THE IMPACT OF DIETARY PROTEIN IN COMPLEMENTARY FOODS ON INFANT GROWTH AND IRON STATUS IN A POPULATION FACING DOUBLE BURDEN OF MALNUTRITION

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A thesis submitted for the degree of Doctor of Philosophy

UCL

DECLARATION

I, Kulnipa Kittisakmontri confirm that the work presented in this thesis in my own. Where information has been derived from other sources, I confirm that this has been indicated in this thesis.

Signature:

Date: 22-11-2021

Acknowledgement

“Studying for a PhD reminds me of when I decided to run my first marathon in Amsterdam three years ago. Whilst training myself day-in day-out I had lots of injuries, breaks in training and doubts along the way, but with the support of those close to me, I could pull myself together again and again. Eventually, when I crossed the finishing line, I couldn’t avoid tears of pride and happiness, as all the hard work was over and I was a different person than the one who started this journey”

Let’s get started on my journey

Before the race, I would like to pay tribute to the generosity of the Anandhamahidol Foundation under the Royal Patronage of Her Royal Highness Princess Maha Chakri Sirindhorn that has provided me with a scholarship since 2017. Without this support, I would not have come this far and my journey would have been more difficult and stressful.

0 – 10 km: Running the first 10 kilometres was quite daunting and made me deflated every time I realised how far I was from the finish line. Likewise, the first year of my degree was scary as it was the first time I had to study abroad, using a foreign language and living in an unfamiliar environment. However, with kind support and wonderful guidance from my supervisors, Professor Mary and Dr Julie, I was encouraged and started to believe in my ability and what I could achieve by starting with small steps, so I kept going without thinking too much of the long distance ahead of me.

11 – 20 km: Moving to the next 10 kilometres was the perfect time in that I could run better as all my muscles were warmed up. However, I had to be alert to the directions along the way as at this stage, if I took the wrong direction, it might take too long to come back, or my race might be ended by exceeding the cut-off time. This stage was like the second year of my degree when I went

back to Thailand for the field work. There were many people who pointed me in the right direction at this right time. Professor Jonathan was one of them, helping me with the isotope technique and suggesting the great idea of using conditional growth. There were nurses and my colleagues at Chiang Mai University hospital and Chomthong hospital who provided me full support. Special thanks go to all the health professionals at the well-baby clinic at the Health Promoting hospital where I was given not only tremendous help, but also received love, lunch and laughs every time I did my data collection there. Finally, I am grateful to all the infants and their families who took part in this study. Without them and their cooperation, my data collection would not have been so successful.

21 – 30 km: After running energetically through the half-way point, this was the time when I was struggling and had to deal with stomach cramps. I had to stop running but I did not stop moving; I kept walking until I felt better and could start running again. This period reminds me of the third year of my study when I had to enter huge amounts of research data and felt overwhelmed about how to manage them. Additionally, some laboratory analyses could not be done as I had expected. That was the most painful and slowest part of my study. Fortunately, with helpful advice from my supervisors I worked out how to narrow down my research data to a manageable amount. Furthermore, my colleagues involved in the laboratory analyses also gave me their full support and tried to help me out. These credits should go to my colleagues at the UCL Great Ormond Street Institute of Child Health, Simon and Adriana, my Thai colleagues at the Faculty of Medicine, Ramathibodi Hospital, Mahidol University as well as Professor Susan and Dr Lucas from Cambridge University. Their support reduced my anxiety, so I could begin to run again toward the finish line.

31 – 42.195 km: Before the race, I had really hoped that I would finish my first marathon in approximately 4 hours. However, things turned out differently as I developed chronic pain in my right knee and foot. This pain gradually accumulated with every kilometre. It was very disappointing and emotional to realise that I would not finish this race in my time goal, but when I saw

someone laying down in the road receiving intravenous fluids just a few kilometres away from the finish line, I realised how lucky I was, at least to still have the chance to finish the race. The final distance was similar to the fourth year of my study when the COVID-19 pandemic hit the global community very hard. Its impact was beyond anyone's expectation. Nevertheless, with lots of support and kindness from the people around me, I finally gathered my energy and completed the thesis just before 4.5 years - which is surprisingly similar to my finishing time at the Amsterdam marathon (4.42 hours).

Throughout the race, I am very grateful for endless help and support from Professor Mary Fewtrell, my primary supervisor who is not only an excellent supervisor in academic life but also my mentor in non-academic life. As a marathon runner, she gave me lots of tips and was the first person who knew my official marathon time. I also feel thankful for my subsidiary supervisor, Dr. Julie Lanigan, who helped and guided me through many difficult times with her expertise and kindness. Moreover, my life as a PhD student would not have been complete without my lovely friends, Sarah, Amna and Jinyue who offered their help and support throughout my time in London. More importantly, I must thank my family in Thailand especially my mother, my father, my younger sister and brother. Whenever I felt down or upset, they were always there for me. Their unconditional love and tremendous support were the most powerful tool encouraging me to the finish line of this PhD journey. Finally, I would like to thank my husband, Anon, who had to balance his time doing his own PhD project with taking very good care of me. His understanding and endless love gave me strength whenever I needed it.

I found only one thing that is totally different between running a marathon and studying for a PhD. For marathon, I can finish more races as long as I keep running, but for PhD degree, this will be the first and only one that I am willing to complete.

Abstract

Dietary protein is a key macronutrient for infant growth, especially during complementary feeding (CF). Inadequate intake contributes to undernutrition. However, evidence from high-income countries suggests high protein intake, especially of animal-based protein (ABP), may increase obesity risk. Determining optimal protein intake and sources during CF is thus challenging, particularly in lower-income settings where the double burden malnutrition (DBM; co-existence of under- and overnutrition) is common; and considering that animal-source foods are a good source of iron during this period of high requirements. This thesis aimed to investigate how protein quantity and source during CF influence infant growth and iron status in a setting experiencing the DBM, and to explore potential mechanisms. A multi-centre, prospective cohort was conducted in 145 healthy-term infants in Chiang Mai, Thailand. Dietary intakes and anthropometric measurements were collected at 6, 9 and 12M. At 12M, blood samples were analysed for iron status, serum IGF-1, IGFBP-3 and plasma branched-chain amino acids (BCAA). Protein consumption exceeded recommendations at 9-12M. Both dairy and non-dairy ABP were positively associated with weight-for-age and weight-for-length z-scores after adjusting for type of milk-feed and non-protein calories, with no effect on linear growth. Dairy ABP showed a stronger association than non-dairy ABP, consistent with its higher association with IGF-1, IGFBP-3 and BCAA. Protein intake did not differ significantly between iron-sufficient versus iron-deficient (ID) infants, but consumption of ≥ 3 tablespoons of liver/week was associated with a nearly 80% reduction in ID. The findings highlight differential effects of protein source on infant growth and iron status. Unlike dairy protein, non-diary ABP may promote growth without greatly increasing IGF-1, IGFBP-3 and BCAA which are implicated in increased obesity risk whilst also representing a good source of iron. The findings could help develop interventions for testing in randomised trials to establish causal relationships and mechanisms and contribute to improving CF protein recommendations.

Impact statement

The first thousand days of life starting from conception until the second birthday is a critical period when several metabolic pathways can be programmed. Malnutrition - whether undernutrition or overnutrition - during this period can result in long-term effects on later health, so studies focusing on improving early-life nutrition have the potential to bring great benefit to individuals and populations, including reducing the risk of obesity, diabetes, cardiovascular diseases and cancers. However, there are many unanswered questions.

I investigated the impact of dietary protein during the weaning (complementary feeding) period on infant growth and iron status. For many years, researchers studying this topic have generally focussed on either undernutrition or overnutrition. In high-income settings, most studies indicate that “too much” protein in early life is linked to overweight/obesity in older children and adults. By contrast, in resource-limited countries, researchers generally examine the effect of “too little” protein, both in terms of quantity and quality, on undernutrition. There has been little research to define the ideal amount and type of protein for infants in situations where both under and overnutrition coexist – so-called double burden malnutrition. I therefore investigated this issue in a population at risk of double-burden malnutrition in Northern Thailand.

The key results from my study indicate that not only the amount, but also the type of protein plays an important role in infant growth. Higher consumption of protein from infant formula and cow’s milk was related to increased weight without affecting length gain. Protein from other animal sources (for example, eggs or meat) showed a smaller effect on weight gain, while there was no impact of plant-based protein. I also investigated underlying mechanisms linking dietary protein and infant growth. A high percentage of energy provided by dietary protein (%PE) from formula and cow’s milk was related to higher

plasma levels of branched-chain amino acids (BCAA) which stimulate secretion of growth factors, resulting in rapid weight gain. Other animal-based proteins had less effect on this pathway while plant-based protein had no effect found. Interestingly, higher %PE from breast milk was related to lower plasma BCAA, clearly supporting the continuation of breastfeeding beyond 6 months. My results also suggest that avoiding cow's milk before 12 months and providing a tablespoon of liver three times per week could optimise both growth and iron status.

To my knowledge, this is the first evidence from a middle-income country confirming the association between high protein intake in early life and rapid weight gain already reported in high-income settings. This study underscores distinctive effects of different protein sources on growth and provides supporting data on underlying mechanism. These key findings not only contribute novel information to this field of research but can also be applied to public health programmes, especially in countries where double burden malnutrition is prevalent.

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List of Definitions

1) **Stunting**

   Stunting is defined as LAZ < 2 SD below the median of the WHO growth standards for children aged under 5 years[1]

2) **Wasting**

   Wasting is defined as WLZ < 2 SD below the median of the WHO growth standards for children aged under 5 years[1]

3) **Underweight**

   Underweight is defined as WAZ < 2 SD below the median of the WHO growth standards for children aged under 5 years[1]

4) **Overweight**

   Overweight is defined as WLZ > 2 SD above the median of the WHO growth standards for children aged under 5 years[2]

5) **Obesity**

   Obesity is defined as WLZ > 3 SD above the median of the WHO growth standards for children aged under 5 years[2]

6) **Complementary feeding**

   Complementary feeding is all solid and liquids foods other than breast milk or infant and follow-on formula[3, 4]

7) **Exclusive breastfeeding**

   Exclusive breastfeeding means the infant receives only breast milk without anything else except for water and necessary medication

8) Predominant breastfeeding

Predominant breastfeeding means that breast milk is more than 50% of daily milk intake after 6 months of age

9) Iron deficiency

Iron deficiency is defined by at least one of the following serum ferritin (if erythrocyte sedimentation rate, ESR ≤ 10 mm/h) or serum ferritin less than 30 μg/ L (if ESR > 10 mm/h) or serum transferrin saturation less than 16%[5]

10) Iron deficiency anaemia

Iron deficiency anaemia is defined as iron deficiency combined with haemoglobin less than 11.0 g/ dL[5]

11) Main caregiver/ Primary caregiver

Main caregiver/ primary caregiver is defined as the person who spends the most time looking after the infant on daily basis

List of Abbreviations

A

ABP	Animal-based protein
ANOVA	Analysis of variance
ASEAN	The Association of South-East Asian Nations
ASFs	Animal-source foods

B

BCAA	Branched chain amino acids
BF	Breastfeeding
BM	Breast milk
BMI	Body mass index
BMIZ	Body mass index-for-age z-score

C

CBC	Complete blood count
CF	Complementary feeding
CHOP	The European Childhood Obesity Project
CI	Confidence interval
CMU	Chiang Mai University Hospital
CTH	Chomthong Hospital

D

DAG	Directed acyclic graph
DBM	Double burden of malnutrition
DCC	Delayed cord clamping
DRIs	Dietary Recommended Intakes

E

EAA	Essential amino acids
EBF	Exclusive breastfeeding
ESR	Erythrocyte sediment rate
EU	European

F

FAO	The Food Agriculture Organisation
FFQ	Food frequency questionnaire

G

GH	Growth hormone

H

HAZ	Height-for-age z-score
Hb	Haemoglobin
HP	High protein intake group
HPH	Health Promoting Hospital

I

ID	Iron deficiency
IDA	Iron deficiency anaemia
IGF-1	Insulin-like growth factor 1
IGFBP-3	Insulin-like growth factor binding protein 3
Ile	Isoleucine
INMUCAL	The Institute of Nutrition, Mahidol University Calculation
IOM	The Institute of Medicine (United States)
IQR	Interquartile range

K

KS	Kolmogorov-Smirnov test
Kw	Weighed Kappa

L

LAZ	Length-for-age z-score
Leu	Leucine
LMICs	Low- and middle-income countries
Ln	Natural log
LOA	Limit of agreement
LP	Low protein intake group

M

MICS	The Multiple Indicator Cluster Survey
MP	Median protein intake group
mTOR	Mammalian target of rapamycin

N

NEAA	Non-essential amino acids
NHES	The Thai National Health and Examination Survey
Non-BM	Non-breast milk
NSO	The Thai National Statistics Office

O

OR	Odds ratio

P

PBP	Plant-based protein
PEM	Protein energy malnutrition
PW	Protein weight ratio

R

r	Correlation coefficient
RCT	Randomised controlled trial

S

SD	Standard deviation
SDS	Standard deviation score
SEANUTS	The South-East Asia Nutrition Survey
SF	Serum ferritin
SI	Serum iron
SMILING	The Sustainable Micronutrient Interventions to Control Deficiencies and Improve Nutritional status and General health in Asia

T

TIBC	Total iron binding capacity
TSAT	Serum transferrin saturation

U

UN	The United Nations
USDA	The United States Department of Agriculture

V

Val	Valine

W

WAZ	Weight-for-age z-score
WHO	The World Health Organisation
WLZ	Weight-for-length z-score

Others

β	Regression coefficient
%CHO	Percentage of energy provided by dietary carbohydrate
%Fat	Percentage of energy provided by dietary fat
%PE	Percentage of energy provided by dietary protein
24-HR	24-hour food recall
3-DFR	3-day food record

Outputs from the research undertaken for this PhD

Data from my PhD research has contributed 3 published articles to date, 1 oral presentation at an international conference, 2 poster presentations at international conferences, 2 poster presentations at the UCL Great Ormond Street (GOS) Institute of Child Health (ICH) competition and 1 MSc dissertation which I co-supervised.

Published peer-reviewed papers

1) **Kittisakmontri K**, Fewtrell M, Roekworachai K, Phanpong C, Lanigan J. Complementary feeding: Attitudes, knowledge, and practices of urban families in northern Thailand. Nutr Diet 2019; 76(1): 57-66

2) **Kittisakmontri K**, Lanigan J, Wells JCK, Fewtrell M. The impact of dietary protein in complementary foods on infant growth and body composition in a population facing the double burden of malnutrition: Protocol for multicenter, prospective cohort study. JMIR Res Protoc 2020; 9(9): e18112

3) **Kittisakmontri K**, Lanigan J, Sangcakul A, Tim-Aroon T, Meemaew P, Wangaueattachon K, Fewtrell M. Comparison of 24-hour recall and 3-day food records during the complementary feeding period in Thai infants and evaluation of plasma amino acids as markers of protein intake. Nutrients 2021; 13(2): 653

Oral presentation

1) **Kittisakmontri K**, Lanigan J, Fewtrell M. Dietary Protein Intake during transitional period and its impact on infant growth in area facing double burden of childhood malnutrition. The 7th International Conference on Nutrition and Growth (virtual meeting) on 27-29 August 2020

Poster presentations

1) **Kittisakmontri K**, Fewtrell M, Roekworachai K, Phanpong C, Lanigan J. **Attitudes and current practices in complementary feeding of Thai urban families.** The 51st Annual Meeting of the European Society for Paediatric Gastroenterology, Hepatology and Nutrition. Geneva, Switzerland on May 9-12, 2018.

2) **Kittisakmontri K**, Lanigan J, Fewtrell M. Dietary protein intake during complementary feeding and its impact on infant growth in area facing double burden of childhood malnutrition. Poster competition at UCL GOS ICH open day 2019. (Awarded Specially Commended)

3) **Kittisakmontri K**, Lanigan J, Fewtrell M. Prevalence of iron deficiency in breastfed infants and how feeding practices can optimise their iron status. Poster competition at UCL GOS ICH open day 2020.

4) **Kittisakmontri K**, Lanigan J, Fewtrell M. Iron status of Thai infants during complementary feeding and dietary factors promoting a good outcome. The 6th World Congress of Paediatric Gastroenterology, Hepatology and Nutrition (virtual meeting) on 2-5 June 2021.

MSc dissertation (MSc Clinical and Public Health 2019/20)

1) Carolina Clavijo Bravo

A dissertation entitled “Adherence to national complementary feeding recommendations and its predictors among families of infants in Chiang Mai, Thailand: A secondary data analysis” (Awarded a distinction)

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Chapter 1

Introduction

In this first chapter of my thesis, I describe what inspired me to conduct my PhD project. As I had been working as a paediatrician and interested in the field of childhood nutrition for some time, I noticed that the prevalence of overweight and obesity in young children increased very rapidly while at the same time, I still gave a consult to many families who had toddlers who were underweight or had faltering growth. This paradoxical scenario was almost in equal measure during my practice. I wondered what was causing this phenomenon but did not find a convincing explanation. At that time, studies in Thai infants and young children mostly focused on either breastfeeding or micronutrient deficiencies while obesity was the most common research topic for older children and adults. Following discussions with my current supervisors and my colleagues in Thailand, I designed a cross-sectional study which gave some further ideas on issues related to the role of different dietary proteins during complementary feeding, contributing to the design of my prospective study.

1.1 Background

The double burden of malnutrition (DBM), the co-existence of under- and over-nutrition that can take place whether in the same populations, households or individuals[6], is an emerging public health concern in low- and middle-income countries (LMICs)[7]. A combination of under- and over-nutrition in early life may not only affect short-term growth and development but also has a significant impact on long-term health outcomes. A body of evidence demonstrates that the first thousand days of life, from conception to the second birthday, is a critical window for metabolic programming and several factors are involved in this process, particularly early life nutrition[8]. Therefore, encouraging healthy eating during this window of opportunity is considered as a promising way to

prevent later health problems such as obesity and obesity-related diseases. When focusing on a specific time frame, the transition from a solely liquid-based diet to solid food during the so-called complementary feeding (CF) period is considered as the most challenging. During this time infants still grow rapidly, and breast milk alone can no longer provide adequate amounts of energy, protein, and other essential nutrients to meet the infant's requirements. As a result, complementary foods have a vital role in promoting healthy growth and development, and have been suggested by the World Health Organisation (WHO)[9] as a targeted double-duty action to ameliorate the DBM.

In the context of complementary foods, dietary protein is one of the most important components. Protein is necessary to provide calories and essential amino acids (EAA) to promote somatic growth. In addition, protein-rich foods also contain many key micronutrients to fulfil nutritional gaps, for example, iron and zinc in red meat. According to existing evidence, protein restriction can lead to undernutrition ranging from severe forms such as kwashiorkor to mild forms of growth faltering, while over-consumption can contribute to overweight and obesity. Although the recommendations for total protein intake have existed for a very long time since 1957[10], there is currently insufficient data to make recommendations about what constitutes an appropriate proportion of different protein sources. Each source of dietary protein – animal-based (meat, dairy, egg, etc.) and plant-based (legumes, pulses, cereal, etc.) - has its distinctive amino acid composition as well as specifically dominant or limited micronutrients. Therefore, without consideration of protein quality, infants and young children may still be at risk of over- or undernutrition despite an adequate total protein intake.

Furthermore, until now, there is no recommended upper limit of daily protein intake for infants and young children in LMICs[11] even though the numbers of overweight/ obese children under five years of age in these populations is on the rise[12]. It should be noted that if high protein intake in early life may contribute to overweight/ obesity in western countries, this phenomenon can also happen elsewhere around the world. Particularly, in middle-income

countries where the dietary pattern has been westernised and the DBM is prevalent, research in this area would be helpful to optimise protein intake for those specific populations. The issue of “too much” protein in early life now needs more attention in LMICs.

Thailand is a middle-income country where the DBM is apparent[13, 14]. According to the latest National Health Examination Survey (NHES), the prevalence of stunting is still unacceptable whilst the rate of overweight and obesity in younger children is rising[15]. In addition, the recent nutritional survey also reported that Thai infants and young children consume a higher amount of dietary protein than recommended[16]. Unfortunately, similar to the situation in LMICs, the issue of high protein intake in early life is overlooked by Thai authorities and researchers. Reliance on the old studies and the longstanding concept that Thai infants and children still consume low quality protein and are less likely to receive animal source foods (ASFs) in their diets can lead to ineffective interventions or nutritional programmes. Therefore, contemporary data is essential, and we need to consider issues related to protein intake from every aspect.

The primary objectives of this research are therefore to investigate how the intake of different amounts and sources of dietary protein during the CF period influence the growth of Thai infants, and to investigate possible mechanisms underlying the effects of different protein sources (especially ASFs versus plant-based foods) on somatic growth. The research will specifically investigate whether, as suggested by some observational studies, dairy protein is more strongly associated with growth and adiposity than non-dairy animal-based protein (ABP) or plant-based protein (PBP). Moreover, the study will investigate potential mechanistic pathways through the growth hormone (GH)-Insulin-like growth factor 1 (IGF-1) and plasma amino acids. In addition to the primary outcomes, this study also investigates how various protein sources affect infant iron status, because iron deficiency (ID) and iron deficiency anaemia (IDA) are also the most common nutritional problem in this age group.

1.2 Overview of the thesis

The organisation of this thesis is as follows: **Chapter 2** provides comprehensive background information on the DBM, using Thailand as an example, and a literature review covering dietary protein in complementary foods, growth outcomes and mechanisms underlying their associations; issues requiring further research are also highlighted: **Chapter 3** reports the key findings from a cross-sectional study and highlights how the results contributed to the design of the main longitudinal study: **Chapter 4** presents the study hypotheses, research methodology and how I measured all outcomes for the main study; dietary assessment methods and the programme used to estimate nutrient intakes are also described in this chapter: **Chapter 5** provides information on statistical analyses including sample size calculation, and the statistical techniques used in this study: **Chapter 6** describes how I dealt with the dietary data gathered at three time points; agreement between two dietary assessing methods is also reported: **Chapter 7: Results 1** reports the demographic data of infants and their family characteristics; prevalence of the DBM at all levels including individual, household and population: **Chapter 7: Results 2** shows descriptive results including feeding practices and nutrient intakes during the CF period: **Chapter 7: Results 3** reports the main outcomes of the study starting by comparing the protein intake of Thai infants with the national recommendations; common sources of dietary protein during CF; the effect of different amounts of protein intake on growth outcomes; and the effect of different protein sources on growth outcomes: **Chapter 7: Results 4** focuses on associations between dietary protein and iron intake and iron status of Thai infants; identifies dietary factors promoting normal iron status; and answers the question “do infants with normal iron status eat too much protein?”: **Chapter 7: Results 5** shows the associations between protein intake and blood biomarkers including GH, IGF-1, Insulin-like growth factor binding protein 3 (IGFBP-3) and plasma amino acids: Finally, **Chapter 8** summarises all key findings from Chapter 7, Results 1-5; discusses strengths and weakness of the research; and identifies the research implications for public health policy, clinical practices and future research.

Chapter 2

Literature review

This chapter is divided into 7 parts; 1) Addressing the DBM in Thailand; 2) Describing differences in dietary patterns and nutrient intakes between higher- and lower-income countries focusing on CF; 3) Describing differences between animal- and plant-based proteins; 4) Discussing how dietary protein may relate to the DBM; 5) Reviewing evidence for associations between dietary protein and growth and body composition of infants and children; 6) Reviewing evidence supporting an association between dietary protein, plasma amino acids and the GH-IGF axis. 7) Reviewing evidence on the role of epigenetics via microRNAs on growth mechanisms.

2.1 The double burden of malnutrition in LMICs, using the current situation in Thailand as an example

In 2016, the global prevalence of stunting in children under five years of age slightly decreased (21.7%; from 198 to 155 million) while the prevalence of overweight more rapidly increased (36.7%; from 30 to 41 million) compared to 2000[17]. Interestingly, both extremes of malnutrition can take place in the same population, in the same household or even in the same individuals, so-called stuntingoverweight [6, 18]. Among the countries where the DBM is prevalent, Thailand may be a good example because, although the prevalence of acute severe protein energy malnutrition (PEM) and some micronutrient deficiencies (i.e., vitamin A and iodine deficiency) have been greatly improved, the prevalence of stunting and underweight has changed little since the 1990s while ID/ IDA is still a major public health problem[13]. In addition, overweight and obesity is constantly increasing in Thai children especially in early childhood[14].

Data from Thai infants and children aged less than two years showed that the prevalence of stunting gradually accumulated by 6%, 6.9%, 9.5%, 14.6% and 16.6% at birth, 6, 12, 18 and 24 months of age, respectively, but the peak incidence was at aged 6 months[19]. When focusing on childhood overweight/ obesity, the prevalence has continued to increase in pre-school children[14]. Recently, the fifth Thai Nutritional Health and Examination Survey (NHES) 2014-5[15] reported that the prevalence of overweight/ obesity in pre-school children was 14.9 % compared to 13.2% in the previous NHES[20]. The most recent survey, the Multiple Indicator Cluster Survey (MICS) led by UNICEF in 2019[21], demonstrated that percentages of all forms of malnutrition including stunting, wasting and overweight/ obesity in under-five children have increased around 4% since the previous survey in 2016[22].

The DBM is not only found in urban families where westernized foods are more available, but it is also reported in suburban and rural communities. In 2013, the South East Asia Nutrition Survey (SEANUTS)[16] reported that prevalence of undernutrition, overweight/ obesity in Thai children aged 6 to 36 months of age tended to be higher in rural households than in urban areas, however, these differences were not statistically significant (stunting 10.6 vs 6.4%; underweight 6.7 vs 2.5%; overweight 4.9 vs 3.1%; obesity 2.2 vs 1.1% in rural and urban, respectively). Following the SEANUTS, the latest NHES also showed that there was no significant difference in rates of malnutrition among under-fives who lived in municipal and non-municipal areas[15].

When considering other forms of undernutrition, micronutrient deficiencies, the results from the SEANUTS demonstrated that iron deficiency was the main issue showing a high prevalence across all age groups of Thai children (6 months to 12.9 years old)[16]. Apart from iron, two studies reported that 25% of infants aged 4-6 months and 57% of school age children had serum Zinc below the sufficient level[23]. In addition, other micronutrients including calcium and vitamin D are currently becoming of more concern in Thailand[24].

Taken together, the body of evidence supports the existence of the DBM in both urban and rural areas of Thailand. This suggests that the habitual diet

and feeding practices among Thai families, especially for infants and young children, are sub-optimal and should be addressed.

2.2 Dietary patterns and nutrient intakes of infants and young children

The CF period is the most challenging and critical period of the first few years of life. At this time, infants are still growing rapidly while their primary diet, breast milk, cannot fulfil their nutrient requirements alone. During the transition from a solely liquid-based diet to family solid foods, multiple nutritional problems can arise if 'inappropriate' or 'inadequate' feeding practices are used. Energy and protein intakes that are mismatched to requirements can result in either under- or overnutrition. Likewise, micronutrient gaps might widen and affect both short- and long-term health outcomes if complementary foods provide 'suboptimal' amounts of those essential micronutrients. Therefore, tracking of dietary patterns and feeding practices during the CF period could be beneficial and may provide important clues on how best to overcome the DBM effectively.

Diets in high-income countries usually include a wide variety of foods from animal sources which make up most of the protein in Western style diets[25]. Conversely, the coverage of animal source foods in developing regions is lower. Sixty to seventy per cent of the total daily protein intake in high-income countries comes from animal sources in contrast to only 20-40% in developing regions[26]. Unsurprisingly, these dietary patterns are also reflected in the CF practices. Studies from the United States and European countries found that ASFs, especially milk and dairy products, are major sources of dietary protein which covered 70-80% of total protein intake of infants aged 6 to 12 months while meats became more common than dairy intake in the second year of life[27, 28].

On the other hand, in lower-income settings, the introduction of dairy foods, meat and other ASFs during the CF period is usually delayed or they are offered less frequently because staple diets are commonly based on cereals or plant-based[13, 29, 30]. In 1998, Gibson et al[31] reported that complementary

foods consumed by infants aged 9 to 11 months in developing countries including Thailand mainly relied on cereals and plant-based foods and contained only small amounts of dairy products and ASFs. A decade later, the 4th NHES 2008-9[32] reported that “dairy products” provided slightly more energy (30%) for children aged 1-3 years compared with “grains and starchy products” (27%) and “meat, poultry and meat products” (14%). Regarding dietary protein, this survey reported that more than 90% of preschool children consume more dietary protein than the Thai recommendations. In addition, when considering protein sources, “dairy products” were a primary source providing around 33% of protein intake, followed by “meat, poultry and meat products” (21%) and “grains and starchy products” (14%). In total, combined ASFs (i.e., dairy, meats, eggs, fish and seafood) made up around 70% of protein consumption in the Thai children aged 1-3 years. According to the 4th NHES 2008-9, we can assume that the dietary pattern of Thai children is now changing toward a western pattern. However, there is no up-to-date study demonstrating energy and nutrient intakes during the CF period in Thai infants.

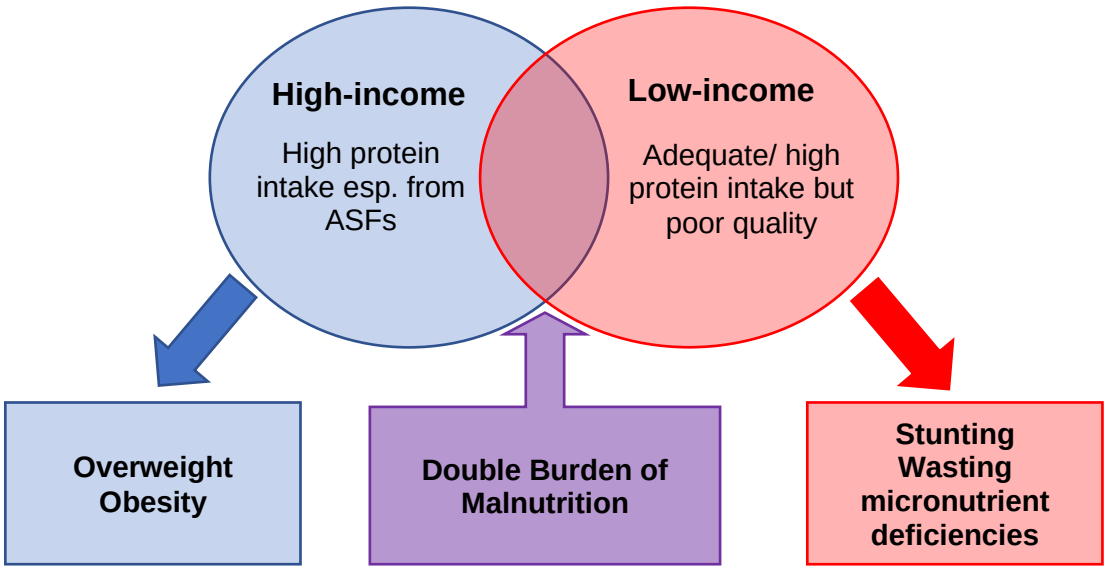
At present, there is only one study from a neighbouring country that might be representative of the current situation in Thailand. Similar to Thailand, Indonesia is now facing a socioeconomic transformation and the DBM is highly prevalent in the population especially in young children[33]. Diana et al[34] showed that breastfed infants aged 9 and 12 months consumed higher amounts of total protein from complementary foods than the national recommendation while delaying introduction of ASF was still common practice (<10% of infants received ASF at 6 months of age). However, the use of ASFs rapidly increased to 68.4 and 86.6%, at 9 and 12 months of age respectively. Protein intake from different sources was not reported in this study.

When focusing on nutrient intakes, data from the Sustainable Micronutrient Interventions to control Deficiencies and Improve Nutritional Status and General Health in Asia (SMILING) project showed that habitual diets of infants and young children aged 6 to 23 months who lived in five countries of South-East Asia (e.g., Thailand, Vietnam, Indonesia, Cambodia and Lao’s PDR) were deficient in many micronutrients, especially iron, zinc and calcium[35].

In contrast to micronutrients, the survey reported that energy and protein intake during the CF period were acceptable in those countries.

Taken together, the aforementioned studies demonstrate that dietary patterns during the CF period are quite different between high- and low-income settings. Notably, for some LMICs where the DBM is highly prevalent, the dietary pattern seems to be westernised to some degree. In this specific circumstance ASFs, especially dairy products and meats, are the major contributors of energy and protein intake. However, in the same population, concerns that poor-quality protein from plant-based foods might provide inadequate amounts of problematic micronutrients, contributing to a high prevalence of ID/ IDA and other micronutrient deficiencies, might lead to the promotion of greater consumption of ASFs. This paradoxical scenario has raised an interesting issue (Figure 2.1). What would happen to young children who already consume ASFs as a main food source, if recommendations and public health programmes move towards encouraging more ASFs to reduce the burden from micronutrient deficiencies. It seems that “dietary protein” is a main focus of this issue, but it is impossible to find the best solution for this conflict without understanding the differences between ABP and PBP and how they affect growth/ nutritional status.

Figure 2.1 The paradoxical scenario in LMICs countries facing the DBM



2.3 Dietary protein and differences between animal- and plant-based protein

When compared with other components in complementary foods, protein is the nutrient whose intake increases the most. The %PE increases from about 5 to 15% when exclusively breastfed infants are introduced to complementary foods[36]. Apart from the noticeable change in quantity, infants are also exposed to a great variety of new dietary proteins such as cow's milk, meats, eggs, cereals, grains, legumes and so on. Similar to the major food sources, dietary protein also has been divided into two main sources, ABP and PBP.

At present, the best indicator of protein quality is still debated. However, the protein-digestibility-corrected amino acid score (PDCAAS) is one of the indicators commonly used. Generally, this score depends on the limiting EAA (mg/g protein) divided by the requirement for this amino acid and corrected for digestibility[37]. Overall, with PDCAAS truncated to 100% the value of proteins is in the order: ABP (meats, milk, eggs) ≥ legume (soya) proteins > cereal proteins (rice, wheat)[38]. The percentage of true digestibility of ABP is roughly around 95% while cereal-based proteins (e.g., rice, maize and wheat) have lower true digestibility of 85-88%[38]. Although soya protein has the highest true digestibility (91%) and PDCAAS among all PBP, it still has limiting amino acids, namely methionine and cysteine. When consider the proportion of protein per weight unit of food, amino acid composition and EAA content varies between ABP and PBP[38]. Less ABP is needed to meet protein requirements compared with PBP. While ABP provide the full complement of EAA, almost all types of PBP are deficient in some, especially lysine, methionine, threonine and tryptophan[26]. Furthermore, leucine, which has important effects on somatic growth is also found predominantly in ABP[39].

Beyond the amino acid pattern and the issue of EAA composition, another crucial element which differs between protein sources is the micronutrient content. Among all problematic micronutrients, iron is the most important mineral that is deficient in the diet of infants across the world. Iron stores at the time of birth in full term infants can cover iron needs only for 4 to 6 months.

Therefore, complementary feeding is essential to provide dietary iron and bridge this deficit. To support rapid growth and the doubling of blood volume from 4 to 12 months of age, the requirements of exogenous iron from the diet are higher than at any other time in life on a body weight basis[40]. In addition, ID with or without anaemia causes adverse outcomes in both the short- and long-term. Neurological development can be permanently affected even if the deficient state or anaemia is treated.

In comparison with non-haem iron in PBP, the bioavailability of haem iron, the major form of iron in ABP, is superior and its absorption is less inhibited by other minerals or foods. In addition, the high percentage of mineral inhibitors such as phytic acid and fibre in plant-based foods may also decrease iron absorption[41]. For these reasons, the WHO and many guidelines recommend the introduction of ASFs in particular red meat during the CF period to meet the iron gap alongside the promotion of breastfeeding. However, in some countries, iron-/ multiple micronutrient-fortified baby foods and/ or universal iron supplementation have been introduced in an attempt to reduce the prevalence of iron deficiency and iron deficiency anaemia in this age group.

There is evidence from both observational studies and randomised-controlled trials (RCTs) on the impact of meat on iron status. Recently, a cross-sectional study in South Korean children aged 8 to 15 months found that inadequate red meat intake increased the odds ratio (OR) of iron deficiency after adjusting for other confounders (OR 1.7; 95% confidence interval (CI) 1.0-2.7)[42]. Interestingly, giving nutritional messages encouraging meat consumption at 6 months of age also showed a positive effect on haemoglobin and haematocrit at 12 months of age in Colombian infants[43]. In addition, red meat is not the only protein source which might have beneficial effects on iron status. An RCT conducted in Cambodian infants also found that infants consuming food combined with small fish and edible spiders had no differences in plasma ferritin, soluble transferrin receptor and haemoglobin when compared with infants receiving iron fortified corn-soy blend products[44]. More clinical trials also reported that infants consuming “higher” amounts of meat during the CF period had favourable levels of haemoglobin concentration compared to those

with “lower” intakes[45, 46]. However, only one RCT found that there was an increased risk of developing marginal iron status in breastfed infants who received a “lower” amount of meat in take in the second half of infancy[46]. The other RCT did not find significant differences in iron status between groups[45].

It would be unethical to perform an RCT comparing iron status in infants who receive ASFs and non-fortified plant-based foods because a superior effect of meat would be expected. Nevertheless, many RCTs show similar results in term of iron status and haemoglobin concentration between a meat group and a fortified cereal group[44, 47-49]. Noticeably, Qasem and colleagues[47] reported that infants who were randomized to receive iron-fortified cereal had almost seven times higher iron intake compared to infants in the meat puree group. Unsurprisingly, the cereal group also had significantly higher faecal iron in the stool, which was considered as unabsorbed iron, compared to the meat puree group. As free iron is a high potency oxidant causing intense inflammation, this finding should be highlighted. Although this study found faecal reactive oxygen species and faecal calprotectin (as markers of oxidative stress) were not significantly different between the fortified cereal and meat puree group, it should be noted that gut microbiota differed significantly between these groups. In addition to this study, Krebs at al [49] also reported that infants who received pureed meat had more butyrate-producing Clostridium bacteria than the group which was randomized to consume iron-fortified cereal. Butyrate is a short-chain fatty acid providing energy to colonocytes and maintains physiological functions in colon. A RCT conducted in Sweden reported that iron sufficient infants randomly allocated to receive ferrous sulfate drops (6.6 mg Fe/day) for 45 days had lower abundance of probiotics, *Lactobacillus* sp. and *Streptococcus,* but higher abundance of *Clostridium* and *Bacteriodes* compared with infants receiving a high-iron-fortified formula with prebiotic, galacto-oligosaccharides. Moreover, faecal calprotectin indicating intestinal inflammation was also positively correlated with *Clostridium difficile* in both high-iron-fortified formula and iron supplement groups[50]. Although the clinical significance of these findings is currently unclear, this issue needs to be considered and further investigated. Globally, single or multiple-micronutrient-fortification seems to be an attractive option to fulfil nutritional gaps in many

countries. However, if such strategies may have an effect on gut microbiota, this may be of concern.

Apart from the differences discussed above, each protein source also has a different impact on biological functions associated with the supply of nitrogen and amino acids in the body[38]. Scientific evidence indicates an impact of dietary protein on growth during early life through one of the major regulators of growth regulation, the mammalian target of rapamycin (mTOR)[51]. From this perspective, it is important to investigate associations between dietary protein during the weaning period and growth in early life which may be relevant for initiatives targeting childhood malnutrition.

2.4 Emerging topics related to dietary protein in complementary foods: a chance to reduce the double burden of malnutrition

Although dietary protein is needed to support healthy growth, in the context of complementary foods this topic has received less attention when compared to micronutrient problems. Since the mid-1970s, the role of protein intake in nutritional interventions has been overlooked due to an assumption that children living in developing countries consumed adequate amounts of protein[10]. Deficiencies of dietary protein and amino acids contribute to growth faltering or, in a more severe form, kwashiorkor. However, after improving education, food availability, food safety, basic sanitation and primary health care, the prevalence of PEM is now lower than in the past. As a result, dietary protein has been less emphasized as a major concern for infants and young children.

In recent years, much of the focus on improving growth in lower income settings has been on improving micronutrient intake and status. Although a systematic review focusing on interventions during the CF period in LMICs found that provision of micronutrient-fortified foods/ supplementation markedly increased the intake of relevant micronutrients, the effect on growth outcomes was still less than expected[52]. These findings suggest that improving micronutrient intake is not the only factor needed to improve growth in early

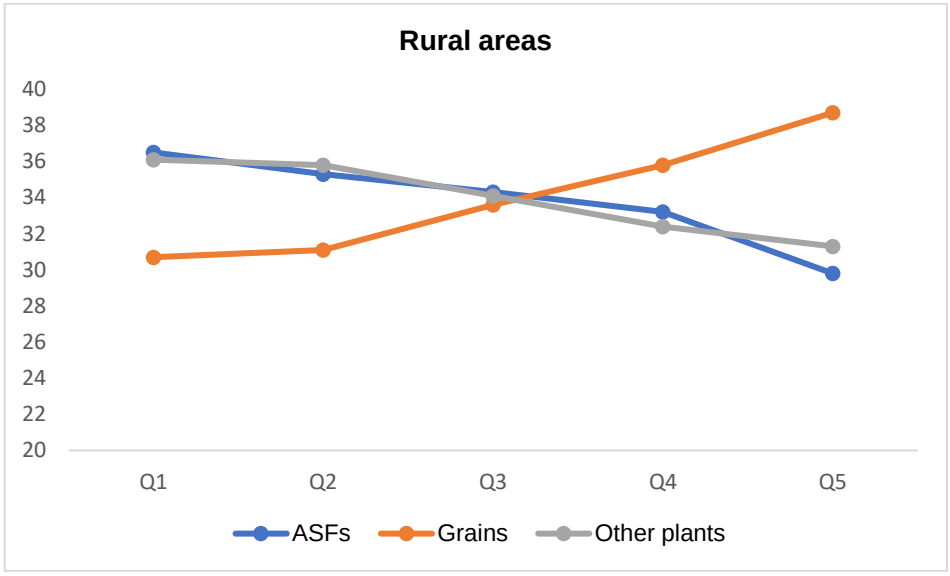
life. Other components in complementary foods might also play this role and need to be highlighted. Given the discussion in the previous section, it is timely to reconsider “dietary protein” as a potential factor to prevent all forms of malnutrition.

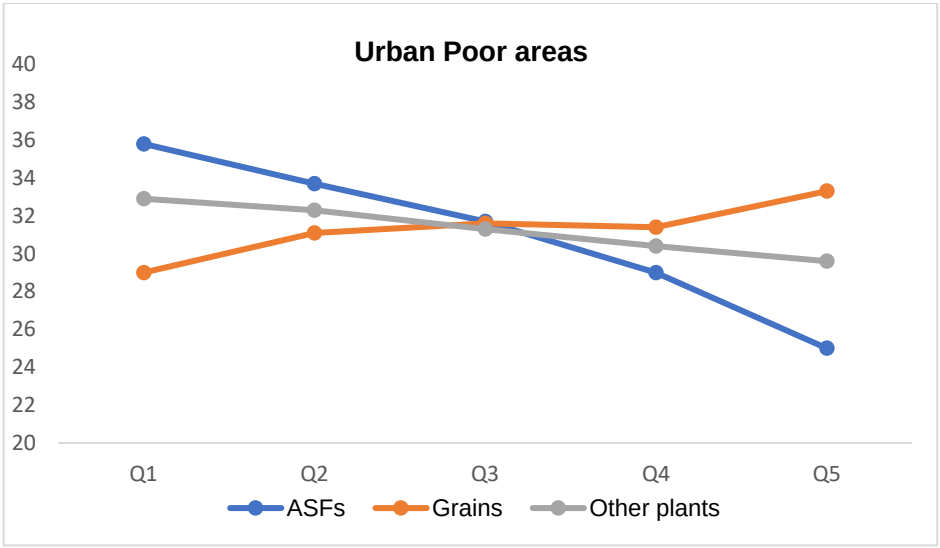
Evidence of a possible link between dietary protein and undernutrition

It is well-established that protein deficiency results in mild to severe forms of acute PEM, kwashiorkor, therefore in this section I will focus on the possible link between dietary protein and stunting, a chronic form of undernutrition, in which the role of protein is still unclear. Although many factors can contribute to stunting, there are several scientific studies demonstrating a link to dietary protein. A study in a Thai population showed that stunted children consumed slightly higher dietary protein than a non-stunted group (median intake 1.78 vs 1.68 g/kg/day, respectively) but the intake of ABP in the stunted children was less than that in the non-stunted group (median intake 18 vs 21.3 g/day, respectively)[53]. However, the results might not reflect consumption during the CF period as this study was conducted in school age children. Furthermore, diet was assessed cross-sectionally and therefore prospective associations of diet with growth could not be considered.

Interestingly, Sari et al[54] gathered national data from 1999 to 2003 and reported that the prevalence of stunting in Indonesian children aged 0 to 59 months (n > 500,000 from 40,000 households) was negatively associated with the household expenditure on ASFs (i.e., milk, meats, eggs), while a positive association was observed in the household expenditure for grains (i.e., rice and other staples) in both rural and urban poor areas (figure 2.2). It could be assumed that a higher expenditure reflects a higher consumption. Therefore, these outcomes might be alternatively interpreted as showing that higher consumption of ASFs was associated with reduced stunting while higher intake of grains/ cereals showed the opposite trends. Overall prevalence of stunting in this population ranged between 30-48.1% in rural and 30.1-41.6% in the urban poor area. Although this evidence did not directly indicate the role of dietary protein in stunted children, it has underscored the different impact of ASFs and plant-based foods on this outcome.

Figure 2.2 Comparison of the associations between rate of stunting and the household expenditure (Q1 – lowest, Q5 - highest) for different food sources[54]





Corresponding to the previous studies, Semba and colleagues[55] also showed a possible link between dietary protein and stunting. They reported that among children aged 12 to 59 months who lived in rural Malawi, 62% were stunted and had lower EAA in their plasma compared with those who were non-stunted. In addition, among 20 serum metabolites with the most significant correlation, the results showed that all branched-chain amino acids (BCAA) including leucine, isoleucine and valine as well as the amino acids limited in PBP (e.g., threonine, tryptophan, methionine, and lysine) were positively

correlated with height-for-age z-score (HAZ). As EAA only come from food, it can be assumed that dietary protein may be a 'missing piece' and might be a key intervention to prevent stunting. However, further study is needed to support this assumption.

Evidence of a possible link between dietary protein and overnutrition

Convincing evidence also highlights the association between dietary protein and childhood overweight and obesity[56]. Since the mid-1990s, dietary protein has been considered as a significant growth promoting factor, and evidence from developed country settings emphasizes that "high protein intake" may be associated with more rapid growth in early life and this impact may be long lasting through adulthood[57]. For example, an observational study reported that every 10 g higher protein intake at 12 months old was associated with a 0.05 standard deviation scores (SDS) higher in body mass index (BMI) at 6 years old[58]. Furthermore, there is robust evidence from a large RCT conducted in five European countries which showed that infants who received high protein formula during first year of life had higher BMI at 2 and 6 years old compared with infant formula containing lower protein or breastfeeding as a control group[59, 60]. Among the different sources of dietary protein, dairy protein has the strongest impact on infant and young child growth followed by a more modest effect of other animal-based proteins such as meats and egg[61]. However, evidence for the source of protein that has the most effect during the CF period is limited and more research warranted. In a global context, there is no evidence of an association between high protein intake and rapid growth in low- and middle-income countries[61].

Taken together, for countries facing the DBM, there is some evidence that dietary protein in complementary foods could be a key factor to overcome both under- and over-nutrition in young children. Future research should investigate not only the amount of protein intake related to growth outcomes but also the source of dietary protein, ideally using rigorous dietary assessment methods. It cannot be assumed that a high intake of PBP will affect infant and young child growth in the same way as ABP. Furthermore, as the prevalence of

overweight/ obesity in children aged under five has constantly escalated in LMICs, we cannot ignore the fact that a high protein intake in early life might be contributing to this problem. Therefore, both “too little (amount & quality)” and “too much” protein intake should be addressed in equal measure for countries facing the DBM. To explain why each protein source has a different impact on nutritional status, it is necessary to understand their impacts on growth and body composition.

2.5 Impact of different protein sources on growth and body composition

Growth differences between vegan, vegetarian and omnivorous children are a proxy to investigate the effect of different protein sources. Studies report that children who are vegan tend to be ‘leaner’ and ‘shorter’ than omnivorous and other type of vegetarian peers[62-64]. However, these are observational studies and there are many differences between vegan/ vegetarian and omnivorous children which need to be considered as confounders. In the general population, studies that investigate how different sources of dietary protein influence infant and child growth fall into two main groups. The first group comes from low-income regions where an association between protein source and undernutrition (stunting, wasting, underweight and micronutrient deficiencies) is targeted. The other group consists of studies conducted in high-income settings where the association between high protein intake and risk of overweight and obesity has been widely investigated.

From the first perspective, there are many observational studies and RCTs attempting to clarify the effect of different protein sources on linear growth and weight gain in low-income settings. In some observational studies, intake of animal-based protein during the CF period is significantly associated with improvement of linear growth and weight gain in infants and young children[65-67]. The consumption of meat is associated with a reduced risk of stunting, by 36%, after adjusting for other confounding factors[67]. However, a study in Central and South African children aged 12 to 36 months found that only milk intake had consistently positive correlations with HAZ in seven countries while meat and egg intake was associated with linear growth in only one country[68].

For the CF trials, a RCT conducted in rural China, Tang and colleagues showed a beneficial effect of adding 60 g/day of meat to the usual diet for a year. The final analysis showed that the intake of animal-based protein was doubled in the meat group (average intake 16 ± 9 vs 10 ± 8 g/day in meat and control group, respectively) and this group showed a positive effect on change in length-for-age z-score (LAZ) and increased length[69]. In addition, two RCTs provided dietary proteins from animal sources that were available in their countries; the results showed favourable effects on weight gain and linear growth if egg was given[70], while small fish and edible insects were not effective[44].

On the other hand, large RCTs conducted in multiple developing countries in Africa and South Asia found that there were no differences in any growth parameters at 12 and 18 months of age between groups of infants who had been receiving lyophilized beef since 6 months of age compared with those who consumed a micronutrient-fortified rice-soy cereal. Unexpectedly, the percentage of stunting increased from 33% to 50% at 12 and 18 months, respectively[71]. Instead of providing food as an intervention, Olaya et al[43] conducted a RCT using simple messages provided to the children's caregivers when they started complementary foods at 6 months. The message, "offer meat at least 3 times a week along with continued breastfeeding and daily intake of fruits and vegetables", was successful in increasing meat intake and improving haemoglobin but again there was no effect on growth parameters.
In conclusion, although ABP, especially dairy protein, tend to favour linear growth and weight gain, results from LMICs are inconsistent[44, 71-73]. To understand the actual effect of ABP on growth in children living in lower-income settings, issues specific to each country such as dietary pattern, food security, local feeding practices, basic sanitation, and other malnutrition-related factors should be considered.

When focusing on the second perspective, the majority of studies from western countries demonstrate associations between higher protein intake and rapid weight gain, altered body composition as well as risk of obesity. Several studies found that higher intake of ABP during the CF period was

associated with significantly higher weight gain and BMI in pre-school and school age children compared with lower intake or plant-based diets[72-75]. Gunther and colleagues[73] reported that dairy protein intake at 12 months old was positively associated with percentage of body fat at 7 years old, but there was no significant effect of meat and cereal protein intake.

For linear growth, a cross-sectional study in Danish children reported that high milk intake was associated with greater length in toddlers. Within the group of ABP, different impacts on growth were observed between dairy products and others (i.e., red meat, poultry, and egg) while there was no effect of plant-based protein on growth[76]. To investigate causality, three RCTs were conducted in the United States and Canada. Only one study, by Tang and colleagues[77] found that the infants who were given beef puree (versus fortified cereal) as the first complementary food had greater changes in length-for-age z-score (LAZ) and weight-for-age z-score (WAZ) at 9 months of age, although weight-for-length z-score (WLZ) was not different. In the other RCTs, there was no growth difference between animal-based and fortified-cereal-based groups[47, 78]. However, the periods of intervention were shorter in these last two studies (only 1 to 2 months compared to approximately 4 months in Tang et al[69]). Therefore, for western populations, although ABP introduced during weaning may have the potential to promote rapid growth and affect body composition, it is unclear whether this applies to all ABP or only dairy protein. Evidence that non-dairy ABP induces rapid growth or increase body fatness is still inconclusive.

Taken together, evidence from both perspectives indicates that ABP particularly dairy protein may provide both beneficial and negative impacts on infant and child growth. However, the important question of how to optimise growth by offering ABP without promoting 'excessive' growth is still unknown especially for the populations manifesting both under- and overnutrition. Regarding recommendations for protein intake, the "safe levels" have been well-established and revisited in all dietary guidelines for many years while the "upper limits" for protein consumption have been provided more recently and only for infants and young children living in western countries[4, 79-81].

For LMICs, this issue has never been raised despite increasing overweight/ obesity in toddlers and preschool children. Therefore, more studies related to high protein intake and risk of overweight/ obesity in LMICs are needed. Furthermore, scientific knowledge explaining the association between dietary protein and growth through human biology and physiology is also required. In order to provide effective interventions, we need to understand the underlying mechanisms that can explain clinical outcomes.

2.6 Possible mechanisms linking dietary protein and somatic growth through plasma amino acids and the GH-IGF axis

It is known that several nutrients directly influence growth during the first year of life while growth hormone and genetic factors increase their roles after that period. However, the GH–IGF axis is still strongly modulated by nutritional signals[82]. GH is essential for normal balanced somatic growth from birth to adult stature. In studies using malnutrition as a model, intake of adequate dietary protein and calories is critical to restore serum IGF-1 after fasting[83]. Amino acids are the most important component of dietary protein for growth and are associated with IGF and insulin secretion. Convincing evidence from in vitro and in vivo studies shows that several amino acids, but especially BCAAs, are potent stimulators[84-88].

Based on a body of evidence, “the Early Protein Hypothesis” was proposed, suggesting that higher protein intake during early life results in rapid growth and increased risk of childhood obesity or even metabolic diseases in adults[57]. Supporting evidence for this hypothesis was obtained from a large, multi-centre RCT performed in five European countries, the Childhood Obesity Project (CHOP). The results from this study indicate that infants who are fed with high protein infant formula during first year of life have higher BMI Z-scores and increases of serum BCAA, IGF-1 and urinary C-peptide (representing insulin synthesis) compared with breastfed infants and infants fed with lower protein formula[59, 60, 89]. Focussing on protein from unfortified cow’s milk, a non-randomized trial was performed in Danish boys aged 8 years old. At the same protein intake (approximately 53 g/day), boys who consumed

1.5 L of milk daily had higher serum IGF-1, Insulin-like growth factor binding protein (IGF-BP) 3 and double the increase of fasting serum insulin when compared to the meat group. However, this study had many limitations including small sample size, short intervention (7 days), lack of a control group and non-randomization[90].

In the context of complementary feeding, Hoppe et al[76] reported a positive correlation between serum IGF-1 and animal-based protein intake in children aged 2.5 years. However, when separating sources of dietary protein, again, only milk was significantly associated with serum IGF-1 concentrations whereas meat and plant-based protein had no such association. In contrast, a RCT conducted in the United States showed conflicting results. Tang and colleagues[77] did not find any differences in serum IGF-1, IGF-BP3 as well as other obesity-related biomarkers in 9M infants who had received the diet interventions, meat puree or iron-fortified cereal since they were about 5 months of age. Surprisingly, these findings did not correspond to the favourable outcomes on WAZ and LAZ in the meat group however, the small sample size might be considered as an important limitation in this study. Interestingly, while ABP may promote growth by inducing the GH-IGF axis, a correlation between plant-based proteins and serum IGF-1 has not been reported.[74, 76, 77, 91]

Fourteen percent of the amino acids in whey protein are leucine, a potent stimulator of IGF-1 secretion, compared to only 8% of the amino acids in meat protein; this is the highest concentration among dietary protein sources[92]. In addition, when compared with PBP, the EAA especially BCAA are higher in most animal sources while the PBP contain more non-essential amino acids (non-EAA) but have limited amounts of some essential ones[93]. In order to avoid a lack of EAA especially for young children requiring higher amounts of EAA compared with older children, it is necessary to use a great variety of PBP[94]. From these distinct characteristics, the impact on the GH-IGF axis would be expected to differ for ABP and PBP.

There are several studies demonstrating gender differences in the associations between protein intake and the GH-IGF axis. For example, Joslowski et al[91] and Thorisdottir et al[74] found that intake of ABP had a different impact on IGF-1 in boys and girls. The study also reported that boys who habitually consumed more ABP when they were 6 to 24 months of age had significantly 'lower serum IGF-1' when they reached pre-pubertal age[91]. This effect was not found in girls. According to Thorisdottir et al[74], serum IGF-1 at 6 years of age was positively associated with intake of dairy protein during infancy but only in female participants.

From current evidence, dietary protein may influence infant growth by two possible mechanisms mediated by key amino acids. The first mechanism may occur by stimulating IGF-1 and insulin secretion while the other promotes somatic growth through sending direct signals to mTOR. The mTOR is a central regulator of cellular metabolism, growth, proliferation and survival by complex signalling cascades that is directly stimulated by amino acids[95]. Of all amino acids, mTOR is most sensitive to leucine, arginine and glutamine[96, 97] Understanding the linkage between dietary protein and these two mechanisms would be an important basis for dietary protein recommendations for infant and young children.

2.7 Possible mechanisms linking dietary protein and somatic growth: mTOR pathway and microRNAs

In brief, the mTOR is a conserved serine/ threonine protein kinase that plays a critical role as a principal conductor of metabolism, growth, cell proliferation and aging through the life cycle. Although there are two specific complex forms of mTOR called mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2), the mTORC1 is the master controller of cellular growth and metabolism[98]. The inputs from diverse environmental and nutritional cues can activate or inhibit the mTORC1 signal and subsequently regulate multiple pathways of central and peripheral organ targets. Similar to mTOR, microRNAs have a wide range of functions in the cell cycle including proliferation, differentiation, apoptosis and autography[99]. Moreover, a growing

body of evidence has shown a close relationship between them and the mTOR pathway in most cancer types, many other diseases and under physiological conditions[100, 101]. This interplay is necessary for fine tuning of diverse cellular functions as well as the epigenetic changes corresponding to both intrinsic and extrinsic exposures[101, 102]. MicroRNAs are present in both target organs and several extracellular fluids. Weber et al[103] found that twelve sources of body fluids contain several microRNAs, including breast milk and plasma. Moreover, because they are carried by extracellular vesicles, exosomes, they are remarkably stable from freezing-thawing cycles and resist the ribonuclease enzyme[102]. There is increasing interest in the role of microRNAs as novel, simple and highly sensitive biomarkers for diagnosis, prognostic prediction, adjunctive therapy and investigation of epigenetic mechanisms[104, 105].

The microRNAs are a class of short (18-22 nucleotides) non-coding RNAs that regulate the expression of a wide variety of genes by silencing targeted messenger RNAs (mRNAs), so-called post-transcriptional translation[106]. Accumulating evidence indicates that microRNAs participate in tissue crosstalk through intracellular and extracellular molecules and could control the expression of other cross-talk molecules. At the same time, microRNAs per se might be regulated by those molecules as well[107].

From current evidence, there is a high possibility that microRNAs also play a major role in the regulation of somatic growth. Several studies reveal that various types of microRNAs can influence body growth through controlling longitudinal bone growth, the GH-IGF axis and a growth-limiting genetic program[108]. In an animal model, miR-140 knockout mice had a significantly shorter tibia length when compared to wild type. In particular, if microRNA let-7 was also suppressed, the reduction of bone length and body size were more severe[109]. According to recent studies, there are other microRNAs such as miR-199a, miR-145 and miR-675 that may affect bone development by regulating chondrocyte differentiation[108].

Studies demonstrate that some microRNAs in maternal blood are associated with the birth weight of the offspring[110]. Furthermore, emerging evidence also suggests that many microRNAs found in placental tissue are significantly related to pregnancy outcomes, for example, an association between miR17-92 cluster and macrosomia[111]. Moving to the post-natal period, interesting evidence suggests that there are a huge number of microRNAs in human milk[112, 113]. Recently, an in vitro study reported that human milk microRNAs in exosomes can pass through the gastrointestinal tract without deterioration and are taken up by human intestinal cells[114]. It can be hypothesised that the diverse microRNAs would affect several physiological functions including post-natal growth as well as transferring messages from mother to baby. However, the role of microRNAs in breast milk is currently speculative and more information is still needed.

Given that some ASFs, such as egg and milk, are basically growth products designed to promote species-specific growth during embryogenesis and infancy, it might be expected that these transfer many growth factors and genetic-related components from mother to offspring. This may explain why consumption of these foods could affect human growth. However, further study is needed to develop this theoretical idea. Although there are various factors which may influence the biogenesis of microRNAs, nutritional factors are expected to play a crucial role. Emerging evidence proposes that mature microRNAs might be synthesized endogenously or obtained from food sources such as breast milk[109]. Dietary compounds per se may alter the expression of endogenous microRNAs[115, 116]. The results from in vitro and animal studies reveal that administration or restriction of macro- or micronutrients has a significant impact on the expression of microRNAs[117]. For example, a study showed that supplementation of EAAs including BCAAs was associated with increasing levels of microRNAs-1 and microRNA-206 in skeletal muscle[118].

When focusing on different sources of dietary protein and their impact on microRNA profiles, surprisingly, only one study performed in healthy adults was found. Tarallo and colleagues[119] demonstrated significant increases of

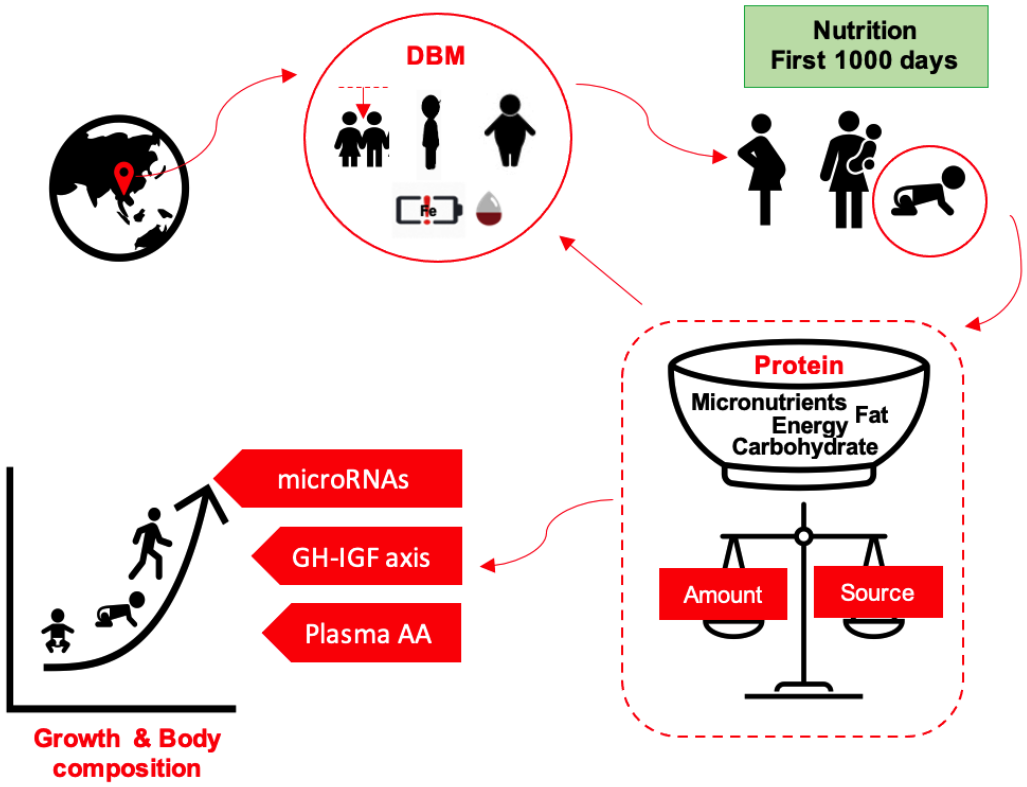
miR-92a expression in stools and plasma of vegan individuals compared with vegetarian participants, with the lowest values in omnivores. Interestingly, the miR-92a in both stools and plasma was also associated with lower BMI. In addition, more specific dietary sources such as cheeses and dairy consumption showed a strongly negative correlation to miR-92a in both samples while intake of meat, processed meat and fish was also inversely related to miR-92a but only in plasma. In stool samples, miR-16 and miR-21 were associated with meat and fish intake in an opposite direction compared to vegetable consumption. Although other microRNAs did not show consistent or similar tends in stool and plasma, this is the first evidence showing a correlation between different protein sources in the diet and microRNA expression in humans.

To my best knowledge, there is no study reporting an association between different sources of dietary protein and their impacts on expression of microRNAs in the context of complementary feeding. Despite increasing research on microRNAs in breast milk, it is still not clear whether or how microRNAs influence infant growth and epigenetic changes in infants[120].

In summary, this literature review has shown that Thailand could be considered representative of LMICs facing the DBM particularly in younger populations. The relevant issues of dietary protein during the CF period are also highlighted and discussed in this review. However, results are inconclusive for some issues. Figure 2.3 is an infographic summarising what was discussed in this chapter.

The following chapter will present detailed information and results from my observational study that contributed to the design of my prospective study.

Figure 2.3 Summary of the literature review



Chapter 3

A cross-sectional study investigating parental practices related to complementary feeding in Thailand

Some data presented in this chapter have been published as “Complementary feeding: Attitudes, knowledge and practices of urban families in northern Thailand” in journal “Nutrition and Dietetics” volume 76, issue 1 page 57-66 in 2019[121]. I was the principal investigator for this study. I designed the questionnaire used in the study with help from my supervisors. I also entered the research data, performed the statistical analyses and wrote the manuscript.

Based on the literature review in the previous chapter, I conducted an observational study exploring common feeding practices during the CF period among Thai families living in Chiang Mai as a target population for my PhD research. Before I started this cross-sectional study, I had read the relevant studies about CF aiming to define the most appropriate intervention that could be used in a prospective RCT aiming to improve CF practices. At that time, there was no recent report of complementary feeding practices among Thai infants and young children. Although the national surveys in Thailand regularly reported prevalence of malnutrition, breastfeeding rate and age of introduction of CF, they did not provide any information about dietary intakes, common food sources or nutrient intakes during the CF period. Moreover, studies in infants and young children at that time were more likely to focus on micronutrient deficiency, especially iron deficiency, than on dietary intakes in general. I also noticed that macronutrients were generally overlooked, despite the national survey reporting an increasing prevalence of overweight/ obesity in young children[15] which might be associated with high protein intake during the CF period. Overall, I concluded that I did not have sufficient evidence to design an appropriate CF intervention for my target population.

As a result, I decided to conduct a cross-sectional study to identify the most problematic issues during the CF period that could be the target of a future nutritional intervention for this population. At that time, I assumed that Thai infants and young children might consume too little dietary protein from ASFs due to the high prevalence of ID/ IDA. In addition, the SMILING project also reported that intakes of iron, zinc, calcium and other micronutrients from complementary foods in 5 South East Asian countries including Thailand were inadequate for infants and young children[35]. Following the results from the SMILING project, the Thai authorities were quite keen to promote the idea of adding more ASFs, especially red meat, liver, and other iron-rich ASFs to overcome these micronutrient deficiencies.

In the next paragraphs I describe the cross-sectional study, including background, research methodology, key findings, discussion, and conclusion.

3.1 Background

There is very little information on current feeding practices particularly in terms of what sources of dietary protein are provided to Thai infants during complementary feeding. A study in 1992 by Jackson et al[122] reported that the amount of dietary protein consumed by infants who lived in Chiang Mai, Thailand during weaning met the average daily intake recommended by WHO in 1985[123] however, protein source and quality was not mentioned in this study.

In 2013, a study from Rojroongwasinkul et al[16] using data from single 24-hour recalls reported that Thai infants and young children aged 0.5 – 2.9 years old who lived in urban areas consumed significantly higher amounts of dietary protein compared to those from rural settings (35.4 vs 32.6 g/day, $p < 0.05$). The average protein intake per body weight was 3.2 and 3.1 g/kg/day for urban versus rural groups, respectively, and protein consumption of both groups was 50% higher than the Thai dietary recommended intakes (DRIs) for protein at that time[124] which suggested 15-18 g/day or 1.4-1.9 g/kg/day for this age

group. Similar to the research by Jackson et al, this study did not report on sources of dietary protein or protein quality given to infants/ young children.

Given the lack of recent information on this topic, this cross-sectional study was conducted to investigate the feeding practices of families in Chiang Mai, Thailand during the CF period. The study also aimed to record the knowledge and attitudes of parents or caregivers toward complementary foods, especially different types of dietary protein, which could help us to understand the factors influencing feeding practices and identify potential targets for future interventions to improve CF practices.

3.2 Study design and participants

A cross-sectional study was conducted at three well-baby clinics in Chiang Mai, Thailand during October to November 2016. A sample size calculation was not performed, and respondents were included from families that had attended each well-baby clinics during the study period. Ethical approval was obtained from the Ethics committee of the Faculty of Medicine, Chiang Mai University (Ethical approval number was PED-2559-04304).

Parents or primary caregivers of healthy term infants aged less than 18 months old who agreed to participate this study were asked to answer a self-administered questionnaire while they were visiting the well-baby clinics for infant immunisation and health surveillance. If the respondent was illiterate, another family member or health professional was asked to help them complete the questionnaire. The questionnaire was anonymous and contained no sensitive questions, therefore informed consent was not required according to the Ethics committee at the Faculty of Medicine, Chiang Mai University.

3.3 Questionnaire

The self-administered questionnaire was designed by gathering information from scientific evidence and knowledge at that time combined with my experiences as a doctor in a well-baby clinic in Thailand for nearly a decade.

This questionnaire was reviewed and adjusted by my current supervisors to ensure that it covered all relevant topics and was not too lengthy. The main questions related to CF were based on when, what, and how complementary foods were introduced/ fed. The actual questionnaire was written in Thai language and the English version is shown in Appendix 1. Due to the limited time available for the respondents in well-baby clinics, the questionnaire used only closed-end questions for parents to choose answers by ticking boxes. However, for some questions parents could give more than one choice and also specify more detail in case their answer was different from the given options.

The body of the questionnaire was divided into four main sections. The first section included general characteristics of respondents and their children, for example their relationship to the infant, family type (nuclear or extended) and household income. The second section contained questions related to basic knowledge and attitudes of respondents toward infant and young child feeding. The last two sections focused on practical and behavioural aspects of complementary feeding. As infant age varied from 0-18 months, only respondents of infants aged 6-18 who had introduced complementary foods were asked to answer the last two parts of this questionnaire.

3.4 Key findings

I collected data from 108 respondents of whom two-thirds had provided complementary foods to their children and answered the sections covering practical and behavioural aspects. Most respondents were either the mother (62%) or father (31.5%) of the infant or toddler. All were literate and more than half were from a middle-class family (Table 3.1).

Table 3.1 Characteristics of respondents

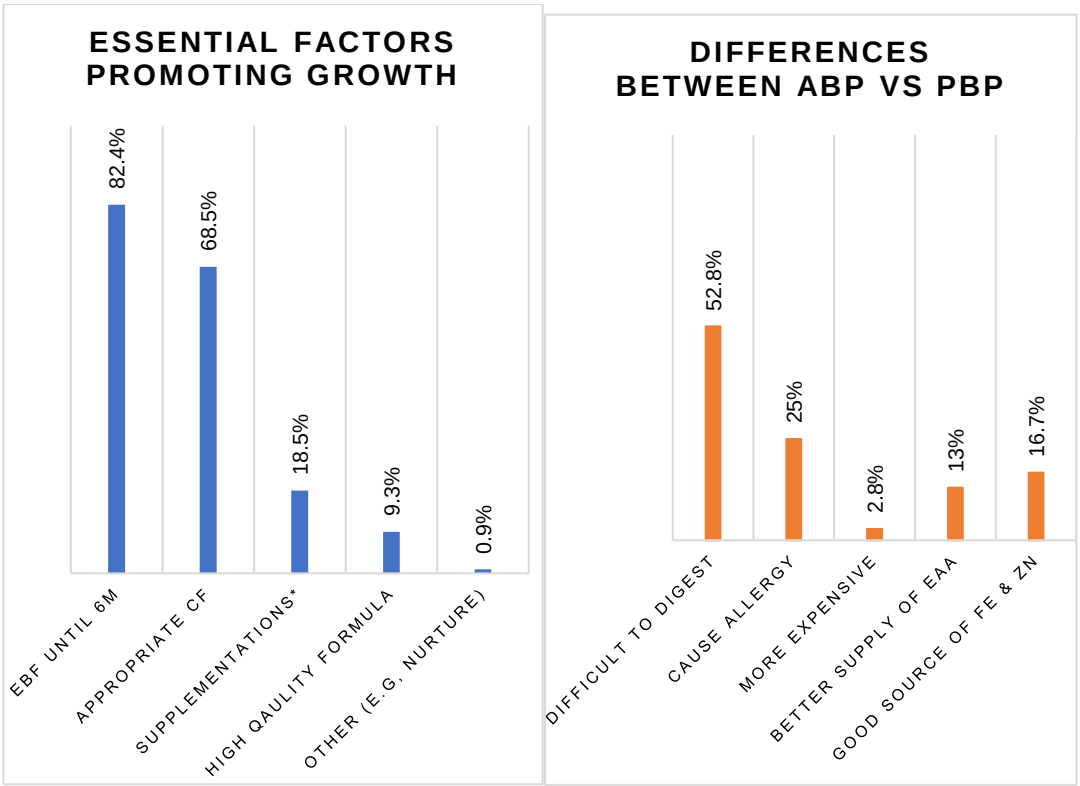
Respondents' characteristics	Results (n = 108)
Relationship to infants/ children, n (%)	
- Mother	67 (62.0)
- Father	34 (31.5)
- Grandparent	6 (5.6)
- Other family members	1 (0.9)
Age of respondents (years old), n (%)	
- less than 20	4 (3.7)
- 20-29	31 (28.7)
- 30-39	63 (58.3)
- 40-49	6 (5.6)
- ≥ 50	2 (1.9)
- Unspecified	2 (1.9)
Educational attainment, n (%)	
- Below bachelor's degree	36 (33.3)
- Bachelor's degree and above	71 (65.7)
- Unspecified	1 (0.9)
Occupation, n (%)	
- Housewife	26 (24.1)
- Agricultural section	1 (0.9)
- Government employee	21 (19.5)
- Other	59 (54.6)
Family type, n (%)	
- Nuclear	46 (42.6)
- Extended	62 (57.4)
Living location, n (%)	
- Downtown	51 (47.2)
- Suburban area	57 (52.8)
Family income* (monthly), n (%)	
- Low	12 (11.1)
- Middle	55 (50.9)
- High	39 (36.1)
- Unspecified	2 (1.9)

**Considering the minimum daily wage and average monthly income of populations in Chiang Mai during period of data collection in 2016*[1,2]

[1] National Wage Committee's notification on minimum wage rate No. 8, 2015-2017
[2] Data sheet of Average monthly income per household by region and province: 2002-2019 [Access 24 April 2021] available at http//: statbbi.nso.go.th/staticreport/page/sector/en/08.aspx

Regarding basic knowledge and attitudes toward complementary feeding, only two-thirds of respondents agreed that complementary foods are important for infant growth while 82.4% of them agreed that exclusive breastfeeding until 6 months of age has beneficial effects on growth and developmental outcomes. Interestingly, although 81.5% of the respondents indicated there is some difference between ABP and PBP, they reported more negative effects of ABP than positive ones. 52.8% and 25% of the respondents believed that ABP were difficult to digest and increased the risk of food allergy, whilst only around 15% agreed that ABP were a good source of essential amino acids and some micronutrients, especially iron and zinc (Figure 3.1).

