamin B_{12} is solely available in animal products, such as meat, liver, fish, eggs and dairy products (398) (399). Vegans and vegetarians are thus particularly at risk of deficiency.

7.1.1 Vitamin B_{12} physiology

Vitamin B_{12}, also known as cobalamin, is a water-soluble vitamin. In food, vitamin B_{12} is found bound to dietary proteins from which the vitamin is released by gastric pepsin in the acidic environment of the stomach. The released vitamin then binds to haptocorrin, a glycoprotein produced by the salivary gland which shields vitamin B_{12} from acid degradation (398, 405, 406) (Figure 26). In the duodenum haptocorrin is degraded and vitamin B_{12} binds to the gastric intrinsic factor (IF). In the ilium, the IF-B_{12} complex is taken up by the cubam receptor for lysosome processing, and vitamin B_{12} is transported to the blood by multidrug resistance protein 1 (MDR1) (398). Vitamin B_{12} then binds to transcobalamin, the blood carrier of the vitamin needed for transport and uptake of vitamin B_{12} by cells (406, 407). About 20% of vitamin B_{12} in plasma is bound to transcobalamin (called holotranscobalamin), and the remainder is bound to haptocorrin, which is taken up by protein receptors in the liver (398, 406). Holotranscobalamin is taken up by the cells, and enters lysosomes where it is degraded and vitamin B_{12} is released and converted to its cofactor forms. Around 60-80% of ingested vitamin B_{12} is excreted through faeces (398).

The average body vitamin B_{12} is estimated to be 2-3 mg in healthy adults, and around half of this is found in the liver, bound to haptocorrin (408, 409). Some of this stored liver vitamin B_{12} is continuously excreted in bile (around 0.15 % of body storage per day), where the majority is reabsorbed and enters the enterohepatic circulation (398, 406). This is a mechanism to preserve vitamin B_{12}, which means that depletion of vitamin B_{12}, and any onset of vitamin B_{12} deficiency, may take years in adults (399).

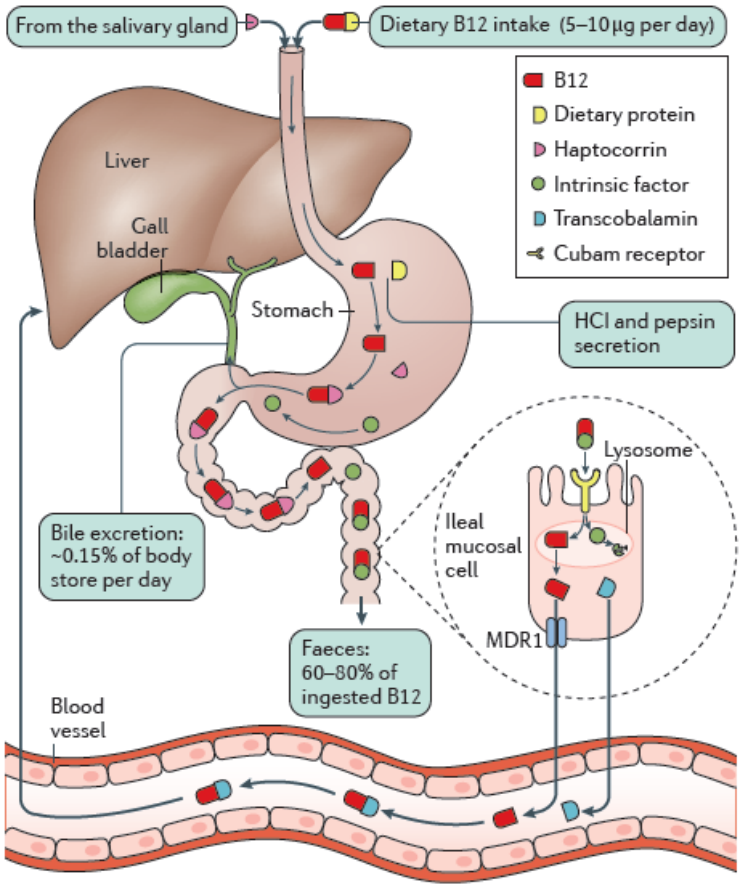

Figure 26. Vitamin B_{12} absorption

From Green et al (2017) (398).

7.1.2 Vitamin B_{12} physiology during pregnancy and lactation

Vitamin B_{12} is transferred across the placenta to the foetus, however there is limited data about this transplacental transfer (410). It has been suggested that the placenta is capable of binding vitamin B_{12} to placental haptocorrin and transcobalamin, making vitamin B_{12} available to the foetus and maternal circulation (410).

In human breast milk vitamin B_{12} is bound almost exclusively to haptocorrin, and only a small amount is bound to transcobalamin (84, 406). The transfer of vitamin B_{12} from transcobalamin in plasma to haptocorrin in milk is thought to occur by a receptor-mediated system, however little is known about the mechanisms that mediate the uptake in mammary cells (84).

7.1.3 Vitamin B_{12} deficiency

Inadequate vitamin B_{12} consumption leads to vitamin B_{12} deficiency, however other causes of the deficiency also exists, such as insufficient vitamin B_{12} absorption (pernicious anaemia), genetic factors, or gastrointestinal infections (398). The health consequences of vitamin B_{12} deficiency are shown in Table 27, and can have severe consequences for infants, children and adults (398). Vitamin B_{12} serves as a cofactor in important cellular processes; and very importantly in two enzymatic reactions (i) the folate-dependent conversion of homocysteine to methionine and (ii) mitochondrial metabolism (398). The first process is important for the maintenance of DNA synthesis and methylation, and the latter for the oxidation of fatty acids and the breakdown of ketogenic amino acids (404).

Table 27. Vitamin B_{12} deficiency consequences. From Green et al (2017) (398).

Population group	**Consequences of vitamin B_{12} deficiency**
Infants/children	Neural tube defects Stunting Cerebral atrophy Hypotonia Developmental delays
Adult/elderly	Contribution to cardiovascular disease Contribution to neurodegenerative disorders Contribution to the development of diabetes mellitus Megaloblastic or macrocytic anaemia Cognitive impairment Depression Bone disease Hearing loss Macular degeneration

The risk of vitamin B_{12} deficiency disorders has been shown to increase if folate status is high (411). The mechanisms that causes excess folate to interfere with vitamin B_{12} metabolism and worsen the health outcomes of vitamin B_{12} deficiency are not yet known (411). This is a cause for concern in populations with high prevalence of vitamin B_{12} deficiency and high exposure of folic acid fortifications or supplement use (404). More research in this area is needed to confirm the effect of folic acid on vitamin B_{12} deficiency.

7.1.4 Vitamin B_{12} in human milk

Vitamin B_{12} is bound to haptocorrin in breast milk (84), where it exists in two forms: apo-haptocorrin (apo-HC), the unsaturated form, and holo-haptocorrin, which is saturated with vitamin B_{12} (412). In breast milk, the majority of vitamin B_{12} is present in the unsaturated form (412).

Vitamin B_{12} concentration has been reported highest in colostrum and transitional milk (176, 413, 414) with a decline as lactation progresses to mature milk (175, 176, 414, 415). A recent review reported that the decline seen in mature milk was more pronounced in women who had a high concentration of vitamin B_{12} in early lactation (92). Two recent studies have however reported an increase in vitamin B_{12} concentration in mature milk (175, 180).

One study found that hindmilk contained slightly higher vitamin B_{12} concentrations than foremilk (180), whereas others have not detected this difference (274, 414). Hampel et al (2017) (274) reported diurnal changes for vitamin B_{12}, however the variance was considered small. Two studies reported no difference in vitamin B_{12} concentration in breast milk between mothers who had given birth pre-term and at term (129, 416), and one study found maternal age to be negatively associated with breast milk vitamin B_{12} concentrations (179).

As described in Chapter 2, vitamin B_{12} concentration in breast milk is affected by maternal vitamin B_{12} supplementation, and mixed findings have been reported on the association between maternal status and breast milk vitamin B_{12} concentration. Based on this, breast milk vitamin B_{12} is likely driven more by maternal intake than status (417, 418).

7.1.5 Assessment of vitamin B_{12} status

The methods commonly recommended to assess vitamin B_{12} status in humans are (i) serum/plasma vitamin B_{12}, (ii) serum holotranscobalamin (holoTc) (iii) plasma total homocysteine (tHcy) and (iiii) serum methylmalonic acid (MMA) (398). The use of at least two of these measurements is recommended to diagnose vitamin B_{12} deficiency, most commonly vitamin B_{12} concentration and MMA or tHcy as the second test (419, 420). Recently this approach has been developed further, where a minimum of two of the four biomarkers are included in a model which calculates a combined vitamin B_{12} (cB_{12}) value (421). This is recognised as a reliable method to diagnose vitamin B_{12} status, however more work is needed to confirm the cut-off for deficiency and the clinical usefulness of this index (421).

Serum/plasma vitamin B_{12}

Serum or plasma total vitamin B_{12} concentration, usually expressed as pmol/l, is the biomarker most often used to assess vitamin B_{12} status (422). The concentration includes the vitamin B_{12} bound to transcobalamin (the active form) and the vitamin B_{12} bound to haptocorrin. This biomarker reflects long-term vitamin B_{12} status in individuals, and is also used as a biomarker for population groups (404). It does not reflect current or immediate vitamin B_{12} intake, as it can take up to several months to react to an increased dietary vitamin B_{12} intake or to a low dose supplement (404). The biomarker is relatively insensitive, especially at low concentrations, and so concentrations below the lower limit of the reference interval indicate *probable* vitamin B_{12} depletion or deficiency, whereas a concentration above the limits indicates replete vitamin B_{12} status (398). The advantage of this biomarker is that the main laboratory method (protein-binding assay) is low in cost and widely available (398, 421).

Serum holotranscobalamin (holoTc)

Serum holoTc concentration is a measurement of only the transcobalamin-bound vitamin B_{12} in blood. It was proposed as a more sensitive maker of vitamin B_{12} status, than total serum or plasma vitamin B_{12} concentrations due to its metabolic functions. However, a recent study showed that this biomarker is only marginally better than total vitamin B_{12} (423). HoloTc is more sensitive to recent intake, and will respond to vitamin B_{12} intake within hours (404).

Plasma total homocysteine (tHcy)

Plasma total tHcy is used as a biomarker for vitamin B_{12} status, as tHcy is increased if vitamin B_{12} status is poor. This is due to vitamin B_{12}'s involvement in methionine synthase where tHcy is converted to methionine. However, tHcy is also increased when folate, riboflavin or vitamin B_6 status is poor, making this biomarker less relevant in populations which are at risk of several micronutrient deficiencies (422).

Serum methylmalonic acid (MMA)

Serum MMA concentration is the most sensitive single vitamin B_{12} biomarker, and has been referred to as the gold standard (424). MMA is a side-reaction product of methylmalonyl CoA metabolism, a process where vitamin B_{12} serves as a cofactor. MMA increases with poor vitamin B_{12} status, and is not affected by the status of other B-vitamins (404). It can detect vitamin B_{12}

stores in the liver, but not recent vitamin B_{12} intake. The largest disadvantage of this biomarker is the high cost of laboratory analysis (424).

7.1.6 Criteria for adequate vitamin B_{12}

Criteria for adequate vitamin B_{12} status for the adult population are available for all four biomarkers (398) (Table 28). The intervals and cut-offs for the vitamin B_{12} biomarkers are however still debated, as there is a lack of literature linking these criteria to functional outcomes (404). The deficiency cut-off (<148 pmol/l) for vitamin B_{12} concentration includes both clinical and sub-clinical vitamin B_{12} deficiency, and is based on research showing that below this cut point MMA and tHcy concentrations start to markedly increase (425). This criteria for vitamin B_{12} adequacy has been endorsed by the Institute of Medicine (IOM) (1998) (417) and the World Health Organization (WHO) (426) (however the WHO use <150 pmol/l instead of <148 pmol/l).

Table 28. Vitamin B_{12} biomarkers' criteria for deficiency, from Green et al (2017) **(398)**.

Biomarker; unit	**Tentative reference interval**	**Tentative cut-off value for B_{12} deficiency**	**Tentative cut-off value for B_{12} repletion**
Vitamin B_{12}; pmol/l	200-600	<148	>221
Holotranscobalamin; pmol/l	40-100	<35	>40
Homocysteine; µmol/l	8-15	>15	<8
Methylmalonic acid; µmol/l	0.04-0.37	>0.37	<0.27

The reference interval covers 95% of vitamin B_{12}-replete individuals. The deficiency cut-offs includes both clinical and sub-clinical deficiency.

Pregnancy

The criteria for deficiency in the adult population (Table 28) have often been applied to pregnant women. However, several studies have described a consistent physiological decrease in maternal vitamin B_{12} concentration across the duration of a normal pregnancy (175, 427-431), as illustrated in Figure 27. A plausible explanation for this includes hemodilution, hormonal changes, vitamin B_{12} transfer to the foetus and changes in vitamin B_{12} binding proteins during pregnancy (177, 430, 432, 433). Further, MMA increases during pregnancy in healthy women (Figure 27), which could be indicative of mild vitamin B_{12} depletion in the last stage of pregnancy (398, 427). MMA increase is also greater in women with a low holoTC at pre-conception (427).

Because of these marked physiological changes in vitamin B_{12} concentration during pregnancy the use of adult criteria for vitamin B_{12} deficiency during pregnancy is debated (434). Establishing specific reference ranges for vitamin B_{12} adequacy and cut-offs for deficiency in pregnancy are needed (398).

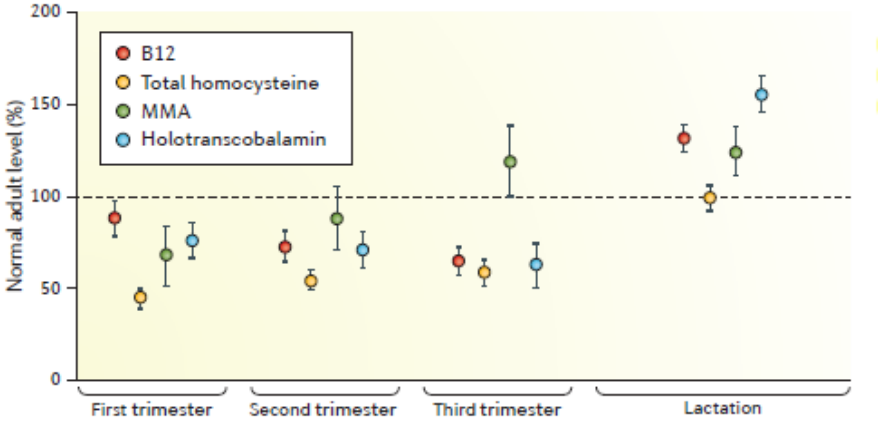

Figure 27. Biomarkers of vitamin B_{12} status during pregnancy and lactation.

From Green et al (2017) (398). Levels of vitamin B_{12} biomarkers presented as a percentage of normal levels based on a population of 207 non-pregnant, non-lactating women (18-40 years). Data during pregnancy for vitamin B_{12} and total homocysteine are based on Greibe et al (2011) (430); data on MMA are based on Murphy et al (2007) (427); data on vitamin B_{12} status during lactation are based on Bjorke-Monsen et al (2008) (435); data on holotranscobalamin levels during pregnancy are based on Murphy et al (2007) (427), and during lactation on Bae et al (2015) (177). Error bars represent the 10th and 90th percentiles from the geometric mean.

Lactation

Maternal serum vitamin B_{12} increases again during lactation, and the concentration is likely to be higher than before pregnancy (177) (Figure 27). This increase in vitamin B_{12} concentration between pregnancy and lactation is believed to represent the improvement of mobilization of maternal vitamin B_{12} stores for transfer of the vitamin to the milk (398).

Infancy

Many studies have applied the adult criteria for vitamin B_{12} deficiency (Table 28) to infants (434). This has been criticised and reference intervals using data from a longitudinal study of well-nourished Norwegian infants (n=364), have been proposed instead (436). In this study, values of

vitamin B_{12} biomarkers changed significantly from birth to two years of age and were higher in non-breastfed children compared to breastfed (436). Table 29 details the tentative reference interval developed by Hay et al (2008) (436) for breastfed infants according to age.

Table 29. Reference intervals for vitamin B_{12} status of breastfed infants according to age, from Hay et al (2008) **(436).**

	Tentative reference interval			
	At birth	6 months	12 months	24 months
Vitamin B_{12}; pmol/l	120-686	121-517	165-580	183-621

7.1.7 Vitamin B_{12} intake requirements during pregnancy, lactation and infancy

Pregnancy and lactation

Vitamin B_{12} requirements increase during pregnancy and lactation. In pregnancy maternal vitamin B_{12} transferred to the foetus is primarily from newly absorbed vitamin B_{12}, and thus maternal stores are a less important source (437). The IOM (1998) (417) suggests an additional 0.2 µg/day of vitamin B_{12} during pregnancy to ensure foetal accumulation of 0.1-0.2 µg/day. This results in an estimated average requirement (EAR) of 2.2 µg/day for pregnant women (Table 30). In lactation, requirements are increased to 2.4 µg/day to account for the transfer of the vitamin to breast milk (417).

Table 30. Recommended dietary vitamin B_{12} intake (µg/day) for pregnant, lactating women and infants between 0-6 months by IOM (417) and WHO/FAO (438).

	Pregnancy		**Lactation**		**Infancy**	
	IOM	WHO	IOM	WHO	IOM	WHO
Vitamin B_{12}	EAR: 2.2 RDA: 2.6	- RDA: 2.6	EAR: 2.4 RDA: 2.8	- RDA: 2.8	AI: 0.4	AI: 0.4

EAR, estimated average requirement (the EAR is the daily dietary intake level of a nutrient expected to satisfy the needs of 50% of the population group. An intake less than the EAR is considered to be inadequate). RDA, recommended dietary allowance (the RDA is defined as equal to the EAR plus twice the coefficient of variation to cover the needs of 97-98% of the population group). AI, average intake (dietary intake believed to be adequate for everyone in the demographic group to maintain health, established where no sufficient data to establish EAR are available). IOM, Institute of Medicine, WHO, World Health Organization.

Infancy

Neonates are particularly vulnerable to vitamin B_{12} deficiency, because infants are born with limited liver vitamin B_{12} storage, especially if maternal status and intake during pregnancy were poor. Infants born to vitamin B_{12}-deficient mothers are at high risk of developing symptoms of vitamin B_{12} deficiency within a few months (439). This is different to how deficiency manifests in adults, who may tolerate a low intake of vitamin B_{12} for several years before symptoms arise. Symptoms such as failure to thrive, apathy, feeding difficulties and developmental regression have been reported in infants aged 4-10 months (439). An infant born to a vitamin B_{12}-replete mother has vitamin B_{12} liver stores of approximately 25-30 µg (440, 441). These stores are adequate to sustain infant vitamin B_{12} needs in the first few months after birth (442).

Vitamin B_{12} requirements for infants are poorly defined. The IOM (1998) (417) and the WHO (438) recommend an average intake (AI) of 0.4 µg/day in the first six months of life (Table 30). This AI is based on the median breast milk vitamin B_{12} concentration of 0.42 µg/l from only nine, unsupplemented Brazilian mothers (414) and an average breast milk intake of 0.781 l/day reported by a test-weighing study (375). However, the validity of the method used to quantify breast milk vitamin B_{12} concentration in this study has been questioned (92). Recent research has shown that it is necessary to remove breast milk vitamin B_{12} from apo-HC to obtain valid concentrations, which was not done in this study (281).

An earlier study, conducted in 1990 in the United States, proposed a cut-off of <362 pmol/l (approximately <0.49 µg/l) to define breast milk B_{12} deficiency (182). This cut-off was based on 19 vegetarian and omnivorous women's breast milk vitamin B_{12} concentration and the threshold for when an increase in infant urinary MMA occurred. However, the samples collected in this study were spread out over a wide range of months (between 2 and 14 months postpartum), potentially introducing bias to the results due to changes in breast milk composition across lactation. In addition, this study was conducted in the 1990's where outdated laboratory methods where used, possibly limiting the accuracy of the vitamin B_{12} measurements. Several studies have used this cut-off, but a recent Canadian study (181) showed that in vitamin B_{12}-replete mother-and-infant-pairs, 50% had breast milk vitamin B_{12} concentrations below 362 pmol/l, suggesting a clear overestimation of deficiency by using this cut-off.

7.2 Vitamin B_{12} in the first 1000 days

Vitamin B_{12} is essential during all life stages, but especially during pregnancy and lactation, when the risk of infant vitamin B_{12} deficiency is high (434).

7.2.1 Pregnancy vitamin B_{12} status and infant health outcomes

Few studies examine the effect of maternal vitamin B_{12} status during pregnancy on infant and childhood health outcomes. Poor maternal vitamin B_{12} status has been linked to neural tube defects in infants (443, 444), still birth (79), pre-term birth (445, 446) and intrauterine growth restriction (79, 447). The available data on low birth weight is not convincing (448). A recent systematic review concluded that the link between maternal vitamin B_{12} status during pregnancy and low birth weight warrants further investigations through high quality designed studies (448). Neumann et al (2013) (79) reported no association between vitamin B_{12} intake during pregnancy and infant growth at six months of age.

In relation to infant brain development, a recent randomised controlled trial (RCT) did not find any effect of maternal B_{12} supplementation during pregnancy and early lactation on infant brain development at nine months of age (449). The authors did however find that high maternal tHcy status in pregnancy was associated with poorer performance on language based assessments, and on fine motor development. In terms of long-term effects of maternal vitamin B_{12} status during pregnancy on childhood cognition, an observational study in India showed that children of mothers with a low vitamin B_{12} status in early pregnancy did less well in attention and memory tests at nine years of age compared to children whose mother had a higher vitamin B_{12} status in early gestation (450).