Figure 3.1 Knowledge and attitudes of respondents on complementary foods (respondents could make more than 1 choice, n = 108)



**Vitamins and mineral supplementations; ABP – animal-based protein; PBP – plant-based protein; EBF – Exclusive breastfeeding; M – Months old; EAA – essential amino acids; FE – Iron; ZN – Zinc*

When considering feeding practices, I found that almost all of the infants had received some form of breastfeeding, whether exclusive or combined breast milk with infant formula during the first 6 months, but the percentages using formula or unfortified cow's milk were higher in the older age groups. The majority of respondents provided first complementary foods when the infant reached 6 months of age. Less than 5% of the infants were given other types of foods (apart from drinking water, medication, or infant formula) before 6 months (Table 3.2).

In terms of food type, table 3.2 shows that mashed banana and home-made rice porridge were commonly used as the first weaning food and only 17% of respondents offered commercial iron-fortified baby foods to their children. On the contrary, ASFs were more likely to be delayed. Some respondents reported delaying the introduction of ASFs until their children were 8 months of age. The most common first ASF introduced to infants was egg yolk. Although the respondents thought that meat and other ASFs were appropriate for their baby, the percentages providing other ASFs were still limited. These findings seem to correspond well with aforementioned knowledge and attitudes toward ABP. Regarding cow's milk and dairy products, 12.5% of the respondents reported using unfortified cow's milk during the complementary feeding period while other dairy products such as cheese, yogurt or butter were rarely consumed. When focusing on the composition of meals, rice was usually a staple, typically combined with some vegetables, one type of ABP and vegetable oil depending on the infant's age. Toddlers tended to have a greater variety of food groups compared with infants. However, this cross-sectional study did not attempt to quantify nutrient intake.

Table 3.2 Feeding practices during the weaning period of infants and young children aged 6-18 months (n = 72)

Feeding practices	6 – 8M (n = 23)	9-12M (n = 24)	13-18M (n = 25)
Current type of milk feeding, n (%)			
- Only breast milk	12 (52.2)	7 (29.2)	7 (28.0)
- Combined breast milk and formula	3 (13.0)	6 (25.0)	5 (20.0)
- Only formula	8 (34.8)	7 (29.2)	10 (40.0)
- Unfortified cow's milk	0	3 (12.5)	2 (8.0)
- Unspecified	0	1 (4.2)	1 (4.0)
Continue consuming breast milk along with CF, n (%)	15 (65.2)	13 (54.2)	12 (48.0)
Timing of first introduction of CF (months old), median age (range)	6 (5-6)	6 (3-7)	6 (4-6)
First complementary foods, n (%)			
- Rice porridge	13 (56.5)	15 (62.5)	16 (64.0)
- Mashed banana	11 (47.8)	6 (25.0)	12 (48.0)
- Commercially fortified infant foods	4 (17.4)	4 (16.7)	0
- Fruit juices	1 (4.3)	0	0
- Others (e.g., pumpkin)	1 (4.3)	1 (4.2)	0
Timing of first introduction of ASFs, n (%)			
- 6-7 months	16 (69.6)	12 (50.0)	12 (48.0)
- 8-9 months	2 (8.7)	10 (41.7)	10 (40.0)
- > 9 months	-	2 (8.3)	2 (8.0)
- Unspecified	1 (4.3)	0	1 (4.0)
Type of commonly introduced ABP, n (%)			
- Egg yolk	16 (69.6)	19 (79.2)	17 (68.0)
- Fish	6 (26.1)	6 (25.0)	7 (28.0)
- Liver	4 (17.4)	4 (16.7)	5 (20.0)
- Pork	4 (17.4)	6 (25.0)	1 (4.0)
- Whole eggs	3 (13.0)	2 (8.3)	5 (20.0)
- Chicken	2 (8.7)	3 (12.5)	1 (4.0)
- Beef	0	0	17 (68.0)

M – months old; CF – complementary foods; ASFs – animal source foods; ABP – animal-based protein

3.5 Discussion

The main outcomes from this cross-sectional study provide a clearer picture of current knowledge, attitudes, and practices of parents regarding infant and young child feeding in the target population living in Chiang Mai, Thailand. Despite the appropriate introduction of complementary foods at 6 months of age, some inappropriate feeding practices were found in this cross-sectional study. The major concerns related to their attitudes and feeding practices around ASFs as many parents had negative perceptions about ABP and were not aware that they are a good source of essential amino acids, iron and zinc. The “Thai traditional weaning styles” such as first introduction of meat-free complementary foods containing only mashed banana with rice and delayed introduction of ABP was also reported in 1992 by Jackson et al[122]. In addition, another study revealed that complementary foods in LMICs including Thailand usually provided inadequate amounts of calcium, iron and zinc while having a high content of anti-nutrients such as phytic acid which reflected the widespread use of cereal-based porridge as a main complementary food[31]. Although the SEANUTS reported adequate protein intake amongst Thai infants and toddlers[35], it seems that protein quality might be overlooked and this could increase the risk of some forms of malnutrition, for example, stunting and ID/ IDA.

Some concerns were also found related to milk feeding, as 12.5% of the respondents reported provision of unfortified cow’s milk to their infants aged less than 12 months while the Thai recommendations on CF suggest avoiding this type of milk until the infant is 12 months old[125]. In addition, the study also demonstrated increasing use of infant and follow-on formula whereas the breastfeeding rate decreased during the weaning period. Although milk is known as a potent growth promoting factor preventing undernutrition and improving linear growth in many studies especially in low-income settings, there is also strong scientific evidence indicating its negative effects including increasing risk of overweight/ obesity[126].

3.6 Conclusions and contributions from the cross-sectional study to planning the main research project

Taken together, I saw potential links between dietary protein in complementary foods and the double burden of malnutrition in my target population. For undernutrition (i.e., underweight, wasting, stunting and iron deficiency), delayed introduction of ABP and provision of a low variety of ASFs during this transitional period may be the key drivers of sub-optimal growth, whereas replacing breast milk by infant or follow-on formula as well as unfortified cow's milk during this period may contribute to increased risk of overweight and obesity. Nevertheless, without detailed information on nutrient intake and infant growth, we cannot draw firm conclusions about the impact of dietary protein in complementary foods on the double burden of malnutrition in Thai population and further investigation is still needed.

Although a RCT would be the ideal study design to investigate if the amount or type of dietary protein is causally related to infant growth and nutritional status, I concluded that the available data were insufficient to define an appropriate intervention. Furthermore, current evidence on whether all sources of ABP – dairy and non-dairy - have the same effect on infant growth is unclear. It would be unacceptable to design an intervention providing more ABP such as meat or organ meat to Thai infants with the aim of preventing undernutrition, if there is a possibility this could lead to an increased risk of overweight/ obesity. Therefore, I decided to collect more data in a prospective cohort study focusing specifically on collecting detailed information on the intake and impact of different protein sources on infant growth, body composition and iron status which could be used to design the most appropriate intervention for a future RCT.

In the next chapter, I describe the main hypotheses, research methodology and how I measured the outcomes for my project which is the main focus of this thesis. In addition, I also present a brief background of Chiang Mai province, the main location of this cohort study, and reasons why this province was considered as a representative of a city undergoing transformation where westernised diets meet conventional feeding practices.

Chapter 4

A Prospective cohort study: Hypotheses, Research Methodology and Outcome Measurements

As discussed in the last chapter, I decided to conduct a prospective cohort study in order to investigate dietary and nutrients intakes of my target population in greater detail, in particular the impact of amount and sources of dietary protein on infant growth and iron status. This chapter describes the main research questions and relevant hypotheses. Research methodology and outcome measurements including anthropometry and laboratory measurements are also described in this chapter.

4.1 Research Questions

1) How much dietary protein and other essential nutrients are consumed by Thai infants in Chiang Mai compared to national and international recommendations?

2) What are the associations between amounts or sources of dietary protein during complementary feeding and the growth outcomes of Thai infants?

3) What are the impacts of different protein sources – animal and plant-based foods - during complementary feeding on the iron status of Thai infants?

4) What possible mechanism(s) could explain a link between dietary protein and growth in early life?

4.2 Research Objectives and Hypotheses

1) To describe current nutrient intakes, especially dietary protein, among Thai infants during the CF period and compare these to Thai Dietary Reference Intakes (DRIs) and global recommendations.

2) To investigate the association between the amounts and different protein sources consumed by Thai infant aged 6 to 12 months (M) and conditional growth at 12M **to test the hypothesis that ABP in recommended quantities can promote infant growth with lower risk of malnutrition than similar amounts of PBP.**

3) To compare the impact of different protein sources provided to infants between 6M to 12M on their body composition at 12M **to test the hypothesis that higher consumption of ABP particularly dairy protein may increase body fatness during infancy.**

4) To investigate the effects of different protein sources, animal- versus plant-based protein typically introduced during the CF period, on the iron status of Thai infants at 12M **to test the hypothesis that timely introduction and frequent intake of ABP especially liver and red meat, would improve iron status and prevent ID/ IDA.**

5) To investigate possible mechanism(s) underlying an influence of dietary protein on growth in early life through hormonal, metabolomic and epigenetic processes **to test the hypothesis that high consumption of ABP may be associated with (1) increase of EAA especially BCAA which would positively correlate with plasma levels of growth GH, IGF-1 and IGFBP-3 (2) patterns of circulating microRNA.**

4.3 Research Methodology

4.3.1 Study design

This study was a prospective, multi-centre, cohort study

4.3.2 Ethics approvals

The project obtained Ethics approvals from the Ethics committee at University College London, United Kingdom (Approval ID: 12551/ 001) and the Ethics committee at the Faculty of Medicine, Chiang Mai University, Thailand (Approval ID: PED-2561-05287) (Appendix2-3). All well-baby clinics in the participating hospitals, namely Chiang Mai University (CMU) hospital, Health Promoting hospital (HPH) and Chomthong hospital (CTH) gave official permission for the principal researcher to conduct this study at their clinics.

4.3.3 Study location

Having lived in Chiang Mai for fifteen years since I was an undergraduate student at Chiang Mai University in 2002, I can say that Chiang Mai has developed very rapidly compared with other provinces in Thailand. In the Northern region, Chiang Mai is the most developed city and also the most attractive location for both Thai tourists and foreigners (Figure 4.1). However, unlike Bangkok, it is continuing in a transitional process where urban and rural areas still coexist. In recent years, most areas in downtown have been replaced by skyline buildings, hotels, superstores, and urban neighbourhoods where people are living a fast-paced life and facing traffic jams every day. On the other hand, only few hours drive from the city centre, you are surrounded by nature and people still live simple lives in villages surrounded by mountains.

Urbanisation has changed not only the physical elements of the city, but also affects the way people live and eat. In the city centre, there are several international chain restaurants serving expensive coffee and high-calorie drinks, fried chicken, pizza and burgers on almost every street corner. Rice and sticky rice which are staple local foods are sometimes replaced by chips and bread. Nowadays, frozen/ ultra-processed foods, milk, cheeses and other dairy products are regularly consumed by people of all ages. Although, these

changes are usually found in the city centre, the urban neighbourhoods are now expanding to more areas in Chiang Mai.

Given the aforementioned issues, it is unsurprising that feeding practices and dietary intakes among infants and young children are also changing toward a western diet. Infant formula and dairy products are available in many shops and stores. Moreover, ASFs are also affordable and available in many forms which make it more convenient for families to provide more ASFs to their children. Nevertheless, many families, especially those who live in suburban and rural areas, still consume local diets containing staples and some vegetables/ fruits with/ without ASFs.

Taken together, it is quite clear that infants and young children in Chiang Mai are now facing a transformation of their diets from local to global. Therefore, they could be a good example for populations in many LMICs. These considerations helped me choose the location of the study sites for my main study. Based on the opening time and distance between each clinic, finally, I chose three well-baby clinics from two hospitals in the city center; Chiang Mai University Hospital – CMU, Health Promoting Hospital – HPH and one in a suburban area; Chom Thong Hospital - CTH (Figure 4.2)

Figure 4.1 Attractive places for tourists in Chiang Mai

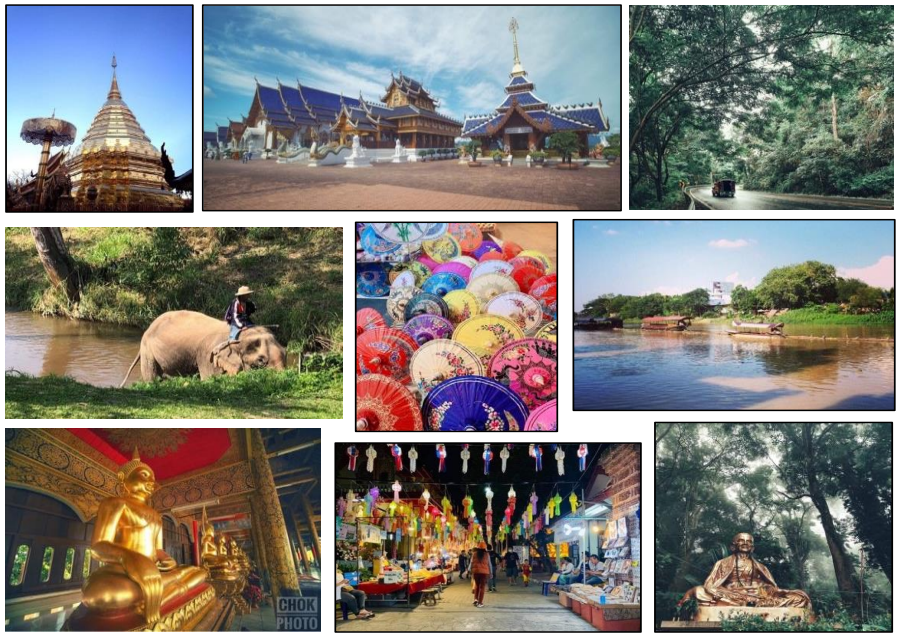


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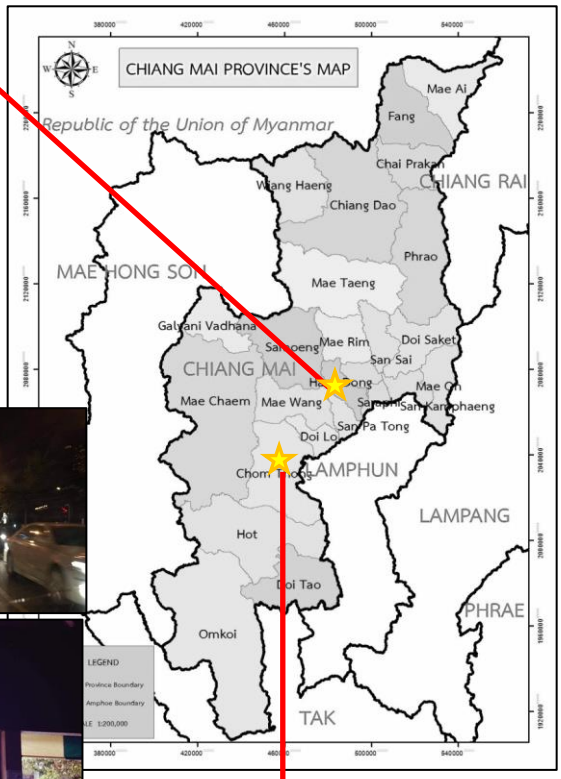
Figure 4.2 Study sites and scenery of urban and suburban area in Chiang Mai

Urban area

Chiang Mai University Hospital

Health Promoting Hospital



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Suburban area

Chom Thong Hospital

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4.3.4 Participants, Recruitment and Eligibility criteria

The target population for this study were healthy term Thai infants aged 4 months who attended one of the three well-baby clinics with their parents or legal guardian for regular immunisation and health surveillance as part of Thailand's National Immunisation Programme for infants and children.

The recruitment process started in June 2018 when all three hospitals gave official permission for access to their well-baby clinics and other hospital facilities. I first introduced my study protocol to the clinic staff, most of them nurses, before asking them to screen infants in the target group for me when the enrolment started. The clinic staff assessed whether the infant was born term, healthy and was aged between 4-6 months and sent parents/ legal guardians of eligible infants to me so I could provide more detailed information and answer their queries. If parents or legal guardians showed interest in participating in the study, I then asked them for contact details and gave them the information sheet. All families who showed interest were allowed at least 48 hours to make their final decision before being contacted again via a phone call. If parents or legal guardians decided to participate, the next appointment was booked, and written informed consent was collected at that time. Finally, 221 infants met the eligibility criteria, and their parents were given the study information.

Eligibility criteria were as follows,

Inclusion criteria

1) Full-term (gestational age $\geq$ 37 weeks) singleton infant aged 4 to 6 months
2) Birth weight $\geq$ 2,500 grams

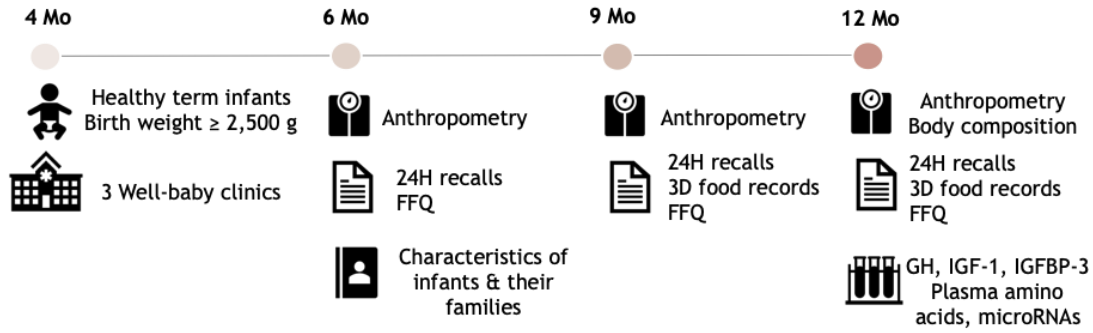
Exclusion criteria

1) Infant with underlying poor-health or chronic disease likely to affect their growth
2) Known case of PEM or recovery from PEM
3) Infant receiving any medication regularly, except for vitamins and mineral supplementation

4.3.5 Study period, Follow-up protocol and Data collection

Enrolment took place between June 2018 and September 2018. Data collection was completed in May 2019. The follow-up protocol is illustrated in Figure 4.3.

Figure 4.3 Follow-up protocol



24H recall – 24-hour food recall; 3D food record – 3-day food record; FFQ – food frequency questionnaire; GH – growth hormone; IGF-1 – insulin-like growth factor 1; IGFBP-3 – insulin-like growth factor binding protein 3

After enrolment, there were 3 visits for data collection from each participant at 6, 9 and 12 months of age. Apart from data collection, at the first visit, I also provided a brief session teaching the parent or caregiver to estimate infant dietary intake using their household utensils, and how to write the 3-day dietary record properly.

First visit: 6 months

1) Demographic data

   Family and parents - Family type, monthly income, parental age, parental education, parental occupation, parent-reported weight and height

   Infants - Prenatal diagnosis, gestational age at birth, mode of delivery, sex, birth weight and height, post-natal problem, type of milk feeding, duration of breastfeeding, age at first introduction of complementary foods, general health of infants including frequency of illness, feeding problems and food allergy

2) Anthropometric measurements

   Body weight and recumbent length

3) Dietary data

   - a 24-hour food recall (24-HR)

   - a semi-quantitative food frequency questionnaire (FFQ)

Second visit: 9 months

1) Anthropometric measurements

   Body weight and recumbent length

2) Dietary data

   - a 3-day food record (3-DFR)
   - a 24-HR
   - a semi-quantitative FFQ

3) Other information

   Type of milk feeding, feeding problems, acute illness or hospitalisation

Third visit: 12 months

1) Anthropometric measurements

   Body weight and recumbent length

2) Dietary data

   - a 3-day food record (3-DFR)
   - a 24-HR
   - a semi-quantitative FFQ

3) Other information

   Type of milk feeding, feeding problems, acute illness, or hospitalisation

4) Biological samples

   - Blood samples for serum GH, IGF1, IGFBP-3, plasma amino acids, serum iron (SI), total iron-binding capacity (TIBC), ferritin, erythrocyte sediment rate (ESR), complete blood count (CBC) and microRNAs
   - Urine samples for deuterium analysis

4.3.6 Blood sample collection, plasma preparation and storage

Non-fasting venous blood samples were collected when infants reached 12 months of age by health professionals at each well-baby clinic. Local anaesthesia using 0.5% lidocaine gel was offered before puncture, but only parents decided whether they used it or not. In total, 5 ml of venous blood was drawn and separated into 5 tubes depending on the analysis to be performed. Table 4.1 shows the type of collecting tube and blood volume required for each test. All blood samples were kept in a fridge until they were analysed the same day or prepared as serum or plasma samples for further analysis following

storage. Some photographs illustrating how I handled infant blood samples are included in appendix 4.

Table 4.1 Blood samples collection

Tube no.	Blood volume	Analytic method
1. EDTA tube	2 ml	ESR, plasma amino acids
2. EDTA tube	1 ml	microRNAs
3. EDTA microtube	0.5 ml	complete blood count
4. Clotted blood tube	1 ml	serum GH, IGF-1, IGFBP-3
5. Heparinised microtube	0.5 ml	SI, TIBC, ferritin

EDTA – ethylenediaminetetraacetic acid; ESR – erythrocyte sediment rate; GH – growth hormone; IGF-1 – insulin-like growth factor 1; IGFBP-3 – insulin-like growth factor binding protein 3; SI – serum iron; TIBC – total iron-binding capacity

Plasma preparation and storage

Blood tests for investigating iron status (i.e., ESR, complete blood count, SI, TIBC, ferritin) were done on the day of blood sample collection. For analysis of GH, IGF-1 and IGFBP-3, serum was separated from clotted blood at room temperature using 3000 rpm centrifuge for 10 minutes and kept in a –20^{O}C freezer. Plasma for evaluating amino acid concentrations and microRNAs were prepared using the same centrifugal force and duration but at 4^{O}C and immediately frozen at –80^{O}C until further analyses.

4.3.7 Deuterium dilution technique: Dosing, sample collection and storage

Infant body composition was measured by the isotope dilution technique using deuterium ($^{2}H_2$), a stable isotope of hydrogen (^{1}H) with a neutron in its nucleus. By using this technique, the total body water (TBW) is estimated by the dilution of deuterium into the body water pool[127]. Detailed information is described in section 4.5: outcome measurements and analysis methods.

Dosing

At 12M, the dose of deuterium oxide was calculated according to infant body weight (0.1 g per kg $^{2}H_2O$ body weight), prepared as a solution (in water or juice) and orally administered to infants during their final visit.

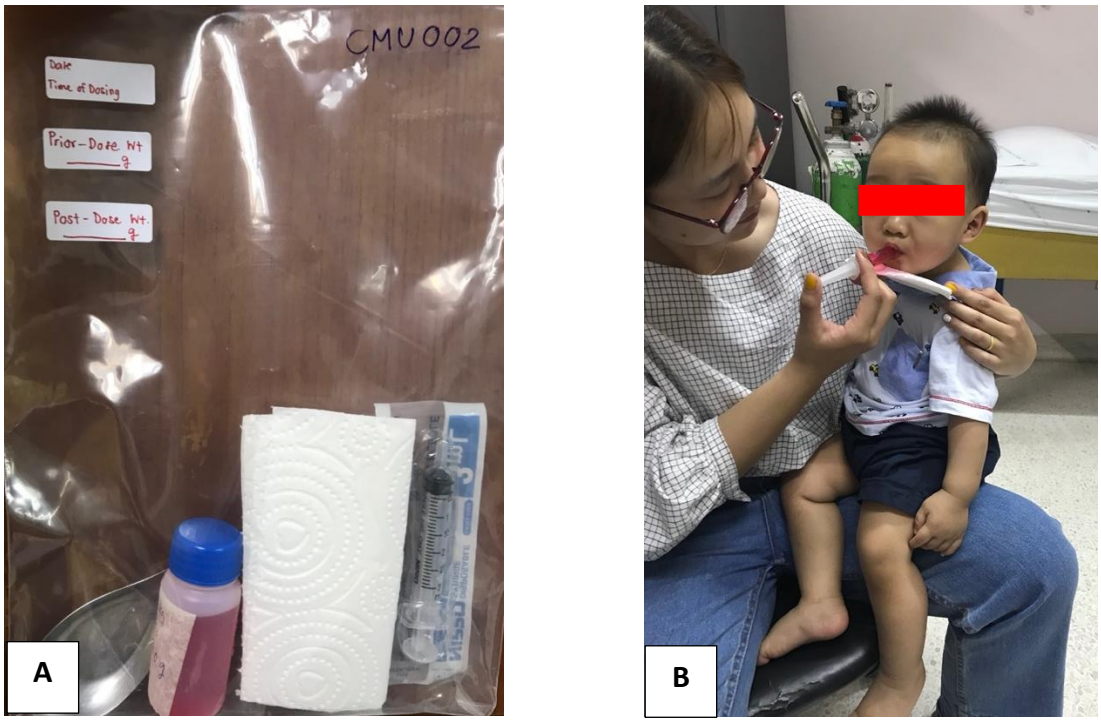


Figure 4.4

(A) The standard dosing set included deuterium solution, napkins, syringe and a spoon (either of them can be chosen for oral administration). Participant ID was labelled on the upper corner of the plastic bag.

(B) Oral administration using a syringe. Weighed napkins were placed near the infant's mouth to collect any spillage.

Sample collection

Urine samples were obtained by using a urine collecting tube/ bag or in some cases by placing cotton balls in the infant's nappy at three specific times, namely before dosing (prior to dose or baseline sample) and after dosing at 6 and 24 hours. All drinking fluids (e.g., water, milk, soup, etc.) were recorded by parents after dosing and before the second urine sample was collected at 6 hours. Appendix 5 shows details for this analysis.

Storage

Urine samples were stored at -80^{o}C until analysis.

4.4 Dietary assessment methods and estimation of nutrient intake

4.4.1 Dietary assessment methods

In general, a first and foremost step when investigating the association between dietary consumption and health outcomes is collecting valid data on nutrient intake data from the target population. Although a multiple day-weighed record is recommended as the gold standard approach, this method is time consuming, burdensome, more expensive, and incurs a higher drop-out rate compared with other dietary assessment methods[128]. Therefore, I selected more convenient tools which are acceptable for an infant population[129-131].

Three types of dietary assessment methods were used in this study to meet two objectives.

1) To quantify nutrient intakes of infants on a daily basis at each visit, I used a 24-HR and an estimated 3-DFR.
2) To assess habitual intake of some specific foods consumed by Thai infants, I used a semi-quantitative FFQ.

24-HR

This method was an interview-based dietary assessment collected by a trained healthcare professional at each study visit. The report form of the 24-HR consisted of study ID, date and time of data collection and dietary data including type and amount of food consumed by infants over 24 hours on the day before the interview (Appendix 6). During the interview, an interviewer showed standard household utensils to aid an estimation of amount of dietary intakes.

Estimated 3-DFR

In contrast to the 24-HR and FFQ, this method was used to collect dietary intake only at the second and third visit. I did not used this method to collect dietary intake at the first visit because my cross-sectional study (more details in Chapter 3) showed that Thai infants were rarely given complementary foods before 6M and their dietary patterns were therefore less variable than at 9M and 12M, so I considered that the 24-HR and FFQ would provide adequate

information. Furthermore, it would reduce the burden on parents so, they would be willing to complete the records at 9 and 12 months.

The report form for the 3-DFR was adapted from one validated by Lanigan et al[4] with the author's permission and translated into Thai language by a Thai native speaker. The 3-DFR contains four main parts. The first part shows 2-dimensional pictures of various types of spoons with labels. The second part includes some examples of dietary records and is followed by the recording section. This section has free spaces for caregivers to complete the date, mealtimes and details of food consumption. The recipe section is the last part, where caregivers can freely describe the recipes recorded in the third part if they are used in multiple meals or contain many ingredients (Appendix 7).

To improve accuracy of the quantitative data, all parents and caregivers received a brief session teaching them how to estimate dietary intake using their household utensils and how to record the 3-DFR properly.

Semi-quantitative food frequency questionnaire

Given the lack of a validated FFQ suitable for obtaining dietary data in Thai infants, I developed the FFQ used in this study by gathering food items consumed by Thai infants and toddlers aged 0 – 3 years old from a national survey, the Food Consumption Data of Thailand in 2016[132]. Finally, 130 food items were selected and classified into 11 different groups namely,

1) Milk and dairy products
2) Meat and meat products
3) Eggs
4) Cereals and cereal-based products
5) Legumes
6) Vegetables
7) Fruits
8) Beverage and snacks
9) Sugar and sweets
10) Oils and seasonings
11) Dietary supplementation

The quantity and frequency of consumption of each item were recorded by a field researcher at each visit. The aim of the FFQ was to record information on the habitual dietary intake of each infant between the study visits (Appendix 8).

As I was the only field researcher, both 24-HR and FFQ were recorded by me. A small number of infants, on average 3 (range 1-7), visited each well-baby clinic at each appointment, thus I was able to collect all the information. However, in cases of time-constraints, some 24-HR were obtained later by a telephone call within 24-72 hours.

4.4.2 Nutrient analysis software and Dietary composition databases

The study mainly used the Thai food composition software called the INMUCAL-Nutrients programme, version 4.0 (2018)[133] developed by the Institute of Nutrition, Mahidol University, Thailand, to convert dietary data to nutrient intakes. Micronutrients including calcium, phosphorous, iron, zinc, vitamin A, vitamin B1, vitamin B2 and vitamin C were reported along with calories and macronutrients. In some cases when nutrient profiles were not available in this software, other reliable sources such as the United States Department of Agriculture (USDA)[134] or the Food and Agriculture Organisation of the United Nations (FAO)[135] were used instead.

For some commercial products for which nutritional information was unavailable in the INMUCAL-Nutrients software, parents were asked to bring or show packages from the products to a field researcher to find out more details.

4.5 Outcome measurements and analysis methods

According to the aforementioned hypotheses, the study outcomes were divided into three main categories including evaluation of infant growth and body composition, assessment of infant iron status and finally, investigation of underlying mechanisms linking dietary protein intake and infant growth. Anthropometric measurements were carried out at all study visits while the

rest of the outcomes (i.e., body composition, iron status, plasma amino acids and microRNAs) were measured when infants were 12M.

4.5.1 Anthropometric measurements, Standard deviation scores (z-scores), and Conditional growth

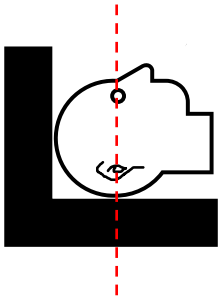
Infant growth was presented as both linear and ponderal growth. Recumbent length was reported in centimetres while kilograms were used to report infant body weight. Table 4.2 shows the measuring tools used in this study. All measuring tools were regularly calibrated throughout the study period. The trained health professionals at each well-baby clinic measured and recorded body weight and length. In the first few weeks of data collection, I observed while they were measuring weight and length to make sure that they understood how to measure weight and length correctly. In addition, I also re-checked the records and asked for re-measurement if the reported figures were in doubt.

Table 4.2 Anthropometric measurements

Parameters	Measuring tools	Methods
Recumbent length*,**	A standard wood board accurate to 0.1 cm	Infant's body is in a supine position with head against a fixed board while the body is parallel to the board's axis following the Frankfurt Plane position (Figure 4.5) and both legs were straightened
Body weight*	An electronic scale† accurate to 0.01 kg	Infant wears a nappy with other accessories being removed

**Both parameters were measured once, but if figure was unusual, the measurement was repeated; **There were 2 people involving in this measurement. A health professional was a recorder while one parent/ caregiver held infants in a straight position; †The models used at each clinic were KROTRON® SKS-2001SB (Chiang Mai University hospital), SECA® 374 (Chomthong hospital) and TSCALE® M101 (Health-promoting hospital). The precision of these scales was ± 5 grams and all scales were regularly calibrated with the same weight.*

Figure 4.5 Frankfurt Plane Position



All measured weights and lengths were converted into standard deviation scores (z-scores) for the infant's age and sex using the WHO software (Anthro version 3.2.2) which incorporates data from the WHO growth standard 2007[136]. All growth parameters, namely weight-for-age z-score (WAZ), weight-for-length z-score (WLZ), body mass index z-score (BMIZ) and length-for-age z-score (LAZ) were used as indicators of infant growth in this study. Although the WHO recommends that weight-for-length should be used to determined wasting and overweight for children aged less than 2 years, a recent study showed that infant BMI had higher positive predictive value than weight-for-length for childhood obesity[137] thus, I presented both indices. Definitions of nutritional status are described in "List of Definitions (page 16)".

In addition to the standard deviation scores, I also presented the growth outcomes in the form of "conditional growth". Conditional growth is considered a better indicator representing growth at any given age regardless of the influence of previous body size[138, 139]. Infants who are born bigger tend to be heavier when they are older than infants having a lower birth weight, with a similar pattern for linear growth. Therefore, to investigate the impact of dietary protein in complementary foods on infant growth, conditional growth at 12M is preferable as it removes the influences of birth weight and body weight at 6 months of age from the final growth outcomes. It essentially indicates whether an infant grows more or less than expected over a period of time, taking into account its measurement at the start. This approach is widely used in research investigating how growth in early life affects later body size and body composition, including long term health effects such as hypertension and diabetes[140-143].

Conditional growth, so-called conditional body size, is a residual between a measured growth parameter, such as body weight or length, at a given age and the expected value at that age given the earlier body size. A linear regression model is used to calculate the expected growth. For example, to calculate conditional length at 12 months (M) using length at 6M, I firstly use a linear regression model with measured length at 12M as the outcome and measured length at 6M as the predictor. The simplest equation would be:

$$\text{Expected Length 12M} = a + b\,(\text{Length 6M})$$

If a is a constant when length at 6M is equal to zero
b is a regression coefficient indicating change in the expected length at 12M corresponded to every 1 cm increase in length at 6M

The next step is to calculate conditional length at 12M of age by subtracting expected length from measured length at 12M as shown below:

$$\text{Conditional length 12M} = \text{Measured length 12M} - \text{Expected length 12M}$$

In this study, I also standardized all conditional growth measurements by dividing them by the standard deviation to demonstrate how they deviate from the median. Interpretations of conditional growth are shown in the following table. Figure 4.4 is an example of conditional WLZ at 12M using my data set.

Table 4.3 Interpretations of conditional growth

If conditional growth is	Interpretation
Negative value	Infant grows less than expected for the given population which might suggest increased risk of undernutrition
Around Zero	Infant grows as expected compared to the growth pattern of the given population
Positive value	Infant grows more than expected for the given population which might suggest increased risk of overweight/ obesity

Figure 4.6 Correlation between conditional WLZ at 12M and regression line

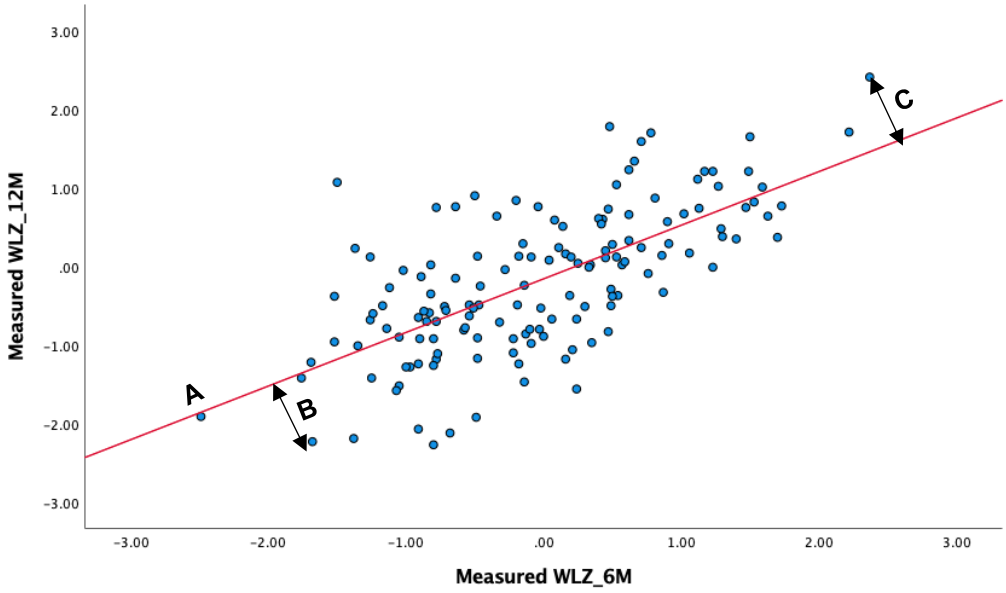


Fig 4.6 The **red line** represents a regression (median) line formulated by using measured weight-for-length z-score (WLZ) 6 months old (M) to predict WLZ at 12M; **Double headed arrows** represent residuals (conditional WLZ 12M); **A** is a residual with a value equal to zero (growth as expected); **B** has a negative value (growth less than expected); **C** has a positive value (growth more than expected). The conditional growth value indicates the magnitude of deviation from expected growth.

4.5.2 Body composition

In this study, I used the deuterium dilution technique to determine total body water (TBW) of infants and thus to estimate fat free mass (FFM), assuming a FFM hydration of 79%. Dilution techniques are based on the equation[144]:

$$C_1V_1 = C_2V_2 = \text{Constant}$$

C is the tracer (Deuterium oxide) concentration; V is the volume

When the infant is given a known amount of deuterium oxide (C_1 x V_1) which is diluted in a given body compartment, the volume of the body compartment (V_2) can be calculated by dividing the constant value by the concentration of deuterium in that body compartment (C_2) after equilibrium has been reached (mostly around 3-5 hours after dosing). Details of dosing, sample collection and storage were described in section 4.3.7.

Deuterium enrichment of urine samples is measured using isotope ratio mass spectrometry (IRMS, Gasbench-Delta XP system, Thermofisher Scientific, Bremen, Germany) at UCL GOSH ICH. The data are used to calculate the deuterium space (N) using the back-extrapolation method (Formula 1). As deuterium exchanges in the body with hydroxyl groups from other molecules, thus the deuterium space has to be corrected for the non-aqueous dilution (4-5%)[144]. Formula 2 shows TBW corrected by hydrogen space. Based on assumption that 79% of FFM in infants is water, FFM is finally calculated by using Formula 3. Other parameters namely fat mass (FM), fat free mass index (FFMI), fat mass index (FMI) and percentage of body fat (%BF) can be calculated using Formulae 4 to 7, respectively.

<u>Formula using to calculate body composition</u>

1) Deuterium space (N) = AT/ a (Ed – Et/ Es – Ep)

   A is the dose given to infants, **T** is the volume of tap water in which the dose is diluted, **a** is the portion of dose diluted, **E** is the isotope enrichment of: **d** – dose; **t** – tap water; **s** – post-dose and **p** – pre-dose
2) TBW (kg) = Deuterium space (N) x hydrogen space (0.96)
3) FFM (kg) = TBW/ 0.79
4) FM (kg) = Body weight (kg) – FFM
5) FFMI (kg/ m^2) = FFM/ length $(m)^2$

6) FMI (kg/ m^2) = FM/ length $(m)^2$
7) %BF = 100 x (FM/ Body weight)

4.5.3 Anaemia and iron status

Definitions of anaemia, iron deficiency (ID) and iron deficiency anaemia (IDA) have been described in the “List of Definitions”. Several tests were used to evaluate these outcomes, namely complete blood count (CBC), serum iron (SI), total iron binding capacity (TIBC), serum ferritin (SF) and erythrocyte sediment rate (ESR). Details of the tests, machines and quality controls are shown in Table 4.4. For anaemic infants who had normal iron status, peripheral blood smears were examined by a paediatrician under supervision of a paediatric hematologist to look for Thalassemia or other causes of anaemia. All of these tests were carried out at the Chiang Mai University Hospital’s laboratory centre, Chiang Mai, Thailand on the day of blood sample collection.

Table 4.4 Laboratory tests investigating anaemia and iron status

Lab results	Quality control and precisions	Measuring Techniques	Machines
Hb (g/dL)	ISO15189	Automated haematological analyser	Sysmex® XN 9000, Sysmex UK Ltd., UK
SI (µmol/L)	Intra-assay ≤ 1.1% Inter-assay ≤ 1.8%	Chemiluminescent	Cobas® modular analyser, Roche Diagnostics, F. Hoffmann-La Roche Ltd., Germany
TIBC (µmol/L)	Intra-assay ≤ 2.4% Inter-assay ≤ 4.7%		
SF (µg/L)	Intra-assay ≤ 9.5% Inter-assay ≤ 13%		
ESR (mm/h)	Built-in internal quality control	Automated system for direct determination of ESR	VES-MATIC cube 30, DIESSE Diagnostica Senese S.p.A., Italy

Hb – haemoglobin; SI – serum iron; TIBC – total iron binding capacity; SF – serum ferritin; ESR – erythrocyte sediment rate

4.5.4 Plasma amino acid analysis

High-performance liquid chromatography was used to measure plasma concentrations of amino acids (Biochrom® 30+ amino acid analyzer (Biochrom Ltd., United Kingdom)). Plasma samples that were aliquoted into 1 ml tubes were stored in a -20ºC freezer until analysis. The plasma samples were initially deproteinized with 6% sulfosalicylic acid (1:1 v/v). After mixing, the samples were centrifuged at 10,000 rpm for 10 minutes. Then 80 μL of the supernatant from each sample was added to 20 μL of Norleucine as an internal standard and the pH adjusted to 2.2 with lithium hydroxide before analysis according to the method of Shapira et al[145]. Free amino acids were determined by ion exchange chromatography using the automatic amino acid analyser. A series of lithium buffer solutions were run through a lithium column containing the amino acids in solution. Individual amino acids were eluted according to their pH. Post-column derivatization with ninhydrin was utilized to elicit a spectrum of colours at different wavelengths (440 and 570 nm). Data analysis was performed using the software EZChrome Elite (SIM GmbH, Germany).

This plasma amino acid analysis was performed by the laboratory centre at the Faculty of Medicine Ramathibodi Hospital, Mahidol University, Bangkok, Thailand. Quality control for this test has been regularly performed under the quality control schemes of the European Research Network for evaluation and improvement of screening, diagnosis and treatment of Inherited disorders of Metabolism (ERNDIM) aiming to assure and standardise procedures for diagnosis, treatment and monitoring of inherited metabolic diseases.

4.5.5 Growth hormone, IGF-1 and IGFBP-3

These laboratory results were analysed by using a solid-phase, enzyme-labeled chemiluminescent immunometric assay using the IMMULITE® 2000 systems (Siemens Healthcare Diagnostics Products Inc., United States). The stored serum was thawed and aliquoted into 3 tubes containing 25, 20 and 5 μL for GH, IGF-1 and IGFBP-3 analysis, respectively. Two-hundred beads coated with anti-GH, anti-IGF-1 and anti-IGFBP-3 were placed in each corresponding tube. The different amounts of alkaline phosphatase suggested

by the company's leaflets were used to conjugate antibody in buffer for the tests. Serum concentrations of GH, IGF-1 and IGFBP-3 were analysed using a photomultiplier tube to detect photon beams from reactions between antibody and antigen complexes. The intra- and inter-assay variation of all tests were less than 8%. These tests were done at the laboratory centre, Department of Paediatrics, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand. In case of a suspected error, the laboratory test was repeated for a confirmation.

Chapter 5

Statistical Analyses

Apart from the sample size estimation, two main statistical analysis sections are presented in this chapter - namely descriptive and analytical parts. The descriptive results present demographic data of infants and their families as well as nutrient intake and feeding practices during the weaning period including both milk and complementary foods consumed by our population. The prevalence of malnutrition at both levels, individual and household, are also shown in numbers and percentages.

The second part of the analyses contains the majority of key results as the main hypotheses required analytical methods to investigate whether dietary protein affected infant growth and iron status. Furthermore, relationships between growth outcomes and proposed underlying mechanisms also needed more complex statistical methods such as multivariable linear regression models. I also used a directed acyclic graph (DAG) to select co-variates for the regression model instead of the previous approach selecting covariates simply based on prior knowledge or data. The use of DAG may increase causal inference.

Statistical analyses in this thesis were performed using IBM SPSS version 26.0 (Armonk, New York: IBM Corp) licensed for University College London. Significance was accepted if the p-value was less than 0.05 or 95%CI of OR did not include 1.

5.1 Sample size estimation

As no directly comparable data were available on which to base a sample size estimation at the time of conceptualisation of this cohort, four different approaches were used to estimate the number of participants required.

Method 1: Using two-different means formula

Aim: To detect a significant difference in the **total protein intake** (g/day) between stunted (n = 58) and non-stunted subjects (n = 169) (based on Thai infants and young children)

Mean difference = 4.7 g/day[53] and SD = 8.4[53]

If power 80% and significance level = 0.05

Sample size required = 102 → +15% dropouts = 117

Method 2: Using two-different means formula

Aim: To detect a significant difference in the **WLZ** between infants aged 6M (n = 230) and 12M (n = 190) based on data from Indonesian infants

Mean difference = 0.5 z-scores[34] and SD = 1.0[34]

If power 80% and significance level = 0.05

Sample size required = 126 → +15% dropouts = 148

Method 3: Using two-different means formula

Aim: To detect a significant difference in the **WLZ at 12 months of age** compared between infants who receive red meat frequently (n = 38) and infants who receive meat less frequently during the CF period (n =38)

Mean difference = 0.5 z-scores[43] and SD = 1.0[43]

If power 80% and significance level = 0.05

Sample size required = 126 → +15% dropouts = 148

Method 4: Formula for multiple regression analysis

Aim: to calculate the minimum required **sample size for a study using multiple regression analysis**, given the desired probability level, the number of predictors in the model, the anticipated effect size, and the desired statistical power level[146].

Anticipated effect size (f^2) = 0.15 (medium effect)

Numbers of predictors* = 10 (from previous studies)

Power of study 80%, significance level 0.05

Sample size required = 118 → +15% dropouts = 140

*_Predictors_ = sex, birth weight, maternal BMI, maternal education, family income, total caloric intake, duration of breastfeeding, type of dietary proteins, total protein intake, baseline growth parameters

Considering all these approaches,**150 participants** was considered to be an adequate target.

5.2 Normality test of data distribution

For continuous variables, I evaluated the normality of each variable using the Kolmogorov-Smirnov statistic and histogram prior to performing statistical tests. Normal distribution was defined if the p-value from the Kolmogorov-Smirnov test was non-significant ($p \geq 0.05$), the Q-Q plot was normal, and its histogram shown a symmetrical, bell-shaped curve. For normally distributed variables, I chose a parametric test whereas either data transformation or a non-parametric test was considered in case of extreme skewness (skewness > 1.0) based on the objective and assumptions made by the given statistical method.

5.3 Transformation of data using natural log

Transformation of data is considered as a method to turn skewed continuous variables into a normal distribution in order to better meet the assumptions of several parametric techniques. It involves mathematical modification such as square root, inversion and logarithm[147].

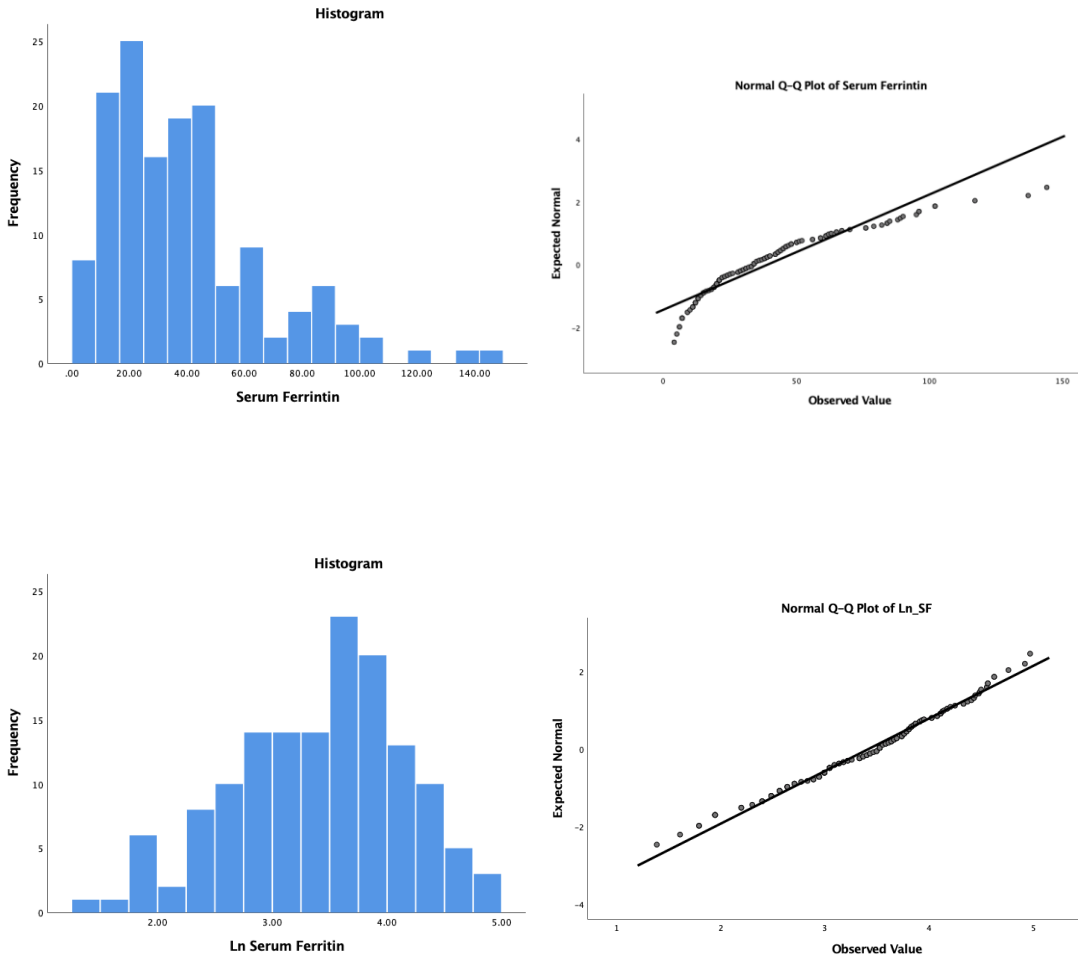
In this study, I preferred using log e, so-called natural log (Ln) to transform the extremely skewed variables. From a statistical perspective, it is unnecessary to back-transform the Ln transformed data in order to interpret on the original scale, as a difference of Ln corresponds to a fractional difference on the original scale. In other words, the transform y = 100 Ln (x) leads to differences, standard deviations (SD), and regression coefficients (β) of y that are equivalent to symmetric percentage (sympercent, s%) differences, SD and β

of x^{148}. For example, if the β of simple linear regression model predicting Ln (serum ferritin) by daily protein intake equals 10. It can be interpreted to mean that every 1 g increase of daily protein intake is associated with a 10% increase in serum ferritin. Table 5.1 and Figure 5.1 demonstrate an example of Ln transformation of serum ferritin from my actual data.

Table 5.1 Normality test of serum ferritin before and after Ln transformation

Normality test	Serum Ferritin	Ln Serum ferritin
Kolmogorov-Smirnov	p-value < 0.001	p-value 0.06
Skewness	1.352	- 0.347

Figure 5.1 Histograms and normal Q-Q plots of serum ferritin before and after Ln transformation



Ln – natural log; Q-Q plot – quantile to quantile plot

5.4 Descriptive analyses

Categorical variables such as infant sex, prevalence of malnutrition, exclusive breastfeeding rate, family's socioeconomic status, and educational attainment of parents are shown as numbers and percentages.

Continuous variables used to describe characteristics of infants and their families, nutrient intakes as well as details of feeding practices that were not involved in the comparison or exploration of relationship between variables were analysed as mean $\pm$ SD or median with interquartile range (IQR) depending on their data distributions.

5.5 Comparisons of independent groups and related samples

For categorical variables, I used Chi-square test to explore whether observed frequencies or proportions of cases were different between two independent variables. In rare cases where less than 80% of cells had expected frequencies of 5 or more, I reported the results of Fisher's exact probability test instead.

For continuous variables, tests for comparison between independent and related variables were chosen based on the number of variables. Non-parametric tests were used if a continuous variable showed extreme skewness. If there were at least 3 groups in a comparison, post-hoc analysis using Bonferroni test was applied to identify which pairs were significantly different. The statistical tests used to compare continuous variables are shown in Table 5.2.

Table 5.2 Statistical tests used to compare mean differences between independent groups and related samples

Statistical tests	Parametric	Non-parametric
Dependent groups • 2 groups • ≥ 3 groups	Student's t-test One-way ANOVA	Mann-Whitney U test Kruskal-Wallis test
Related samples • 2 time points • ≥ 3 time points	Paired t-test Repeated ANOVA	Wilcoxon Signed Rank test Friedman test

ANOVA – Analysis of Variance

5.6 Correlation analysis

Correlation analysis was used to describe strength and direction of the linear relationship between two variables whether it was a continuous level (parametric test - Pearson's correlation) or ordinal level (non-parametric test - Spearman's correlation).

Correlation analysis was used to help me;

1) Decide whether I should investigate the relationship between two variables of interest by using a linear regression model. For example, if I found a positive correlation between daily protein intake (independent variable) and WLZ (dependent variable), this suggested that I could explore this relationship in more detail by using linear regression.
2) Describe relationships between nutrient intakes determined using two dietary assessment methods which I describe in more detail in section 5.9.

According to Cohen[149], strength of correlations can be interpreted as follows;

Small – if correlation coefficient (r) = 0.10 to 0.29

Medium – if r = 0.30 to 0.49

Large – if r = 0.50 to 1.00

5.7 General Linear Models (GLM) and selection of confounding factors

The majority of research hypotheses were tested by GLM including regression analysis, Analysis of Variance (ANOVA) and factor analysis. Types of regression analysis were chosen based on characteristics of independent variables (predictors) and dependent variables (outcomes) according to table 5.3. ANOVA models were considered to determine differences of outcomes according to different groups or categories. In a similar way to regression analysis, confounding factors in the form of categorical or numeric variables can also be entered into an ANOVA model to control for their influence.

The following three steps were used as to investigate whether protein intake could affect the outcomes (i.e., infant growth and iron status)

1) Selection of confounding factors by DAG

   Confounding factors are variables intervening in the causal assumption between predictors and outcomes of interest as they influence both predictors and outcomes. In order to select them appropriately, I used the technique called DAG. This technique provides the minimum set of confounders to enter in multivariable regression analyses. More details of this technique are described later in this section.

2) Univariate regression analyses

   This step was used to explore the effect of each predictor and potential confounder on particular outcomes. For potential confounders, this analysis will help to identify statistically significant confounders that should be included in the final model. With this approach, one predictor/ confounder was entered to predict one outcome. The statistical methods used in this step were simple linear regression, logistic regression and univariate ANOVA.

3) Multivariable regression analyses

Multivariable regression analysis was used for all statistical analyses investigating impacts of protein intake on the outcomes (i.e., infant growth and iron status). Potential confounders were entered into the multivariable regression model as controlled variables in order to demonstrate the actual effect of protein intake on infant growth and iron status. There were two models at this step. The first model included all confounders suggested by the DAG while the final model included only confounders that were statistically significant in the univariate regression model/ the first model as co-variates of the main predictor. The statistical methods used in this step were multiple/ multivariate linear regression and multivariate ANOVA.

Table 5.3 Types of regression analysis used in this study

Regression Models	Predictors		Outcomes	
	Characteristics	Number	Characteristics	Number
Linear				
- Simple	Continuous	1	Continuous	1
- Multiple	Continuous	≥ 2	Continuous	1
- Multivariate	Continuous	≥ 2	Continuous	≥ 2
Logistic				
- Binary	Category/Continuous	≥ 1	Binary	1
- Multinominal	Category/Continuous	≥ 1	≥ 3 Categories	1

Directed Acyclic Graphs (DAG)

As a cohort study cannot demonstrate causal relationships between predictors and outcomes by its nature, it is very important to consider which variables should be controlled for when investigating the impact of a predictor on a particular outcome. Although causality cannot be established in an observational study, careful control of potential confounders can allow greater causal inference to be made. Therefore, I used DAG to identify potential confounders of the causal assumptions between protein intake (predictor) and my outcomes of interest (infant growth and iron status).

What is a DAG?

DAGs, so called causal graphs are graphical tools/ diagrams illustrating the key concepts of exposure/ predictor, outcomes, causation, confounding and bias[150]. In other words, they provide a structural relation between variables and distinguish causal effects between predictor and particular outcome from bias paths relating to confounders[151]. As suggested by the title, a path linking two variables has to be directed from one variable to another variable by a unidirectional arrow (Figure 5.2) while the paths linking ≥ 3 variables need to be acyclic, meaning that arrows are not allowed to go from one variable to another to form a closed loop (Figure 5.2). In a DAG, an arrow pointing from A to B means that A affects B. The complexity of a DAG depends on how many variables are involved in the causal path between the predictor and the outcome; the information usually comes from published scientific evidence and prior knowledge. For example, if we would like to see the effect of protein intake on infant growth, we must identify other variables that affect either protein intake or infant growth. Some of those variables might end up as confounding factors affecting both protein intake and infant growth and need to be adjusted for in the multivariable regression model.

Why is the DAG a preferable approach for selecting co-variates?

In general, causal inference in observational studies is limited due to biases. A bias is a systematic error that contributes to misinterpretation of the actual relationship between the predictor and the outcome[150] and should be distinguished from a random error or lack of precision[152] which can be eliminated by increasing sample size. Confounding bias is one type of bias that causes spurious relationships between predictor and outcomes. The bias caused by a confounder, a variable that affects both the exposure and the outcome, can be reduced by controlling for this variable[153]. In contrast to confounders, mediators and colliders involved in the casual path do not need to be controlled for. A mediator is a variable that lies on the casual path between the predictor and the outcome while a collider is a variable being a

common effect of both predictor and outcomes[153]. While controlling for confounders can reduce bias, adjusting for colliders can increase bias[153, 154]. For mediators, controlling for these variables may or may not result in bias, depending on the research question. If the researcher aims to investigate only direct effects of the predictor on the outcomes, the mediator should be controlled for, but if the researcher looks for the total effect, controlling for the mediator might diminish the total effect of the predictor on the outcomes[153]. Examples of a confounder, mediator and collider are shown in Figure 5.3.

According to this information, if we use the common previous approach by selecting co-variates from significant correlations or significant outcomes from published articles, without considering the type of variable or relationships between variables, this might lead to unrecognized bias by controlling for the wrong covariates. Using the DAG can reduce subjectivity in deciding what variables should be adjusted for and improve the casual inference when investigating the effect of the predictor on the outcome. Figure 5.4 illustrates the difference between using the common previous approach and DAG approach to select co-variates for multivariable regression models.

Creating and using the DAG

In this thesis, I chose the widely used free browser-based software called “Digitty.net” version 3.0 (2020) developed by German scientists[155]. I have created two DAGs, one for growth outcomes and another for iron status shown in Chapters 7 Results 3 and 4, respectively. After entering all causal paths for the relevant variables in a section called “the Model code”, the software automatically created two exposure-outcome adjustment sets, one group for the total effect (including effect via mediator) and one for the direct effect (mediator effect is removed), in order to assess potential causality between the predictor and the outcome, based on the assumption that no other confounders are at play.

Figure 5.2 Example of casual paths between variables

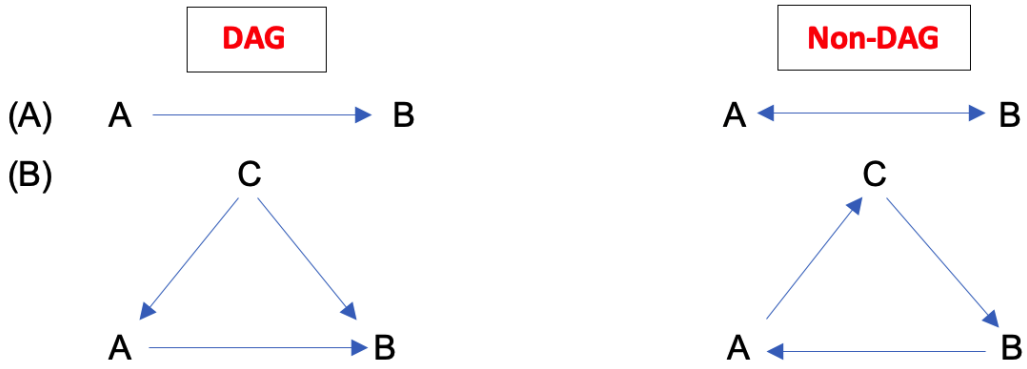
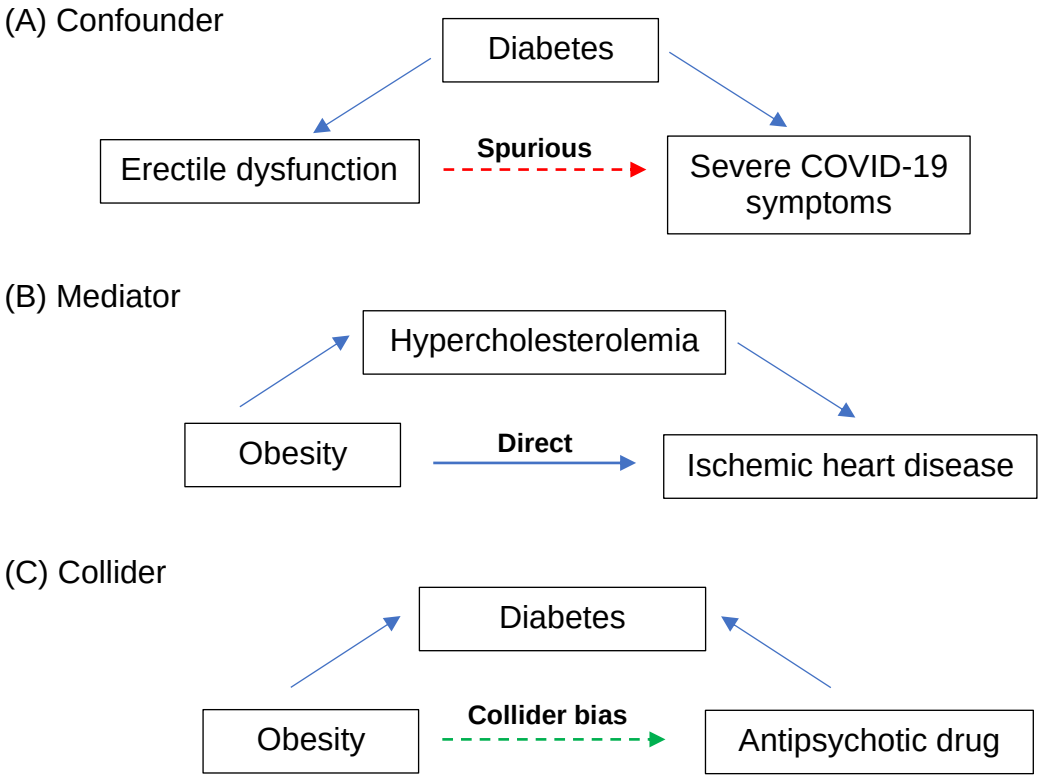
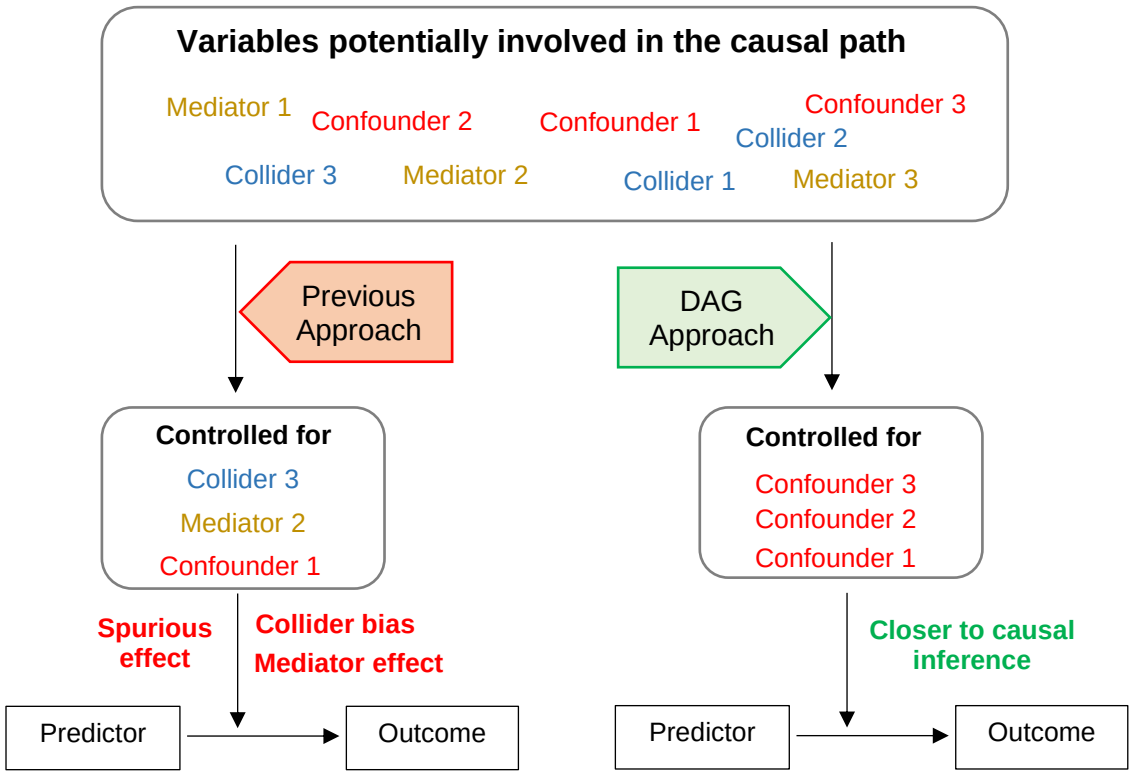


Figure 5.3 Example of confounder, mediator and collider



This figure illustrates type of co-variate; (A) diabetes is a confounder causing both erectile dysfunction and severe COVID-19 symptoms. If diabetes is not adjusted for in the model, there might be a spurious association (red dash line) between erectile dysfunction and severe COVID-19 symptoms; (B) hypercholesterolemia is a mediator between obesity and ischemic heart disease. However, obesity also directly affects ischemic heart disease via other pathways such as systemic inflammation. If researchers control for hypercholesterolemia, they will miss the total effect of obesity on ischemic heart via mediator; (C) diabetes is a common outcome between obese people and people taking antipsychotic drugs. If diabetes is adjusted for, it might open a new path (collider bias) suggesting that obesity results in taking antipsychotic drugs.

Figure 5.4 The difference between the commonly used previous approach and DAG approach



5.8 Statistics used to determine agreement between 24-HR and 3-DFR

According to previous evidence, 3-4 days of dietary data are ideally required to provide an accurate assessment of nutrient intake in infants[156,157]. Therefore, dietary data from 3-DFR seemed to be more appropriate than 24-HR. However, for cases where 3-DFR were missing or incomplete, I wanted to be able to use dietary data from the 24-HR instead.

In order to justify using dietary data from 24-HR in this way, the agreement between the 24-HR and 3-DFR needed to be clarified. In this thesis, I applied 6 statistical tests to analyse the agreement between these two methods which represented agreements at both group and individual level (Table 5.4)

The outcomes of the agreement analysis between 24-HR and 3-DFR are presented in the next chapter (Chapter 6, section 6.4).

Table 5.4 Statistical tests and interpretation criteria used to determine agreement between 24-HR and 3-DFR

Statistical test	Interpretation criteria[158]	
	acceptable to good outcome	poor outcome
Group level		
1) Paired t-test	p-value > 0.05	≤ 0.05
2) Percent difference	< 10%	> 10%
3) Weighed Kappa (Kw)	Kw > 0.2	≤ 0.2
4) Bland-Altman analysis	Narrow LOA Absent of bias	Wide LOA Present of bias
Individual level		
1) Correlation coefficient (r)	r ≥ 0.3	< 0.3
2) Cross-classification (quartiles)	Same quartile >50% Opposite quartile <10%	<50% >10%

LOA – Limit of Agreement

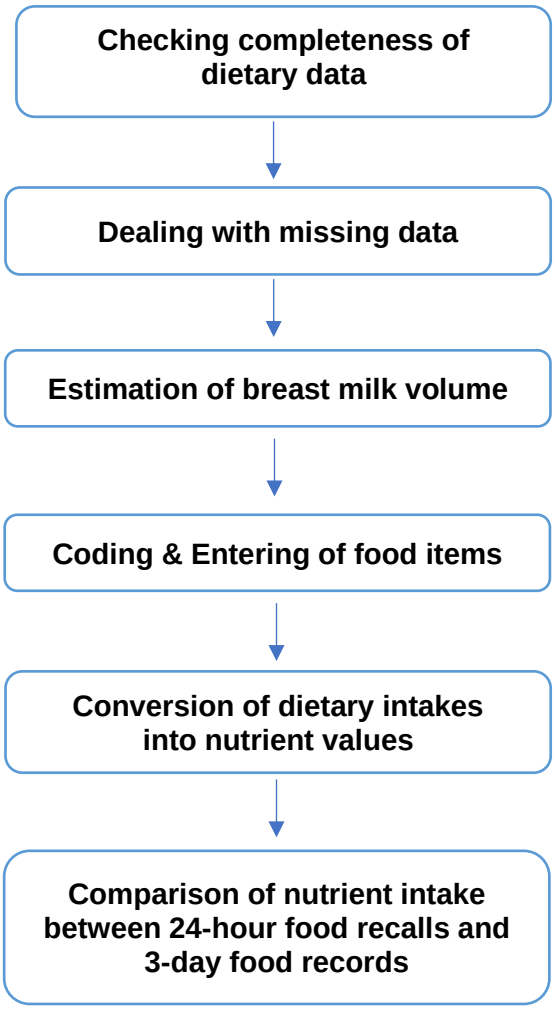
In the next chapter I will describe how I managed my research data and also present the outcomes from statistical analyses of agreement between 24-HR and 3-DFR, which is an essential first step before investigating the main hypotheses.

Chapter 6

Management of dietary data

Dietary data were an important part of data collection and required a number of steps to turn dietary consumptions into estimated nutrient intakes. A primary aim of the estimated nutrient intakes was to use them as a key predictor or co-variate in further analyses, for example, protein intake, non-protein calories, iron intake. This chapter will provide details of how the dietary data were handled, transformed, and selected for further analyses. Figure 6.1 gives an overview of this chapter.

Figure 6.1 An overview of the important steps in this chapter



6.1 Completeness of dietary data and dealing with missing data

As described in Chapter 4 section 4.4, dietary data were collected by three dietary assessment methods including a 24-HR, 3-DFR and FFQ; however, at 6 months only the 24-HR and FFQ were used. Each subject was expected to provide three data sets (at 6, 9 and 12 months old) for the 24-HR and FFQ but only two data sets (at 9 and 12 months old) for the 3-DFR. In total, there were 8 sets of dietary data that needed to be checked for their completeness.

Completeness of dietary data

As the 24-HR and FFQ were obtained by interview, they were unlikely to be left blank unless parents did not have time to finish the interview due to their availability at the clinics. In these cases, the field researcher contacted them later within the same week and there were only rare cases where parents refused or were unavailable for phone interview. In contrast to these methods, the completeness of the 3-DFR was more problematic as there were more missing data, unclear handwriting and unrealistic portion sizes. Therefore, the completeness of dietary data from the 3-DFR were double checked by the field researchers at the clinics (missing data, legibility) and within the same week (portion size). The flow chart (Figure 6.2) shows how each problem was managed during data collection.

Although the completeness of dietary data was carefully handled during data collection, there were still small numbers with missing data as shown in the table below (Table 6.1). The total number of 145 comes from the final number of infants after exclusion of dropouts (n = 4) and one infant who had developed multiple food allergies during the study period (n = 1) which limited his consumption of protein sources (Figure 6.3).