7.2.2 Infancy vitamin B_{12} status and health outcomes

The importance of vitamin B_{12} intakes during infancy has also been explored. Kvestad et al (2015) (402) observed in their RCT an effect of vitamin B_{12} supplementation on gross motor development in Indian infants, and similar effects were observed in an RCT of Norwegian infants (451). Another observational study linked higher vitamin B_{12} status in 12-18 month old Indian children to higher mental development scores (403). An observational study investigating long-term effect linked vitamin B_{12} status in infancy to cognitive functioning in later childhood in Nepalese children (401). They reported that a higher cB_{12} score (combining plasma vitamin B_{12},

tHcy and MMA) during infancy was associated with a higher Ages and Stages Questionnaire score five years later, indicative of better cognitive function.

The period of exclusive breastfeeding (EBF) is a particularly vulnerable period. Healthy, breastfed infants have lower vitamin B_{12} status than non-breastfed infants, possibly reflecting an altered, but appropriate, vitamin B_{12} status associated with breastfeeding (431, 436, 451). However, mothers with depleted vitamin B_{12} stores, and a habitual low intake of animal source foods, are at risk of low breast milk vitamin B_{12} concentration, from which the exclusively breastfed infant is at risk of neurological and growth deficits (439). This has been shown in case studies of vegan or vegetarian mothers who were exclusively breastfeeding their infants. The deficient infants presented with irritability, anorexia and failure to thrive before being treated with a high dose intramuscular and oral vitamin B_{12}. In 40-50% of the cases cognitive and developmental retardation persisted (439, 452). Torsvik et al (2015) (453) recently reported that breastfeeding compared to formula feeding was associated with poorer gross motor development at six months of age in Norwegian infants with a sub-optimal birth weight (2000-3000 g). More studies are needed on the relationship between breast milk vitamin B_{12} concentrations, infant vitamin B_{12} status and postnatal outcomes.

7.3 Vitamin B_{12} status of mothers, breast milk and infants

Ensuring optimal vitamin B_{12} intakes in exclusively breastfed infants in populations where habitual intake of animal food sources is limited, is of great importance. As highlighted in Chapter 2, maternal vitamin B_{12} supplementation during lactation has a positive effect on breast milk vitamin B_{12}. However, few intervention studies have investigated the effect of maternal supplementation during pregnancy. In fact most supplementation studies conducted on this topic have (i) not investigated the effect of supplementation exclusively during pregnancy (none of the 7 studies identified investigated the effect during pregnancy only), and (ii) not investigated the effect simultaneously on breast milk vitamin B_{12} concentration and infant status (only 2 out of 7 studies have investigated this (175, 176)). Most available interventions have investigated the effect longitudinally across the first six months of lactation (4 out of 7 studies (161, 163, 175, 176)). Essentially, no study has investigated how maternal supplementation during pregnancy impact breast milk vitamin B_{12} concentration and infant vitamin B_{12} status across the first six months postpartum, highlighting the need for future research.

This Chapter presents an original analysis investigating; (i) the effect of an vitamin B_{12}-containing multiple micronutrient supplement during pregnancy on breast milk vitamin B_{12} concentration and infant vitamin B_{12} status during in the first six months of life in rural Gambia; (ii) determinants of breast milk vitamin B_{12} concentration and (iii) estimated infant vitamin B_{12} intake.

7.4 Methods

Data and samples for this analysis were collected as a part of the ENID trial and the ENID-Bone extension to the ENID trial. Maternal plasma (from baseline, 30 weeks' gestation and 12 weeks postpartum), breast milk (8, 12 and 24 weeks postpartum) and infant plasma (cord blood, 12 and 24 weeks postpartum) from participating women and infants in the tablet arm of ENID (Iron-folic acid (FeFol) and multiple micronutrients (MMN)) were used. Maternal plasma vitamin B_{12}, breast milk vitamin B_{12} concentration and infant plasma vitamin B_{12} concentration were analysed. Participant recruitment, maternal supplementation, data and sample collection methodologies are all described in full in Chapter 4 (page 117).

7.4.1 Sample analysis

Plasma and breast milk samples were aliquoted and frozen until analysis. Plasma and breast milk samples were transported to USDA Western Human Nutrition Research Centre (WHNRC), Davis, California, USA, for analysis.

Plasma vitamin B_{12} concentration

Plasma vitamin B_{12} was measured by chemiluminescence immunoassay on Cobas e411 (Roche Diagnostic Corp., Indianapolis, the United States). The samples were defrosted, vortexed and put on the instrument for analysis. Roche V0, V1, V2 Varia controls were used with each assay run to ensure that parameters of the instrument and the reagents were within Roche's predetermined range. All sample preparations were carried out under subdued light and on ice to protect the analytes against degradation. Tables 31 and 32 show the inter-assay variability for the quality controls of maternal and infant plasma vitamin B_{12}. The coefficients of variation (CV) obtained are of acceptable quality.

Table 31. Inter-assay variability of the quality controls for maternal plasma vitamin B_{12}

	Maternal (low)	Maternal (medium)	Maternal (high)
n	12	12	12
Mean (pmol/l)	161.4	365.3	774.5
SD	12.8	15.5	26.4
CV (%)	7.9	4.3	3.4

SD, standard deviation; CV, coefficient of variation; n, sample size.

Table 32. Inter-assay variability of the quality controls for infant plasma vitamin B_{12}

	Infant (low)	Infant (medium)	Infant (high)
n	7	7	7
Mean (pmol/l)	174.1	370.6	782.2
SD	19.5	25.4	23.2
CV (%)	11.2	6.9	3.0

SD, standard deviation; CV, coefficient of variation; n, sample size.

Breast milk vitamin B_{12} concentration

Breast milk B_{12} was measured using an IMMULITE 1000 Vitamin B_{12} solid-phase, competitive chemiluminescent enzyme immunoassay, which is an in-house method based on a published method (454). Breast milk sample preparation was carried out under subdued light to avoid degradation. Breast milk aliquots were defrosted and the samples were centrifuged for 10 minutes at 14000 rpm and 4 ºC to remove fat and solids from the sample, and 200 µl of the whey fraction was transferred to a polypropylene tube for analysis. Pooled breast milk from one apparently healthy donor was used as a quality control. Table 33 presents the inter-assay variability of the quality controls reported. The CVs obtained are of high quality.

Table 33. Inter-assay variability of the quality controls for breast milk vitamin B_{12}

	Vitamin B_{12} (ID 1)	Vitamin B_{12} (ID 2)	Vitamin B_{12} (ID 3)	Vitamin B_{12} (ID 4)
n	4	4	4	4
Mean (pmol/l)	87.5	91.5	95.5	99.5
SD	1.3	1.3	1.3	1.3
CV (%)	1.5	1.4	1.4	1.3

SD; standard deviation, CV; coefficient of variation, n; sample size

7.4.2 Statistical analysis

The same statistical approach was used as described in Chapter 6 (section 6.4.2), with the exception that maternal plasma vitamin B_{12}, breast milk vitamin B_{12} concentration and infant plasma vitamin B_{12} were investigated.

Outliers were defined as data with residuals >3 standard deviations (SD) from the mean in the mixed effects models and were excluded from the analysis (maternal plasma vitamin B_{12} n=3 data points removed, breast milk vitamin B_{12} n=2, infant plasma vitamin B_{12} n=2). In addition the data points that fell below the minimum detection concentration for breast milk vitamin B_{12} concentration (n=74) were excluded from the analysis. This was done to decrease the variance in the residuals, and to make them normally distributed; with these 74 data points in the model, the residuals were far from normally distributed even when logged transformed.

Individual breast milk vitamin B_{12} concentrations were converted from pmol/l to µg/l by multiplying the concentration by 0.0013554 (calculated by using the molar weight of vitamin B_{12}, which is 1355.4 g/mol).

7.5 Results

For this analysis women and children from the FeFol and MMN arms of the ENID trial were included, representing a total of 381 mother and infant pairs (Figure 19 in Chapter 6, Section 6.5). Baseline characteristics of the study population according to intervention arm are presented in Table 21, in Chapter 6 (Section 6.5).

Mean (SE) maternal plasma vitamin B_{12} concentration at baseline was 332 (8.7) pmol/l, and this did not differ between supplement groups (p=0.6, Table 34). At baseline, 7% (21/289) had a vitamin B_{12} concentration lower than 148 pmol/l indicating deficiency, and 16% (45/289) had a concentration between 148-221 pmol/l, indicating vitamin B_{12} depletion.

7.5.1 Supplement effect on vitamin B_{12} status and breast milk concentration

Maternal B_{12} status

Maternal MMN supplementation significantly improved maternal plasma vitamin B_{12} concentration compared with FeFol (p=0.02, Table 34, Figure 28). At 30 weeks' gestation mean

(SE) plasma vitamin B_{12} for the FeFol group was 264 (12.6) pmol/l and 311 (12.5) pmol/l for the MMN group, and were significantly different (p=0.008). Between baseline and 30 weeks' gestation, vitamin B_{12} concentration decreased in the FeFol group (p<0.001) whereas the decrease in the MMN group was non-significant (p=0.2). At 30 weeks' gestation, 42% (56/132) in the FeFol group had plasma vitamin B_{12} concentrations ≤221 pmol/l and 31% (41/133) in the MMN group (p=0.05).

Between 30 weeks' gestation and 12 weeks postpartum, plasma vitamin B_{12} concentration increased in both supplement groups (significant for the FeFol group, p=0.03, not for the MMN group, p=0.8). The difference in concentration between the groups was not significant at 12 weeks postpartum (p=0.2). Mean vitamin B_{12} concentration at 12 weeks postpartum was 304 (8.3) pmol/l, and 20% (63/320) had a vitamin B_{12} concentration lower than 148 pmol/l and 11% (36/320) had a concentration between 148-221 pmol/l at 12 weeks postpartum.

Breast milk vitamin B_{12} concentration

For breast milk vitamin B_{12} concentration, no evidence was found for an overall time by supplement interaction (p=0.9, Table 34, Figure 29), and no evidence for a difference in concentration between the two supplement groups that was consistent over time (p=0.4). Median (IQR) breast milk vitamin B_{12} concentration at 8 weeks postpartum was 179 (100, 233) pmol/l in the FeFol group and 183 (144, 259) pmol/l in the MMN group, with an overall median of 181 (121, 249) pmol/l.

Breast milk vitamin B_{12} concentration decreased between 8 and 12 weeks postpartum for both groups, however this decrease was not significant (FeFol group, p=0.3, MMN group, p=0.2). Between 12 and 24 weeks postpartum breast milk vitamin B_{12} concentration increased significantly for both supplement groups (p<0.001). Median breast milk vitamin B_{12} at 24 weeks was 184 (149, 256) pmol/l for the FeFol group, and 207 (152, 290) pmol/l for the MMN group. No significant difference was found in breast milk vitamin B_{12} concentration by supplement group at any of the three time-points.

Infant vitamin B_{12} status

Maternal MMN supplementation during pregnancy did not significantly improve infant plasma vitamin B_{12} concentration over time compared to FeFol (no evidence for an overall time by supplement interaction, p=0.2, Table 34, Figure 30). Median infant cord plasma vitamin B_{12}

concentration was 353 (224, 530) pmol/l and 368 (270, 604) pmol/l for the FeFol and MMN groups, respectively, and decreased to 263 (179, 357) pmol/l in the FeFol group and to 300 (205, 375) pmol/l in the MMN group at 12 weeks postpartum. A significant difference in cord vitamin B_{12} concentrations between the two groups was observed at birth ($p=0.02$), which remained to 12 weeks postpartum ($p=0.01$). The difference in plasma vitamin B_{12} concentration observed at birth and at 12 weeks postpartum disappeared at 24 weeks postpartum ($p=0.4$). Infant plasma vitamin B_{12} concentration significantly decreased between birth and 24 weeks postpartum for both supplement groups ($p<0.001$). At 24 weeks of age 5% (17/327) of infants had a plasma vitamin B_{12} concentration below 121 pmol/l.

Table 34. Maternal plasma B_{12}, breast milk B_{12} and infant plasma B_{12} concentrations according to maternal supplement group and time-point, derived from mixed effects models.

Mothers	Baseline	p-value *	30 weeks' gestation	p-value *	12 weeks postpartum	p-value *	p-value **
Plasma vitamin B_{12} concentration (pmol/l) [a]							
MMN	326.6 (12.1)		311.3 (12.5)		314.3 (11.6)		
FeFol	336.4 (12.3)		264.1 (12.6)		293.2 (11.8)		
Difference between supplement groups (pmol/l) [b]	-9.82 (17.2)	0.6	47.19 (17.8)	0.008	21.17 (16.6)	0.2	0.02
Breast milk	8 weeks postpartum		12 weeks postpartum		24 weeks postpartum		
Breast milk vitamin B_{12} concentration (pmol/l) [a]							
MMN	183.3 (144.0, 259.0)		169.0 (110.6, 234.7)		206.7 (151.5, 289.9)		
FeFol	179.1 (99.6, 233.3)		154.3 (86.8, 214,0)		184.2 (148.7, 256.2)		
Difference between supplement groups (%)[b]	12.4 (-6.7, 35.4)	0.2	9.8 (-8.2, 31.3)	0.3	9.7 (-7.8, 29.0)	0.3	0.9 (0.4)
Infants	Birth (cord blood)		12 weeks postpartum		24 weeks postpartum		
Plasma vitamin B_{12} concentration (pmol/l) [a]							
MMN	368 (270, 604)		300 (205, 375)		239 (185, 332)		
FeFol	353 (224, 530)		263 (179, 357)		236 (185, 332)		
Difference between supplement groups (%)[b]	16.0 (2.9, 30.8)	0.02	14.0 (2.9, 26.4)	0.01	5.0 (-5.4, 16.4)	0.4	0.2

Data presented for each supplement groups are medians (IQR) (non-normally distributed data), or means (SE) (normal distributed data). The means are derived from the mixed effect models, and the medians are derived from the raw data. For maternal plasma vitamin B_{12}, the difference between the two supplement groups is presented as the difference in mean (95% CI), and for breast milk vitamin B_{12} and infant plasma vitamin B_{12}, the differences are presented as the percentage (95% CI) difference in mean concentrations between groups, calculated by exponentiating coefficients from the log transformed model. All data were log-transformed, except for maternal plasma vitamin B_{12}.

[a] To convert plasma and breast milk vitamin B_{12} concentration to µg/l multiply the concentration with 0.0013554

[b] FeFol group is the referent group

* This p-value tests the difference in concentration between supplement groups at the given time-point

** This p-value tests the difference in concentration between the two supplement groups depending on time; in other words the p-value tests an overall time by supplementation interaction. For the breast milk vitamin B_{12} analysis the p-value presented in brackets is the overall supplement effect independent of time.

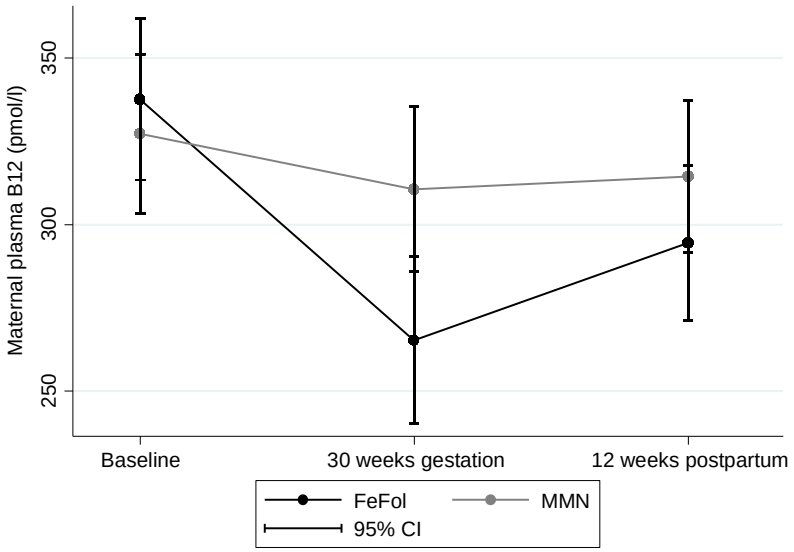

Figure 28. Longitudinal maternal mean plasma vitamin B_{12} concentration (pmol/l) according to supplement group at baseline, 30 weeks' gestation and 12 weeks postpartum.

Data were normally distributed and analysed using a mixed effects model. The overall time by supplement interaction p=0.02.

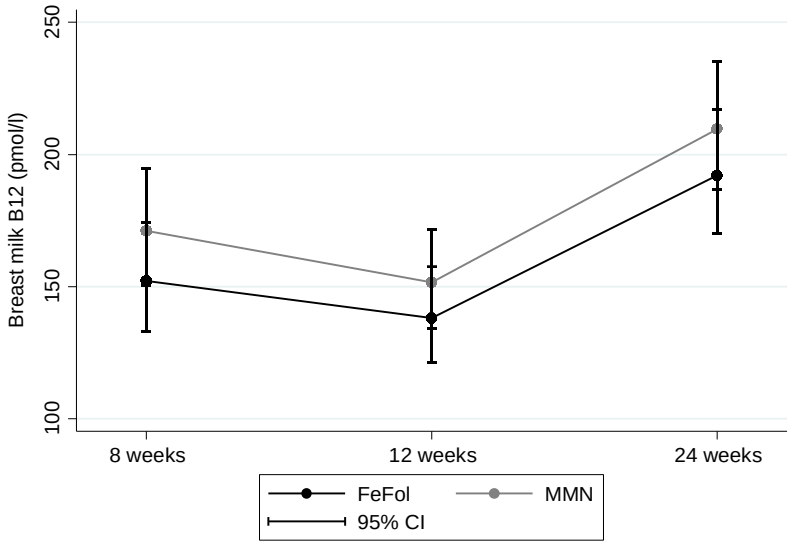

Figure 29. Longitudinal breast milk vitamin B_{12} concentration (pmol/l) (geometric means) according to supplement group at 8, 12 and 24 weeks postpartum.

Data were log-transformed and analysed using a mixed effects model. Values on y-axis are unlogged. The overall time by supplementation interaction p=0.9. The overall time by supplementation effect (consistent over time) p=0.4.

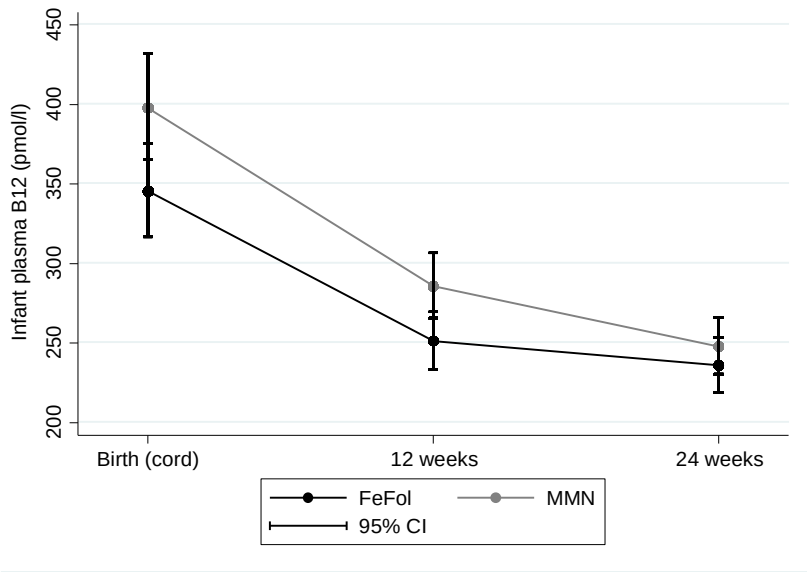

Figure 30. Longitudinal infant plasma vitamin B_{12} concentration (pmol/l) (geometric means) according to supplement group at birth, 12 and 24 weeks postpartum.

Data were log-transformed and analysed using a mixed effects model. Values on y-axis are unlogged. The overall time by supplementation interaction p=0.2.

7.5.2 Determinants of breast milk vitamin B_{12} concentration

Maternal plasma B_{12} concentration at 12 weeks postpartum was weakly associated with breast milk vitamin B_{12} concentration in samples collected at the same time (crude coefficient (log-transformed): 0.001, 95% CI: 0.0001, 0.001, p=0.02, r=0.15) (Figure 31). Maternal plasma vitamin B_{12} concentration in early pregnancy (baseline) was not associated with breast milk vitamin B_{12} concentration at 12 weeks postpartum (crude coefficient (log-transformed): 0.001, 95% CI: -0.0001, 0.0014, p=0.09, r=0.11) (Figure 32). Data from both supplement groups were included in this analysis as there was no difference in maternal plasma vitamin B_{12} concentration or breast milk vitamin B_{12} concentration between the two supplement groups at either timepoint. Breast milk vitamin B_{12} concentration at 12 weeks postpartum was also positively associated with maternal height (p=0.02) (Table 35). When adding maternal height as a potential confounder of the relationship between maternal plasma vitamin B_{12} at 12 weeks postpartum and breast milk vitamin B_{12} concentration, the association remained (adjusted coefficient (log-transformed): 0.001, 95% CI: 0.0001, 0.001, p=0.03, r=0.18).

Mothers who were exclusively breastfeeding their infant at six months postpartum did not have a different breast milk vitamin B_{12} concentration compared to mothers who were not exclusively breastfeeding (data not shown).

Infant vitamin B_{12} status at 12 weeks postpartum was driven by both maternal plasma vitamin B_{12} and breast milk vitamin B_{12} (Figure 33). Based on crude estimates, maternal plasma was a stronger predictor (r=0.38, p<0.001) of infant status than breast milk vitamin B_{12} (r=0.14, p=0.02). Data from both supplement groups were included in this analysis.