Figure 6.2 Flow chart demonstrating how the researcher managed to complete dietary data for the 3-day food record (3-DFR)

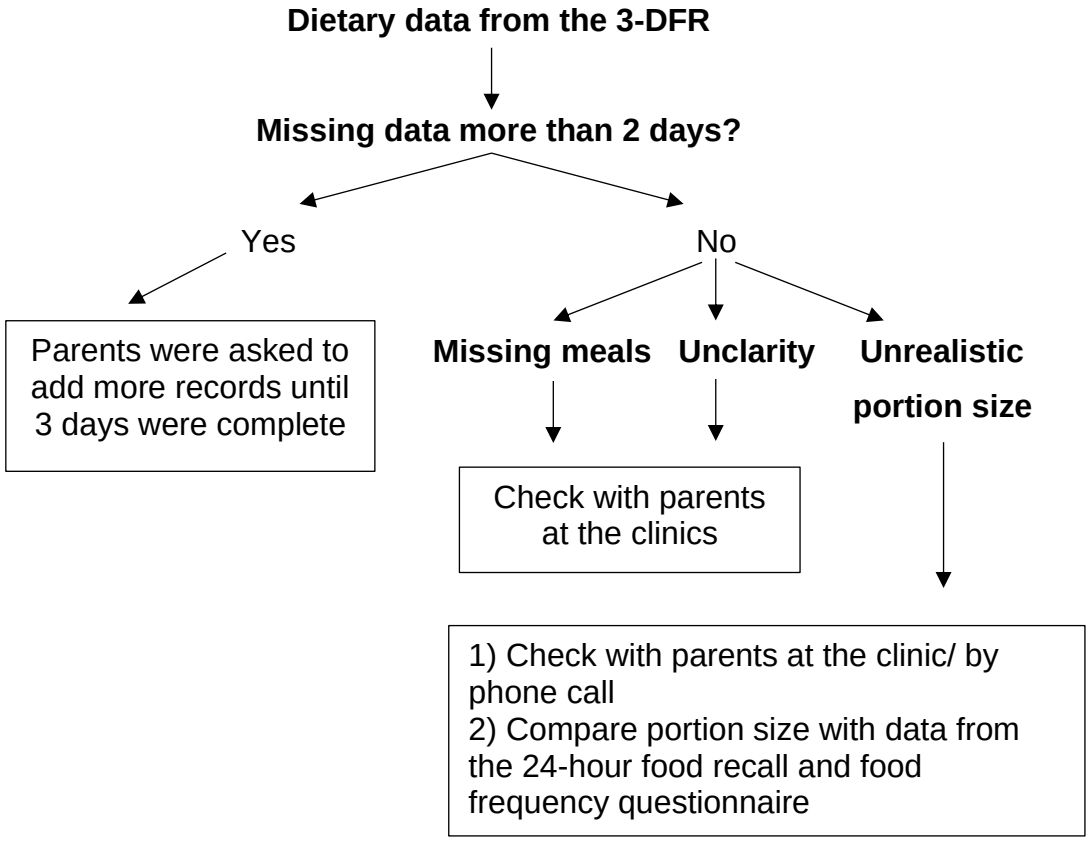
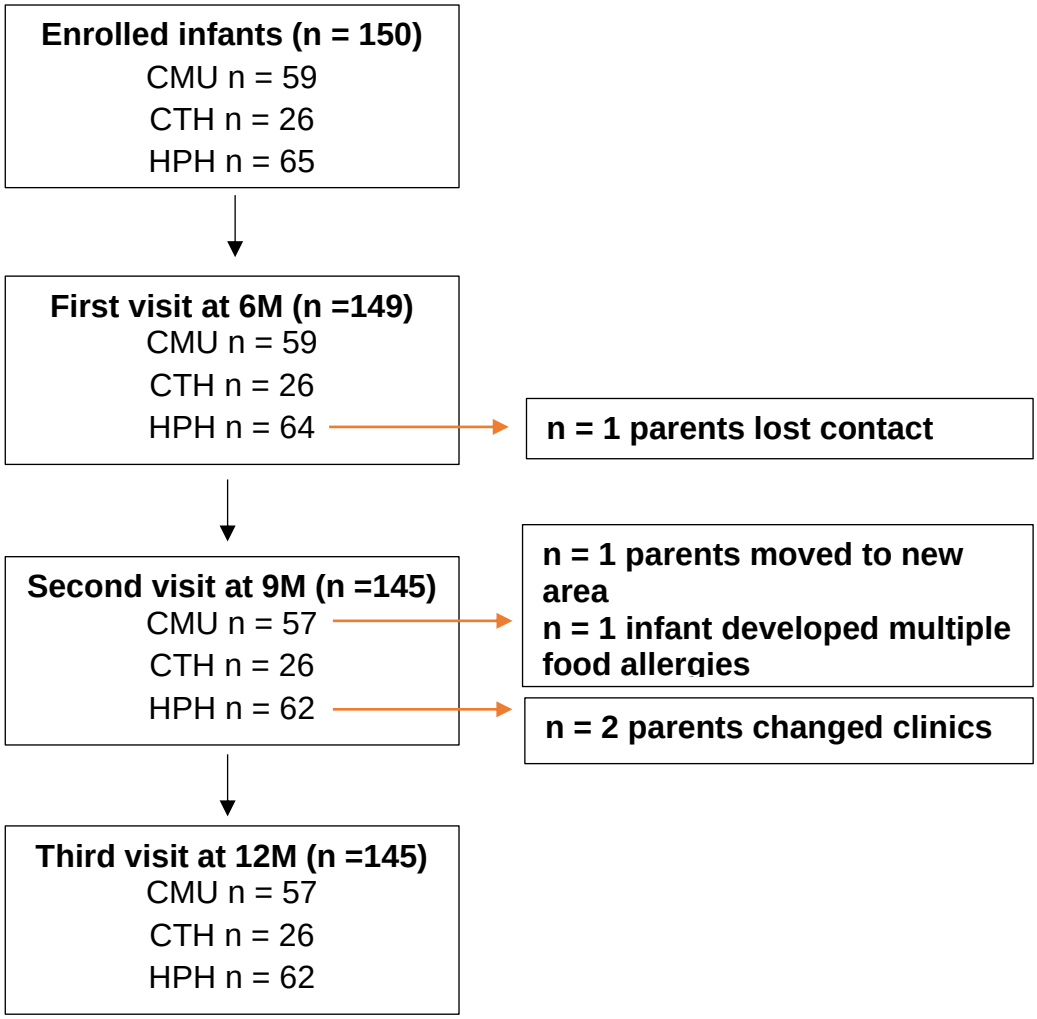


Table 6.1 Completeness of dietary data at 6, 9 and 12 months (n = 145)

Dietary methods	Completeness, n (%)		
	6M	9M	12M
3-DFR	-	130 (89.7%)	125 (86.2%)
24-HR	145 (100%)	142 (97.9%)	144 (99.3%)
FFQ	145 (100%)	142 (97.9%)	144 (99.3%)

M – months old; 3-DFR – 3-day food record; 24-HR – 24-hour food recall; FFQ – food frequency questionnaire

Figure 6.3 Flow chart showing the number of participants from enrolment to the end of the protocol



CMU *– Chiang Mai University Hospital*

CTH *– Chom Thong Hospital;*

HPH *– Health Promoting Hospital*

Dealing with missing data

Given the very small number of dropouts and exclusions during the study period (3.3%), in general, I dealt with missing data by using pairwise deletion to avoid faulty assumptions that could result from replacing the missing data by other statistical techniques. However, dietary data needed more careful management as they had to be converted into nutrient intakes which were essential for further analyses. In this thesis, dietary data at 6 months came from the 24-HR while at 9 and 12 months old they were based on the 3-DFR

as it can help to avoid recall-bias and previous studies[156, 157] also suggested that several days of records were required to achieve more accurate results for infant food consumption. As the results of analyses in section 6.5 demonstrated good agreement between the 3-DFR and 24-HR, I decided to replace missing data from the 3-DFR with dietary data from the 24-HR. By using this approach, complete dietary data were obtained for all infants at 9 and 12 months of age.

After preparing the dietary data as described above, there was still one significant issue to resolve. As milk intakes for some breastfed infants were recorded in the form of timing (minutes per breast feed), this needed to be converted into milk volume before being entered into the INMUCAL-Nutrients programme to calculate nutrient intakes. The next section describes the methods I used to estimate breast milk volumes from duration of breastfeeding.

6.2 Estimation of breast milk volume in breastfed infants

In this cohort, parents were asked to record the volume of milk consumed by their infant unless it was breastfeeding. Mothers who breastfed their babies were asked to record the duration of each breastfeeding-episode from the beginning until the infant completely stopped sucking.

I applied two techniques used in previous studies to estimate the volume of breast milk from duration of breastfeeding.

1) According to Lanigan et al[129], if the duration of breastfeeding (per episode) was at least 10 minutes, the volume of breast milk was estimated to be approximately 135 g and 100 g for 6-7- and 8–12-month-old infants, respectively. If the duration of breastfeeding was less than 10 minutes, the volume of breast milk was calculated proportionally.
2) An algorithm was developed by Olaya GA[159] using information from a previous study[160] and her unpublished data. The volume of breast milk was estimated using data on the number of breastfeeds, duration of each episode and infant appetite.

I used the average volume from these two methods as my final result. After dietary data had been prepared, the next step was to encode and enter all food items into the INMUCAL-Nutrients programme.

6.3 Coding and entering of food items

All food items from the 24-HR and 3-DFR were coded according to the suggestions in the manual of INMUCAL-Nutrients version 4.0. Four main sections including food groups, meals, cooking methods and unit were coded as shown in table 6.2. Identification data, sex, age and health status were required before entering data into the platform (Figure 6.4).

Dietary data from each participant were entered into the 5 separate files based on dietary assessment methods and infant age. Those files included dietary data from the 24-HR at 6, 9 and 12M and from the 3-DFR at 9 and 12 months. Figure 6.4 demonstrates the appearance of the pages from the platform where food items were entered. For the 3-DFR, the programme also allows the user to input more than one day of food records by specifying numbers in the specific box (figure 6.5). Although the INMUCAL-Nutrients version 4.0 is the most up-to-date programme and covers almost every food item usually consumed by Thai infants, it still lacks data for some items. In those cases, the nutrient compositions were taken from the nutrition facts (on commercial foods) or other food composition databases such as the United States Department of Agriculture (USDA) and Food Agriculture Organisation of the United Nation (FAO/ UN).

Table 6.2 Food groups and codes of food items

Food groups	Codes		
	Meals	Cooking methods	Unit
01 Cereals **02** Starchy roots & tubers **03** Legumes, Nuts & Seeds **04** Vegetables **05** Fruits **06** Meat & meat products **07** Finfish and shellfish **08** Eggs **09** Milk and milk products **10** Fat and oils **11** Sugars, syrups and confectionery **12** Condiments **13** Beverages, Alcoholic **14** Beverages, Non-Alcoholic **15** Snack foods, Puff products **16** Fast foods **17** Local dishes, mixed food **18** Dessert **19** Insects **20** Miscellaneous **21** Baby foods **22** Medical foods	**SB** = Snack before breakfast **BR** = Breakfast **SM** = Snack mid-morning **LU** = Lunch **SA** = Snack mid-afternoon **DI** = Dinner **SD** = Snack after dinner/ bedtime	**BL** = Blanch **BO** = Boiling **FR** = Frying **GR** = Grill **RA** = Raw **RE** = Ready to eat **SF** = Stir fry **ST** = Steaming	**KG** = Kilogram **GR** = Gram **LD** = Ladle **CU** = Cup **TB** = Tablespoon **TS** = Teaspoon **LA** = Large size **MI** = Medium size **SM** = Small size

Figure 6.4 Appearance of the identification page

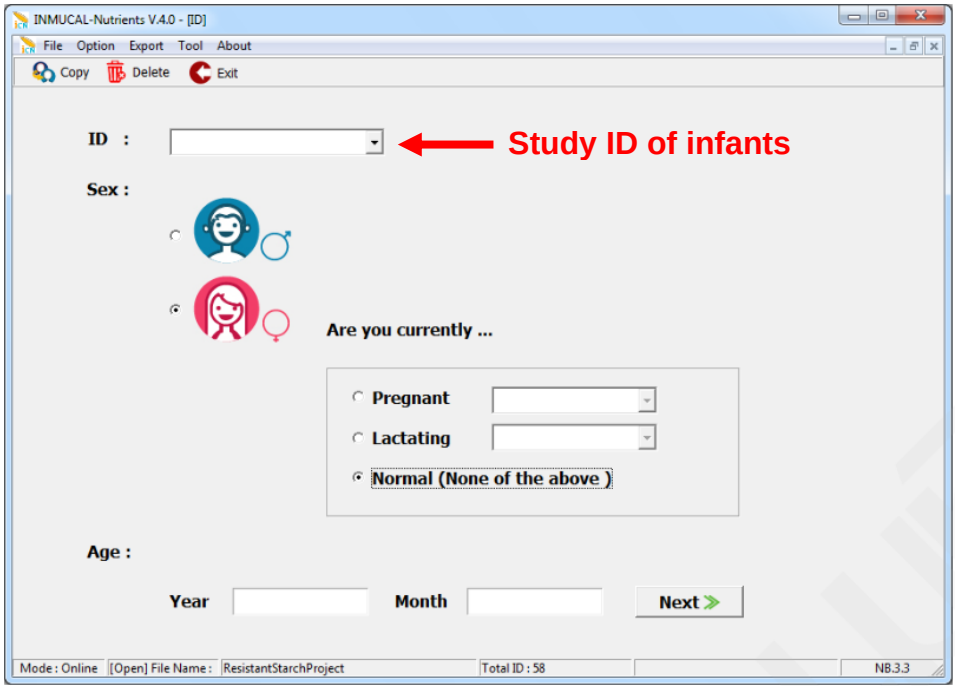
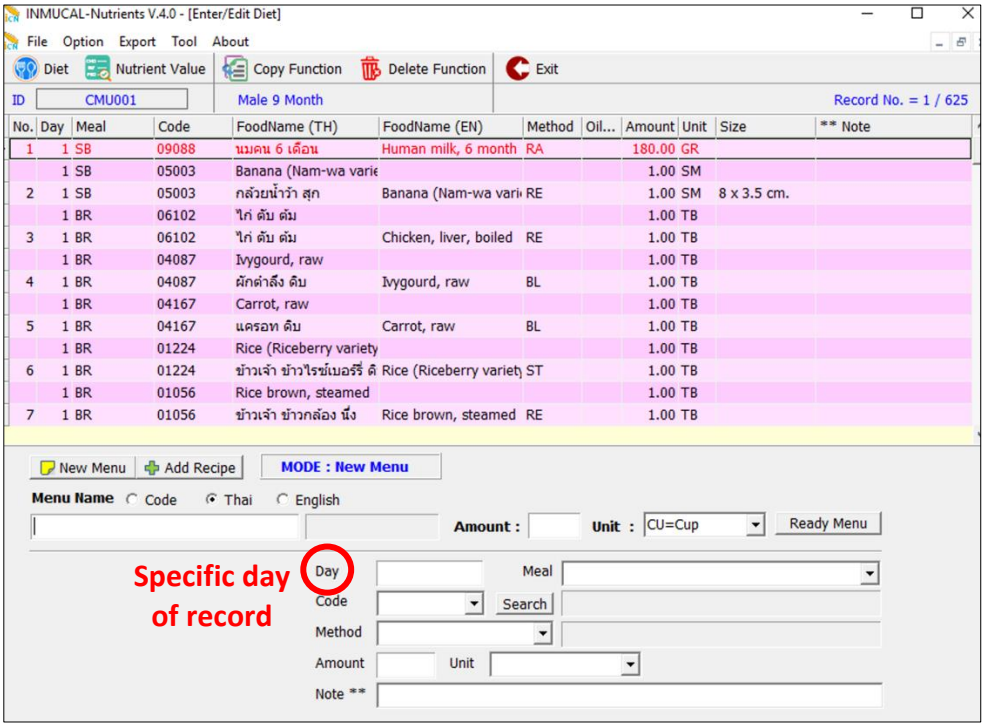


Figure 6.5 Appearance of the data entry platform



6.4 Conversion of dietary data into nutrient values

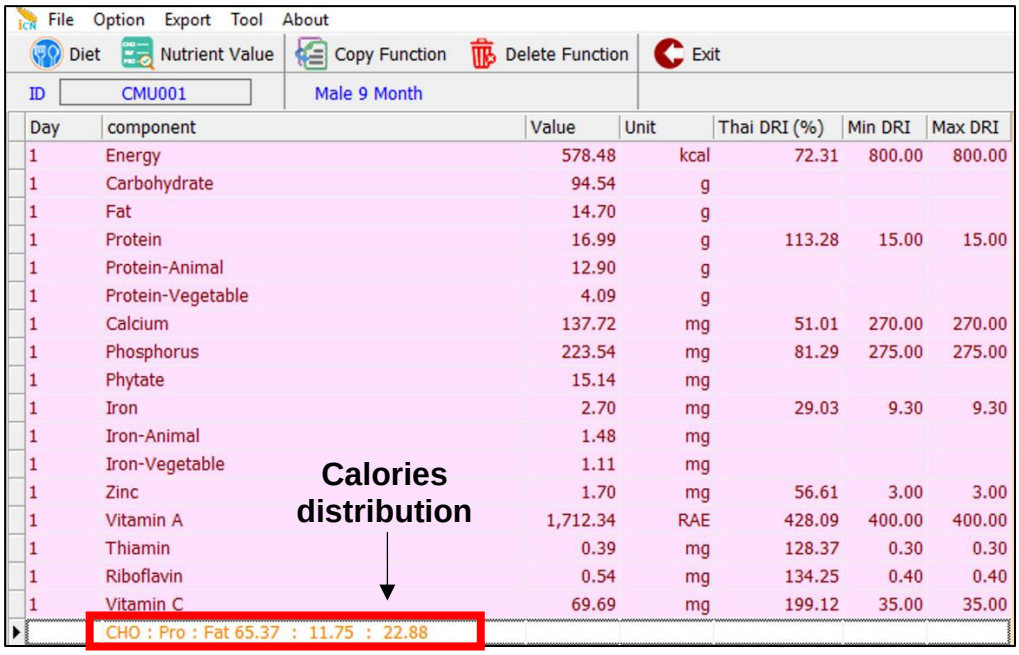
Dietary data entered into the INMUCAL-Nutrients programme were converted into nutrient intakes automatically. Nutrients including energy, calorie distribution, macronutrients, micronutrients and phytate were reported in the Nutrient Value page (Figure 6.6). Both protein and iron were also separately reported by food source; animal- and plant-based foods. Additionally, the nutrient values were compared with the recommended intakes using the previous version of Thai DRIs (version 2003) and reported as percentages.

The nutrient values for all infants could be exported into a Microsoft Excel spreadsheet shown in Figure 6.7. In some cases when food items were not included in the database, nutrient values from nutrition facts or other databases were manually added later to the Excel spreadsheets. However, there was still a problem for phytate because its content was absent from the nutrition facts of commercial products. Therefore, I had to exclude phytate from further analyses.

The completed nutrient data in the Excel spreadsheets were exported to SPSS for checking normality and outliers. Extreme or ambiguous values were managed by checking for errors that could have occurred during data collection and processing. Only final verified data were used to investigate agreement between the 24-HR and 3-DFR.

The next section will demonstrate the agreement between the 24-HR and 3-DFR for nutrient intake at 9 and 12 months of age using the statistical tests described in Chapter 5, section 5.8. In this thesis, I report agreement for energy, protein and iron intake which are key exposures for my study.

Figure 6.6 Appearance of nutrient value page



Day	component	Value	Unit	Thai DRI (%)	Min DRI	Max DRI
1	Energy	578.48	kcal	72.31	800.00	800.00
1	Carbohydrate	94.54	g			
1	Fat	14.70	g			
1	Protein	16.99	g	113.28	15.00	15.00
1	Protein-Animal	12.90	g			
1	Protein-Vegetable	4.09	g			
1	Calcium	137.72	mg	51.01	270.00	270.00
1	Phosphorus	223.54	mg	81.29	275.00	275.00
1	Phytate	15.14	mg			
1	Iron	2.70	mg	29.03	9.30	9.30
1	Iron-Animal	1.48	mg			
1	Iron-Vegetable	1.11	mg			
1	Zinc	1.70	mg	56.61	3.00	3.00
1	Vitamin A	1,712.34	RAE	428.09	400.00	400.00
1	Thiamin	0.39	mg	128.37	0.30	0.30
1	Riboflavin	0.54	mg	134.25	0.40	0.40
1	Vitamin C	69.69	mg	199.12	35.00	35.00

Figure 6.7 An example of nutrient values in the Excel spreadsheet

ID	Sex	Status	PregLact	Age (year)	Age (month	TotalDay	ENER (kcal)	CHO (g)	FAT (g)	PRO (g)	PROA (g)	PROV (g)
CMU001	M	N	0	1	0	3	1178.771	145.2565	47.99177	41.45483	35.1025	6.35233
CMU002	M	N	0	1	0	3	1063.432	160.3275	29.42906	39.3151	17.527	21.7881
CMU003	F	N	0	1	0	3	925.9052	136.0987	26.22781	36.36519	28.51148	7.853709
CMU004	F	N	0	1	0	3	696.0474	78.14611	30.41309	27.43638	22.25091	5.18547
CMU005	F	N	0	1	0	3	411.589	58.6317	12.81562	15.4304	12.35558	3.07347
CMU006	M	N	0	1	0	3	440.1288	56.83593	14.2008	21.24454	17.24828	3.996259
CMU007	M	N	0	1	0	3	564.6176	74.43222	18.64109	24.77977	23.20266	1.577109
CMU008	M	N	0	1	0	3	523.2179	61.66603	22.43118	18.66833	16.28741	2.380452
CMU009	F	N	0	1	0	3	483.9108	62.92223	19.47962	14.22639	11.54997	2.676423
CMU010	M	N	0	1	0	3	581.7678	79.90227	18.70384	23.45611	20.2161	3.24001

CA (mg)	P (mg)	PHYT (mg)	FE (mg)	FEA (mg)	FEV (mg)	ZN (mg)	VITA (RAE)	VITB1 (mg)	VITB2 (mg)	VITC (mg)
1153.395	894.2804	22.62424	12.72488	10.18167	2.290318	6.398373	1116.543	0.276608	0.259284	154.9203
540.4492	284.7039	61.11697	5.361807	1.139051	4.094088	2.277736	26.7976	1.865258	2.005671	25.71338
279.0734	445.2144	18.86472	4.117639	2.255459	1.852671	2.392353	294.7071	0.475597	0.547151	176.2388
237.309	352.9884	5.64667	3.139947	2.075892	0.975915	2.824645	495.5422	0.375925	0.575913	33.20098
96.64411	169.2282	30.7981	2.16006	1.125943	1.032196	1.319863	1114.533	0.267989	0.271758	28.70562
90.96877	208.7295	13.3298	2.700542	2.012201	0.688341	1.754863	2395.158	0.251963	0.609258	28.73577
511.8628	510.0616	0.421993	5.794385	5.267913	0.418199	5.08275	295.3733	0.438105	1.033329	85.35294
236.1745	189.9803	2.234804	2.30733	1.702067	0.604622	1.563532	851.8967	0.326431	0.374808	28.6019
247.2017	232.2722	0.264617	4.017798	3.448442	0.456441	3.013841	1291.344	0.409234	0.685019	74.23082
371.1164	418.7889	16.14278	6.561127	5.81819	0.720601	3.796531	345.3231	0.486939	0.863801	88.25739
		13.424	13.70277	12.67769	0.312642	6.709003	1005.459	0.864882	1.883625	104.1605

Calories distribution **% THAI DRI**

CHO_D	PRO_D	FAT_D	ENER_P	PRO_P	CA_P	P_P	FE_P	ZN_P	VITA_P	VITB1_P	VITB2_P	VITC_P
49.29082	14.06713	36.6420	117.8771	230.3046	230.679	194.4088	219.3945	319.9186	279.1357	55.32169	51.85681	387.3008
60.30569	14.78801	24.906	106.3432	218.4172	108.0898	61.89215	92.44494	113.8868	6.6994	373.0516	401.1343	64.28346
58.79591	15.7101	25.4939	92.59052	202.0288	55.81467	96.78574	70.99378	119.6176	73.67677	95.11947	109.4302	440.5971
44.90847	15.76695	39.3245	69.60474	152.4244	47.4618	76.73661	54.13702	141.2323	123.8856	75.185	115.1826	83.00245
56.98082	14.99593	28.0232	41.1589	85.72444	19.32882	36.78873	37.24241	65.99314	278.6331	53.59787	54.3515	71.76404
51.65388	19.30755	29.0385	44.01288	118.0252	18.19375	45.37597	46.56107	87.74313	598.7895	50.39268	121.8517	71.83942
52.73105	17.55508	29.7138	56.46176	137.6654	102.3726	110.883	99.90319	254.1375	73.84334	87.62098	206.6657	213.3824
47.14365	14.27193	38.5844	52.32179	103.7129	47.2349	41.30006	39.78155	78.17661	212.9742	65.28613	74.96158	71.50476
52.0114	11.75951	36.2290	48.39108	79.03551	49.44033	50.49397	69.27238	150.6921	322.8361	81.84688	137.0037	185.5771
54.93754	16.12747	28.9349	58.17678	130.3117	74.22328	91.04107	113.1229	189.8266	86.33079	97.38777	172.7603	220.6435
50.52177	13.23383	36.244	102.3271	188.0806	197.7219	164.5411	236.2547	335.4501	251.3648	172.9764	376.7249	260.4012

6.5 Dietary data agreement between the 24-HR and 3-DFR

Although I used two dietary assessment methods to collect food consumption of infants at 9 and 12 months, only nutrient intakes from the 3-DFR were considered as the primary resource for further analyses, because at least 3 days of records are ideally required to provide accurate dietary data. Nevertheless, as the aforementioned section showed higher percentages of missing data from 3-DFR, it was necessary to replace the missing data by using nutrient values from the 24-HR (10% and 14% of nutrient values at aged 9 and 12 months, respectively). In order to fill those gaps, the agreement between the methods should be acceptable.

In this section, I used various statistical tests to demonstrate the agreement between 24-HR and 3-DFR at both individual and group level. The agreements for total energy intake (kcal/day), total protein intake (g/day), percentage of protein energy distribution (%PE) and iron intake (mg/day) are reported here. At individual level of agreement, I used Pearson's correlations and cross-classifications while results from paired t-tests, percentage of mean difference, weighted kappa (Kw) and Bland-Altman analysis were used to test the agreement at group level. The next paragraphs describe the outcomes from these analyses.

Agreement of nutrient intake at individual level

According to table 6.3, the correlation coefficients of all selected nutrients were good to excellent (range from 0.63 to 0.90) and scatter plots also demonstrated positive correlations between the two methods (Figure 6.8). Considering the results from the cross-classification, percentages of the same quartile for all nutrients reached an acceptable value (more than 50%) and also showed very low percentages of the opposite quartiles (acceptable value is less than 10%) for all selected nutrients at both 9 and 12 months. These outcomes indicated acceptable agreement between the 24-HR and 3-DFR at individual level.

Agreement of nutrient intake at group level

There was no difference in the paired t-tests and the percentages of mean difference were low (less than 10%) which reflected acceptable agreement between the two methods (Table 6.4). In addition, the weighted kappa also showed acceptable agreements (more than 0.2) as shown in table 6.5. The Bland-Altman plots did not show direction of bias for the selected nutrients (Figure 6.9) and the majority of mean differences were between the lower and upper limits of agreement (LOA). Although the mean differences for all selected nutrients were small, the LOA representing the range of mean differences were quite wide for most nutrients (Table 6.5). These reflected the fact that nutrient intakes of some infants differed quite markedly between the two methods.

Although the results demonstrated acceptable to good agreement between the two methods at individual and group level for almost all statistical analyses apart from some issues on LOA, it was still unclear how reliable they were as I had collected dietary data once at each visit. However, to investigate this point, the next paragraph will show the variation in energy and protein intake of infants using data from the individual days of the 3-DFR.

Variation of total energy and protein intake from 3-DFR

Energy and protein intake from the three different days at 9 and 12 months old from the 3-DFR were compared. Energy consumption from milk intake and complementary foods are presented separately. Repeated ANOVA measurement was used to determine these variations. As shown in table 6.6, there were no significant differences in energy and protein intake over three separate days and daily intakes were almost the same at both 9 and 12 months old, although more variation was found at 12 months of age. Energy intakes whether from milk or complementary foods also showed very small variations from day to day. These findings indicated that infants have limited variation in food consumption from day to day which could explain the good agreement between 24-HR and 3-DFR.

Taken together, these results indicate acceptable agreement for the selected nutrients between 24-HR and 3-DFR at both 9 and 12 months of age in this population whether at individual or group level. Therefore, for further statistical analyses, when nutrient values from 3-DFR were missing, I subsequently used the nutrient values from 24-HR.

The full report, including the dietary agreement for other nutrients, namely carbohydrate, fat, calcium, phosphorus, zinc, vitamin B1, vitamin B2 and vitamin C has been published elsewhere[161]. The results also indicate acceptable to good agreement for those nutrients with a few exceptions for some micronutrients. For example, vitamin A showed lower Kw and percentages in the same quartile than acceptable values and its LOA were wider compared with other nutrients at both 9 and 12 months old.

The next chapter will present the demographic data and prevalence of double burden of malnutrition at individual, household, and population level in the study population.

Table 6.3 Pearson's correlation coefficients and cross-classification of selected nutrients between 24-HR and 3-DFR

Nutrients	Correlation coefficients (r)*		% same quartiles		% opposite quartiles	
	9M	12M	9M	12M	9M	12M
Energy (kcal/d)	0.81	0.75	54.8	53.2	0	1.6
Protein (g/day)	0.79	0.74	57.1	50.8	0.8	1.6
%PE	0.63	0.67	54.5	50.8	3.6	1.6
Iron (mg/d)	0.90	0.84	57.9	59.7	0	0

**All p-value < 0.001; M – months*

Figure 6.8 Scatter plots demonstrating relationships between nutrient intakes between the 24-hour food recalls (24-HR) and 3-day food records (3-DFR)

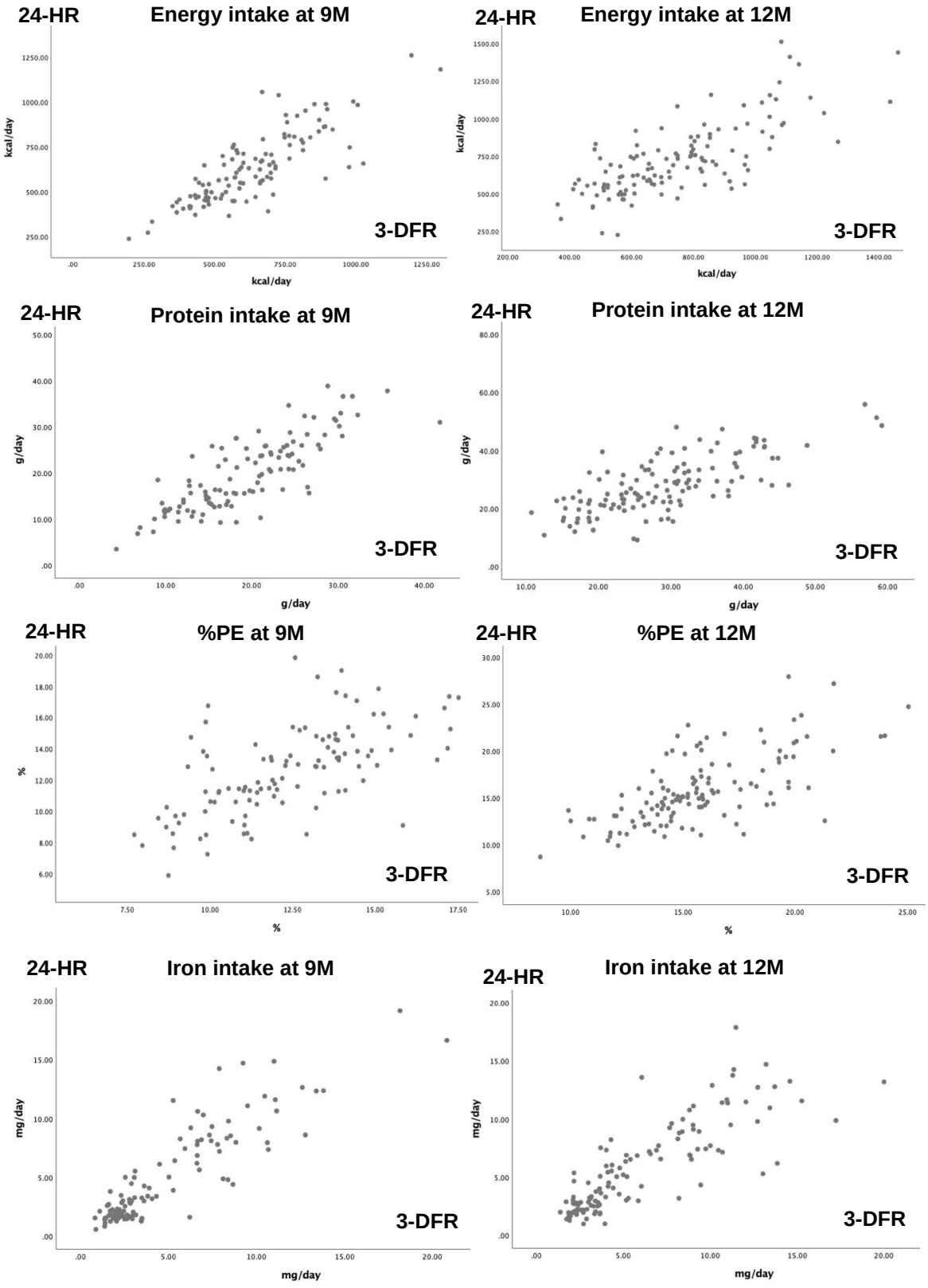


Table 6.4 Paired t-test and mean difference for selected nutrients compared between 24-HR and 3-DFR

Nutrients	9M			12M		
	mean ± SD	**Δmean (%)**	***p***	**mean ± SD**	**Δmean (%)**	***p***
Energy (kcal/d)						
- 24-HR	624.5±193.9	-5.6	0.60	725.5±236.9	-21.6	0.14
- 3-DFR	630.0±191.4	(0.9)		747.0±222.5	(2.9)	
Protein (g/d)						
- 24-HR	19.8±8.4	-0.4	0.41	28.4±10.5	-0.9	0.21
- 3-DFR	20.2±7.9	(2.0)		29.3±10.1	(3.1)	
%PE						
- 24-HR	12.4±2.8	-0.3	0.36	15.9±3.7	0.2	0.57
- 3-DFR	12.7±2.6	(2.4)		15.7±3.0	(1.3)	
Iron (mg/d)						
- 24-HR	5.0±4.0	0.1	0.76	5.9±4.0	-0.3	0.15
- 3-DFR	4.9±3.8	(2.0)		6.2±4.3	(4.8)	

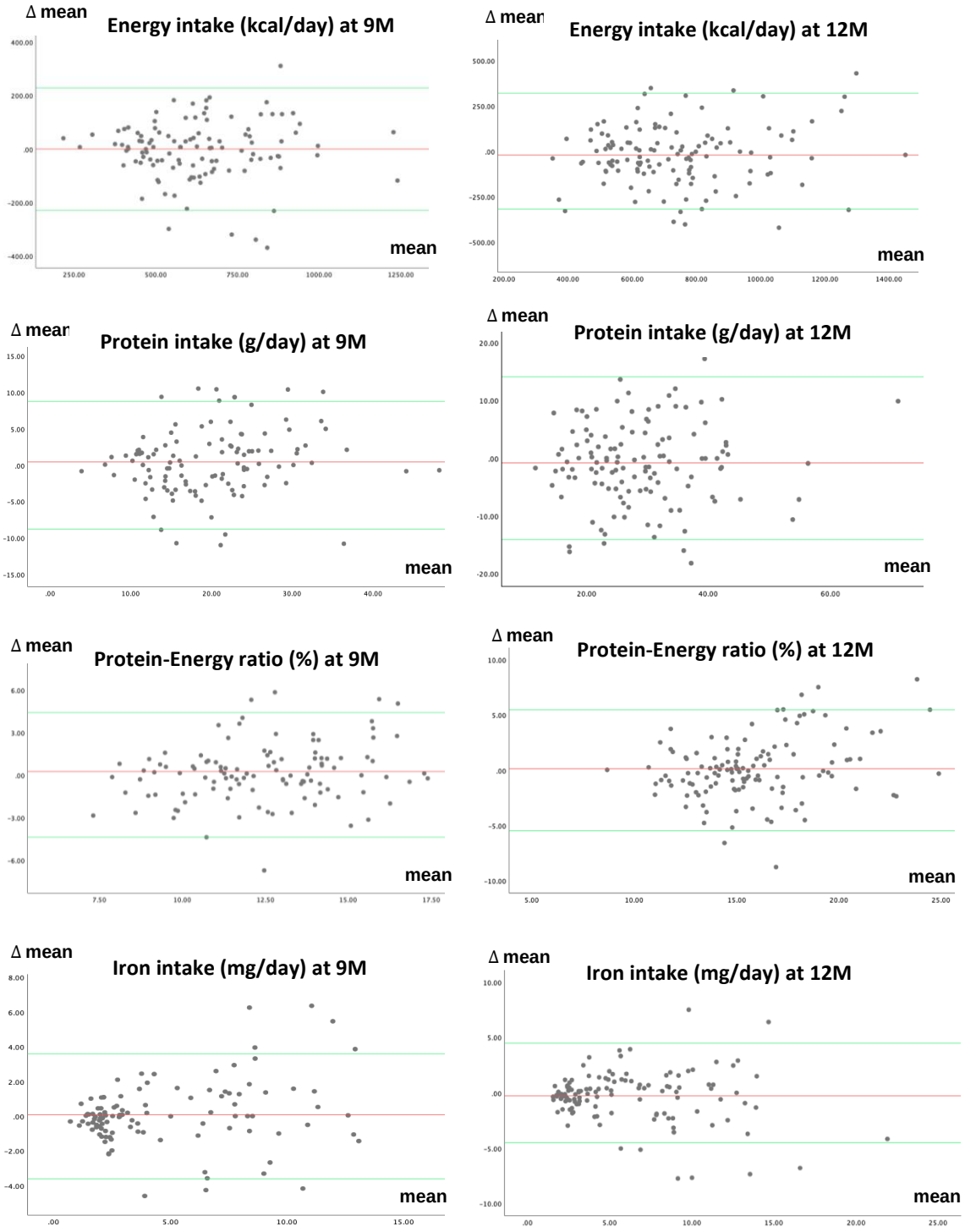
Δ ***mean*** *– mean differences*
M – months old; SD – standard deviation; 24-HR – 24 hour-food recall; 3-DFR – 3 day-food record

Table 6.5 Weighted kappa and Bland-Altman limit of agreement of selected nutrients compared between 24-HR and 3-DFR

Nutrients	Kw*		LOA (Δmean, ±1.96SD)	
	9M	**12M**	**9M**	**12M**
Energy (kcal/d)	0.33	0.30	-5.6, ±233.7	-21.6, ±318.9
Protein (g/d)	0.35	0.28	-0.4, ±10.7	-0.9, ±14.5
%PE	0.27	0.28	-0.3, ±5.5	0.2, ±5.5
Iron (mg/d)	0.36	0.37	0.1, ±3.5	-0.3, ±4.7

**All p-value < 0.001; M – months old; Kw – weighted kappa; LOA – limit of agreement*

Figure 6.9 Bland-Altman plots for selected nutrients



This figure shows the Bland-Altman plots for selected nutrients at aged 9 (left column) and 12 months (M) (right column). The x axes represent mean values for nutrient intake from 24-hour food recalls and 3-day food records while the y axes represent mean differences between these two methods. The red line indicates a zero difference when the mean difference between two methods is zero regardless of mean nutrient intake. The green lines represent $\pm$ 1.96 standard deviation of mean differences reflecting the upper and lower limit of agreement.

Table 6.6 Change in average energy and protein intakes over 3 days at 9M and 12M based on the 3-day food records

Nutrients	Day 1 mean±SD	Day 2 mean±SD	Day 3 mean±SD	p-value
Total energy (kcal/d)				
- 9M	613.2±202.5	618.4±197.9	633.0±228.3	0.12
-12M	746.2±238.0	730.7±234.1	744.5±255.9	0.90
Energy from milk (kcal/d)				
- 9M	366.2±161.6	356.8±158.0	365.0±182.5	0.89
- 12M	346.6±169.7	342.7±162.9	350.8±187.1	0.64
Energy from complementary foods (kcal/d)				
- 9M	248.3±119.4	262.3±128.1	268.7±143.3	0.06
- 12M	402.5±165.4	390.6±158.0	396.8±159.8	0.62
Protein intake (g/d)				
- 9M	19.7±8.6	19.8±8.1	20.2±9.4	0.36
- 12M	29.0±10.5	28.6±10.9	29.3±11.3	0.70

M – months old; SD – standard deviation

Chapter 7: Results 1

Demographic data and Prevalence of the Double Burden of Malnutrition in the study population

In this chapter, demographic data of infants and their families are described to provide a clear picture of the study population. Subsequently, I present the prevalence of the DBM found in this cohort from individual to population level. In the last section, key findings and interesting points are summarised and discussed.

R1.1 Demographic data of participants and their family characteristics

Of 145 infants, there were equal numbers of boys and girls. All were born full-term with appropriate birth weight and length, although some mothers had been diagnosed with prenatal problems such as gestational diabetes or anaemia. Around two-thirds of infants were the first-born child and one-third were delivered by caesarean section (Table R1.1).

Considering parents' characteristics (Table R1.2), mean ages of mothers and fathers were around 30 years old. Although average heights of both parents were similar to the Thai population, the mean BMI especially for fathers approached the cut-off BMI for overweight in Asian populations[162]. Almost all parents had completed the compulsory education in Thailand (secondary school) and more than half of both parents graduated with a bachelor's or higher degree. Interestingly, nearly 98% of mothers were working and the majority of them were in the private sector or self-employed.

Consistent with the high rate of working mothers, the results shown in table R1.3 are consistent with the characteristics of middle-class families where both parents share childcare responsibilities and contribute financially.

Nevertheless, nearly two-thirds of infants lived in an extended family and grandparents were also involved in childcare. More than 80% of the families reported incomes ranging between the lowest rate calculated from the minimum daily wage and the highest rate which was about twice the average monthly income of Thai families according to figures from Thai National Statistics Office (NSO) in 2019[163].

Table R1.1 Demographic data of infants (n =145)

Characteristics	Results
Gender, male (n, %)	73 (50.3)
Gestational age, week (mean ± SD)	38.8 ± 1.0
Route of delivery (n, %)	
- Vaginal delivery	96 (66.2)
- Caesarean section	49 (33.8)
Child order (n, %)	
- First born	93 (64.1)
- Second child	48 (33.1)
- Third/Forth child	4 (2.8)
Birth weight, grams (mean ± SD)	3,156 ± 364
Birth length, cm (mean ± SD)	49.3 ± 1.9
Birth head circumference, cm (mean ± SD)	33.3 ± 1.4
Maternal screening (n, %)	
- Iron deficiency anaemia/ other anaemia	5 (3.5)
- Gestational diabetes	13 (9.0)
- Viral hepatitis B carrier	3 (2.1)
- Hypertension	2 (1.4)

SD – standard deviation

Table R1.2 Parental characteristics

Characteristics	Results
Parental age, years old (mean ± SD)	
- Mothers	29.8 ± 5.7
- Fathers	32.0 ± 5.9
Parental height, cm (mean ± SD)	
- Mothers	157.7 ± 5.7
- Fathers	170.4 ± 5.8
Parental BMI, kg/m^2 (mean ± SD)	
- Mothers	22.8 ± 4.0
- Fathers	24.7 ± 3.6
Maternal educational attainment (n, %)	
- No formal education	2 (1.4)
- Primary school	5 (3.5)
- Secondary school	52 (35.9)
- College	17 (11.7)
- Bachelor	61 (42.1)
- Postgraduate	8 (5.5)
Paternal educational attainment (n, %)	
- No formal education	3 (2.1)
- Primary school	9 (6.2)
- Secondary school	49 (33.8)
- College	24 (16.6)
- Bachelor	52 (35.9)
- Postgraduate	6 (4.1)
Employed parents (n, %)	
- Fathers	142 (97.9)
- Mothers	94 (64.8)
Maternal occupation (n, %)	
- Government officer	26 (17.9)
- State enterprise	16 (11.0)
- Private section	59 (40.7)
- Self-employment	34 (23.5)
- Agriculture	6 (4.1)

Paternal occupation (n, %)	
- Government officer	24 (16.6)
- State enterprise	5 (3.5)
- Private section	41 (28.3)
- Self-employment	21 (14.5)
- Agriculture	2 (1.4)

SD – standard deviation

Table R1.3 Family characteristics

Characteristics	Results
Dietary data provider (n, %)	
- Mothers	134 (92.4)
- Fathers	6 (4.1)
- Grandparents	17 (11.7)
- Others	2 (1.4)
Main caregivers (n, %), choose more than 1	
- Mothers	139 (95.9)
- Fathers	11 (7.6)
- Grandparents	61 (42.1)
- Others	11 (7.6)
Number of main caregivers (n, %)	
- 1 person	77 (53.1)
- 2 people	54 (37.2)
- ≥ 3 people	14 (9.7)
Family type (n, %)	
- Nuclear type	50 (34.5)
- Extended type	95 (65.5)
Family house (n, %)	
- Owner	57 (39.3)
- Rental	24 (16.6)
- Others (e.g., employer's accommodation)	64 (44.1)
Number of rooms in family house (n, %)	
- 1 room	15 (10.3)
- 2 rooms	59 (40.7)
- 3 rooms	48 (33.1)
- ≥ 4 rooms	23 (15.9)

Source of drinking water (n, %)	
- Unfiltered water	3 (2.1)
- Filtered water	26 (17.9)
- Buying from dealer	116 (80.0)
Main financial providers (n, %), choose more than 1	
- Mother	92 (63.5)
- Father	140 (96.6)
- Grandparents	13 (9.0)
- Others	2 (1.4)
Number of main financial providers (n, %)	
- 1 person	52 (35.9)
- 2 people	83 (57.2)
- 3 people	7 (4.8)
- ≥ 4 people	3 (2.1)
Family income per month*#, Thai Baht (n, %)	
- less than 10,000	11 (7.6)
- 10,000-29,999	65 (44.8)
- 30,000-49,999	51 (35.2)
- ≥ 50,000	18 (12.4)

Minimum wage in Chiang Mai was 320 Baht per day during period of data collection[164]
#Average monthly income of Thai families reported by the National Statistical Office of Thailand 2019 was 26,018 Baht[163]

As Thailand is an upper-middle income country according to the World Bank's classification[165], the socioeconomic characteristics of my study population were similar to a majority of Thai families especially, for those who live in urban areas. In addition, parents' occupations reflected socioeconomic transition as there were only a small proportion of parents still working in the agricultural sector, with most working in the private sector which included factories or other industries. In the next section I present data on the prevalence of the DBM from individual to population level.

R1.2 Prevalence of the double burden of malnutrition

According to the WHO, the DBM can manifest at three levels including individuals, households and populations[6]. I examined the prevalence of the DBM at all levels, although the prevalence might be underestimated for

parents as I did not obtain data on non-communicable diseases and their anthropometry data were self-reported. The DBM requires the co-existence of two opposite extremes of nutrition within individuals, households or populations. In this thesis I categorised wasting, underweight, stunting/ short stature and iron deficiency as undernutrition, and overweight and obesity as overnutrition.

As shown in table R1.4, the DBM was found at individual, household, and population levels, except for infants individually. The prevalence of infant malnutrition shown in this table was assessed when the infants were age 12 months. 2.8% and 0.7%, respectively, of mothers and fathers were short and overweight/ obese indicating the DBM at individual level. The most common form of the DBM present at the household level was the combination of iron deficient infants living with overweight/ obese parents followed by stunted/ wasted/ underweight infants who lived with overweight/ obese parents. Interestingly, more than two-thirds (71%) of the infants with ID/ IDA lived with overweight/ obese parents.

At population level, the number of infants with undernutrition was higher than those who were overweight whilst the opposite was found in parents. More than one-third of mothers and almost two-thirds of fathers were overweight/ obese while only small proportions had undernutrition. Although the prevalence of infant malnutrition was quite small, Figure R1.1 shows that the percentages increased over time, except for overweight/ obesity. At 12M, the percentages of all forms of undernutrition were higher than at 6M and 9M while the percentages for overweight/ obesity were stable over 6M.

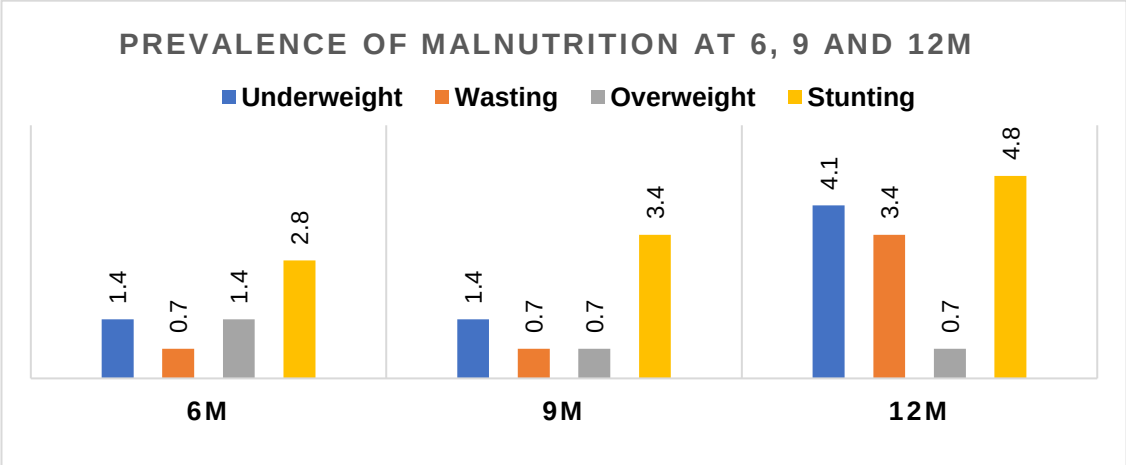
For undernutrition, the prevalence of wasting and underweight was stable between aged 6-9M. However, prevalence trebled at 12M while the prevalence of stunting increased over the CF period. Furthermore, the number of infants with ID/ IDA was very high (43% of all infants). Altogether, around 1 in 2 infants in this cohort suffered from at least one form of malnutrition at 12M.

Table R1.4 Prevalence of double burden of malnutrition

Type of DBM	**Prevalence**, n (%)
Individual level	
- Infants (n =145)	0
- Mothers (n = 145)	4 (2.8)
- Fathers (n = 138)	1 (0.7)
Household level	
- Wasted/ underweight/ stunted infants living with overweight/ obese parent	8 (5.5)
- Infants with ID/ IDA living with overweight/ obese parent	44 (30.3)
- Overweight infant living with underweight parent	1 (0.7)
Population level	
Infant	
- Underweight	6 (4.1)
- Wasting	5 (3.5)
- Overweight	1 (0.7)
- Stunting	7 (4.8)
- ID	35 (24.1)
- IDA	27 (18.6)
Mother	
- Underweight*	9 (6.2)
- Overweight/ Obese*	55 (37.9)
- Short stature† (height < 147.2 cm)	6 (4.1)
Father	
- Underweight*	6 (4.3)
- Overweight/ Obese*	89 (64.5)
- Short stature† (height < 157.7 cm)	1 (0.7)

**Cut-off BMI in Asian populations[162]; †Defined by less than -2SD of average height of Thai female and male adults[166]; ID – Iron deficiency; IDA – Iron deficiency anaemia*

Figure R1.1 Prevalence of malnutrition at 6, 9 and 12 months of age (M) (n = 145)



R1.3 Summary of key results and discussion

Key results

- The prevalence of infant stunting, wasting and underweight increased with age and was highest at 12 months.
- During infancy, the prevalence of overweight/ obesity was less than that of undernutrition.
- Nearly 1 in 2 of the infants in this cohort had at least one form of malnutrition whether under- or overnutrition while ID/ IDA was the most common form of malnutrition.
- The DBM is evident in this Thai population at all levels, although there were no stunted-overweight infants.

The results presented in this chapter provided a clear picture of the study population and the prevalence of DBM. The demographic data are consistent with the socioeconomic transition in Thailand as the results showed a good number of highly educated parents, a high percentage of working mothers, a majority of families with a moderate income and changing employment from agriculture to industrial sectors. Similar to many LMICs experiencing the transition this can lead to public health problems, including the double burden of malnutrition, which is evident in this cohort at all levels except for individual infants. Noticeably, more than one-third of all infants were living in households where the DBM existed.

Focusing on global figures for childhood malnutrition, in 2020, the prevalence of stunting in children under-five was still highest (144 million) followed by wasting (47 million) and obesity (38 million)[167]. Although, the numbers for all forms of malnutrition slightly decreased compared with the estimations in 2017[47], the reduction of stunting and wasting are still far behind the global targets for 2025 proposed by the World Health Organisation (WHO)[168]. These figures also indicate that the DBM is a huge burden in many countries across the world.

Globally, prevalence of the DBM varies from country to country[169-171], but the trends are accelerating more rapidly among middle-income countries where socioeconomic and nutrition transition are most rapid[172]. A recent systematic review of nationally representative data in under-five children showed that 5 out of 93 LMICs worldwide are facing the DBM at national level as the prevalence of both under- and overnutrition in those countries exceeds the international threshold: 20% stunting and 10% overweight/ obesity[173], while the median prevalence of stunted-overweight children who are under-five in LMICs is 1.4% (IQR 0.9 – 2.6)[171].

More specifically, among eight country members in the Association of South East Asian Nations (ASEAN) including Thailand, Rachmi et al[174] demonstrated the DBM in infants and children was present at all three levels. The prevalence

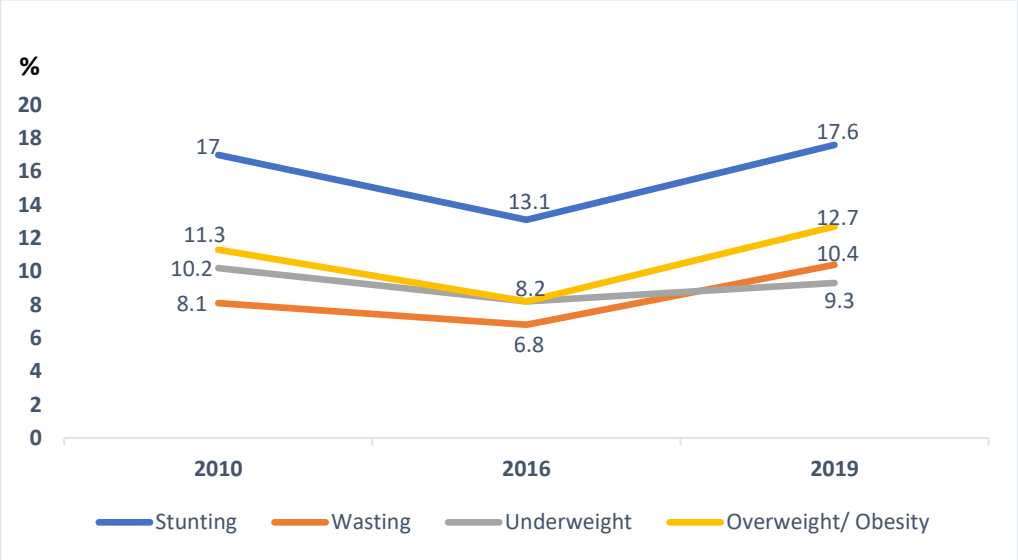
of DBM at national level varied among ASEAN countries while at household level, the figures were between 5% (Vietnam) and 30.6% (Indonesia). Only two countries reported the prevalence of DBM at individual level. The figures for stunted-overweight children were 1.2% and 7.2% in the recent surveys in Vietnam and Indonesia, respectively[174]. In this paper, during 2011 - 2017, the prevalence of undernutrition in Thai children and adolescents gradually decreased while the percentages of overweight/ obesity constantly increased. The DBM at household and individual level was not reported in the Thai population in this article or in other studies.

It is well recognised that Thailand was successful in dramatically reducing the prevalence of acute severe wasting and other forms of undernutrition during the 1980s to mid-1990s due to implementation of several nutritional programmes and improvement of primary health services[13]. However, in the last few decades, the percentage of ID/ IDA has remained high while the prevalence of stunting has reached a plateau which is around 10-12% and underweight remains at 10-15% in the paediatric population. In contrast, the rate of overweight/ obesity has been rising, with higher figures found in younger generations[20]. In 2014, Thailand was recognised as a country where both wasting and overweight in under-five children were overlapping burdens at national level[175].

Figure R1.2 shows the comparison of prevalence of malnutrition among the three latest national surveys in Thai children using the WHO growth standard 2007 and a consistent definition of malnutrition[16, 21, 22]. This line graph demonstrates that the prevalence of stunting, wasting and underweight among children aged under-five was unchanged, despite a small drop in 2016 while the percentages of overweight/ obesity reached the highest figure in 2019. Unlike the results from these national surveys, the prevalence of undernutrition and overweight in our cohort were measured earlier and over a narrower age range (6-12 months vs 0-59 months) which might explain the relatively low percentages compared to those surveys. In addition, the exclusion of low-birth weight and preterm infants who are at risk of undernutrition, and the high

proportion of breastfed infants which may mitigate risk of overweight, could result in reduced rates of both under and over-nutrition.

Figure R1.2 Comparison of the prevalence of stunting, wasting, underweight and overweight/ obesity among under-five children in 3 Thai national surveys[16, 21, 22]



Nevertheless, to my best knowledge, this is the first study presenting the prevalence of DBM at household and individual level in a Thai population. Although DBM was absent in infants at individual level, the results for household DBM were still interesting. Considering the household DBM based on only anthropometric data in this cohort, the prevalence of malnourished infants who lived with at least one parent who had the opposite form of malnutrition was about 6.2% which was similar to the prevalence in some countries in ASEAN (5% in Vietnam and 8% in Malaysia)[174]. Furthermore, this may be the first study considering the combination of infants with ID/ IDA and overweight/ obese parents as one type of household DBM.

As iron deficiency is the most common micronutrient deficiency and a major public health problem regardless of a country's socioeconomic status, the high proportion of this type of household DBM found in this cohort may provide a new perspective for primary prevention of ID/ IDA in infants and young

children. As shown in table R1.4, the high prevalence of infants with ID/ IDA living with overweight/ obese parents in this cohort highlights the importance of considering family diet and parental eating behaviours when considering the prevention and treatment of iron deficiency in infants and young children. Energy-dense foods with poor nutritional value can lead to overweight/ obesity and the family diet is known to be highly influential for infant feeding.

In this cohort, the prevalence of ID and IDA were still high, although the figures were slightly lower than the latest study conducted between 2016-2017 in Thai infants aged between 9-12 months of age which reported 34% had ID and 25.7% IDA[176]. Over a decade, the situation of IDA in Thai infants, especially for breastfed infants has not improved[177] despite the implementation of a universal iron screening and iron supplementation programme for infants aged 6-12 months in public hospitals by the Thai government in 2013[178].

Taken together, data presented in this chapter confirm the DBM as a current threat for the Thai population. It seems likely that all forms of malnutrition might share some common aetiologies and be linked to the other forms in some way. Therefore, understanding the DBM holistically by identifying its "shared drivers" - factors that can lead to either under- or overnutrition - should be a good starting point to develop more effective interventions or nutritional policies that simultaneously reduce all forms of malnutrition, so-called "double-duty actions". According to the WHO, the shared drivers of DBM could be biological, environmental or socioeconomic factors and five potential candidates for double-duty actions were proposed in 2017 (Figure R1.3)[9]. However, there is no suggestion from the WHO which actions should be prioritised.

Pradeilles and colleagues[179] gathered all factors related to all forms of malnutrition through the existing conceptual frameworks, and finally identified 83 out of 207 factors that could be shared drivers of DBM at all levels. In this review, the authors also compared the percentages of shared drivers that were addressed by the double-duty actions proposed by the WHO. The results

showed that "**Regulation of marketing**" addressed 65.1% of shared drivers followed by "**Promotion of appropriate early and complementary feeding in infants"** (53%). "**Maternal nutrition and antenatal care programmes**" and "**School food programmes and policies**" contained 43.4% and 41% of shared drivers, respectively while "**Initiatives to promote and protect exclusive breastfeeding in the first 6 months, and beyond**" came last, addressing 24% all of shared drivers.

From this review, it seems that complementary feeding related programmes or interventions could potentially alleviate the DBM and should be prioritised as a potential solution. Nevertheless, it is necessary to understand the current situation and feeding practices in a given population before planning interventions. The next chapter will provide more details of complementary feeding in the study population including feeding practices, characteristics of complementary foods and nutrient intakes. These results might help identify the factors that underlie the DBM in this population.

Figure R1.3 Five potential candidates for double-duty actions identified by the WHO

Chapter 7: Results 2

Feeding practices and Nutrient intakes during the complementary feeding period

As discussed in the previous chapter, interventions or policies targeting optimal complementary feeding are potentially a double-duty action that could reduce all forms of malnutrition and also establish a good foundation for health throughout life. However, many factors related to complementary feeding can impact the infant's nutritional status, contributing to malnutrition, whether under- or overnutrition. Therefore, it is necessary to understand current practices and actual nutrient intakes of the targeted population as this information will help determine the most problematic issues that should be modified in order to diminish the DBM. The first two sections of this chapter will describe feeding practices of the parents and nutrient intakes of the infants from my cohort, reflecting the current situation in Chiang Mai, Thailand. The last section will highlight key findings and discuss these in detail.

R2.1 Complementary feeding practices

When considering complementary feeding, there are three common questions that are widely asked in terms of feeding practices. These questions are when (timing of first introduction), what (type of foods given to infants) and how (amount of food, number of meals, combination of food groups, type and amount of milk, etc.). The following sub-sections will provide this information for my study population.

R2.1.1 Age of first introduction of complementary foods

Consistent with my cross-sectional study (Chapter 3), parents usually introduced first complementary food when their infants were 4 to 6 months old (n = 141, 97.2%) and rarely started CF before 4 months (n =3, 2.1%) or later than 6.5 months (n = 1, 0.7%). Rice, as a Thai staple, was the first

complementary food provided to infants, followed by fruits and vegetables while ASFs were introduced later (Table R2.1). These findings were in line with the first foods chosen by parents. According to table R2.1, 20% of parents started weaning their child with ASFs and only 17.9% first introduced commercial foods which are usually fortified with iron and some micronutrients.

Table R2.1 Introduction of complementary feeding†

Variables	Results
- Age (months) at first introduction of CF, (mean ± SD)	5.7 ± 0.6
- Age of introduction each food group (mean ± SD)	
Rice	5.7 ± 0.6
Fruits	5.8 ± 0.6
Vegetables	5.9 ± 0.5
Eggs	6.0 ± 0.5
Meats	6.3 ± 0.9
Fishes	6.5 ± 0.9
Dairy products *(excluding infant/ follow-on formula)*	9.9 ± 2.2
- First complementary foods * (n, %)	
Rice	67 (46.2)
Fruits	49 (33.8)
Rice added to breast milk	32 (22.1)
ASFs	29 (20.0)
Commercial baby foods	26 (17.9)
Vegetables	17 (11.7)

†*Data were based on the study record form; *Parents could choose more than one food group if they used a combination; CF – complementary foods, SD – standard deviation; ASFs – animal-source foods*

R2.1.2 Milk feeding practices

Along with complementary foods, milk is a key component in meeting energy and essential nutrient requirements for infants during this transitional period. Two main types of milk were given to the study infants, breast milk and formula. As shown in table R2.2, 44.1% were exclusively breastfed until 6 months of age and more than 80% of these infants continued to consume only

breast milk along with complementary foods. However, the majority of infants discontinued breastfeeding at around 9 months of age based on the average duration of any breastfeeding. Sixty percent of the infants were given infant/ follow-on formula during the CF period, and it was concerning that nearly 15% of the infants consumed unfortified cow's milk, despite the Thai authorities suggesting it should be avoided until 12 months[180]. Unsurprisingly, since solid food had been introduced, the average milk intakes fell by 26% at 9 months and 35% at 12 months compared with the average volume at 6 months.

Table R2.2 Milk feeding practices

Variables	Results
Breastfeeding practices	
- Exclusive BF until 6M (n, %)	64 (44.1)
- Providing only breast milk alongside CF until 12M (n, %)	53 (36.6)
- Any BF (n, %)	144 (99.3)
- Duration of exclusive BF, months (mean ± SD)	4.4 ± 2.0
- Duration of predominant BF, months (mean ± SD)	8.4 ± 4.4
- Duration of any BF, months (mean ± SD)	9.2 ± 3.9
Formula and dairy products	
- Number of infants receiving formula feeding (n, %)	87 (60)
- Duration of formula feeding, months (median, IQR)	3 (0, 9)
- Number of infants receiving cow's milk* before 12M (n, %)	21 (14.5)
Average intake of breast milk/ formula ml/day (mean ± SD)	
- 6M†	832.2 ± 182.5
- 9M‡	613.0 ± 198.6
- 12M‡	542.2 ± 198.0

†*The average intake was based on the 24-hour food recalls;* ‡*Average intakes were mainly based on the 3-day food records; *Unfortified cow's milk; BF – breastfeeding; CF – Complementary food; SD – standard deviation; M – months old*

R2.1.3 Meal composition

Before demonstrating the results from my cohort, some terminology should be explained. In the Thai context, mealtimes are divided into main meals (i.e., breakfast, lunch and dinner) and additional meals, so-called snack times.

According to the Thai complementary feeding recommendations, the number of main meals vary based on infant age while snacks should be provided 1-2 times a day. The Thai CF recommendations were based on the OPTIFOOD linear programme estimating appropriate complementary foods for infants and young children to optimise nutrient intakes during the CF period[181] (Appendix 11). Traditionally, only one course is served in each main meal for Thai infants. It usually consists of a staple (i.e., rice/ sticky rice/ noodle/ rice porridge), a dietary protein (e.g., egg, liver, pork, poultry, etc), and some vegetables. Although some parents offered fruits as a part of the main meal, a more common practice is to give them as a snack in a separate meal. In this cohort, milk intake (i.e., breast milk, formula, unfortified cow's milk) was counted independently from main meals and snacks while other dairy products (e.g., cheese, yoghurt) were counted as snacks according to common feeding practices among Thai parents.

As shown in table R2.3, the number of meals increased with age. At 9 months of age, the median number of main meals reached three times a day which was similar to their family members. When considering dietary composition of each main meal, at 6M, only half of parents provided ASFs to their children, but the ratio was dramatically increased at 9 and 12 months when almost all of them offered ASFs in every main meal. The dietary composition of each main meal was more diverse when infants reached 9 months old. The Thai complementary feeding recommendations suggest that parents should add ½ teaspoon of vegetable oil per day, but less than a quarter of families complied with this advice.

Likewise, snacks were also given more often and were more varied at 9 and 12 months. Fruits were the most common snack across age groups, but western snacks (i.e., bread/ pastry and dairy products) were increasingly offered especially in older infants. Although parents hardly ever offered sweets/ sugary beverages to their infants, other common snacks such as Thai commercial snacks may also contain some added sugar.

Table R2.3 Number of daily meals and meal composition†

Variables	6M	9M	12M
Numbers of meals per day, (median, IQR)	1 (1, 2)	4 (2, 3)	5 (4, 5)
Numbers of main meals per day (median, IQR)	1 (1,1)	3 (2, 3)	3 (3, 3)
Food composition in main meal*, (n, %)			
- Staple	94 (64.8)	143 (98.6)	145 (100)
- ASFs	72 (49.7)	144 (99.3)	144 (99.3)
- Vegetables	76 (52.4)	135 (93.1)	138 (95.2)
- Fruits	31 (21.4)	71 (49.0)	60 (41.4)
- Legumes/ nuts	5 (3.4)	18 (12.4)	19 (13.1)
- Commercial baby foods	37 (25.5)	9 (6.2)	1 (0.7)
- Added oils	3 (2.1)	36 (24.8)	26 (17.9)
Food groups† in each main meal, (n, %)			
- 1 group	17 (11.7)	3 (2.1)	2 (1.4)
- 2 groups	64 (44.1)	31 (21.4)	46 (31.7)
- 3 groups	49 (33.8)	92 (63.4)	89 (61.4)
- ≥ 4 groups	6 (4.1)	20 (13.8)	10 (6.9)
Number of snacks per day, (median, IQR)	0 (0,0)	1 (1, 2)	2 (1, 2)
Type of snacks*, (n, %)			
- Fruits	33 (22.8)	120 (82.8)	124 (85.5)
- Vegetables	1 (0.7)	22 (15.2)	21 (14.5)
- Traditional Thai snacks	1 (0.7)	28 (19.3)	42 (29.0)
- Commercial snacks	1 (0.7)	24 (16.6)	34 (23.4)
- Bread/ pastry	0	36 (16.6)	55 (37.9)
- Sugary sweets/ beverage	0	0	4 (2.8)
- Dairy products	1 (0.7)	8 (5.5)	26 (17.9)
- Others	0	13 (9.0)	21 (14.5)

†*Data were based on the study record form and food frequency questionnaires; *Parents can choose more than one group; † Food groups include carbohydrate (rice, sticky rice, noodle, etc.), protein (ASFs, nuts, legumes), fat (cooking oils, butter, lard, etc.), vegetables, and fruits; IQR – interquartile rank; M – months old*

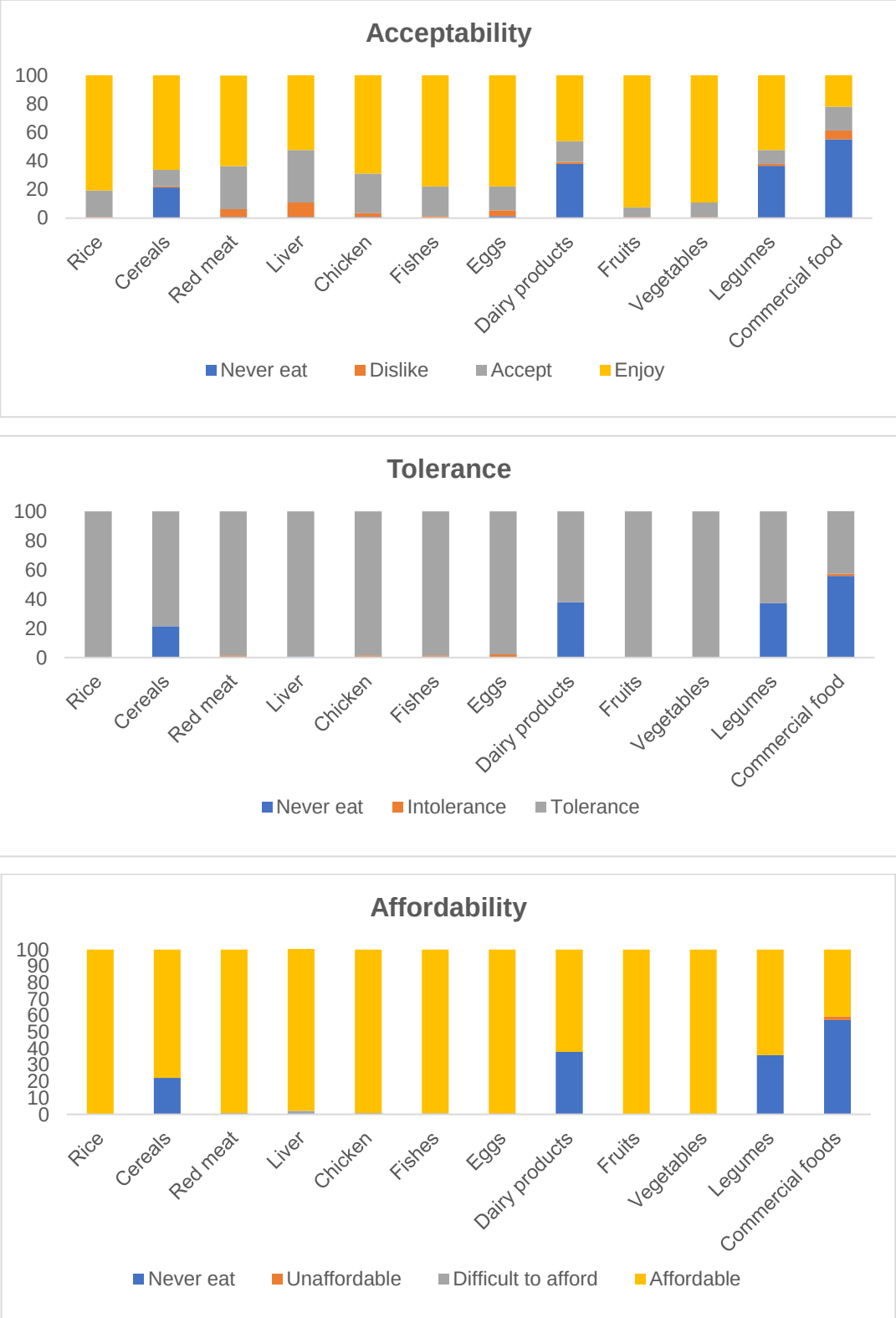
R2.1.4 Acceptability, Tolerance and Affordability of complementary foods

Other aspects of complementary foods must be considered in order to understand feeding practices and dietary patterns. These aspects include food preferences, food intolerances, and food price. These factors could result in limited food diversity in some specific areas/ populations.

In this section, the bar charts in figure R2.1 demonstrate the “Acceptability”, “Tolerance” and “Affordability” towards complementary foods among our study population from 6 to 12 months of age. In general, there was no significant problem in terms of acceptability, food tolerance and affordability. When focusing on acceptability, liver was more likely to be reported as being disliked by infants while other ASFs such as red meat, chicken, fishes and eggs were more accepted. In addition, the majority of infants were reported to enjoy eating rice, fruits and vegetable and around 40-50% of infants had no experience with dairy products, legumes and commercial baby foods. In terms of food tolerance, although 2% of parents reported non-specific rashes in their children after egg consumption, no serious reactions were reported. Furthermore, there was almost 100% affordability for all food groups given to the infants during the complementary feeding period.

Although most feeding practices seemed to be appropriate, there were some concerns related to ASFs as the introduction was slightly delayed in some families and the establishment of ASFs as a daily main meal occurred at older ages. A considerable number of infants received formula and unfortified cow’s milk during complementary feeding. Those practices can contribute to either too little or too much intake of dietary protein, thus it is important to know how they affect the nutrient intakes of the infants.

Figure R2.1 Acceptability, tolerance and affordability of complementary foods



R2.2 Nutrient intakes during complementary feeding

In this section, dietary consumptions were converted into nutrient intakes and subsequently compared with the Dietary Reference Intake for Thais (Thai DRI) launched in 2020[182]. The outcomes demonstrate the dynamic changes of nutrient intakes from 6 to 12 months of age and also illustrate the differences between actual intakes of the study population and the latest Thai DRI.

R2.2.1 Nutrient intakes of the study population

Overall, the results showed that the intake of most nutrients increased with infant age but to different degrees. Protein intake dramatically increased 60-80% from 6 to 9 months and around 25% from 9 to 12 months, whether expressed as percent protein-energy (%PE) or protein weight ratio (PW). At 12 months, the protein intake was twice the intake at 6 months and around 90% of dietary protein came from ASFs. Consumption of protein from plant-based foods was nearly 7 times lower than protein from ASFs at 12 months. Energy consumption increased by 42% from 6 to 12 months while there were small changes in percentages of energy distribution from carbohydrate (%CHO), and fat (%fat) as shown in table R2.4.

The same pattern of older infants having higher nutrient intakes was also found for micronutrients, except for vitamin A intake. Unlike other micronutrients, the peak of vitamin A intake was at 9 months and then fell by 42% at 12 months. Interestingly, the iron intake also increased sharply from 6 to 9 months (1.2 times) and tripled at 12 months. Similar to protein intake, ASFs were the main sources of iron during this period. The findings for protein and iron intake were consistent with the results in the previous section (section R2.1.3) that showed increasing provision of ASFs in the main meal at 9 and 12 months of age.

Although the data shown in table R2.4 demonstrate the dynamic changes of nutrient intakes during the transitional period, it is very difficult to define the adequacy of these nutrient intakes unless they are compared with the national recommendations. This information is provided in section R2.2.2.