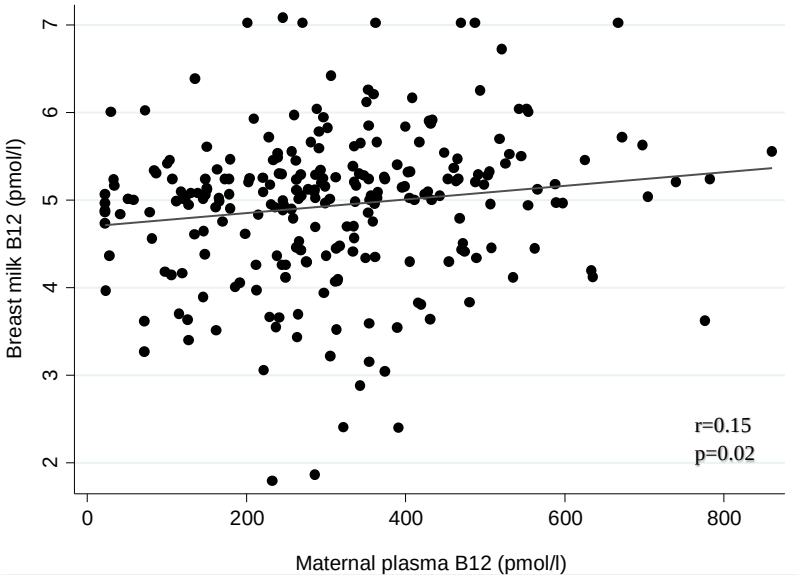

Figure 31. Association between maternal plasma vitamin B_{12} and breast milk vitamin B_{12} at 12 weeks postpartum (n=252).

Breast milk vitamin B_{12} values were log-transformed before analysis. The grey line is the linear regression fit.

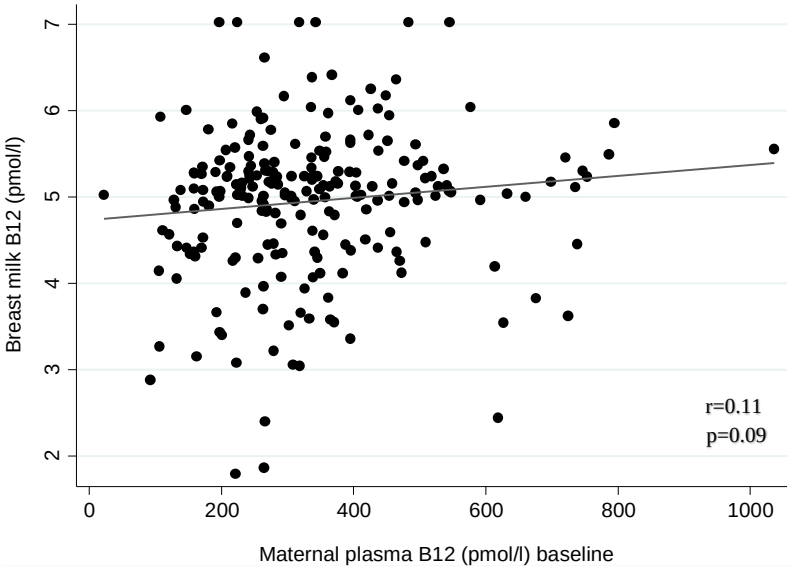

Figure 32. Association between maternal plasma vitamin B_{12} at baseline (early pregnancy) and breast milk vitamin B_{12} at 12 weeks postpartum (n=228).

Breast milk vitamin B_{12} values were log-transformed before analysis. The grey line is the linear regression fit.

Table 35. Determinants of breast milk vitamin B_{12}

	n	Breast milk vitamin B_{12} 12 weeks postpartum
Maternal age, years	301	-0.14 (0.01)
Maternal weight, kg	300	0.0004 (0.01)
Maternal height, cm	301	0.02 (0.01)*
Maternal BMI (kg/m^2)	300	-0.01 (0.02)
Parity		
Primiparous	42	5.13 (0.93)
Multiparous (≥ 1 previous pregnancy)	259	4.96 (0.85)
Maternal education [a]		
No education	226	4.98 (0.85)
Low (1-7 years)	40	5.15 (0.92)
Medium (8-14 years)	35	4.83 (0.90)
Gestational age at birth (weeks)	298	-0.04 (0.03)
Season of sample collection		
Dry season (Nov to May)	188	5.01 (0.07)
Wet season (June to Oct)	109	4.95 (0.08)
Village		
Core villages [b]	76	4.94 (0.08)
Outreach villages	225	5.00 (0.06)
Ethnicity		
Mandinka	250	4.96 (0.06)
Other	51	5.09 (0.99)
Infant sex		
Female	151	5.02 (0.07)
Male	150	4.95 (0.07)
Infant age at 12 week visit	293	0.001 (0.01)

Non-normal distributed data were log transformed. Continuous data are presented as logged beta coefficient (SE), categorical data as logged means (SD). Continuous data were analysed using linear regression and categorical data using *t*-tests or ANOVA.

* Evidence for a difference between outcome and exposure variable $p \leq 0.05$.

[a] Maternal education was defined as completed years of either English or Arabic schooling

[b] Core villages are: Keneba, Jali, Kantong Kunda and Manduar situated close to the MRC Keneba. Outreach villages are the remaining 24 villages in West Kiang. n; sample size.

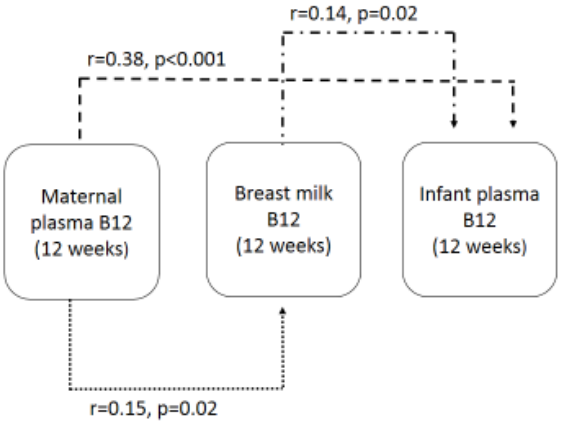

Figure 33. Associations between maternal plasma vitamin B_{12}, breast milk vitamin B_{12} and infant vitamin B_{12} at 12 weeks postpartum.

Analysed by linear regression.

7.5.3 Estimated infant vitamin B_{12} intake

In exclusively breastfed infants, vitamin B_{12} intake is equal to excretion in breast milk. Table 36 shows the estimated average infant vitamin B_{12} intakes (µg/day) at three time-points. The estimated median intake of vitamin B_{12} for exclusively breastfed infants was 0.17 µg/day at 12 weeks postpartum. Ninety-one percent (255/281) of infants had an estimated vitamin B_{12} intake below the AI of 0.4 µg/day at 12 weeks postpartum.

Table 36. Estimated average daily vitamin B_{12} intake for exclusively breastfed infants (µg/day) at varying time-points [a]

	8 weeks postpartum		12 weeks postpartum		24 weeks postpartum
Total (n=266)	0.192 (0.128, 0.263)	Total (n=281)	0.172 (0.093, 0.226)	Total (n=189)	0.206 (0.161, 0.281)

Data are medians (IQR). Only exclusively breastfeed infants were included.

* Difference in medians between supplement groups, $p \leq 0.05$. Analysed by Mann-Whitney test.

[a] Calculated as the concentration (µg/l) excreted in breast milk*0.782 l/day.

[b] Total = data from both supplement groups, n; sample size.

7.6 Discussion

Supplementing pregnant Gambian women with a multiple micronutrient supplement, containing 5.2 µg/day of vitamin B_{12}, resulted in higher maternal plasma vitamin B_{12} concentration during pregnancy and a higher infant cord blood and infant plasma concentration at 12 weeks postpartum. No evidence of an effect was detected on maternal postpartum status or on breast milk vitamin B_{12} concentrations across the first six months of lactation, suggesting that the positive effect of supplementation on maternal status during pregnancy was not sustained into lactation.

To the best of my knowledge, this is the first intervention study to have supplemented women during pregnancy only and investigated the effect on breast milk and infant plasma vitamin B_{12} concentrations. Duggan et al (2014) (175) and Siddiqua et al (2015) (176), randomised and supplemented Indian and Bangladeshi women, respectively, during both pregnancy and lactation, making it impossible to determine if the observed effects both studies found on breast milk concentrations were due to pre- or postnatal supplementation or a combination of both.

In this study in The Gambia, maternal supplementation starting in early pregnancy had a positive effect on maternal plasma vitamin B_{12} concentration alongside a reduced prevalence of mothers with depleted vitamin B_{12} concentrations in late gestation. MMN supplementation maintained maternal plasma vitamin B_{12} concentrations across pregnancy, whereas a large drop was observed in the FeFol group. This decrease in plasma B_{12} levels of unsupplemented women is consistent with observations from the existing literature in both well- and poorly-nourished populations (175, 427-431). Further, a study reported that despite equivalent vitamin B_{12} intakes, women in the third trimester of pregnancy had a 21% lower serum B_{12} levels than non-pregnant women (177). This observed decrease during late pregnancy is likely due to normal physiologic changes, including hemodilution, an increased supply of vitamin B_{12} from the mother to the foetus (177), and potentially also due to a change in vitamin B_{12} binding proteins during pregnancy (430). The stage at which this decline becomes problematic for the mother's and infant's vitamin B_{12} status, and the level at which it is beneficial to prevent a decline are not yet known.

The results obtained in this analysis indicate that a supplement containing 2xRDA of vitamin B_{12} can maintain maternal plasma B_{12} levels in early pregnancy in this population, and thus prevent a decline. Duggan et al (2014) (175) also reported a maintenance, and even an increase, of vitamin B_{12} status with vitamin supplementation across pregnancy. In this study indian women were

supplemented from 14 weeks gestation with 50 µg/day (50% had low or depleted status in early pregnancy), and baseline maternal plasma B_{12} concentration was 160 pmol/l, which in the supplemented group increased to 216 pmol/l and 184 pmol/l in the second and third trimester, respectively. This suggests that a high dose of vitamin B_{12} supplementation led to increased vitamin B_{12} plasma levels during pregnancy among highly deficient Indian women. However, the cut-off currently used to determine vitamin B_{12} deficiency during pregnancy is the same that is used for the general adult population (398). This is not appropriate because of the marked physiological changes in status during pregnancy (434). Without a suitable cut-off for vitamin B_{12} deficiency in pregnancy it is not possible to determine if 2xRDA in this analysis or 50 µg/day in the study by Duggan et al (2014) (175) are sufficient in increasing maternal plasma to levels that are adequate to build up infant stores for healthy development. More research is needed on optimal vitamin B_{12} status during pregnancy.

The effect of supplementation during pregnancy was not sustained in the women during lactation in this rural Gambian population. However, maternal plasma concentrations were non-significantly higher in the MMN group compared to the FeFol group at 12 weeks postpartum, likely due to the carry-over effect of the supplement. Further, plasma concentrations increased between 30 weeks' gestation and 12 weeks postpartum (significantly for the FeFol group, non-significantly for the MMN group). This observed increase for both groups indicates an improved mobilisation of maternal stores during lactation for transfer of the vitamin to breast milk, and a return to pre-pregnancy vitamin B_{12} levels. This increase in status during lactation is consistent with other findings (177, 431). Bae et al (2015) (177) reported higher plasma vitamin B_{12} concentrations during lactation compared to concentrations during both pre-pregnancy and pregnancy in well-nourished women with equivalent vitamin B_{12} intakes. The size of the increase in plasma vitamin B_{12} concentration during lactation, and whether the concentration surpasses pre-pregnancy levels, is hypothesised to depend on maternal status before pregnancy along with intake and depletion of stores during pregnancy (398).

In this analysis, maternal supplementation did not have an effect on breast milk vitamin B_{12} concentrations across the first six months of lactation. Evidence suggests that supplementing women during lactation improve maternal circulating concentrations of vitamin B_{12}, increasing breast milk concentrations and thus benefitting the infant. For instance, an RCT supplemented Bangladeshi women with 250 µg/day of vitamin B_{12} during pregnancy and until 3 months postpartum (almost 70% of women had low or depleted vitamin B_{12} status at baseline) (176). This study found a higher increase in maternal plasma B_{12}, breast milk and infant plasma B_{12} at

3 months postpartum compared to the placebo group (maternal plasma: 416 pmol/l vs. 242 pmol/l, breast milk: 245 pmol/l vs. 170 pmol/l, infant plasma: 328 pmol/l vs. 200 pmol/l). Similar results were observed in a comparable study of vitamin B_{12} deficient Indian women (175). In this study a supplement of 50 μg/day given during both pregnancy and lactation improved maternal vitamin B_{12} status during pregnancy, breast milk vitamin B_{12} concentrations and infant status at six weeks postpartum compared to a placebo (breast milk: 136 pmol/l vs. 87 pmol/l, infant plasma: 199 pmol/l vs. 139 pmol/l). This indicates that in populations with high maternal vitamin B_{12} deficiency, continuing supplementation during lactation improves both maternal, breast milk and infant vitamin B_{12} status beyond the metabolic adaptations that naturally occur during this period. In contrast, an observational study of well-nourished Danish women found that maternal supplementation during lactation (1-18 μg/day) from 3 weeks to 9 months postpartum did not increase maternal plasma vitamin B_{12} concentrations in well-nourished Danish women (455). Despite clear methodological differences between these two studies, this could suggest that only deficient and depleted lactating women benefit from postnatal supplementation.

An increase in breast milk concentration was observed between 12 and 24 weeks postpartum in both groups in this analysis. This increase could potentially be driven by the improved mobilisation of maternal stores for transfer to breast milk between 3 and 6 months postpartum. However, the few existing studies available on this suggest that the recovery of plasma vitamin B_{12} levels from pregnancy to lactation occurs earlier than between 3 to 6 months of lactation (431, 455). In Australian women, pregnancy vitamin B_{12} levels were exceeded at around 14 weeks postpartum, and only a small increase in maternal plasma vitamin B_{12} was observed between 14 and 27 weeks postpartum (431). Further, maternal serum vitamin B_{12} did not change between 3 weeks to 9 months postpartum in a Danish cohort, suggesting that the recovery of maternal plasma vitamin B_{12} occurred before 3 weeks postpartum (455).

Two other studies have observed a similar increase in mature milk in late lactation (175, 180). Duggan et al (2014) (175) reported an increase of 68 pmol/l to 80 pmol/l between 3 and 6 months postpartum in unsupplemented Indian women, and a Danish observational study reported an increase of 290 pmol/l to 440 pmol/l between 4 and 9 months postpartum (180). Only the Danish study analysed maternal plasma vitamin B_{12} concentration at the same timepoints, and they found no significant difference in maternal plasma B_{12} concentrations between 4 and 9 months. This suggests that maternal vitamin B_{12} status is not directly linked to the increase in breast milk concentrations during the same time-period. Other researchers have hypothesised this increase in Danish women's vitamin B_{12} concentrations in mature milk is caused by a difference in infant

milk intake (92). At four months postpartum, the majority of the Danish infants were exclusively breastfed, and at nine months all had started complementary feeding, which is generally accompanied by a decrease in milk intake. In this Chapter's analysis a difference in breast milk B_{12} concentration was however not found between exclusively and not-exclusively breastfed children at six months postpartum. Furthermore, no published study has to date investigated the impact breast milk volume has on breast milk vitamin B_{12} concentration. More research is needed to establish why breast milk vitamin B_{12} in both well- and poorly nourished populations has been reported to increase in late lactation.

Infant vitamin B_{12} concentrations in cord blood were significantly different between the two supplement groups. This concurs with other studies that have found that maternal vitamin B_{12} status during pregnancy is highly reflective of foetal vitamin B_{12} status at birth (176, 456). Siddiqua et al (2015) (176) supplemented pregnant women with 250 µg/day of vitamin B_{12} during pregnancy and lactation and reported a higher infant cord blood vitamin B_{12} concentration compared to placebo (555 pmol/l vs. 208 pmol/l). Further, in this analysis it was found that infant plasma vitamin B_{12} declined across infancy in both supplement groups, which is in agreement with other studies' results (180, 436). This suggests that supplementing mothers during pregnancy has beneficial effects on infant status at birth.

In this analysis, significant differences in infant plasma vitamin B_{12} were also observed at 12 weeks postpartum, likely due to a carry-over effect of the supplementation during pregnancy. An explanation for this could be that infants born to supplemented mothers were born with a higher vitamin B_{12} liver storage that lasted until 12 weeks postpartum. This is supported by an observational analysis in deficient Indian mothers; higher maternal vitamin B_{12} status during pregnancy were associated with increased infant vitamin B_{12} status and a decreased prevalence of deficiency at six weeks of age (457). This highlights the importance of maternal supplementation during pregnancy in poorly nourished population, as the beneficial effect is likely to lasts beyond the neonatal period.

7.6.1 Vitamin B_{12} deficiency, intake and determinants

According to IOM plasma vitamin B_{12} cut-off for deficiency, the Gambian women in this analysis were considered mildly vitamin B_{12}-deficient in early pregnancy. Seven percent of the included women were below 148 pmol/l (deficiency) and 16% between 148-221 pmol/l (depletion). At 30 weeks' gestation the percentage of vitamin B_{12} deficiency and depletion was higher, also in the

MMN group (31%). However, interpretation on maternal vitamin B_{12} status during pregnancy should be made with caution. As already described, it is not appropriate to use adult cut-offs during pregnancy, and furthermore plasma vitamin B_{12} is an insensitive biomarker, especially at low concentrations (398). To accurately define maternal vitamin B_{12} status during pregnancy, and the effectiveness of 2xRDA on maternal status during pregnancy, a second (or preferably more) biomarker, such as MMA or tHcy, is needed. Future studies should include several biomarkers in order to correctly assess maternal status during pregnancy.

At 12 weeks postpartum the median vitamin B_{12} breast milk concentration was 154 pmol/l in the FeFol group and 169 pmol/l in the MMN group. The adequacy of breast milk vitamin B_{12} concentration is difficult to determine as no appropriate cut-off for deficiency is available. If using the IOM's recommended AI for infants, more than 90% of exclusively breastfed infants had an estimated intake below 0.4 µg/day, at 12 weeks postpartum in both groups. The AI is however based on poor data from a study with a small sample size (n=9), and where outdated analytical quantification methods were used (414). Furthermore, actual breast milk intakes were not obtained in this analysis, and the daily vitamin B_{12} intake of exclusively breastfed infants is thus based on an estimate. In vitamin B_{12}-replete Canadians whose breast milk samples were analysed using the same laboratory technique, concentrations of 698 pmol/l at 8 weeks postpartum were observed (181). In comparison, the breast milk vitamin B_{12} concentrations found in rural Gambian women are considered very low. However, limited comparable data is available from well-nourished women using appropriate laboratory techniques, making it difficult to conclude on breast milk vitamin B_{12} adequacy in this rural Gambian population. Establishing references values for breast milk vitamin B_{12} adequacy along with vitamin B_{12} intake requirement and long-term outcomes is needed.

The infants in this analysis were on the contrary considered vitamin B_{12} sufficient, when applying the reference interval developed by Hay et al (2008) (436), who measured healthy Norwegian mother-and-infant-pairs. Only 5% of infants in this study were below the lower reference value of 121 pmol/l for breastfed infants at six months of age. Nevertheless, the Norwegian study did not measure maternal status or breast milk B_{12} concentrations which makes the interpretation of these infant reference values difficult. It is also possible that the lower reference value of 121 pmol/l proposed by Hay et al (2008) (436) is too low. For instance, in the study by Siddiqua et al (2015) (176) mothers in the placebo group had high prevalence of deficient and depleted plasma B_{12} concentrations during both pregnancy and lactation, and infants in this group had a median plasma B_{12} concentration of 200 pmol/l at three months postpartum. Further in the study by

Duggan et al (2014) (175) infant plasma concentration was 139 pmol/l at 6 weeks postpartum in the placebo group of highly deficient Indian women. More studies are needed to confirm or reject the appropriate use of the reference interval developed by Hay et al (2008) (436).

When infant vitamin B_{12} status (200 pmol/l at three months postpartum) in this analysis is compared with the findings of Siddiqua et al (2015) (176), who analysed plasma samples using the same laboratory technique, the Gambian infants' status is considered moderately low. However, there is a lack of literature linking infant vitamin B_{12} status to functional outcomes, making it hard to determine if the values reported in these Gambian infants are adequate for healthy development.

Maternal status during lactation in this analysis was associated with breast milk vitamin B_{12} concentration, after adjusting for potential confounders. This is in agreement with some previous studies (177, 180-182) (See Chapter 2). Maternal status during pregnancy was not associated with vitamin B_{12} concentration in mature milk, and no other study has been identified measuring this (Table 1 in Chapter 2).

Maternal status during lactation was a stronger predictor of infant vitamin B_{12} status than breast milk concentrations in this analysis. This is in agreement with a study by Deegan et al (2012) (458), who found that in Guatemala, infant vitamin B_{12} status at 12 months of age was predicted by maternal status rather than breast milk concentrations, implicating a long-term effect of maternal pregnancy status. It is possible that maternal status is a better indicator of infant status in the first year of life, as infants born to replete mothers are born with ~25-30 µg vitamin B_{12} storage (441), protecting them from inadequacy in early infancy. This hypothesis is also supported by the effect of supplementation on infant postnatal plasma B_{12} and not on breast milk concentrations observed in this analysis.

7.6.2 Strengths and limitations

These findings expand current knowledge around maternal vitamin B_{12} status during pregnancy and lactation, breast milk vitamin B_{12} and infant vitamin B_{12} status in a population with low habitual intake of dietary vitamin B_{12}. Limited studies have covered the full period through pregnancy, lactation and infancy and measured status concurrently. This analysis exposed the need to focus on maternal vitamin B_{12} status during both periods of pregnancy and lactation, as both are important for maternal and infant vitamin B_{12} status. Further, this analysis has identified

the need of additional research on definitions of vitamin B_{12} deficiency during pregnancy, lactation and infancy, and the development of a vitamin B_{12} infant EAR recommendation during the period of EBF.

A key strength of this study was the use of advanced laboratory techniques to measure vitamin B_{12} in breast milk. Concentrations were quantified using the most up to date analytical method, removing vitamin B_{12} from apo-HC, which is necessary to obtain a reliable concentration of vitamin B_{12} in breast milk (281).

One limitation of this study was that only one biomarker to assess maternal and infant vitamin B_{12} status was collected. Plasma vitamin B_{12} is insensitive to especially low concentrations and should not be used as a single biomarker to assess status. Analysing MMA or holoTc simultaneously with plasma vitamin B_{12} would have given a more reliable indication of vitamin B_{12} status in this population.

7.6.3 Conclusion

In conclusion, a daily multiple micronutrient supplementation (containing 5.2 µg/day vitamin B_{12}) given during pregnancy to rural Gambian women increased maternal vitamin B_{12} status during pregnancy and had a carry-over effect on infant plasma vitamin B_{12} status in the first 12 weeks of life. Supplementation did not have an effect on breast milk vitamin B_{12} concentrations, suggesting maternal vitamin B_{12} status during pregnancy might be a stronger predictor of infant status than breast milk vitamin B_{12} concentration in the first 12 weeks postpartum.

Chapter 8

Thiamin, riboflavin and vitamin B_6

This Chapter presents how a thiamin, riboflavin and vitamin B_6-containing multiple micronutrient supplement given during pregnancy influences maternal status during pregnancy and breast milk composition in rural Gambia. The background begins with a review of vitamin B_1, B_2, B_6 physiology and deficiency in human milk. This is followed by a description of assessment of status of the three B-vitamins, definitions of adequate status, intake requirements and epidemiological evidence for the importance of these vitamins. The focus of the background is on all three periods of pregnancy, lactation and infancy. The background is followed by a short introduction to the research area, methods, results and discussion.

8.1 Background

Thiamin (vitamin B_1), riboflavin (vitamin B_2) and vitamin B_6 have been described in the literature as neglected vitamins of public health importance (459). Assessment of these vitamins is generally not included in population studies, as they are difficult to measure (77), and because information about the consequences of marginal or subclinical deficiencies are lacking (459). As a result, the global prevalence of thiamin, riboflavin and vitamin B_6 deficiency is uncertain (77).

Deficiencies of these B-vitamins may be of concern especially in resource-poor settings where dietary intake of animal products and fruits and vegetables are low (459). The prevalence of thiamin deficiency is likely to be high where diets are high in refined or polished grains (460). Deficiency of all three B-vitamins often occurs simultaneously within the same population, as the food sources of the vitamins are similar (459). Additionally, riboflavin is involved in the

metabolism of folic acid, pyridoxine, vitamin D and K, and a low riboflavin status can thus have implications for the status of a variety of other vitamins (461).

8.1.1 B-vitamin physiology

Thiamin

Thiamin, also known as vitamin B_1, is a water-soluble vitamin. A continuous dietary intake of the vitamin is needed to sustain adequate levels as the vitamin is to a large degree not stored in any tissue (462). In the diet thiamin mainly exists in the phosphorylated form (463), which is converted into free thiamin before absorption. Thiamin is absorbed as thiamin ion (T+) in the small intestine by active and passive transport following uptake from the gastrointestinal tract and transportation by the blood to several tissues and organs. Active transport occurs at low concentrations of thiamin and passive diffusion at higher concentrations (462, 464). After uptake into the cell, thiamin is phosphorylated mostly to thiamin pyrophosphate (TPP), and some of the TPP is further converted to thiamin triphosphate (TTP) (464). Both free thiamin and thiamin monophosphate (TMP) circulate in the blood, bound to albumin. All tissues can take up free thiamin and TMP and phosphorylate them to TPP and TTP (465). Thiamin is excreted in the urine which occurs when there is excess concentration of T+ and TMP (464).

TPP is the most abundant form of thiamin in the body, and makes up more than 80% of total thiamin (464). Thiamin is involved in the extraction of energy from carbohydrate sources, and in amino acid metabolism where TPP is the essential cofactor for enzymes involved in glucose and amino acid metabolism. Thiamin is also involved in the regulation of neuronal communication, and in several functions in the immune system (464).

Riboflavin

Riboflavin, also known as vitamin B_2, is an essential water-soluble vitamin. Dietary riboflavin is absorbed and processed in the small intestine. Prior to absorption riboflavin from dietary sources, which exists most abundantly as flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), are hydrolysed to free riboflavin (466). When absorbed the conversion of free riboflavin to its co-enzyme form mainly takes place in the cytoplasm, where first FMN is generated by phosphorylation and then most of the FMN is further reformed to FAD (467). Free riboflavin can also be regenerated from FMN and FAD (466). There is little or no storage of riboflavin in the

body, and any surplus is excreted in the urine, in the form of free riboflavin and its catabolites (467).

FMN and FAD play a key role in the conversion of folic acid and vitamin B_6, and in a variety of processes involving carbohydrates, lipids and amino acids (463). Free riboflavin has limited biological activity (466). In healthy individuals, the most abundant form in plasma is FAD (468).

Vitamin B_6

Vitamin B_6 is the collective term for pyridoxal (PL), pyridoxamine (PM) and pyridoxine (PN) (463). All vitamers also exist in their phosphorylated forms; pyridoxal-5'-phosphate (PLP), pyridoxamine-5'-phosphate (PMP) and pyrodixine-5'-phosphate (PNP) (469). 4-pyrodoxic acid (4-PA) is the catabolic product of vitamin B_6 metabolism. It is metabolically inactive, and is excreted in the urine (470, 471). In the diet, vitamin B_6 exits both in the free and phosphorylated forms, however before absorption all vitamers are hydrolysed to the free form (463). The phosphorylation of PL, PM and PN to PLP, PMP and PNP, respectively occurs primarily in the liver. PL and PLP are the predominantly vitamers of vitamin B_6 in the human body (470).

PLP is the most biological active form of the vitamin, and acts as a cofactor in many biologically processes, such as amino acids transformations, and in the metabolism of carbohydrates and lipids (463). PLP is also involved in one-carbon metabolism, where it acts as a coenzyme for four different enzymes (470). Dietary sources of vitamin B_6 are meat, cereal grains, vegetables and nuts (470).

8.1.2 B-vitamin physiology during lactation

The transport mechanisms involved in thiamin, riboflavin and vitamin B_6 secretion into milk are generally unknown. For thiamin and vitamin B_6 there is no literature available on the transport mechanisms involved in secretion of these vitamins into milk (84). However, one recent study found that of all the thiamin vitamers present in milk, only free thiamin concentration increased across lactation, suggesting an active transport mechanism (472). TMP may either be actively transported into milk, or originate from phosphorylation of free thiamin or hydrolysis of TPP (472).

For riboflavin a little more is known. Free riboflavin and FAD are secreted into milk, and the multidrug transporter breast cancer resistance protein (BCRP/ABCDG2) is said to mediate the

free riboflavin transport (473). During pregnancy and lactation BCRP is induced and, via an ATP-dependent mechanism, free riboflavin is transported into milk. It is also possible that FAD is transported by a BCRP-independent active transporter, however not enough is known about this mechanism (473). A recent study conducted in humans suggests that FAD is supplied into the milk at a constant rate across lactation, and when supplements are taken, an increase in free riboflavin occurs, suggesting that free riboflavin is the driving force for the change in total riboflavin content in breast milk (472). There seems to be a preferential secretion of the free form into breast milk by BCRP.

8.1.3 B-vitamin deficiency

Thiamin

Thiamin deficiency may result from inadequate dietary intake of the vitamin, as well as from decreased absorption (462). Consumption of foods high in thiaminases (for instance specific plants and fish) or antithiamin compounds (for instance fermented fish, tea leaves and betel nut) and extended cooking of foods can also contribute to the deficiency (460). Pregnant women and infants are the population groups at highest risk of deficiency, and severe thiamin deficiency can lead to infantile beri-beri which in some affected populations is the leading cause of infant mortality (149, 150, 474). Infantile beri-beri often occurs among breastfed infants around three months of age, which without treatment can lead to infant death within a few hours (474). Maternal thiamin deficiency during pregnancy also places the infants at risk of infantile beri-beri, as the foetus is relying on placental thiamin transfer during the third trimester of pregnancy (153). This is known because infants born to thiamin sufficient mothers have up to three times higher thiamin concentration in umbilical cord blood compared to maternal venous blood at birth (475). Beri-beri is characterised by oedema, cardiac failure and neurological symptoms (476).

Limited studies have investigated how thiamin deficiency affects infant growth and development. It has been hypothesised that thiamin deficiency is linked to intrauterine growth restriction (IUGR) with one study reporting that mothers with normal pregnancies had higher erythrocyte thiamin concentrations compared to mothers experiencing IUGR (477). Further studies on the effect that maternal thiamin deficiency has on infant brain development in high-risk populations are warranted (460).

Riboflavin

Riboflavin deficiency results in a range of clinical abnormalities, including degenerative changes to the nervous system, endocrine dysfunction, anaemia and skin disorders (466), but isolated clinical deficiency of riboflavin is often not recognisable by any physical feature (461). In addition to insufficient dietary intakes of riboflavin, deficiency can also be caused by certain endocrine abnormalities, for instance thyroid hormone insufficiency (461). Haste et al (1991) (478) found that riboflavin intake during pregnancy was positively associated with birth weight, which is one of the few studies in the literature that has investigated the effect of riboflavin intake on infant growth and development.

Vitamin B_6

A low dietary intake of vitamin B_6 and, as a consequence, vitamin B_6 deficiency, is often unheard of in the general population (470). However, at risk groups still exist, and among those are pregnant and lactating women (470). Symptoms of vitamin B_6 deficiency have been reported to include weakness, sleeplessness and a loss of appetite (469). In early stages of development the vitamin is vital, especially during central nervous system development (469). A few studies have also linked vitamin B_6 status in infants to poor growth (479-481). For instance, infants born to mothers supplemented with vitamin B_6 during pregnancy had a higher birth weight (479). Additionally, infant plasma PLP concentrations at birth predicted infant growth (480), and a slower length-for-age velocity and a poorer weight gain was recorded in infants with low vitamin B_6 status compared to infants with an adequate status (481). Good quality studies are needed assessing vitamin B_6 supplementation on infant outcomes to establish the importance of this vitamin (77, 482).

8.1.4 B-vitamins in human milk

The most prevalent form of thiamin in breast milk is free thiamin and TMP (156, 472). TPP has also been found in milk, however only in small concentrations (472). For riboflavin, the most prevalent vitamers in breast milk are free riboflavin and FAD (472, 483). For vitamin B_6, PL is the most dominant form in milk, with possible contributions of PN, pyrodoxamine, pyridocal-phosphate or pyridoxamine-phosphate, however all in relatively small quantities (164, 484).

Two longitudinal studies have shown an increase in thiamin concentration in breast milk from transitional to mature milk (129, 151), although a more recent study observed a decrease in

concentration from transitional to mature milk (153). There is a lack of longitudinal studies investigating changes in riboflavin concentration across lactation, the available studies reported stable concentrations over the first several weeks of lactation (129, 151, 158). Breast milk vitamin B_6 concentration increases sharply in the first few weeks of lactation followed by a gradual decline in late lactation in unsupplemented women in longitudinal studies (129, 163, 165, 485).

Hampel et al (2017) (274) found higher thiamin and riboflavin concentrations in hindmilk versus foremilk, however the differences were small. No difference in vitamin B_6 was reported (274). The same research group reported diurnal changes for all three B-vitamins, with riboflavin having the largest change in concentrations (274). In addition riboflavin and vitamin B_6 had the highest range of concentrations throughout one day when a supplement was provided, compared to the remainder of the B-vitamins. An increase in concentration of more than 200% of the daily median was reported within four hours after the supplement was consumed for both vitamins (274). The authors concluded that without consumption of supplements, the morning, early and late afternoon and evening hours are acceptable representative times for breast milk collection when investigating these vitamins.

As described in Chapter 2, maternal thiamin, riboflavin and vitamin B_6 status and intake influence breast milk concentrations. For thiamin, maternal supplementation during lactation had an effect on breast milk concentration of women with a poor thiamin status, but this effect was often not seen in well-nourished women (see Chapter 2). Similar results were found in rats where thiamin concentration in milk decreased when fed a diet low in thiamin, but did not change with a diet high in thiamin (486). This suggest a tight regulation of thiamin transfer into milk at high intakes, and the existence of a preferential transport of thiamin into breast milk when maternal status is poor. The regulation of thiamin in human milk is however a complex process involving several vitamin-specific transport systems, that as already described, is yet to be fully understood (84).

Some researchers have found breast milk thiamin and vitamin B_6 concentrations to be associated with premature delivery, with lower concentrations in preterm versus term milk (129, 487). This association was not observed for riboflavin (129). Data on other environmental influences of thiamin, riboflavin and vitamin B_6 concentrations in breast milk are lacking.

8.1.5 Assessment of B-vitamin status and criteria for adequacy

Thiamin status is reliably assessed by measuring erythrocyte thiamin diphosphate or erythrocyte thiamin transketolase activity and erythrocyte thiamin transketolase activity coefficient (aETK) (156, 488, 489). There is generally a lack of established and agreed upon cut-offs for these biomarkers except for aETK, with values higher than 1.25 implying deficiency (417).

The traditionally used method for measuring riboflavin status is by the erythrocyte glutathione reductase activation coefficient (EGRAC) assay (490), which measures the ratio of glutathione reductase activity in the presence and absence of added flavin adenine dinucleotide. It is measured in red blood cells, and reflects long-term riboflavin status (461). An EGRAC higher than 1.4 defines deficiency (417). Plasma and serum concentration of free riboflavin, FAD and FMN have also more recently been used to assess riboflavin status, however cut-offs for deficiency has not yet been established (490).

For vitamin B_6, plasma PLP concentration is the most commonly used biomarker to measure status (491). Some studies have measured total plasma B_6, a mixture of a combined measurement of all vitamin B_6 derivatives, and as a ratio of concentrations in plasma (PLP+PL+PA) (492). However, these biomarkers were recently found to not assess vitamin B_6 status accurately (491). Analytical methods used for assessment of plasma PLP include HPLC and LC-MS/MS based assays. It has been proposed that a cut-off for plasma PLP of <20 nmol/l in the adult population indicates vitamin B_6 deficiency (417), a concentration in the range of 20-30 nmol/l indicates marginal status, and >30 nmol/l suggests vitamin B_6 sufficiency (470). A cut-off for plasma PLP to indicate vitamin B_6 deficiency during pregnancy has not been defined.

The most reliable method to assess breast milk concentration of all three vitamins is ultra-performance liquid-chromatography tandem mass spectrometry (UPLC-MS/MS) (469). For thiamin, high-performance liquid-chromatography-fluorescence detection (HPLC-FLD) has furthermore been considered a valid method (472). The research group at USDA Western Human Nutrition Research Centre (WHNRC) in California, developed both of these analytical techniques (472, 493). Riboflavin and vitamin B_6 are analysed simultaneously, improving resolution, speed and sensitivity compared to other analysis methods where each vitamin are analysed separately (494).

8.1.6 Intake requirements during pregnancy, lactation and infancy

Pregnancy and lactation

The dietary requirement of thiamin, riboflavin and vitamin B_6 all increase during pregnancy and lactation to account for the vitamin transfer to the placenta, for foetal uptake and for the transfer of the vitamins to breast milk during lactation (417).

The estimated average requirement (EAR) recommended by the Food and Nutrition Board of the Institute of Medicine (IOM) of the U.S National Academy of Sciences during pregnancy is 1200 µg/day for thiamin and riboflavin (417) (Table 37). The EAR for vitamin B_6 during pregnancy is estimated at 1600 µg/day. Studies have found plasma vitamin B_6 to decrease during pregnancy, especially in the third trimester compared to non-pregnant women (495, 496), with the drop in plasma concentration being driven by a drop in PLP. This drop is likely due to physiological changes during pregnancy, however this has not been fully investigated (417). The cut-off for plasma PLP of <20 nmol/l along with an assumed bioavailability of vitamin B_6 of 75% from the diet was used in the calculations of the EAR (417).

Table 37. Recommended dietary thiamin, riboflavin and vitamin B_6 intake (µg/day) for pregnant, lactating women and infants between 0-6 months by IOM (417) and WHO/FAO (438)

	Pregnancy		**Lactation**		**Infancy**	
	IOM	WHO	IOM	WHO	IOM	WHO
Thiamin	EAR: 1200 RDA: 1400	- RDA: 1400	EAR: 1200 RDA: 1400	- RDA: 1500	AI: 200	AI: 200
Riboflavin	EAR: 1200 RDA: 1400	- RDA: 1400	EAR: 1300 RDA: 1600	- RDA: 1600	AI: 300	AI: 300
Vitamin B_6	EAR: 1600 RDA: 1900	- RDA:1900	EAR:1700 RDA 2000	- RDA: 2000	AI: 100	AI: 100

EAR, estimated average requirement (the EAR is the daily dietary intake level of a nutrient expected to satisfy the needs of 50% of the population group); RDA, recommended dietary allowance (the RDA is defined as equal to the EAR plus twice the coefficient of variation to cover the needs of 97-98% of the population group); AI, average intake (dietary intake believed to be adequate for everyone in the demographic group to maintain health, established where no sufficient data to establish EAR are available); IOM, Institute of Medicine; WHO, World Health Organization.

Infancy

Infant thiamin, riboflavin and vitamin B_6 requirements in the first six months of life is presented in Table 37. The World Health Organization (WHO) based their recommendations on those from the IOM (438). The infant requirements are poorly defined, and they are all based on an average

intake (AI) and not on an EAR, which is preferable (374). An AI should only be interpreted as a guideline for dietary nutrient intake.

For thiamin, the infant AI of 200 µg/day is based on the mean breast milk concentration of 210 µg/l from two studies conducted during the 1980s in the United States including only 24 women (151, 152), and an infant intake of 0.781 litres of milk per day (375). The AI was rounded up from 164 to 200 µg/day. For riboflavin, the infant AI was previously based on the same two studies used to determine thiamin AI (151, 152), but in 1990 it was highlighted that these two studies failed to detect FAD in breast milk, underestimating the total riboflavin concentration needed (483). Thereafter the AI was set to 300 µg/day based on five women's breast milk roboflavin concentrations (483) and microbiological determined riboflavin concentration in milk (497) (350 µg/l). For vitamin B_6 the recommended infant AI is based on a study from 1976 including only 19 American women's breast milk vitamin B_6 concentration (130 µg/l) (166).

8.2 B-vitamin status of mothers and concentrations in breast milk

Limited studies have examined the effect of maternal thiamin, riboflavin and vitamin B_6 status during pregnancy on breast milk concentrations (see Chapter 2). This makes it difficult to conclude on how adequate infant intake of these vitamins are ensured during the period of exclusive breastfeeding (EBF), especially in settings where the lactating mother is at risk of deficiency. Apart from being of low-quality design, most identified interventions on this topic have (i) not investigated the effect of supplementation or fortification exclusively during pregnancy (only 1 out of 12 studies have investigated this (150)), and (ii) only half have investigated the effect of supplementation or fortification longitudinally across the first six months of lactation (6 out of 12 studies have investigated the longitudinal effect (151, 157, 159-161, 163)). Essentially no randomised controlled trial (RCT) investigating the effect of supplementation during pregnancy on breast milk thiamin, riboflavin and vitamin B_6 across the first six months of lactation has been conducted, highlighting the need for further research.

The existing literature available on supplementation or fortification interventions on thiamin, riboflavin or vitamin B_6 during pregnancy, consist of two studies (148, 150). The most recent study, conducted in 2016, found perinatal consumption of thiamin fortified fish sauce increased maternal thiamin status, breast milk concentration and infant thiamin status in rural Cambodian women (148). However, this study supplemented women during both pregnancy and lactation, which makes it impossible to conclude if the effect observed was due to pre- or postnatal thiamin

intake. McGready et al (2001) (150) did, as the only identified intervention study, supplement women during pregnancy only. However, in this study thiamin-deficient women were supplemented and the control women were thiamin-replete, making comparisons in breast milk concentrations across the two groups problematic. Neither of the two studies described here (148, 150) investigated longitudinal effects on breast milk concentration or infant status.

This Chapter presents an original analysis investigating; (i) the effect of a thiamin, riboflavin and vitamin B_6 containing multiple micronutrient supplement given during pregnancy on breast milk thiamin, riboflavin and vitamin B_6 concentrations; (ii) determinants of breast milk thiamin, riboflavin and vitamin B_6 concentrations and (iii) estimated infant intakes of these B-vitamins.

8.3 Methods

Data and samples for this analysis were collected as a part of the ENID trial and the ENID-Bone extension to the ENID trial. Maternal plasma (from baseline and 30 weeks' gestation) and breast milk (8, 12 and 24 weeks postpartum) were used from participating women and infants in the tablet arm of ENID (Iron-folic acid (FeFol) and multiple micronutrients (MMN)). Maternal status was only assessed in around half of the mothers from the tablet arm (n=183), selected according to availability of samples. Maternal plasma riboflavin and vitamin B_6 were analysed, whereas maternal plasma thiamin and FAD were not. Plasma thiamin concentrations were not analysed because the development of the quantification method was not successful at the Medical Research Council (MRC) Elsie Widdowson Laboratory (EWL). FAD was analysed but was later discarded because obtained values were incorrect due to unviable standard values. Further, infant status of these three B-vitamins were not assed. Participant recruitment, maternal supplementation, data and sample collection methodologies are all described in full in Chapter 4 (page 117).

8.3.1 Sample analysis

Plasma and breast milk samples were aliquoted and frozen until analysis. Plasma samples were transported to MRC EWL (Cambridge, UK) and breast milk samples were transported to USDA WHNRC, Davis, California, the United States, for analysis.

Plasma riboflavin and vitamin B_6 *concentration*

Plasma riboflavin concentration was measured by a liquid chromatography-tandem mass spectrometry (LCMS/MS) method, developed specifically for this study, inspired by existing methods (493, 498). The samples and plasma controls were defrosted on a rotary mixer for a minimum of 15 minutes. For each sample an aliquot (200 µl) of plasma was combined with trichloroacetic acid (10%) (100 µl) in a screw cap plastic tube (2 ml) and briefly mixed on a vortex mixer. Internal standard (an aqueous solution of isotopically labelled riboflavin) (20 µl) was added and the mixture briefly vortex mixed once more. Each tube was centrifuged for 15 minutes to spin down the protein precipitate. The resultant aqueous layer was transferred into an amber vial and injected onto the LCMS/MS. Pooled plasma was used for quality control. All sample handling procedures were conducted under subdued light. It was only possible to measure plasma riboflavin concentration and not FAD in this analysis.