Table R2.4 Nutrient intakes of infants at 6 to 12 months (n = 145)

Variables	6M†	9M‡	12M‡
Total energy, kcal (mean ± SD)	529.8 ± 145.1	639.5 ± 203.7	750.8 ± 215.4
%E distribution, (mean ± SD)			
- Carbohydrate	47.6 ± 2.8	50.6 ± 5.3	48.0 ± 7.0
- Protein	7.8 ± 1.5	12.6 ± 2.7	15.6 ± 3.0
Animal-based	7.4 ± 1.4	10.8 ± 2.8	13.6 ± 3.1
Plant-based	0.4 ± 0.3	1.8 ± 1.0	2.1 ± 0.7
- Fat	44.6 ± 2.6	36.7 ± 5.4	36.4 ± 5.7
Protein weight ratio, g/kg (mean ± SD)	1.4 ± 0.6	2.5 ± 1.0	3.2 ± 1.1
Calcium intake, mg (mean ± SD)	295.6 ± 156.5	347.0 ± 264.5	464.4 ± 285.8
Phosphorus intake, mg (mean ± SD)	181.4 ± 109.9	326.7 ± 197.0	461.3 ± 226.8
Iron intake, mg (median, IQR)			
- All food sources	1.6 (1.1, 5.8)	3.5 (2.2, 7.6)	4.8 (2.9, 9.2)
- Animal-based	1.4 (1.0, 5.4)	2.4 (1.5, 6.8)	3.9 (1.8, 7.9)
- Plant-based	0.1 (0.04, 0.2)	0.9 (0.4, 0.9)	0.8 (0.6, 1.2)
Zinc intake, mg (median, IQR)	1.5 (1.2, 3.8)	2.4 (1.6, 4.5)	3.5 (2.2, 5.4)
Vitamin A, retinol activity equivalents (median, IQR)	474.0 (386.5, 617.5)	1060 (486.3,1736.6)	606.0 (382.9, 1115.5)
Vitamin B1, mg (median, IQR)	0.1 (0.1, 0.4)	0.3 (0.2, 0.5)	0.4 (0.3, 0.7)
Vitamin B2, mg (median, IQR)	0.3 (0.2, 0.9)	0.6 (0.4, 1.2)	1.0 (0.5, 1.3)
Vitamin C, mg (median, IQR)	40.8 (32.6, 80.6)	66.6 (41.0, 98.2)	64.2 (40.2, 101.6)

†Data were based on the 24-hour food recalls; ‡Data were mainly based on the 3-day food records; %E – percentage of energy distribution; SD – standard deviation; IQR – interquartile rank

R2.2.2 Comparison of nutrient intakes between the study population and the Thai DRI 2020

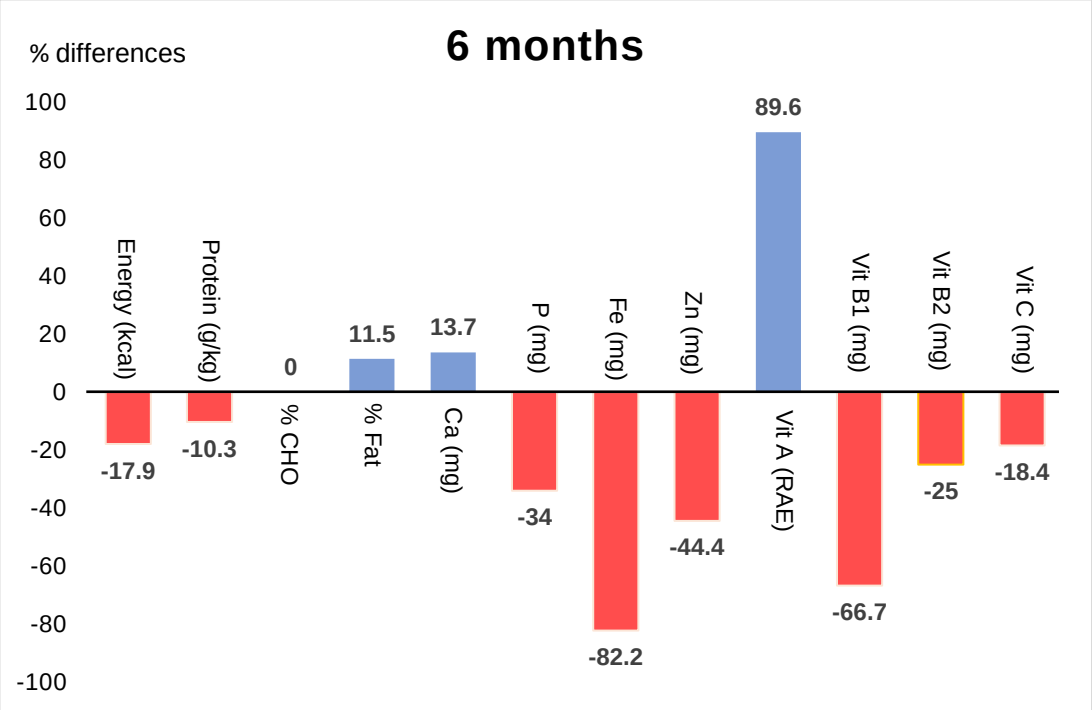
In the Thai DRI 2020[182], the reference intakes of infants during the complementary feeding period were separated into two age groups: 6-11 months and 1-3 years of age. Therefore, in this cohort, I have compared nutrient intakes at 6 and 9 months with the Thai DRI for infants aged 6-11 months and young children aged 1-3 years. Detailed information of the Thai DRI for both age groups are in appendix 12.

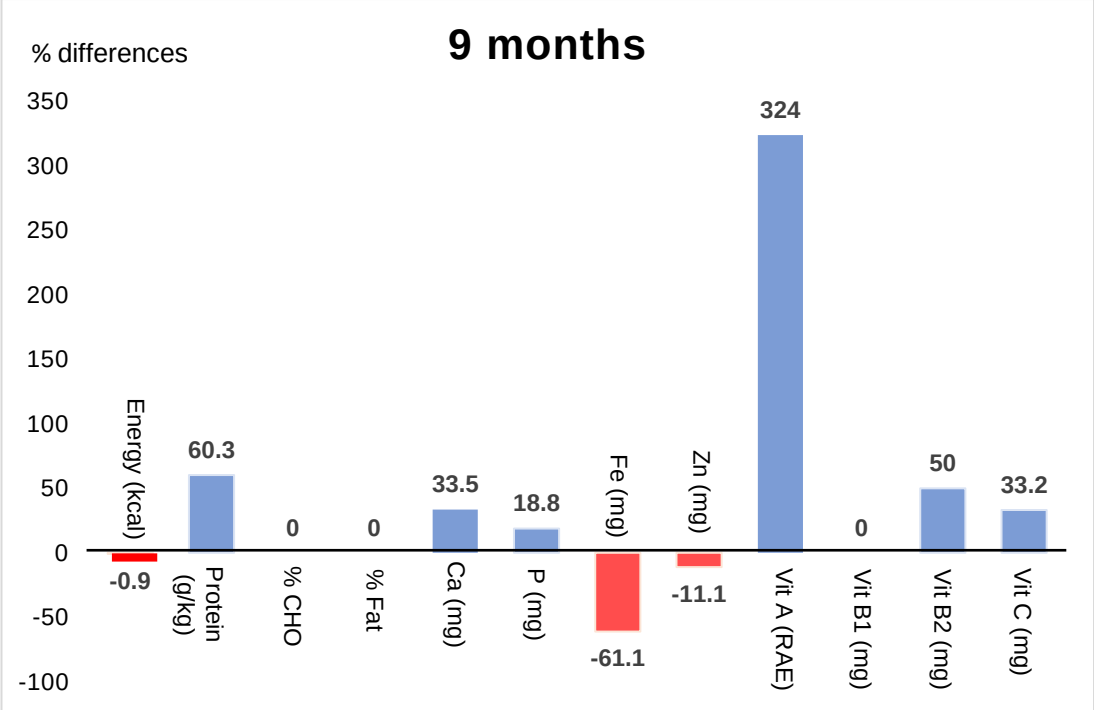
The bar charts in figure R2.2 demonstrate the percent differences between the average nutrient intakes found in our cohort and Thai DRI 2020. A red bar illustrates percent difference when the nutrient intake found in this cohort is lower than the Thai DRI 2020 while a blue bar represents a higher intake. Overall, the outcomes in this section were consistent with the results in the previous sections of this chapter. At the beginning (6 months of age), nutrient intakes were mostly lower than the reference intakes but started to improve at 9 and 12 months old, except for %fat, calcium and vitamin A. However, %fat and calcium intake were just a bit higher than the Thai DRI while vitamin A intake was nearly 90% higher than the recommendation at 6 months. In addition, vitamin A intakes were consistently higher than the Thai DRI while energy, iron and zinc intakes were still lower than the Thai DRIs throughout the weaning period.

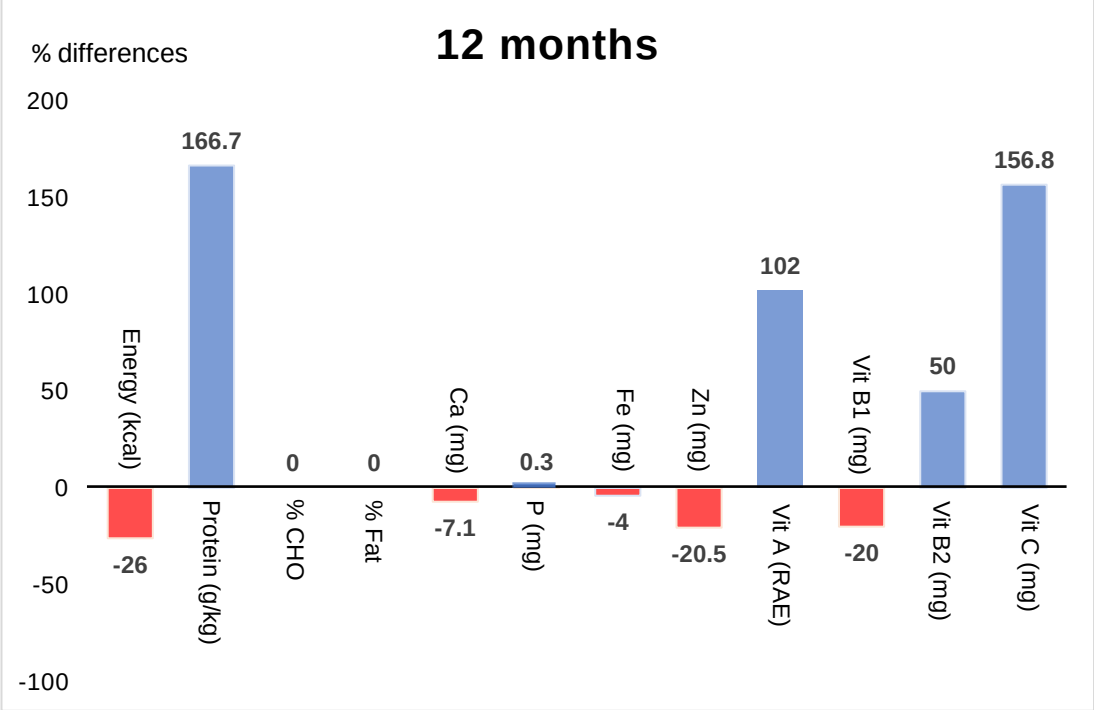
Noticeably, consistent with the results in section R2.1.3 and R2.2.1 which showed increasing consumption of ASFs over the weaning period, protein and iron intakes also increased dramatically at 9 and 12 months old. Protein intake started from a small percent lower than the Thai DRI but was significantly above the reference values at age 9 months (60%) and 12 months (166%), whereas the iron intake was still 82%, 61% and 4% lower than the reference intake at age 6, 9 months and 12 months of age, respectively. Although ASFs are also a better source of zinc compared to plant-based foods, the infants still received less zinc from the diet than the recommendation, indicating zinc is a problem nutrient among the study population. Vitamin C is the only vitamin

reported in this cohort for which the DRI at 1-3 years old is less than that at 6-11 months old: 25 vs 40 mg/day, respectively, thus it was unsurprising to see a higher intake of vitamin C than the Thai DRI at 12 months.

Figure R2.2 Comparison of nutrient intakes with Thai Dietary Recommended Intake (DRI) 2020







%CHO – percentage of energy provided by dietary carbohydrate; %Fat – percentage of energy provided by dietary fat; Ca – calcium; P – phosphorus; Fe – iron; Zn – zinc; RAE – retinol activity equivalents

R2.3 Summary of key results and discussion

Key results

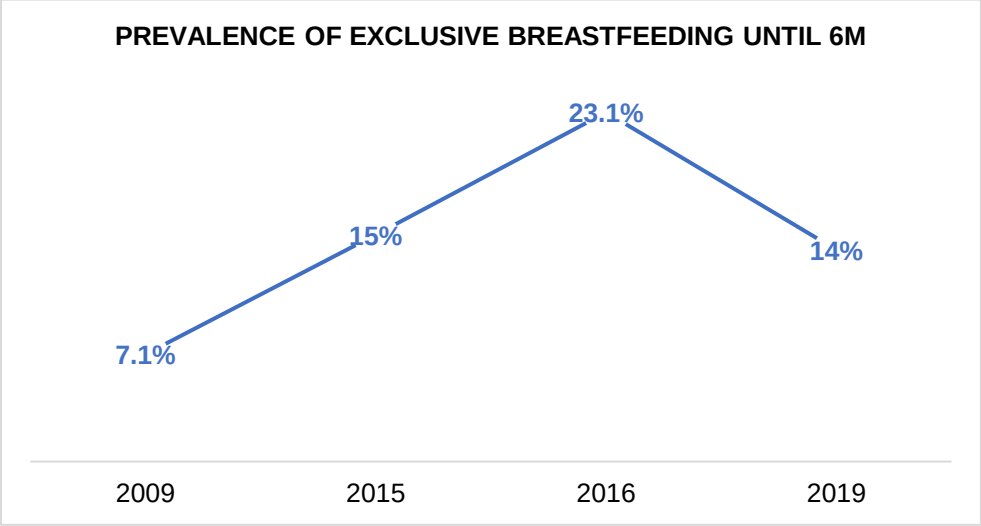
- Thai infants were introduced to complementary foods at an appropriate time and received an age-appropriate number of meals daily.
- There was a delay in the introduction of ASFs in some families.
- Dietary composition in the early stages of weaning was less diverse and only half of infants were offered ASFs at 6 months old.
- The consumption of ASFs was greater at 9 and 12 months old which contributed to the rapid increase in protein consumption and higher intake of iron and zinc during this period.
- Consumption of animal-based protein increased from 6 to 12 months and provided nearly 90% of dietary protein.
- From 9 months onwards, there was a rapid increase in dietary protein that may be partly attributed to increasing provision of formula and cow's milk as breastfeeding was usually discontinued at around this age.

The results presented in this chapter reflect the paradoxical practices of protein consumption especially protein from ASFs among Thai families. While some infants were introduced late to animal-based protein, other infants were exposed to high protein intake throughout the complementary feeding period, particularly those receiving infant formula.

Similar to the results from this cohort, recent studies have reported more appropriate timing of introduction of complementary feeding among LMICs as the majority of infants receive complementary foods at approximately 6 months of age[183, 184] which follows the WHO recommendations[185, 186]. In the Thai context, over a decade ago Jackson et al[122] reported that Thai parents living in Chiang Mai province introduced complementary foods to infants as early as 7 days after birth and the median age of introduction of complementary foods was about 1 months old. By contrast, more recent studies in Thai infants including our cross-sectional study demonstrate that most infants are given complementary foods at around 6 months old[121, 187, 188]

As shown in figure R2.3, the percentages of infants who are exclusively breastfeeding until 6 months have been rising since 2009 despite a small drop in the latest national survey. However, the figures are less than the percentage of infants exclusively breastfed until 6 months in our cohort (44.1%). One of the study sites, the Health Promoting hospital, is a baby-friendly hospital where breastfeeding is intensively promoted and use of infant formula is very restricted, which may affect the overall percentage of exclusive breastfeeding found in our study. The figure from our cohort is also similar to the global prevalence (41%) reported by the WHO/ UNICEF but still far below the target of 70% for 2030[189]. The combination of these positive factors for infant growth, namely timely introduction of complementary feeding with a higher rate of exclusive breastfeeding until 6 months of age, might suggest that the prevalence of DBM should be ameliorated over the next decade. However, as the DBM is still present, there might be other specific issues underlying this burden. The following paragraphs will discuss whether other key findings could be linked to the DBM.

Figure R2.3 Comparison of prevalence of exclusive breastfeeding until 6 months among the national surveys in Thailand during 2009 to 2019[15, 21, 22, 190]



Other key results from this cohort potentially relevant for the DBM can be divided into two distinctive groups. Firstly, the provision of a low variety of food groups at an early stage of weaning and delaying introduction of ASFs might contribute to undernutrition. Secondly, consuming high amounts of dietary protein in particular dairy protein during the complementary feeding period may lead to overweight/ obesity. These findings are supported by several studies.

Previous studies reported that the diversity of complementary foods is positively correlated with attained length of infants and may reduce the risk of stunting[191-193]. Additionally, in countries where undernutrition (i.e., wasting, underweight and stunting) is prevalent, the variety of complementary foods is usually low[194-196]. In particular for the South and South-East countries facing high prevalence of undernutrition, infants and young children consumed a lower variety of food groups including legumes and nuts, dairy products, meat and organs compared with the total intakes of all infants living in 42 LMICs[196]. According to the WHO infant and young child feeding indicator[197], the diversity of dietary intake can predict dietary quality and adequacy of micronutrient intake among infants and young children[198]. Noticeably, in Asian populations, ASFs such as flesh meat, eggs, fish and dairy products are not typically introduced as the first complementary foods, although they have more impact on dietary quality than other food groups[183, 199].

Delaying introduction of ASFs is another factor that can contribute to undernutrition. Although eggs were introduced from the start of the weaning process in our cohort, other ASFs were delayed until infants reached 7-8 months old in some families. This finding is consistent with the result from my previous study showing that 36% of Thai parents offered ASFs after infants were 7 months old[121]. This issue can cause more serious problems for breastfed infants as ASFs provide many essential nutrients, especially iron and zinc, that can fill the nutrient gap after 6 months of age when breast milk alone cannot meet infant requirements for these nutrients. A prospective cohort study assessing dietary intakes of Indonesian infants aged 6 to12

months also showed that consumption of ASFs was related to a decreased risk of stunting[200] while another study reported that stunted children consume ASFs less than non-stunted children[201]. Rice-based complementary foods traditionally given to Asian infants including Thai infants generally contain low energy[200] and inadequate amounts of "problem micronutrients" such as iron, zinc, calcium, vitamin A and iodine[31, 202]. Therefore, without an appropriate amount of ASFs introduced in a timely fashion, infant growth could be compromised and might lead to undernutrition. Conversely, a rapid increase and excess intake of dietary protein, especially from formula and cow's milk, may also be undesirable.

As shown in table R2.4, protein is the only macronutrient that consistently increases throughout the complementary feeding period. Noticeably, protein intakes were significantly higher than the Thai DRIs at 9 and 12 months when the majority of Thai mothers had discontinued breastfeeding, according to the average duration of 'any breastfeeding' (Table R2.2). When combined with premature exposure to unfortified cow's milk and providing dairy products as snacks, it can be assumed that high protein intakes at 9 to 12 months result not only from non-dairy ASFs, but that dairy protein is a main contributor. For infants who are predominantly formula-fed, their protein intakes might be too high for a longer duration compared with breastfed infants.

The increasing intake of animal-based protein reported in this cohort is quite different from the previous reports in Thai infants[31, 203] but closer to the situation in high-income settings where protein in complementary foods mainly comes from dairy products and other ASFs[28, 204]. According to Damianidi et al[28], the protein intake among infants in 5 European (EU) countries often exceeds the EU recommendations from 9 months old and the main source of protein in the first 2 years of life is dairy protein from infant formula. Likewise in the US, a country with a high prevalence of childhood obesity, the main protein sources from 6-12 months of age are also infant formula and milk[204]. Undoubtedly, the relationship between high protein intake and risk of later overweight/ obesity is widely supported by the evidence from high-income countries[57, 205-208] while there is still a lack of data from LMICs[61].

Considering protein intake among the younger populations in LMICs including Thailand where a rapid socioeconomic transition and change in eating style towards western diets have already taken place, most studies on complementary feeding report that infants receive adequate or even high amounts of dietary protein[122, 201, 209-212]. Although protein quantity is adequate, there is still a concern about protein quality in these populations. Researchers from LMICs are more likely to investigate the impact of consumption of ASFs on infant growth in the context of preventing undernutrition[44, 67, 69]. As the current evidence suggests that increased consumption of ASFs can improve linear growth and weight gain in infants, many LMICs tend to encourage parents to provide more ASFs, in particular milk and dairy products, to promote infant and child growth[25, 213].

Recently, the South-East Asian Nutrition Surveys (SEANUTS)[214] reported that infants and children aged 6 months to 12 years old in four nations (Thailand, Indonesia, Malaysia and Vietnam) consumed milk and dairy products regularly on a daily basis, especially infants and children less than 6 years of age, except for Thai children where almost all were regular consumers of milk regardless of their age. In addition, while higher maternal education and socioeconomic status are positively associated with dairy consumption in most countries, Thailand is the only nation where dairy consumption is common across all income and education groups. Furthermore, this survey also reported the amount of dairy protein intake compared to the local recommendations. The results showed that even children who consumed less than 1 dairy consumption (one dairy consumption equals 100 g of UHT milk/ 50 g of yoghurt/ 10 g of condensed milk/ 5 g of cheese on daily basis) had a protein intake that was 119% of the recommendations and this reached 179% for children having at least 2 dairy consumptions. It seems that protein intake during the complementary feeding period in Thai infants has become westernised and is moving closer to the pattern seen in high-income countries. However, it should be noted that there is no UL for protein intake, therefore the assumption that protein intakes above the DRI could contribute to adverse outcomes such as overweight/ obesity still needs more robust evidence.

When considering adequacy of nutritional intake within the study population it is important to note that comparisons with Thai DRIs may not provide an accurate assessment. There are a number of reasons for this. Dietary assessment is subject to bias as it relies on reports of intake that may or may not be accurate. Commonly, these reports rely on recall and in the case of children are based on parental observations, which introduces further bias. Day-to-day variation in intake also complicates interpretation of dietary intake data. Even if food records were completely accurate, the extent to which they reflect habitual intake is uncertain. Further, when nutrient intakes are measured in a population there is considerable inter-individual variation. Defining inadequacy as an intake less than or equal to the DRIs could also lead to an overestimation as the DRIs describe intakes that are adequate for the majority of the population and the prevalence of inadequate intake depends on the shape and variation of the usual intake distribution, not on the average intake[215]. The risk of deficiency in an individual at a given intake may vary from almost no risk to almost absolute certainty of risk. However, for most nutrients, if the average intakes are greater than the DRIs, the prevalence of inadequacy could be assumed to be acceptably low[216].

In summary, the results presented in this chapter suggest that dietary protein is potentially a “shared contributor” of the DBM in the study population. Some infants might consume too little protein, both in quantity and quality, due to delayed introduction of ASFs and this, combined with a low variety of food groups may result in at least one form of undernutrition (e.g., stunting, wasting, underweight and iron deficiency). Conversely some infants, especially those fed formula, might consume too much protein which might increase the risk of childhood overweight/ obesity. However, the assumption that “dietary protein” might be a “shared driver” of the DBM still needs confirmation. In the next chapter, I will examine the relationship between dietary protein amount and source and infant growth outcomes.

Chapter 7: Results 3

Amount and sources of dietary protein during complementary feeding and their associations with infant growth

The aim of this chapter is to investigate the impact of dietary protein in complementary foods on infant growth. Protein intakes among Thai infants during the complementary feeding period will first be compared to international recommendations and the recent national survey in Thailand and South-East Asian (SEA) countries. Associations between protein intake and growth outcomes will be examined, taking into account both quantity and quality (protein sources). In addition, although causality cannot be proven in this observational study, I try to improve causal inference in the relationship between protein intake and growth by using a DAG to identify relevant confounders. The key results of this chapter are expected to address two important questions: (1) whether the impact of protein intake during complementary feeding on the growth of infants in LMICs is similar to that in high-income settings. (2) which protein source(s) have the most impact on growth.

R3.1 Amount of protein intake and comparisons with international recommendations and the recent survey in SEA countries

In the last chapter I compared the amount of dietary protein consumed by the infants in this cohort with the Thai DRIs. In this chapter, I compared protein intake reported at 6 to 12 months old with the international recommendations (Table R3.1). Protein intakes shown as protein-weight (PW) ratio at 9 and 12 months old were markedly higher than the international recommendations. At 6 months, protein intake was just above the international recommendations but then reached around 2 and 3 times higher at 9 and 12 months, respectively (Figure R3.1).

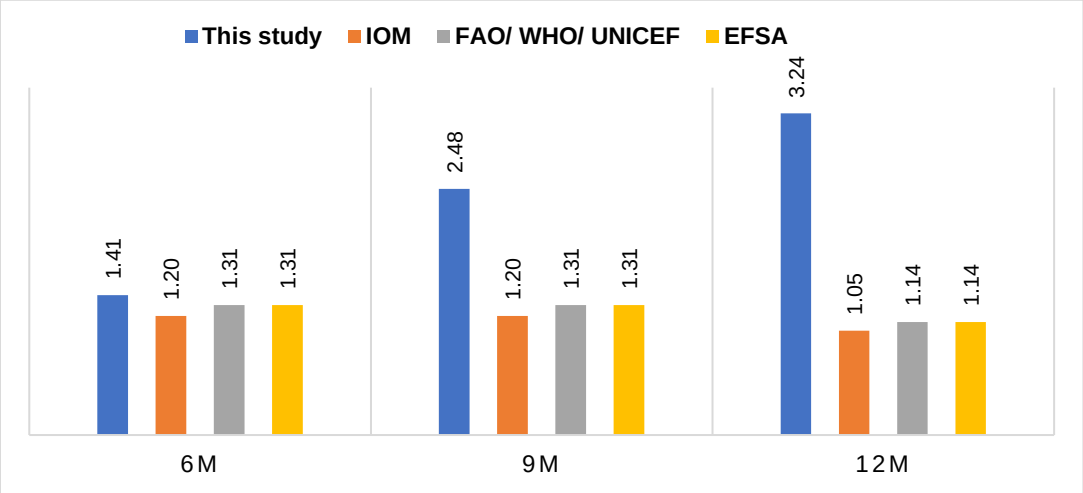
When considering the results of Thai infants aged 6 to 12 months from the SEANUTS[217] in table R3.2 (unpublished data), although protein intake expressed as total protein, PW ratio and %PE were quite similar to my study, infants from my cohort consumed slightly more ABP and less PBP. While energy and other macronutrients were also slightly different, daily iron intake was 25% lower in my study. The next section will provide the details of food sources that were commonly used as complementary foods to provide a clearer picture of the main protein sources given to infants in my study.

Table R3.1 Comparison of protein intake between this cohort and the international recommendations

Data	6 months	9 months	12 months
This study† (2018-9)	(mean ± SD)	(mean ± SD)	(mean ± SD)
Total daily intake, g/d	10.49 ± 4.03	20.52 ± 8.66	29.24 ± 9.98
PW ratio, g/kg/d	1.41 ± 0.56	2.48 ± 1.04	3.24 ± 1.05
%PE, %	7.78 ± 1.46	12.64 ± 2.73	15.61 ± 3.03
Thai DRIs (2020), PW ratio	1.56		1.20
IOM (USDA, 2005)[81], PW ratio	1.20		1.05
WHO/ FAO/ UNICEF (2007)[11], PW ratio	1.31‡		1.14‡
EFSA (2017)[218], PW ratio	1.31*		1.14*

*†Data were based on the 24-hour food recalls (6 months) and the 3-day food records (9 and 12 months); ‡Safe levels (Average intake + 1.96SD); *Population reference intake (PRI); PW ratio – protein weight ratio; %PE – percentage of energy provided by dietary protein; SD – standard deviation; DRIs – Dietary Recommended Intakes; IOM – Institute of Medicine; USDA – The United States Department of Agriculture; WHO – The World Health Organisation; FAO – The Food and Agriculture Organisation; UNICEF – The United Nations Children's Fund; EFSA – The European Food Safety Authority*

Figure R3.1 Comparison of the protein-weight* (PW) ratio between this cohort and the international recommendations



**Unit of PW ratio is g/kg/day; IOM – the Institute of Medicine; FAO – the Food and Agriculture Organisation; WHO – the World Health Organisation; UNICEF – the United Nations Children's Fund; EFSA – the European Food Safety Authority*

Table R3.2 Comparison of average nutrient intakes of Thai infants from 6 to 12M between this cohort and the South-East Asian Nutrition Survey (SEANUTS)[219]

Nutrient intakes (mean ± SD)	**Our study**[†] (n = 145)	**SEANUTS** (n = 135)
Total energy (kcal/d)	639.6 ± 150.6	684.6 ± 232.5
Total protein, mg/d	19.9 ± 6.4	20.4 ± 10.2
- ABP (% of total intake)	17.7 ± 5.9 *(88.8%)*	15.8 ± 9.4 *(77.5%)*
- PBP (% of total intake)	2.5 ± 1.4 *(12.8%)*	3.8 ± 2.4 *(18.7%)*
PW ratio, g/kg/d	2.4 ± 0.7	2.5 ± 1.3
%Energy distribution		
%PE	12.0 ± 0.5	11.7 ± 3.6
%CHO	48.8 ± 3.5	54.4 ± 7.4
%Fat	39.2 ± 3.2	33.9 ± 6.7
Total iron intake (mg/d)	5.0 ± 3.3	6.7 ± 4.4

[†]Data were mainly based on the 3-day food records; SD – standard deviation; ABP – animal-based protein; PBP – plant-based protein; PW ratio – protein weight ratio; %PE – percentage of energy provided by dietary protein; %CHO – percentage of energy provided by dietary carbohydrate; %Fat – percentage of energy provided by dietary fat

R3.2 Sources of dietary protein in complementary foods of the study population

As shown in table R3.3, food sources were divided into 3 groups based on the protein sources of interest; dairy ASFs, non-dairy ASFs and plant foods. Infants consumed fewer varieties of ASFs, both non-dairy and dairy compared with plant foods at 6 months. However, this improved at 9 and 12 months. Iron-rich foods such as red meat and liver were less likely to be given to the infants on a daily basis compared to eggs which contain less well-absorbed non-haem iron. Noticeably, various types of dairy products were provided at 12 months, but the most common product was still infant formula. Although the plant-based foods included a greater variety of food items, most of them were subtypes of "rice" which is a staple in Thai dishes. The most common food items from each food source that had been given to the infants on a daily basis since age 6 months were hens' egg, formula and polished rice.

The following sections examine whether the amount and source of dietary protein in complementary foods influences infant growth in my cohort.

Table R3.3 Ranking of food sources providing dietary protein to Thai infants on a daily basis at each age (n = 145)

Food sources	6M†	9M†	12M†
Non-dairy ASFs (% of all infants)			
1st	Hen egg (15.9)	Hen egg (51.7)	Hen egg (52.4)
2nd	Pork (1.4)	Pork (18.6)	Pork (22.1)
3rd	Chicken liver; River fish (0.7)	Chicken; River fish (6.2)	Chicken (4.1)
4th	-	Sea fish (4.8)	Pork liver (2.1)
5th	-	Pork liver (4.1)	Chicken liver; Duck egg (1.4)
Dairy products (% of all infants)			
1st	Infant formula (31.1)	Follow-on formula (26.9)	Follow-on formula (38.7)
2nd	-	Infant formula (20)	Others* (20)
3rd	-	Others (0.7)	Cow's milk (9.7)
4th	-	-	Infant formula (6.2)
5th	-	-	Yoghurt (1.4)

Plant-based foods (% of all infants)			
1st	Polished rice (44.2)	Polished rice (56.6)	Polished rice (75.9)
2nd	Commercial baby food† (25.5)	Rice porridge (27.6)	Glutinous rice (34.5)
3rd	Banana (22.3)	Banana (18.6)	Brown rice (22.8)
4th	Brown rice (13.1)	Brown rice; Orange (17.2)	Orange (17.9)
5th	Pumpkin (11.1)	Pumpkin (11.1)	Rice porridge (15.8)

*†Data were based on the food frequency questionnaires; *Cheese, Milk ice-cream, Fermented milk; †Cereal-based; ASFs – animal source foods; M – months old*

R3.3 Categorisation of High, Median and Low protein intake groups

Before investigating the association between protein intake and growth, it is necessary to explain how I classified the cohort into high, median and low protein intake groups. I first needed to decide the best measure of protein to use in the analyses - whether total intake (g/day), PW ratio (g/kg/day) or %PE. Finally, I chose "%PE" to represent protein intake for 4 reasons:

1) There is a major disadvantage to using "total protein intake" because larger infants consume more protein. It might therefore be very difficult to conclude whether protein intake affects weight gain, rather than a bigger infant just eating relatively more food and protein.
2) The PW ratio also leads to another problem if the main outcome is the weight-related z-score. The following equations show that there is an inverse association between PW ratio and WAZ or WLZ via the infant weight when these variables are included in the same regression model.

$$PW = \frac{\text{Total protein intake (g/day)}}{\text{Individual weight (kg)}}$$

$$WAZ = \frac{\text{Individual weight} - \text{mean weight of infants at same age}}{\text{Standard deviation (SD)}}$$

$$WLZ = \frac{\text{Individual weight} - \text{mean weight of infants at the same length}}{\text{Standard deviation (SD)}}$$

3) The %PE gives information on protein density which reflects one aspect of diet quality and has been used as a reference for the target population to balance risk of protein deficiency[220] and excess[221]. In

addition, the literature in this field usually describes the association between protein intake and outcomes in the form of %PE[73, 206, 222] so it might be easier to compare my findings with those studies using this measure.

4) As the %PE is calculated based on energy intake, it is per se adjusted for total food consumption. In other words, the %PE can represent the protein intake regardless of body size and energy intake of each individual.

The next point to consider was how to determine cut-off levels of %PE to categorise high, median and low protein intake. Although western authorities suggest that 15% is a safe level in terms of reducing risk of obesity for infants[221], there is no strong evidence to support recommendations for protein intake in infants from other ethnic groups in order to reduce risk of overweight/ obesity. The previous international recommendations were more focused on protein deficiency[223]. Almost three decades ago, experts recommended that the safe level of %PE should be 6.9-7.1% and 6.2-7% based on the protein quality for infants aged 6-9 and 9-12 months, respectively[224] while the latest one from the Joint WHO/ FAO/ UNU experts suggested that the safe level should be 7.6% and 7.8% for female and male infants[225].

Due to a lack of cut-off levels for high protein intake in global populations, I decided to categorise protein intake groups by using data from this cohort. Table R3.4 shows the similar values for %PE from 6 to 12 months for both means $\pm$ SD and median with IQR. However, the number of infants in each protein group using cut-off values from the median with IQR was more balanced than using cut-off values from means $\pm$ SD. Finally, high, median and low protein intakes were defined as %PE $\geq 75^{th}$ percentile, 25^{th} to 75^{th} percentile and $\leq 25^{th}$ percentile, respectively. Using these criteria, in the next section I examine growth z-scores and nutritional status among infants in these protein intake groups. Noticeably, the means of PW ratio exceeded the recommendations shown in table R3.1 when infants were 9M and 12M, even in the low protein intake group where the means for PW ratio at 9M and 12M were 1.8 and 2.7 g/kg/day, respectively.

Table R3.4 Categorisation of infants based on percent protein-energy from 6 to 12M

Protein intake	Results†	
	means ± SD	median (IQR)
Percent protein-energy (%PE)	12.01 ± 1.63	12.02 (10.86, 12.92)
Cut-off value by %PE		
High	≥ 13.64[a]	≥ 12.92[d]
Median	10.39 – 13.63[b]	10.87 – 12.91[e]
Low	≤ 10.38[c]	≤ 10.86[f]
Number of infants in each protein group (n = 145)		
High	20 (13.8%)	36 (24.8%)
Median	102 (70.3%)	73 (50.4%)
Low	23 (15.9%)	36 (24.8%)

†Data were mainly based on the 3-day food records; a ≥ means + SD; b < means – SD to < means + SD; c ≤ means – SD d ≥ P_{75}; e < P_{25} to < P_{75}; f ≤ P_{25}; %PE – percentage of energy provided by dietary protein; SD – standard deviation; IQR – interquartile rank

R3.4 Comparison of anthropometry and prevalence of malnutrition among protein intake groups

In this section, I present the analyses with protein intake as a categorical variable based on the %PE from 6 to 12 months old as explained in section R3.3. The outcome measures were infant growth (z-scores) analysed as 1continuous variables and nutritional status as categorical variables. I will present the dose-response effect of %PE on infant growth in the next section.

Interestingly, as shown in table R3.5, the comparisons of all growth z-scores between groups showed a consistent pattern throughout the study period, although with varying degrees of statistical significance. Values were highest in infants from the high protein (HP) group, followed by the median protein (MP) group, while the infants from the low protein (LP) group had the lowest values. Notably, there were two opposite trends in terms of changes in weight-related parameters (WAZ, WLZ and BMIZ) from 6 to 12M. While the z-scores for all weight-related parameters increased over time in the HP group, a

downward trend was found in both MP and LP groups. The gap in weight-related z-scores between the HP and other groups increased with age. There was no significant difference between groups at 6 months, while at 9 months WAZ of the HP group was significantly higher than the other two groups and at 12 months all weight-related parameters were significantly higher in the HP group compared to MP and LP groups.

Infants in the HP group had positive values for all weight-related conditional growth parameters, suggesting that they had gained more weight than expected between 6 and 12 months. Their values were also significantly different compared to the conditional WAZ, WLZ and BMIZ of the infants from the other two groups. Although all growth parameters were higher in the MP group, they were not significantly different to those of the LP group. Interestingly, differences in LAZ among infants the protein groups were not significant, except at 6 months. However, at this age, differences between pairs in the post-hoc analysis were not significant. Additionally, the conditional LAZ showed only small values in all groups suggesting length gain deviated little from the expected values regardless of protein intake.

As shown in table R3.6, the prevalence of all forms of malnutrition did not differ significantly between protein intake groups although there was a trend towards higher percentages of infants with undernutrition in the LP group.

Table R3.5 Comparison of anthropometry among protein intake groups

Growth anthropometry	High (n=36)	Median (n=73)	Low (n=36)	p-value (*p*)*	*p*** H vs L	*p*** H vs M	*p*** M vs L
6M[#]							
WAZ	-0.14	-0.40	-0.50	0.18	0.23	0.39	1.00
WLZ	0.02	-0.06	-0.05	0.91	1.00	1.00	1.00
BMIZ	-0.08	-0.14	-0.16	0.93	1.00	1.00	1.00
LAZ	-0.15	-0.55	-0.66	0.04	0.09	0.06	1.00
9M[#]							
WAZ	0.03	-0.46	-0.59	**0.003**	**0.004**	**0.01**	1.00
WLZ	0.14	-0.22	-0.24	0.08	0.17	0.12	1.00
BMIZ	0.09	-0.24	-0.26	0.13	0.27	0.19	1.00
LAZ	-0.17	-0.48	-0.69	0.06	0.05	0.29	0.81
12M[#]							
WAZ	0.10	-0.45	-0.60	**0.001**	**0.001**	**0.003**	1.00
WLZ	0.25	-0.30	-0.39	**0.004**	**0.008**	**0.009**	1.00
BMIZ	0.29	-0.19	-0.31	**0.01**	**0.02**	**0.04**	1.00
LAZ	-0.19	-0.55	-0.64	0.08	0.11	0.17	1.00
Conditional[#]							
WAZ	0.54	-0.13	-0.28	**0.001**	**0.001**	**0.002**	1.00
WLZ	0.58	-0.14	-0.30	**<0.001**	**<0.001**	**0.001**	1.00
BMIZ	0.46	-0.13	-0.27	**0.002**	**0.004**	**0.009**	1.00
LAZ	0.07	-0.01	-0.04	0.90	1.00	1.00	1.00

***ANOVA;* ****Post-hoc analysis using Bonferroni's test;* [#]Data were shown as mean values; *H – high intake group; M – median intake group; L – low intake group; WAZ – weight-for-age z-score; WLZ – weight-for-length z-score; BMIZ – body mass index z-score; LAZ – length-for-age z-score*

Table R3.6 Prevalence of malnutrition among protein intake groups

Prevalence n (%)	**HP** (n=36)	**MP** (n=73)	**LP** (n=36)	***p-value**
Underweight				
6M	0	1 (1.4%)	1 (2.8%)	0.73
9M	0	1 (1.4%)	1 (2.8%)	0.60
12M	0	3 (4.1%)	3 (8.3%)	0.18
Wasting				
6M	0	0	1 (2.8%)	0.22
9M	0	1 (1.4%)	0	0.61
12M	1 (2.8%)	4 (5.5%)	0	0.33
Stunting				
6M	0	1 (1.4%)	3 (8.3%)	0.06
9M	0	2 (2.7%)	3 (8.3%)	0.14
12M	0	3 (4.1%)	4 (11.1%)	0.08
Overweight				
6M	1 (2.8%)	0	1 (2.8%)	0.36
9M	1 (2.8%)	0	0	0.22
12M	0	0	1 (2.8%)	0.22

***Fisher's exact test; HP – high protein-intake group; MP – median-intake group; LP – low protein-intake group; M – months old*

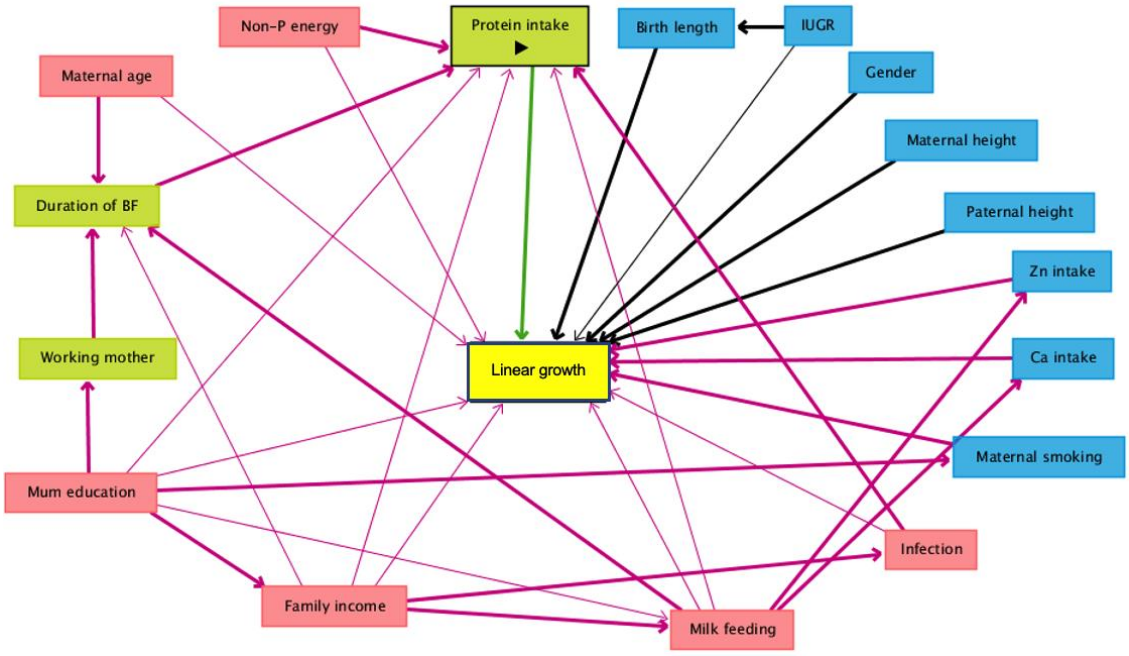
R3.5 DAG and selection of co-variates for regression analysis

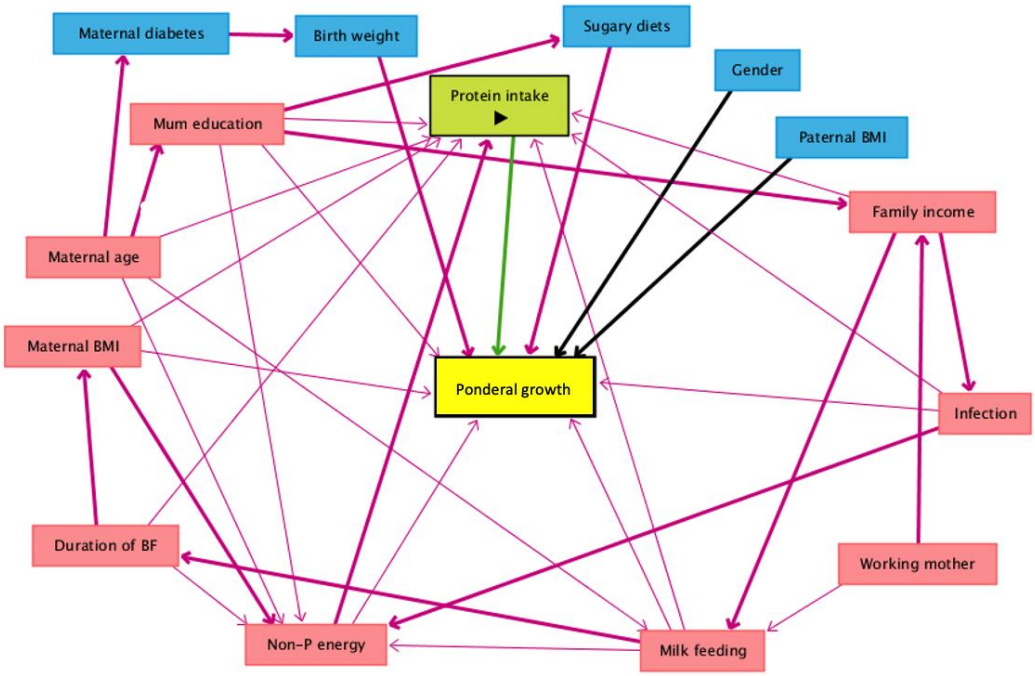
Before moving to the main analyses showing the dose-response between protein consumption and growth outcomes, it is necessary to describe how I selected co-variates for inclusion in the regression analysis.

I started by reviewing the relevant literature to identify which factors influence infant growth, whether linear or ponderal. After identifying all relevant factors from the literature, I used "DAGitty.net" (more detailed information on DAG was provided in Chapter 5) to create two separate DAGs, one for linear growth and one for ponderal growth, using all variables collected in my study with the potential to confound or mediate the relationship between protein intake and infant growth. This programme allowed me to enter the proposed causal path between my predictor (protein intake) and the outcomes of interests (linear and ponderal growth) as well as associations between other variables, in a

window called “model code” (Appendix 13). After all relations had been established in the model code, the programme automatically created the DAG and suggested the “minimal sufficient adjustment sets” excluding all non-casual paths and leaving all casual paths that should be controlled for as “co-variates” for the impact of protein intake on the growth outcomes (linear or ponderal growth). These suggested co-variates were assumed to be “confounders” of the causal path between protein intake and growth outcomes. According to the DAGs in figure R3.2, there was no mediator lying in the casual path between protein intake and growth outcomes, thus the minimum set of factors for total effect and direct effect were similar for these DAGs. The set of suggested co-variates that should be controlled for is shown in table R3.7.

Figure R3.2 The directed acyclic graph for linear and ponderal growth





Green box: predictor; Yellow box: main outcomes; Red box: potential confounders; Blue box: other variables; Green arrow: casual path; Red arrow: bias path

Table R3.7 Selected co-variates suggested by DAG

Linear growth (conditional LAZ)	**Ponderal growth** (conditional WAZ, WLZ, BMIZ)
1) Type of milk feeding 2) Non-protein energy (6-9M and 9-12M) 3) Maternal age 4) Maternal education 5) Infection (Frequency of illness) 6) Family income	1) Type of milk feeding 2) Non-protein energy (6-9M and 9-12M) 3) Maternal age 4) Maternal education 5) Infection (Frequency of illness) 6) Maternal BMI

WAZ – weight-for-age z-score; WLZ – weight-for-length z-score; BMIZ – body mass index z-score; BMI – body mass index; LAZ – length-for-age z-score; M – months old

R3.6 Associations between protein intake and growth outcomes

There were three main steps for the analyses in this section. I started with a univariate regression analysis to see whether the predictor (%PE) and other co-variates were associated with the outcomes (i.e., conditional growth for WAZ, WLZ, BMIZ and LAZ), and then I entered both predictor and suggested co-variates from the DAG into the first multiple regression model. Finally, I used a second multiple regression model to determine effects of %PE on conditional growth adjusting only for significant co-variates found from the univariate regression analyses and the first model. Henceforth, for the regression analyses, I chose only the average %PE from 9-12 months old as the main predictor because during this time, dietary recommendations from the Thai complementary feeding guideline (Appendix 11) are the same and protein intake is more established and diverse compared to the early stage of CF. In addition, the supportive evidence also indicates that key food sources providing energy and macronutrients are fairly similar between 9 and 12 months[226] and diets at 9M are strongly related to risk of childhood stunting[200]. I chose to use conditional growth as it is free of bias from previous body size and represents whether infants grow more or less than expected based on the growth pattern of this specific population.

The outcomes from the univariate regression analyses shown in table R3.8 demonstrated that the %PE from 9-12 months was a significant positive predictor of all conditional weight-related z-scores while there was no relation between %PE from 9-12 months and conditional LAZ. According to table R3.9, the univariate regression models investigating correlations between other co-variates and growth outcomes showed that “Type of milk feeding from 9-12M”, “Non-protein energy 6-9M” and “Non-protein energy 9-12M” were significantly and positively associated with conditional weight-related z-scores. In other words, “Formula feeding” and increasing intake of “Non-protein energy 6-9M” or “Non-protein energy 9-12M” may promote greater weight gain. “Type of milk feeding from 9-12M” showed a significant association with conditional LAZ.

In multiple regression analyses, only the %PE from 9-12 months of age showed consistent results, being significantly associated with all conditional weight-related z-scores regardless of non-protein energy intake and type of milk feeding (Table R3.11). By contrast, neither %PE nor other co-variates were associated with conditional LAZ. The higher adjusted R^2 in the second multiple regression models suggest that the model predicted a higher proportion of the outcome overall compared to the first model.

In terms of the dose-response association between %PE and infant growth, these analyses suggest that a 1% increase in %PE from 9-12 months is associated with an increase in conditional weight-related z-scores by 0.22 – 0.27 z-scores, regardless of non-protein energy consumption during the CF period and type of milk feeding.

Taken together, the key outcomes reported in section R3.4 and R3.6 strongly support the hypothesis that higher protein intake during the CF period is associated with more rapid infancy weight gain. The next section will investigate whether all protein sources have the same effect on growth.

Table R3.8 Univariate regression analyses investigating association between %PE from 9-12 months old and conditional growth

Predictor	Outcomes	Results		
		Adj R^2	**ß**	**p-value**
%PE	Conditional WAZ	0.08	0.29	**< 0.001**
9-12M	Conditional WLZ	0.09	0.31	**< 0.001**
	Conditional BMIZ	0.06	0.25	**0.002**
	Conditional LAZ	- 0.006	0.04	0.66

%PE – percentage of energy provided by dietary protein; Adj R^2 – adjusted coefficient of determination; ß – regression coefficient; M – months old; WAZ – weight-for-age z-score; WLZ – weight-for-length z-score; BMIZ – body mass index z-score; LAZ – length-for-age z-score; M -months old

Table R3.9 Univariate regression analyses investigating associations between selected co-variates and growth outcomes

1) Main outcomes: Ponderal growth (i.e., conditional WAZ, WLZ, BMIZ)

Co-variates	Outcomes	Results		
		Adj R^2	ß	p-value
Type of milk feeding 9-12M*	Conditional WAZ	0.05		
	Combined		0.02	0.91
	Formula		0.53	**0.008**
Breast milk[1]	Conditional WLZ	0.04		
Combined	Combined		0.27	0.19
Formula	Formula		0.55	**0.006**
	Conditional BMIZ	0.03		
	Combined		0.34	0.09
	Formula		0.50	**0.01**
Non-Protein energy 6-9M	Conditional WAZ	0.09	0.002	**< 0.001**
	Conditional WLZ	0.06	0.002	**0.002**
	Conditional BMIZ	0.05	0.002	**0.006**
Non-Protein energy 9-12M	Conditional WAZ	0.06	0.002	**0.001**
	Conditional WLZ	0.03	0.002	**0.03**
	Conditional BMIZ	0.02	0.001	0.05
Maternal education* (graduated & above: Yes[1]/No)	Conditional WAZ	- 0.013	0.04	0.82
	Conditional WLZ	- 0.014	- 0.01	0.94
	Conditional BMIZ	- 0.012	- 0.08	0.62
Frequency of illness	Conditional WAZ	- 0.006	- 0.03	0.72
	Conditional WLZ	- 0.006	- 0.03	0.72
	Conditional BMIZ	- 0.006	- 0.03	0.71
Maternal BMI	Conditional WAZ	- 0.006	- 0.007	0.73
	Conditional WLZ	- 0.007	<0.001	1.00
	Conditional BMIZ	- 0.007	- 0.002	0.92
Maternal age	Conditional WAZ	- 0.007	0.001	0.96
	Conditional WLZ	- 0.007	<0.001	0.99
	Conditional BMIZ	- 0.003	0.01	0.48

**Categorical variables; [1]reference category; Adj R^2 – adjusted coefficient of determination; ß – regression coefficient; WAZ – weight-for-age z-score; WLZ – weight-for-length z-score; BMIZ – body mass index z-score; BMI – body mass index; M – months old*

2) Main outcome: Linear growth (conditional LAZ)

Co-variates	Results		
	Adj R^2	**ß**	**p-value**
Type of milk feeding 9-12M*	0.03		
Breast milk[1]		NA	NA
Combined		- 0.44	**0.03**
Formula		0.04	0.86
Non-Protein energy 6-9M	0.007	0.001	0.16
Non-Protein energy 9-12M	0.01	0.001	0.09
Maternal education* (graduated & above: Yes[1]/No)	- 0.004	0.13	0.43
Frequency of illness	- 0.007	- 0.02	0.82
Family Income* (≥30,000 Baht/ month: Yes[1]/ No)	0.003	- 0.20	0.23
Maternal age	0.002	-0.02	0.26

**Categorical variables; [1]reference category; NA – Not analysis; BF – breastfeeding; Adj R^2 – adjusted coefficient of determination; ß – regression coefficient; LAZ – length-for-age z-score; M -months old*

Table R3.10 Summary of significant co-variates from univariate regression analyses

Outcomes	Significant Covariates
Ponderal growth	Type of Milk from 9-12M Non-Protein energy 6-9M Non-Protein energy 9-12M
Linear growth	Type of Milk from 9-12M

M – months old

Table R3.11 Multiple linear regression analyses investigating association between %PE from 9-12 months and conditional growth outcomes

1) Main outcome: Conditional WAZ

Predictors **Model 1** Adj R^2 0.112	**ß**	***p***	**Predictors** **Model 2** Adj R^2 0.134	**ß**	***p***
%PE 9-12M	0.23	**0.005**	/	0.25	**0.002**
Type of milk 9-12M	0.03	0.80	/	0.03	0.81
Non-Protein energy 6-9M	0.21	0.14	/	0.20	0.15
Non-Protein energy 9-12M	0.07	0.62	/	0.08	0.56
Frequency of illness	- 0.03	0.73			
Maternal education	0.08	0.35			
Maternal BMI	- 0.01	0.45			
Maternal age	0.01	0.92			

WAZ – weight-for-age z-score; M – months old; BMI – body mass index; Adj R^2 – adjusted coefficient of determination; ß – regression coefficient; p – p-value

2) Main outcome: Conditional WLZ

Predictors **Model 1** Adj R^2 0.104	**ß**	***p***	**Predictors** **Model 2** Adj R^2 0.127	**ß**	***p***
%PE 9-12M	0.26	**0.002**	/	0.27	**0.001**
Type of milk 9-12M	0.10	0.35	/	0.10	0.32
Non-Protein energy 6-9M	0.22	0.13	/	0.21	0.13
Non-Protein energy 9-12M	- 0.07	0.61	/	- 0.07	0.60
Frequency of illness	- 0.03	0.70			
Maternal education	0.07	0.43			
Maternal BMI	- 0.04	0.60			
Maternal age	0.01	0.91			

WLZ – weight-for-length z-score; M – months old; BMI – body mass index; Adj R^2 – adjusted coefficient of determination; ß – regression coefficient; p – p-value

3) Main outcome: Conditional BMIZ

Predictors **Model 1** Adj R^2 0.078	**ß**	***p***	**Predictors** **Model 2** Adj R^2 0.080	**ß**	***p***
%PE 9-12M	0.22	**0.01**	/	0.22	**0.009**
Type of milk 9-12M	0.10	0.37	/	0.10	0.34
Non-Protein energy 6-9M	0.19	0.20	/	0.17	0.23
Non-Protein energy 9-12M	- 0.05	0.72	/	- 0.05	0.73
Frequency of illness	- 0.01	0.89	[redacted]		
Maternal education	0.11	0.23			
Maternal BMI	- 0.05	0.56			
Maternal age	0.07	0.46			

BMIZ – body mass index z-score; M – months old; BMI – body mass index; Adj R^2 – adjusted coefficient of determination; ß – regression coefficient; p – p-value

4) Main outcome: Conditional LAZ

Predictors **Model 1** Adj R^2 - 0.02	**ß**	***p***	**Predictors** **Model 2** Adj R^2 – 0.012	**ß**	***p***
%PE 9-12M	0.02	0.86	/	0.02	0.68
Type of milk 9-12M	- 0.12	0.30	/	0.02	0.83
Non-Protein energy 6-9M	0.05	0.72	[redacted]		
Non-Protein energy 9-12M	0.17	0.25			
Frequency of illness	- 0.01	0.91			
Maternal education	- 0.06	0.54			
Maternal age	- 0.08	0.39			
Family income	0.12	0.20			

LAZ – length-for-age z-score; M – months old; Adj R^2 – adjusted coefficient of determination; ß – regression coefficient; p – p-value

R3.7 Associations between consumption of different protein sources and growth outcomes

In these analyses, protein sources were divided into three main groups namely, milk, non-dairy ASFs, and plant-based foods. Milk included breast milk, formula, cow's milk, and other dairy products while non-dairy ASFs referred to eggs, flesh meat, organs, seafoods, and other animal products. The last protein source was plant-based foods covering cereals, vegetables, fruits, legumes, nuts and seeds.

In this section, I used a similar approach beginning with univariate regression models to investigate associations between each protein source and conditional growth z-scores. As the co-variates were the same set used in section R3.6, they were also included in the multiple linear regression models as the previous analyses.

According to the results of univariate regression analyses shown in table R3.12, milk protein was the only protein source significantly associated with all conditional weight-related parameters. However, as breastfeeding is associated with a reduced risk of childhood obesity while formula and cow's milk are associated with increased risk of rapid weight gain, I performed further analyses separating %PE from breast milk and non-breast milk (i.e., formula, cow's milk, other dairy products). To avoid excluding data from infants who were given only breast milk or formula/ cow's milk, I created a new variable called "**Difference between %PE between non-breast milk (non-BM) and breast milk (BM)**" – so called "Different %PE from milk". This was a continuous variable, calculated by subtraction of %PE from BM from %PE from non-BM. The value of this variable could be

- **Negative** – when infants received %PE from non-BM < BM
- **Zero** – when infants received %PE from non-BM = BM
- **Positive** – when infants received %PE from non-BM > BM

Higher values of this variable therefore indicated more %PE from non-BM.

There were significant positive associations between the different %PE between non-BM and BM and conditional weight-related z-scores suggesting that increasing %PE from non-BM is associated with increased conditional weight-related z-scores. There was no significant association with conditional LAZ (Figure R3.3). In addition, it should be noted that the standardised regression coefficients (ß) were higher than using %PE from both types of milk. Altogether, these findings indicate that the protein intake from non-BM could be the main contributor to the results found in the univariate regression models (Table R3.12).

In the multiple regression models, it was interesting that %PE from both milk/dairy and non-dairy ASFs was positively associated with conditional WAZ and WLZ, independent of other co-variates shown in table R3.13. Noticeably, only %PE from non-dairy ASFs was significantly associated with conditional BMIZ while the p-value for the association between %PE from milk and conditional BMIZ in the multiple linear regression analysis was just above the statistical significance threshold (p = 0.07).

Table R3.13 shows higher regression coefficients from the multiple linear regression models focusing on %PE from milk and non-dairy ASFs as main predictors compared to the previous analyses when protein sources were not considered. The key outcomes (using model 2) can be interpreted as follows,

- A 1% increase in daily PE **from milk/dairy** from 9-12 months may increase the conditional **WAZ and WLZ by 0.47 and 0.40 z-scores**, respectively after adjusting for other protein sources, non-protein energy consumption and type of milk feeding.
- A 1% increase in daily PE **from non-dairy ASFs** from 9-12 months may increase the conditional **WAZ, WLZ, BMIZ by 0.27, 0.32 and 0.25 z-scores**, respectively after adjusting for other protein sources, non-protein energy consumption and type of milk feeding.

In this cohort, none of these protein sources was associated with conditional LAZ and there was no relationship between %PE from plant-based foods and conditional growth at 12 months.

Altogether, the significant results in this section underscore the impact of protein sources on infant growth during the complementary feeding period. It is therefore necessary to consider both the amount and source of protein when investigating associations between protein intake and growth. The final section of this chapter will summarise the key findings and discuss them in the context of other published data, highlighting the new knowledge added by this cohort, as well as the limitations and suggestions for further research.

Table R3.12 Univariate regression analyses investigating associations between %PE from different protein sources from 9-12 months and conditional growth

Predictor	Outcomes	Results		
		Adj R^2	**ß**	**p-value**
%PE from Milk/ dairy	Conditional WAZ	0.059	0.61	**0.002**
	Conditional WLZ	0.046	0.54	**0.006**
	Conditional BMIZ	0.032	0.47	**0.02**
	Conditional LAZ	- 0.002	0.17	0.39

%PE – percentage of energy provided dietary protein; WAZ – weight-for-age z-score; WLZ – weight-for-length z-score; BMIZ – body mass index z-score; LAZ – length-for-age z-score; Adj R^2 – adjusted coefficient of determination; ß – regression coefficient

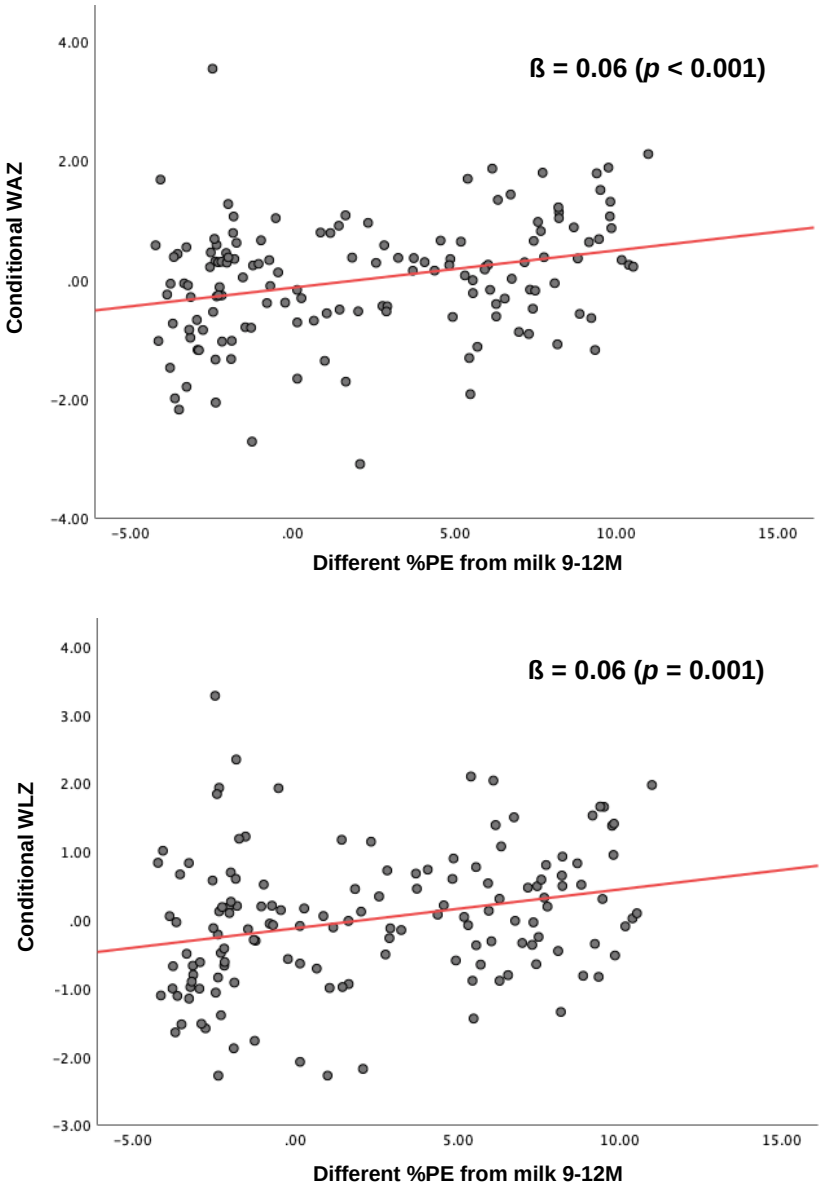
Predictor	Outcomes	Results		
		Adj R^2	**ß**	**p-value**
%PE from Non-dairy ASFs	Conditional WAZ	- 0.006	0.09	0.70
	Conditional WLZ	0.000	0.21	0.34
	Conditional BMIZ	- 0.004	0.14	0.53
	Conditional LAZ	- 0.006	- 0.07	0.76

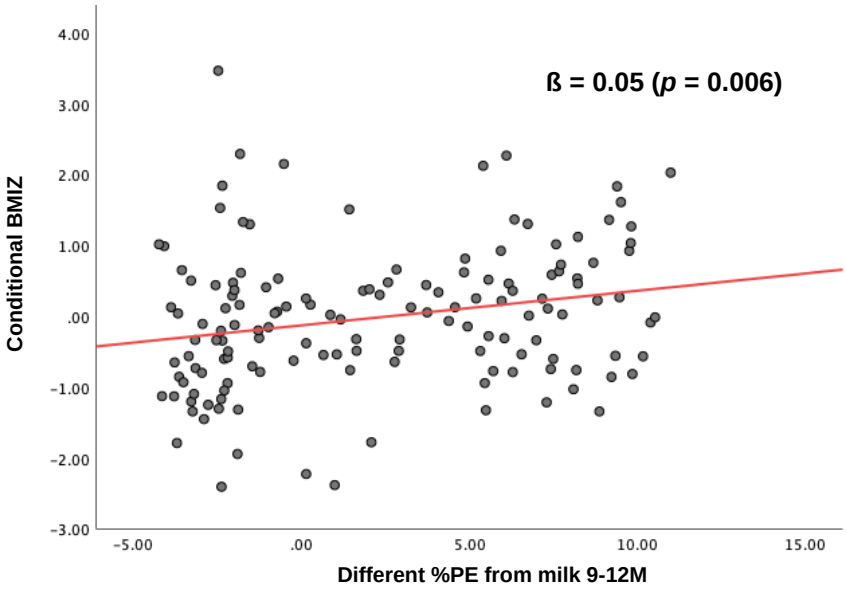
%PE – percentage of energy provided dietary protein; WAZ – weight-for-age z-score; WLZ – weight-for-length z-score; BMIZ – body mass index z-score; LAZ – length-for-age z-score; Adj R^2 – adjusted coefficient of determination; ß – regression coefficient

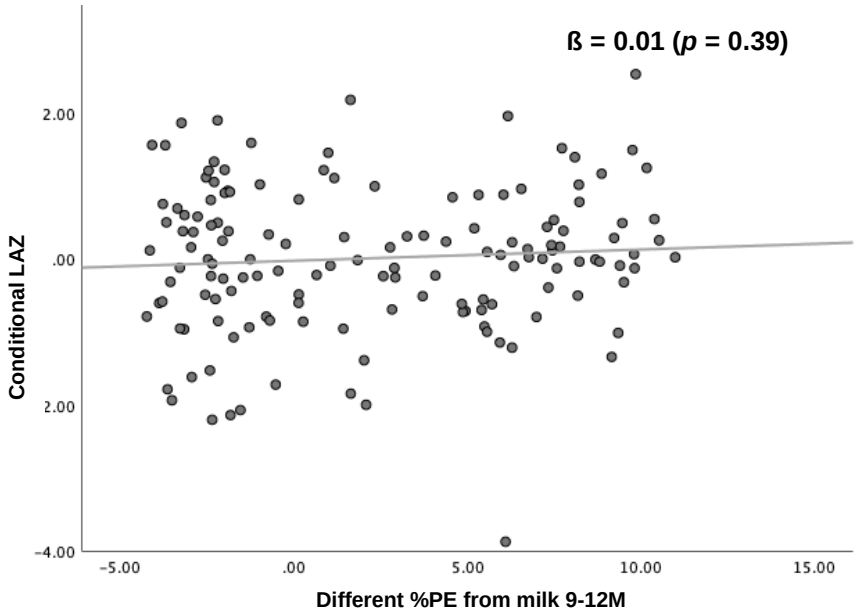
Predictor	Outcomes	Results		
		Adj R^2	ß	p-value
%PE from Plant-based foods	Conditional WAZ	- 0.003	- 0.05	0.70
	Conditional WLZ	- 0.004	- 0.04	0.49
	Conditional BMIZ	- 0.004	- 0.04	0.55
	Conditional LAZ	- 0.005	- 0.03	0.59

%PE – percentage of energy provided dietary protein; WAZ – weight-for-age z-score; WLZ – weight-for-length z-score; BMIZ – body mass index z-score; LAZ – length-for-age z-score; Adj R^2 – adjusted coefficient of determination; ß – regression coefficient

Figure R3.3 Scatter plots showing simple linear regressions of the different %PE between non-breast milk and breast milk, and conditional growth







%PE – percentage of energy provided by dietary protein; WAZ – weight-for-age z-score; WLZ – weight-for-length z-score; BMIZ – body mass index z-score; LAZ – length-for-age z-score; ß – regression coefficient; p – p-value

Table R3.13 Multiple linear regression analyses investigating associations between %PE from different sources from 9-12 months and conditional growth outcomes

1) Main outcome: Conditional WAZ

Predictors Model 1 Adj R^2 0.104	**ß**	***p***	**Predictors Model 2** Adj R^2 0.119	**ß**	***p***
%PE from Milk/ dairy	0.41	**0.02**	/	0.47	**0.004**
%PE from Non-dairy ASFs	0.25	**0.02**	/	0.27	**0.01**
%PE from Plant-based foods	0.11	0.33	/	0.13	0.22
Type of milk 9-12M	- 0.09	0.54	/	- 0.09	0.56
Non-Protein energy 6-9M	0.21	0.15	/	0.18	0.50
Non-Protein energy 9-12 M	0.06	0.65	/	0.02	0.95
Frequency of illness	- 0.03	0.76			
Maternal education	0.07	0.39			
Maternal BMI	- 0.05	0.53			
Maternal age	0.04	0.69			

WAZ- weight-for-age z-score; ASFs – animal source foods; BMI – body mass index; M – months old; Adj R^2 – adjusted coefficient of determination; ß – regression coefficient; p – p-value

2) Main outcome: Conditional WLZ

Predictors Model 1 Adj R^2 0.092	**ß**	***p***	**Predictor Model 2** Adj R^2 0.103	**ß**	***p***
%PE from Milk/ dairy	0.36	**0.04**	/	0.40	**0.02**
%PE from Non-dairy ASFs	0.30	**0.004**	/	0.32	**0.002**
%PE from Plant-based foods	0.11	0.33	/	0.12	0.25
Type of milk 9-12M	0.05	0.73	/	0.06	0.67
Non-Protein energy 6-9M	0.22	0.13	/	- 0.02	0.95
Non-Protein energy 9-12M	- 0.08	0.59	/	0.09	0.75
Frequency of illness	- 0.03	0.73			
Maternal education	0.07	0.44			
Maternal BMI	- 0.04	0.63			
Maternal age	0.02	0.82			

WLZ- weight-for-length z-score; ASFs – animal source foods; BMI – body mass index; M – months old; Adj R^2 – adjusted coefficient of determination; ß – regression coefficient; p – p-value

3) Main outcome: Conditional BMIZ

Predictors Model 1 Adj R^2 0.065	**ß**	***p***	**Predictors Model 2** Adj R^2 0.058	**ß**	***p***
%PE from Milk/ dairy	0.30	0.09	/	0.31	0.07
%PE from Non-dairy ASFs	0.24	**0.02**	/	0.25	**0.02**
%PE from Plant-based foods	0.10	0.38	/	0.10	0.36
Type of milk 9-12M	0.06	0.72	/	0.08	0.60
Non-Protein energy 6-9M	0.19	0.20	/	0.001	1.00
Non-Protein energy 9-12 M	- 0.06	0.69	/	0.22	0.83
Frequency of illness	- 0.01	0.91	[redacted]		
Maternal education	0.11	0.23			
Maternal BMI	- 0.05	0.59			
Maternal age	0.08	0.42			

BMIZ- body mass index z-score; ASFs – animal source foods; BMI – body mass index; M – months old; Adj R^2 – adjusted coefficient of determination; ß – regression coefficient; p – p-value

4) Main outcome: Conditional LAZ

Predictors Model 1 Adj R^2 - 0.028	**ß**	***p***	**Predictors Model 2** Adj R^2 -0.020	**ß**	***p***
%PE from Milk/ dairy	0.13	0.46	/	0.17	0.34
%PE from Non-dairy ASFs	<0.001	1.00	/	0.02	0.84
%PE from Plant-based foods	- 0.002	0.98	/	0.02	0.89
Type of milk 9-12M	- 0.22	0.17	/	- 0.09	0.51
Non-Protein energy 6-9M	0.05	0.76	[redacted]		
Non-Protein energy 9-12 M	0.17	0.25			
Frequency of illness	- 0.01	0.92			
Maternal education	- 0.06	0.49			
Maternal age	- 0.06	0.57			
Family income	0.12	0.22			

LAZ- length-for-age z-score; ASFs – animal source foods; M – months old; Adj R^2 – adjusted coefficient of determination; ß – regression coefficient; p – p-value

R3.8 Summary of key results and discussion

Key results

- The study infants consumed much higher protein than the Thai DRIs and the international recommendations from 9 to 12 months of age.
- Consumption of ABP increased significantly from 6 to 12 months while intake of PBP changed little throughout the CF period.
- The most common ASFs for the study infants were eggs and formula while red meats and liver were given less often to the infants on a daily basis.
- High %PE had a significant impact on infant weight but not length gain at 12 months.
- There were dose-response associations between %PE and weight-related parameters (WAZ, WLZ and BMIZ).
- Milk, particularly formula and cow's milk, had the highest impact on infant weight gain followed by ABP from non-dairy sources.
- There was no evidence of an association between %PE and linear growth, or between PBP and growth parameters in this cohort.

According to these key findings, protein intake during the complementary feeding period among Thai infants is now shifting toward the "Western style" of consuming higher amounts of dietary protein, especially from ASFs, rather than relying solely on plant-based foods as reported in a previous study[31]. I also found that protein intake expressed in several ways (i.e., total protein (g/d), PW ratio and %PE) in our cohort was similar to the results from the SEANUTS survey[217]. The average %PE from 6 to 12 months old in the cohort was almost twice the safe level suggested by the WHO and was 50% higher than the previous figure reported a few decades ago[227]. More specifically, Thai infants aged 9-12 months tended to receive the same amount of dietary protein reported by researchers from high-income settings where %PE in this age group was around 15%[28, 228].

There was evidence that protein quality as well as quantity has been westernised. In our cohort, more than 80% of dietary protein during the CF period came from ASFs which, again, did not differ from the results found in the SEANUTS. When compared with a study before 2000, there was a major change of protein sources provided during the complementary feeding period. In 1998, Gibson et al[31] reported that complementary foods in LMICs including Thailand were mainly based on plant-based foods such as cereals, starchy roots and tubers accompanied with a small quantity of ASFs which, in general, provided inadequate amounts of essential micronutrients such as iron, zinc, and calcium. According to this study dried fish was the only ASF in Thai complementary foods[31]. Since 2000, there have been no published studies showing the common protein sources in complementary feeding diets of Thai infants.

The results from this cohort provide some evidence against the longstanding assumption that infants and young children in LMICs mainly receive poor quality protein from plant-based foods and consume less ASFs[10, 229, 230]. By focusing on this concept, studies from LMICs tend to promote consumption of ASFs while overlooking a potential impact of high protein intake, particularly ABP, on the risk of childhood obesity in these populations. Currently, there are no studies from LMICs investigating associations between high protein intake during infancy and risk of overweight/ obesity[61] nor any nutritional programmes in LMICs that take this issue into account[231], even though there is strong evidence that overweight and obesity are prevalent at early ages in many countries around the world, regardless of their economic status[12]. In Thailand, the prevalence of overweight/ obesity in under-five children, has increased from 8.2% to 12.7% according to the MICS 2015-6[22] and 2019[21], respectively. Although several factors might contribute to this situation, the provision of “too much” protein during the CF period should be considered as one possible factor. Further studies are needed to confirm the findings from this cohort.

To our knowledge, this is the first study demonstrating the impact of high protein intake during infancy on rapid weight gain in a population outside EU countries. By categorising infants into 3 groups based on their protein intakes from 6 to 12 months old, the outcomes clearly showed that the infants who consumed protein ≥13 %PE gained significantly more weight (i.e., WAZ, WLZ and BMIZ) from 6 to 12 months compared with the infants who consumed less dietary protein, while the LAZ between those groups were not different. In addition, the use of conditional growth reduces bias from previous body size and could strengthen the observed effect of dietary protein on body weight change during the complementary feeding period. The average %PE of the infants in the HP group was 14% compared to 12% and 10% in the MP and LP, respectively. Although the average %PE in the HP group was lower than other reports from western countries, the key findings from our cohort were still in line with a majority of studies in European populations showing a positive effect of high protein intake during infancy on weight gain, not linear growth.

Over recent years, the association between high protein intake during infancy and rapid weight gain or childhood obesity has been shown in several observational studies[73-75, 206, 222, 232] while a causal effect of high consumption of protein from infant and follow-on formula during the first year of life on childhood obesity was shown in a large, multi-centre, double-blinded RCT conducted in 5 EU countries[233]. Unlike the observational studies investigating the effect of protein intake during infancy on BMI at preschool[232] and school age[73, 74, 206, 222], my study has shown that the effect of high protein intake in particular between 6-12 months on child growth can be seen at an earlier age. Although Koletzko et al[59] also reported that at 12 months, the infants who consumed high protein formula had higher WAZ, WLZ, and BMIZ than those who received lower protein formula or breastfed infants, it is difficult to draw conclusions about the effect of protein intake during the complementary feeding period per se as the intervention started before the introduction of complementary feeding and significant effects on weight and BMI z-scores were observed as early as 6 months of age. Focussing on linear growth as the main outcome, there is only one study from Denmark showing high protein

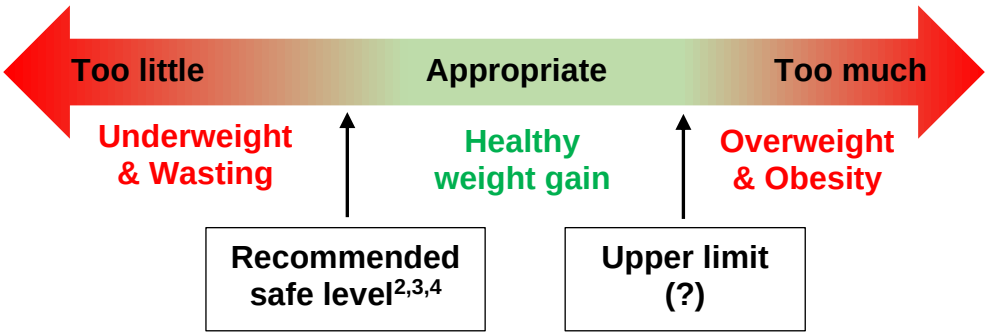
intake at 9 months was correlated with length at 10 years of age. However, this correlation was not significant after adjusting for the infant's body size at 9 months[206]. The EU authorities suggest limiting protein intake during the first years of life within 15%PE[4, 80] and this concern has also translated to a reduction in the protein content of both infant and follow-on formulas that are available in EU countries[234].

In addition to the convincing evidence presented in the previous paragraph, there is a study from a LMIC relevant to the impact of dietary protein on growth in more vulnerable populations. Bhargava et al[235] analysed data from the Cebu Longitudinal Health and Nutritional Surveys conducted in metropolitan Cebu in the Philippines from 1983 to 2005. They used dynamic random effect models to investigate the influence of various variables including the child's dietary intake on multiple outcomes such as birth outcomes, growth and morbidity between ages 2-24 months, weight and height from 8-19 years old and final adult height at 22 years old. The results showed that protein intake was positively associated with weight but not length between from 2-24 months, while calcium intake was positively associated with linear growth. The average intake of protein (means $\pm$ SD) reported in this study was 12.3 $\pm$ 12.1 g/day (11%PE) and 21.6 $\pm$15.6 (12%PE) at 1 and 2 years of age, respectively. In contrast to young children, protein intakes during school age and adolescence (8-19 years old) were positively associated with both weight and height ($p < 0.05$). However, this study did not include information on dietary intake during infancy, and the average energy intake in this population at 1 year of age (434 kcal/d) was very low compared with the recommendations[236] which may indicate that they were undernourished.

Taken together, it seems that increasing protein intake in early life can promote weight gain, regardless of predisposing nutritional status. If we see this effect as a spectrum, either "too little" or "too much" protein can result in malnutrition, but in two very different forms, "underweight/ wasting" and "overweight/ obesity" (figure R3.4). In the context of LMICs, the outcome of "too much" protein intake is usually obscured by reporting it as a "catch-up growth" or

“better weight gain” because of a concern about undernutrition. This bias may lead to a lack of scientific studies generating evidence to make a proper recommendation on the upper limit of protein intake for infants and young children in LMICs. However, with a decreasing rate of undernourished populations and displacement of local foods by westernised diets in many LIMCs, rapid weight gain from high protein intake should be seen as a nutritional problem, not a satisfactory outcome. In particular for countries where the DBM is prevalent and numbers of overweight/ obese under-five year olds are increasing, optimising protein intake at an early age might be one potential solution for these problems. In order to optimise the consumption of dietary protein for infants and young children, information on the “dose-response” effect of protein intake on weight gain and identification of the most influential protein source(s) should be considered.

Figure R3.4 Effect of protein intake on weight gain



In this cohort, the multiple linear regression analyses suggested a “dose-response” effect of protein intake on weight gain. A 1% increase in daily PE between 9-12 months was associated with a 0.40 SDS increases in the conditional WLZ, after adjusting for duration of predominant breastfeeding, non-protein energy consumption and type of milk feeding. This means that an increase of 8% PE would be associated with a 3 SDS greater increase in WLZ than expected for an infant of the same initial size. Ideally, if infants and young children consume dietary protein following the recommendations[3], they should have a healthy weight gain and fulfil their growth potential. In this case, when more than 3%PE is added, it may contribute to increasing risk of overweight by increasing WLZ $\geq$ +1SDS[2] of their previous growth potential.

Interestingly, Michaelsen et al[227] reported that most studies focusing on the impact of dietary protein in the first two years of life on NCD risk in adulthood showed a significant association between high protein intake and growth at 12 months and reported a %PE around 13%. In addition, the upper limits of %PE for infants and young children who are living in the European countries recommended by Agostoni et al[79] and the EU authorities[4, 80] are 14 and 15% of total energy intake, respectively. However, such figures are lacking for other populations, especially for those are suffering from the DBM.

The outcomes found in this cohort are in line with several studies[58, 72-76, 237-8] even though some studies did not find a significant impact of meat protein on weight-related parameters[76]. More specifically, for milk protein, the results from my cohort also suggested that protein from formula and cow's milk were a potent promoter of weight-related z-scores while protein from breast milk seemed to lessen this effect. However, we cannot conclude that breast milk has a negative impact on weight gain because infants who consumed only breast milk along with complementary foods still grew normally during the study period. Altogether, the strong effect of milk protein found in this cohort is more likely to reflect the effect of dairy protein than protein from breast milk.

Compared to milk protein, non-dairy ABP including flesh meats, poultry, organ meats, eggs and other meat products showed a small but still significant impact on weight gain in our population. A trial comparing meat and dairy protein (e.g., infant yoghurt, cheese, whey powder) in formula-fed infants at age 5 to 12 months found that infants in the "dairy group" gained significantly more in WLZ compared to the "meat group" while the latter group had significantly higher LAZ[239]. In this RCT, energy and total protein intake at baseline, 10M and 12M were not different between two groups. Corresponding with these results, a recent meta-analysis also showed that provision of ABP to term infants or young children, whether formula or food-based animal protein can increase weight gain, (weighted mean difference; WMD were +0.14 kg; 95%CI 0.07, 0.21, I^2 81.9% in the formula trial comparing higher and lower formula and +0.09 kg; 95%CI 0.06, 0.13 I^2 85.8% in food-based animal

protein supplementation compared with usual diet or plant-based foods)[240]. Nevertheless, neither formula or food-based animal protein showed a significant effect on height (WMD were +0.01 cm; 95%CI -0.07, 0.08, I^2 75.7% for formula and -0.02, 95%CI -0.06, 0.01, I^2 97.8 for food-based animal protein). The authors concluded that effects of ABP are relatively modest depending on "baseline characteristics of undernutrition", "background of diets", and "corresponding doses and durations of supplementation"[240].

Unlike infants and young children in high-income countries where dairy consumption is relatively high, non-dairy ABP could be a good alternative choice for populations in LMICs, in particular for breastfed infants. Tang et al[77] suggested that a high intake of meat protein is a safe recommendation for breastfed infants who are in particular need of good sources of iron and zinc during the CF period. In addtion, Allen et al[230] stated that meat consumption can increase weight, muscle mass and cognitive performance among children in low-income areas. Apart from flesh meat, Iannotti et al[70] reported that infants aged 6 to 9 months who were randomised to eat one egg a day for 6 months had significantly higher LAZ and WAZ at 12 months of age, although these effects were absent at two year follow-up[241].

Moreover, when this intervention was repeated in Malawian infants, the study reported non-significant outcomes[242]. Despite some negative evidence, meats and other non-dairy ABP are still nutritient-dense foods for younger populations in LMICs. However, for some countries where the prevalence of overweight/ obesity in young children is on the rise, trade-offs between possible negative effects of this portein source on weight gain and benefits for iron/micronutrient status should be considered. Furthermore, it should be noted that 'extra' weight gain could be either fat mass or fat free mass, and we cannot assume that weight gain from increasing intake of non-dairy ABP during the CF period will contribute to increasing body fatness without further evidence. I will be able to address this issue when my collected samples for body composition are analysed. Until now, only high protein intake from formula during infancy has been shown to be strongly associated with increased body fatness of young children[243].

Last but not least, when focusing on the negative findings in this cohort, the results did not demonstrate an impact of dietary protein on linear growth nor associations between PBP and growth parameters. These findings are in agreement with the majority of studies in this research area. According to the results from the European Childhood Obesity Project (CHOP) trial, no impact of high protein formula on linear growth was observed at 2 and 6 years old[59, 60]. Likewise, the observational studies that found the promoting effect of high protein intake on body weight also reported non-significant effects on length/ height of their study populations[72, 74, 240]. Although some studies found an effect of high protein intake on linear growth, this effect was still weaker than that for body weight/ BMI[75, 206]. More importantly, the studies showing negative results had measured linear growth between 2 and 6 years[59, 60, 72, 74, 240] while the positive studies had measured height at 9 and 10 years[75, 206].

According to Victora et al[244], HAZ of young children around the world falls significantly from 1 month until 24 months of age, and are then quite stable with some small fluctuations until 59 months. In contrast to young children, at around prepuberty, height velocity starts to increase again. It is possible that during the period of linear growth faltering, an effect of high protein intake might be difficult to observe compared with the prepuberty period when height velocity begins to increase. In addition, as evidence also shows that both childhood obesity and high protein intake especially from dairy products and meats are related to an earlier onset of puberty[245-248], it is possible that at age 9-10 years, some of the children in those studies could already be in puberty. This could explain why an impact of high protein intake during infancy on height has been observed in older children. Unfortunately, pubertal stage was not reported in these studies[75, 206].

The finding that PBP intake was not associated with growth parameters in my cohort was in accordance with the high protein studies conducted in the western populations[58, 75, 76, 237]. In a recent systematic review, the authors concluded that neither type nor amount of fortified infant cereals influence growth, body size, body composition or overweight/ obesity, however, it was not robust evidence[249]. We know that PBP has limited amounts of some

essential amino acids (e.g., lysine, methionine, and tryptophan)[250] and low bioavailability of key micronutrients such as iron and zinc[41] which are involved in growth mechanisms, and this may explain why PBP does not appear to influence growth. Nevertheless, we should consider that the absence of an effect could also be due to other factors such as the ratio of PBP in local diets and type of PBP used in any specific population. Considering the ratio of ABP and PBP in our cohort, while more than three-quarters of protein intake was ABP, just around 20% of dietary protein came from plant sources.

In addition, when considering the type of PBP in this cohort, most of the infants consumed cereals, vegetables and fruits which, in fact, comprise only small amounts of protein and essential amino acids compared with legumes[251]. Some scholars call legumes “the poor man’s meat”[252]. According to a double-blind RCT in rural Malawi, supplementation of cow pea or common bean flour between age 6 to 12 months resulted in less linear growth faltering compared with the control group who were given a traditional corn-soy blend[253]. Altogether, the non-significant results for PBP in my study should therefore be interpreted carefully. For countries where different types of PBP are main components of the local diet, outcomes might be different from our findings, thus further studies are still needed to demonstrate whether some types of PBP could promote growth.

In conclusion, this chapter underscores the effect of high protein intake on rapid weight gain during the CF period and also demonstrated “dose-response” relations between dietary protein and weight-related parameters. For those countries having high intake of ASFs and increasing prevalence of overweight/ obesity among under-five children, the upper limits of protein intake during the CF period should range between 13 to 16%PE depending on the background diet. If formula and dairy protein are a main protein source, the upper limits should favour a lower percentage as the results from this cohort showed that dairy protein was the most potent factor associated with higher weight-related z-scores. On the contrary, a higher figure might be acceptable for breastfed infants or nutritionally vunerable children with a greater need for iron, zinc and other essential micronutrients from non-dairy

ASFs. Although the impact of high protein intake on infant growth in this cohort is clear and consistent, there are still more questions left to clarify.

- Do infants consuming higher ABP have better iron status?
- Can breastfed infants given high amounts of ABP achieve normal iron status without increasing their risk of overweight/ obesity?
- Why does high protein consumption contribute to rapid weight gain? (what are the underlying mechanisms?)

These issues were investigated in my study and the results are presented in the following chapters.

Chapter 7: Results 4

Protein intake and iron status

As mentioned previously, in a country where the DBM is prevalent, it is more complicated to determine the most appropriate recommendation for ASFs, considering that overconsumption of ABP may increase the risk of overweight/ obesity in young children. The results from Chapter 7, Results 3 clearly showed that protein from both formula/ dairy products and non-dairy ASFs was positively associated with weight gain during the CF period although with different effect sizes. Both types of ASF are a good source of iron, which is often limited in the diet of infants during the complementary feeding period. However, infant formula contains non-haem iron which is less easily absorbed than the haem iron present in non-dairy ASFs. This is important when considering how to balance positive and negative effects of different ASFs on growth and iron status.

To address this issue, this chapter aims to explore the effect of the amount and source of protein on the iron status of infants during the CF period. The %PE is used as a marker of “protein intake” in this chapter to make the results comparable with the outcomes in Chapter 7, Results 3, while iron status is discussed using both continuous variables: serum ferritin (SF), transferrin saturation (TSAT), hemoglobin (Hb) and categorical variables: ID, IDA, normal iron status. The definitions of ID/ IDA were described in the “List of definitions” (page 16)

In this chapter, I first present protein sources (i.e., milk/ dairy, non-dairy ASFs and plant-based foods) contributing dietary iron for infants during the CF period and correlations between the intake of different protein sources and iron intake in infants receiving only breast milk alongside complementary foods, as these infants are at risk of ID/ IDA. I describe the identification of potential confounders for multiple regression analyses using the DAG

approach. I also investigate whether iron-sufficient infants always had “too high” protein intake, and how breastfed infants can achieve normal iron status using a food-based approach.

The distribution of all dependent variables entered into regression analyses was tested using the Kolmogorov-Smirnov (KS) method. Apart from SF, other variables showed a normal distribution (Hb: p-value from KS test = 0.41) or mild skewness (TSAT: p-value from KS test = 0.002, skewness = 0.61) which were acceptable for the regression model. However, SF was extremely skewed (p-value from KS test < 0.001, skewness = 5.31). When I excluded one participant with a SF of 405 ng/ml due to recent recovery from acute diarrhea, I found that the skewness was still 1.35. Therefore, I transformed SF using natural logs and the final result was more acceptable (p-value from KS test = 0.06, skewness = - 0.35). Ln SF was used in the statistical analyses presented in this chapter.

R4.1 Associations between protein intake from different sources and iron intake

In this section, both protein and iron intake were obtained from daily food consumption using the INMUCAL-Nutrients programme. The main objective was to investigate the association between protein intake from three different sources: milk (i.e., formula, breast milk, and dairy products), non-dairy ASFs (i.e., meats, organ meats, eggs, fishes, poultry, meat products) and plant-based foods, and iron intake. It should be noted that the INMUCAL-Nutrients programme does not separate haem and non-haem iron, thus the iron content from ASFs includes both forms.

As shown in figure R4.1, milk was the main contributor of total iron intake at both 6-9 and 9-12 months, however the proportions of iron intake from non-dairy ASFs and plant-based foods increased in the later period. Considering that the iron content is relatively low in breast milk, I therefore performed sub-group analyses in infants receiving only breast milk along with complementary foods (n = 45). As shown in table R4.1, protein from breast milk was negatively associated with total iron intake, while non-dairy ABP was the main contributor of total iron intake in this group.

Figure R4.1 Proportion of iron intake from different food sources from aged 6-9 and 9-12 months (n =145)

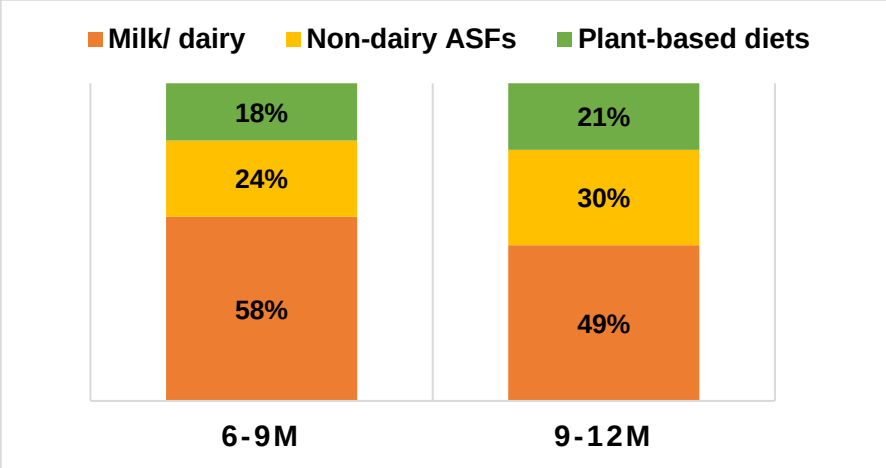


Figure R4.1 demonstrates proportions of iron from different food sources: orange bars (milk and dairy products); yellow bars (Non-dairy animal source foods - ASFs); Green bars (plant-based foods). At both stages of complementary feeding, milk is the main contributor of dietary iron followed by non-dairy ASFs and plant-based foods.

Table R4.1 Pearson's correlations between %PE from different sources and iron intake in infants who received only breast milk along with complementary foods (n = 45)

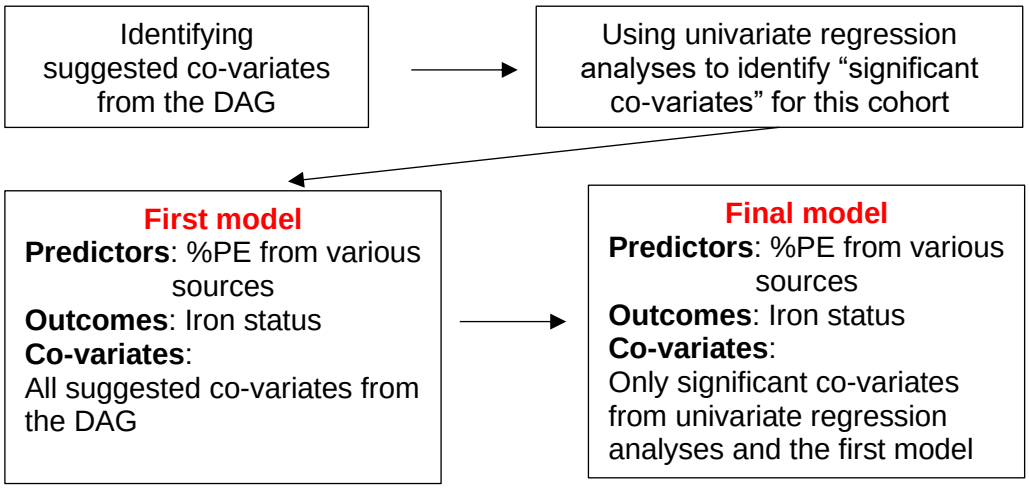
%PE from	Total iron intake		Iron intake from ASFs	
	r	*p*	r	*p*
During 6-9M				
• Breast milk	- 0.36	**0.01**	- 0.22	0.16
• Non-dairy ASFs	0.58	**<0.001**	0.63	**<0.001**
• Plant-based foods	0.37	**0.01**	-	-
During 9-12M				
• Breast milk	- 0.37	**0.012**	- 0.22	0.16
• Non-dairy ASFs	0.60	**<0.001**	0.69	**<0.001**
• Plant-based foods	0.21	0.16	-	-

All nutrient intakes were mainly based on the 3-day food records; %PE – percentage of energy provided by dietary protein; r – correlation coefficient; p – p-value; M – months old; ASFs – animal source foods

R4.2 DAG and selection of co-variates for regression analysis

Before moving to the main analyses, it is very important to carefully select covariates for the multiple regression models. A DAG was used again to decide which variables were potential confounders. The final result obtained after entering the relationships between variables into the web-based software, DAGitty.net (more details were described in Chapter 5), are shown in figure R4.2. The minimal sufficient adjustment set suggested by the DAG and their representatives are presented in table R4.2. Although we know that each protein source mainly influences iron status via its iron content (a mediator), the "minimal sufficient adjustment set" from this DAG reflects the total effect of different protein sources on iron status, including the effect via a mediator.

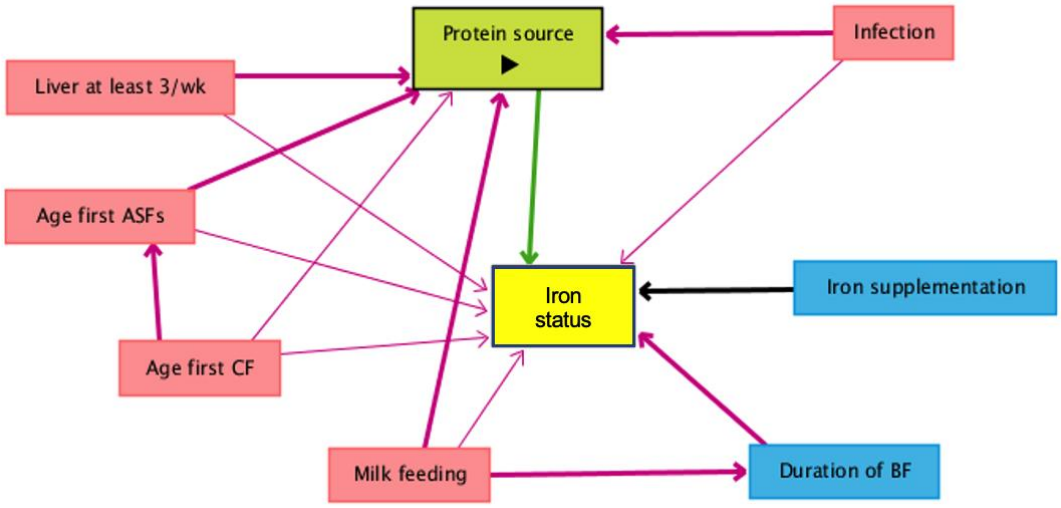
In total, there were 10 variables that should be included in the multiple regression analyses (called the first model). All of these variables were initially tested in univariate regression models to examine whether they were associated with iron status in my cohort. The outcomes of the univariate regression analyses are presented in table R4.3. According to this table, "**type of milk feeding during 6-12M**", "**provision of liver ≥ 3 times/ week at 12M**" and **"Plasma ESR >10 mm/h"** were significantly associated with iron status while "**age of introduction of egg**" was significantly associated with Hb. All of these significant variables were controlled for in the final multiple regression models (table R4.4). The steps of the analyses are shown below;



Notably, the univariate regression analyses confirmed the contrasting effects of breastmilk and formula on iron status. When comparing with breast milk, infant formula was positively associated with all parameters of iron status. There was also a significant positive association between frequent consumption of liver at age 12 months and iron status.

The next section shows the results from multiple regression analyses predicting iron status by protein intake from different sources.

Figure R4.2 Directed acyclic graph predicting infant iron status



Green box: predictor; Yellow box: main outcomes; Red box: potential confounders
Blue box: other variables; Green arrow: casual path; Red arrow: bias path

This figure illustrates the causal path between protein intake from different sources and iron status. The main predictor (independent variable) was the protein intake from different sources while the main outcome (dependent variable) was iron status. The red boxes represent variables that could be confounders as they affect both predictor and main outcome. The blue boxes are other variables affecting iron status in infants and young children that are not associated with protein intake from different sources. All of these variables were suggested by previously published articles.

Table R4.2 Suggested co-variates by the DAG

Suggested co-variates by DAG	Representatives
1) Age of first introduction of CF	• Age of introduction of CF (months)
2) Age of first introduction of non-dairy ASFs	• Age of introduction of meat (months) • Age of introduction of egg (months) • Age of introduction of fish (months)
3) Type of milk feeding (category)	• Type of milk feeding during 6-12M - Breast milk - Combined feeding - Formula
4) Type of milk feeding(continuous)	• Amount of unfortified cow's milk intake* at 12M (ml/day)
5) Provision of liver ≥3 times/ week (category)† - Yes - No	• Provision of liver ≥3 times/week at 9M • Provision of liver ≥3 times/week at 12M
6) Infection and inflammation	• Frequency of illnesses (times) • Plasma ESR > 10 mm/h

***This was consumption of unfortified cow's milk including UHT milk and pasteurized milk at 12M. There were few reports of infants receiving these types of milk at 6,9M.*
†Infants rarely consumed liver ≥3 times/week at 6M.
DAG – directed acyclic graph; M – months old; CF – complementary food; ASFs – animal source foods; ESR – erythrocyte sediment rate

Table R4.3 Univariate regression analyses between covariates suggested by the DAG and iron status

Suggested variables by DAG	**Outcomes**			
	Ln SF ß (*p*)	**TSAT** ß (*p*)	**Hb** ß (*p*)	**Iron status‡** Exp (ß) (95%CI)
Age of introduction of CF	- 0.02 (0.86)	0.04 (0.97)	- 0.07 (0.61)	ID 1.05 (0.55, 2.00) IDA 1.32 (0.59, 2.96)
Age of introduction of meat	0.12 (0.15)	- 0.99 (0.21)	- 0.07 (0.51)	ID 0.75 (0.42, 1.36) IDA 1.39 (0.83, 2.33)
Age of introduction of egg	0.01 (0.96)	- 0.63 (0.61)	**- 0.33 (0.04)**	ID 0.75 (0.34, 1.66) IDA 1.22 (0.50, 2.98)
Age of introduction of fish	0.03 (0.61)	- 0.75 (0.26)	- 0.08 (0.35)	ID 0.96 (0.62, 1.50) IDA 1.14 (0.73, 1.81)
Type of milk feeding during 6-12M (cat)*	**0.49 (<0.001)**	**2.77 (<0.001)**	**0.44 (<0.001)**	*Combined feeding* ID 1.15 (0.43, 3.05) IDA 0.38 (0.23, 1.97) ***Formula*** ID 0.67 (0.23, 1.97) **IDA 0.04 (0.01, 0.34)**
Amount of unfortified cow's milk intake[1]	<0.001 (0.47)	- 0.005 (0.18)	0.001 (0.09)	ID 0.96 (0.62, 1.50) IDA 1.14 (0.73, 1.81)
Provision of liver ≥ 3/week at 9M[2] (cat)	0.10 (0.41)	1.05 (0.39)	- 0.21 (0.19)	ID 0.55 (0.23, 1.22) IDA 0.68 (0.28, 1.62)
Provision of liver ≥ 3/week at 12M (cat)†,[2]	0.05 (0.68)	**2.62 (0.04)**	0.04 (0.82)	**ID 0.30 (0.12, 0.75)** IDA 0.41 (0.16, 1.09)
Frequency of illness	0.12 (0.16)	- 0.06 (0.51)	0.03 (0.75)	ID 1.25 (0.89, 1.75) IDA 1.00 (0.69, 1.46)
Plasma ESR > 10 mm/h	**0.20 (0.02)**	- 0.05 (0.55)	- 0.14 (0.10)	ID 1.19 (0.45, 10.66) IDA 1.96 (0.74, 5.17)

*‡ Reference group = Normal iron status; ***** Reference group = Breast milk; **†** Reference group = No; [1]Data were mainly based on the 3-day food records; [2]Data were based on the food frequency questionnaires; DAG – directed acyclic graph; Ln – natural log; SF -serum ferritin; TSAT – transferrin saturation; Hb – haemoglobin; ß = regression coefficient; Exp (ß) = exponential of regression coefficient; p = p-value; M – months old; ID – iron deficiency; IDA – iron deficiency anaemia; CF – complementary feeding; ESR – erythrocyte sediment rate*

Table R4.4 Summary of co-variates significantly associated with iron status in univariate regression models

Outcomes	Significant co-variates
Ln SF	• Type of Milk feeding • Plasma ESR > 10 mm/h
TSAT	• Type of Milk feeding • Provision of liver ≥ 3 times/week at 12M
Hb	• Type of Milk feeding • Age of introduction of egg
ID/ IDA	• Type of Milk feeding • Provision of liver ≥ 3 times/week at 12M

Ln – natural log; SF -serum ferritin; TSAT – transferrin saturation; Hb – haemoglobin; M – months old; ESR – erythrocyte sediment rate

R4.3 Associations between protein intake from different sources and iron status

This section mainly focusses on the effect of protein intake from different food sources on iron status of infants at 12 months. I began with univariate regression analyses showing associations between the main predictors: %PE from milk, non-dairy ASFs and plant-based foods, and the main outcomes: Ln SF, TSAT, Hb and iron status (i.e., normal iron status, ID, IDA). In these univariate regression analyses, I divided protein intake into 2 periods using average intakes at 6-9 and 9-12 months, to investigate whether there was any difference between the early and later stages of the CF period.

As shown in table R4.5, only %PE from milk was significantly associated with iron status, whether using continuous or categorical variables. Unlike the effect of %PE on growth, the results showed similar impact of %PE during both periods. The results suggest that increasing consumption of milk protein may improve iron status of infants at 12 months of age, increasing SF, TSAT, Hb and reducing the risk of having ID/IDA. However, as previous sections (R4.1 and R4.2) highlighted the opposite associations between breast milk and

formula and iron status, I separated the two types of milk feeding using the same variable that was described in Chapter 7, Result 3, "**Different %PE between non-breast milk (non-BM) and breast milk (BM)**" or so-called "**Different %PE from milk**". Although non-BM intake 9-12 months included protein from both formula and unfortified cow's milk, formula was the main source of protein as only 13% of infants had received more than 100 ml/day of unfortified cow's milk during this period and none of them were given unfortified cow's milk at 6 and 9 months. Therefore, it can be assumed that the higher values of this variable reflect higher %PE from formula while negative values represent higher %PE from BM. Scatter plots (figure R4.3) showed positive associations between the different %PE from milk during both periods and for all indicators of iron status. In other words, if infants consumed relatively more %PE from formula than BM during the CF period, they tended to have higher SF, TSAT and Hb at 12 months of age. However, adjustment for potential confounders is needed to draw further conclusions.

Table R4.5 Univariate regression analyses between %PE from different food sources and iron status

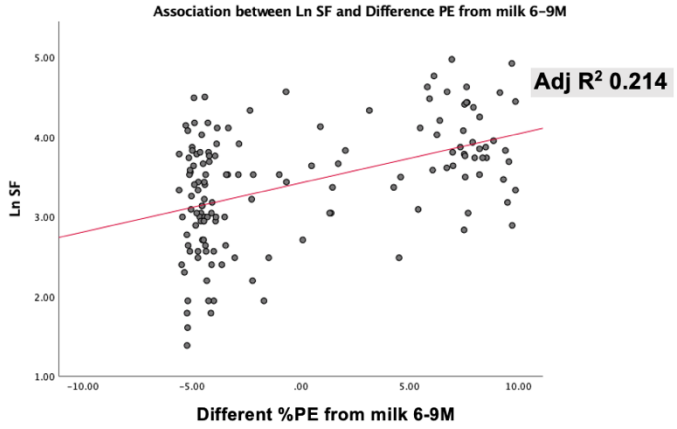
Outcomes	Predictors[1]	Results		
		Adj R^2	ß	*p*
Ln SF*	**(1) %PE from different sources 6-9M**			
	• Milk/ dairy	0.168	**0.17**	**<0.001**
	• Non-dairy ASFs		- 0.02	0.66
	• Plant-based foods		- 0.06	0.72
	(2) %PE from different sources 9-12M			
	• Milk	0.148	**0.11**	**0.002**
	• Non-dairy ASFs		- 0.03	0.21
	• Plant-based foods		0.06	0.60
TSAT*	**(1) %PE from different sources 6-9M**			
	• Milk/ dairy	0.102	**1.46**	**0.002**

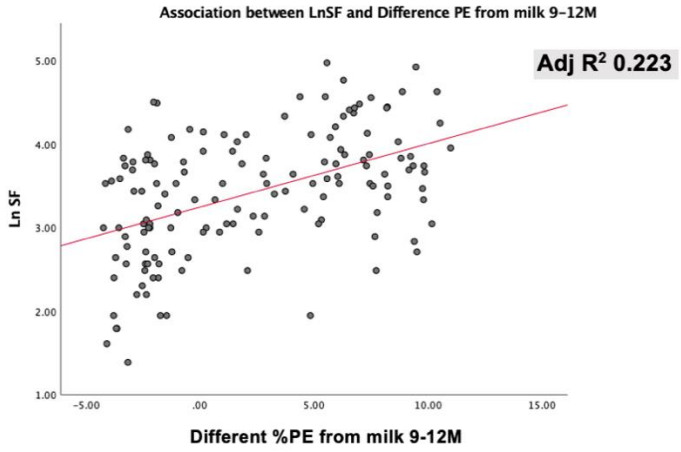
	• Non-dairy ASFs		- 0.40	0.32
	• Plant-based foods		0.96	0.46
	(2) %PE from different sources 9-12M			
	• Milk/ dairy	0.096	**1.28**	**0.001**
	• Non-dairy ASFs		0.01	0.97
	• Plant-based foods		0.16	0.14
Hb*	**(1) %PE from different sources 6-9M**			
	• Milk/ dairy	0.112	**0.21**	**<0.001**
	• Non-dairy ASFs		- 0.05	0.32
	• Plant-based foods		0.23	0.17
	(2) %PE from different sources 9-12M			
	• Milk/ dairy	0.113	**0.17**	**<0.001**
	• Non-dairy ASFs		0.01	0.71
	• Plant-based foods		0.10	0.37
Dependent	**Independent**	**Exp (ß)**	**95% CI**	***P***
ID/ IDA†	**(1) %PE from different sources 6-9M**			
	• Milk/ dairy	**0.67**	0.49,0.91	**0.01**
	• Non-dairy ASFs	1.24	0.97, 1.60	0.09
	• Plant-based foods	0.61	0.26, 1.46	0.27
	(2) %PE from different sources 9-12M			
	• Milk	0.81	0.65, 1.01	0.06
	• Non-dairy ASFs	1.03	0.88, 1.20	0.73
	• Plant-based foods	0.73	0.39, 1.34	0.31

Simple linear regression analysis †Binary logistic analysis, the reference value was infants who had normal iron status thus, the Exp(ß) represented odds ratio of having ID/ IDA; ‡Natural log transformation has been applied for iron intake from different sources due to extreme skewness; [1]All predictors (%PE) were mainly based on the 3-day food records; %PE – percentage of energy provided by dietary protein; Ln – natural log; SF – serum ferritin; TSAT – transferrin saturation; Hb – haemoglobin; ID – iron deficiency; IDA – iron deficiency anaemia; Adj R^2 – adjusted coefficient of determination; ß – regression coefficient; Exp (ß) – exponential of regression coefficient; CI – confidence intervals; p – p-value; M – months old; ASFs – animal source foods

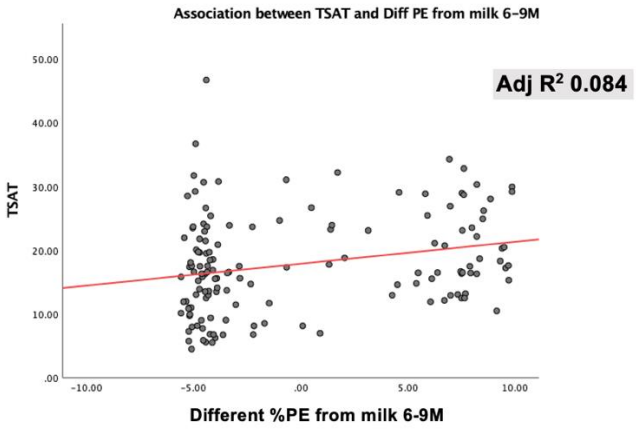
Figure R4.3 Associations between **different %PE from milk** at aged 6-9 and 9-12 months, and **iron status**

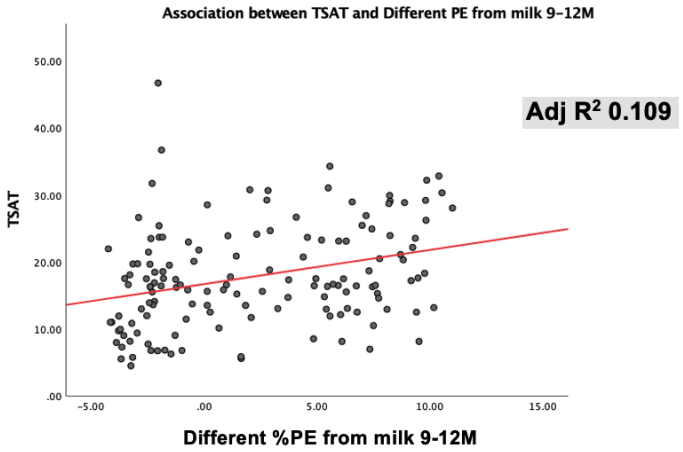
A) **Natural log of serum ferritin (Ln SF)**



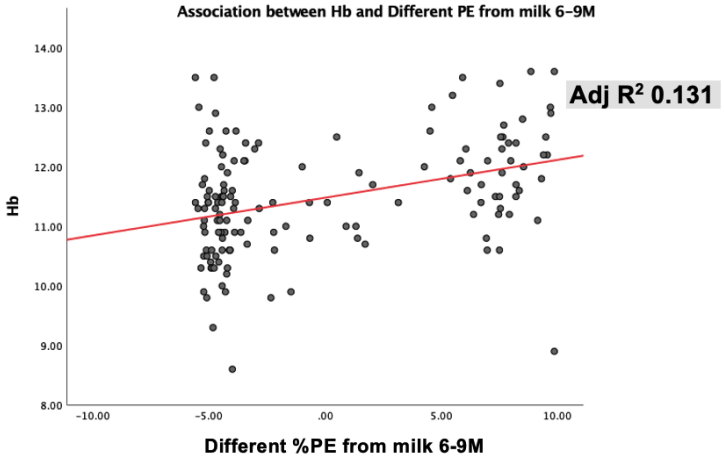


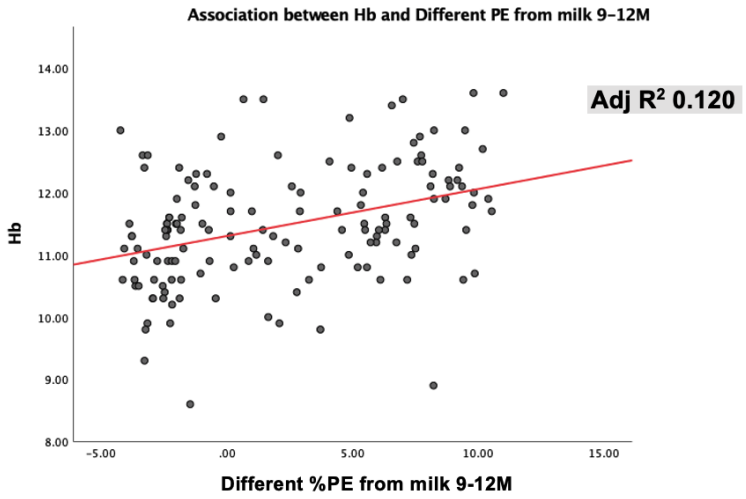
B) **Transferrin saturation (TSAT)**





C) **Haemoglobin (Hb)**





These scatter plots demonstrate associations between "Difference of %PE between non-breast milk (non-BM) minus %PE from breast milk (BM)" at 6-9M or 9-12M and blood concentrations of serum ferritin, transferrin saturation, and haemoglobin at 12M. The R^2 values represent the proportion of the variance of Ln SF, TSAT, and Hb explained by the different %PE from milk.

I concluded that it was reasonable to use protein intake from the whole CF period (6-12 months of age) in the subsequent regression analyses instead of two separate periods, because the univariate models showed similar results for associations between protein intake during both periods and iron status.

In the multiple regression analyses %PE from different sources was not a significant predictor of iron status using continuous variables. As shown in table R4.6, %PE from milk during the CF period was positively associated only with TSAT while no significant effects were found for SF, Hb and ID/ IDA. A 1% increase in %PE from milk between 6-12 months was associated with 0.45% increase of TSAT at 12M. However, according to the final model controlling for %PE from other diet sources, the type of milk feeding, unfortified cow's milk intake and provision of liver ≥ 3 times/ week at 12 months were significant predictors for iron status while plasma ESR was significantly associated only with SF which is well known as an acute phase reactant.

Table R4.7 shows the models using categorical variables for iron status. There were no significant associations between %PE from each protein source and iron status (Table R4.6, Table R4.7). Apart from the main predictor, protein intake, it was interesting that two variables: **"amount of unfortified cow's milk intake at 12M"** and **"provision of liver ≥ 3 times/ week at 12M"** were significantly associated with infant iron status in both models. The results showed a negative impact of unfortified cow's milk on iron status while the provision of liver ≥ 3 times/ week showed some benefits. The findings can be interpreted as follows:

- Every 100 ml increase in the intake of **unfortified cow's milk** at 12 months was associated with a **24% decrease in TSAT** (table R4.6, final model) independent of protein intake, type of milk feeding, regular consumption of liver and inflammation.

- Every 100 ml increase in the intake of **unfortified cow's milk** at 12 months was associated with **40% increasing** r**isk of ID and IDA**

(adjusted odd ratios in table R4.7B) regardless of protein intake, type of milk feeding, regular consumption of liver and inflammation.

- Receiving **liver ≥ 3 times/ week** at age 12 months was associated with a **0.2% increase in TSAT** (Table R4.6, final model) and with a **78% and 70% reduction in the risk of ID and IDA** respectively (Table R4.7, final model), after adjusting for protein intake, type of milk feeding, and unfortified cow's milk intake at 12 months old.

In addition, the results from the multiple regression analyses showed an association between type of milk feeding and SF. Receiving a combination of breast milk and formula or only formula during the CF period was associated with higher SF compared to receiving breast milk. "Age at introduction of egg" was the only variable predicting Hb level in the final multiple regression model; earlier introduction of egg was associated with higher Hb at 12 months.

The results suggest that protein intake during the CF period had less impact on infant iron status than other variables such as the consumption of unfortified cow's milk and frequent provision of liver to infants. However, higher consumption of milk protein especially from formula during the CF period was still significantly associated with increasing TSAT, while using formula as the main milk also showed a positive association with SF. Notably, protein from either formula or cow's milk can contribute to "too much" protein intake leading to rapid weight gain, but the effects on iron status were obviously different. While high protein intake from formula may improve iron status, increasing consumption of cow's milk which also provides very high protein intake may lead to ID/ IDA. These conflicting findings led to the next section that will address the question: Do infants with normal iron status eat too much protein?

Table R4.6 Multiple regression analyses predicting iron status (continuous data) by %PE from different source during the CF period

Outcomes	Predictors and Co-variates	First model		Final model*	
		Adj R^2 0.270		Adj R^2 0.284	
		ß	*p*	ß	*p*
Ln SF	(1) %PE Milk/ dairy[1]	0.14	0.39	0.15	0.36
	(2) %PE Non-dairy ASFs[1]	- 0.10	0.29	- 0.08	0.39
	(3) %PE Plant-based foods[1]	- 0.002	0.98	0.01	0.94
	(4) Type of milk 6-12M	**0.37**	**0.005**	**0.37**	**0.003**
	(5) Amount of unfortified cow's milk Intake at 12M[1]	- 0.15	0.07	- 0.16	0.05
	(6) Provision of liver ≥ 3/week at 9M[2]	0.12	0.14	-	-
	(7) Provision of liver ≥ 3/week at 12M[2]	0.04	0.59	-	-
	(8) Age of introduction of CF	- 0.07	0.42	-	-
	(9) Age of introduction of egg	0.09	0.33	-	-
	(10) Age of introduction of meat	0.09	0.31	-	-
	(11) Age of introduction of fish	- 0.04	0.64	-	-
	(12) Frequency of illness	0.07	0.38	-	-
	(13) Plasma ESR > 10 mm/h	**0.14**	**0.07**	**0.16**	**0.03**
TSAT		Adj R^2 0.139		Adj R^2 0.160	
		ß	*p*	ß	*P*
	(1) %PE Milk/ dairy[1]	**0.41**	**0.02**	**0.45**	**0.01**
	(2) %PE Non-dairy ASFs[1]	- 0.04	0.67	- 0.02	0.88
	(3) %PE Plant-based foods[1]	0.10	0.36	0.09	0.41
	(4) Type of milk 6-12M	0.05	0.74	0.01	0.95
	(5) Amount of unfortified cow's milk Intake at 12M[1]	**- 0.23**	**0.01**	**- 0.24**	**0.006**
	(6) Provision of liver ≥ 3/week at 9M[2]	- 0.001	0.99	-	-
	(7) Provision of liver ≥ 3/week at 12M[2]	**0.20**	**0.03**	**0.20**	**0.01**
	(8) Age of introduction of CF	- 0.004	0.97	-	-

	(9) Age of introduction of egg	0.07	0.49	-	-
	(10) Age of introduction of meat	- 0.07	0.44	-	-
	(11) Age of introduction of fish	- 0.10	0.31	-	-
	(12) Frequency of illness	- 0.05	0.56	-	-
	(13) Plasma ESR > 10 mm/h	- 0.07	0.43	- 0.09	0.27
		Adj R^2 0.160		Adj R^2 0.169	
		ß	*p*	ß	*P*
Hb	(1) %PE Milk/ dairy[1]	0.16	0.36	0.21	0.20
	(2) %PE Non-dairy ASFs[1]	0.01	0.89	0.02	0.81
	(3) %PE Plant-based foods[1]	0.07	0.53	0.10	0.34
	(4) Type of milk 6-12M	0.22	0.10	0.22	0.09
	(5) Amount of unfortified cow's milk Intake at 12M[1]	0.04	0.66	-	-
	(6) Provision of liver ≥ 3/week at 9M[2]	- 0.17	0.05	- 0.14	0.08
	(7) Provision of liver ≥ 3/week at 12M[2]	0.13	0.13	-	-
	(8) Age of introduction of CF	0.09	0.32	-	-
	(9) Age of introduction of egg	**- 0.23**	**0.02**	**- 0.18**	**0.03**
	(10) Age of introduction of meat	- 0.02	0.80	-	-
	(11) Age of introduction of fish	- 0.02	0.86	-	-
	(12) Frequency of illness	0.06	0.48	-	-
	(13) Plasma ESR > 10 mm/h	- 0.16	0.05	- 0.16	0.05

Final model included main predictors (protein intake from different sources), significant co-variates from univariate analyses in table R4.4 and the significant co-variates from the first models; [1]Data were mainly based on the 3-day food records; [2]Data were based on the food frequency questionnaires; %PE – percentage of energy provided by dietary protein; Ln – natural log; SF – serum ferritin; TSAT – transferrin saturation; Hb – haemoglobin; Adj R^2 – adjusted coefficient of determination; ß – regression coefficient; p – p-value; M – months old; ASFs – animal source foods; CF – complementary feeding; ESR – erythrocyte sediment rate

Table R4.7 Multiple regression analyses predicting iron status (categorical data) by %PE from different source during the CF period

A) First model

Predictors And Co-variates	ID		IDA	
	Exp(ß)	95%CI	Exp(ß)	95%CI
(1) %PE Milk/ dairy[1]	0.68	0.37, 1.25	0.79	0.36, 1.70
(2) %PE Non-dairy ASFs[1]	1.10	0.78, 1.55	1.20	0.86, 1.67
(3) %PE Plant-based foods[1]	1.17	1.00, 1.01	0.77	0.18, 3.24
(4) Type of milk 6-12M*				
- Combined BM and formula	1.47	0.37, 5.98	0.22	0.04, 1.07
- Formula	1.79	0.21, 15.19	**0.03**	0.001, 0.73
(5) **Amount of unfortified cow's milk intake at 12M[1]**	**1.01**	**1.002, 1.01**	**1.01**	**1.001, 1.01**
(6) Provision of liver ≥ 3 /week at 9M[†,2]	0.57	0.22, 1.48	0.69	0.23, 2.02
(7) **Provision of liver ≥ 3/week at 12M[†,2]**	**0.19**	**0.06, 0.67**	**0.27**	**0.08, 0.96**
(8) Age of introduction of CF	2.02	0.77, 5.31	2.22	0.72, 6.86
(9) Age of introduction of egg	0.40	0.12, 1.35	0.45	0.12, 1.72
(10) Age of introduction of meat	0.66	0.30, 1.46	1.98	0.89, 4.37
(11) Age of introduction of fish	1.12	0.62, 2.03	0.95	0.48, 1.88
(12) Frequency of illness	1.33	0.88, 2.02	0.98	0.59, 1.61
(13) Plasma ESR > 10 mm/h[#]	1.82	0.59, 5.65	3.29	0.93, 11.59

***Reference group = Breast milk; †Reference group = No; #Reference group = No; [1]Data were mainly based on the 3-day food records; [2]Data were based on the food frequency questionnaires; %PE – percentage of energy provided by dietary protein; CF – complementary feeding; ID – iron deficiency; IDA – iron deficiency anaemia; ASFs – animal source foods; M -months old; BM – breast milk; CF – complementary feeding; ESR – erythrocyte sediment rate; Exp (ß) – exponential of regression coefficient; CI – confidence intervals*

B) Final Model

Independent variables & covariates	ID		IDA	
	Exp(ß)	95%CI	Exp(ß)	95%CI
(1) %PE Milk/ dairy[1]	0.66	0.38, 1.14	0.81	0.41, 1.62
(2) %PE Non-dairy ASFs[1]	1.01	0.75, 1.37	1.12	0.82, 1.55
(3) %PE Plant-based foods[1]	1.06	0.33, 3.44	0.83	0.21, 3.31
(4) Type of milk 6-12M*				
- Combined BM and formula	1.44	0.42, 5.00	0.39	0.10, 1.54
- Formula	1.92	0.30, 12.48	0.06	0.003, 1.18
(5) Amount of unfortified cow's milk intake at 12M[1]	**1.004**	**1.001, 1.01**	**1.004**	**1.001, 1.01**
(6) Provision of liver ≥ 3/week at 12M†,[2]	**0.22**	**0.08, 0.62**	**0.30**	**0.10, 0.92**

***Reference group = Breast milk; †Reference group = No; [1]Data were mainly based on the 3-day food records; [2]Data were based on the food frequency questionnaires; %PE – percentage of energy provided by dietary protein; CF – complementary feeding; ID – iron deficiency; IDA – iron deficiency anaemia; ASFs – animal source foods; M -months old; BM – breast milk; BF – breastfeeding; CF – complementary feeding; Exp (ß) – exponential of regression coefficient; CI – confidence intervals*

R4.4 Do infants with normal iron status eat too much protein?

ASFs, especially red meats and organ meats, are good sources of iron, but also provide high protein content that could contribute to rapid weight gain. This raises the concern that promoting ASFs to infants living in a country where both overweight/ obesity and ID/IDA are prevalent in young children is still appropriate. Furthermore, as shown in section R4.3, infants with high protein intakes from milk might not always have satisfactory iron status if they consume unfortified cow's milk instead of formula, while consumption of non-dairy ABP did not significantly affect iron status with the exception of regular consumption of liver. Therefore, it is interesting to consider how we can optimise infant iron status without increasing the risk of overweight/ obesity.

Tables R4.8 and R4.9 show protein intake during the CF period in infants with normal iron status, ID and IDA at 12 months, considering both quantity and sources of protein.

- Quantity of protein

The results in table R4.8 clearly show that there were no differences in daily protein intake among infants with normal iron status, ID and IDA. %PE from all diets were similar for all groups and also less than 15% throughout the CF period. In addition, when categorising infants based on %PE using the same criteria used in Chapter 7, Results 3 (high, median, and low), the proportions of infants in the protein intake groups did not differ significantly between infants with normal iron status or ID/ IDA. Notably, for infants with IDA, the percentage of infants consuming high, median and low protein were nearly equal. Altogether, in terms of protein quantity, the results showed that the infants with normal iron status did not consume more protein than infants with ID/IDA suggesting it is possible to achieve normal iron status without increasing the risk of rapid growth, overweight/ obesity due to excess intake of dietary protein. However, protein sources should be taken into account.

- Sources of dietary protein

Iron-sufficient infants received significantly more protein from milk, in particular formula, than infants with IDA throughout the CF period, but intake of milk protein was not different compared to the ID group. The amounts of milk consumed at all time points also confirmed that the type of milk was important. The patterns of milk intake were similar but in the opposite direction for formula and breast milk. The highest intake of formula was found in infants with normal iron status, with intermediate intake in infants with ID and lowest intake in infants with IDA. The reverse pattern was found for breast milk consumption. Notably, infants with normal iron status consumed less unfortified cow's milk at age 12 months compared to infants with ID/ IDA but the difference was significant only between the normal and ID groups.

Considering other protein sources, infants with IDA unexpectedly consumed a significantly higher amount of protein from non-dairy ASFs than infants with normal iron status at 6-9 months (Table R4.8). However, this difference was absent at 9-12 months. Intake of protein from plant-based foods was the only

source with very similar figures across all iron status groups throughout the whole CF period. Taken together the results suggest that the consumption of different protein sources may affect the iron status of infants to some extent, especially for milk protein. While infants with normal iron status tended to consume a higher amount of formula during the CF period, the average intakes of breast milk and cow's milk were higher in ID and IDA groups.

To summarise the findings addressing whether infants with normal iron status eat too much protein:

1) Infants with different iron status consumed similar amounts of dietary protein throughout the whole period of CF.
2) Infants with normal iron status consumed a higher amount of formula and the lowest amount of unfortified cow's milk during the CF period compared to ID infants.
3) Infants with ID/ IDA consumed larger amounts of breast milk throughout the CF period and tended to have higher %PE from non-dairy ASFs compared to infants with normal iron status.

It was disappointing to find that infants who consumed more breast milk alongside CFs as recommended might not achieve normal iron status despite a higher intake of protein from non-dairy ASFs. As breast milk clearly has more benefits for infants than disadvantages, I next explored the dietary factors promoting normal iron status in infants consuming predominantly breast milk during the complementary feeding, to address whether it would be possible to improve the iron status of these infants by using a diet-based approach.

Table R4.8 Comparison of %PE among infants based on their iron status

Variables	Average ± SD		
	Normal (n = 83)	**ID** (n = 35)	**IDA** (n = 27)
%PE from all diets*			
• 6-9M	10.3 ± 1.2	10.2 ± 1.9	10.2 ± 1.5
• 9-12M	14.2 ± 2.3	14.1 ± 2.1	14.0 ± 2.4
% PE from milk*			
• 6-9M[1]	**6.3 ± 1.8**	5.6 ± 1.4	**4.9± 0.8**
• 9-12M[2]	**5.7 ± 2.5**	5.3 ± 2.0	**4.2 ± 2.0**
%PE from non-dairy ASFs*			
• 6-9M[2]	**2.9 ± 1.7**	3.5 ± 1.4	**4.1 ± 1.5**
• 9-12M	6.6 ± 2.8	6.9 ± 2.4	7.8 ± 2.3
%PE from plant-based foods*			
• 6-9M	1.1 ± 0.6	1.1 ± 0.5	1.3 ± 0.4
• 9-12M	1.9 ± 0.8	1.9 ± 0.5	2.1 ± 0.5
Amount of milk intake			
6M†			
• Breast milk[2,3]	**502.8 ± 412.5**	539.3± 371.1	**777.5± 262.2**
• Formula[2,3]	**341.0 ± 409.4**	274.4± 414.7	**33.3± 124.6**
9M*			
• Breast milk[2,3]	**279.8 ± 275.6**	320.9± 283.3	**510.9± 231.7**
• Formula[2]	**363.1 ± 397.9**	256.9± 324.2	**61.5± 203.6**
12M*			
• Breast milk[2]	**142.6 ± 190.9**	218.0± 254.3	**327.9± 309.5**
• Formula[2]	**360.7 ± 321.1**	243.6± 306.3	**120.4± 215.2**
• Unfortified cow's milk[4]	**35.7 ±126.8**	**125.0± 264.3**	62.8± 178.7

[1] Post-hoc analysis (Bonferrini's) Normal vs IDA: p < 0.001
[2] Post-hoc analysis (Bonferrini's) Normal vs IDA: p < 0.05
[3]Post-hoc analysis (Bonferrini's) ID vs IDA: p < 0.05
[4]Post-hoc analysis (Bonferrini's) Normal vs ID: p < 0.05
**Data were mainly based on the 3-day food records; † Data were based on the 24-hour food recalls; %PE – percentage of energy provided by dietary protein; SD – standard deviation; ID – iron deficiency; IDA – iron deficiency anaemia; M – months old; ASFs – animal source foods*

Table R4.9 Association between iron status and protein intake groups

Iron status Protein intake	Normal (n = 83)	ID (n = 35)	IDA (n = 27)	*p**
High	19 (22.9%)	9 (25.7%)	8 (29.6%)	0.63
Median	45 (54.2%)	18 (51.4%)	10 (37.0%)	
Low	19 (22.9%)	8 (22.9%)	9 (33.3%)	

**Chi-square test*
ID – iron deficiency; IDA – iron deficiency anaemia; p – p-value

R4.5 Dietary factors promoting normal iron status in infants consuming predominantly breast milk during complementary feeding

To select infants for the subgroup analyses in this section I began by targeting breastfed infants who consumed only breast milk along with complementary foods. However, there were insufficient infants in this group (n=45) for the planned analyses. I then compared the duration of exclusive breastfeeding (EBF) and predominant BF between iron-deficient infants and infants with normal iron status. The duration of predominant BF was significantly longer in iron-deficient infants compared to infants with normal iron status while the duration of EBF was not different between these two groups (Appendix 14). Therefore, I decided to select subjects based on the duration of predominant BF for further analyses, including all those who had been breastfed $\geq$ 6 months (n = 94). The average duration of EBF and predominant BF for these infants was 5.5 $\pm$ 1.0 and 11.3 $\pm$ 1.3 months, respectively.

Several variables shown in table R4.10 were considered as dietary factors influencing iron status and classified into 4 groups based on their characteristics. The further analyses are presented in order considering milk intake (group 1), protein intake from complementary foods (group 2), age of introduction of CF and ASFs (group 3), and frequency of provision of iron-rich foods (group 4).

Table R4.10 Dietary variables selected for investigating their impact on iron status in predominantly breastfed infants

Dietary protein*,[1]	Frequency of provision of iron-rich foods[2]	Other diet-related factors
1) %PE all diets 6-12M	1) Pork at 6M	1) Duration of EBF
2) PW all diets 6-12M	2) Pork liver at 6M	2) Duration of predominant BF
3) %PE non-dairy ASFs 6-9M	3) Commercially fortified baby food at 6M	3) Age of introduction of CF 4) Age of introduction of meat
4) %PE non-dairy ASFs 9-12M	4) Pork at 9M 5) Pork liver at 9M	5) Age of introduction of egg 6) Age of introduction of fish
5) %PE plant-based foods 6-9M	6) Commercially fortified baby food at 9M	7) Amount of BM at 6M[3] 8) Amount of BM at 9M[1]
6) %PE plant-based foods 9-12M	7) Chicken liver at 9M 8) Pork at 12M 9) Pork liver at 12M	9) Amount of BM at 12M[1] 10) Amount of formula at 6M[3] 11) Amount of formula at 9M[1]
	10) Commercially fortified baby food at 12M 11) Chicken liver at 12M	12) Amount of BM at 12M[1] 13) Amount of unfortified cow's milk/ dairy products at 12M[1]

***** *%PE from milk was not included as amount of milk could be a proxy for it; [1]Data were mainly based on the 3-day food records; [2]Data were based on the food frequency questionnaires; [3]Data were based on the 24-hour food recalls; %PE – percentage energy provided dietary protein; M – months old; EBF – exclusive breastfeeding; BF – breastfeeding; CF – complementary feeding; BM - breast milk; ASFs – animal source foods*

As shown in table R4.11, breastfed infants with normal iron status consumed a lower amount of breast milk compared with the ID/ IDA group but the difference was only significant at 12 months of age. Considering formula and cow's milk intake, although there was no significant difference, breastfed infant with ID/ IDA tended to consume less formula throughout the CF period but more cow's milk at 12 months of age than breastfed infants with normal iron status.

In addition, when performing regression analyses to predict iron status by milk intake at different ages (Table R4.12), only the amount of formula at 6M showed a significant positive association with SF (p = 0.02) while both intake of breast milk and cow's milk at 12 months of age showed negative

associations with TSAT ($p < 0.05$). Furthermore, consuming a higher amount of breast milk and cow's milk at 12 months of age were also associated with increasing risk of ID ($p = 0.02$). All these findings could be interpreted as follows:

For infants who are predominantly breastfed for at least 6 months

- Every 100 ml increase in intake of formula at 6M is associated with a 20% increase in SF at 12 months
- Every 100 ml increase in intake of breast milk or unfortified cow's milk at 12 months old is associated with 1% decrease in TSAT at 12M.
- Every 100 ml increase in intake of breast milk or cow's milk at 12 months old is associated with a 100% increase in the risk of being ID/ IDA.

Group 1: Milk intake

Table R4.11 Comparison of average amount of milk consumed by iron sufficient and iron-deficient infants

Amount of milk intake (ml/day), means ± SD	**Iron status**		***p***
	Normal (n=47)	**ID/ IDA** (n=47)	
Breast milk at 6M*	805.7 ± 192.8	787.8 ± 195.3	0.65
Breast milk at 9M†	476.0 ± 195.9	507.9 ± 206.7	0.44
Breast milk at 12M†	**248.9 ± 194.8**	**348.6 ± 261.3**	**0.04**
Formula at 6M*	32.6 ± 118.2	8.5 ± 41.5	0.19
Formula at 9M†	88.5 ± 233.1	57.2 ± 199.1	0.49
Formula at 12 M†	197.8 ± 241.7	128.5 ± 231.4	0.16
Unfortified cow's milk at 12M†	15.7 ± 55.4	64.7 ± 184.3	0.09

**Data were based on the 24-hour food recalls; †Data were mainly based on the 3-day food records; SD – standard deviation; ID – iron deficiency; IDA – iron deficiency anaemia; p – p-value; M – months old*

Table R4.12 Multiple regression analyses predicting iron status by amount of milk intake during the CF period

Outcomes	Predictors Amount of milk intake (ml/day)	ß	*p*
Ln SF	**6M***		
	• Breast milk	<0.001	0.71
	• **Formula**	**0.002**	**0.02**
	9M†		
	• Breast milk	- 0.001	0.17
	• Formula	<0.001	0.73
	12M†		
	• Breast milk	- 0.001	0.17
	• Formula	<0.001	0.30
	• Unfortified cow's milk	< -0.001	0.97
TSAT	**6M***		
	• Breast milk	0.003	0.52
	• Formula	0.01	0.16
	9M†		
	• Breast milk	- 0.001	0.82
	• Formula	0.004	0.48
	12M†		
	• **Breast milk**	**- 0.01**	**0.01**
	• Formula	< - 0.001	1.00
	• **Unfortified cow's milk**	**- 0.01**	**0.03**
Hb	**6M***		
	• Breast milk	< 0.001	0.40
	• Formula	0.001	0.20
	9M†		
	• Breast milk	< 0.001	0.73
	• Formula	0.001	0.25
	12M†		
	• Breast milk	- 0.001	0.32
	• Formula	< 0.001	0.98
	• Unfortified cow's milk	0.001	0.30

Outcomes	Predictors Amount of milk intake	ID		IDA	
		Exp(ß)	*p*	Exp(ß)	*p*
ID/ IDA Reference: normal iron status	**6M***				
	• Breast milk	1.00	0.29	1.00	0.58
	• Formula	0.99	0.25	1.00	0.30
	9M†				
	• Breast milk	1.00	0.54	1.00	0.37
	• Formula	1.00	0.93	1.00	0.80

	12M† • **Breast milk** • Formula • **Unfortified cow's milk**	**1.01** 1.00 **1.01**	**0.02** 0.28 **0.02**	1.00 1.00 1.00	0.10 0.72 0.44

**Data were based on the 24-hour food recalls; †Data were mainly based on the 3-day food records; CF – complementary feeding; Ln – natural log; SF – serum ferritin; TSAT – transferrin saturation; Hb – haemoglobin; ID – iron deficiency; IDA – iron deficiency anaemia; M – months old; ß - regression coefficient; p – p-value; Exp (ß) – exponential of regression coefficient; CI – confidence intervals*

As shown in table R4.13, neither %PE from all diets nor from each food source were different among breastfed infants with different iron status. Table R4.14 also shows that age of introduction of CF and other ASFs were not different between breastfed infants with normal iron status and those with ID/ IDA.

As shown in table R4.15, eggs were the most common ASFs that parents regularly provided to breastfed infants over the entire period of CF. At 6 months, iron-rich ASFs were not often given to breastfed infants, with less than 10% regularly receiving iron-rich ASFs such as liver. Although the regular provision of meats (i.e., pork, chicken) improved at 9 and 12 months, the percentages with regular consumption of chicken/ pork liver was still low. Only around a quarter of parents reported providing either chicken/ pork liver to their children ≥ 3 times/ week even though this is suggested by the Thai CF recommendations (Appendix 11). Moreover, it should be noted that iron-fortified baby food was not popular in this group. As shown in figure R4.4, iron-fortified baby foods were often used only at 6 months with lower percentages at 9 and 12 months.

In line with the outcomes for the whole study population which suggested benefits for encouraging provision of liver ≥ 3 times/week, table R4.16 suggests that breastfed infants with normal iron status were more likely to receive liver ≥ 3 times/week at age 9 and 12 months compared to those with ID/IDA, even though the difference at 9 months was not statistically significant ($p = 0.07$). Following on from these findings, the results from table R4.17 show that it is not just frequency that is important; regular consumers also ate larger portion sizes compared to those who were offered liver < 3 times/ week.

According to table R4.17, regular consumers usually ate approximately 1 tablespoon (tbs) each meal compared to roughly around a half a tbs in less-frequent consumers.

When different frequencies of consumption were considered (i.e., never eat, once/ twice a month, once/ twice a week, ≥ 3 times/week, daily, twice a day; Table R4.18), more frequent liver consumption at 9 months was associated with a significantly reduced risk of ID/ IDA at 12 months in these breastfed infants. Although not statistically significant at 12 months, the odd ratios of ID/ IDA were still less than 1 for regular consumption of chicken/ pork liver at 12 months old.

In summary, the significant findings in this section are generally in line with current knowledge/ recommendations; for example, cow's milk should be avoided before 12 months and formula had a more favourable impact on iron status than breast milk. However, these analyses provide further evidence to support these recommendations. Furthermore, there was a substantial practical point resulting from these analyses as the results suggest that provision of one tablespoon of liver at least 3 times/ week as early as possible during the CF period could reduce risk of ID/ IDA for breastfed infants.

Group 2: Protein intake from complementary foods

Table R4.13 Comparison of average %PE from complementary foods between iron-sufficient and iron-deficient infants

%PE* (Average ± SD)	**Iron status**		***p***
	Normal (n = 47)	**ID/ IDA** (n =47)	
All diets 6-12M	11.7 ± 1.6	11.6 ± 1.5	0.87
From non-dairy ASFs 6-9M	3.4 ± 1.8	3.9 ± 1.5	0.12
From non-dairy ASFs 9-12M	7.5 ± 2.9	7.5 ± 2.4	0.90
From plant-based foods 6-9M	1.2 ± 0.5	1.3 ± 0.4	0.24
From plant-based foods 9-12M	2.1 ± 0.8	2.1 ± 0.5	0.96

**Data were mainly based on the 3-day food records; %PE – percentage of energy provided by dietary protein; SD – standard deviation; ID – iron deficiency; IDA – iron deficiency anaemia; p – p-value; M – months old; ASFs – animal source foods*

Group 3: Age of introduction of CF and ASFs

Table R4.14 Comparison of age of first introduction of CF and other ASFs between iron-sufficient infants and iron-deficient infants

Age of introduction months old, (means ± SD)	**Iron status**		***p***
	Normal (n = 47)	**ID** (n =47)	
CF	5.7 ± 0.6	5.8 ± 0.6	0.28
Egg	6.0 ± 0.4	6.0 ± 0.5	0.24
Meat	6.2 ± 0.5	6.4 ± 0.9	0.26
Fish	6.4 ± 0.8	6.6 ± 1.0	0.95

CF – complementary feeding; ASFs – animal source foods; SD – standard deviation; ID – iron deficiency; p – p-value

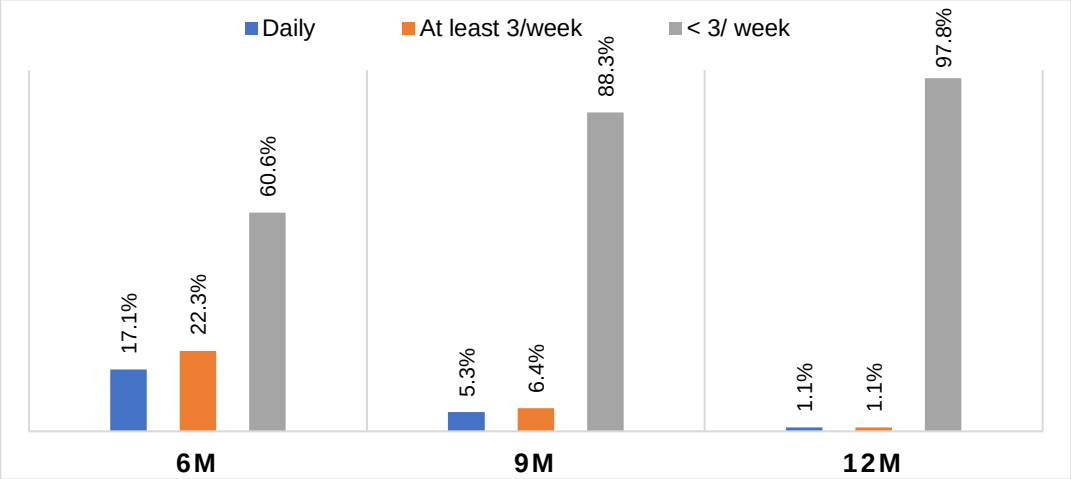
Group 4: Frequency of provision of iron-rich foods

Table R4.15 Percentage of infants receiving common ASFs ≥ 3 times/week at 6, 9 and 12 months

Rank of common ASFs	**6M*** (n =94)	**9M*** (n =94)	**12M*** (n =94)
1st	Egg, hen 47.9%	Egg, hen 83%	Egg, hen 90.4%
2nd	Pork liver 6.4% Chicken liver 6.4%	Pork 58.5%	Pork 76.6%
3rd	Pork 5.4%	Chicken 42.6%	Fish 39.4%
4th	Fish 3.2%	Fish 41.5%	Chicken 37.2%
5th	Chicken 2.1%	Chicken liver 29.9%	Chicken liver 22.3%
6th	-	Pork liver 29.8%	Pork liver 21.3%

** Data were based on the food frequency questionnaires; ASFs – animal source foods; M – months old*

Figure R4.4 Percentages of infants receiving iron-fortified baby foods (n = 94)



The bar charts illustrate the percentages of infants who received iron-fortified baby foods during the CF period (6-12 months of age). The blue bars represent daily consumption, orange bars show percentages of infants consuming this type of food ≥ 3 times/ week and grey bars present percentages of those who received < 3 times/ week.