To determine plasma PLP a reverse-phase high performance liquid chromatography (HPLC) method with post column derivatisation and fluorimetric detection was used. The chromatographic conditions employed are based upon a published method (499). The samples were previously processed for riboflavin analysis and the same extracts were injected onto the HPLC for PLP analysis. Pooled plasma (at two concentration levels) were used as quality controls. All sample handling procedures were conducted under subdued light. Table 38 details the inter-assay variability of the quality controls for the plasma vitamers. The coefficients of variation (CV) obtained are of acceptable quality.

Table 38. Inter-assay variability of the quality controls for plasma riboflavin and vitamin B_6.

	Riboflavin	**PLP (level 1)**	**PLP (level 2)**
n	26	24	24
Mean (µg/l)	5.9	12.1	6.8
SD	0.7	0.5	0.4
CV (%)	12.3	4.1	5.2

PLP, pyridoxal-5-phosphate; SD, standard deviation; CV, coefficient of variation; n, sample size.

Plasma riboflavin concentrations that fell below the minimum detection concentration (2.5 nmol/l) was reported in the analysis as 1.25 nmol/l, which is half of the minimum detection concentration. This was the case for five data-points at 8 weeks postpartum. No samples fell below the minimum detection concentration for plasma PLP.

Breast milk thiamin, riboflavin and vitamin B_6 concentration

TPP, TMP and free thiamin were measured as their respective thiochrome esters by HPLC-FLD as previously described (472). After protein precipitation and fat-removal, samples were subjected to a pre-column thiochrome derivatization prior to analysis. Pooled breast milk from one apparently healthy donor was used as quality control. All sample preparations were carried out under subdued light and on ice to protect the analytes against degradation.

The concentration of total riboflavin (free riboflavin and FAD) and total vitamin B_6 (PL and PN) in breast milk were assessed by UPLC-MS/MS. The B-vitamins were analysed simultaneously. Samples were subjected to protein precipitation and non-polar constituents were removed by liquid-liquid extraction before analysis. As internal standards $^{13}C_4$,$^{15}N_2$-riboflavin and $^{2}H_3$-pyridoxal hydrochloride were added to the samples for quantification. Pooled breast milk from one apparently healthy donor was used as a quality control. All sample preparations were carried out under subdued light and on ice to protect the analytes against degradation. Table 39 details the inter-assay variability of the quality controls for all breast milk vitamers. The CVs obtained are of acceptable quality.

Table 39. Inter-assay variability of quality controls for breast milk thiamin, riboflavin and vitamin B_6.

	Free ribo-flavin	**FAD**	**PL**	**PN**	**TPP**	**TMP**	**Free thiamin**
n	186	186	186	186	61	61	61
Mean (µg/l)	459.9	45.1	403.2	1.8	22.5	123.0	44.6
SD	26.9	5.0	22.7	0.2	2.3	11.5	4.0
CV (%)	5.8	11.2	5.6	8.9	10.4	9.3	8.9

FAD, flavin adenine dinucleotide; PL, pyridoxal; PN, pyridoxine; TPP, thiamin pyrophosphate; TMP, thiamin monophosphate; SD, standard deviation; CV, coefficient of variation; n, sample size.

Total breast milk thiamin concentration was used in this analysis, which was calculated as the sum of combined free thiamin, TMP and TPP concentrations based on molecular weights: total thiamin = free thiamin + (TMP x 0.871) + (TPP x 0.707). Total breast milk riboflavin concentration was calculated as the sum of the combined free riboflavin and FAD concentrations, based on molecular weights: total riboflavin = free riboflavin + (FAD x 0.479), and total breast milk B_6 concentration, was calculated as the sum of PL and PN: total vitamin B_6= PL+PN. The reason why total vitamin B_6 concentration is not based on molecular weights, is because PL and PN's molecular weights are almost identical (PL=167.2 g/mol, PN=169.2 g/mol). Samples where

breast milk vitamin B_6 concentrations fell below the minimum detection concentration (1 µg/l), were reported as 0.5 µg/l, which is half of the minimum detection concentration. This was the case for six data points at 8 weeks postpartum. No concentrations for breast milk thiamin or riboflavin fell below the minimum detection concentration.

8.3.2. Statistical analysis

The same statistical approach was used as described in Chapter 6 (section 6.4.2), with the exception that maternal plasma riboflavin, plasma PLP and breast milk thiamin, riboflavin and vitamin B_6 concentrations were investigated.

Outliers were defined as data with residuals >3 standard deviations (SD) from the mean in the mixed effect models and were excluded from the analysis (maternal plasma riboflavin n=3 data points removed, maternal plasma PLP n=0, breast milk thiamin n=3, breast milk riboflavin n=1, breast milk total vitamin B_6 n=11).

8.4 Results

For this analysis women and children from the FeFol and MMN arms of ENID were included, representing a total of 384 mother and infant pairs (Figure 19 in Chapter 6, Section 6.5). Baseline characteristics of study population according to intervention arm are presented in Table 12, in Chapter 6 (Section 6.5).

Median (IQR) plasma riboflavin concentration was 9 (5, 16) nmol/l at baseline, and did not differ between supplement groups (p=0.3, Table 40). Median (IQR) plasma PLP concentration was 15 (12, 20) nmol/l at baseline, and did not differ between supplement groups (p=0.5, Table 40). At baseline, 75% (129/173) of the women in both supplement groups had PLP concentrations below 20 nmol/l.

8.4.1 Supplement effect on maternal status and breast milk concentration

Maternal riboflavin and vitamin B_6 status

Maternal MMN supplementation significantly improved maternal plasma riboflavin concentration compared with FeFol (p<0.001, Table 40, Figure 34). At 30 weeks' gestation median plasma riboflavin for the FeFol group was 7 (5, 11) nmol/l and 20 (11, 28) for the MMN

group. Between baseline and 30 weeks' gestation, plasma riboflavin concentration increased in the MMN group (p<0.001) and did not change in the FeFol group (p=0.2).

Maternal MMN supplementation had a significant effect on plasma PLP concentration compared with FeFol (p<0.001, Table 40, Figure 35). At 30 weeks' gestation the median plasma PLP for the MMN group was 15 (10, 22) nmol/l and 8 (6, 10) nmol/l for the FeFol group. Between baseline and 30 weeks' gestation, plasma PLP concentration decreased in the FeFol group (p<0.001), and did not change in the MMN group (p=0.2). At 30 weeks' gestation, 71% (60/85) of the women from the MMN group and 99% (83/84) from the FeFol group had PLP concentrations below 20 nmol/l/.

Breast milk thiamin, riboflavin and vitamin B_6 concentrations

Thiamin

Free thiamin was the main form of thiamin in breast milk at 8 weeks postpartum, constituting 52% of total thiamin. TMP was present in smaller amounts, 41% of total thiamin and TPP even less, constituting only 7% of total thiamin. Table 41 details the vitamer distribution according to supplement group.

No evidence was found for an overall time by supplement interaction (p=0.7, Table 40, Figure 36), and no evidence for a difference between the two supplement groups that was consistent over time (p=0.2). Mean (SE) breast milk total thiamin concentration at 8 weeks postpartum was 117 (1.9) µg/l for both groups combined.

Breast milk thiamin concentration was similar at 8 and 12 weeks postpartum for both supplement groups, however between 12 and 24 weeks postpartum, the concentration significantly decreased in both groups (FeFol: p=0.003, MMN: p<0.001). Mean breast milk thiamin at 24 weeks was 107 (2.6) µg/l for the FeFol group and 100 (2.5) µg/l for the MMN group.

Riboflavin

The major form of riboflavin in breast milk was FAD, which contributed to 83% of the total riboflavin concentration at 8 weeks postpartum. Free riboflavin made up the remaining 17% (Table 41).

No evidence was found for an overall time by supplement interaction (p=0.6, Table 40, Figure 37), however there was a significant difference in breast milk riboflavin concentration that was consistent over time, with a higher concentration in the MMN group (p=0.003). Median breast milk total riboflavin concentration at 8 weeks postpartum was 228 (170, 283) µg/l in the FeFol group and 265 (207, 332) µg/l in the MMN group.

Between 8 and 12 weeks postpartum, total breast milk riboflavin concentration increased in both supplement groups (p<0.001 for both groups). Between 12 and 24 weeks postpartum, breast milk total riboflavin concentration did not change (FeFol: p=0.8, MMN: p=0.4). Median breast milk total riboflavin at 24 weeks was 283 (200, 373) µg/l in the FeFol group and 300 (207, 422) µg/l in the MMN group.

Vitamin B_6

The major form of vitamin B_6 in breast milk was PL, which contributed to about 99.6% of the total vitamin B_6 breast milk concentration at 8 weeks postpartum, with PN making up the remainder (Table 41).

There was no evidence for an overall time by supplement interaction (p=0.5, Table 40, Figure 38), and no evidence for a difference between the two supplement groups that was consistent over time (p=0.6). Median breast milk total vitamin B_6 at 8 weeks postpartum was 21 (13, 32) µg/l in both groups combined.

Breast milk total vitamin B_6 concentration significantly decreased between 8 and 12 weeks postpartum for both supplement groups (FeFol: p=0.05, MMN: p=0.004), and between 12 and 24 weeks postpartum, the concentration significantly increased in both supplement groups (p<0.001). Median breast milk total vitamin B_6 at 24 weeks was 30 (18, 46) µg/l for the FeFol group and 28 (17, 42) µg/l for the MMN group.

Table 40. Maternal and breast milk B-vitamin concentrations according to maternal supplement group, derived from individual mixed effects models

Mothers	Baseline	p-value *	30 weeks' gestation	p-value *	12 weeks postpartum	p-value *	p-value **
Plasma riboflavin concentration (nmol/l) [a]							
MMN	10.4 (5.34, 17.1)		19.8 (11.1, 28.0)				
FeFol	7.98 (4.65, 15.8)		7.15 (4.56, 10.9)				
Difference between supplement groups (%) [b]	14.5 (-10.9, 47.3)	0.3	135.0 (82.9, 201.8)	<0.001			<0.001
Plasma PLP concentration (nmol/l)							
MMN	15.5 (11.4, 20.6)		15.2 (9.6, 21.9)				
FeFol	14.6 (11.9, 19.4)		7.8 (5.7, 10.1)				
Difference between supplement groups (%)	4.24 (-8.7, 19.0)	0.5	92.2 (68.1, 119.7)	<0.001			<0.001
Breast milk	8 weeks postpartum		12 weeks postpartum		24 weeks postpartum		
Breast milk total thiamin concentration (µg/l) [c]							
MMN	114.9 (2.6)		113.3 (2.5)		100.4 (2.5)		
FeFol	118.9 (2.7)		117.1 (2.7)		107.4 (2.6)		
Difference between supplement groups (µg/l)	-3.99 (-11.4, 3.4)	0.3	-3.76 (-11.0, 3.4)	0.3	-7.01 (-14.1, 0.08)	0.05	0.7 (0.2)
Breast milk total riboflavin concentration (µg/l) [d]							
MMN	265.3 (206.6, 331.6)		309.1 (233.2, 408.0)		299.5 (206.5, 421.5)		
FeFol	227.7 (169.5, 282.8)		285.5 (202.4, 361.5)		282.8 (200.2, 372.5)		
Difference between supplement groups (%)	17.9 (7.04, 29.9)	0.001	14.1 (3.71, 25.4)	0.007	11.0 (1.03, 44.8)	0.03	0.6 (0.003)
Breast milk total B_6 concentration (µg/l) [e]							
MMN	21.4 (11.9, 31.0)		17.6 (12.2, 25.8)		28.2 (17.0, 42.2)		
FeFol	21.4 (13.8, 33.5)		19.5 (11.6, 28.2)		30.0 (17.8, 46.3)		
Difference between supplement groups (%)	-4.02 (-18.7, 13.3)	0.6	-9.48 (-22.9, 6.3)	0.2	1.12 (-13.7, 18.5)	0.9	0.5 (0.6)

Data presented on concentrations are medians (IQR) (non-normally distributed data), or means (SE) (normal distributed data). The means are derived from the mixed effects models, and the medians are derived from raw data. For breast milk total thiamin, the difference in concentration between the two supplement groups is presented as the difference in means (95% CI), and for maternal plasma and breast milk riboflavin and vitamin B_6, the differences are presented as the percentage (95% CI) difference in mean concentrations between groups, calculated by exponentiating coefficients from the log transformed model. All data were log-transformed, except for breast milk thiamin concentration.

[a] Plasma riboflavin is free riboflavin only. It was not possible to include plasma FAD in this analysis.

[b] FeFol group is the referent group
[c] Total breast milk thiamin concentrations are expressed as free thiamin, and are calculated as the sum of combined free thiamin, TMP and TPP concentrations based on molecular weights: total thiamin=free thiamin + (TMP x 0.871) + (TPP x 0.707). To convert total thiamin concentration to nmol/l divide total thiamin with 0.26535.
[d] Total breast milk riboflavin concentrations are expressed as free riboflavin and are calculated as the sum of combined free riboflavin and FAD concentrations based on molecular weights: total riboflavin=free riboflavin + (FAD*0.479). To convert total breast milk riboflavin concentrations to nmol/l divide total breast milk riboflavin with 0.37637.
[e] Total breast milk vitamin B_6 concentrations are calculated as the sum of PL and PN: total B_6=PL+PN (it is not based on molecular weights, as PL and PN's molecular weights are almost identical). To convert total breast milk vitamin B_6 concentrations to nmol/l, divide total breast milk vitamin B_6 with 0.1672.
* This p-value tests the difference in concentration between supplement groups at the given time-point.
** This p-value tests the difference in concentration between the two supplement groups depending on time; in other words the p-value tests an overall time by supplementation interaction. For the breast milk analyses the p-value presented in brackets is the overall supplement effect independent of time.
PLP, pyridoxal-5-phosphate; FeFol, Iron-folic acid; MMN, multiple micronutrient

Table 41. Breast milk vitamer distribution according to supplement group.

	Breast milk B_6		Breast milk thiamin			Breast milk riboflavin	
	PL	PN	Free thiamin	TMP	TPP	Free riboflavin	FAD
FeFol							
8 weeks	100%	0%	52%	41%	7%	17%	83%
12 weeks	99%	1%	53%	40%	6%	10%	90%
24 weeks	100%	0%	47%	46%	6%	9%	91%
MMN							
8 weeks	100%	0%	51%	42%	7%	16%	84%
12 weeks	99%	1%	55%	38%	6%	11%	89%
24 weeks	100%	1%	47%	47%	6%	9%	91%

Example of calculations: Breast milk PL (%): (mean breast milk PL/mean total breast milk B_6)*100. Breast milk TMP (%): ((mean TMP x 0.871)/ total breast milk thiamin)*100. Breast milk FAD (%): ((mean FAD*0.479)/mean total breast milk riboflavin)*100.
FAD, flavin adenine dinucleotide; PL, pyridoxal; PN, pyridoxine; TPP, thiamin pyrophosphate; TMP, thiamin monophosphate; FeFol, Iron-folic acid; MMN, multiple micronutrient.

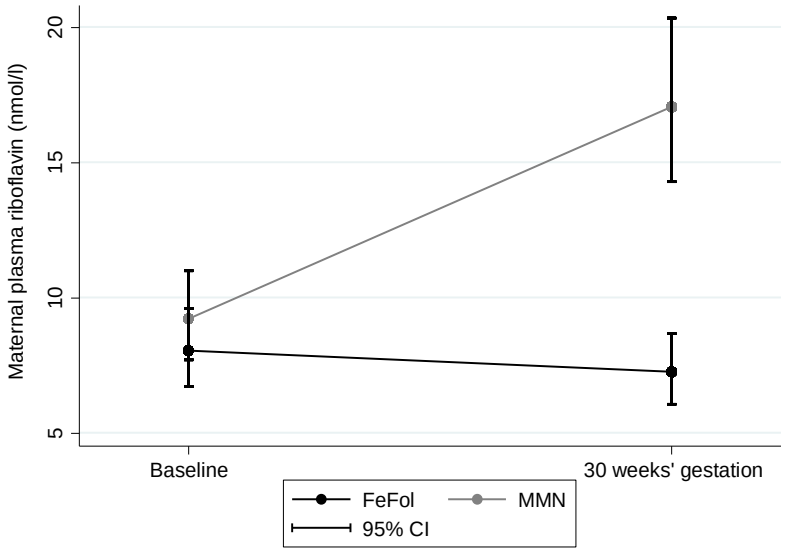

Figure 34. Longitudinal maternal plasma riboflavin concentration (nmol/l) (geometric means) according to supplement group at baseline and 30 weeks' gestation.

Data were log-transformed and analysed using a mixed effects model. Values on y-axis are unlogged. The overall time by supplementation interaction p<0.001.

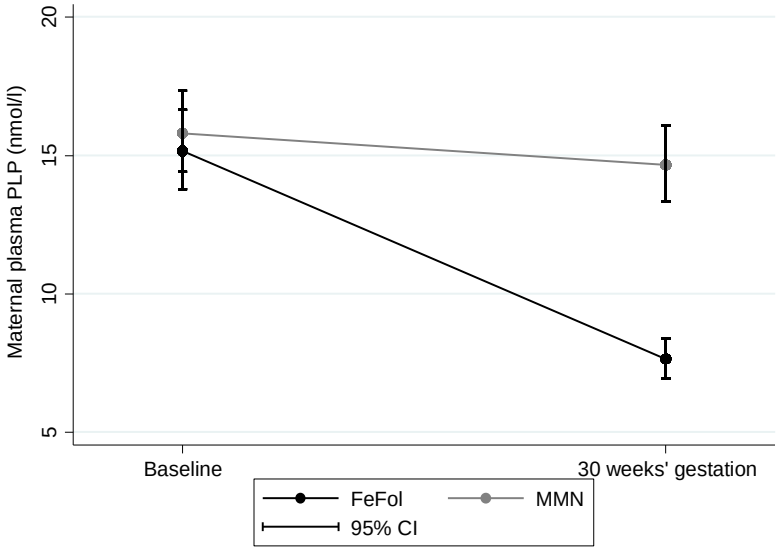

Figure 35. Longitudinal maternal plasma PLP concentration (nmol/l) (geometric means) according to supplement group at baseline and 30 weeks' gestation.

Data were log-transformed and analysed using a mixed effects model. Values on y-axis are unlogged. The overall time by supplementation interaction p<0.001.

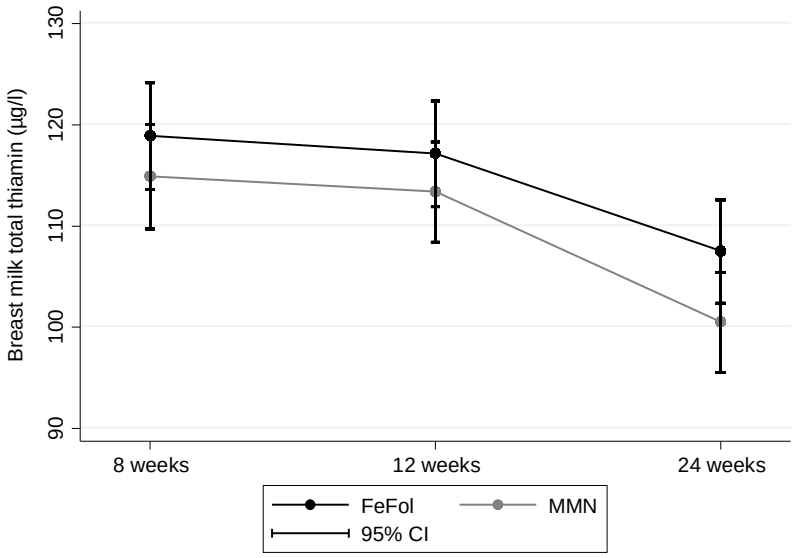

Figure 36. Longitudinal breast milk total thiamin concentrations (µg/l) (means) according to supplement group at 8, 12 and 24 weeks postpartum.

Data were normally distributed and analysed using a mixed effects model. The overall time by supplementation interaction p=0.7. The overall time by supplementation effect (consistent over time) p=0.2

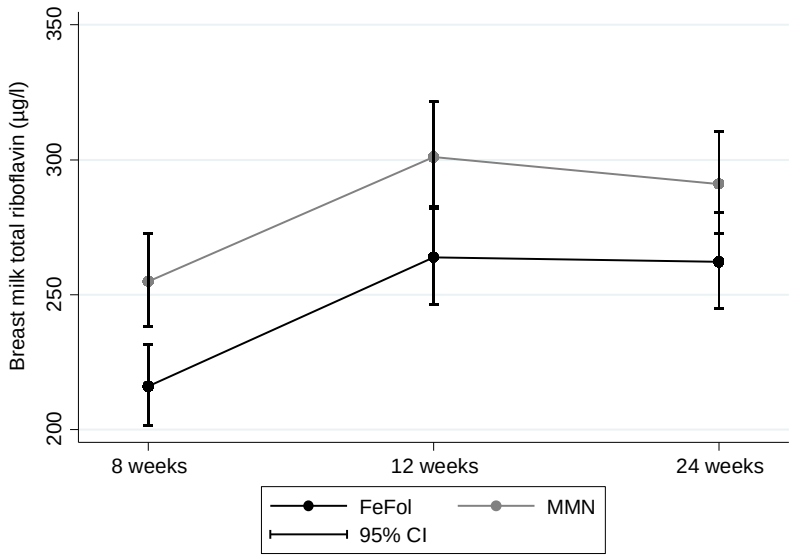

Figure 37. Longitudinal breast milk total riboflavin concentrations (µg/l) (geometric means) according to supplement group at 8, 12 and 24 weeks postpartum.

Data were log-transformed and analysed using a mixed effects model. Values on y-axis are unlogged. The overall time by supplementation interaction p=0.6. The overall time by supplementation effect (consistent over time) p=0.003.