Table R4.16 Comparison of percentage of infants consuming iron-rich foods ≥ 3 times/week between iron-sufficient and iron-deficient infants at 6-12 months

Iron-rich ASFs	Normal (n =47)	ID/ IDA (n=47)	*p*
6M†			
• Iron-fortified baby food	11 (23.4%)	10 (21.3%)	1.00*
• Pork	1 (2.1%)	4 (8.5%)	0.24#
• Pork liver	4 (8.5%)	2 (4.3%)	0.68#
• Chicken liver	2 (4.3%)	4 (8.5%)	0.68#
9M†			
• Pork	27 (57.4%)	28 (59.6%)	0.83*
• Chicken	19 (40.4%)	21(44.7%)	0.83*
• Pork liver	16 (34.0%)	12 (25.5%)	0.37*
• Chicken liver	18 (38.3%)	10 (21.3%)	0.07*
12M†			
• Pork	35 (74.5%)	37 (78.7%)	0.63*
• Chicken	16 (34.0%)	19 (40.4%)	0.67*
• **Pork liver**	**14 (29.8%)**	**6 (12.8%)**	**0.04***
• Chicken liver	13 (27.7%)	8 (17%)	0.22*

*†Data were based on the food frequency questionnaires; *Chi-square; #Fisher's exact test; ASFs – animal source foods; ID – iron deficiency; IDA – iron deficiency anaemia; M – months old; p – p-value*

Table R4.17 Comparison of average portion size of liver between regular and less-frequency consumptions at 9 and 12 months

Provision of liver at age	**Portion size** (tablespoon/ meal), means ± SD		***p***
	Regular consumer (≥ 3 times/week)	**Less-frequent consumers** (< 3 times/week)	
9M*	**1.0 ± 0.3**	0.4 ± 0.5	**< 0.001**
12M*	**1.1 ± 0.6**	0.5 ± 0.5	**< 0.001**

**Data were based on the food frequency questionnaires; M – months old; SD – standard deviation; p – p-value*

Table R4.18 Multiple logistic regression predicting iron status by provision of iron-rich foods

Predictors* Frequency of provision of	**Outcomes**			
	ID		**IDA**	
	Exp (ß)	*p*	Exp (ß)	*p*
6M				
• Iron-fortified baby food	1.07	0.67	0.97	0.83
• Pork	1.45	0.25	1.80	0.05
• Pork liver	1.18	0.58	0.98	0.96
• Chicken liver	1.25	0.43	1.03	0.92
9M				
• Pork	0.99	0.97	1.23	0.40
• Chicken	1.27	0.37	0.94	0.81
• **Pork liver**	1.00	0.98	**0.61**	**0.03**
• **Chicken liver**	**0.46**	**0.002**	0.70	0.10
12M				
• Pork	0.89	0.68	0.93	0.81
• Chicken	1.61	0.11	1.28	0.38
• Pork liver	0.90	0.65	0.94	0.76
• Chicken liver	0.84	0.40	0.79	0.26

**Data were based on the food frequency questionnaires; ID – iron deficiency; IDA – iron deficiency anaemia; M – months old; Exp (ß) – exponential of regression coefficient; p – p-value*

R4.6 Summary of key results and discussion

Key results

- %PE from formula is highly correlated with daily iron intake while %PE from non-dairy ABP and PBP showed lower correlations.
- When adjusted for potential confounders, %PE from formula during the CF period was positively associated with TSAT, but not SF, Hb, and iron status of infants, while no significant associations between non-dairy ABP or PBP intake and iron indicators were observed.
- Infants with different iron status consumed the same amounts of daily %PE which were <15% during the CF period. However, those with normal iron status received the highest amount of formula and consumed the least amount of breast milk and unfortified cow's milk.
- Consumption of unfortified cow's milk had a significantly negative impact on TSAT and iron status while provision of liver at least 3 times per week consistently showed a protective effect on ID/ IDA regardless of type of milk feeding, infection/ inflammation and %PE from different sources during the CF period.
- For breastfed infants, avoiding unfortified cow's milk and regular consumption of one tablespoon of liver at least 3 times per week as early as possible during the CF period may improve iron status and reduce the risk of ID/ IDA.

The key findings from this chapter are clear and consistent with current scientific evidence. The results from multiple regression analyses strongly confirmed a negative impact of an early introduction of unfortified cow's milk before the first birthday and the protective effect of regular consumption of iron-rich ASFs such as liver on infant iron status, especially for breastfed infants who are more susceptible to ID/ IDA than those who receive formula during the CF period. However, what I found most interesting are the results demonstrating a relationship between dietary protein and iron status, which is the main focus of this section.

At present, there are two separate research focuses related to ASFs. While some researchers extensively investigate the positive impact of iron from ASFs on infant iron status, others emphasise the downside of too high intake of protein from ASFs increasing the risk of overweight/ obesity. These opposing considerations might be less problematic in high-income and low-income countries where the prevalence of ID/IDA and overweight/ obesity are quite different in magnitude, but they present difficulties in middle-income countries where both nutritional problems are equally problematic. More importantly, in countries where iron-fortified complementary foods are not commonly used, it is very challenging to determine the best trade-off between ID/IDA and overweight/obesity when making recommendations for the consumption of ASFs.

In high-resource settings where the prevalence of ID/ IDA in infants and young children are relatively low[254] and meat supplements during the CF period do not show any significant effect on iron status[46, 48, 49, 255-6], decreasing consumption of ASFs to reduce protein intake seems to be a reasonable strategy to reduce the burden of overweight/ obesity in younger generations. Recently, the first RCT focusing on this practice has been conducted in Sweden. Johansson et al[257] randomly allocated term-born infants aged 4-6 months into two groups. Baseline demographic data were not different between groups and average duration of exclusive breastfeeding was approximately 4 months in both groups. Parents in the intervention group received some advice to make protein-reduced complementary foods based on Nordic-grown, season-based regional foods enriched with fish, plant-based foods and vegetable oils while reducing added sugar, saturated fat, meat/ meat products at the same time. The control group used conventional weaning foods. At 9 months, protein intake was significantly lower in the intervention group compared to the control group (average daily protein intakes were 15.9 vs 21.5 g/day, respectively, $p < 0.001$) while all growth parameters and laboratory results representing iron status were not different between the two groups. However, it should be noted that during the intervention period, infants in both groups were receiving unlimited amounts of iron-fortified complementary foods and formula. In addition, %PE at 9 months of both

groups were less than 15%[3]. Therefore, in high-resource settings, reducing consumption of meat/ meat products seems to be an appropriate strategy to promote healthy weight gain without compromising infant iron status.

When considering the situation in resource-limited countries where the prevalence of anaemia and ID/ IDA in infants and young children are extremely high and have serious consequences[2], encouraging consumption of iron-rich ASFs such as red meat and organs is one of many principal food-based programmes used to alleviate this burden[5]. In those countries facing the highest burden of ID/ IDA in children aged less than 5 years[254], the prevalence of overweight/ obesity is far below that for undernutrition, especially stunting[258]. Therefore, apart from problems related to affordability and availability, iron-rich ASFs seem to be better choices than iron-fortified foods or iron supplementation as they contain highly absorbable iron and various essential nutrients that could promote growth in general[229]. To my best knowledge, there is no study reporting an association between intake of ASFs and overweight/ obesity among infants and young children from these specific settings. Altogether, in resource-poor resource countries, promotion of iron-rich ASFs along with breastfeeding seems to be an appropriate practice resulting in better iron status without increasing prevalence of overweight/ obesity.

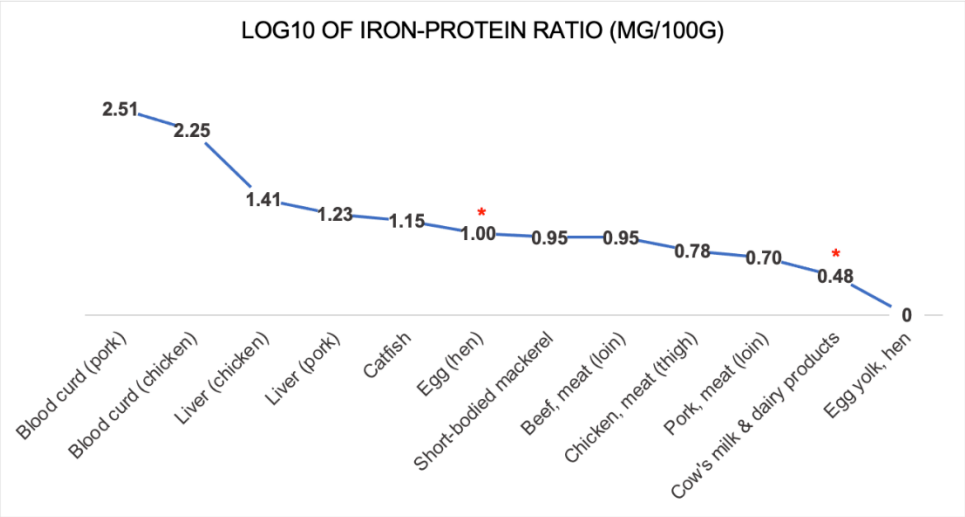
In between these two situations many mainly middle-income countries are facing the DBM and struggling with both nutritional problems. Although ID/ IDA is more of a concern as a major burden in late infancy and toddler life, there is increasing evidence that a high protein intake from ASFs during this period may accelerate the prevalence of overweight/ obesity in older age groups. This led me to consider how protein intake from different sources affects infant iron status, and whether we can identify the best strategy to provide an appropriate amount of ASFs to achieve normal iron status whilst also promoting healthy weight gain by optimising protein intake for this specific population. Although

[3] Data not shown in the original article, but could be calculated from the daily intake of energy and macronutrients shown in table 3 of the original article

provision of iron-fortified foods or micronutrient powders and iron supplementation for anaemic infants identified from universal screening sounds more feasible, there is still some debate on the risk of iron overload in countries with a high prevalence of Thalassemia like Thailand[259]. In addition, more recent studies consistently show a negative impact of unabsorbed iron on gut microbiota and inflammatory state in humans[47, 260-2], thus it is worth investigating alternative ways of solving this issue.

I began by examining correlations between dietary protein and iron intake. It was unsurprising to see that milk protein was highly correlated with iron intake, as the main contributor of milk protein in this cohort was formula. Both protein and iron content in formula are relatively high and variations of both nutrients among commercial brands are low compared to non-dairy ASFs and plant-based foods. However, the correlation between non-dairy ABP and iron intake was still good ($r = 0.5\text{-}0.6$, $p < 0.001$) and also increased at 9-12 months when consumption of iron-rich ASFs increased. As shown in figure R4.5, the iron-protein ratio (mg iron/ g protein) is quite different among ASFs, ranging from 0 to 3.27. This index was calculated based on nutrient composition data from the INMUCAL-Nutrients programme estimating this ratio per 1 tbs of cooked ASFs/ 1 boiled egg. A higher value of this index represents a higher iron content, but lower protein content which would be preferable in order to provide adequate iron without excess intake of protein, although it does not take into account the haem or non-haem iron content and absorbability of each ASF. Noticeably, the ASFs having higher values for this index such as blood curd and liver are also considered as excellent sources of haem iron which is more absorbable. Kongkachuichai et al[263] reported that cooked blood curd was the “best source” of haem iron compared to other ASFs commonly consumed by the Thai population. Blood curd contains haem iron, roughly around 75% of its iron content. Liver is also a good source of iron as total iron content is just slightly below blood curd and 18-24% of its iron content is haem iron. Interestingly, the study also reported high iron content in catfish and short-bodied mackerel which are available and affordable in Thailand. Based on the iron-protein ratio, some ASFs seem to be more preferrable than others.

Figure R4.5 Comparison of the log 10 of iron-protein ratio (mg/ 100 g) among various animal-source foods commonly used in Thailand



This figure demonstrates the log10 of iron-protein ratio of each animal-source food (ASFs) that are commonly used in Thailand. The red asterisk (*) indicates ASFs containing only non-haeme iron. Cooked blood curd is an only food source that provides highest dietary iron per 100 g of protein.

Although several studies try to propose food-based models aiming to meet dietary iron requirements for the late infancy and toddler period, the results are rather disappointing. A systematic review reported that among 8 studies using modeled diets based on locally available complementary foods in LMICs, none of them could provide adequate amounts of iron and zinc to infants and toddlers aged 6 to 23 months[264]. However, Vitta and Dewey[265] is the only study demonstrating that all nutrient gaps could be fulfilled in the best-case scenario when chicken liver is consumed daily. This information supports the key finding of my cohort that regular consumption of liver at least 3 times per week during the CF period significantly reduced risk of being ID and IDA at 12 months old by 78% and 70%, regardless of milk feeding, duration and protein intake from different sources. However, there is some evidence that an increasing iron intake does not always improve infant iron status[49, 266].

As shown in section R4.4, %PE from all diets among infants with different iron status were almost the same and did not exceed the proposed upper limits (%PE less than 15%) in all age groups. In addition, when I classified infants

based on their protein intake, there were no differences in percentages of ID/IDA observed among high, median and low protein intake groups. These results suggest that, for iron status, the total protein intake is less important than the source of dietary protein offered to infants during the CF period.

In contrast to protein from formula, intake of non-dairy ABP and PBP did not show any significant associations with iron status. Moreover, infants with ID/IDA had even higher %PE from non-dairy ASFs than iron-sufficient infants during the CF period. However, these results should not discourage provision of non-dairy ASFs, but more attention should be paid to the type of ASFs that are consumed, especially for predominantly breastfed infants. According to table R4.17, egg was the first non-dairy ASFs introduced to infants at an early stage of CF in both iron-sufficient and iron-deficient groups. Age of introduction of meat and fish were slightly delayed in iron-deficient infants compared to infants with normal iron status, even though there was no statistical significance. Additionally, a high proportion of infants regularly consumed egg throughout the CF period. At 6 months, apart from egg, other non-dairy ASFs were rarely consumed while the consumption of pork, chicken and fish increased at aged 9 and 12 months. Noticeably, very small numbers of infants regularly consumed iron-rich foods such as liver at an early stage of CF and the percentages were still lower than other ASFs at aged 9 and 12 months. Therefore, it is not surprising that protein intake from non-dairy ASFs was not associated with iron status. Unless iron-rich ASFs are regularly consumed from the early stages of CF and widely used in the population, protein intake from ASFs might not have any apparent impact on infant iron status.

Although I did not separately analyse the effect of “protein intake” from unfortified cow’s milk on iron status, the strong association between “amount” of unfortified cow’s milk and all parameters representing iron status confirms its negative impact. Consistent with several studies and recommendations[42, 267-269], it is clear that infants should avoid this type of milk during the transitional period whether they are formula-fed or breastfed. Unfortified cow’s milk not only has low iron content resulting in iron deficiency, but it also provides too much protein that increases the risk of overweight/ obesity at later ages. On

the other hand, liver whether pork or chicken liver, seems to be a preferable choice compared to other non-dairy ASFs, as the results from this cohort consistently suggest a protective effect against ID/ IDA. This result was in accordance with a cross-sectional study reporting that Thai infants aged 9-12 months who were iron-deficient consumed lower amounts of meat and liver[270] while another study showed that delaying introduction of meat significantly increased the risk of iron depletion[176]. In addition, with an intake of one tbs at least 3 times per week combined with its iron-protein ratio, it is unlikely that protein intake from this amount and frequency of liver would be “too much” for infants. Unfortunately, I could not investigate the association between blood curd and iron status due to the very small number of infants that had consumed it in this cohort.

Although liver consumption seems to be promising way to achieve normal iron status, there could be some concern about vitamin A intake. Regular consumption of liver, whether chicken or pork liver, at least 3 times/ week is more likely to provide vitamin A exceeding the ULs suggested by the Institute of Medicine of the United States (based on the INMUCAL-Nutrients programme: 1 tbs of cooked chicken and pork liver provides 1,344 and 2,859 μg of vitamin A respectively). The tolerable upper level (UL) of vitamin A for infants aged 0-12 months suggested by the Institute of Medicine, United States (600 μ g/ day of preformed vitamin A) is based on proportional calculation by infant’s body weight using the adult UL (3,000 μg/ day) which is presumably not the best way to establish the UL[271]. Acute toxicity can occur in children at an intake of 1500 IU/ kg/ day, equivalent to 450μg/kg/day[272]. Until now there is no report of vitamin A toxicity caused by consumption of chicken or pork liver in infants. However, further studies should consider the effect of these food sources on the risk of hypervitaminosis A.

In addition to hypervitaminosis A, several studies demonstrated that heavy metals especially toxic elements such as lead (Pb), Cadmium (Cd), Mercury (Hg), Arsenic (As) and Nigel (Ni) accumulate in liver and kidney more than in meat from poultry or pork[273-278]. Nookabkaew et al[279] reported that both

chicken or porcine liver contained high amounts of toxic elements especially As, Cd and Pb compared to other complementary foods that were available in Thailand. As all toxic heavy metals are harmful to human health and can result in many serious consequences[280] (e.g., cancers, renal failure, immunological problem, liver disease, cardiovascular problem, hematopoiesis dysfunction and neurodevelopment deterioration), food safety should be considered when encouraging parents to give liver to their children more frequently. However, there is a lack of clinical studies investigating the association between liver consumption and health outcomes in infants or children.

Last but not least, although I did not discuss the effects of delayed cord clamping (DCC) and iron supplementation in this chapter, I considered the associations between these factors and infant iron status. Regarding DCC, there was no official guideline/ national programme in place at the time of my data collection. Although some hospitals (CMU and HPH) reported using this procedure in vaginally born babies, timing varied between health professionals and was not recorded. This procedure was not performed in CTH (hospital in the suburban area). I used study site as a (crude) indicator for DCC and the prevalence of ID/ IDA was not significantly different among the hospitals (Appendix 15). For iron supplementation, the Thai authorities have launched universal anaemia screening by using the haematocrit at 6 and 12 months and providing weekly iron supplementation (12.5 mg elemental iron/ week) for infants aged 6-12 months. However, this programme was not followed by health professionals at the well-baby clinic of CMU, while both HPH and CTH had adopted the programme. As mentioned before, there were no differences in the prevalence of ID/ IDA among infants from different clinics. Furthermore, the iron status of supplemented and unsupplemented infants was not significantly different (Appendix 16). It should be noted that, according to parental report, only around 45% of infants given the supplementation complied with weekly iron supplementation.

In summary, I have shown that infants in a middle-income country like Thailand can achieve normal iron status without high intake of dietary protein during the CF period. Most importantly, food sources should be taken into account. As shown in figure R4.6, I proposed 4 outcome scenarios considering both the amount of protein and iron intake. Scenario A is the worst-case scenario when the infant mainly consumes unfortified cow's milk along with ASFs containing low iron content. This scenario could result in both overweight/ obesity and ID/ IDA, so-called DBM at individual level. Infants in scenario B regularly consume formula along with regular consumption of iron-rich ASFs. In this scenario, infants would usually be iron-sufficient but would be more susceptible to overweight/ obesity if they consume large amounts of high-protein formula. In scenario C, infants are breastfed and consume solely/ predominantly breast milk whilst receiving ASFs that contain low iron content. This scenario can contribute to ID/ IDA and may increase the risk of being wasted or stunted if protein and iron intake are too low. Lastly, scenario D could be considered the 'ideal' scenario where infants are mainly breastfed and regularly consume iron-rich ASFs such as liver in adequate amounts and with adequate frequency during the CF period. This scenario could produce the most preferable outcomes.

In addition to scenario D, to prevent ID/ IDA more effectively, DCC and iron supplementation should be considered as public health policies for a high-risk population as suggested by the WHO[281-2]. However, it should be highlighted that without good compliance and adherence these policies are unlikely to successfully decrease the prevalence of ID/ IDA in infants and young children.

Figure R4.6 Scenarios derived from protein and iron intake during the complementary feeding period observed in my cohort

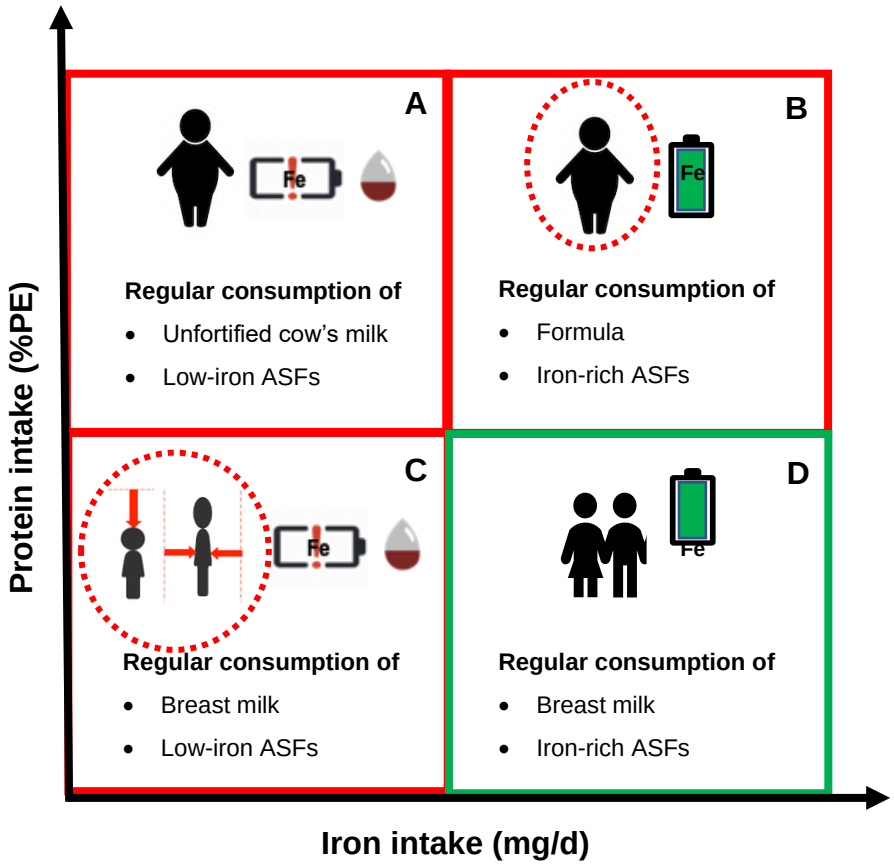


Figure R4.6 demonstrates four outcome scenarios based on protein and iron intake. The best-case scenario is scenario D when infants are breastfed and regularly consume adequate amount of iron-rich, animal source foods (ASFs) during the complementary feeding (CF) period while scenario A is the worst-case scenario when infants early exposure to unfortified cow's milk during the CF period and consume only ASFs with low iron content. The scenario A will result in the double burden of malnutrition at individual level when both overweight/ obesity co-exists with iron deficiency/ iron deficiency anaemia. The red dashed circles represent increasing risk of being over- or undernutrition. For scenario B, infants are at risk of overweight/ obesity if they consume large amount of formula while in scenario C, risk of being stunted/ wasted is higher for infants who consume large amount of breast milk but eat small amount of ASFs.

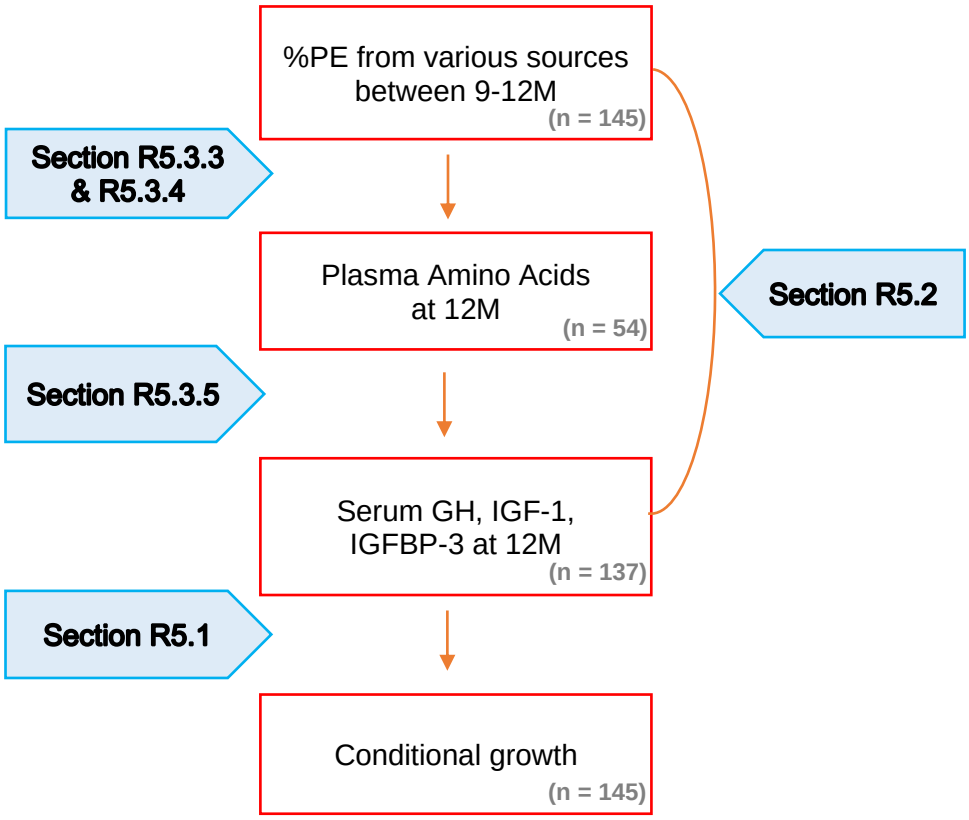
Chapter 7: Results 5

The proposed mechanisms underlying the impact of dietary protein on infant growth

In Chapter 7, Results 3, I demonstrated a significant association between protein intake especially ABP on growth outcomes. In this chapter, I consider potential mechanisms for this association. My hypothesis is that high protein intake, especially from ASFs, may result in altered plasma amino acid concentrations which could promote growth via the GH-IGF axis. In figure R5.1, I describe the sequence of analyses performed to test this hypothesis. I started by establishing whether the GH-IGF axis was associated with conditional growth in my cohort, followed by investigating associations between %PE from 9-12 months, already shown to be strongly associated with weight-related conditional growth in Chapter 7, Results 3, and serum GH, IGF-1 and IGFBP-3. After that, I used data from subgroup analysis, selecting only infants who had consumed milk protein, non-dairy ABP and PBP in the highest and lowest quartiles, to address whether the intake of different protein sources contributes to different growth outcomes through stimulating the GH-IGF axis, mediated by particular amino acids (e.g., branched-chain amino acids – BCAA or essential amino acids – EAA).

Specifically, I hypothesised that a high intake of both dairy and non-dairy ABP from complementary foods may increase some amino acids, in particular BCAA: leucine (Leu), isoleucine (Ile) and valine (Val), as suggested by previous evidence in 6-month-old infants[89], and that this would result in stimulation of IGF-1 and its binding-protein which promotes more rapid infant weight gain. Notably, of 145 infants, 137 (94.5%) provided sufficient blood samples for the analysis of GH, IGF-1 and IGFBP-3.

Figure R5.1 Steps used to test the hypothesis



This figure illustrates how my hypothesis was tested step by step. The first step was to show an impact of growth-promoting factors on conditional growth. In the second step, I investigated whether a high protein intake was associated with concentrations of growth-promoting factors. Finally, the last two steps investigated the link between high protein intake and the GH-IGF axis via a potential mediator, plasma amino acids.

R5.1 Association between growth outcomes and the GH-IGF axis

The role of the GH-IGF axis in growth is well-established, but as a first step in my analyses, I checked that the expected associations between growth-promoting factors and growth outcomes were present in my cohort. As shown in table R5.1, there were significant positive correlations between serum levels of IGF-1 and all conditional growth parameters including WAZ, WLZ, BMIZ and LAZ, while the concentration of IGFBP-3 was also positively associated with all growth parameters except conditional LAZ. None of these growth parameters were correlated with concentrations of GH.

Although growth outcomes were not different in males and females, concentrations of IGF-1 and IGFBP-3 were significantly higher in females (table R5.2). Nevertheless, as shown in figure R5.2, regression lines predicting weight-related conditional growth (i.e., WAZ, WLZ and BMIZ) by concentration of IGF-1 in both sexes were almost the same, reflecting similar regression coefficients, while IGFBP-3 in female infants predicted these growth parameters slightly better than in male infants. According to figure R5.2 (D), the regression lines were almost horizontal in male infants indicating no associations between the concentration of IGF-1 or IGFBP-3 and conditional LAZ, while in female infants, only IGF-1 concentration showed a slightly positive association with conditional LAZ.

Altogether, these results are in line with the current knowledge that IGF-1 and its most common binding protein, IGFBP-3 play a key role in growth. Furthermore, the results also demonstrated sex dimorphism of the GH-IGF axis consistent with evidence suggesting that girls usually gain weight more rapidly than boys in the first 2 years of life[283-284]. These results were a first and fundamental step for my further analyses. If a high intake of ABP contributes to rapid weight gain, it is expected to positively influence the GH-IGF axis as well. In the next section, I will test this assumption.

Table R5.1 Pearson's correlations between conditional growth and serum GH, IGF-1 and IGFBP-3 at 12 months

Conditional	Correlation coefficient (r)		
	GH (ng/mL)	IGF-1 (ng/mL)	IGFBP-3 (µg/mL)
WAZ	- 0.02	**0.36***	**0.33***
WLZ	- 0.04	**0.32***	**0.33***
BMIZ	0.05	**0.24†**	**0.27†**
LAZ	- 0.10	**0.19†**	0.10

***p <0.001; †p < 0.05; GH – growth hormone; IGF-1 – insulin-like growth factor 1; IGFBP-3 – insulin-like growth factor binding protein 3; WAZ – weight-for-age z-score; WLZ – weight-for-length z-score; BMIZ – body mass index z-score; LAZ – length-for-age z-score*

Table R5.2 Comparison of conditional growth and serum GH, IGF-1, IGFBP-3 between male and female infants

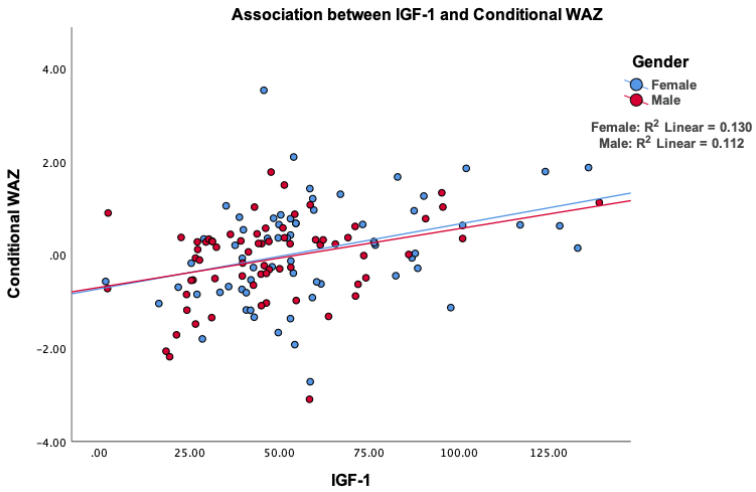
Variables (Means±SD)	**Male**	**Female**	***p****
	(n = 74)	**(n = 71)**	
Conditional WAZ	- 0.11 ± 0.90	0.11 ± 1.08	0.17
Conditional WLZ	- 0.06 ± 0.91	0.06 ± 1.08	0.48
Conditional BMIZ	- 0.04 ± 0.91	0.01 ± 1.07	0.79
Conditional LAZ	- 0.15 ± 1.01	0.16 ± 0.96	0.06
	(n = 71)	**(n = 66)**	
GH (ng/mL)	4.60 ± 7.67	4.32 ± 4.76	0.81
IGF-1 (ng/mL)	**47.95 ± 23.73**	**59.98 ± 28.41**	**0.01**
IGFBP-3 (µg/mL)	**2.50 ± 0.76**	**2.81 ± 0.56**	**0.01**

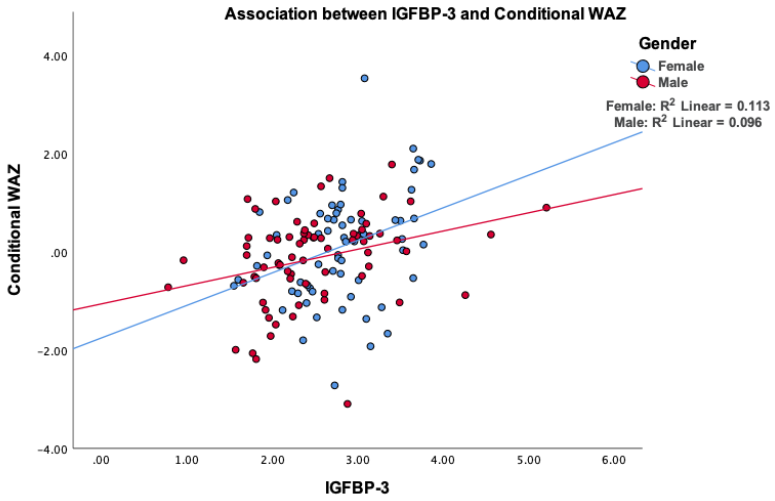
***Student's t-test; GH – growth hormone; IGF-1 – insulin-like growth factor 1; IGFBP-3 – insulin-like growth factor binding protein 3; WAZ – weight-for-age z-score; WLZ – weight-for-length z-score; BMIZ – body mass index z-score; LAZ – length-for-age z-score; SD – standard deviation; p – p-value*

Figure R5.2 Scatter plots showing associations between conditional growth and serum insulin-like growth factor 1 (IGF-1), insulin-like growth factor binding protein 3 (IGFBP-3) separated by infant sex

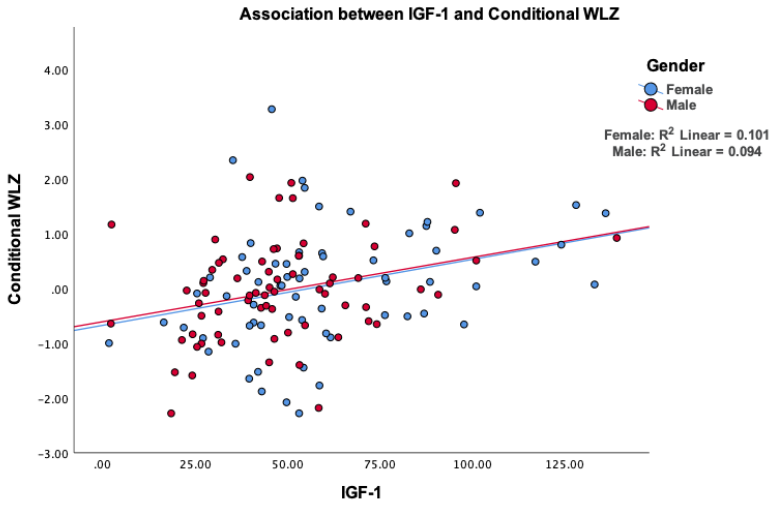
- Predictors: serum IGF-1, IGFBP-3
- Outcomes: Conditional growth

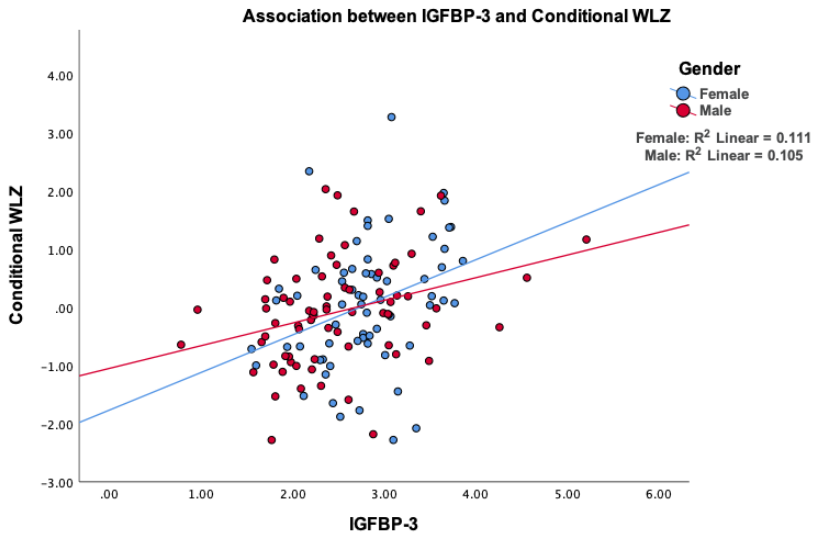
(A) Conditional weight-for-age z-score (WAZ)



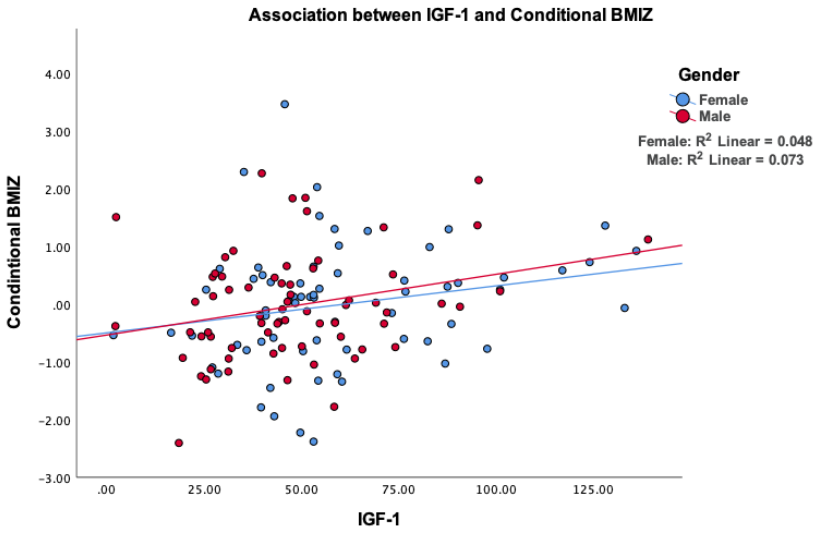


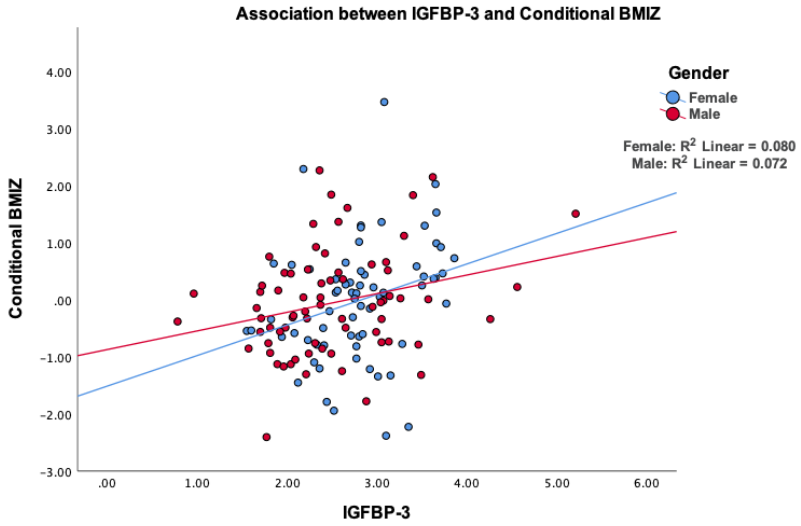
(B) Conditional weight-for-length z-score (WLZ)



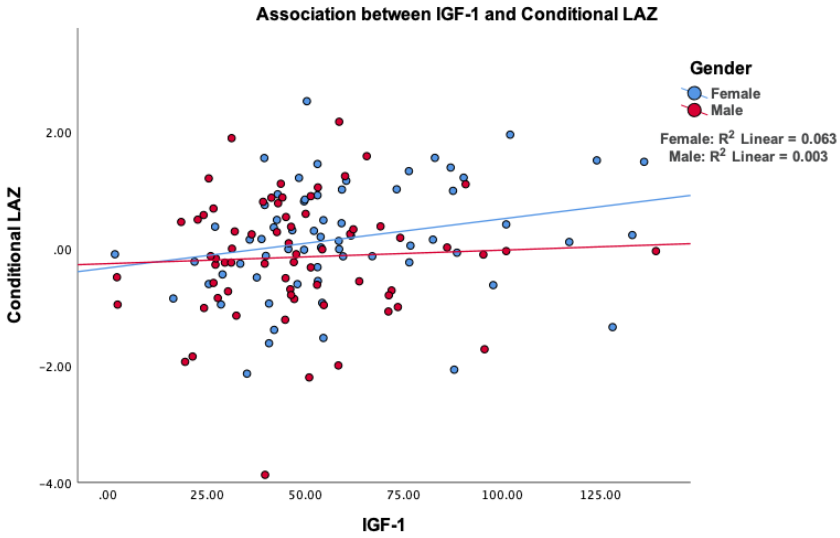


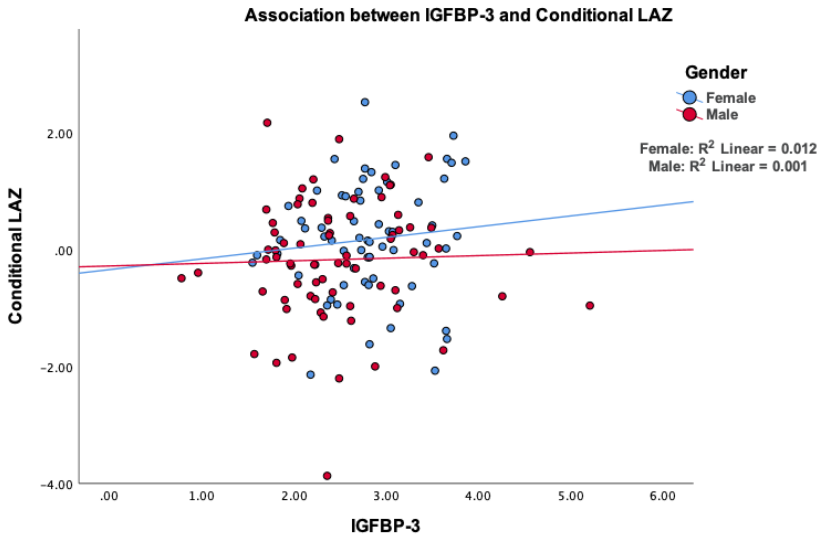
(C) Conditional body mass index z-score (BMIZ)





(C) Conditional length-for-age z-score (LAZ)





R5.2 Association between protein intake and the GH-IGF axis

In the last section, I reported that concentrations of IGF-1 and IGFBP-3 which are growth promoting factors were positively associated with conditional growth, especially weight-related z-scores (i.e., WAZ, WLZ and BMIZ). I next investigated whether IGF-1 and IGFBP-3 could be mediators of the association between high intake of ABP and rapid weight gain found in Chapter 7, Results 3. Table R5.3 shows correlation coefficients (r) between %PE and concentrations of GH, IGF-1 and IGFBP-3. Only %PE from milk/ dairy was significantly correlated with IGF-1 and IGFBP-3. However, when using the variable **"Different %PE from milk"** (as I used previously in Chapter 7, Results 3 and 4, where a positive value means higher %PE from formula and cow's milk while a negative value reflects higher protein from breast milk), the results indicated that this correlation reflects protein from formula and cow's milk rather than protein from breast milk.

When I compared concentrations of IGF-1 and IGFBP-3 among protein intake groups using the same criteria used in Chapter 7, Results 3, there was a trend that infants in the high protein intake group had the highest levels of IGF-1 compared with median and low protein intake group, although there was no statistical significance (p = 0.09). Combined with the correlations shown in

table R5.3, it seemed that without specifying protein source, the %PE in general showed less effect on IGF-1 and IGFBP-3 than milk protein.

I therefore selected %PE from different food sources to examine their associations with IGF and IGFBP-3 using scatter plots. As shown in figure R5.4, again, only %PE from milk/ dairy was positively associated with concentrations of IGF-1 and IGFBP-3 and this effect was mainly attributable to protein from formula and cow's milk (Figure R5.4B). Focusing on figure R5.4 (C) and (D), associations between other protein sources and IGF-1 and IGFBP-3 concentrations tended to be negative but the %PE from non-dairy ABP or PBP explained less than 5% and 2% of IGF-1 and IGFBP-3 concentrations in female and male infants, respectively. More importantly, it should be noted that female infants tended to have higher serum IGF-1 and IGFBP-3 compared to male subjects when they consumed similar amounts of milk/ dairy protein.

Taken together, these results are in agreement with my hypothesis that high %PE was positively associated with concentrations of IGF-1 and IGFBP-3, but strong and consistent associations were only shown for milk/ dairy protein. Additionally, sex dimorphism was observed again in this section.

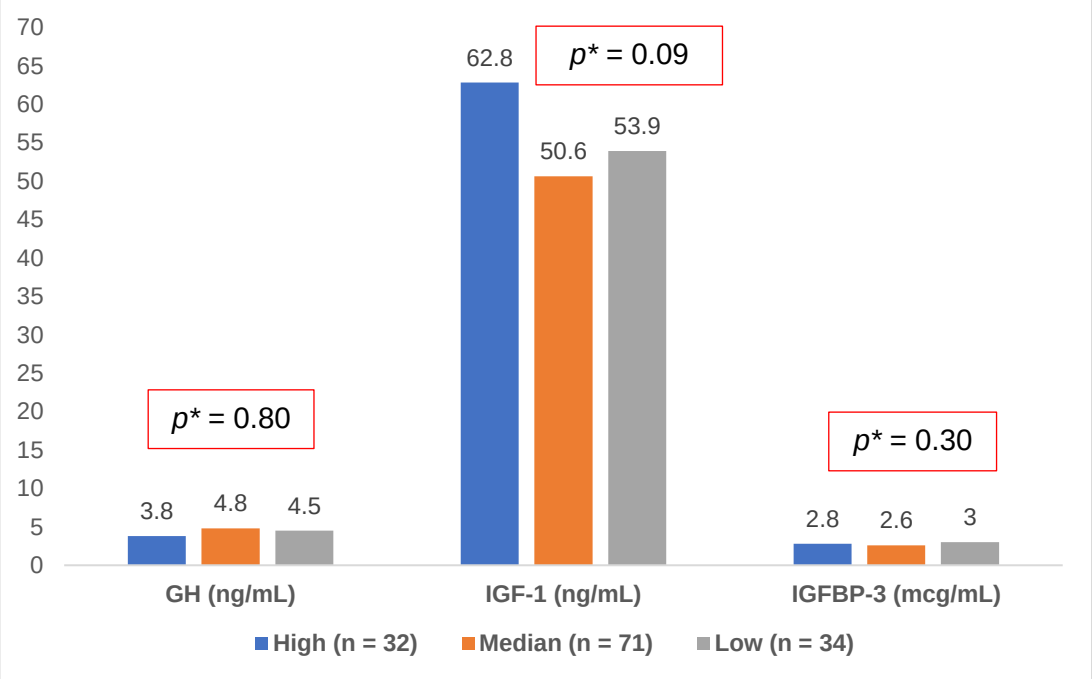
So far, I have shown associations between dietary protein, the GH-IGF axis and growth outcomes. In the next section, I will investigate the role of plasma amino acids as a potential mediator in these associations.

Table R5.3 Pearson's correlation between %PE from 9-12 months and serum GH, IGF-1 and IGFBP-3 at 12 months (n = 137)

Protein intake* (%PE)	Correlation coefficient (r)		
	GH	IGF-1	IGFBP-3
All diets	- 0.12	0.11	0.13
Milk/ Dairy	- 0.10	**0.33†**	**0.21‡**
Different %PE between Non-BM vs BM	- 0.10	**0.38†**	**0.21‡**
Non-dairy ASFs	- 0.02	- 0.16	- 0.04
Plant-based foods	0.02	- 0.11	- 0.09

*****Data were mainly based on the 3-day food records; †p <0.001; ‡p = 0.02; %PE – percentage of energy provided by dietary protein; GH – growth hormone; IGF-1 – insulin-like growth factor 1; IGFBP-3 – insulin-like growth factor binding protein 3; WAZ – weight-for-age z-score; WLZ – weight-for-length z-score; BMIZ – body mass index z-score; LAZ – length-for-age z-score; BM – breast milk; ASFs – animal source foods*

Figure R5.3 Mean serum GH, IGF-1 and IGFBP-3 at 12 months among protein intake groups

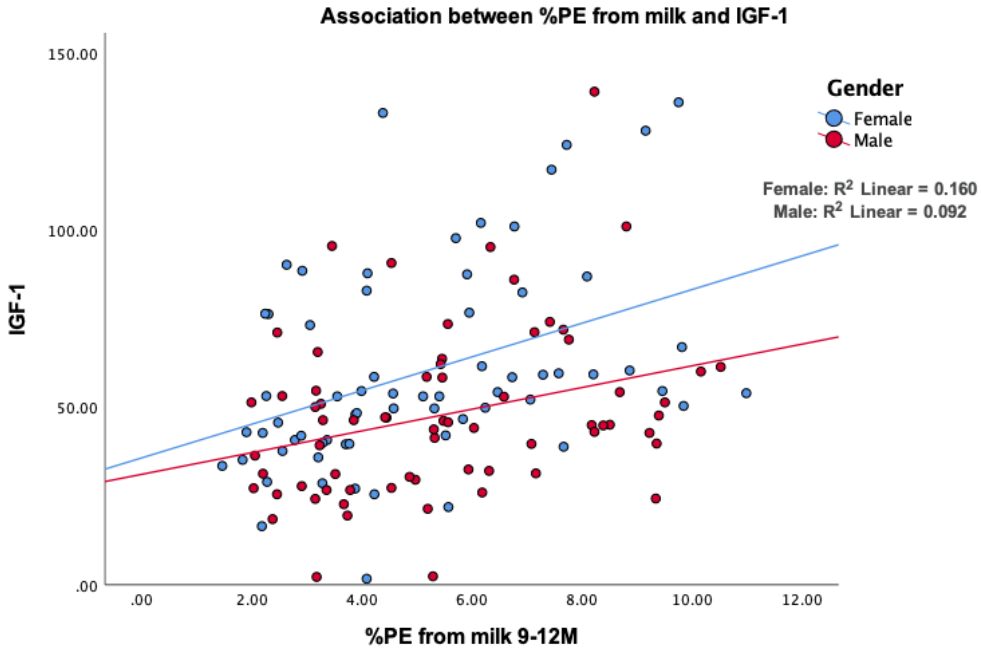


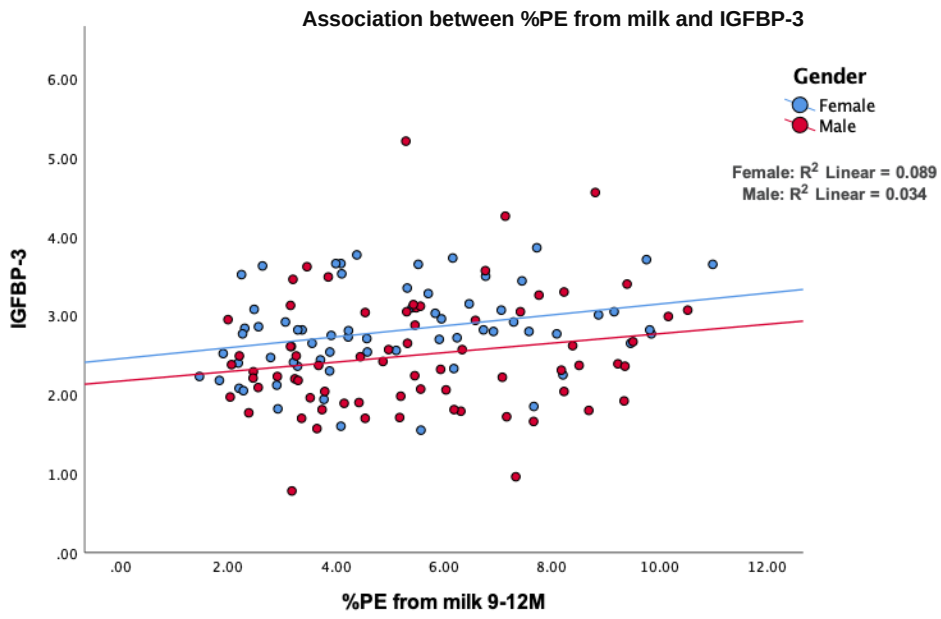
*p-values from One-way ANOVA analyses
GH – growth hormone; IGF-1 – insulin-like growth factor 1; IGFBP-3 – insulin-like growth factor binding protein 3

Figure R5.4 Scatter plots showing simple linear regression between %PE from different sources from 9-12 months and serum insulin-like growth factor 1 (IGF-1), insulin-like growth factor binding protein 3 (IGFBP-3) at 12 months (M) separated by infant sex

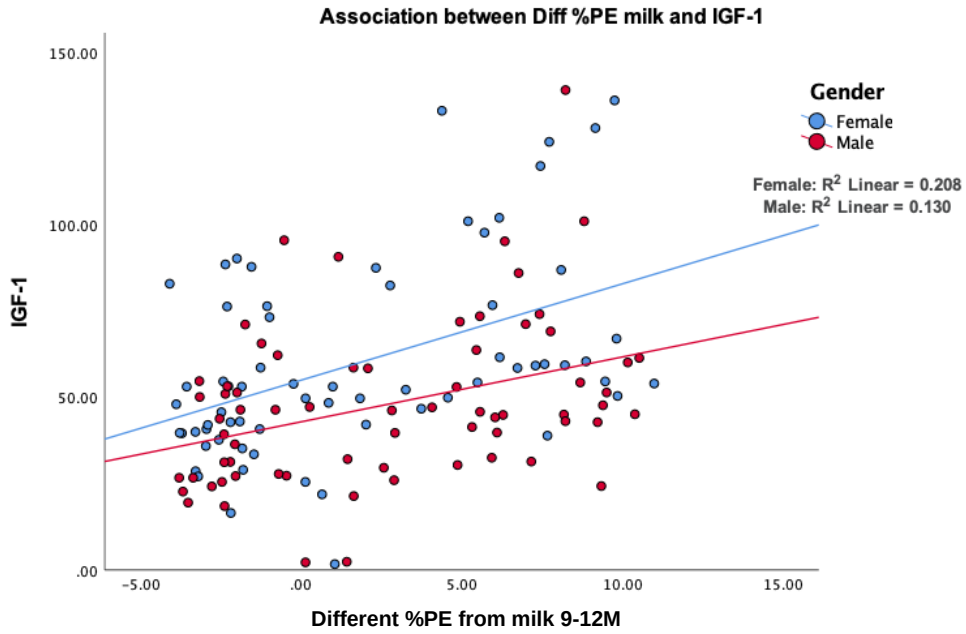
- Predictors: %PE from different sources
- Outcomes: serum IGF-1, IGFBP-3

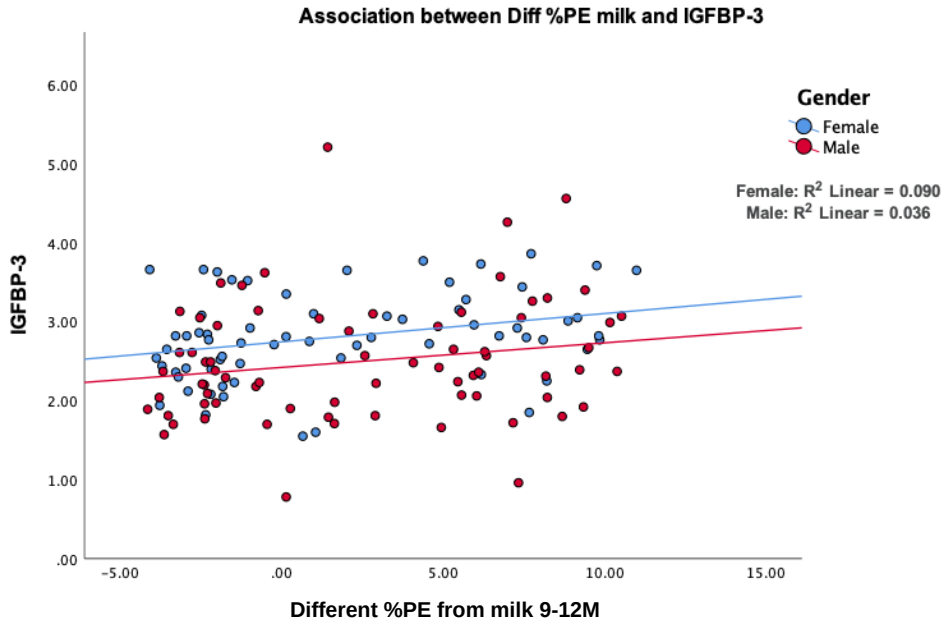
(A) %PE from milk/ dairy



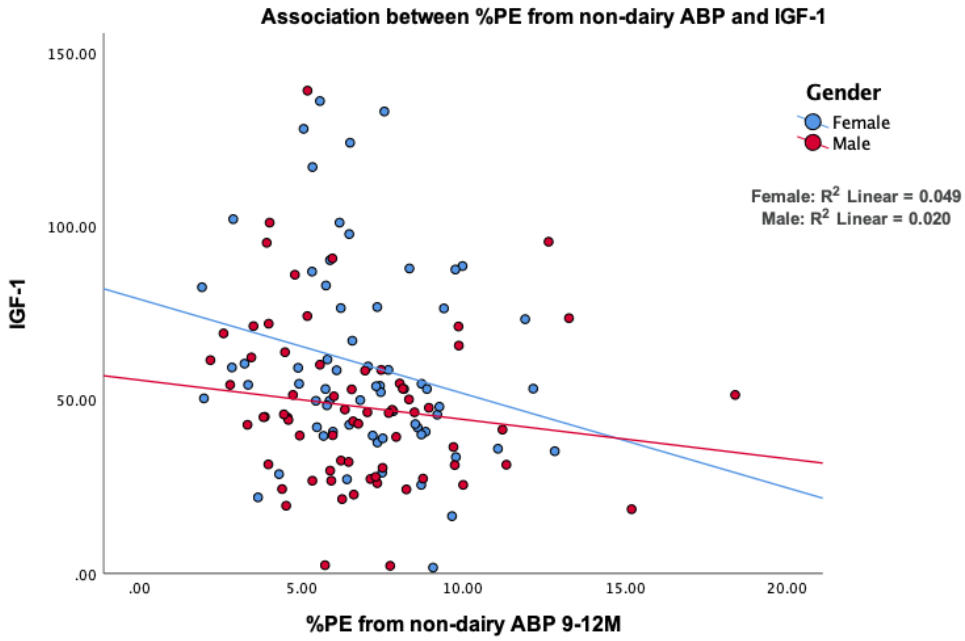


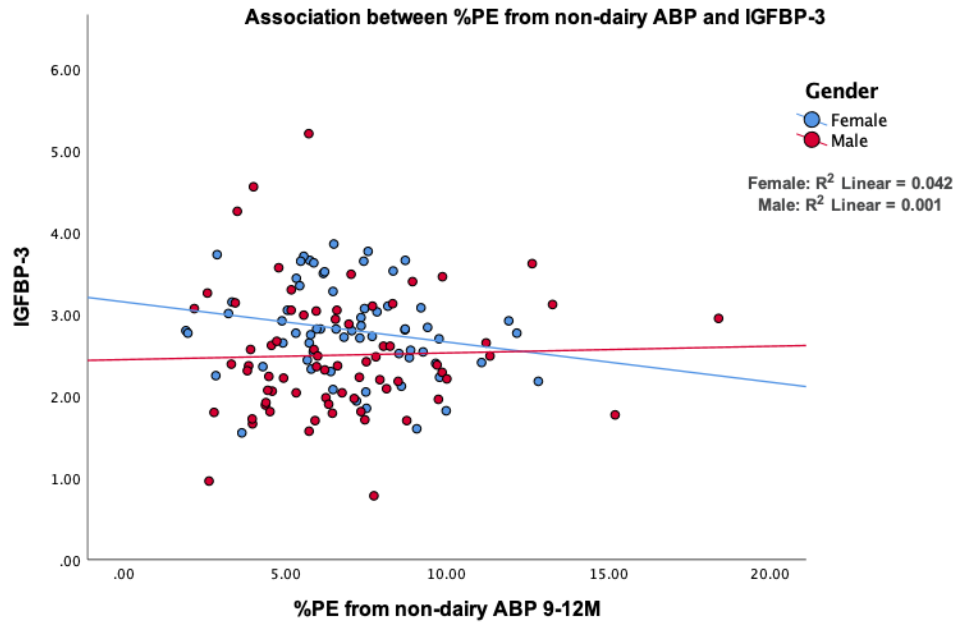
(B) Different %PE from milk



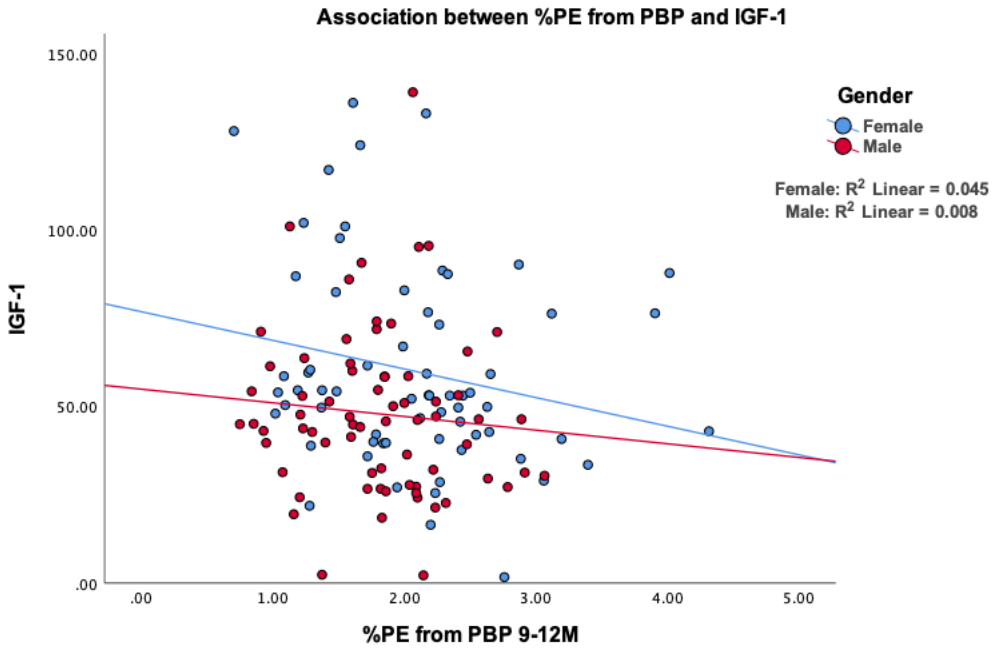


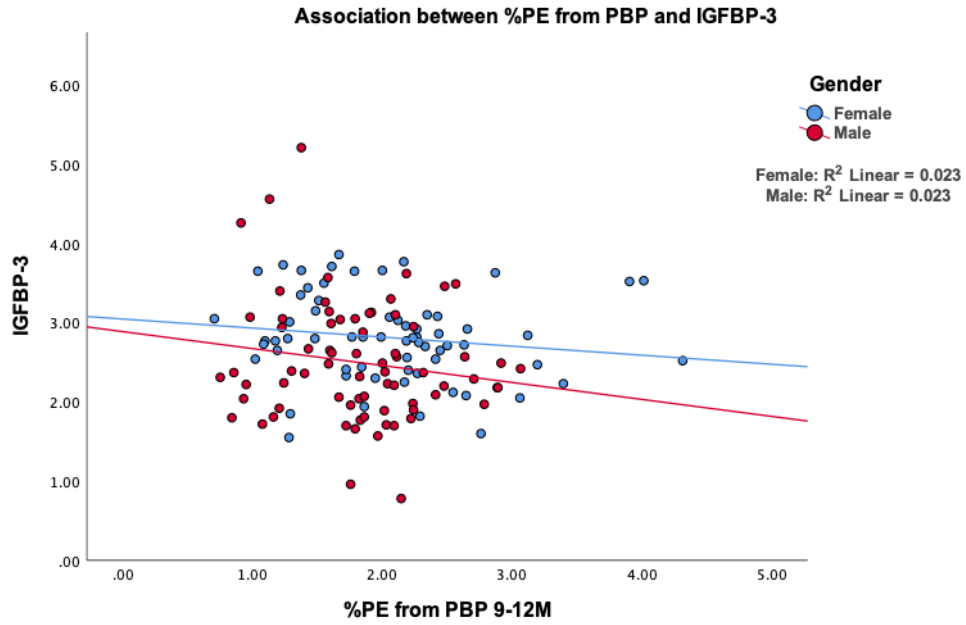
(D) %PE from Non-dairy animal-based protein (ABP)





(D) %PE from Plant-based protein (PBP)





R5.3 Subgroup analysis of plasma amino acids

Before demonstrating the outcomes, I will describe how I selected infants for subgroup analysis of amino acids, which was necessary because of limited time and funding.

R5.3.1 Selection criteria

Two batches of samples were chosen for plasma amino acids analysis. The first batch was analysed to test feasibility and the second batch aimed to increase the sample size to increase the power for the statistical analyses.

- The first batch: plasma amino acids were analysed between October 2019 to February 2020
- The second batch: plasma amino acids were analysed between August and September 2020

Step 1: The inclusion criteria used for sample selection were:

1) Blood samples for plasma amino acids analysis were available.
2) Dietary data were completed and of good quality.
3) Plasma samples were clear (cloudy plasma indicates that the blood sample was drawn shortly after a meal which may lead to misinterpretation)

The number of infants included at this step was **106** (73% of all infants)

Step 2: Classification of protein intake groups

- I used figures of %PE at 12 months of age to classify protein intake groups.
- The %PE from each protein source was divided into three groups as shown in table R5.4.

Step 3: Selection of the first batch for plasma amino acids analysis

I classified the protein intake groups into 6 groups as shown in table R5.5 (A). The high and low protein intake groups for each protein source were infants who consumed protein from that source in the highest and lowest quartile

whilst having intakes of other protein sources in the median quartile. Using this approach, **29 samples** were chosen for the first batch analysis.

Step 4: Selection of the second batch for plasma amino acids analysis
Given the promising results observed in the first batch samples (high consumption of milk protein associated with some EAA) combined with the positive effect of ABP on infant growth, I selected more samples for analysis to increase the number of samples from infants who received high and low protein from dairy and non-dairy ABP. Given the insignificant associations between PBP and growth, I selected samples for the second batch regardless of PBP intake. Finally, **25 more samples** were selected for the second plasma amino acids analysis. In total, **54 samples** were analysed for plasma amino acids.

To avoid unrecognised biases, I checked whether this subgroup is representative of the whole cohort. Table R5.6 demonstrates similar values between groups including key characteristics, protein and non-protein energy intake at 9-12 months, which suggests that the selected group was indeed representative.

Table R5.4 Classification of protein intake groups from each protein source using %PE at age 12 months

%PE from*	**Low intake** (less than P_{25})	**Median intake** (P_{25} - P_{75})	**High intake** (more than P_{75})
Milk/ dairy	< 2.81	2.81 – 7.29	> 7.29
Non-dairy ASFs	< 5.91	5.91 – 10.14	> 10.14
Plant-based foods	< 1.64	1.64 – 2.45	> 2.45

**Data were mainly based on the 3-day food records; %PE – percentage of energy provided by dietary protein; ASFs – animal source foods; P_{25} – 25th percentile; P_{75} – 75th percentile*

Table R5.5 Selection of subgroup for plasma amino acids analysis

(A) First batch (n = 29)

Protein intake groups	%PE from*		
	Milk/ dairy	Non-dairy ASFs	Plant-based foods
High Milk (n = 5)	**High**	Median	Median
Low Milk (n = 5)	**Low**	Median	Median
High Meat (n = 5)	Median	**High**	Median
Low Meat (n = 4)	Median	**Low**	Median
High Plant (n = 5)	Median	Median	**High**
Low Plant (n = 5)	Median	Median	**Low**

**Data were mainly based on the 3-day food records; %PE – percentage of energy provided by dietary protein; ASFs – animal source foods*

(B) Second batch (n = 25)

Protein intake groups	%PE from*		
	Milk/ dairy	Non-dairy ASFs	Plant-based foods
High Milk (n = 4)	**High**	Median/ Low	High/Median/Low
Low Milk (n = 6)	**Low**	Median/ Low	High/Median/Low
High Meat (n = 10)	Median/ Low	**High**	High/Median/Low
Low Meat (n = 5)	Median/ Low	**Low**	High/Median/Low

**Data were mainly based on the 3-day food records; %PE – percentage of energy provided by dietary protein; ASFs – animal source foods*

Table R5.6 Comparison of some characteristics and nutrient intakes between subgroup (n = 54) and all infants (n = 145)

Variables	Subgroup	All infants
Sex		
Female, number (%)	26 (48.1)	71 (49.0)
Type of milk feeding 9-12M, number (%)		
• Breast milk	22 (40.7)	45 (31.0)
• Combination	18 (33.3)	49 (33.8)
• Formula	14 (25.9)	51 (35.2)

Duration of predominant breastfeeding (months), means ± SD	8.4 ± 4.4	9.1 ± 4.2
%PE during aged 9-12M*, means ± SD		
• All diets	14.3 ± 2.5	14.1 ± 2.3
• Milk/ dairy	4.5 ± 1.9	5.3 ± 2.4
• Non-dairy ASFs	7.5 ± 2.5	6.9 ± 2.6
• Plant-based foods	2.2 ± 0.7	2.0 ± 0.7
Non-protein energy during aged 9-12M (kcal/d), means ± SD	564.7 ± 167.0	596.3 ± 154.2

**Data were mainly based on the 3-day food records; SD – standard deviation; ASFs – animal source foods; M – months old*

R5.3.2 Characteristics of infants in each protein intake group

There were 3 main groups called milk, meat and plant groups based on protein sources. In each group, infants were divided into high and low intake of each protein source as described in section R5.3.1. The results from table R5.7 demonstrate that there were no breastfed infants in the high milk group and vice versa for solely formula-fed/ cow's milk-fed infants in the low milk group. The %PE between high and low intake of each protein source were clearly different in particular for the milk and meat groups. Apart from the high milk and high meat groups, overall %PE were still less than 15%.

Table R5.7 Characteristics of selected subjects for plasma amino acids analysis divided each protein intake groups (n = 54)

Characteristics	**Milk group**		**Meat group**		**Plant group**	
	High (n=9)	**Low** (n=11)	**High** (n=15)	**Low** (n=9)	**High** (n=5)	**Low** (n=5)
Sex Female, n (%)	5 (55.6)	4 (36.4)	8 (53.3)	4 (44.4)	3 (60.0)	2 (40.0)
Milk feeding at 12M, n (%)						
- Breast milk	0	11	7 (46.7)	3 (33.3)	1 (20.0)	1 (20.0)
- Combined	2 (22.2)	(100.0)	4 (26.7)	0	2 (40.0)	2 (40.0)
- Formula	4 (44.4)	0	3 (20.0)	5 (55.6)	2 (40.0)	2 (40.0)
- Unfortified CM	3 (33.3)	0	1 (6.7)	1 (11.1)	0	0

Duration of predominant BF (months), means ± SD	7.3±4.8	11.6±1.2	10.1±3.7	7.2±5.2	6.6±5.4	10.0 ±3.1
%PE during aged 9-12M*	**Milk group**		**Meat group**		**Plant group**	
	High	**Low**	**High**	**Low**	**High**	**Low**
All diets	16.0±1.3	12.2±1.3	16.6±1.9	11.5±0.9	14.5±1.8	13.7±2.5
Milk/ Dairy	**7.3±1.5**	**2.9±0.6**	3.7±1.4	4.8±1.5	4.5±1.7	5.2±1.5
Non-dairy ASFs	6.7±1.4	7.2±1.4	**10.2±2.5**	**4.5±1.0**	7.4±1.1	7.1±0.9
Plant-based foods	1.9±0.3	2.2±0.5	2.4±0.6	2.2±1.2	**2.6±0.6**	**1.5±0.4**

**Data were mainly based on the 3-day food records; BF – breastfeeding; SD – standard deviation; ASFs – animal source foods; M – months old*

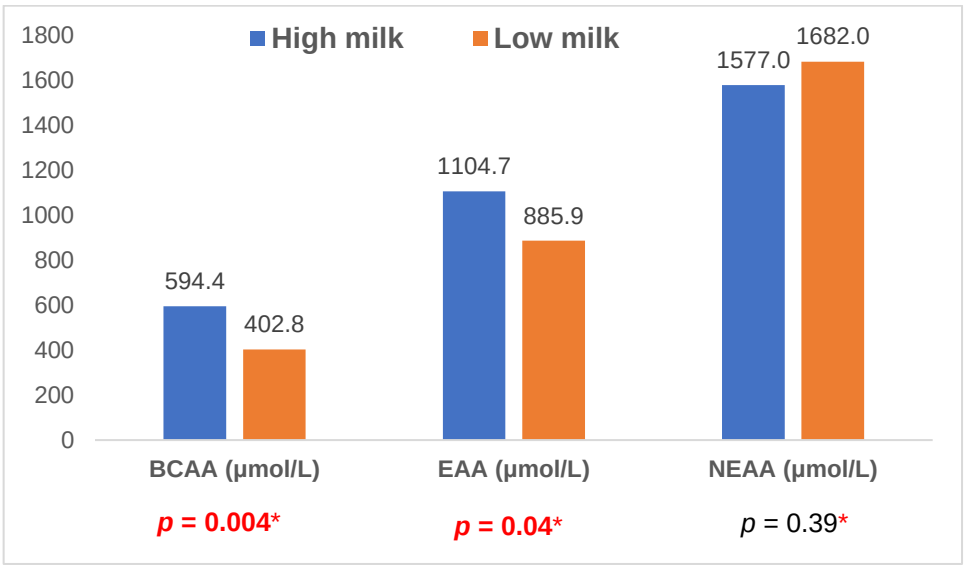
R5.3.3 Comparisons of plasma amino acids among protein intake groups

In this section, plasma concentrations of amino acids have been combined into three variables representing branched-chain amino acids (BCAA: Leu, Ile and Val), essential amino acids (EAA: Leu, Ile, Val, methionine, phenylalanine, threonine, tryptophan, lysine, histidine) and non-essential amino acids (NEAA: alanine, arginine, asparagine, aspartate, cysteine, glutamate, glutamine, glycine, proline, serine and tyrosine).

When comparing these variables between high and low protein intake from each source, significant differences in plasma amino acids were only found for the milk group. As shown in figure R5.5 (A), concentrations of both BCAA and EAA were significantly higher in the high milk group ($p = 0.004$, and 0.04, respectively). Although concentrations of BCAA and EAA of infants in the high meat group were higher than those in the low meat group, differences were not statistically significant (Figure R5.5 B). In contrast to the milk and meat groups, infants who consumed high PBP had lower plasma concentrations of BCAA, EAA and NEAA compared with the low plant group but the different was not statistically significant.