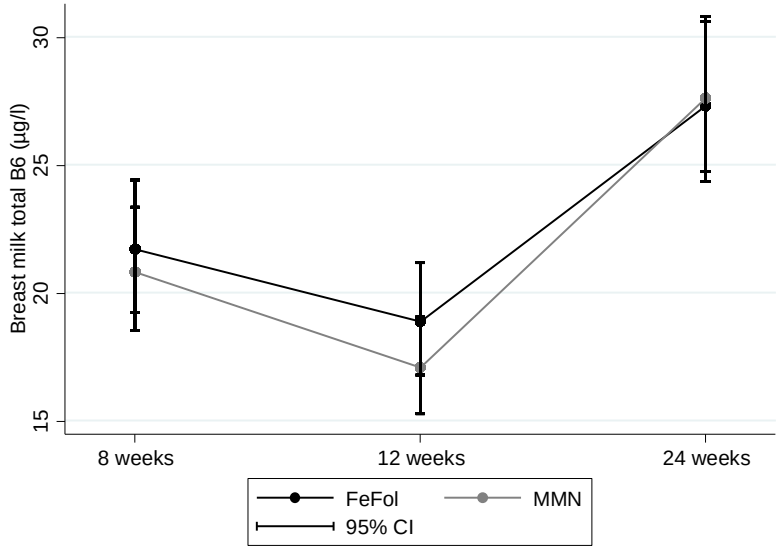

Figure 38. Longitudinal breast milk total vitamin B_6 concentration (µg/l) (geometric means) according to supplement group at 8, 12 and 24 weeks postpartum.

Data were log-transformed and analysed using a mixed effects model. Values on y-axis are unlogged. The overall time by supplementation interaction p=0.5. The overall time by supplementation effect (consistent over time) p=0.6.

8.4.2 Determinants of breast milk thiamin, riboflavin and vitamin B_6 concentrations

Breast milk total thiamin at 12 weeks postpartum was positively associated with maternal age ($p<0.001$) and seasonality ($p<0.001$), when looking at the crude estimates (Table 42). Data from both supplement groups were included in this analysis, because no difference in thiamin concentration in breast milk between groups was found. However, if only including mothers from the FeFol group the associations remained (data not shown).

Maternal breast milk riboflavin was not associated with maternal riboflavin status in early pregnancy (baseline) (crude coefficient (log-transformed): 0.058, 95%CI: -0.045, 0.163, p=0.3, r=0.121) (Figure 39). Breast milk total riboflavin at 12 weeks postpartum was associated with maternal age (p=0.003), gestational age at birth (p=0.01) and seasonality (p=0.03), based on the crude associations (Table 42). Only data from the FeFol group were included in these analyses, because a difference in riboflavin concentration at 12 weeks postpartum was found.

Maternal breast milk vitamin B_6 was associated with maternal PLP concentration in early pregnancy (baseline) (crude coefficient (log-transformed): 0.250, 95%CI: 0.017, 0.483, p=0.04, r=0.142) (Figure 40). Data from both supplement groups were included in this analysis, as there was no difference in plasma PLP at baseline or breast milk vitamin B_6 at 12 weeks postpartum between supplement groups. If only including mothers from the FeFol group, the association however disappeared (data not shown). Breast milk total vitamin B_6 was further negatively associated with maternal age (p=0.05), based on the crude estimates (Table 42). After adjusting the association between breast milk total vitamin B_6 and maternal PLP for maternal age, the association was slightly attenuated, with borderline significance (adjusted coefficient (log-transformed): 0.222, 95%CI: -0.013, 0.457, p=0.06, r=0.168) (Table 43).

None of the three B-vitamin concentrations in breast milk were associated with EBF to six months; mothers who were EBF their infant at six months postpartum did not have a different breast milk concentration compared to mothers who were not exclusively breastfeeding at six months (data not shown).

Table 42. Determinants of breast milk thiamin, riboflavin and vitamin B_6 concentrations at 12 weeks postpartum.

	n	Breast milk thiamin	n	Breast milk riboflavin [a]	n	Breast milk vitamin B_6
Maternal age, years	337	1.10 (0.26)*	160	0.02 (0.006)*	337	-0.011 (0.006)*
Maternal weight, kg	336	0.17 (0.19)	159	-0.002 (0.002)	336	-0.004 (0.004)
Maternal height, cm	337	-0.37 (0.30)	160	0.002 (0.006)	337	-0.012 (0.006)
Maternal BMI (kg/m^2)	336	0.78 (0.52)	159	-0.008 (0.01)	336	-0.003 (0.01)
Parity						
Primiparous	44	108.8 (30.73)	17	5.39 (0.10)	44	3.07 (0.11)
Multiparous (≥ 1 previous pregnancy)	293	115.4 (33.03)	143	5.61 (0.04)	293	2.86 (0.04)
Maternal education [b]						
No education	255	115.8 (33.40)	127	5.63 (0.47)	256	2.89 (0.74)
Low (1-7 years)	46	115.5 (30.32)	23	5.37 (0.54)	46	2.85 (0.62)
Medium (8-14 years)	36	104.4 (30.16)	10	5.54 (0.36)	35	2.94 (0.56)
Gestational age at birth (weeks)	333	-0.049 (1.08)	159	0.06 (0.02)*	333	-0.005 (0.02)
Season of sample collection						
Dry season (Nov to May)	205	119.9 (2.21)*	105	5.53 (0.05)*	204	2.86 (0.69)
Wet season (June to Oct)	128	105.7 (2.92)	54	5.71 (0.06)	129	2.91 (0.73)
Village						
Core villages [c]	85	115.8 (37.22)	39	5.63 (0.07)	85	2.82 (0.08)
Outreach villages	252	114.1 (31.20)	121	5.57 (0.04)	252	2.91 (0.71)
Maternal ethnicity						
Mandinka	280	114.5 (1.97)	138	5.58 (0.04)	280	2.86 (0.70)
Other	57	114.7 (4.21)	22	5.66 (0.10)	57	3.03 (0.69)
Infant sex						
Female	164	112.8 (2.46)	75	5.59 (0.06)	164	2.89 (0.06)
Male	173	116.2 (2.58)	85	5.59 (0.05)	173	2.89 (0.05)
Infant age at 12 week visit	329	-0.46 (0.26)	158	0.005 (0.005)	329	0.001 (0.005)

Non-normal distributed data were log transformed (breast milk riboflavin and breast milk vitamin B_6). Continuous data are presented as logged beta coefficient (SE), categorical data as logged means (SD). Continuous data were analysed using linear regression and categorical data using *t*-tests or ANOVA.

* Evidence for a difference between outcome and exposure variable $p \leq 0.05$.

[a] only data from the FeFol group was included in this analysis

[b] Maternal education was defined as completed years of either English or Arabic schooling

[c] Core villages are: Keneba, Jali, Kantong Kunda and Manduar situated close to the MRC Keneba. Outreach villages are the remaining 24 villages in West Kiang.

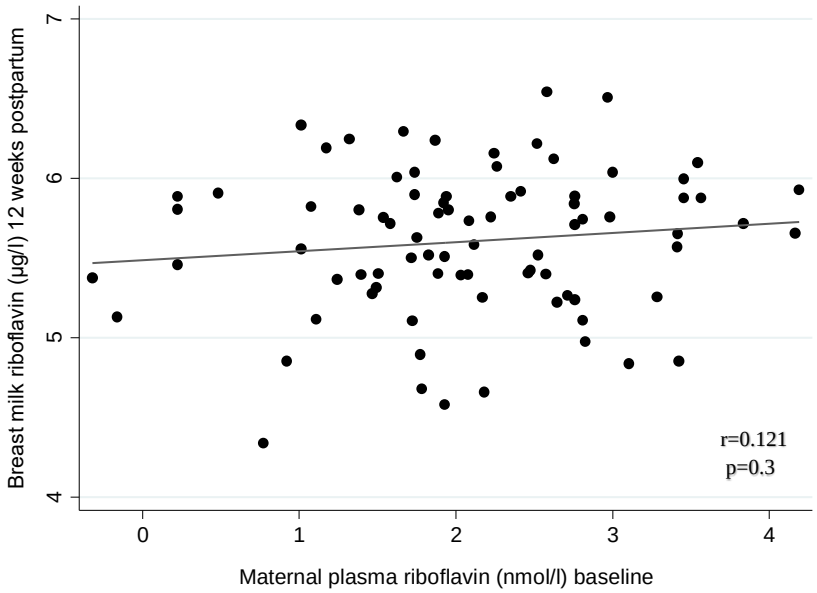

Figure 39. Association between maternal plasma riboflavin at baseline (early pregnancy) and breast milk riboflavin at 12 weeks postpartum (n=82).

All values were log-transformed before analysis. The grey line is the linear regression fit. Only mothers from the FeFol group was included in this regression analysis.

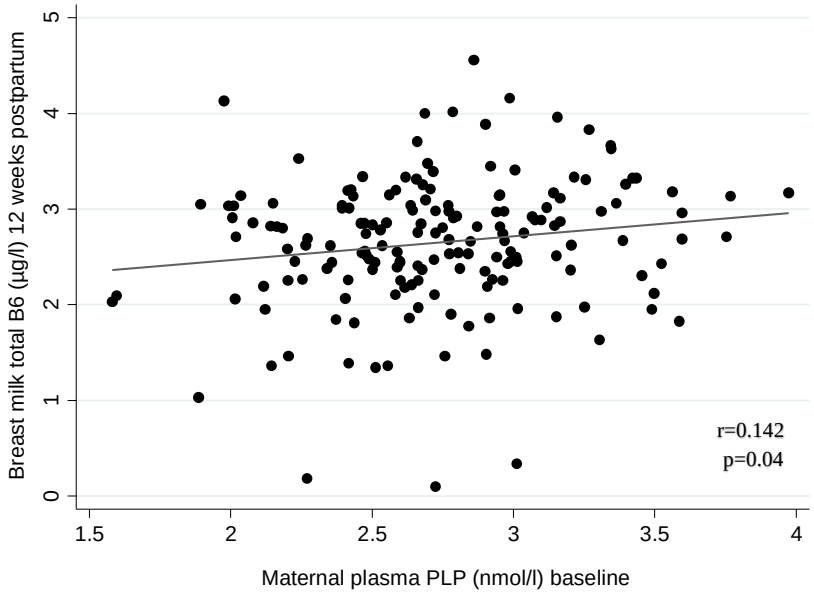

Figure 40. Association between maternal plasma PLP at baseline (early pregnancy) and breast milk total B_6 at 12 weeks postpartum (n=171).

All values were log-transformed before analysis. The grey line is the linear regression fit.

Table 43. Adjusted regression of breast milk total vitamin B_6 at 12 weeks postpartum and maternal PLP at baseline (early pregnancy).

	n	β-coefficient	SE	95% CI	p-value
Maternal plasma PLP in early pregnancy	171	0.222	0.119	-0.013, 0.457	0.06
Maternal age	171	-0.013	0.008	-0.030, 0.004	0.1

The β-coefficient presented for each of the determinants are all adjusted for the remaining variables in the table. Breast milk total vitamin B_6 was logged transformed.
SE, standard error; CI, confidence interval; n, sample size; PLP, pyridoxal-5-phosphate.

8.4.3 Estimated infant B-vitamin intake

In exclusively breastfed infants, thiamin, riboflavin and vitamin B_6 intakes are equal to excretion in breast milk. Table 44 details the estimated average infant thiamin, riboflavin and vitamin B_6 intakes (μg/day) at three time-points.

The estimated median intake of thiamin for infants was 90 μg/day at 12 weeks postpartum. All infants from both supplement groups (312/312) had an estimated thiamin intake below the AI of 200 μg/day at 12 weeks postpartum. The estimated median infant intake of riboflavin in the FeFol groups was 221 μg/day at 12 weeks postpartum. Eighty-one percent (120/148) of the infants from the FeFol group and 71% (116/163) from the MMN group had an estimated riboflavin intake below the AI of 300 μg/day at 12 weeks postpartum. The estimated median intake of infant vitamin B_6 was 14 μg/day at 12 weeks postpartum. Almost 100% (311/312) of the infants from both supplement groups had an estimated vitamin B_6 intake below the AI of 100 μg/day at 12 weeks postpartum.

Table 44. Estimated average daily thiamin, riboflavin and vitamin B_6 intake for exclusively breastfed infants (μg/day) at varying time-points.

B_1 intake [a]	8 weeks postpartum		12 weeks postpartum		24 weeks postpartum
Total [b] n=304	90.8 (69.1, 110,4)	Total n=312	88.8 (71.8, 106.5)	Total n=190	78.4 (64.6, 98.6)
B_2 intake [a]	8 weeks postpartum		12 weeks postpartum		24 weeks postpartum
FeFol n=150	179.2 (132.6, 221.2)	FeFol n=148	220.9 (156.4, 282.2)	FeFol n=95	221.1 (156.6, 282.0)
MMN n=256	207.4 (160.6, 258.4)*	MMN n=163	239.1 (180.9, 319.1)*	MMN n=94	249.2 (185.7, 354.8)*
Total n=306	190.4 (146.5, 242.7)	Total n=311	234.2 (169.0, 297.1)	Total n=189	229.5 (167.5, 315.3)
B_6 intake [a]	8 weeks postpartum		12 weeks postpartum		24 weeks postpartum
Total n=298	16.7 (9.9, 24.7)	Total n=312	14.1 (9.2, 21.7)	Total n=190	22.6 (13.5, 39.2)

Data are medians (IQR). Only riboflavin intake is presented according to supplement group, as this is the only vitamin with a difference in breast milk concentration according to supplement group.

* Difference in medians between supplement groups, $p \leq 0.05$. Analysed by Mann-Whitney test.

[a] Calculated as the concentration excreted in breast milk*0.782 l/day

[b] Total = data from both supplement groups.

FeFol, iron-folic acid; MMN, multiple micronutrients; n, sample size.

8.5 Discussion

A multiple micronutrient supplement given daily during pregnancy, containing 2.8 mg of each of thiamin, riboflavin and vitamin B_6, resulted in higher maternal plasma riboflavin and PLP during pregnancy and a modestly higher riboflavin concentration in breast milk across the first six months of lactation. No effect was seen on breast milk thiamin or vitamin B_6 concentrations across the first six months of lactation.

To the best of my knowledge, this is the first intervention study to have supplemented women during pregnancy and investigated the effect on breast milk thiamin, riboflavin and vitamin B_6 concentrations across the first six months of lactation. The study by McGready et al (2001) (150), is the only identified intervention that supplemented women during pregnancy only. The authors supplemented thiamin-deficient pregnant women (100 mg/day thiamin), and the control group were unsupplemented thiamin-replete women (defined as having no physical symptoms of deficiency), which makes it difficult to compare breast milk thiamin concentrations according to supplement use in this study. In this study by McGready et al (2001) (150), the median thiamin breast milk concentration was 128 µg/l in the supplemented group and 117 µg/l in the unsupplemented group (not significantly different) at three months postpartum, possibly illustrating that supplementation during pregnancy can increase breast milk thiamin for thiamin-deficient women to the same concentration as thiamin-replete women. No comparable interventions studies were identified on riboflavin or vitamin B_6. More studies on breast milk composition of these investigated B-vitamins and the effect of maternal supplementation during pregnancy are warranted, especially in populations where maternal status is considered marginal.

In this analysis, maternal plasma riboflavin concentration significantly increased with supplementation, whereas PLP was constant across pregnancy in the MMN group, and decreased in the FeFol group. This observed decrease in PLP during pregnancy in the FeFol group is consistent with the findings from other studies of unsupplemented pregnant women (495, 496, 500). This drop was also observed in maternal plasma vitamin B_{12} concentrations during pregnancy in this rural Gambian population (Chapter 7, Section 7.5.1), suggesting similar mechanisms for vitamin B_{12} and B_6 during pregnancy. However, it is not clear why this decrease occurs. Possibly, it is due to the same hypothesised reasons reported for vitamin B_{12}: haemodilution, and/or an increased supply of vitamin B_6 from the mother to the foetus (177).

The maintenance of PLP concentration in the MMN group across pregnancy in this Gambian population indicates that 2xRDA (2.8 mg/day) is sufficient in this population to prevent a decline in PLP. An intervention study in well-nourished American women found that at least 5 mg/day of vitamin B_6 was needed to prevent a decrease in PLP (500). With 5 mg/day PLP concentration increased from 32 nmol/l at baseline (<22 weeks' gestation) to 47 nmol/l at 30 weeks' gestation. A dose of 2.6 mg/day was not able to prevent the PLP decline (500). This indicates that regardless of a considerably lower baseline vitamin B_6 status in this rural Gambian population (15 nmol/l), a lower dose was needed to maintain PLP across pregnancy compared to well-nourished American women. Nevertheless, the trial by Schulster et al (1984) (500) only had a sample size of 10 or less in their supplement arms, decreasing the accuracy of the reported results.

Further, it is not known if this decrease in PLP levels across pregnancy constitute vitamin B_6 deficiency, and what the desired maternal PLP status is during pregnancy to prevent maternal, foetal and infant deficiency. Existing literature report that a decrease in PLP levels during pregnancy is potentially due to normal physiologic changes, as the decrease has previously been reported in studies of healthy and vitamin B_6-replete women (495, 496, 500). One study further found an increase in plasma PL during pregnancy compared to non-pregnant controls, offsetting the PLP decrease (495). PL may serve as an available source of vitamin B_6 to meet the increased metabolic demands during pregnancy suggesting that an adequate total vitamin B_6 concentration is of more importance than an adequate PLP concentration. More knowledge is needed about which concentrations of PLP, PL and total vitamin B_6 that constitutes deficiency during pregnancy.

The longitudinal pattern of the three breast milk B-vitamin concentrations identified in this analysis were all different in this population. Total breast milk thiamin concentrations were stable between weeks 8 and 12 of lactation and decreasing between 12 and 24 weeks of lactation. Riboflavin concentrations increased in early lactation (between 8 and 12 weeks postpartum) and then remained stable between 12 and 24 weeks. Finally, vitamin B_6 concentration decreased modestly in early lactation followed by an increase in late lactation (between 12 and 24 weeks postpartum). Few longitudinal studies exist on thiamin, riboflavin and vitamin B_6 concentrations in mature milk, which makes it difficult to conclude if these patterns are normal physiological changes, or if they are driven by maternal nutritional status. Nevertheless, vitamin B_6 seems to behave like vitamin B_{12} as described in Chapter 7 (see section 7.6), with an increase in concentrations around six months postpartum, indicating that similar mechanisms occurs for

these two vitamins. As discussed in Chapter 7, this could potentially be due to recovery of maternal status during lactation.

The longitudinal pattern of breast milk thiamin concentrations seen in this analysis may reflect good maternal status in pregnancy; enough to maintain a good supply in breast milk in early pregnancy until 12 weeks postpartum. Supporting this is the findings by McGready et al (2001) (150), who reported milk concentrations from thiamin-replete women living near the Thai/Burmese border. The authors reported milk thiamin concentrations of 117 µg/l at 12 weeks postpartum, identical to the concentrations found in this study (117 µg/l in the FeFol group). This is the only available study of thiamin-replete women where breast milk concentrations were measured around three months postpartum. However, these women were categorised as thiamin-replete based only on the absence of physical symptoms of thiamin deficiency. These women could thus be mild or moderately deficient. Further, the decline observed in breast milk thiamin concentration between 3-6 months of lactation in this analysis could indicate that maternal status during pregnancy is not sufficient to maintain adequate thiamin supply to the infant across the entire duration of EBF in this population.

The change observed in breast milk riboflavin concentrations across the first six months of lactation may, as seen with vitamin B_6 and B_{12}, reflect recovery of maternal status during lactation. This is however difficult to determine in this analysis, firstly because maternal riboflavin status was not measured during lactation, and secondly because no study has investigated the difference in riboflavin status in non-pregnant, pregnant and lactating women. More studies are needed on longitudinal changes in B-vitamin concentrations across lactation in replete-populations.

8.5.1 Deficiency, intake and determinants of breast milk B-vitamin concentrations

In this population, maternal vitamin B_6 status during pregnancy was low, with 75% of the women being below the cut-off for deficiency (PLP <20 nmol/l) in early pregnancy (baseline). High rates of deficiency were observed in both supplement groups at 30 weeks' gestation, even in the MMN group (71%). This suggests that 2xRDA is not sufficient in increasing vitamin B_6 concentrations to sufficient levels in this rural Gambian population. Nevertheless, the reliability of PLP as a vitamin B_6 status biomarker has been questioned, and especially during pregnancy as plasma fluctuations are not taken into account (470).

The existing literature on breast milk concentrations of the three investigated B-vitamins are sparse. As a consequence, the infants' AIs for thiamin, riboflavin and vitamin B_6 are derived from poor data. If using the existing AIs, almost all exclusively breastfed infants in this rural Gambian population had estimated thiamin and vitamin B_6 intakes below the respective AIs at 12 weeks postpartum and more than 80% had estimated riboflavin intakes below the AI. This is however estimates, as actual breast milk intakes were not obtained in this analysis. A study from 2012, using the same laboratory techniques to measure breast milk concentrations as in this analysis, analysed breast milk thiamin, riboflavin and vitamin B_6 from five different countries (high and low-income countries) and highlighted the high variance of concentrations in breast milk across regions (493). Almost all of the median breast milk concentrations reported in this study were lower than the concentration required to reach the infant AIs (493). This large discrepancy between recent analysed milk concentrations and the infant AIs are likely explained by a combination of less sensitive analytical techniques for quantification and a small sample size. Infant requirements of all three B-vitamins need to be revisited with the intention of deriving EARs for exclusively breastfed infants.

In comparison to other studies, breast milk thiamin concentrations are considered sufficient up until 12 weeks postpartum in this rural Gambian population, as already described. With riboflavin, concentrations in milk are considered moderately low in this population in comparison with the only available study measuring mature milk concentrations in a replete population (158). This study reported riboflavin breast milk concentrations of 996 nmol/l (375 µg/l) at 6 weeks postpartum in riboflavin-replete Spanish women (EGRAC <1.2 in third trimester). This is moderately higher than this study's findings of 265 µg/l in the MMN group and 228 µg/l in the FeFol group at 8 weeks postpartum, suggesting that 2xRDA supplementation during pregnancy is likely not sufficient in increasing riboflavin concentrations in breast milk to adequate concentrations. However, this study by Ortega et al (1999) (158) did not use the same analytical techniques to determine concentration, decreasing the comparability of the results.