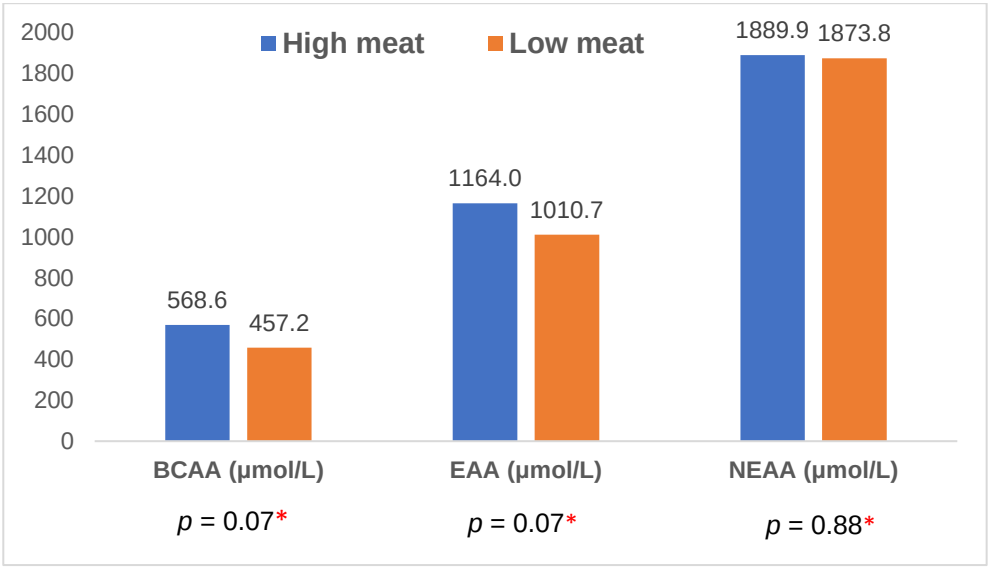
Figure R5.5 Comparison of average plasma concentrations of branched chain amino acids (BCAA), essential amino acids (EAA) and non-essential amino acids (NEAA) among protein intake groups

(A) Milk group (n = 20)



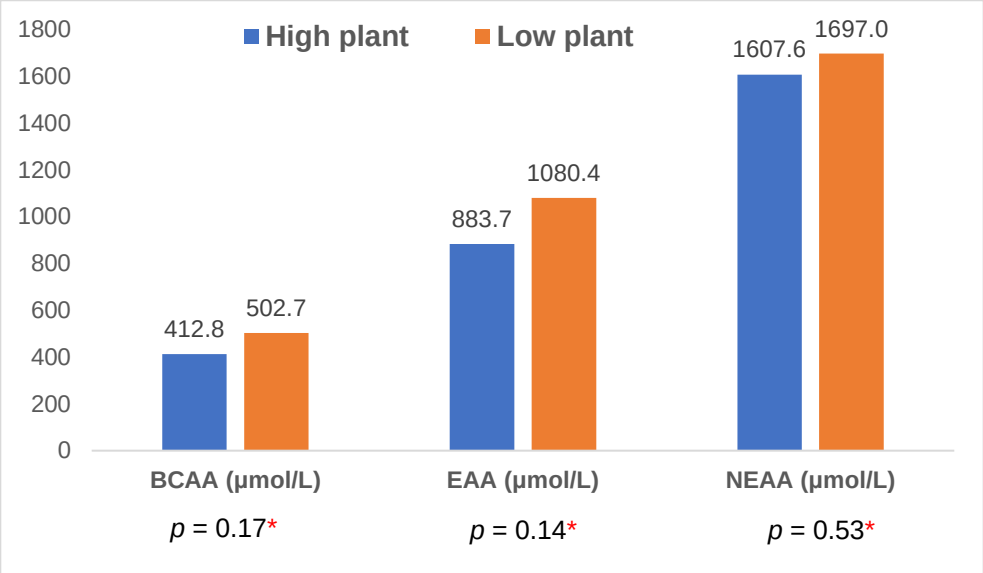
Student's t-test

(B) Meat group (n = 24)



Student's t-test

(C) Plant group (n = 10)



**Student's t-test*

R5.3.4 Association between plasma amino acids and dietary protein

From the previous section, we can see that protein from milk and meat were potentially associated with plasma BCAA and EAA. I next pooled the data from the high and low protein groups for each protein source to investigate dose-response relationships between the %PE from different sources and plasma amino acids.

As shown in table R5.8, only %PE from milk/ dairy was significantly correlated with plasma BCAA (correlation coefficient (r) = 0.35). In addition, I did multivariate regression analyses to examine which specific BCAA could be predicted by %PE. As I had previously demonstrated (Figure R5.5 B) that infants in the high meat group had higher plasma BCAA with a borderline p-value (p = 0.07) compared to the low meat group, I decided to include %PE from non-dairy ASFs as a predictor along with %PE from milk/dairy in the multivariate regression model. As shown in table R5.9, the first model showed that plasma concentration of Leu and Val increased when infants consumed more %PE from milk/ dairy. The results suggested that a 1% increase in %PE from milk/ dairy from 9-12 months was associated with 8.15 and 16.85 μmol/L increase in plasma Leu and Val at 12 months, respectively, regardless of the %PE from non-dairy ASFs.

However, it was not clear whether all types of milk have the same effect on plasma Leu and Val. To address this, I separated infants based on their type of milk feeding from 9-12 months when considering associations between %PE from milk/ dairy and plasma levels of each BCAA. Figure R5.6 (left column) clearly illustrates the contrasting associations between protein from formula and breast milk and plasma BCAA. While increasing %PE from formula/ cow's milk showed positive associations with all BCAA, especially Val and Leu, (considering steep slopes and higher adjusted R^2), increasing %PE from breast milk showed the opposite. Interestingly, when focusing on associations between %PE from non-dairy ASFs and plasma levels of all BCAA (right column of figure R5.6), all scatter plots and lines were in the same direction suggesting increasing consumption of %PE from non-dairy ASFs was positively associated with plasma Leu, Ile and Val whether infants were fed with breast milk or formula. However, it should be noted that formula-fed infants had the highest plasma levels of all BCAA regardless of how much protein from non-dairy ASFs had been consumed, followed by those fed a combination of milk types, whilst plasma levels of all BCAA were lowest in breastfed infants. Therefore, it was very clear that protein from formula and cow's milk had a significant and great impact on plasma BCAA.

Next, I test the hypothesis that plasma amino acids are associated with the GH-IGF axis.

Table R5.8 Pearson's correlation between %PE from 9-12 months and plasma concentration of BCAA, EAA, NEAA (n = 54)

Plasma levels (μmol/L)	**Correlation coefficient (p-value)**		
	%PE Milk/ dairy*	%PE Non-dairy ASFs*	%PE Plant-based foods*
BCAA	**0.35 (0.01)**	0.04 (0.78)	0.01 (0.93)
EAA	0.17 (0.22)	0.09 (0.54)	0.14 (0.31)
NEAA	- 0.22 (0.11)	0.11 (0.44)	0.25 (0.07)

**Data were mainly based on the 3-day food records; %PE – percentage of energy provided by dietary protein; ASFs – animal source foods; BCAA – branched chain amino acids; EAA – essential amino acids; NEAA – non-essential amino acids*

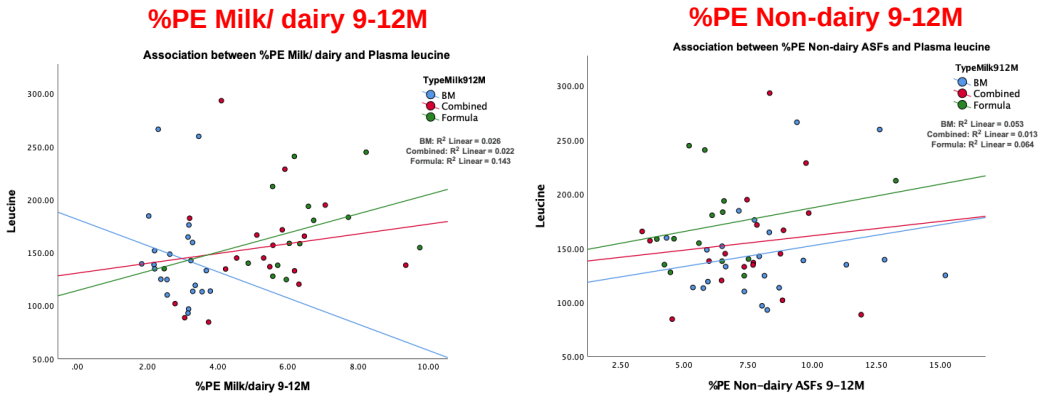
Table R5.9 Multivariate regression analysis predicting plasma Leu, Ile and Val by %PE from milk/ dairy and non-dairy ASFs (n = 54)

Predictors	Outcomes (μmol/L)		
	Plasma Leu Adj R^2 0.077	**Plasma Ile** Adj R^2 0.028	**Plasma Val** Adj R^2 0.115
• %PE Milk/dairy*			
ß	**8.15**	3.56	**16.85**
95% CI	**1.34,14.95**	- 1.40,8.53	**4.88, 28.82**
• %PE Non-dairy ASFs*			
ß	4.47	3.21	9.19
95% CI	- 0.77, 9.70	- 0.61,7.03	- 0.02, 18.39

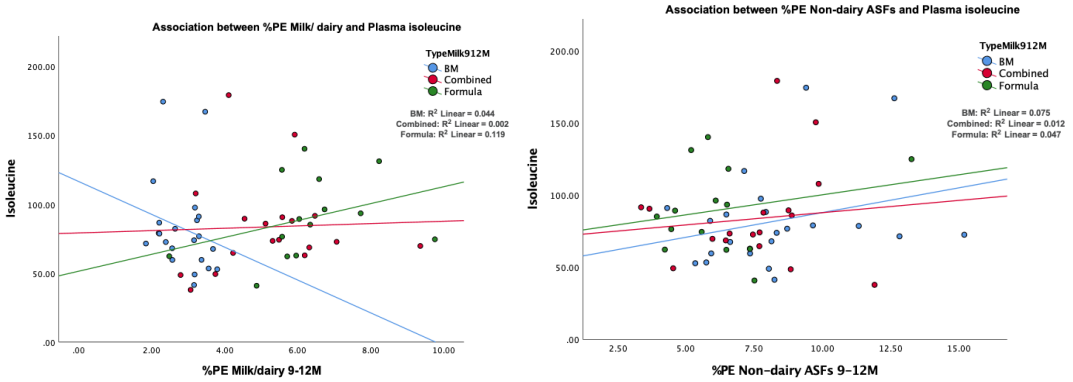
**Data were mainly based on the 3-day food records; %PE – percentage of energy provided by dietary protein; ASFs – animal source foods; ß – regression coefficient; CI – confidence intervals; Adj R^2 – adjusted coefficient of determination; Leu – leucine; Ile – isoleucine; Val – valine*

Figure R5.6 Scatter plots demonstrating association between %PE from animal source foods (ASFs) including both milk and non-dairy ASFs and all branched chain amino acids separated by type of milk feeding during aged 9-12 months (M) (n = 54)

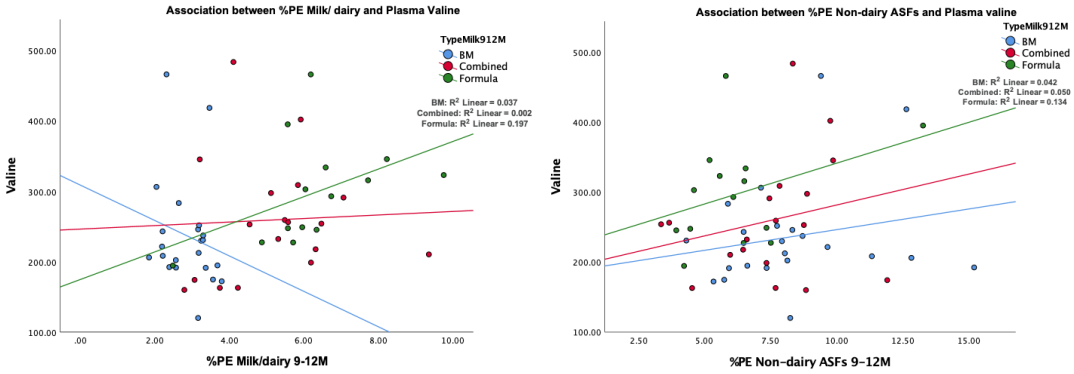
(A) **Plasma Leucine**



(B) **Plasma Isoleucine**



(C) **Plasma Valine**



These scatter plots demonstrate associations between %PE from ASFs from 9-12 months and plasma Leu, Ile, and Val separated by type of milk intake during the same period. Protein intake in the left column is %PE from milk/ dairy while the right column is %PE from non-dairy ASFs. The blue dots and lines represent solely breastfed infants; red dots and lines represent infants who consumed both breast milk and formula; green dots and lines represent solely formula-fed infants.

R5.3.5 Association between plasma amino acids and the GH-IGF axis

So far, in section R5.3, I have demonstrated that high consumption of protein from formula and cow's milk was strongly associated with plasma BCAA, especially Leu and Val, with a dose-response effect on plasma Leu and Val. Therefore, in this last section of hypothesis testing, to confirm the plausibility of my proposed mechanism, I investigated associations between plasma BCAA and the GH-IGF axis.

As shown in table R5.10, plasma BCAA were highly correlated with serum IGF-1 and IGFBP-3 (r = 0.51 and 0.38, respectively). Plasma EAA also showed significant associations with IGF-1 and IGFBP-3 while there no correlations between plasma amino acids and GH or plasma NEAA and all

growth-promoting factors. When considering results from multivariate regression analysis predicting changes in serum GH, IGF-1 and IGFBP-3 by plasma Leu, Ile and Val, only plasma Val was significantly associated with IGF-1 and IGFBP-3 after adjusting for infant sex (Table R5.11). The dose-response effect of plasma Val on IGF-1 and IGFBP-3 suggests that a 1 μmol/L increase in plasma Val was associated with a 0.29 ng/ mL increase in serum IGF-1 and a 0.01 μg/ mL (or 10 ng/ mL) increase in serum IGFBP-3, regardless of infant sex.

Plasma Leu showed the same regression coefficient, but no statistical significance was observed. However, when considering the effect of all BCAA on this model through adjusted R^2, the figures indicated that all BCAA and infant sex can explain 32.8% and 27.7% of the variability in serum IGF-1 and IGFBP-3, respectively. These outcomes are clearly illustrated again by figure R5.7 where all plasma BCAA were similarly associated with serum IGF-1 and IGFBP-3, but plasma BCAA have a greater impact on serum IGF-1 than IGFBP-3 considering the slopes of the lines.

Taken together, the results in section R5.3 clearly and consistently indicate that higher consumption of protein from formula and cow's milk was strongly associated with increasing plasma BCAA, especially Leu and Val. However, only plasma Val has shown a dose-response effect on serum IGF-1 and IGFBP-3 regardless of infant sex. Combining these outcomes with the results from previous sections, we can now see the link between variables more explicitly.

Table R5.10 Pearson's correlations between plasma BCAA, EAA, NEAA and serum GH, IGF-1, IGFBP-3 (n = 54)

Plasma (μmol/L)	**Correlation coefficient (p-value)**		
	GH (ng/ mL)	IGF-1 (ng/ mL)	IGFBP-3 (μg/ mL)
BCAA	- 0.11 (0.45)	**0.51 (<0.001)**	**0.38 (0.01)**
EAA	- 0.13 (0.36)	**0.40 (0.003)**	**0.30 (0.03)**
NEAA	- 0.19 (0.16)	- 0.06 (0.68)	0.07 (0.64)

BCAA – branched chain amino acids; EAA – essential amino acids; NEAA – non-essential amino acids; GH – growth hormone; IGF-1 – insulin-like growth factor 1; IGFBP-3 – insulin-like growth factor binding protein 3

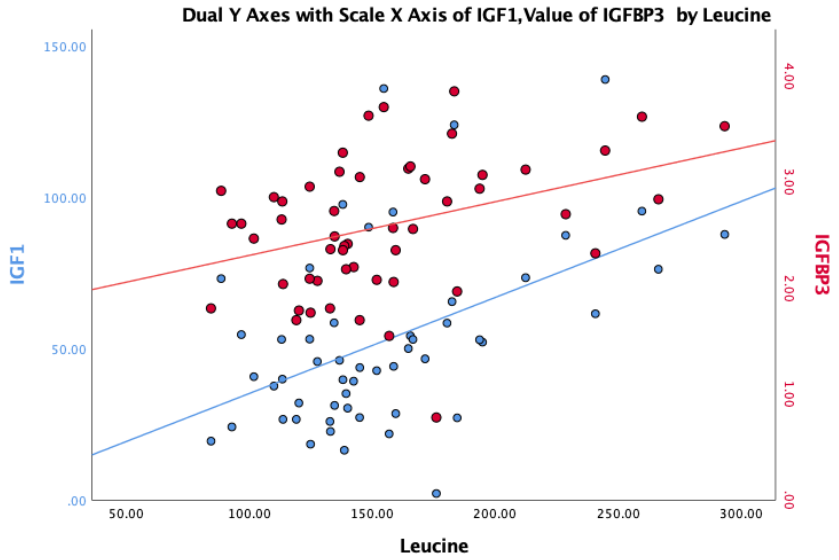
Table R5.11 Multivariate regression analysis predicting serum IGF-1 and IGFBP-3 by plasma Leu, Ile and Val (n = 54)

Predictors (μmol/L)	**Outcomes***			
	IGF-1 (ng/ mL) Adj R^2 0.328		**IGFBP-3** (μg/ mL) Adj R^2 0.277	
	ß	95% CI	ß	95% CI
Plasma Leu	0.29	- 0.24, 0.82	0.01	- 0.01, 0.02
Plasma Ile	- 0.70	- 1.38, - 0.01	- 0.02	- 0.03, 0.01
Plasma Val	**0.29**	**0.04, 0.54**	**0.01**	**0.001, 0.01**

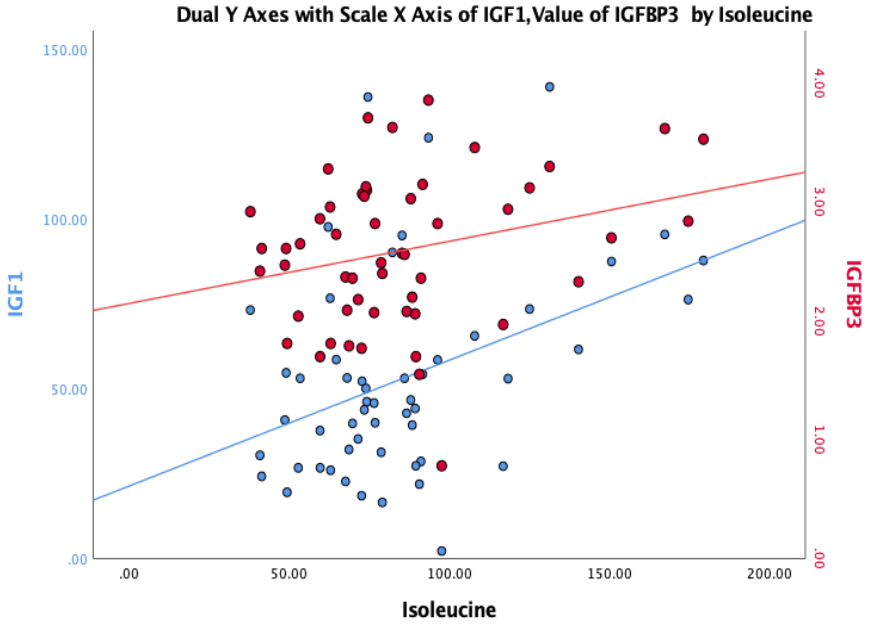
***Adjusted for sex of infants*
GH – growth hormone; IGF-1 – insulin-like growth factor 1; IGFBP-3 – insulin-like growth factor binding protein 3; Adj R2 – adjusted coefficient of determination; ß – regression coefficient; CI – confident intervals; Leu – leucine; Ile – isoleucine; Val - valine

Figure R5.7 Association between each plasma branched-chain amino acids and the GH-IGF axis

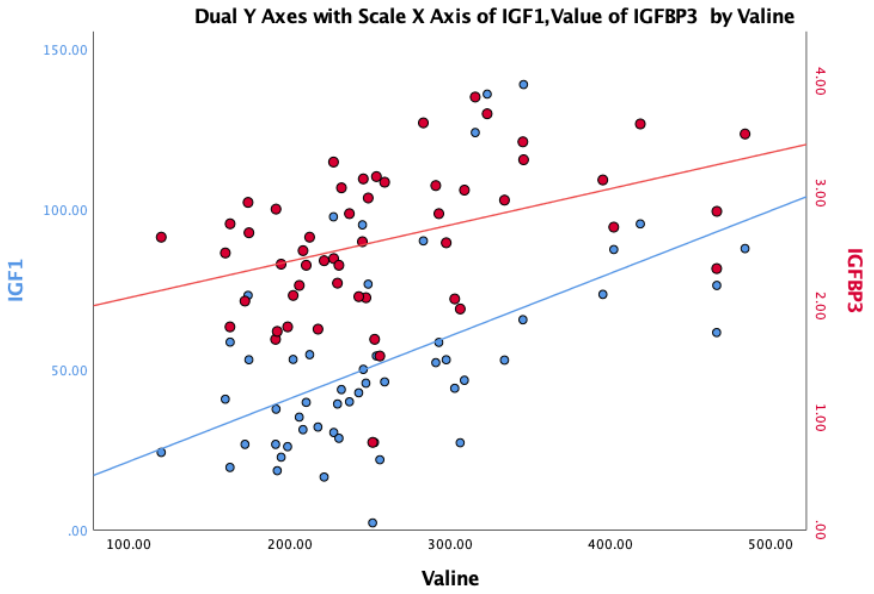
(A) Plasma leucine predicting concentration of serum insulin-like growth factor 1 (IGF-1) and insulin-like growth factor binding protein 3 (IGFBP-3)



(B) Plasma isoleucine predicting concentration of IGF-1 and IGFBP-3



(C) Plasma valine predicting concentration of IGF-1 and IGFBP-3



These scatter plots demonstrate associations between %PE from animal source foods (ASFs) from 9-12 months and plasma leucine, isoleucine, and valine separated by type of milk intake during the same period. Protein intake in the left column is %PE from milk/ dairy while the right column is %PE from non-dairy ASFs. The blue dots and lines represent solely breastfed infants; red dots and lines represent infants who consumed both breast milk and formula; green dots and lines represent solely formula-fed infants.

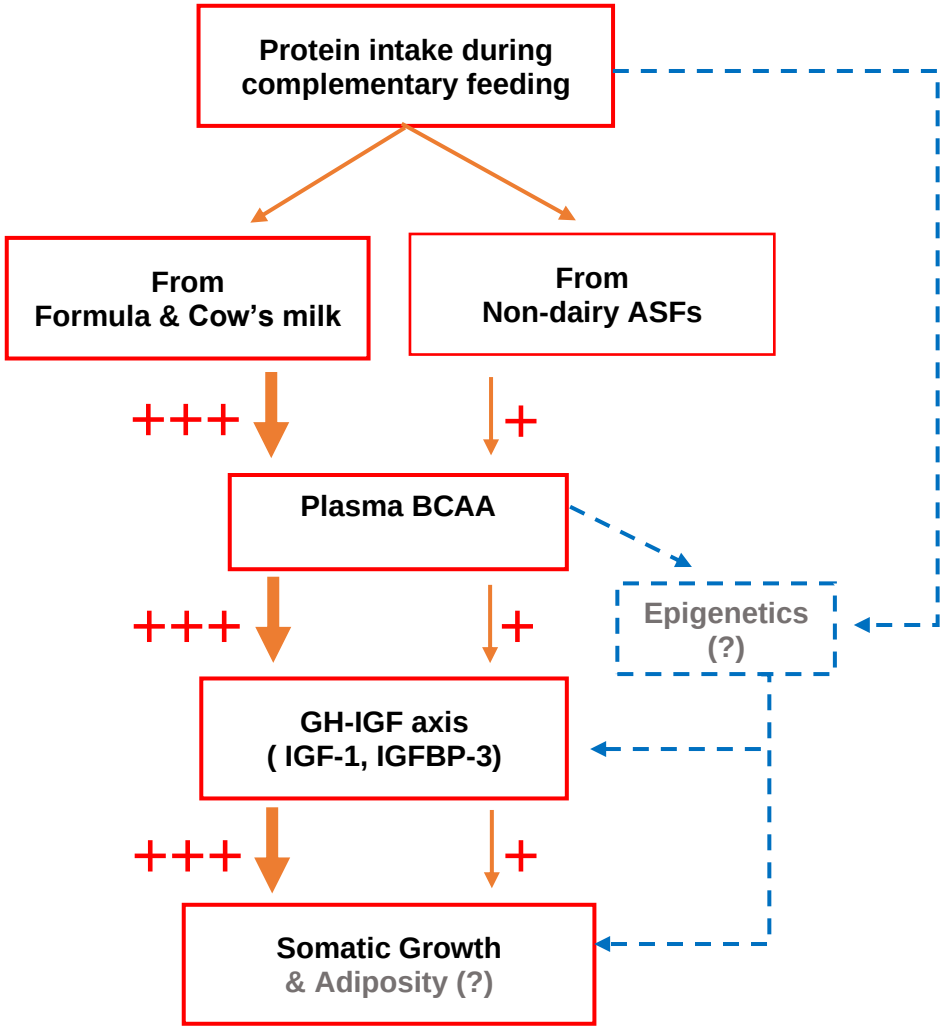
R5.4 Proposed underlying mechanism: how dietary protein is linked to growth outcomes via plasma amino acids and the GH-IGF axis

In this section, all key findings have been summarised in a final figure demonstrating the proposed mechanism based on my results.

The findings were very consistent, even though the analyses used a range of dependent/ independent variables and analytic methods, which increases my confidence in the proposed mechanism. As shown in figure R5.8, I postulated that high consumption of protein from formula and cow's milk during the CF period plays a major role in promoting rapid weight gain through increasing plasma BCAA concentrations which can stimulate the GH-IGF axis, resulting in greater secretion of IGF-1 and IGFBP-3 and thereby promoting somatic growth. Protein from non-dairy ASFs may influence growth via this same mechanism but with less potency. However, without the results from body composition and microRNAs analysis, I cannot confirm an impact of high

intake of ABP specifically on adiposity, or the potential role of epigenetic mechanisms. Unfortunately, these analyses were delayed because of the COVID-19 pandemic and are not available at the time of writing.

Figure R5.8 The proposed mechanism underlying the impact of high protein intake during the complementary feeding period on growth



This figure is the proposed mechanism underlying the impact of high protein intake during the complementary feeding on infant growth. Based on results in this chapter, high protein intake from formula and cow's milk is positively associated with plasma branched chain amino acids (BCAA) which could stimulate the growth hormone (GH) - insulin-like growth factor (IGF) axis and result in excessive secretion of IGF-1 and IGFBP-3 into blood circulation promoting somatic growth and causing rapid weight gain if protein intake is too high. Protein from non-dairy animal source foods (ASFs) might affect growth via the same mechanism but with less potency.

R5.5 Summary of key results and discussion

Key results

- Serum concentrations of IGF-1 and IGFBP-3 were positively correlated with high intake of protein from formula/ cow's milk and weight-related z-scores.
- There was evidence of sex dimorphism in the GH-IGF axis as female infants had significantly higher levels of IGF-1 and IGFBP-3 than male infants, although growth outcomes between sexes were not significantly different.
- High intake of protein from formula/ cow's milk was significantly associated with increasing plasma BCAA.
- Plasma BCAA were highly correlated with blood concentration of IGF-1 and IGFBP-3 but only plasma Val showed a dose response effect on both IGF-1 and IGFBP-3.
- Although statistical significance was not observed, increasing intake of breast milk showed an inverse association with all BCAA while higher consumption of %PE from non-dairy ASFs tended to be associated with higher levels of each BCAA.

Evidence demonstrating associations between dietary protein from different food sources and the GH-IGF axis/ Metabolomics

The results from this chapter are consistent with a large scale RCT conducted in 5 European countries, the so-called European Childhood Obesity Project (CHOP) that demonstrated an important role of increasing plasma BCAA as a link between high protein intake during early life and rapid weight gain or risk of being overweight/ obese in later life[89], via the GH-IGF-1 axis. This RCT randomly allocated infants into 2 intervention groups receiving higher or lower protein formula from age 3 months until 12 months without controlling for complementary feeding and followed the growth of these infants up to 6 years of age. At 2 and 6 years of age, infants in the higher protein group had

significantly higher BMIZ compared to the lower protein group and breastfed infants as reference group without any significant differences for linear growth[59, 60].Combining clinical outcomes with results from metabolomic analyses[285] and other laboratory test[89] (e.g., serum amino acids, total and free IGF-1, IGFBP-2, IGFBP-3, blood glucose and urea, urinary creatinine and c-peptides), they finally proposed the “Early protein hypothesis” built upon these key findings. The hypothesis postulates that high protein intake in excess of metabolic demands during early childhood may increase insulin-releasing amino acids, especially BCAA, whether in blood or tissue, resulting in secretion of insulin and IGF-1 that could trigger weight gain and induce adipogenesis[233]. However, as blood samples were obtained once when subjects were around 6 months of age, this hypothesis can only refer to protein from formula and not dietary protein in general, because most infants in the RCT were unlikely to have been consuming a variety of dietary proteins, or even to have started complementary foods at that time[286].

Evidence explaining the association between high protein intake during the CF period and rapid growth, or risk of overweight/ obesity remains limited and inconclusive. In 2004, Hoppe et al[206] reported that protein intake at 9 months of age was strongly associated with body weight and length but not body fatness of children at aged 10 years. However, they did not find an association between protein intake at 9 months and serum IGF-1 at 10 years old. Only a positive correlation between serum IGF-1 and body weight at 10 years old ($r = 0.31$, $p < 0.05$) was observed. This cohort study did not analyse serum IGF-1 at 9 months.

Following the aforementioned large RCT, there was a single-centre RCT called the Early Protein and Obesity in Childhood study (EPOCH)[287] comparing the effect of high protein and low protein formula on the IGF-1 axis beyond 6 months of age. This RCT showed that infants who were randomly assigned to exclusively consume either high protein formula or low protein formula for the first 4 months of life had higher serum IGF-1 and IGFBP-3 at 4 and 9 months compared to a reference group, breastfed infants, but no differences were observed between the two formula groups. The high protein

group had significantly higher BMI than breastfed infants at 4 and 6 months, although no differences were seen after 9 months of age. There were no differences in BMI between the high and low protein groups from birth until 60 months of age. It should be noted that after 9 months, the protein intake did not differ between high and low protein groups, although no data on complementary feeding were reported. Although formula-fed infants had higher IGF-1 and IGFBP-3 at 9 months, the effects on BMI were not consistent with the previous large RCT. This might reflect the fact that the sample size calculation was based on detecting differences in IGF-1 concentration between the two formula groups, so the trial may not have had statistical power to detect differences in BMI. Another RCT examining the effects of %PE from formula and cow's milk on growth and serum IGF-1 between 9 and 12 months also found no significant differences between groups, although a higher %PE was positively associated with serum IGF-1 after adjusting for sex and breastfeeding duration[288].

Recently, a short report suggested an association between protein intake and serum IGF-1 during the CF period. Tincu et al[289] conducted a cross-sectional study investigating whether high protein intake is associated with body size, serum IGF-1, urea and glucose of Romanian infants aged 12 months (n = 75). The prevalence of overweight/ obesity was 14.2% for 12-month-old infants which is higher than the prevalence reported from my study (less than 1%). They also found that protein intake was positively correlated with body size (both weight and length) as well as serum urea and IGF-1. Additionally, infants who consumed protein ≥ 2.5 g/kg/day had higher serum IGF-1, urea and glucose than infants who consumed less protein (all p-values <0.05) and moreover, at 12 months of age, 70.5% of the variance of serum IGF-1 could be explained by protein intake. Nevertheless, very limited information is available from this short report, and this study did not investigate the impact of different protein sources.

Interestingly, there is a relevant recent study from the United States investigating the effect of different sources of ABP (i.e., dairy products and meats) on growth and metabolomic outcomes during the CF period. Tang et

al[239] reported that formula-fed infants randomly allocated to either a dairy or meat group (n = 32 each group) and receiving similar %PE from 5 to 12 months (10%PE and 15%PE at 5 and 12 months old, respectively) showed distinctive growth patterns. While formula-fed infants in the dairy group, who mainly consumed products such as cheeses and yoghurt, had greater change in WLZ over time (change of WLZ during 5-12M: +0.76 SDS, p <0.001 in the dairy group vs +0.30, p = 0.55 in meat group), the meat group had a significantly greater increase in length. These specific patterns continued until aged 24 months in a follow-up study[290], even though the intervention ended at 12 months. However, there were no significant differences in serum IGF-1, IGFBP-3 and metabolomic profiles between the two groups[239, 291]. Although a differential effect of dairy and non-dairy ABP on the GH-IGF-1 axis and metabolomic profiles was not shown, this study demonstrated that serum IGF-1 and IGFBP-3 increased significantly over time from 5 to 12 months of age in line with increasing %PE[239]. Furthermore, increasing protein intake by provision of ABP was positively associated with blood concentrations of EAA and short chain acylcarnitine such as acylcarnitine C4, C5 and C5:1, which are derivatives from BCAA metabolism. These findings were similar to outcomes from the CHOP study mentioned earlier. A principal components analysis showed a positive relationship between all BCAA and phenylalanine (the second component) and change in LAZ from 5 to 12 months which was stronger in the meat group than the dairy group. There were no associations found between this component and change in WAZ or WLZ. Disappointingly, this study did not report on associations between metabolomic profiles and growth-promoting factors, and breastfed infants were not included as a reference group.

In contrast to the aforementioned studies, my study only focused on the CF period and considered all sources of dietary protein including different sources of milk. The comprehensive analyses from my study showed similar findings to the CHOP study, indicating the impact of high protein intake on rapid weight gain via the GH-IGF-1 axis stimulated by increasing plasma EAA, especially BCAA. Nevertheless, I have extended the findings of that trial by providing evidence that the same mechanism postulated by Koletzko et al[233] continues

into late infancy. More importantly, my study also highlights that only high protein intake from ABP, especially dairy protein, is associated with rapid weight gain during infancy through this proposed mechanism, while consumption of PBP seems to have no effect at all.

As expected, protein from formula and cow's milk clearly affects growth via the GH-IGF axis[292-294]. Apart from infancy, several studies conducted in children also found that high consumption of cow's milk is associated with increasing levels of serum IGF-1 and its binding proteins[76, 295-297]. Increasing consumption of cow's milk can enhance final adult height[298-299] and improve linear growth in children, especially for those who are at risk of undernutrition[300-302], and this is explained by the many IGF-1 receptors in growth plate of long bone[303]. It is therefore unclear why high protein intake from formula and cow's milk is more likely to promote weight gain and increase adiposity[58, 243, 304] than to promote linear growth in well-nourished infants. There are several possible explanations based on current evidence.

1) One possible reason suggested by the CHOP study is that high protein consumption not only stimulated IGF-1 secretion but may also have resulted in increased insulin concentrations. This was supported by the observation that the highest levels of C-peptide: creatinine ratio (an end product of insulin metabolism) were found in the urine of infants who received high protein formula. As insulin plays a major role in lipogenesis, it could potentially result in increasing weight and fat mass rather than length gain.

2) Because of structural homology between IGF-1 and insulin and their receptors[305], an excessive secretion of IGF-1 induced by high milk protein intake could also stimulate the insulin receptor and might contribute to lipogenesis.

3) IGF-1 itself also has a direct effect on adipose tissue in particular for adipocyte differentiation[306], thus IGF-1 may play a critical role in lipid accumulation as well[307].

4) Apart from indirectly inducing rapid weight gain and adiposity through increasing secretion of insulin/ IGF-1 axis, high protein intake from formula could lead to a saturation of the BCAA degradation which may result in excessive amounts of BCAA in blood circulation[285]. As current evidence shows that high plasma BCAA concentrations may have an inhibitory effect on β-oxidation[308] and increase lipogenesis[309], this might be another explanation for why "too much" protein intake from formula can affect weight gain and adiposity especially in early life.

Moving to other protein sources, consistent with the results shown in this chapter, most other studies did not find a significant association between meat intake and serum IGF-1 and/or its binding proteins[74, 76, 77, 293]. Although non-dairy ABP such as meats and eggs are considered as high-quality protein providing all EAA that are necessary for growth in general, they show less effects on the GH-IGF axis compared to dairy protein. If we consider the proposed mechanism that high amounts of BCAA seem to be a potent stimulator of the GH-IGF axis, thus the lower content of BCAA found in non-dairy ABP compared to dairy protein[37, 310] might be a reason why it stimulates less IGF-1 secretion. As shown in figure R5.6, although increasing consumption of non-dairy ABP was positively associated with plasma levels of BCAA, the coefficients of determination (R^2) predicting change in plasma Leu, Ile and Val by %PE were highest in those who consumed high protein from formula and cow's milk. Focusing on PBP, almost all studies consistently report that it has no impact on growth[58, 74, 76, 311], fat mass[312] or the GH-IGF axis[91, 313-314] in infants and children. This is not surprising given longstanding knowledge that most types of PBP have lower protein content and contain lower amounts of EAA[250], particularly lysine/tryptophan (in cereals) and sulphur-containing amino acids (in legumes)[315], compared to ABP.

Sex dimorphism of the GH-IGF axis

The sex dimorphism of serum IGF-1 and IGFBP-3 found in my study, regardless of type of milk feeding, is also consistent with findings from the

CHOP study mentioned previously. In secondary analyses of that dataset, Closa-Monasterolo et al[316] reported that serum IGF-1 and IGFBP-3 at 6 months of age were higher in female infants than males. Furthermore, they showed that the response to high protein intake from formula tended to be stronger in females than male infants. Consistent with my study, they did not find an interaction between sex and the nutritional intervention on growth parameters. Other studies mentioned previously also reported the same finding[239, 289]. Undoubtedly, this specific issue of sex differences in the IGF-1 axis has been well-recognised by many studies[74, 317] as well as in Thai infants and children[318].

Plasma BCAA: a mediator between dietary protein and the GH-IGF-1 axis

The discussion so far partly illustrates how plasma BCAA could be a mediator between protein intake and the GH-IGF axis. Although I also showed positive correlations between the sum of plasma EAA and serum IGF-1 and IGFBP-3, the coefficients were less than those observed for plasma BCAA. Additionally, apart from those three BCAA, I did not find other individual EAA that were significantly associated with IGF-1 and IGFBP-3 (data not shown). Therefore, I will focus mainly on the plausibility of BCAA as part of this proposed mechanism. The BCAA including Leu, Ile and Val have been suggested as a potential mediator linking high protein intake in early life to rapid growth for many years. Rolland-Cachera et al[205] hypothesised that high protein from formula may contribute to higher blood concentrations of BCAA, IGF-1 and insulin leading to rapid weight gain, increasing adiposity and risk of later obesity. The association between high protein formula and increasing plasma BCAA is supported by other studies conducted before the CHOP study[319-322]. However, the strongest evidence supporting this hypothesis came from metabolomic analyses of the CHOP study using blood samples at age 6 months. Kirchberg et al[285] reported that BCAA including Leu, Ile and Val were the most discriminant metabolites between infants receiving high and low protein formula. Furthermore, they also found that the degradation products of BCAA (i.e., short-chain acylcarnitine C4 and C5) reached a saturation point when plasma BCAA was too high. These results indicate when plasma levels

of BCAA exceed the metabolic capacity, it may result in undesirable effects such as overactivation of the insulin/ IGF-1 axis leading to rapid growth and increased adiposity.

For other protein sources, Tang et al[291] reported that plasma BCAA increased between 5 and 12 months regardless of whether formula-fed infants were randomly allocated into a meat-based or a dairy-based group, with no significant difference between groups. Some evidence in adults also shows that high meat intake is positively associated with plasma BCAA, however, the results are not consistent in different populations[323]. Regarding PBP, Lonnerdal et al[322] reported that adding cereal 25g/day as complementary food for formula-fed infants at 4 months did not affect plasma amino acids at 7 months while infants who received high protein formula had significantly higher levels of Val, Leu and histidine at 7 months old. Furthermore, a recent RCT in India showed that consumption of legume-based protein for a month was positively associated with plasma BCAA in stunted children. Although meat and legume-based protein tends to increase plasma BCAA, more studies are needed in infants and children to make a firm conclusion.

At present, there are two main concepts explaining how BCAA potentially promote somatic growth. The first one is accordance with the “Early protein hypothesis” from the CHOP study and the main findings from my study, and proposes that BCAA may influence growth and adiposity via the IGF-1 and/ or insulin pathway. The second concept suggests that BCAA, especially Leu, may directly stimulate the mammalian target of rapamycin (mTOR) pathway which plays a central role in cellular metabolism, growth, proliferation and survival[98, 324-326]. Regarding the first concept, a study using an animal model showed that a high protein diet and BCAA-supplemented diet can improve fetal growth by increasing gene and protein expressions of IGF-1 and IGF-2 in fetal liver[88], while a clinical study in formula-fed infants showed that plasma BCAA positively affect both plasma insulin and IGF-1, but it explained more of the variance of plasma insulin after controlling for sex, weight and feeding group[327]. Furthermore, based on scientific evidence some researchers have postulated that plasma amino acids, especially Leu, from milk intake can either

stimulate the mTOR pathway directly or indirectly via IGF-1 and insulin[328]. The increased interest in the signalling pathway between amino acids and mTOR is not only relevant to high protein intake and the risk of overweight/ obesity, but may also explain the important role of EAA in undernourished populations[51]. Therefore, we can see the potential importance of better understanding mechanism in order to study the impact of dietary protein at both extremes on growth during infancy. However, more clinical studies are needed to explore the signalling pathway between plasma BCAA and mTOR.

Breast milk and its potential role reducing risk of overweight/ obesity via effects on plasma amino acid concentrations

Before concluding, I would like to emphasise the negative association observed between %PE from breast milk and plasma BCAA. This might be a protective effect of breast milk preparing infants for increased %PE from non-dairy ABP during the CF period. According to Lonnerdal et al[329] the true protein content in human milk declines from birth, becoming stable at around 4-6 months of age, which is an appropriate time for the introduction of complementary foods. When non-dairy ASFs with higher protein content are introduced alongside continued breastfeeding, I assume that plasma amino acids, especially BCAA, would be more appropriate than when such foods are introduced alongside infant formula, resulting in more 'optimal' growth without over-activation of this proposed mechanism. However, further research with a larger sample size is needed to investigate whether over- consumption of non-dairy ABP has the same adverse effects on growth via this proposed mechanism in breastfed infants as in those consuming formula and cow's milk.

Conclusions and ongoing laboratory work

In summary, I have shown that the same mechanism proposed by Koletzko et al[233] in relation to formula-feeding, namely that high protein intake in excess of metabolic demands during early childhood may increase insulin-releasing amino acids, especially BCAA, resulting in secretion of insulin and IGF-1 that

could trigger weight gain, continues until later infancy. However, I found that protein from formula and cow's milk is the main factor driving this effect. The impact of non-dairy ABP was not as robust, and I found no effect of PBP which is in line with the non-significant association between PBP and growth outcomes reported in Chapter 9. It should be noted that my study population rarely consumed legumes which are a better protein source than cereals, fruits and vegetables; thus a further study in populations consuming more legumes should be considered.

Last but not least, because of the COVID-19 pandemic causing a delay in body composition and microRNA analyses, I do not currently have data on infant adiposity (as an outcome) or microRNAs (as a potential part of the underlying mechanism). As recent in vitro studies and studies in animal models demonstrate a strong interplay among protein intake, somatic growth, amino acids, insulin/ IGF-1 axis, microRNAs, and the mTOR pathway[84, 108, 324-325, 330-331] outcomes from the microRNA analyses in this cohort will represent an important addition to this research field.

Chapter 8

General discussion and Conclusions

In the final chapter of this thesis, I first summarise and collate the key findings of my research. This section starts with an overview including a public health perspective, then addresses dietary protein intake patterns and the impact of dietary protein during the CF period on infant growth and iron status, before concluding by discussing potential mechanisms that may underpin the clinical outcomes. I also revisited the hypotheses described in Chapter 4 to see whether they were confirmed by my research. Finally, I discuss the strengths and limitation of the research, the implications for practice and policy, as well as remaining research gaps and suggestions for future research.

8.1 Overview of main findings

- Nutritional status and the DBM

Although the participants in this relatively small prospective cohort cannot be considered nationally representative, the characteristics of infants and their families are typical for Thai middle-class, nuclear families with good standards of literacy and sanitation. In Chapter 7, Results 1, I showed that the DBM was present at all levels, from individual to population. In this cohort, nearly 1 in 2 infants suffered from at least one form of malnutrition. The most common nutritional problem in infants was iron deficiency which affected 42.7% of the cohort. Interestingly, 71% of iron deficient infants were living with at least one parent who was overweight/ obese indicating a high prevalence of DBM at household level. Although the prevalence of other forms of undernutrition including wasting, underweight and stunting were quite low, they increased with age. Only one infant was overweight. However, the combination of high percentages of parental overweight/ obesity (37.9% of mothers and 64.5% of fathers) in this cohort with an increasing prevalence of overweight/ obesity in

children aged under five from a recent national survey[15,21], is of concern. I next investigated the contribution of complementary feeding practices to infant nutritional status focusing on protein intake and source.

- Patterns of dietary protein intake

The main analyses in Chapter 7, Results 2 identified two contrasting feeding issues related to dietary protein. On the one hand, some parents delayed introduction of ASFs, and most families reported providing a low variety of ASFs during the early stage of CF (6 to 9 months old). These practices could lead to iron deficiency and undernutrition, especially for breastfed infants. On the other hand, at later stages of CF (9 to 12 months old), breastfeeding was discontinued and replaced by formula in most infants. At this time, protein intake especially from ASFs greatly increased and exceeded both national and international recommendations. Moreover, nearly 15% of infants received unfortified cow's milk at this stage. These feeding practices could contribute to rapid weight gain. Overall, it is plausible that feeding practices relating to dietary protein could contribute to the DBM, so I next investigated how dietary protein from different sources affects infant growth and iron status.

- Impact of dietary protein in complementary foods on infant growth

In Chapter 7, Results 3, using multiple regression analyses to predict conditional growth by %PE from different food sources, the results consistently showed that %PE from dairy protein (formula, cow's milk and dairy products) was the strongest factor positively associated with weight-related z-scores (i.e., WAZ, WLZ and BMIZ). There was also a dose-response relationship after adjusting for type of milk-feed, non-protein calories and breastfeeding duration. Likewise, non-dairy ABP including meats and eggs was also positively associated with weight-related z-scores, but the effect was less than for dairy protein. There was no effect of PBP on any of the growth parameters and none of the protein sources showed an association with linear growth.

The finding that ASFs are positively associated with weight-related Z-scores raises some practical issues since these foods are also a good source of iron. I therefore investigated whether infants could achieve adequate iron status without excess weight gain which might increase their risk of becoming overweight/ obese.

- Impact of dietary protein in complementary foods on iron status

As shown in the Chapter 7, Results 4, consumption of unfortified cow's milk during the CF period was associated with poor iron status even after adjusting for protein intake from other ASFs, type of milk fed and inflammation. It also showed dose-response relationships with both SF and TSAT as well as increasing the risk of ID and IDA by 100% and 40%, respectively for every 100 ml increase of cow's milk consumption. In contrast to cow's milk, higher protein intake from formula and regular consumption of liver (one tablespoon at least 3 times/week) were significant predictors of normal iron status. More importantly, I found that average %PE were less than 15% and almost equal among iron-sufficient and iron-deficient infants, which indicated that infants with normal iron status did not eat more protein than infants with ID/ IDA, and their protein intake was still within the proposed upper limit. Finally, I created 4 theoretical scenarios based on the results from my study. The best-case scenario balancing risk of being ID/ IDA and overweight/ obese is a breastfed infant who receives iron-rich ASFs such as liver regularly and soon after introduction of CF. For the worst-case scenario, infants having the highest risk of ID/IDA and overweight/ obesity are those who mainly consume unfortified cow's milk along with iron-poor ASFs during the CF period. This scenario may lead to the DBM at individual level when the infant has both ID/IDA and overweight/ obesity at the same time. Although intake of formula favours better iron status, its adverse effect on weight gain should be considered. Therefore, to balance the risk of ID/IDA and overweight/obesity in infants and young children, avoiding consumption of unfortified cow's milk during the CF period and promoting regular consumption of iron-rich ASFs alongside breastfeeding from the start of complementary feeding are the most appropriate measures to prevent the DBM at individual level. Unlike dairy protein, non-diary ABP may

promote growth without greatly increasing IGF-1, IGFBP-3 and BCAA which are implicated in increased obesity risk, whilst also providing a good source of iron.

My findings clearly show the impact of ABP on infant growth and iron status. I next explored some of the potential mechanisms for the effect on growth.

- Potential mechanisms explaining how dietary protein influences growth during the complementary feeding period

The laboratory tests were selected based on longstanding knowledge that somatic growth is controlled by the GH-IGF axis and dietary protein the only source of EAA in humans. Based on the “early protein hypothesis" proposed by the CHOP study[223], I assumed that the same mechanism may continue until late infancy, but unlike the CHOP study, I was also able to investigate the impact of different protein sources. The key findings in Chapter 7, Results 5 were consistent with the early protein hypothesis; a high %PE from formula and cow’s milk was the strongest factor associated with higher plasma levels of BCAA and growth-promoting factors, serum IGF-1 and IGFBP-3, while non-dairy ABP also stimulated this pathway, but with less potency. There was no effect of PBP on plasma amino acids nor growth-promoting factors. More importantly, I found that increasing %PE from breast milk could be a protective factor associated with lower plasma BCAA when %PE from non-dairy ABP increases during the CF period. The consistency of the results, especially between clinical and laboratory outcomes, provide convincing support for the proposed mechanism demonstrated in Chapter 7, Results 5, although more studies with larger sample size are needed to support this proposed mechanism.

8.2 Revisiting the hypotheses

(1) Association between the amounts and different protein sources consumed by Thai infant aged 6 to 12 months and conditional growth at 12M

Hypothesis: ABP in “recommended” quantities can promote infant growth with lower risk of malnutrition than similar amounts of PBP.

The results from this cohort partly support the hypothesis – as ABP showed positive association with conditional growth while there was no association found between PBP and conditional growth. However, as my study population rarely consumed legumes, which are known to be better PBP than cereals/ fruits/ vegetable, the results cannot be generalized to all PBP. Additionally, as most of the study population consumed higher ABP than the “recommended” quantities during the CF period, it was difficult to fully test this hypothesis.

(2) Compare the impact of different protein sources provided to infants between 6M to 12M on their body composition at 12M

Hypothesis: Higher consumption of ABP particularly dairy protein may increase body fatness during infancy.

The results from this cohort: awaiting body composition analyses.

(3) Effects of different protein sources, animal- versus plant-based protein typically introduced during the CF period, on the iron status of Thai infants at 12M

Hypothesis: timely introduction and frequent intake of ABP especially liver and red meat, would improve iron status and prevent ID/ IDA

The results from this cohort support this hypothesis

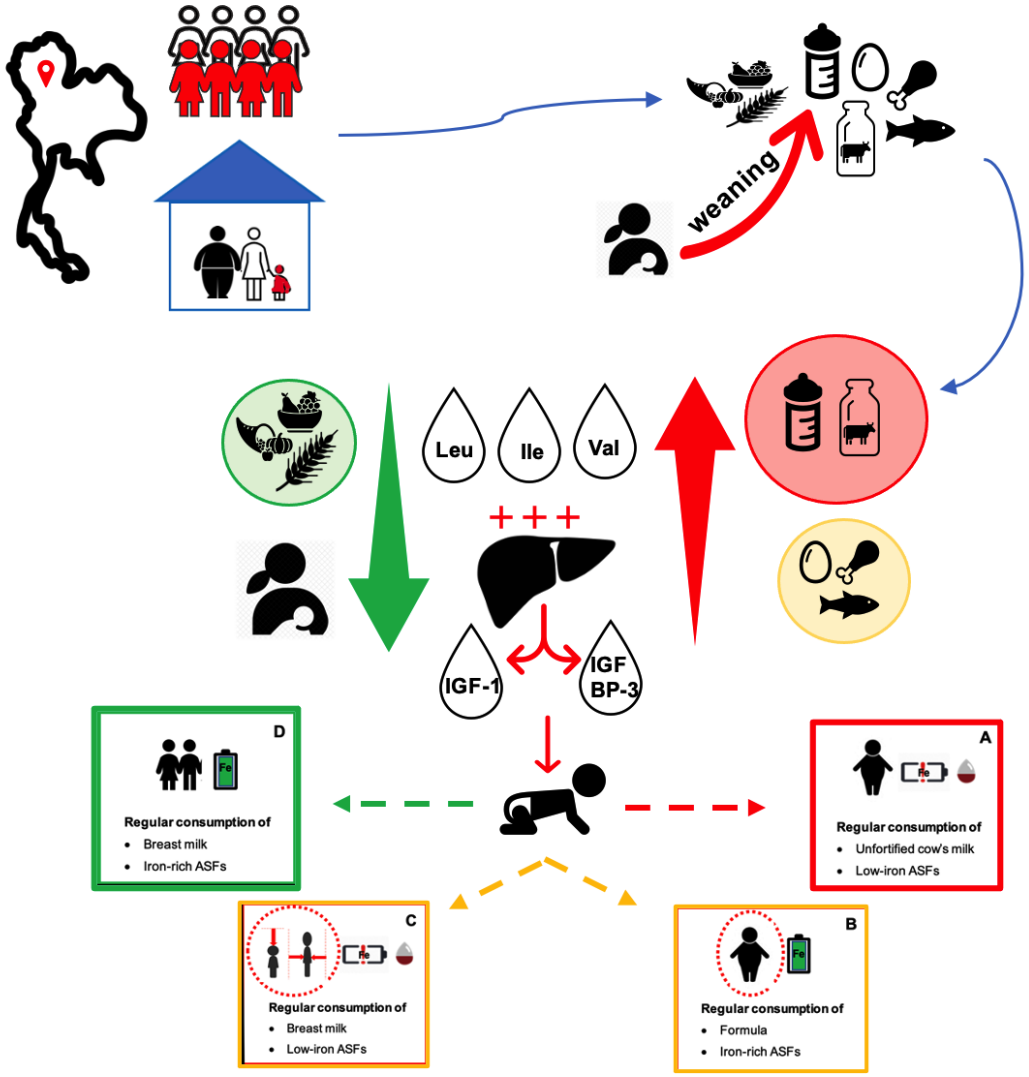
(4) The possible mechanism underlying an influence of dietary protein on growth in early life through hormonal, metabolomic and epigenetic processes

Hypothesis: High consumption of ABP may be associated with (1) increase of EAA especially BCAA which would positively correlate with plasma levels of growth GH, IGF-1 and IGFBP-3 (2) patterns of circulating microRNA

The results from this cohort: (1) Support (2) awaiting microRNAs analyses

Before moving to the other sections of this chapter, I have gathered all key results that I have discussed into one infographic (Figure 8.1).

Figure 8.1 Summary of all key findings in this thesis



8.3 Strengths and limitations

This research has several strengths. Firstly, it provides the first evidence from LMICs demonstrating the impact of high protein intake in early life on accelerating weight gain during infancy. It may remind researchers in LMICs especially those who are working in countries where the DBM is prevalent that protein intake from ASFs can present problems whether infants consume “too

little" or "too much". Furthermore, this is the first study to show that the "early protein hypothesis" applies to infants from a non-European population. Secondly, the drop-out rate was only 3% - well below the 15% allowed for in the sample size calculation – thus providing increased statistical power. Thirdly, completeness of dietary intake data was very high: more than 85% for the 3-DFR and almost 100% for the 24-HR; and the reliability of dietary data was acceptable as all caregivers were trained to use their household utensils to estimate food intake before recording the 3-DFR. Fourthly, as the nutrient composition programme used in this cohort, the INMUCAL-Nutrients programmed, has been developed based on the nutrient composition of local Thai foods and ingredients, this reduced the chance of estimation errors arising from the use of less relevant food composition tables. Another strength is data quality, especially for dietary data as I used 3 dietary assessment tools at three consecutive ages. Furthermore, the main outcomes were objective and measured under standardized conditions; for example, all body weights and lengths were measured by health professionals according to protocols and laboratory tests were performed in an accredited laboratory. Lastly, the good agreement between clinical and laboratory outcomes were strengths of this cohort, and the main results were clear and consistent throughout the thesis, despite the use of different statistical approaches.

Nevertheless, this research also has some limitations. First and foremost, the results from a cohort study cannot indicate causal relationships between predictors and outcomes, although DAGs were used to carefully select the 'minimum adjustment set' of potential confounders for the regression models, which is argued to improve casual inference. The next limitation relates to the generalizability of my findings on the lack of impact of PBP on growth outcomes and biomarkers. As my study population rarely consumed legumes, which are known to be a better source of essential amino acids than cereals, fruits and vegetables, I cannot generalise the findings to populations in which legumes are more widely consumed by infants and young children. Thirdly, the selection of a smaller number of participants for plasma amino acids might have decreased the power of the study to detect significant associations between the intake of non-dairy ABP and plasma BCAA. Moreover, as infants

consumed a variety of protein sources, I could not investigate associations between the amino acid composition of different complementary foods and plasma amino acids. Lastly, with limited laboratory resources and funding, I was not able to investigate other potential mediators of the effect of protein on growth such as insulin, other metabolites or the mTOR pathway. However, as microRNAs analysis is now underway, I hope to gain some indirect evidence relating to other potential metabolic pathways via epigenetic mechanisms.

8.4 Research implications

The results from this study can be applied to both clinical and public health policy. However, generalization of these findings to other populations is dependent on several factors, for example, the burden of under- and overnutrition, breastfeeding rates, local staples, availability and affordability of ASFs, and availability of iron-fortified complementary foods.

- Clinical implications

  Breastfed infants – infants should receive iron-rich ASFs as soon as the first solid foods are introduced, especially for infants exclusively breastfed for 6 months. Adequate amount of ASFs should be provided to infants every day but liver or other ASFs containing equivalent haem iron must be regularly provided - at least 3 tablespoons per week. Unfortified cow's milk must be avoided until 12 months of age.

  Formula-fed infants – when solid food is introduced, the carers should consider reducing the volume of formula proportionally to increasing the intake of other ASFs. If possible, carers should use lower-protein formula instead of high-protein formula. Regular consumption of iron-rich ASFs should be encourage but unfortified cow's milk must be avoided until 12 months of age.

- Policy implications

1) Breastfeeding should be encouraged beyond 6 months of age
2) Promote provision of iron-rich ASFs as soon as introduction of complementary feeding
3) Emphasise consumption of liver or other ASFs containing equivalent haem iron at least 3 tablespoons per week
4) Recommend avoiding unfortified cow's milk until 12 months old
5) Reduce protein content in follow-on formula

When considering the current Thai CF recommendations (Appendix 11), the first and the third points above are recommended, but the regular consumption of liver at least 3 times a week may not be adequately emphasised. In addition, a result from the MSc dissertation using some of my data showed that most family did not adhere to this recommendation[332]. For the second implication, although ASFs are encouraged at early stages of CF, the recommendations do not specify iron-rich ASFs. Finally, there is no recommendation to avoid cow's milk during infancy.

8.5 Remaining knowledge gaps and suggestions for future research

I mentioned earlier in this thesis that before conducting my research, there was insufficient information available to design an appropriate intervention for a clinical trial to improve complementary feeding practices and infant outcomes in my target population. However, some key results from this thesis may be used to develop interventions for testing in future randomized trials. For example, the effect of provision of at least 3 tablespoons of liver per week on infant iron status, especially in breastfed infants, or a study to investigate the most appropriate ratio between protein from formula and non-dairy ASFs to decrease the risk of overweight/ obesity in formula-fed infants. In addition, I plan to consider a follow-up study of participants from this cohort to investigate the longer-term effect of protein intake during the CF period on their growth and adiposity.

As discussed previously, the proposed mechanisms investigated in this thesis may not be the only underlying mechanisms explaining the effect of high protein intake in early life on growth. There is still a lack of evidence from clinical research to confirm whether BCAA directly stimulate the mTOR pathway or indirectly stimulate the mTOR pathway via the IGF-1 axis. Furthermore, although some studies suggest that the GH-IGF1 axis might be programmed by high protein consumption in early life[299], there is no convincing evidence to clarify which epigenetic processes might control this process. I hope that the microRNAs result from my cohort may inform future clinical studies on this topic.

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Appendices

Appendix 1 A questionnaire used in the cross-sectional study

1

Complementary feeding: Attitudes, Knowledge and Practices

1. Respondent's information

1.1) Relationship with infant or toddler

□ mother □ father □ other (please, specify……)

1.2) Age of respondent

□ < 20 y □ 20-29 y □ 30-39 y □ 40-49 y □ > 50 y

1.3) Educational level

□ primary school □ secondary school □ high school

□ bachelor's Degree □ higher Bachelor's degree

1.4) Occupation

□ housewife □ government employee □ framer □ other (specify).....

1.5) Do you live in the city? □ yes □ no (sub-urban/ rural)

1.6) Household income (monthly)

□ less than 10,000 Baht □ 10,000 – less than 30,000 Baht

□ 30,000- less than 50,000 Baht □ ≥ 50,000 Baht

1.7) Number of children in family

□ 1 □ 2 □ 3 □ others (Specify).....

1.8) Number of family members

□ less than 3 □ 3 □ 4 □ ≥ 5

2. Child's information

2.1) Your child is □ first child □ second child □ Other (specify).....

2.2) Gender □ male □ female

2.3) Age of your child

□ 2 months □ 4 months □ 6 months □ 9 months

□ 12 months □ >12 months (specify)..... months

2.4) Who is/ are primary caregiver of infants?

□ mother □ father □ grandparent (specify).....
□ babysitter □ nursery

3. Complementary feeding: Attitudes and Knowledge

3.1) What is an appropriate age for first introduction of complementary food?

□ less than 4 months (specify)..... □ 4 - before 6 months

□ 6 months □ after 6 months (specify).....months

3.2) Appropriate age for 2nd main meal should be started at..... months

3.3) Appropriate age for 3rd main meal should be started at..... months

3.4) Do you agree that animal protein should be the first complementary food for infant?

□ Yes □ No because.....

3.5) Are animal proteins different from plant proteins?

□ Yes because (you can choose more than one choice) □ No

□ difficult to digest □ higher allergenicity

□ high cost □ better supply of essential amino acids

□ a good source of iron and zinc

3.6) What is the most reliable source of information about infant and young child feeding for complementary feeding

□ healthcare professionals □ media/ internet □ friends
□ maternal and child handbook □ maternal and child magazine □ senior relatives
□ official website of the Ministry of Public Health

3.7) How confident you are to feed your child properly? (1= least, 6=most)

□ 1 □ 2 □ 3 □ 4 □ 5 □ 6

3.8) What is/ are the most important factors to promote growth of infant and toddler? (you can choose more than one choice)

□ exclusive breastfeeding at least 6 months
□ high quality infant formula feeding
□ an appropriate practice of complementary feeding
□ vitamins and minerals supplementation
□ others (specify).....

4. Complementary feeding: Practical aspect

4.1) Type of currently milk feeding

□ Breast milk (breastfeeding or expressed breast milk)

□ Combined breast milk and infant formula

□ Infant or follow-on formula

□ Cow's milk

4.2) Feeding breast milk along with complementary feeding

□ yes □ no

4.3) Do you still use bottle feeding for your child? □ yes □ no

4.4) When did you firstly introduce complementary food for your child?

□ less than 4 months (specify)..... □ 4 - before 6 months

□ 6 months □ after 6 months (specify).....months

4.5) How many main meals* are offered each day?

□ 1 □ 2 □ 3 □ ≥ 4

**Combinations of staple food and other food groups to provide a majority of calories and nutrients each day.*

4.6) What is the most appropriate type of the first complementary food?

□ rice porridge □ mashed banana □ commercial food (specify).....

□ fruit juices □ other (specify).....

4.7) Current texture and characteristics of complementary foods

□ soup/liquid □ puree/ mashed food □ chopped food □ Family food

4.8) When did the first time that your child were given animal-based protein?

□ 6-7 months □ 8-9 before 6 months □ 10-12 months □ > 12 months

4.9) What are the animal-based proteins that have been introduced to your child?
(you can choose more than one choice)

□ egg yolk □ whole egg □ chicken □ fish

□ beef □ pork □ liver □ others (specify).....

4.10) What are food groups contained in your child's food each day?
(you can choose more than one choice)

□ carbohydrate (e.g., rice, sticky rice, bread, cereal)

□ protein (e.g., egg, chicken, pork, liver, tofu, beans, lentils, pulses)

□ fat (e.g., vegetable oil, butter, lard)

□ vegetables and fruits

□ dairy products

4.11) Do you give any snacks for your child except milk?
□ yes (□ 1 meal □ 2 meals □ ≥ 3 meals) □ no

4.12) How much does your child drink fruit juice each day?

□ none □ 1-2 Oz □ 3-4 Oz □ > 4Oz

4.13) Do you routinely cook for your child?

□ yes □ no

4.14) Cooking methods used for preparing complementary foods (you can choose more than one choice)

□ boiling □ steaming □ frying □ grilling □ baking

4.15) Do you add any sugar or sauce in complementary food?

□ yes □ no

4.16) Do you add oil in complementary foods?

□ yes □ no

4.17) Do you supplement any vitamins or minerals to your child?

□ yes □ no

5. Complementary feeding: Behavioural aspect

5.1) When do you offer main meal to your child each day?

□ following the same schedule every day

□ when you notice that he/ she is hungry

□ irregular schedule

5.2) How is feeding method mainly use for your child at the moment?

□ feed by caregiver □ two-spoon technique or partial support

□ Self-feeding with closed monitoring

5.3) How often that your child eat with other family members?

□ never □ sometimes □ frequently

5.4) Have you pre-chewed food before feeding your child?

□ yes □ never

5.5) Have you offered food to satisfy your child?

□ yes □ never

Appendix 2 Ethics approval from the University College London

UCL RESEARCH ETHICS COMMITTEE
OFFICE FOR THE VICE PROVOST RESEARCH

31st January 2018

Professor Mary Fewtrell
Department of Population, Policy and Practice
Institute of Child Health
UCL

Dear Professor Fewtrell

Notification of Ethics Approval with Provisos
Project ID/Title: 12551/001: Intake and sources of dietary protein in complementary foods and their impact on infant growth in an area facing double burden of childhood malnutrition

Further to your satisfactory responses to the Committee's comments, I am pleased to confirm in my capacity as Joint Chair of the UCL Research Ethics Committee (REC) that the data collection element of your study has been ethically approved by the UCL REC until **28th February 2019** on condition that:

1. recruitment does not commence until local ethics approval has been secured from the Faculty of Medicine, Chiang Mai University, Thailand with written evidence provided for our records;

2. you supply formal letters of agreement to participate in the study from the three well-baby clinics in Thailand.

Ethical approval is also subject to the following conditions:

Notification of Amendments to the Research
You must seek Chair's approval for proposed amendments (to include extensions to the duration of the project) to the research for which this approval has been given. Ethical approval is specific to this project and must not be treated as applicable to research of a similar nature. Each research project is reviewed separately and if there are significant changes to the research protocol you should seek confirmation of continued ethical approval by completing an 'Amendment Approval Request Form'
http://ethics.grad.ucl.ac.uk/responsibilities.php

Adverse Event Reporting – Serious and Non-Serious
It is your responsibility to report to the Committee any unanticipated problems or adverse events involving risks to participants or others. The Ethics Committee should be notified of all serious adverse events via the Ethics Committee Administrator (ethics@ucl.ac.uk) immediately the incident occurs. Where the adverse incident is unexpected and serious, the Joint Chairs will decide whether the study should be terminated pending the opinion of an independent expert. For non-serious adverse events the Joint Chairs of the Ethics Committee should again be notified via the Ethics Committee Administrator within ten days of the incident occurring and provide a full written report that should include any amendments to the participant information sheet and study protocol. The Joint Chairs will confirm that the incident is non-serious and report to the Committee at the next meeting. The final view of the Committee will be communicated to you.

1

Final Report
At the end of the data collection element of your research we ask that you submit a very brief report (1-2 paragraphs will suffice) which includes in particular issues relating to the ethical implications of the research i.e. issues obtaining consent, participants withdrawing from the research, confidentiality, protection of participants from physical and mental harm etc.

In addition, please:

- ensure that you follow all relevant guidance as laid out in UCL's Code of Conduct for Research: http://www.ucl.ac.uk/srs/governance-and-committees/resgov/code-of-conduct-research
- note that you are required to adhere to all research data/records management and storage procedures agreed as part of your application. This will be expected even after completion of the study.

With best wishes for the research.

Yours sincerely

[redacted]

Dr Lynn Ang
Joint Chair, UCL Research Ethics Committee

Cc: Miss Kulnipa Kittisakmontri & Dr Julie Lanigan

Appendix 3 Ethics approval from the Faculty of Medicine, Chiang Mai University

Research Ethics Committee
Faculty of Medicine Chiang Mai University

AF/04-010/04.0
No. 165 /2018

Certificate of Approval

Name of Ethics Committee : Research Ethics Committee 1
Faculty of Medicine, Chiang Mai University
Address of Ethics Committee : 110 Intavaroros Rd., Amphoe Muang, Chiang Mai, Thailand 50200

Principal Investigator: Kulnipa Kittisakmontri, M.D.
Department of Pediatrics, Faculty of Medicine, Chiang Mai University.

Protocol title: Intake and sources of dietary protein in complementary foods and their impact on infant growth in an area facing double burden of childhood malnutrition

STUDY CODE: **PED-2561-05287** Research ID: **05287**

Sponsor: Childhood Nutrition Research Centre, University College London

Documents filed	Document reference
Research protocol	Research Proposal, version **2**, 28 March 2018
Subject information sheet/ Informed consent documents	Parent information sheet, version 2, 28 March 2018 Parental Informed Consent Form version 1, 18 January 2018
Principal Investigator Curriculum vitae	Version date 18th January 2018
Other	Questionnaire 1 : 24-hour Dietary Recall version 1, 18 January 2018 Questionnaire 2 : Food Frequency Questionnaire version 1, 18 January 2018 Questionnaire 3 : Baby Eating Behaviour Questionnaire (BEBQ) version 1, 18 January 2018 Questionnaire 4 : 3-day Food Records version 1, 18 January 2018

DECISION : [ ] By expedited review

[✓] By full committee-1 meeting 2 /2018 Date: March 22, 2018

No. 165 /2018

Opinion of the Ethics Committee/Institutional Review Board : PLS. CHECK ONE

✓ Approval

Progress report submit every ☐ 3 months ☐ 6 months ☑ 1 year ☐ Other.....

Date of Approval: 30 April 2018 **Expiration Date:** 29 April 2019

This Ethics Committee is organized and operates according to GCPs and relevant international ethical guidelines, the applicable laws and regulations.

[redacted]

Signed : .....

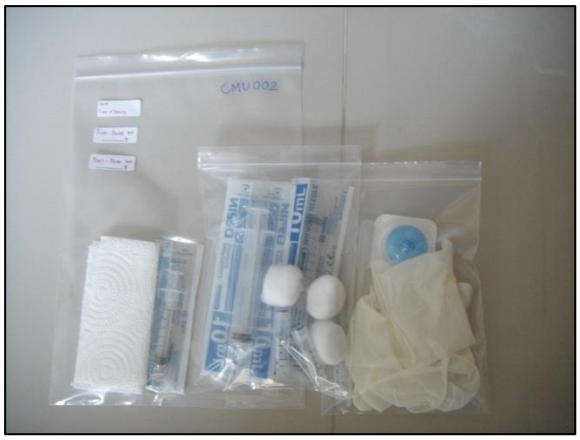
(Emeritus Professor Malai Muttarak, M.D.)

Chairperson, Faculty of Medicine

GENERAL CONDITION OF APPROVAL:

- Please submit the progress report at least once a year except where required more frequent by the REC.
- In particular, approval of this study must be renewed at least three months before the expiration date if work is to continue.
- Prior Research Ethics Committee approval is required before implementing any changes in the consent documents or protocol unless those changes are required urgently for the safely of subjects.
- Any event or new information that may affect the benefit/risk ratio of the study must be reported to the REC promptly
- Any protocol deviation/violation must be reported to the REC

Appendix 4 Blood sample collection, Plasma preparation and Storage



Blood collecting kit

Blood sample collection

Serum preparations

Storage of blood samples for plasma amino acids and microRNAs analysis (-80^{o}C freezer)

Appendix 5 A record form: Deuterium dose and fluid records

แบบบันทึกการดื่มน้ำและนมภายใน 6 ชั่วโมงหลังดื่มไอโซโทปเสถียรของธาตุไฮโดรเจน

แพทย์ระบุ

Study code: CMU/ CTH/ HPH_____ วันที่__/__/__ (Date)

น้ำหนักทารก_____ กิโลกรัม ปริมาณไอโซโทป_____ กรัม (Body weight (kg); Dosage (g))

เวลาให้ดื่มไอโซโทปเสถียร _____:_____ (Dosing time)

*Prior-dose weight_____ g **Post-dose weight _____ g

ผู้ปกครองระบุ

ใส่สำลีในผ้าอ้อมสำเร็จรูปเวลา ___:_____ เวลาเก็บปัสสาวะครั้งที่ 1 ___:_____

ใส่สำลีในผ้าอ้อมสำเร็จรูปเวลา ___:_____ เวลาเก็บปัสสาวะครั้งที่ 2 ___:_____

ใส่สำลีในผ้าอ้อมสำเร็จรูปเวลา ___:_____ เวลาเก็บปัสสาวะครั้งที่3 ___:_____

(Urine collecting time)

ชนิดและปริมาณของอาหารและของเหลวที่ดื่มภายใจ 6 ชั่วโมงหลังดื่มไอโซโทปเสถียรของธาตุไฮโดรเจน

1) ชนิด_____ปริมาณ_____เวลา_____:_____

2) ชนิด_____ปริมาณ_____เวลา_____:_____

3) ชนิด_____ปริมาณ_____เวลา_____:_____

4) ชนิด_____ปริมาณ_____เวลา_____:_____

5) ชนิด_____ปริมาณ_____เวลา_____:_____

6) ชนิด_____ปริมาณ_____เวลา_____:_____

7) ชนิด_____ปริมาณ_____เวลา_____:_____

8) ชนิด_____ปริมาณ_____เวลา_____:_____

9) ชนิด_____ปริมาณ_____เวลา_____:_____

10) ชนิด_____ปริมาณ_____เวลา_____:_____

(Fluid intakes within 6 hours after dosing)

* Weight of syringe/ bottle/ beaker containing stable isotope and tissue in sealed bag before dosing

**Weight of syringe/ bottle/ beaker with remaining amount of stable isotope and used tissue in sealed bag

Appendix 6 A record form: 24-HR

Appendix 1: 24-Hour Dietary Recall Form

Study code: _ _ _/_ _ _/ _ _/ _ _/ _ _ _ _ **Date of interview:** _ _/ _ _/ _ _ _ _ **Appointment:** 1st (6 mo)/ 2nd (9 mo)/ 3rd (12 mo)

Line No.	Time		Location 1 = Home 2 = Nursery 3 = Other	Foods and beverages	Amount* *duration if breastfeeding	Description and Preparation	code
	Hrs/Min	AM/PM					
1							
2							
3							
4							
5							
6							
7							
8							
9							

Appendix 7 A record form: estimated 3-DFR

Food Diary

Parent's name ……..……..

Child's name …….

Daytime contact number …..……..

If you have any problems filling out the diary please do not hesitate to contact **Dr Kulnipa Kittisakmontri**
Tel. +66 896845402

1) To start recording from your child's next main meal.