For vitamin B_6, reported concentrations in rural Gambian women's milk were considered low. In this study milk concentrations were 21 µg/l for both supplement groups at 8 weeks postpartum. Thomas et al (1979) (163) reported vitamin B_6 concentrations of 204 µg/l at 6 weeks postpartum in well-nourished American women, suggesting low milk levels in rural Gambian women. Nevertheless, the study by Thomas et al (1979) (163) had a small sample size (n=17) and used a different analytical technique to quantify milk concentrations than what was used in this analysis,

limiting the comparability of the results. This is however the only study available from a replete population measuring mature milk vitamin B_6 concentrations.

These low breast milk concentrations, even in the MMN supplemented group, imply that to ensure adequate breast milk thiamin, riboflavin and vitamin B_6 concentrations, maternal prenatal supplementation is not sufficient in this population. To increase breast milk concentration, maternal postnatal supplementation is needed. This is supported by a recent RCT, where thiamin fortified fish sauce were provided during pregnancy and early lactation in Cambodia (148). This study reported improved maternal thiamin status, breast milk concentrations and infant status compared to a placebo group (breast milk thiamin: 177 µg/l vs. 144 µg/l) (148). For vitamin B_6, the only available RCT supplemented American women during lactation (from delivery to nine months postpartum) with a multiple micronutrient that between study arms was different in vitamin B_6 concentration (159). One group of women were given a supplement containing 0.5 mg/day pyridoxine and another group 4 mg/day. Postnatal supplementation of 4 mg/day increased maternal vitamin B_6 status and breast milk concentrations compared to 0.5 mg/day (breast milk total vitamin B_6 1317 nmol/l (220 µg/l) vs. 2666 nmol/l (446 µg/l) at six months postpartum). No RCT was found supplementing women with riboflavin during lactation. This indicates that breast milk concentration of thiamin and vitamin B_6 can be increased by maternal supplementation during lactation.

In this analysis, maternal plasma riboflavin in early pregnancy was not correlated with breast milk riboflavin at 12 weeks postpartum. This is in contrast with one available study reporting an association between maternal EGRAC during third trimester of pregnancy and breast milk riboflavin in mature milk (158). The lack of an association in this analysis could be because total plasma riboflavin was not measured. Further, maternal plasma PLP in early pregnancy was correlated with breast milk vitamin B_6, however when adjusting for confounding variables, only evidence for a borderline association remained. No other studies were found investigating this.

Breast milk thiamin concentrations were in this analysis associated with seasonality of breast milk sample collection. This is in agreement with findings from a previous study in rural Gambia (297). Dominguez-Salas et al (2013) (297) reported a marked difference in rural Gambian women's status of several B-vitamins according to season. During the wet season (June-Oct) riboflavin, folate and vitamin B_6 biomarkers (thiamin was not investigated) were lower than during the dry season (Nov-May), mainly due to a low dietary intake of the vitamins.

Further, breast milk riboflavin was positively associated with maternal age and with gestational age at birth in this analysis. No association between the three B-vitamin concentrations in breast milk and mothers who were EBF to six months were found in this analysis.

8.5.2. Strengths and limitations

These findings expand current knowledge around maternal vitamin B_6 status during pregnancy and breast milk thiamin, riboflavin and vitamin B_{12} concentrations in a population with low habitual intake of animal food sources. This analysis exposed the need to focus on these important, but often, neglected micronutrients. This analysis further identified the need for research on vitamin B_6 deficiency during pregnancy, and on the development of infant EAR recommendations for the investigated B-vitamins during the period of EBF.

A key strength of this analysis was the use of advanced laboratory techniques to measure these complex B-vitamins in breast milk, increasing reliability of the obtained results. One limitation of this analysis was that maternal status was only measured in half of the women from the tablet arm of ENID. This was due to low sample availability. However, measuring maternal status in all mothers would likely not have altered the results, because of the highly controlled supplementation and high compliance in this study.

In addition, maternal plasma thiamin and FAD concentrations were not analysed, limiting the interpretation of how maternal thiamin and riboflavin status were affected by supplementation and also the ability to determine if the women had a poor status of these micronutrients. Further, maternal riboflavin was not measured using the gold standard method, EGRAC, as red blood cells were not collected as a part of ENID. Lastly, maternal status of the three B-vitamins during lactation and infant status of the three B-vitamins were not measured, due to low sample availability. This limited the interpretation of the impact of the maternal micronutrient supplement on maternal pstpartum and infant status.

8.5.3 Conclusion

In conclusion, a daily multiple micronutrient supplementation (2.8 mg of each of thiamin, riboflavin and vitamin B_6) given during pregnancy to rural Gambian women increased breast milk concentration of riboflavin, but not thiamin or vitamin B_6 concentrations.

Chapter 9

Discussion

The aim of this thesis was to explore maternal nutrition, breast milk micronutrient composition and infancy growth and nutritional status in a rural Gambian population with a history of poor growth and inadequate micronutrient status. In particular the focus was on the period of exclusive breastfeeding (EBF) and the influence of maternal nutritional status on human milk composition of essential micronutrients.

This research highlighted the importance of maternal nutrition on breast milk micronutrient status, and identified many important gaps in the existing knowledge base. Original research using trial data from rural Gambia showed that a prenatal supplement, containing twice the recommended dietary allowance (RDA) of 15 micronutrients, only moderately increased breast milk concentrations of iodine and riboflavin over the first six months of lactation, and no effect was seen on thiamin, vitamin B_6 and B_{12} concentrations in breast milk. These observations of poor breast milk micronutrient concentrations may impact on the benefit of following the World Health Organization's (WHO) recommendation of EBF to six months in settings where the mother enters pregnancy and lactation with a poor nutritional status. This highlights the need for nutrition interventions during this critical period of growth and development. Further, these observations may explain, in part, the lack of benefit of EBF to six months on growth in this population of Gambian infants. In this final section of this thesis, these findings are put into the context of existing literature and the public health implications of the research are discussed.

9.1 Infant growth

Growth faltering remains highly prevalent across infancy and young childhood in many low-and middle-income settings, including this rural Gambian population (2, 74). Optimal breastfeeding practices, including EBF to six months, is advocated to promote healthy growth (15). However, despite impressive EBF practices in this rural Gambian population, with 32% of infants receiving only breast milk in the first six months of life, substantial growth faltering was observed in early infancy and 26% of children were stunted by two years of age. In this population, EBF to six months had limited impact on the growth of infants in the first two years of life. The observed improvements in weight, length, weight-for-age (WAZ), length-for-age (LAZ) and weight-for-length z-score (WLZ) in the group who were exclusively breastfed to six months were all small in magnitude. This is in agreement with results from other studies conducted in populations living under similarly resource-constrained conditions (49, 67-70). Even though no impact was seen on growth in this setting, it is still recommended to EBF to six months, as EBF has other important health benefits, especially in settings with a high infectious burden.

Several preventative interventions against growth faltering have been implemented in rural Gambia over the years. The Medical Research Council (MRC) have been working in the West Kiang region since the 1940's, and in the 1970's a permanent field station and clinic was set up in rural Keneba, offering the local population and surrounding villages, access to free primary health care. A supplement centre was later implemented providing treatment for severely malnourished children (74). Further this population has benefitted from free access to comprehensive immunisation, malaria treatment, improved water and sanitation facilities (74), health and nutrition education (501, 502), and national coverage of The Baby Friendly Community Initiative (503). These changes have occurred alongside the implementation of a large number of supplementation trials assessing the benefit of a range of interventions during pregnancy and early infancy (288, 299, 305, 504, 505). Despite large improvements in childhood mortality (506) and reductions in the prevalence of diarrhoea, malaria and other infections over the years (74, 288, 507), the scale of malnutrition, in particular stunting, has in this population remained unacceptably high; longitudinal data collected between 2000-2012 illustrated that 30% of infants remain stunted, 22% underweight and 11% wasted (74).

Developing interventions to alleviate growth faltering remains a priority research and programmatic focus globally. Several large trials have tested prenatal supplementation (reviewed in (21)), pre- and postnatal supplementation (508), fortification of infant complementary foods

(509, 510) and a combination of all; maternal pre- and postnatal and infant supplementation (511, 512), but with limited effect. This lack of effect is likely due to the high burden of multiple stressors that impact on growth such as the intrauterine environment, infant and young child feeding practices, hygiene and sanitation, and infections. In environments where these factors are sub-optimal, nutrition interventions alone are unlikely to yield benefit and multiple stressors may need to be targeted simultaneously to advance healthy growth (54, 74, 513). Countries that have gone through an economic transition and poverty reduction have simultaneously experienced rapid declines in stunting prevalence (514), demonstrating that economic development is a key underlying determinant of healthy growth (74).

The consequences of poor growth can further extend to the next generation, as maternal short stature is associated with offspring stunting (4, 5), increasing the risk of an intergenerational cycle of stunting (3). In addition, there is expanding evidence supporting the concept that infant growth is modified by an epigenetically imprinted history of the privious generations' nutritional exposure (515-517). In rural Gambia, the seasonally-driven alterations in food availability expose unborn children to in utero energy and nutrient restriction and this has been shown to impact on several generation's growth (76). This highlights the importance of an in utero environment free from nutritional stressors in order to achieve healthy growth of future generations. It further supports the argument that high-intensity, multifaceted interventions across multiple generations are needed to overcome growth faltering in these settings.

The role of breast milk micronutrients in infant growth has, to some degree, been neglected until recently. No intervention study or trial has, to date, examined the effect of a maternal postnatal supplement on breast milk micronutrient concentrations, with the specific objective to improve micronutrient status and infant growth. Some indirect evidence comes from nutrition intervention trials, where women are randomised to supplements during pregnancy and lactation. Christian et al (2016) (508) supplemented mothers during pregnancy and lactation (to three months postpartum) with a multiple micronutrient supplement (15 micronutrients, 1xRDA) in Bangladesh. Supplementation led to a higher infant LAZ and lower stunting prevalence through three months of age, compared to the control group (iron-folic acid). In contrast, Dewey et al (2017) (512) did not find an effect of a combined maternal pre-and postnatal lipid-based nutrient (LNS) supplement (containing 21 micronutrients and 118 kcal daily) on infant growth between birth and six months of age. However, neither maternal micronutrient status or breast milk micronutrient composition were investigated in either of these trials, making it impossible to

determine if the supplement had any effect on breast milk micronutrient concentrations and how these concentrations were related to infant growth.

Within the context of a resource-constrained environment, poor maternal nutritional status leading to poor breast milk micronutrient composition may be one of a number of contributing factors leading to poor growth in infants and young children. More exploratory research on the underlying biological factors is needed in this area to determine causal determinants of the observed growth faltering, so that targeted interventions can be more effectively developed and implemented. Novel research investigating the impact of maternal pre- and postnatal supplementation on breast milk micronutrient composition and, in turn, infant growth, is warranted.

9.2 Breast milk micronutrients

Micronutrient deficiencies are often referred to as 'hidden hunger' as many children suffering from vitamin or mineral deficiencies do not show symptoms typically associated with malnutrition (518). Nevertheless, suffering from micronutrient deficiencies can have long lasting consequences. For example, iodine deficiency during pregnancy and infancy can lead to growth retardation and reduced cognitive function in the infant (345), while vitamin B_{12} deficiency is linked to neurological, motor and cognitive impairment (398). The combined effect of multiple deficiencies, which is highly likely as several micronutrients are found in the same foods, may contribute to even worse effects (459). Further, it is possible that there are more subtle consequences of moderate deficiencies that are yet to be identified.

In their 2002 evaluation report on the optimal duration of EBF, the WHO concluded that, apart from a possible negative effect of EBF to six months on infant iron status in poorly nourished populations, "the available evidence is grossly inadequate to assess risk of deficiency in other micronutrients" (48). This WHO evaluation has not been updated since and, as highlighted in this thesis, studies conducted since this time are insufficient to make any substantive contribution to the evidence base, other than reinforcing that maternal undernutrition may be a risk factor for poor concentrations of certain micronutrients in breast milk.

The research presented in this thesis highlights a number of factors contributing to the lack of data in this important area. First, breast milk micronutrient concentrations are difficult to measure, and only recently have valid, high sensitivity quantification methods been developed.

Further, the available data on most micronutrients remain limited, and it is not known what the optimal micronutrient concentrations are in breast milk for healthy growth and development of the infant. In addition, it is plausible that because milk concentrations of some micronutrients are very low in breast milk and are not influenced by maternal status or dietary intake, researchers have considered pregnancy as a more important time-period to ensure adequate infant micronutrient status than the early postpartum days, limiting the focus on the lactation period. Iron offers the best example of this, since milk iron concentrations cannot meet infant iron requirements at any stage of lactation (50). Instead, infants are born with an iron endowment obtained from maternal stores during pregnancy, which is able to sustain infant iron requirements during the period of EBF if maternal stores during pregnancy are adequate (50). This might give the impression that maternal status during pregnancy is of more importance than her nutritional status during lactation. However, iron is an exception. As described in Sections 6.1, 7.1, and 8.1 infant postnatal status is, for several micronutrients, dependent on adequate maternal pre- and postnatal micronutrient status.

9.2.1 Maternal nutritional influences on breast milk micronutrients

Chapter 2 of this thesis highlighted a paucity of comparable data to describe the relationship between maternal micronutrient status, dietary intake and breast milk micronutrient composition. Most studies included in the systematic review investigated small numbers of women, often from non-representative population groups. Further, many studies had poorly-defined collection methods and used outdated and likely invalid analytical quantification methods. Nevertheless, in the few examples where data is available from different populations for the same micronutrient, breast milk concentrations are higly variable, suggesting a key role of maternal nutritional status. For example, Figures 41 and 42 show breast milk B-vitamin concentrations at 12 weeks postpartum from 13 different populations of unsupplemented women all analysed at the USDA Western Human Nutrition Research Centre (data presented in this thesis is included, and the remaining data is mostly unpublished). These Figures clearly illustrate the high variability of breast milk micronutrient composition across populations, likely because of different maternal dietary intakes. It further indicates the importance of taking into account maternal nutritional status and intake during lactation, when investigating breast milk micronutrient concentrations.

These figures also highlight that few populations have breast milk B-vitamin concentrations reaching IOM’s average intake (AI) recommendations for infants aged 0-6 months. Even populations with assumed adequate dietary intakes (e.g. Davis, California) have concentrations

below the AIs for most of the B-vitamins. This suggests that the infant AIs are based on poor data and are likely overestimating infant needs.

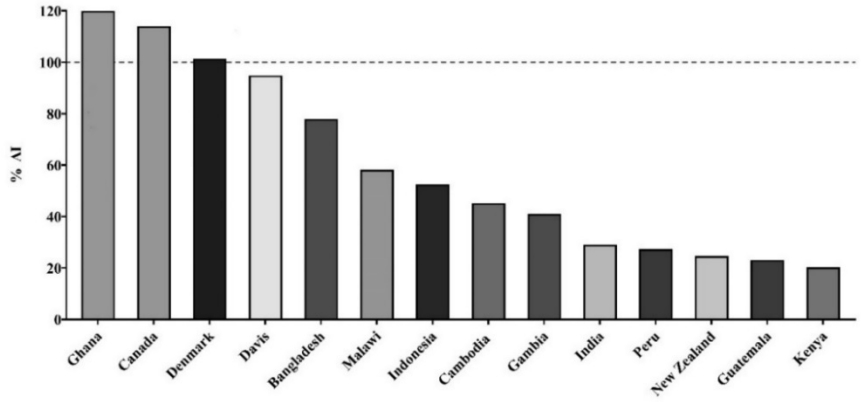

Figure 41. Breast milk vitamin B_{12} concentrations according to infant average intake (AI) recommendations.

The percentage AIs are based on median breast milk concentrations from unsupplemented women at 12 weeks postpartum. All data were analysed at the USDA Western Human Nutrition Research Centre, California USA. Data shared by Dr Lindsay Allen.

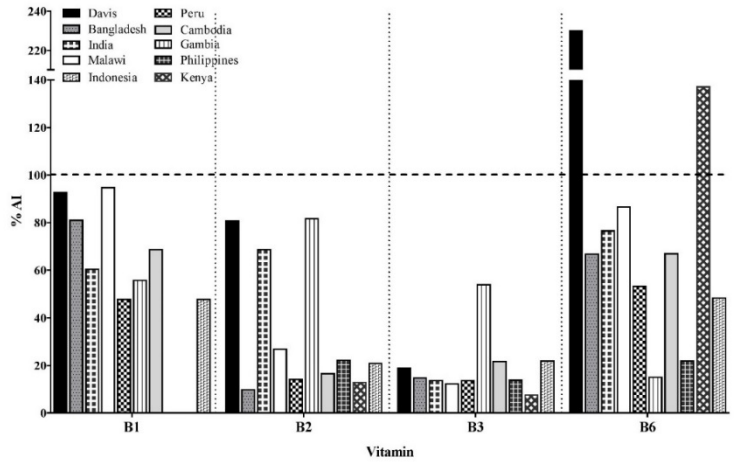

Figure 42. Breast milk vitamin B_1, B_2, B_3 and B_6 concentrations according to infant average intake (AI) recommendations.

The percentage AIs are based on median breast milk concentrations from unsupplemented women at 12 weeks postpartum. All data were analysed at the USDA Western Human Nutrition Research Centre, California USA. Data shared by Dr Lindsay Allen.

9.2.2 Adequacy of micronutrients in breast milk

As highlighted in this thesis (Sections 6.5.1, 7.5.1, 8.4.1), prenatal supplementation with multiple micronutrients (MMN) had a modest effect on breast milk iodine and riboflavin concentrations, but no effect on thiamin, vitamin B_6 or vitamin B_{12}. The increase observed in milk concentrations of iodine and riboflavin were, however, too low to sustain infant requirements during the period of EBF. For iodine, 85% of exclusively breastfed infants from the MMN-supplemented group had estimated intakes at 12 weeks of age below the recently developed estimated average requirement (EAR) (377). Further, when comparing against breast milk concentrations obtained from iodine replete populations (pooled milk concentration from women from China, Croatia and Philippines; median 171 ug/l at 2-26 weeks postpartum) (241), the values from this Gambian population were low (51-57 μg/l at 8-24 weeks postpartum). For riboflavin, there is currently no recommendation for an EAR. However, comparing milk concentrations from the MMN-supplemented group (265 μg/l at 8 weeks postpartum) to concentrations obtained from a study by Ortega et al (1999) (158) of riboflavin replete Spanish women (375 μg/l, at 6 weeks postpartum), concentrations appear moderately low.

No effect of prenatal supplementation was observed on breast milk concentrations of thiamin and vitamins B_6 and B_{12} among this Gambian population at any stage of lactation. For all three of these B-group vitamins, no EAR for infants (0-6 months) exist, only an AI recommendation which is based on poor data. Furthermore, comparable data from both resource-poor and affluent populations are limited. The few studies available have a low sample size and often used different analytical quantification methods. In comparison to the available data, concentrations of breast milk vitamin B_{12} and B_6 were considered low (Sections 7.6 and 8.5), while thiamin appeared to be adequate in the first 12 weeks postpartum, however likely not to 24 weeks (Section 8.5).

Overall breast milk concentrations of iodine, thiamin, riboflavin, vitamin B_6 and B_{12} were considered low across the first six months of EBF in this rural Gambian population, even among women who received a prenatal supplement containing these micronutrients. However, the lack of robust data on breast milk composition of all these micronutrients from well-nourished populations makes it difficult to accurately determine the level of inadequacy in this population.

Despite these concerns, and because of the other known benefits of breastfeeding for both the infant and the mother, breast milk is still considered the optimal food for infants during the first six months of life. However, this thesis highlights the need for high quality data to inform our

knowledge on breast milk concentrations of micronutrients to meet infants' needs. These data would provide a reference which interventions to improve breast milk micronutrient concentrations could be assessed against.

9.2.3 Interventions to improve breast milk micronutrients

To improve breast milk micronutrient concentrations, interventions should be focused on improving maternal status both pre- and postnatally. A prenatal supplement, while effective in improving maternal status during pregnancy and infant micronutrient status at birth for some micronutrients, is not effective in increasing breast milk concentrations and infant status to appropriate levels for the first six months of lactation, as illustrated in this thesis. Despite the paucity of the evidence on the effect of maternal postnatal supplementation on breast milk composition and infant status, the available data would support that a combination of a pre-and postnatal supplementation (175, 176) or a postnatal supplementation alone (148, 159, 227, 395) is effective in improving maternal status, breast milk concentrations and infant status during the months of EBF.

In the first few months of life, infants are dependent on nutrient stores acquired by placental transfer and, in the case of exclusively breastfed infants, nutrient intake from human milk to ensure adequate micronutrient status. Using vitamin B_{12} as an example, if maternal intake of the vitamin during pregnancy is adequate, infants are born with adequate liver storage (440, 441), which can maintain infant B_{12} status in the first few months of life. However, as reported (Section 7.1), these stores become depleted, and breast milk vitamin B_{12} is then needed to sustain infant status across the entire duration of EBF. In situations of maternal deficiency, milk supplies are likely to be inadequate to match this demand, especially if the initial endowment to the infant's liver stores are low, as a consequence of maternal prenatal deficiency. This emphasises the importance of ensuring adequate maternal nutritional status both pre- and postnatally.

To ensure adequate postnatal supply of micronutrients to the infant, it has been suggested that indirect supplementation via the mother may be preferable over direct supplementation to the infant. In a recent RCT of iodine supplementation in Morocco, maternal postpartum supplementation was shown to be superior in improving infant iodine status and postpartum growth, compared to direct supplementation to the infant (227). The authors discussed that this reflect the more natural route of ensuring optimal intake of iodine to infants, with breastfeeding providing iodine in a constant and regulated supply. In addition to optimising infant status,

indirect supplementation via the mother would also offer benefit to maternal iodine status and encourage the maintenance of EBF for longer durations.

In populations at high risk of micronutrient deficiency, improving maternal status both pre- and postnatally would help improve breast milk micronutrient status, and offer short and longer-term benefit to both mother and infant.

9.3 Strengths and limitations

The research presented in this thesis used data and samples from a completed randomised trial in rural Gambia (the ENID Trial) (305). An important strength of ENID to meet the objectives of this thesis was that women and their infants were followed longitudinally from early pregnancy, through lactation and until the children reached two years of age. This allowed a direct investigation of the effect of supplementation on maternal status, breast milk concentrations and infant status, across two equally important periods for healthy growth and development, and in a population at high risk of nutritional deficiency. The data presented in this thesis enhance existing knowledge, especially since much of the existing evidence comes from cross-sectional studies, or from studies that focus on discrete periods during pregnancy or lactation.

The use of a randomised, controlled design in the ENID trial is an important strength for the hypotheses tested, as this design is considered the gold standard of epidemiological studies, and provides strong evidence of causal relationships (519). Participants in ENID were successfully randomised to each trial arm, which along with the successful blinding and allocation concealment, minimised selection bias and confounding (519). This implies that the relationships analysed in the last three Chapters of this thesis, between prenatal supplementation and maternal status, breast milk composition and infant status, can be considered causal. However, a limitation is that the research objectives included in this thesis are secondary, as the initial study was not designed with these specific objectives in mind.

The infant growth data presented in Chapter 5 were analysed longitudinally using complex multilevel models, which is the optimal analytical method to apply when analysing longitudinal growth data (71). However, in this analysis, infant feeding status was used as the exposure variable instead of the trial design, and so the results obtained must be considered as observational. Several limitations and biases are linked to an observational study design, with confounding as one of the greatest (519). Breastfeeding status and growth outcomes are

especially prone to be confounded by social pattern, and even though the results in this thesis were adjusted for socio economic variables (i.e. maternal education), the results may still be affected by residual confounding.

Reverse causality is another important bias that observational studies cannot avoid (519). Growth and breastfeeding are dynamic processes influencing each other, and with an observational study design it is not possible to determine the temporal sequence. In the results presented in this thesis, evidence was found to suggest that poor infant growth determined cessation of EBF, rather than the reverse. However, and because of the ethical implications of randomising infants to either breastfeeding or formula feeding, it is unlikely that future work will be able to fully address this question.

The sample size included in this thesis was large, with power to detect reasonable differences in breast milk composition between the two study arms (Section 4.2), and in infant growth according to breastfeeding practices. In the analysis of breastfeeding practices and growth, the high proportion of infants who remained exclusively breastfed to six months enabled a robust analysis within the limits of the sample size available. Few other settings would have enough women maintaining EBF to six months to allow this analysis within a cohort of this size, as the sample size in the EBF group would be too small for any meaningful analysis.

A limitation of embedding these research questions within ENID is that the intervention of focus was a multiple micronutrient, and not a trial of the individual micronutrients studied (iodine, thiamin, riboflavin, vitamin B_6 or vitamin B_{12}). The results obtained could therefore be influenced by known or unknown interactions between micronutrients. For instance, it is known that, besides iodine, selenium also plays an essential role in thyroid hormone metabolism (520). The extent of any such interactions are not possible to discern. Further, the use of a multiple micronutrient precluded the study of any potential effects of, for example, iodine or vitamin B_{12} on infant functional outcomes, such as growth or cognitive development.

The ENID trial was not set up *a priori* to investigate dose effects of maternal micronutrients given during pregnancy in this rural Gambian setting. The UNIMMAP supplementation, which is 1xRDA of 15 micronutrients, was developed and formulated in 1999 by UNICEF, WHO and United Nations University for use in pregnancy in populations at risk of multiple micronutrient deficiencies, and to potentially replace the recommended iron-folic acid supplement (306). In ENID it was decided to supplement with 2xRDA, as this dose increased birth weight by 177 g compared to 1xRDA in infants born to women from Guinea-Bissau (307). It is therefore of note

that, even at intakes of 2xRDA in ENID, this level of supplementation was not sufficient to increase maternal status to acceptable levels for almost all micronutrients investigated. For example, maternal urinary iodine concentration (UIC) in late pregnancy in the MMN-supplemented group was below the UIC cut-off for adequate iodine intake. This could reflect that, either, the dose given was too low to ensure sufficiency in these women, or that the intervention period was too short to re-build depleted iodine stores. It is possible that the use of a standard formulation of multiple micronutrients, instead of a supplement tailored to meet the needs of the individual micronutrients investigated in this thesis, impacted on the results obtained. Future studies should incorporate a specific focus on dose effects when investigating breast milk micronutrients.

Another potential limitation relates to the method of milk collection. Within ENID, samples were not obtained from a full breast expression, and collection was not standardised according to the infant's last feed, which is advised when investigating breast milk composition (88). Instead, a hand-expressed 5 ml sample was obtained from each breast and the collection was only standardised to a specific time of day (between 9 and 11 am). Thus, the breast milk samples analysed in this thesis most likely had a higher content of foremilk than hindmilk, which is not representative of usual milk content. This could have implications for the measured breast milk iodine concentrations (226), however this would unlikely have had any impact on the overall trial results.

One final limitation was that breast milk volume was not collected in ENID. It is well known that there is a large between-infant variation in daily breast milk intakes, for example ranging from 450 to 1300 ml/day in healthy infants across the first six months of EBF (99). However, the impact of this variation on breast milk concentration of micronutrients is not fully understood. Adding this information into the current analysis would have strengthened the data obtained, and increased our understanding of the relationship between milk volume and breast milk micronutrient concentrations.

9.4 Future work

The research in this thesis has identified that there is limited valid data available on breast milk micronutrient composition during the first six months of lactation when breast milk is recommended as the infant's only source of nutrients. This is especially true for populations from resource-constrained settings, where many women are at risk from micronutrient deficiencies. More research in this area would help ensure interventions were appropriately developed and targeted to ensure infants can be exclusively breastfed to six months, without placing them at risk of micronutrient deficiency.

A more robust evidence base on breast milk micronutrient concentrations from well-nourished populations would further enable the development of infant EAR recommendations where this information is currently lacking. For the B-vitamins investigated in this thesis, only an AI is available. The AIs are based on limited data from studies with small sample sizes. Invalid quantification methods were in most occasions used, and maternal intake or status of the micronutrient were not considered. A longitudinal study with the objective to develop valid reference values for micronutrient concentrations in breast milk and use these values to develop EAR recommendations for infants between 0-6 months, is urgently needed. This research need will be met by a recently initiated longitudinal cohort study (ttrial registration number: NCT03254329) (521), The MILW (Mothers, Infants and Lactation Quality) study will follow healthy unsupplemented mothers from four countries (Denmark, Brazil, Bangladesh and The Gambia) with adequate micronutrient status across the first 8.5 months of lactation. The overall objective of this study is to develop reference values for breast milk micronutrient concentrations across lactation and relate it to maternal and infant nutritional status and infant functional outcomes. These data will allow a reference against which low breast milk micronutrient concentrations can be identified. This will make it possible to intervene with postnatal supplementation where needed and thereby prevent both maternal and infant micronutrient deficiencies during the lactation period.

The data presented from the ENID trial in this thesis represents one of the few examples of a study specifically investigating the effect of a prenatal micronutrient supplement on breast milk micronutrient composition during the first six months of lactation. More data exists from trials of postnatal supplementation, or trials examining the combined effect of pre- and postnatal supplementation. However, there is a lack of data from studies conducted in resource-poor countries. Work to extend the results obtained in this thesis should focus on dose response trials,

examining interventions during both pregnancy and lactation with the aim of establishing the optimal dose required to ensure adequate breast milk micronutrient status in micronutrient deficient populations, such as rural Gambia.

Such a study could further highlight how suitable the universal UNIMMAP supplement is with its predefined content and dose, compared to a context-specific supplement, where the content and dose of the supplement are based on specific needs of the population. A universal multiple micronutrient supplement, such as UNIMMAP, is unlikely to be the most effective solution, specifically as a woman's pre-pregnancy micronutrient status and dietary intake prior to and during pregnancy most likely plays a significant role in how she responds to different doses of a micronutrient supplement.

There is a need for pregnancy and lactation specific cut-offs for deficiency, especially of the B-vitamins, where more work is urgently needed. The cut-off for maternal plasma vitamin B_{12} and B_6 status is currently the same for women of reproductive age, pregnant and lactating women, even though large physiological changes occur during these periods. The cut-offs for infant deficiency is also poor for all the micronutrients in focus in this thesis. There is, for instance, no international endorsed cut-off or reference values for infant serum thyroglobulin concentration, which needs to be both age-appropriate and method-specific (371). The same is the case for infant plasma vitamin B_{12} concentrations where currently adult cut-offs are used for infants, which is not recommended (434).

Finally, further research should focus specifically on the relationship between breast milk micronutrient status and infant growth and development. There is a possibility that poor maternal nutritional status and intake of important micronutrients during lactation play a role in infant growth faltering in The Gambia, however it is unlikely the only cause. Many trials continue to provide conflicting conclusions about the effectiveness of different interventions to improve growth in resource-poor settings and, if any positive effect is seen, the magnitude continues to be moderate. Undoubtedly, more attention needs to be paid to the underlying biological factors and mechanisms that may be involved in growth faltering.

9.5 Conclusion

In conclusion, this thesis expands current knowledge around maternal nutrition during pregnancy and lactation, breast milk micronutrients and infant nutritional status and growth in a rural Gambian population where food availability and nutritional status are poor. Infant growth faltered in this population despite high EBF rates, and EBF to six month did not impact infant weight, length or z-scores in the first two years of life. Maternal nutritional status and dietary intake influenced several breast milk micronutrients, which in a resource-poor setting, such as The Gambia, is likely to have detrimental implications for infant health. A prenatal multiple micronutrient supplementation improved maternal micronutrient status during pregnancy and infant status in the immediate neonatal period. However, a prenatal supplement was not sufficient in increasing breast milk micronutrient concentrations to appropriate levels across the period of EBF, suggesting the need for additional maternal supplementation during lactation. The content and dose of any future recommended pre- and postnatal supplement for use in micronutrient deficient contexts requires further study. Future supplementation needs to be population specific, such that maternal status and food availability are taken into consideration. This thesis further supports the continued promotion of EBF for six months in rural Gambia, to benefit the health of both mothers and children.

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Appendices

Infant Weekly Morbidity Form

Baby's WKNO:	#Name?	**Infant ID:**	#Name?		
Name:	#Name?	**Date of Birth:**	#Name?	**Sex:**	#Name?
Mother:	#Name?	**Father:**	#Name?		
Village:	#Name?	**Compound:**	#Name?		

Age (Weeks): **#Name?** Form Number:

Date of Visit: |__|__|/|__|__|/|__|__|__|__| Visited by: _____

Has it been decided to withdraw the baby from study? Yes ☐ No ☐

If yes, what is the reason? ☐ Mother/Guardian Decision ☐ Moved Away ☐ Died ☐ Other: _____

Date of Withdrawal: |__|__|/|__|__|/|__|__|__|__|

Infant Morbidity

Has the infant been sick in the past 7 days? Yes ☐ No ☐

If yes, please complete the table below:

	YES	NO	Number of Days [0 - 7]	Health facility visited and/or Treatment taken (details)
Diarrhoea (>2 loose stools/day)				
Vomiting (not associated with feeding)				
Cough				
Rapid breathing				
Fever				
Other, Specify).....				

Comments:

IF THE BABY IS CURRENTLY SICK AND REQUIRES URGENT MEDICAL ATTENTION, PLEASE INFORM THE STUDY MIDWIFE OR TREKKING NURSE ON CALL.

Breastfeeding questionnaire

1. Are you currently breastfeeding your infant? Yes ☐ No ☐

2. In the past 7 days, have you given your infant anything other than breast milk? Yes ☐ No ☐

If no, the questions are completed. If yes, please proceed to Question 3.

3. What other foods/drinks have been given, in the past 7 days

Drinks		**Comments**
Water	Once ☐ > Once ☐ Most days ☐ Never ☐	
Glucose water	Once ☐ > Once ☐ Most days ☐ Never ☐	
Cows milk	Once ☐ > Once ☐ Most days ☐ Never ☐	
Powdered milk	Once ☐ > Once ☐ Most days ☐ Never ☐	
Tinned milk	Once ☐ > Once ☐ Most days ☐ Never ☐	
Tea	Once ☐ > Once ☐ Most days ☐ Never ☐	
Other liquids	Once ☐ > Once ☐ Most days ☐ Never ☐	
Semi-solids		
Mono/Jidiyo (circle type)		
Sanyo/mani/kinti/findi/tubanyo/ dukala/Tiakere churo/sunno	Once ☐ > Once ☐ Most days ☐ Never ☐	
Other (details:	Once ☐ > Once ☐ Most days ☐ Never ☐	
.....	Once ☐ > Once ☐ Most days ☐ Never ☐	
.....	Once ☐ > Once ☐ Most days ☐ Never ☐	
.....	Once ☐ > Once ☐ Most days ☐ Never ☐	
Pre-prepared weaning foods (e.g. Cerelac, Nuturelle etc)	Once ☐ > Once ☐ Most days ☐ Never ☐	
Solids (mother to list)	Once ☐ > Once ☐ Most days ☐ Never ☐	
.....	Once ☐ > Once ☐ Most days ☐ Never ☐	
.....	Once ☐ > Once ☐ Most days ☐ Never ☐	
.....	Once ☐ > Once ☐ Most days ☐ Never ☐	

Expected date of submission: 25/04/2013

Appendix 2. Model equations

Model equation for Berkey reed growth model:

$$\text{Weight}_{ij}/\text{length}_{ij} = \beta_{0j} + \beta_{1j}\text{age}_{ij} + \beta_{2j}\text{lnage}_{ij} + \beta_3\text{invage}_{ij} + \beta_4\text{sex}_j + \beta_5\text{sex}_j*\text{age}_{ij} + \beta_6\text{sex}_j*\text{lnage}_{ij} + \beta_7\text{sex}_j*\text{invage}_{ij} + \beta_8\text{FP}_j + \beta_9\text{FP}_j*\text{ age}_{ij} + \beta_{10}\text{FP}_j*\text{lnage}_{ij} + \beta_{11}\text{FP}_j*\text{invage}_{ij} + \beta_{12}\text{sin1}_{ij} + \beta_{13}\text{cos1}_{ij} + \beta_{14}\text{sin2}_{ij} + \beta_{15}\text{cos2}_{ij} + \beta_{16}\text{sin1}_{ij}*\text{age}_{ij} + \beta_{17}\text{cos1}_{ij}*\text{age}_{ij} + \beta_{18}\text{sin2}_{ij}*\text{age}_{ij} + \beta_{19}\text{cos2}_{ij}*\text{age}_{ij} + \beta_{20}\text{m\_height}_j + \beta_{21}\text{m\_bmi}_j + \beta_{22}\text{GA}_j + \beta_{23}\text{parity}_j + \beta_{24}\text{village}_j + \beta_{25}\text{morbidity}_{ij} + e_{ij}$$

$$\beta_{0j} = \beta_0 + \mu_{0j}$$
$$\beta_{1j} = \beta_1 + \mu_{1j}$$
$$\beta_{2j} = \beta_2 + \mu_{2j}$$

$$\begin{pmatrix}\mu_{0j}\\ \mu_{1j}\\ \mu_{2j}\end{pmatrix} \sim N\left\{\begin{pmatrix}0\\0\\0\end{pmatrix}, \begin{pmatrix}\sigma^2_{\mu 0} & . & .\\ \sigma_{\mu 01} & \sigma^2_{\mu 1} & .\\ \sigma_{\mu 02} & \sigma_{\mu 12} & \sigma^2_{\mu 2}\end{pmatrix}\right\} \qquad e_{ij} \sim N(0, \sigma^2_e)$$

Model equation for restricted cubic spline (4 knots):

$$\text{Z-score}_{ij} = \beta_{0j} + \beta_{1j}\text{rs1}_{ij} + \beta_{2j}\text{rs2}_{ij} + \beta_3\text{rs3}_{ij} + \beta_4\text{sex}_j + \beta_5\text{sex}_j*\text{rs1}_{ij} + \beta_6\text{sex}_j*\text{rs2}_{ij} + \beta_7\text{sex}_j*\text{rs3}_{ij} + \beta_8\text{FP}_j + \beta_9\text{FP}_j*\text{rs1}_{ij} + \beta_{10}\text{FP}_j*\text{rs2}_{ij} + \beta_{11}\text{FP}_j*\text{rs3}_{ij} + \beta_{12}\text{sin1}_{ij} + \beta_{13}\text{cos1}_{ij} + \beta_{14}\text{sin2}_{ij} + \beta_{15}\text{cos2}_{ij} + \beta_{16}\text{m\_height}_j + \beta_{17}\text{m\_bmi}_j + \beta_{18}\text{GA}_j + \beta_{19}\text{parity}_j + \beta_{20}\text{village}_j + \beta_{21}\text{morbidity}_{ij} + e_{ij}$$

$$\beta_{0j} = \beta_0 + \mu_{0j}$$
$$\beta_{1j} = \beta_1 + \mu_{1j}$$
$$\beta_{2j} = \beta_2 + \mu_{2j}$$

$$\begin{pmatrix}\mu_{0j}\\ \mu_{1j}\\ \mu_{2j}\end{pmatrix} \sim N\left\{\begin{pmatrix}0\\0\\0\end{pmatrix}, \begin{pmatrix}\sigma^2_{\mu 0} & . & .\\ \sigma_{\mu 01} & \sigma^2_{\mu 1} & .\\ \sigma_{\mu 02} & \sigma_{\mu 12} & \sigma^2_{\mu 2}\end{pmatrix}\right\} \qquad e_{ij} \sim N(0, \sigma^2_e)$$

lnage= ln(age+1)
invage=(1/(age+1)) – 1
rs1, rs2, rs3 = restricted cubic spline functions produced in stata with the command: *mkspline rs = age , cubic nknots(4) displayknots.* (4 knots; 0.008, 0.389, 0.797 and 1.993 years)
FP = feeding practice (EBF-6 or nEBF-6)
sin1= sin(1*2*π*toy)
cos1= cos(1*2*π*toy)
sin2= sin(2*2*π*toy)
cos2= cos(2*2*π*toy), toy= time of year when infant anthropometric measurements were taken.
m_height = maternal height
m_bmi= maternal BMI
GA = gestational age at birth
morbidity = infant morbidity (experienced in the past week)
Z-score_{ij} is the z-score of the infant j at occasion i. β_{0j} is the intercept and β_1j and β_2j are regression coefficients. β_0j, β_1j and β_2j have mixed effects that comprise a sample average fixed effect (β) and a subject specific random effect (u_j), and e_{ij} is the residual error. In the two models above,

the fixed effects together describe the sample average curve and the random effects are individual departures from the intercept and slope of that curve. The formula therefore describes the growth curve of every infant. Because each model parameter takes different values for each infant, each parameter demonstrates variance and there is covariance between parameters. The underlying variance-covariance structure of these data is described by the matrix above. Where, $\sigma_{\mu 0}^2$ $\sigma_{\mu 1}^2$ $\sigma_{\mu 2}^2$ are the variances of the three random effects and $\sigma_{\mu 01}$ $\sigma_{\mu 12}$ $\sigma_{\mu 02}$ are the co-variances between the random effects. μ_{0j} μ_{1j} μ_{2j} are assumed to follow a multivariate normal distribution, the e_{ij} to be normally distributed and μ_{0j} μ_{1j} μ_{2j} are assumed to be independent of e_{ij}.

Appendix 3. Linear regression of WLZ and EBF to six months

Table 1. Crude and adjusted regressions of infant WLZ at birth and EBF to six months

Association	**coefficient**	**p-value**
Crude association between infant birth WLZ and EBF to six months	-0.1926	0.02
Association adjusted for maternal age	-0.1956	0.02
Association adjusted for maternal weight at baseline	-0.1977	0.02
Association adjusted for maternal height	-0.1847	0.02
Association adjusted for maternal BMI	-0.1914	0.02
Association adjusted for maternal education	-0.1969	0.02
Association adjusted for maternal prenatal supplementation	-0.1991	0.02
Association adjusted for parity	-0.1971	0.02
Association adjusted for gestational age at birth	-0.1853	0.03
Association adjusted for infant sex	-0.1931	0.02
Association adjusted for infant season of birth	-0.1958	0.02
Association adjusted for infant diarrhoea incidence	-0.1928	0.02
Association adjusted for infant morbidity incidence	-0.1902	0.02

WLZ, weight-for-length z-score; BMI, body mass index; EBF, exclusive breastfeeding.

Appendix 4. Abstracts, presentations and awards

Abstracts and presentations

March 2015:	Oral presentation at the MRC International Nutrition Group (ING) annual meeting in Keneba, The Gambia. Presentation: *How does maternal nutritional status and dietary intake influence breast milk concentration?*
November 2015:	Abstract and oral presentation at the 9th World Congress on Developmental Origins of Health and Disease (DOHaD) in Cape Town, South Africa. Abstract title: *The impact of exclusive breastfeeding on growth in rural Gambian infants: Data from the ENID trial*.
March 2016:	Abstract and poster presentation at the 18th International Society for Research in Human Milk and Lactation (ISRHML) conference in Stellenbosch, South Africa. Abstract title: *Benefits and determinants of exclusive breastfeeding in a rural West African setting: Data from the ENID trial.*
April 2016:	Oral presentation at the MRC ING annual meeting in Keneba, The Gambia. Presentation: *Multiple micronutrient supplementation in rural Gambian women: impact on micronutrient breast milk concentration and nutritional status.*
October 2016:	Abstract and oral presentation at the Micronutrient Forum Global Conference in Cancun, Mexico (*invited speaker*). Abstract title: *Effects of maternal micronutrient supplementation on breast milk micronutrients in the Gambia – Focus on iodine, B_1, B_2, B_6 and B_{12}.*
October 2017:	Abstract and poster presentation at the IUNS-ICN 21st International Congress of Nutrition in Buenos Aires, Argentina. Abstract title: *Maternal, breast milk and infant vitamin B12 status in rural Gambia.*

Awards

November 2015:	Society for the Study of Human Biology Travel Award (£750)
March 2016:	ISRHML Trainee Travel Award (US$1000)
August 2016:	MRC Doctoral Training Partnership Flexible Supplement. Skills and Partnership Training Funding 2016 (£5000). *Went to ETH Zurich in Switzerland for two months (August and September 2016) to analyse ENID infant and maternal thyroglobulin concentrations with guidance from Dr Maria Andersson and Professor Michael Zimmermann*