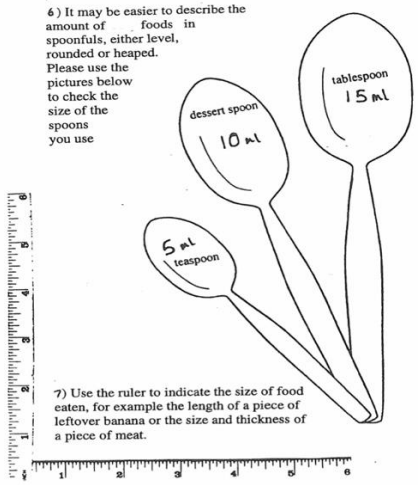
2) Please give as much detail as you can about the brand of food eaten.

3) If you cook some dishes at home, please provide as much detail as you can on the pages provided at the back of this diary.

4) To help us to measure the portion of food eaten by your child we ask you to describe the food in handy measures, for example: number of spoonsful of cereal, a thin slice of bread spread thickly or thinly with butter/spread, a potato the size of an egg.

5) Please measure as much of the leftovers as possible.

6) It may be easier to describe the amount of foods in spoonfuls, either level, rounded or heaped. Please use the pictures below to check the size of the spoons you use



7) Use the ruler to indicate the size of food eaten, for example the length of a piece of leftover banana or the size and thickness of a piece of meat.

Please follow the example shown below:

Example: Morning

Time	Food/drink items	Serving size	Amount left
breakfast 7.00 am	Stream rice	4 tbs	2 tbs
	Boiled egg	1 egg	¼ egg
	Easy-peel orange	1 medium	-none-
	water	60 ml	-none-
midmorning 10.30	Sticky rice with banana	1 piece	-none-
	Follow-on formula	8 Oz	1 oz
lunch 12.00	Fried rice with chicken	4 tbs	1 tbs
	Orange juice (fresh press)	3 Oz	1 Oz

Morning Day 1 (___/___/___)

Time	Food/drink items	Amount offered to child	Amount left
midnight to breakfast			
breakfast			
midmorning			
lunch			

Afternoon Day 1 (___/___/___)

Time	Food/drink items	Amount offered to child	Amount left
Mid afternoon			
teatime			
evening			
bedtime			

****If you give any supplements to your child please, describe more detail**

.....
.....
.....
.....
.....

Morning Day 2 (___/___/___)

Time	Food/drink items	Amount offered to child	Amount left
midnight to breakfast			
breakfast			
midmorning			
lunch			

Afternoon Day 2 (___/___/___)

Time	Food/drink items	Amount offered to child	Amount left
Mid afternoon			
teatime			
evening			
bedtime			

****If you give any supplements to your child please, describe more detail**

.....
.....
.....
.....
.....

Morning Day 3 (___/___/___)

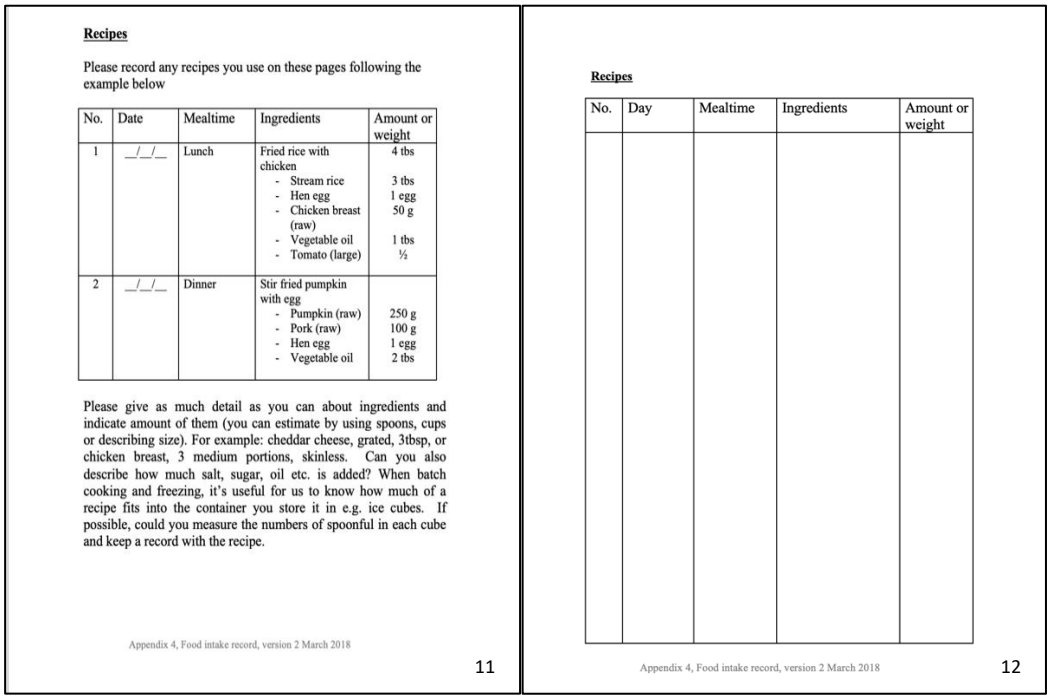
Time	Food/drink items	Amount offered to child	Amount left
midnight to breakfast			
breakfast			
midmorning			
lunch			

Afternoon Day 3 (___/___/___)

Time	Food/drink items	Amount offered to child	Amount left
Mid afternoon			
teatime			
evening			
bedtime			

****If you give any supplements to your child please, describe more detail**

.....
.....
.....
.....
.....



Recipes

Please record any recipes you use on these pages following the example below

No.	Date	Mealtime	Ingredients	Amount or weight
1	__/__/__	Lunch	Fried rice with chicken - Stream rice - Hen egg - Chicken breast (raw) - Vegetable oil - Tomato (large)	4 tbs 3 tbs 1 egg 50 g 1 tbs ½
2	__/__/__	Dinner	Stir fried pumpkin with egg - Pumpkin (raw) - Pork (raw) - Hen egg - Vegetable oil	 250 g 100 g 1 egg 2 tbs

Please give as much detail as you can about ingredients and indicate amount of them (you can estimate by using spoons, cups or describing size). For example: cheddar cheese, grated, 3tbsp, or chicken breast, 3 medium portions, skinless. Can you also describe how much salt, sugar, oil etc. is added? When batch cooking and freezing, it's useful for us to know how much of a recipe fits into the container you store it in e.g. ice cubes. If possible, could you measure the numbers of spoonful in each cube and keep a record with the recipe.

Appendix 4, Food intake record, version 2 March 2018

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Recipes

No.	Day	Mealtime	Ingredients	Amount or weight

Appendix 4, Food intake record, version 2 March 2018

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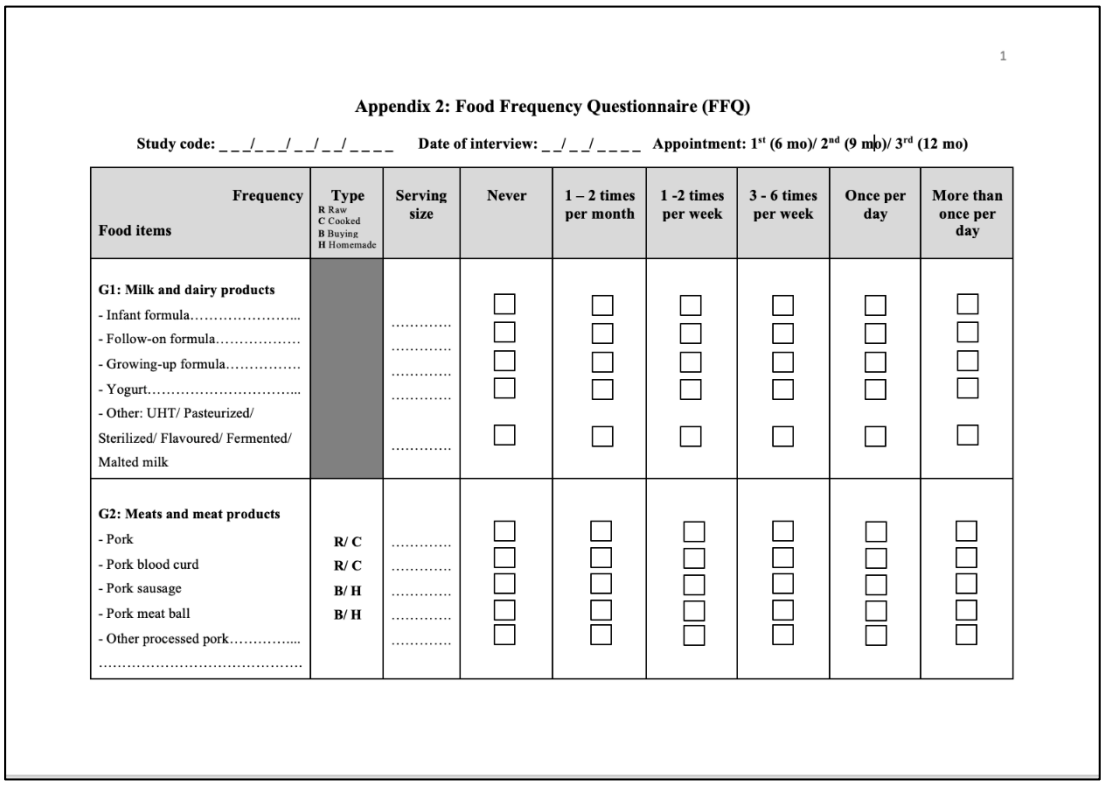
Appendix 8 A record form: semi-quantitative FFQ



1

Appendix 2: Food Frequency Questionnaire (FFQ)

Study code: _ _ _/_ _ _/ _ _/ _ _/ _ _ _ _ Date of interview: _ _/ _ _/ _ _ _ _ Appointment: 1st (6 mo)/ 2nd (9 mo)/ 3rd (12 mo)

Food items / Frequency	Type R Raw C Cooked B Buying H Homemade	Serving size	Never	1 – 2 times per month	1 -2 times per week	3 - 6 times per week	Once per day	More than once per day
G1: Milk and dairy products								
- Infant formula……..		……	☐	☐	☐	☐	☐	☐
- Follow-on formula……		……	☐	☐	☐	☐	☐	☐
- Growing-up formula……		……	☐	☐	☐	☐	☐	☐
- Yogurt……..		……	☐	☐	☐	☐	☐	☐
- Other: UHT/ Pasteurized/ Sterilized/ Flavoured/ Fermented/ Malted milk		……	☐	☐	☐	☐	☐	☐
G2: Meats and meat products								
- Pork	R/ C	……	☐	☐	☐	☐	☐	☐
- Pork blood curd	R/ C	……	☐	☐	☐	☐	☐	☐
- Pork sausage	B/ H	……	☐	☐	☐	☐	☐	☐
- Pork meat ball	B/ H	……	☐	☐	☐	☐	☐	☐
- Other processed pork……..		……	☐	☐	☐	☐	☐	☐
……								

Food items / Frequency	Type (R Raw, C Cooked, B Buying, H Homemade)	Serving size	Never	1 – 2 times per month	1 -2 times per week	3 - 6 times per week	Once per day	More than once per day
- Chicken	R/ C	.....	☐	☐	☐	☐	☐	☐
- Chicken blood curd	R/ C	.....	☐	☐	☐	☐	☐	☐
- Chicken liver	R/ C	.....	☐	☐	☐	☐	☐	☐
- Chicken sausage	B/ H	.....	☐	☐	☐	☐	☐	☐
- River fish…….. ……	R/ C	.....	☐	☐	☐	☐	☐	☐
- Sea fish…….. ……	R/ C	.....	☐	☐	☐	☐	☐	☐
- Canned fish	R/ C	.....	☐	☐	☐	☐	☐	☐
- Canned Tuna	R/ C	.....	☐	☐	☐	☐	☐	☐
- Imitation crab stick	R/ C	.....	☐	☐	☐	☐	☐	☐
- Fish ball	B/ H	.....	☐	☐	☐	☐	☐	☐
- River prawn	R/ C	.....	☐	☐	☐	☐	☐	☐
- Sea prawn	R/ C	.....	☐	☐	☐	☐	☐	☐
- Beef	R/ C	.....	☐	☐	☐	☐	☐	☐
G3: Eggs and products								
- Chicken egg	R/ C	.....	☐	☐	☐	☐	☐	☐
- Duck egg	R/ C	.....	☐	☐	☐	☐	☐	☐

Food items / Frequency	Type (R Raw, C Cooked, B Buying, H Homemade)	Serving size	Never	1 – 2 times per month	1 -2 times per week	3 - 6 times per week	Once per day	More than once per day
- Quail egg	R/ C	.....	☐	☐	☐	☐	☐	☐
- Salted egg	B/ H	.....	☐	☐	☐	☐	☐	☐
- Egg tofu	R/ C	.....	☐	☐	☐	☐	☐	☐
G4: Cereals								
- Stream white rice	B/ H	.....	☐	☐	☐	☐	☐	☐
- Stream brown rice	B/ H	.....	☐	☐	☐	☐	☐	☐
- Stream glutinous rice	B/ H	.....	☐	☐	☐	☐	☐	☐
- Rice porridge	B/ H	.....	☐	☐	☐	☐	☐	☐
- Noodle……..	B/ H	.....	☐	☐	☐	☐	☐	☐
- Egg noodle	B/ H	.....	☐	☐	☐	☐	☐	☐
- Pasta	B/ H	.....	☐	☐	☐	☐	☐	☐
- Commercial cereal……	-	.....	☐	☐	☐	☐	☐	☐
- Cornflake……..	-	.....	☐	☐	☐	☐	☐	☐
- Bread: White/ Whole wheat	B/ H	.....	☐	☐	☐	☐	☐	☐
- Corn	R/ C	.....	☐	☐	☐	☐	☐	☐
- Potato: Mashed/ Fried	B/ H	.....	☐	☐	☐	☐	☐	☐

Food items / Frequency	Type (R Raw, C Cooked, B Buying, H Homemade)	Serving size	Never	1 – 2 times per month	1 -2 times per week	3 - 6 times per week	Once per day	More than once per day
G5: Legumes								
- Peanut	**R/ C**	.....	☐	☐	☐	☐	☐	☐
- Black bean	**R/ C**	.....	☐	☐	☐	☐	☐	☐
- Red bean	**R/ C**	.....	☐	☐	☐	☐	☐	☐
- Mung bean	**R/ C**	.....	☐	☐	☐	☐	☐	☐
- Soya tofu (**Hard**)	**R/ C**	.....	☐	☐	☐	☐	☐	☐
- Soya tofu (**Soft**)	**R/ C**	.....	☐	☐	☐	☐	☐	☐
G6: Vegetables								
- Pumpkin	**R/ C**	.....	☐	☐	☐	☐	☐	☐
- Wax gourd	**R/ C**	.....	☐	☐	☐	☐	☐	☐
- Cucumber/ long cucumber	**R/ C**	.....	☐	☐	☐	☐	☐	☐
- Luffa gourd	**R/ C**	.....	☐	☐	☐	☐	☐	☐
- Tomato	**R/ C**	.....	☐	☐	☐	☐	☐	☐
- Yard long bean	**R/ C**	.....	☐	☐	☐	☐	☐	☐
- Common bean	**R/ C**	.....	☐	☐	☐	☐	☐	☐
- Pea	**R/ C**	.....	☐	☐	☐	☐	☐	☐
- Bean sprout	**R/ C**	.....	☐	☐	☐	☐	☐	☐

Food items / Frequency	Type (R Raw, C Cooked, B Buying, H Homemade)	Serving size	Never	1 – 2 times per month	1 -2 times per week	3 - 6 times per week	Once per day	More than once per day
- Baby corn	**R/ C**	.....	☐	☐	☐	☐	☐	☐
- Carrot	**R/ C**	.....	☐	☐	☐	☐	☐	☐
- Cabbage	**R/ C**	.....	☐	☐	☐	☐	☐	☐
- Cauliflower	**R/ C**	.....	☐	☐	☐	☐	☐	☐
- Chinese cabbage	**R/ C**	.....	☐	☐	☐	☐	☐	☐
- Choi sum	**R/ C**	.....	☐	☐	☐	☐	☐	☐
- Chinese kale	**R/ C**	.....	☐	☐	☐	☐	☐	☐
- Celery tango	**R/ C**	.....	☐	☐	☐	☐	☐	☐
- Spinach	**R/ C**	.....	☐	☐	☐	☐	☐	☐
- Sweet leaf bush	**R/ C**	.....	☐	☐	☐	☐	☐	☐
- Ivy gourd	**R/ C**	.....	☐	☐	☐	☐	☐	☐
- Spring onion	**R/ C**	.....	☐	☐	☐	☐	☐	☐
- Mushroom: Oyster, Orinji,		.....	☐	☐	☐	☐	☐	☐
Golden needle, Straw	**R/ C**	.....	☐	☐	☐	☐	☐	☐
- Seaweed	**R/ C**	.....	☐	☐	☐	☐	☐	☐
G7: Fruits								
- Banana	**R/ C**	.....	☐	☐	☐	☐	☐	☐

Frequency Food items	Type R Raw C Cooked B Buying H Homemade	Serving size	Never	1 – 2 times per month	1 -2 times per week	3 - 6 times per week	Once per day	More than once per day
- Ripen Papaya	R/ C	……	☐	☐	☐	☐	☐	☐
- Pine apple	R/ C	……	☐	☐	☐	☐	☐	☐
- Guava	R/ C	……	☐	☐	☐	☐	☐	☐
- Apple	R/ C	……	☐	☐	☐	☐	☐	☐
- Chinese pear	R/ C	……	☐	☐	☐	☐	☐	☐
- Pear	R/ C	……	☐	☐	☐	☐	☐	☐
- Orange	R/ C	……	☐	☐	☐	☐	☐	☐
- Water melon	R/ C	……	☐	☐	☐	☐	☐	☐
- Ripen Mango	R/ C	……	☐	☐	☐	☐	☐	☐
- Ripen Jackfruit	R/ C	……	☐	☐	☐	☐	☐	☐
- Mangosteen	R/ C	……	☐	☐	☐	☐	☐	☐
- Rambutan	R/ C	……	☐	☐	☐	☐	☐	☐
- Grape	R/ C	……	☐	☐	☐	☐	☐	☐
- Dried fruits	R/ C	……	☐	☐	☐	☐	☐	☐
- Canned fruits	R/ C	……	☐	☐	☐	☐	☐	☐
G8: Beverages and snacks - Fresh fruit juice	B/ H	……	☐	☐	☐	☐	☐	☐

Frequency Food items	Type R Raw C Cooked B Buying H Homemade	Serving size	Never	1 – 2 times per month	1 -2 times per week	3 - 6 times per week	Once per day	More than once per day
- 100% UHT fruit juice	-	……	☐	☐	☐	☐	☐	☐
- Mixed fruit juice	-	……	☐	☐	☐	☐	☐	☐
- Fizzy drink	-	……	☐	☐	☐	☐	☐	☐
- Soy milk	-	……	☐	☐	☐	☐	☐	☐
- Malted/ Cocoa powder (3 in 1)	-	……	☐	☐	☐	☐	☐	☐
- Ice-cream: Milk/ Coconut	B/ H	……	☐	☐	☐	☐	☐	☐
- Crisps	-	……	☐	☐	☐	☐	☐	☐
- Rice based snack	-	……	☐	☐	☐	☐	☐	☐
- Other ready-to-eat snacks	-	……	☐	☐	☐	☐	☐	☐
- Egg cake	B/ H	……	☐	☐	☐	☐	☐	☐
- Spongy cake	B/ H	……	☐	☐	☐	☐	☐	☐
- Toddy palm cake	B/ H	……	☐	☐	☐	☐	☐	☐
- Sweet sticky rice with topping	B/ H	……	☐	☐	☐	☐	☐	☐
- Banana sticky rice	B/ H	……	☐	☐	☐	☐	☐	☐
- Thai coconut pudding	B/ H	……	☐	☐	☐	☐	☐	☐
- Bread bun: Sweet/ Savoury	B/ H	……	☐	☐	☐	☐	☐	☐
- Roti with condense milk & sugar	B/ H	……	☐	☐	☐	☐	☐	☐
- Egg biscuits	B/ H	……	☐	☐	☐	☐	☐	☐
- Crackers/ Wafers/ Breadsticks	-	……	☐	☐	☐	☐	☐	☐

8

Food items / Frequency	Type R Raw C Cooked B Buying H Homemade	Serving size	Never	1 – 2 times per month	1 -2 times per week	3 - 6 times per week	Once per day	More than once per day
G9: Sugar and Sugary snacks								
- Sugar: White/ Brown	**R/ C**	.....	☐	☐	☐	☐	☐	☐
- Condense milk	**R/ C**	.....	☐	☐	☐	☐	☐	☐
- Honey	**R/ C**	.....	☐	☐	☐	☐	☐	☐
- Chocolate sweets	-	.....	☐	☐	☐	☐	☐	☐
- Sweets and toffee	-	.....	☐	☐	☐	☐	☐	☐
- Jelly and gummy	-	.....	☐	☐	☐	☐	☐	☐
G10: Oils and Seasonings								
- Soy-bean oil		.....	☐	☐	☐	☐	☐	☐
- Palm oil		.....	☐	☐	☐	☐	☐	☐
- Rice bran oil		.....	☐	☐	☐	☐	☐	☐
- Avocado oil		.....	☐	☐	☐	☐	☐	☐
- Sun flower oil		.....	☐	☐	☐	☐	☐	☐
- Other oils: sesame, coconut, corn		.....	☐	☐	☐	☐	☐	☐
- Lard oil		.....	☐	☐	☐	☐	☐	☐
- Butter		.....	☐	☐	☐	☐	☐	☐
- Fish sauce		.....	☐	☐	☐	☐	☐	☐
- Soya sauce		.....	☐	☐	☐	☐	☐	☐

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Food items / Frequency	Type R Raw C Cooked B Buying H Homemade	Serving size	Never	1 – 2 times per month	1 -2 times per week	3 - 6 times per week	Once per day	More than once per day
- Flavoured sauce		.....	☐	☐	☐	☐	☐	☐
- Ketchup		.....	☐	☐	☐	☐	☐	☐
- Salt		.....	☐	☐	☐	☐	☐	☐
G11: Supplementations								
- Protein powder……..		.....	☐	☐	☐	☐	☐	☐
- Multivitamin: Syrup/ Suspension ……		.....	☐	☐	☐	☐	☐	☐
- Vitamin B complex……..		.....	☐	☐	☐	☐	☐	☐
- Vitamin C……..		.....	☐	☐	☐	☐	☐	☐
- Elements: Calcium/ Iron/ Zinc		.....	☐	☐	☐	☐	☐	☐
- Other……		.....	☐	☐	☐	☐	☐	☐

Appendix 9 A record form: main data record form

1

Data record form: Part I General information

Hospital: □ **CMU** □ **Chomthong** □ **Health promoting** **Date** ____/____/____

I. Demographic data **Study code: CMU/ CTH/ HPH** __ __ __

Name:

Gender: □ Female □ Male **Date of Birth**___/___/___ **Age**____months

The baby is: □ Only child □ First child □ Second child □ Other (_____)

Maternal screening: □ Normal □ Abnormal (_____)

Gestational age_____weeks

Route of delivery: □ Vaginal delivery □ With forceps/ vacuum □ Caesarean section

Birth weight_____grams **Birth length**_____cm **Head circumference**_____cm

Post-neonatal problem: □ Yes (_____) □ No

Home address:

Telephone number:

Mother's name: _____ **Age:**_____ years

Bodyweight_____ kg **Height**_____ cm **BMI**_____ kg/m^2

Marital status: □ Single □ Married □ Unmarried □ Widowed □ Divorced

Father's name: _____ **Age:**_____ years

Bodyweight_____ kg **Height**_____ cm **BMI**_____ kg/m^2

Who is the main carer for the baby?

□ Mother □ Father □ Grandmother □ Other family member (who_____)

□ Other (who_____)

Who is the main carer preparing foods and feed the baby?

□ Mother □ Father □ Grandmother □ Other family member (who_____)

□ Other (who_____)

II. Socio-economic status

Family income

Who is the main provider financially? □ Father □ Mother □ Other (who_____)

Monthly income (Baht): □ less than 10,000 □ 10,000 – 29,999

□ 30,000 – 49,999 □ more than 50,000

Father's occupation Employed: □ Yes □ No Full-time work: □ Yes □ No Type of work: □ Government □ State enterprises □ Private section □ Self-employed □ Agriculture **Father's education level** □ Uneducated □ Primary school □ Junior high school □ Senior high school □ College certificate □ Bachelor degree □ Higher than bachelor degree	**Mother's occupation** Employed: □ Yes □ No Full-time work: □ Yes □ No Type of work: □ Government □ State enterprises □ Private section □ Self-employed □ Agriculture Working days: _____ days/week Working hours: _____ hours/day **Mother's education level** □ Uneducated □ Primary school □ Junior high school □ Senior high school □ College certificate □ Bachelor degree □ Higher than bachelor degree
Home conditions Home: □ owner □ rent □ other Number of rooms: _____ Separated kitchen: □ Yes □ No Source of drinking and cooking water: □ Stilled water □ Filtered tap water □ Unfiltered tap water □ Other _____	**Family composition** (family members living in the same house) □ Father □ Mother □ Grandfather □ Grandmother □ Aunt □ Uncle □ Children _____ Age: 1. _____ 2. _____ 3. _____ 4. _____ Total number of people in the family_____

III. Mode of feeding since birth

Breast milk: □ Yes □ No	**Exclusively breastfed:** □ Yes □ No Duration_____months	**Breast milk duration** _____months
Infant formula: □ Yes, age of introduction_____months □ No **Which?**_____**for how long**_____days/ months		
Follow-on formula: □ Yes, age of introduction_____months □ No **Which?**_____**for how long**_____days/ months		

Complementary feeding

Age of first introduction_____months

Which foods were introduced? □ Fruit juice □ Mashed banana □ Egg yolk

□ Other (_____)

Why? _____

Who did recommend? _____

Have you ever fed baby by bottle? □ Yes □ No, I used (cup/drinking beaker/_____)

Food	Age of introduce	Preparation	How much	Time (hour)
Fruits				
Meat				
Cereals				
Milk dairy products				
Fish				
Vegetables				
Egg				
Legumes				
Sugar				
Salt/ Fish sauce				
Soya sauce				

IV. History of illness

Date of illness	Diagnosis	Hospitalization	Outcome
		□ **Yes**, _____day(s) □ **No**, any drug? (Y/N) _____	□ Full recovery □ Partial recovery □ Other_____
		□ **Yes**, _____day(s) □ **No**, any drug? (Y/N) _____	□ Full recovery □ Partial recovery □ Other_____
		□ **Yes**, _____day(s) □ **No**, any drug? (Y/N) _____	□ Full recovery □ Partial recovery □ Other_____

Date of illness	Diagnosis	Hospitalization	Outcome
		□ **Yes**, ______day(s) □ **No**, any drug? (Y/N) __________________	□ Full recovery □ Partial recovery □ Other________
		□ **Yes**, ______day(s) □ **No**, any drug? (Y/N) __________________	□ Full recovery □ Partial recovery □ Other________
		□ **Yes**, ______day(s) □ **No**, any drug? (Y/N) __________________	□ Full recovery □ Partial recovery □ Other________
		□ **Yes**, ______day(s) □ **No**, any drug? (Y/N) __________________	□ Full recovery □ Partial recovery □ Other________
		□ **Yes**, ______day(s) □ **No**, any drug? (Y/N) __________________	□ Full recovery □ Partial recovery □ Other________
		□ **Yes**, ______day(s) □ **No**, any drug? (Y/N) __________________	□ Full recovery □ Partial recovery □ Other________
		□ **Yes**, ______day(s) □ **No**, any drug? (Y/N) __________________	□ Full recovery □ Partial recovery □ Other________
		□ **Yes**, ______day(s) □ **No**, any drug? (Y/N) __________________	□ Full recovery □ Partial recovery □ Other________
		□ **Yes**, ______day(s) □ **No**, any drug? (Y/N) __________________	□ Full recovery □ Partial recovery □ Other________
		□ **Yes**, ______day(s) □ **No**, any drug? (Y/N) __________________	□ Full recovery □ Partial recovery □ Other________

Data record form: Part II Growth and Food intake

I. Growth

Date	Age	Weight (g/ kg)	Length (cm)	BMI (kg/m^2)	Head circumference* (cm)	Mid-upper arm circumference* (cm)
	6 months	WAZ WHZ	LAZ	BMIZ	……percentile	MUACZ
	9 months	WAZ WHZ	LAZ	BMIZ	……percentile	MUACZ
	12 months	WAZ WHZ	LAZ	BMIZ	……percentile	MUACZ

**Do the measurement in triplicate and use the mean value*

WAZ-Weight for age z-score; WHZ-Weight for height z-score; LAZ-Length for age z-score; BMIZ-Body mass index z-score; MUACZ-Mid-upper arm circumference z-score

II. Food intake

Food intake will be interviewed by researcher at **6, 9 and 12 months of age** using 24-hours dietary recall *(appendix 1)*, Food frequency questionnaire-FFQ *(appendix 2)*, and Baby eating behaviour questionnaire-BEBQ *(appendix 3)*. Parent will be asked to write the 3-days food record *(appendix 4)* **at 9 and 12 months of age**.

At 6 months

- **How was baby appetite?** □ Normal □ Decreased □ Increased
- **How was the intake?** □ Normal □ Fast □ Slowly
- **Did you offer pre-chewing foods?** □ Yes □ No
- **How was food intake?** □ Usual □ Unusual
- **How was the health status?** □ Healthy □ Unhealthy

If there was some illness, did the illness affect baby appetite? □ Yes □ No

At 9 months

- **How was baby appetite?** □ Normal □ Decreased □ Increased
- **How was the intake?** □ Normal □ Fast □ Slowly
- **Did you offer pre-chewing foods?** □ Yes □ No

- **How was food intake?** □ Usual □ Unusual
- **How was the health status?** □ Healthy □ Unhealthy

If there was some illness, did the illness affect baby appetite? □ Yes □ No

At 12 months

- **How was baby appetite?** □ Normal □ Decreased □ Increased
- **How was the intake?** □ Normal □ Fast □ Slowly
- **Did you offer pre-chewing foods?** □ Yes □ No
- **How was food intake?** □ Usual □ Unusual
- **How was the health status?** □ Healthy □ Unhealthy

If there was some illness, did the illness affect baby appetite? □ Yes □ No

III. Breast milk intake

At 6 months

Did the baby receive breast milk: □ Yes □ No

If yes, □ in the morning □ in the afternoon □ during the night

How many times per day? _____ For how long did you offer _____minutes (approximately)

At 9 months

Did the baby receive breast milk: □ Yes □ No

If yes, □ in the morning □ in the afternoon □ during the night

How many times per day? _____ For how long did you offer _____minutes (approximately)

At 12 months

Did the baby receive breast milk: □ Yes □ No

If yes, □ in the morning □ in the afternoon □ during the night

How many times per day? _____ For how long did you offer _____minutes (approximately)

Total duration of exclusive breastfeeding _____ months

Total duration of predominant breastfeeding _____months

Total duration of any breastfeeding _____months

IV. Acceptability, Tolerance and Affordability

Food	Acceptability			Tolerance		Affordability		
	1 Does not eat	2 Eaten	3 Enjoys eating	1 Tolerate	2 Does not tolerate	1 Yes	2 Difficult to access	3 Cannot afford
Meat - pork - chicken - liver (pork/chicken) - beef - fish (river/sea)								
Egg								
Dairy products - infant formula - follow-on formula - growing-up formula - other dairy product								
Vegetables								
Fruits								
Rice								
Cereals								
Legumes								
Commercial complementary foods								

Data record form: Part III Laboratory report and Body composition

I. Laboratory report

Lab **(Date collected___/____/___)**	**Results**
1. Haemoglobin/ Haematocrit	
2. MCV	
3. Serum ferritin	
4. Serum iron	
5. Serum transferrin	
6. Erythrocyte sediment rate (ESR)	
7. Growth hormone	
8. Insulin-like growth factor 1 (IGF-1)	
9. Insulin-like growth factor binding protein (IGF-BP 3)	
10. Plasma essential amino acids - Leucine - Isoleucine - Valine - Methionine - Phenylalanine - Lysine - Threonine - Tryptophan - Histidine	

II. Results of Body Composition (Date collected ___/___/___) (Analysis ___/___/___)

Body composition	**Results**
Fat mass (FM)	
% Fat mass	
Fat free mass (FFM)	

Appendix 10 An information sheet and a consent form

An inform consent (English version)

Information Sheet for Parents

UCL Research Ethics Committee Approval ID Number: 12551/001

Title of Study: Intake and sources of dietary protein in complementary foods and their impact on infant growth in an area facing double burden of childhood malnutrition

Department: Population, Policy and Practices, Institute of Child Health

Name and Contact Details of the Researcher: Miss Kulnipa Kittisakmontri

E-mail: kulnipa.kittisakmontri.16@ucl.ac.uk Tel. (+44)77 06558053 (UK), (+66)89 6845402 (Thailand)

Name and Contact Details of the Principal Researcher: Professor Mary Fewtrell

E-mail: m.fewtrell@ucl.ac.uk Tel. (+44) 02079052251

You are being invited to take part in a research project conducted by a postgraduate student under the supervision of experienced staff at University College London (UCL). Before you decide it is important for you to understand why the research is being done and what participation will involve. Please take time to read the following information carefully and discuss it with other family members if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part. Thank you for reading this.

1. **What is the purpose of the project?**

After six months of age, breastfeeding alone cannot provide enough nutrients, and complementary foods (so-called weaning foods) are needed to make sure the baby grows and develops normally. Evidence shows that giving too little protein can lead to undernutrition whilst too much may lead to overweight. In addition, the type of protein may also be important. Animal-based proteins are recommended for infants because they are high quality protein (eggs) as well as a good source of calcium (dairy products) and iron (red meat, liver and poultry) compared to plant-based proteins such as rice, cereal, legumes and vegetables. However, we do not know the ideal amount of each type of each protein and this is important in a country like Thailand

In this project, we will investigate the effect of different dietary proteins consumed during the weaning period (from 6 to 12 months) on the growth and the iron status of Thai infants. We will also investigate some of the possible explanations for these effects.

2. **Why has your child been chosen?**

We are recruiting healthy, full-term, singleton infants whose birthweight was at least 2.5kg. If an infant has chronic illness, receives medication regularly or has been diagnosed with malnutrition, they are not eligible for the study.

3. **Do I have to take part?**

It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. However, if you refuse to take part, your child will still receive treatment and care as usual without loss of any benefits.

Whilst there are no other immediate benefits for the families participating in the project, we hope that this work will contribute useful information to improve infant and young child feeding in Thailand.

7. What if something goes wrong?

If you are unhappy with the way the study is conducted and would like to complain, please contact Processor Mary Fewtrell.

Address: Fourth floor, Department of Population, Policy and Practice, Institute of Child Health, University College London, 30 Guildford Street, London United Kingdom, WC1N 1EH
E-mail: m.fewtrell@ucl.ac.uk

However, if you feel that your complaint has not been handled correctly, please, contact the Chair of the UCL Research Ethics Committee by e-mail to ethics@ucl.ac.uk

8. Will my taking part in this project be kept confidential?

All the information that we collect about you and your child during the course of the research will be kept strictly confidential. Only the researcher will access the personal details in order to contact you during the study period. Your baby will be given a study ID number and this will be used during the study. All identifiable data or information that could be linked back to you and your child will be kept separately from other study data. You and your child will not be able to be identified in any reports or publications.

9. Limits to confidentiality

Confidentiality may be limited if your child is diagnosed with anaemia or iron deficiency. If this is found, it is the responsibility of the researcher to refer your baby to a paediatrician for further investigation and treatment.

10. What will happen to the results of the research project?

The results from this research study will be written up as a PhD thesis, published as a scientific paper in peer-reviewed journals and presented at conferences. However, you and your child will not be identified in any report or publication.

The collected data will be stored in safe place where accessibility is restricted to the researchers only. Data might be re-used for additional or subsequent research but your personal data will not be shared or transferred to other organizations or third parties.

11. Data Protection Privacy Notice

The data controller for this project will be University College London (UCL). The UCL Data Protection Office provides oversight of UCL activities involving the processing of personal data, and can be contacted at data-protection@ucl.ac.uk. UCL's Data Protection Officer is Lee Shailer and he can also be contacted at data-protection@ucl.ac.uk.

Your personal data will be processed for the purposes outlined in this notice. The legal basis that would be used to process your personal data will be the provision of your consent. You can provide your consent for the use of your personal data in this project by completing the consent form that has been provided to you.

Your personal data will be processed so long as it is required for the research project. If we are able to anonymise the personal data you provide we will undertake this, and will endeavour to minimise the processing of personal data wherever possible.

During the study period, you have a right to withdraw at any time without giving a reason and without it affecting any benefits. If you decided to withdraw, you will be asked what you wish to happen to the data you have provided up to that point.

4. What will happen to me if I take part?

If you agree, the researcher will phone you after you have had a chance to read the information sheet to answer any questions you may have. If you agree to take part, we will ask you to meet us for 20 to 30 minutes before or after your child visits the paediatrician in the well-baby clinic at 6, 9 and 12 months of age. The study visit will be on the same day as your child's regular check-up appointment so no extra visits are needed.

1. At the first visit (at 6 months), after we have explained the study to you and answered your questions we will ask you to sign a consent form. We will collect information about you (age, education, occupation, family income, marital status) and ask your permission to obtain some details about your pregnancy and your baby from your maternal-child handbook. We will ask you some questions about how you are feeding your baby and the foods that s(he) is eating and given some suggestions for recording food diary. Your baby's weight, length, head and mid-upper arm circumference will be measured.

2. We will ask you to keep a food diary for your baby for 3 days before you come for the second and third visits. You will be asked to write down everything your baby eats and drinks as well as how much. We will give you instructions about how to do this and if you have any questions you will be able to contact us for advice.

3. At the second visit (at 9 months), we will collect your food diary and ask you some more questions about your baby's diet, appetite, and general health. The growth measurements will be repeated.

4. At the final visit (at 12 months), we will collect more information about your baby's diet. In addition, with your permission we will draw a small blood sample – 5ml or about 1 teaspoon - from your baby to check the iron status and test for anaemia. Some of the sample will be frozen so that we can measure some factors that might help explain how different types and amounts of protein in the diet can affect a baby's growth. The sample will be destroyed after the tests have been done. If your baby has anaemia or low iron levels we will tell your paediatrician so that further tests can be done and treatment given if needed.

5. We will also measure the amount of fat and muscle your baby has by giving a drink containing a stable isotope. This is a naturally occurring substance which we all have in our bodies, and this method has been used in hundreds of babies around the world. We will collect a sample of your baby's urine before the drink and again six hours afterwards. This can be done easily by putting cotton wool in the diaper.

All records collected in the study will be kept for at least 5 years. If there is no follow-up study after this project, the records will be destroyed.

5. What are the possible disadvantages and risks of taking part?

There is a possibility that during the blood test your child might experience some discomfort and distress. We will provide the best care for your child by using a local anaesthetic patch to numb the skin and the procedure will be done by experienced nurses or technicians.

6. What are the possible benefits of taking part?

Your baby may benefit from the blood test which will detect any sign of iron deficiency or anaemia which could then be investigated and treated.

If you are concerned about how your personal data is being processed, please contact UCL in the first instance at data-protection@ucl.ac.uk. If you remain unsatisfied, you may wish to contact the Information Commissioner's Office (ICO). Contact details, and details of data subject rights, are available on the ICO website at: https://ico.org.uk/for-organisations/data-protection-reform/overview-of-the-gdpr/individuals-rights/

12. Who is organising and funding the research?

This research project is funded by the Childhood Nutrition Research Centre, UCL Great Ormond Street Institute of Child Health, London, UK. The researcher, Dr Kittisakmontri is funded by a scholarship from the Anandamahidol Foundation under the Royal Patronage of His Majesty the King of Thailand.

14. Contacts for further information

Miss Kulnipa Kittisakmontri (Field researcher in Thailand)

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110, Inthawaroros Rd, Mueang Chiang Mai district, Chiang Mai, Thailand 50200
E-mail: *kulnipa.kittisakmontri.16@ucl.ac.uk* or *kulnipa.k@cmu.ac.th*
Tel. (+66) 5393 5413-15

Professor Mary Fewtrell (Principal researcher)

Address: Fourth floor, Department of Population, Policy and Practice, Institute of Child Health
University College London, 30 Guildford Street, London, United Kingdom, WC1N 1EH
E-mail: *m.fewtrell@ucl.ac.uk*
Tel. (+44) 02079052251

Thank you for reading this information sheet and for considering to take part in this research study.

***If you decide to participate, you will be given a copy of the information sheet and a signed consent form to keep.**

An information sheet (Thai version)

ข้อมูลสำหรับผู้แทนโดยชอบธรรมของอาสาสมัคร
สำหรับการเข้าร่วมในโครงการวิจัย

ชื่อโครงการศึกษาวิจัย : ปริมาณและประเภทของโปรตีนในอาหารตามวัยกับอิทธิพลต่อการเจริญเติบโตของทารกในพื้นที่ซึ่งประสบปัญหาภาวะทุพโภชนาการพร่องและเกินร่วมกัน
หมายเลขโครงการศึกษาวิจัย : PED-2561-05287
ผู้ให้ทุนสนับสนุนการวิจัย : Childhood Nutrition Research Centre, University College London
แพทย์ผู้วิจัยหลัก: แพทย์หญิง กุลนิภา กิตติศักดิ์มนตรี

ท่านได้รับการเชื้อเชิญให้เข้าร่วมการศึกษาวิจัยนี้เนื่องจากผู้อยู่ในความดูแลของท่าน มีสุขภาพสมบูรณ์แข็งและเข้าเกณฑ์การคัดเข้าของการศึกษาวิจัยนี้ ท่านจะได้มีโอกาสและเวลาอ่านข้อมูลข้างล่างก่อน หากท่านมีข้อข้องใจใด ๆ เกี่ยวกับการศึกษาวิจัยนี้ และสิทธิต่าง ๆ กรุณาซักถามจากแพทย์ หรือ ผู้ช่วยแพทย์ที่ทำการศึกษาวิจัย ซึ่งจะเป็นผู้สามารถให้ความกระจ่างได้ นอกจากท่านจะได้รับเอกสารข้อมูลสำหรับผู้แทนโดยชอบธรรมสำหรับการเข้าร่วมในโครงการวิจัยของอาสาสมัคร ฉบับนี้ หากท่านตัดสินใจให้ผู้อยู่ในความดูแลของท่าน เข้าร่วมการศึกษาวิจัย ท่านก็จะได้รับสำเนาใบยินยอมที่เซ็นชื่อกำกับเก็บไว้ 1 ฉบับ เรารู้สึกยินดีที่ท่านได้สละเวลา อ่าน (หรือแพทย์ผู้ศึกษาวิจัยได้อ่านให้ท่านรับทราบ) ข้อมูลดังต่อไปนี้

การศึกษาวิจัยนี้เกี่ยวกับเรื่องอะไร
ที่มาของการศึกษา วัตถุประสงค์ของการศึกษา

ปัจจุบันประเทศไทยประสบปัญหาภาวะทุพโภชนาการในเด็กทั้งภาวะโภชนาการพร่องและภาวะโภชนาการเกิน ถึงแม้ว่าความชุกของปัญหาการขาดสารอาหารอย่างรุนแรงจะดีขึ้นอย่างเห็นได้ชัด แต่ปัญหาน้ำหนักตัวน้อยและเตี้ยยังเป็นปัญหาสำคัญโดยเฉพาะอย่างยิ่งในทารกและเด็กเล็ก ในขณะเดียวกันปัญหาภาวะน้ำหนักเกินและโรคอ้วนมีแนวโน้มเพิ่มขึ้นเรื่อย ๆ ทั้งยังเริ่มเกิดขึ้นในเด็กที่อายุน้อยลงจากเดิม

ปัญหาทุพโภชนาการทั้งพร่องและเกินนี้ไม่ได้ส่งผลกระทบต่อสุขภาพในระยะสั้นเท่านั้นแต่ยังส่งผลต่อปัญหาสุขภาพและพัฒนาการของเด็กในระยะยาวอีกด้วย ดังนั้นโภชนาการในช่วงแรกของชีวิตจึงมีความสำคัญอย่างมากต่อสุขภาพของบุคคลคนหนึ่ง นอกจากการให้นมแม่อย่างเดียวตั้งแต่แรกเกิดจนถึงทารกอายุหกเดือนแล้ว ทารกยังจำเป็นต้องได้รับพลังงานและสารอาหารเพิ่มเติมจาก “อาหารตามวัย” เมื่อทารกอายุตั้งแต่หกเดือนขึ้นไป เนื่องจากพลังงานและสารอาหาร เช่น โปรตีน ธาตุเหล็ก สังกะสี แคลเซียม รวมไปถึงวิตามินหลาย ๆ ชนิดที่ได้รับจากนมแม่อย่างเดียวไม่เพียงพอต่อความต้องการของทารกอีกต่อไป ซึ่งในบรรดาสารอาหารต่าง ๆ โปรตีนนับว่าเป็นสารอาหารหลักที่สำคัญที่สุดสารอาหารหนึ่ง

เป็นที่ทราบกันดีว่าโปรตีนเป็นส่วนประกอบของโครงสร้างหลักของร่างกาย เอนไซม์ และฮอร์โมนมากมายที่จำเป็นในการเจริญเติบโต นอกจากนี้หลักฐานงานวิจัยบ่งชี้ว่าเด็กที่มีภาวะเตี้ยจะมีกรดอะมิโนจำเป็นในเลือดน้อยกว่าเด็กสุขภาพดีทั่วไป ซึ่งกรดอะมิโนจำเป็นเหล่านี้ร่างกายไม่สามารถสร้างเองได้และต้องได้รับจากอาหารประเภทโปรตีนเท่านั้น อย่างไรก็ตามในทางกลับกันการได้รับอาหารประเภทโปรตีนที่มากเกินความจำเป็นก็อาจส่งผลให้ทารกและเด็กเล็กมีการเจริญเติบโตที่รวดเร็วเกินไป ซึ่งเป็นการเพิ่มความเสี่ยงในการเกิดภาวะน้ำหนักเกินและโรคอ้วนตั้งแต่อายุน้อย นอกจากปริมาณจะมีความสำคัญแล้ว ประเภทของโปรตีนก็อาจส่งผลต่อการเจริญเติบโตที่แตกต่างกัน หลักฐานงานวิจัยในปัจจุบันบ่งชี้ว่าทารกและเด็กเล็กที่รับประทานโปรตีนจากสัตว์โดยเฉพาะอย่างยิ่งผลิตภัณฑ์จากนมวัวจะมีแนวโน้มในการเจริญเติบโตรวดเร็วกว่าคนที่ได้รับโปรตีนเหล่านี้ในปริมาณน้อยกว่า

เนื่องจากปัญหาภาวะโภชนาการพร่องและเกินมีแนวโน้มเกิดขึ้นพร้อมกันอย่างเห็นได้ชัดในทารกและเด็กเล็ก อีกทั้งยังไม่มีการศึกษาวิจัยถึงความสัมพันธ์ของปัญหานี้กับอาหารที่เด็กวัยนี้ได้รับมาก่อนในประเทศไทย จึงเป็นที่มาของคำถามงานวิจัยของการศึกษานี้ว่าปริมาณและประเภทของโปรตีนในอาหารตามวัยที่ทารกได้รับในช่วงอายุตั้งแต่หกเดือนจนถึงหนึ่งขวบปีแรกมีอิทธิพลอย่างไรต่อการเจริญเติบโตของทารก โดยวัตถุประสงค์หลักของการศึกษาวิจัยนี้คือการหาปริมาณและประเภทของโปรตีนในอาหารตามวัยที่ทำให้ทารกเจริญเติบโตอย่างเหมาะสมที่สุด เพื่อป้องกันทั้งภาวะโภชนาการพร่องและเกิน นอกจากนี้การศึกษา

ฉบับแก้ไข วันที่ 4 มกราคม 2561

วิจัยนี้ยังดูผลลัพธ์ในเรื่องของธาตุเหล็กในร่างกาย รวมถึงการหากลไกที่เป็นไปได้ในการอธิบายความสัมพันธ์ของโปรตีนในอาหารกับการเจริญเติบโตของทารกอีกด้วย

วิธีการศึกษาวิจัย

การศึกษาวิจัยนี้เป็นการศึกษาแบบสังเกตและติดตามไปข้างหน้า โดยเริ่มบันทึกการรับประทานอาหารและการเจริญเติบโตของทารกตั้งแต่อายุ 6 จนถึง 12 เดือน โดย**ไม่มี**การให้ยาหรือการทดลองทางคลินิกใด ๆ ตลอดระยะเวลาการศึกษา

เหตุผลความจำเป็นที่ผู้อยู่ในความดูแลของท่านได้รับการเชิญเข้าร่วมการวิจัยครั้งนี้

เนื่องจากผู้อยู่ในความดูแลของท่านเป็นทารกสุขภาพดี เกิดครบกำหนดโดยมีน้ำหนักแรกเกิดมากกว่าหรือเท่ากับ 2,500 กรัม

จำนวนอาสาสมัครที่คาดว่าจะเชิญเข้าร่วมโครงการ อย่างน้อย 150 ราย

ผู้อยู่ในความดูแลของท่านจะต้องปฏิบัติตัวอย่างไร

หากท่านตัดสินใจให้ผู้อยู่ในความดูแลของท่าน เข้าร่วมการศึกษาวิจัยนี้ ท่านจะถูกขอร้องให้เซ็นชื่อลงในหนังสือแสดงความยินยอมสำหรับผู้ปกครองของอาสาสมัคร จากนั้น ท่านจะได้รับคำแนะนำเรื่องการบันทึกข้อมูลอาหารจากผู้วิจัย โดยผู้อยู่ในความดูแลของท่าน จะได้รับการติดตามจำนวน 3 ครั้ง เมื่ออายุ 6, 9 และ 12 เดือน ตามวันนัดเพื่อมารับวัคซีนและตรวจสุขภาพ ณ คลินิกเด็กสุขภาพดี โดยไม่มีการนัดนอกเหนือจากวันนัดปกติ ท่านจะถูกขอร้องให้บันทึกข้อมูลอาหารที่ทารกรับประทาน 3 วันต่อสัปดาห์ ก่อนมาพบผู้วิจัยตามนัด เมื่อทารกอายุ 9 และ 12 เดือน

ท่านจะถูกสัมภาษณ์เรื่องข้อมูลการรับประทานอาหารของผู้อยู่ในความดูแลของท่าน โดยผู้อยู่ในความดูแลของท่านจะได้รับตรวจร่างกาย ได้แก่ การชั่งน้ำหนัก วัดความยาว วัดเส้นรอบศีรษะ และเส้นรอบวงต้นแขน ในการนัดติดตามทั้งสามครั้ง

เมื่ออายุ 12 เดือน ผู้อยู่ในความดูแลของท่านจะถูกเจาะเลือดปริมาณ 5 มิลลิลิตร (ประมาณ 1 ช้อนชา) เพื่อตรวจหาความเข้มข้นเลือด ปริมาณธาตุเหล็กสะสมในร่างกาย กรดอะมิโนจำเป็น ฮอร์โมนการเจริญเติบโต รวมไปถึงการเก็บปัสสาวะเพื่อตรวจหาสัดส่วนองค์ประกอบในร่างกาย โดยการเก็บปัสสาวะจะกระทำภายใน 6 ชั่วโมงหลังดื่มน้ำหรือนมที่ผสมสารไอโซโทปเสถียรของธาตุไฮโดรเจน ซึ่งเป็นสารประกอบที่มีตามธรรมชาติและไม่ก่อให้เกิดอันตรายต่อร่างกาย ทั้งยังมีความปลอดภัยในการทำการศึกษาในทารกและเด็กเล็ก

ความเสี่ยงจากการเข้าร่วมการวิจัยนี้

ความเสี่ยงจากการเจาะเลือดทางหลอดเลือดดำอาจทำให้เกิดความเจ็บปวด หรือรอยช้ำหลังการเจาะเลือดได้

มาตรการในการลดความเสี่ยงจากการเข้าร่วมการวิจัยนี้

การเจาะเลือดจะปฏิบัติตามหลักการมาตรฐานโดยพยาบาลหรือนักเทคนิคการแพทย์ผู้ชำนาญ รวมถึงการใช้ยาชาเฉพาะที่ทาบริเวณตำแหน่งที่จะเจาะเลือดเพื่อบรรเทาความเจ็บปวดของผู้อยู่ในความดูแลของท่าน

ผู้อยู่ในความดูแลของท่านจะได้ประโยชน์อะไรจากการศึกษานี้

ผู้อยู่ในความดูแลของท่านจะได้รับการตรวจคัดกรองภาวะขาดธาตุเหล็กหรือภาวะซีดจากการขาดธาตุเหล็ก หากได้รับการวินิจฉัยทั้งสองภาวะนี้ผู้อยู่ในความดูแลของท่านจะได้รับการรักษาอย่างรวดเร็วและเหมาะสมจากแพทย์ หรือหากผู้อยู่ในความดูแลของท่านมีภาวะซีดโดยไม่ได้เกิดจากการขาดธาตุเหล็ก ผู้อยู่ในความดูแลของท่านจะถูกส่งตัวไปพบกุมารแพทย์โรคเลือดเพื่อรับการตรวจวินิจฉัยที่เหมาะสมต่อไป

ค่าใช้จ่ายในการเข้าร่วมวิจัย

ค่าใช้จ่ายอื่น ๆ ที่เกิดจากขั้นตอนการรักษาตามปกติ ท่านจะต้องเป็นผู้รับผิดชอบ ในกรณีที่ผู้อยู่ในความดูแลของท่านมีสิทธิเบิกค่ารักษา กรุณาสอบถามสิทธิ จากแพทย์ผู้วิจัย

ค่าตอบแทน

ท่านและผู้อยู่ในความดูแลของท่านจะไม่ได้รับค่าตอบแทนใด ๆ จากการเข้าร่วมการศึกษาวิจัยนี้

หากผู้อยู่ในความดูแลของท่าน ได้รับบาดเจ็บจากการเข้าร่วมการศึกษาวิจัย

หากผู้อยู่ในความดูแลของท่าน ได้รับบาดเจ็บจากเจาะเลือด ผู้วิจัยจะดูแลรักษาผู้อยู่ในความดูแลของท่านตามมาตรฐาน

หากท่าน/ผู้อยู่ในความดูแลของท่านไม่ต้องการเข้าร่วมการศึกษาวิจัย หรือเปลี่ยนใจระหว่างร่วมศึกษาวิจัย

ท่าน/ผู้อยู่ในความดูแลของท่าน ไม่จำเป็นต้องเข้าร่วมการศึกษาวิจัยนี้หากไม่สมัครใจ หลังจากตัดสินใจจะเข้าร่วมการศึกษาแล้ว ท่าน/ผู้อยู่ในความดูแลของท่าน สามารถจะถอนตัวได้ตลอดเวลา การตัดสินใจของท่าน/ผู้อยู่ในความดูแลของท่าน จะไม่มีผลต่อการรักษาในอนาคต หรือการดูแลอื่นใด

ใครจะรู้บ้างว่าผู้อยู่ในความดูแลของท่าน เข้าร่วมการศึกษานี้

แพทย์ประจำตัวของผู้อยู่ในความดูแลของท่าน (แพทย์เวชปฏิบัติทั่วไป) ควรจะได้รับทราบว่าท่านและผู้อยู่ในความดูแลของท่าน ตัดสินใจเข้าร่วมการศึกษาวิจัยนี้ ข้อมูลของผู้อยู่ในความดูแลของท่าน ที่ถูกบันทึกไว้ระหว่างการศึกษาหรือข้อมูลอื่น ๆ จะถูกเก็บไว้เป็นความลับตลอดเวลา เช่นเดียวกับข้อมูลที่เกี่ยวข้องจากแฟ้มเวชระเบียนของโรงพยาบาล ผู้ที่เกี่ยวข้องกับโครงการวิจัยรวมทั้งคณะกรรมการจริยธรรมการวิจัย สามารถที่จะขอตรวจสอบข้อมูลเหล่านี้ได้ โดยข้อมูลเหล่านี้จะยังเก็บรักษาไว้เป็นเรื่องลับเฉพาะ

การปกป้องรักษาข้อมูล : ข้อมูลใดบ้างที่จะถูกเก็บรวบรวมไว้จากการศึกษานี้

ข้อมูลส่วนตัวที่ท่านและผู้อยู่ในความดูแลของท่าน ไม่ต้องการเปิดเผยจะถูกเก็บรวบรวมไว้และนำมาใช้เพื่อวัตถุประสงค์ทางการวิจัยทางการแพทย์ เฉพาะในส่วนที่เกี่ยวข้องกับการศึกษา โดยจะไม่มีการอ้างถึงชื่อผู้อยู่ในความดูแลของท่าน ในรายงานหรือวารสารใด ๆ โดยผู้วิจัยจะทำทุกวิถีทางเพื่อให้เกิดความมั่นใจว่าข้อมูลส่วนตัวของท่านและผู้อยู่ในความดูแลของท่าน จะถูกปกป้องไว้

หากท่านและผู้อยู่ในความดูแลของท่านมีคำถาม สามารถติดต่อใครได้บ้าง

หากท่านและผู้อยู่ในความดูแลของท่าน มีคำถามหรือมีความวิตกกังวลเกี่ยวกับการศึกษาวิจัยนี้ หรือสงสัยว่าผู้อยู่ในความดูแลของท่าน กำลังได้รับบาดเจ็บจากการเข้าร่วมการวิจัยนี้ กรุณาติดต่อ พญ. กุลนิภา กิตติศักดิ์มนตรี เบอร์โทรศัพท์ 053 945412-5 (ในเวลาราชการ) โทรศัพท์มือถือ 089-68545402 (นอกเวลาราชการ) หรือติดต่อสอบถามเกี่ยวกับสิทธิของอาสาสมัครในการเข้าร่วมโครงการวิจัยได้ที่คณะกรรมการจริยธรรมการวิจัย คณะแพทยศาสตร์ มหาวิทยาลัยเชียงใหม่ 110 ถ.อินทวโรรส ต.ศรีภูมิ อ.เมือง จ.เชียงใหม่ 50200 โทรศัพท์ /แฟกซ์ 0-5394-6643 (ในเวลาราชการ)

A consent from (English version)

CONSENT FORM FOR PARENTS OF A PARTICIPANT IN A RESEARCH STUDY

Please complete this form after you have read the Information Sheet and listened to an explanation about the research.

Title of Study: Intake and sources of dietary protein in complementary foods and their impact on infant growth in an area facing double burden of childhood malnutrition

Department: Population, Policy and Practices, Institute of Child Health

Name and Contact Details of the Researcher: Miss Kulnipa Kittisakmontri
E-mail: kulnipa.kittisakmontri.16@ucl.ac.uk
Tel. 077-0655-8053

Name and Contact Details of the Principal Researcher: Professor Mary Fewtrell
E-mail: m.fewtrell@ucl.ac.uk
Tel. 0207905 Ex 42251

Name and Contact Details of the UCL Data Protection Officer: _____

This study has been approved by the UCL Research Ethics Committee: Project ID number: _____

Thank you for considering taking part in this research. The person organising the research must explain the project to you before you agree to take part. If you have any questions arising from the Information Sheet or explanation already given to you, please ask the researcher before you decide whether to join in. You will be given a copy of this Consent Form to keep and refer to at any time.

I confirm that I understand that by ticking/initialling each box below, I am consenting to this element of the study. I understand that it will be assumed that unticked/ initialled boxes mean that I DO NOT consent to that part of the study. I understand that by not giving consent for any one element that I may be deemed ineligible for the study.

		Tick Box
1.	*I confirm that I have read and understood the Information Sheet for the above study. I have had an opportunity to consider the information and what will be expected of me. I have also had the opportunity to ask questions which have been answered to my satisfaction	
2.	*I understand that I will be able to withdraw at any time	
3.	*I consent to the processing of my age, gender, education, occupation, marital status, monthly income, as well as my child's birth history, dietary records, growth and all	

Consent Form, version 1, January 2018

	laboratory results for the purposes explained to me. I understand that such information will be handled in accordance with all applicable data protection legislation.	
4.	**Use of the information for this project only** *I understand that all personal information will remain confidential and that all efforts will be made to ensure I cannot be identified. I understand that my data gathered in this study will be stored anonymously and securely. It will not be possible to identify me in any publications.	
5.	*I understand that my information may be subject to review by responsible individuals from the University for monitoring and audit purposes.	
6.	*I understand that my participation is voluntary and that I am free to withdraw at any time without giving a reason, without the care or legal rights of my child being affected. *I understand that if I decide to withdraw, any personal data I have provided up to that point will be deleted unless I agree otherwise.	
7.	I understand the potential risks of participating and the support that will be available to me should I become distressed during the course of the research.	
8.	I understand the there is no promise or guarantee of benefits have been made to encourage me to participate.	
9.	I understand that the data will not be made available to any commercial organisations but is solely the responsibility of the researchers undertaking this study.	
10.	I understand that I will not benefit financially from this study or from any possible outcome it may result in in the future.	
11.	I agree that my anonymized research data may be used by the researcher for future research.	
12.	I understand that the information I have submitted will be published as a report and I wish to receive a copy of it. Yes/No	
13.	I hereby confirm that I understand the inclusion criteria as detailed in the Information sheet and explained to me by the researcher.	
14.	I hereby confirm that: (a) I understand the exclusion criteria as detailed in the Information sheet and explained to me by the researcher; and (b) I do not fall under the exclusion criteria.	
15.	I agree that my child will be referred to receive an appropriate care if any unexpected results are found in relation to his/her health.	
16.	I have informed the researcher of any other research in which I am currently involved or have been involved in during the past 12 months.	
17.	I am aware of who I should contact if I wish to lodge a complaint.	

18.	I voluntarily agree to take part in this study.	
19.	**Use of information and blood sample for this project and beyond** I would be happy for the data I provide to be archived in locked cabinet at Department of Paediatrics, Faculty of Medicine at Chiang Mai University, Thailand for at least five years. I understand that other authenticated researchers will have access to my anonymized data. I understand that any blood or urine samples collected from my child which are leftover after the planned analyses have been completed will be destroyed.	

If you would like your contact details to be retained so that you can be contacted in the future by the researchers to invite you to participate in follow up studies to this project, or in future studies of a similar nature, please tick the appropriate box below.

	Yes, I would be happy to be contacted in this way	
	No, I would not like to be contacted	

_____	_____	_____
Name of participant	Date	Signature
_____	_____	_____
Name of witness (If applicable)	Date	Signature
_____	_____	_____
Researcher	Date	Signature

A consent form (Thai version)

หนังสือแสดงความยินยอมสำหรับผู้ปกครองของอาสาสมัคร

(PARENTAL INFORMED CONSENT FORM)

ข้าพเจ้า นาย/นาง/นางสาว..... ซึ่งเป็นผู้ปกครอง/บิดา/มารดาของ อาสาสมัคร ของ ด.ช./ด.ญ. ..... ขอให้ความยินยอมให้ทารกในความปกครองของข้าพเจ้าเข้าร่วมในการศึกษาวิจัยเรื่อง *"ปริมาณและประเภทของโปรตีนในอาหารตามวัยกับอิทธิพลต่อการเจริญเติบโตของทารกในพื้นที่ซึ่งประสบปัญหาภาวะทุพโภชนาการพร่องและเกินร่วมกัน"*

ข้าพเจ้าได้รับข้อมูลและคำอธิบายเกี่ยวกับการวิจัยนี้แล้ว ข้าพเจ้าได้มีโอกาสซักถามเกี่ยวกับการวิจัยนี้และได้รับคำตอบเป็นที่พอใจแล้ว ข้าพเจ้ามีเวลาเพียงพอในการอ่านและทำความเข้าใจกับข้อมูลในเอกสารนี้อย่างถี่ถ้วน และได้รับเวลาเพียงพอในการตัดสินใจว่าจะให้ทารกในความปกครองของข้าพเจาเข้าร่วมการศึกษาวิจัยนี้หรือไม่

ผู้วิจัยมีความยินดีที่จะให้คำตอบต่อคำถามประการใดที่ข้าพเจ้าอาจจะมีได้ ตลอดระยะเวลาการเข้าร่วมการวิจัยครั้งนี้ ผู้วิจัยรับรองว่าจะเก็บข้อมูลเฉพาะที่เกี่ยวกับข้าพเจ้า/ทารกในความปกครองของข้าพเจ้าเป็นความลับ และจะเปิดเผยได้เฉพาะในรูปแบบที่เป็นสรุปผลการวิจัย ผู้วิจัยจะได้ปฏิบัติในสิ่งที่ไม่ก่อให้เกิดอันตรายต่อร่างกาย หรือจิตใจของข้าพเจ้า/ทารกในความปกครองของข้าพเจ้าตลอดการวิจัยนี้ และรับรองว่า หากเกิดมีอันตรายใด ๆ จากการวิจัยดังกล่าว ทารกในความปกครองของข้าพเจ้าจะได้รับการรักษาอย่างเต็มที่

ข้าพเจ้ายินยอมให้ทารกในความปกครองของข้าพเจ้าเข้าร่วมการวิจัยโดยสมัครใจ และสามารถที่จะถอนตัวจากการวิจัยนี้เมื่อใดก็ได้ โดยไม่มีผลกระทบต่อการรักษาพยาบาลที่ทารกในความปกครองของข้าพเจ้าจะได้รับ ถ้าหากทารกในความปกครองของข้าพเจ้าเป็นผู้ป่วย และในกรณีที่เกิดข้อข้องใจหรือปัญหาที่ข้าพเจ้า/เด็กในความปกครองของข้าพเจ้าต้องการปรึกษากับผู้วิจัย **ข้าพเจ้าสามารถติดต่อกับผู้วิจัย คือ พญ. กุลนิภา กิตติศักดิ์มนตรี ได้ที่ ภาควิชากุมารเวชศาสตร์ คณะแพทยศาสตร์ มหาวิทยาลัยเชียงใหม่ โทรศัพท์ที่ทำงาน** (053) 945412-5 **โทรศัพท์เคลื่อนที่** 089-6845402 โดยการลงนามนี้ ข้าพเจ้าไม่ได้สละสิทธิ์ใด ๆ ที่ข้าพเจ้าพึงมีทางกฎหมาย

ลายมือชื่อผู้ปกครอง/บิดา/มารดา _____วัน-เดือน-ปี_____
(_____)

ลายมือชื่อผู้ให้ข้อมูลการวิจัย _____ วัน-เดือน-ปี_____
(_____)

ลายมือชื่อพยาน _____ วัน-เดือน-ปี_____
(_____)

Appendix 11 The Thai complementary feeding recommendations

Age (months)	Carbohydrate (per meal)	Protein (per meal)	Vegetable (per meal)	Fruit (per day)	Oil (per day)	Characteristics of food
6 (1 meal)	Finely mashed rice, 2 tbs	½ Egg yolk alternate with meat, 1 tbs	Boiled vegetable, ½ tbs	Ripe fruit, 1 piece	½ Tea spoon	Finely ground
7 (1 meal)	Soft cooked rice, 3 tbs	½ Whole egg alternate with meat, 1 tbs	Cooked vegetable, 1 tbs	Ripe fruit, 2 pieces	½ Tea spoon	Roughly ground
8 (2 meals)	Soft cooked rice, 4 tbs	½ Whole egg alternate with meat, 1 tbs	Cooked vegetable, 1 tbs	Ripe fruit, 3 pieces	½ Tea spoon	Roughly ground
9–12 (3 meals)	Soft cooked rice, 4 tbs	½ Whole egg alternate with meat, 1 tbs	Cooked vegetable, 1½ tbs	Ripe fruit, 4 pieces	½ Tea spoon	Roughly ground
13–36 (3 meals)	Cooked rice, 6 tbs (1 ladle)	½ Whole egg alternate with meat, 1 tbs	Raw/cooked vegetables, 4 tbs	Fresh fruit, 3 portions[a]	3 Tea spoons	Family food

tbs, table spoon. [a]One portion of fresh fruit = 15 g of carbohydrate from fruit (e.g. 1 portion = 1 medium size orange, 1 apple, ½ guava, ½ Cavendish banana).

6 months old

7 months old

8 months old

9-12 months old

Appendix 12 The Thai DRIs for infants aged 6-12 months (only some nutrients shown in Chapter 7, Result 2)

Nutrients	6-11 months old	12 months
Total energy (kcal/day)	610 (girls) 680 (boys)	980 (girls), 1050 (boys)
Protein (g/kg/day)	1.56	1.20
%Fat	35-40%	35-40%
%CHO	45-65%	45-65%
Calcium (mg/day)	260	500
Phosphorus (mg/day)	275	460
Iron (mg/day)	9	5
Zinc (mg/day)	2.7	4.4
Vitamin A (mcg/day)	250	300
Vitamin B1 (mg/day)	0.3	0.5
Vitamin B2 (mg/day)	0.4	0.5
Vitamin C (mg/day)	50	25

Appendix 13 Examples of data input in the “model mode” of Dagitty.net

1. **Linear growth**

```
dag {
bb="-6.577,-5.248,5.845,5.264"
"Birth length" [pos="0.669,-4.048"]
"Ca intake" [pos="4.509,0.524"]
"Duration of BF" [pos="-5.181,-1.585"]
"Family income" [pos="-2.725,3.956"]
"Linear growth" [outcome,pos="-0.703,0.570"]
"Maternal age" [pos="-4.988,-3.386"]
"Maternal height" [pos="3.498,-2.493"]
"Maternal smoking" [pos="4.268,1.893"]
"Milk feeding" [pos="1.223,4.279"]
"Mum education" [pos="-5.241,2.386"]
"Non-P energy" [pos="-3.171,-4.063"]
"Paternal height" [pos="4.076,-1.493"]
"Protein intake" [exposure,pos="-0.884,-4.078"]
"Working mother" [pos="-5.072,0.524"]
"Zn intake" [pos="4.497,-0.677"]
Gender [pos="2.800,-3.432"]
IUGR [pos="2.077,-4.078"]
Infection [pos="3.197,3.171"]
"Birth length" -> "Linear growth"
"Ca intake" -> "Linear growth"
"Duration of BF" -> "Protein intake"
"Family income" -> "Duration of BF"
"Family income" -> "Linear growth"
"Family income" -> "Milk feeding"
"Family income" -> "Protein intake"
"Family income" -> Infection
"Maternal age" -> "Duration of BF"
"Maternal age" -> "Linear growth"
"Maternal height" -> "Linear growth"
"Maternal smoking" -> "Linear growth"
"Milk feeding" -> "Ca intake"
"Milk feeding" -> "Duration of BF"
"Milk feeding" -> "Linear growth"
"Milk feeding" -> "Protein intake"
"Milk feeding" -> "Zn intake"
"Mum education" -> "Family income"
"Mum education" -> "Linear growth"
"Mum education" -> "Maternal smoking"
"Mum education" -> "Milk feeding"
"Mum education" -> "Protein intake"
"Mum education" -> "Working mother"
"Non-P energy" -> "Linear growth"
```

"Non-P energy" -> "Protein intake"
"Paternal height" -> "Linear growth"
"Protein intake" -> "Linear growth"
"Working mother" -> "Duration of BF"
"Zn intake" -> "Linear growth"
Gender -> "Linear growth"
IUGR -> "Birth length"
IUGR -> "Linear growth"
Infection -> "Linear growth"
Infection -> "Protein intake"
}

2. Ponderal growth

dag {
bb="-6.106,-4.967,5.702,5.358"
"Birth weight" [pos="-1.506,-3.591"]
"Duration of BF" [pos="-3.852,2.713"]
"Family income" [pos="3.791,-1.414"]
"Maternal BMI" [pos="-4.161,0.248"]
"Maternal age" [pos="-3.966,-0.855"]
"Maternal diabetes" [pos="-3.520,-3.622"]
"Milk feeding" [pos="1.732,4.013"]
"Mum education" [pos="-2.902,-2.503"]
"Non-P energy" [pos="-1.884,3.937"]
"Paternal BMI" [pos="3.036,-2.337"]
"Ponderal growth" [outcome,pos="-0.019,0.626"]
"Protein intake" [exposure,pos="-0.168,-2.851"]
"Sugary diets" [pos="1.022,-3.712"]
"Working mother" [pos="3.745,2.939"]
Gender [pos="2.247,-3.168"]
Infection [pos="4.375,1.080"]
"Birth weight" -> "Ponderal growth"
"Duration of BF" -> "Maternal BMI"
"Duration of BF" -> "Non-P energy"
"Duration of BF" -> "Protein intake"
"Family income" -> "Milk feeding"
"Family income" -> "Protein intake"
"Family income" -> Infection
"Maternal BMI" -> "Non-P energy"
"Maternal BMI" -> "Ponderal growth"
"Maternal BMI" -> "Protein intake"
"Maternal age" -> "Maternal diabetes"
"Maternal age" -> "Milk feeding"
"Maternal age" -> "Mum education"
"Maternal age" -> "Non-P energy"
"Maternal age" -> "Protein intake"
"Maternal diabetes" -> "Birth weight"
"Milk feeding" -> "Duration of BF"
"Milk feeding" -> "Non-P energy"

```
"Milk feeding" -> "Ponderal growth"
"Milk feeding" -> "Protein intake"
"Mum education" -> "Family income"
"Mum education" -> "Non-P energy"
"Mum education" -> "Ponderal growth"
"Mum education" -> "Protein intake"
"Mum education" -> "Sugary diets"
"Non-P energy" -> "Ponderal growth"
"Non-P energy" -> "Protein intake"
"Paternal BMI" -> "Ponderal growth"
"Protein intake" -> "Ponderal growth"
"Sugary diets" -> "Ponderal growth"
"Working mother" -> "Family income"
"Working mother" -> "Milk feeding"
Gender -> "Ponderal growth"
Infection -> "Non-P energy"
Infection -> "Ponderal growth"
Infection -> "Protein intake"
}
```

3. Iron status: Hemoglobin, Serum ferritin, Transferrin saturation

```
dag {
bb="-4.488,-5.519,4.334,6.87"
"Age first ASFs" [pos="-2.026,-0.422"]
"Age first CF" [pos="-1.487,1.392"]
"Duration of BF" [pos="1.983,2.625"]
"Iron status" [outcome,pos="0.470,0.485"]
"Iron supplementation" [pos="2.419,0.431"]
"Liver at least 3/wk" [pos="-1.915,-1.964"]
"Milk feeding" [pos="-0.359,2.662"]
"Protein source" [exposure,pos="0.171,-2.599"]
Infection [pos="2.248,-2.617"]
"Age first ASFs" -> "Iron status"
"Age first ASFs" -> "Protein source"
"Age first CF" -> "Age first ASFs"
"Age first CF" -> "Iron status"
"Age first CF" -> "Protein source"
"Duration of BF" -> "Iron status"
"Iron supplementation" -> "Iron status"
"Liver at least 3/wk" -> "Iron status"
"Liver at least 3/wk" -> "Protein source"
"Milk feeding" -> "Duration of BF"
"Milk feeding" -> "Iron status"
"Milk feeding" -> "Protein source"
"Protein source" -> "Iron status"
Infection -> "Iron status"
Infection -> "Protein source"
}
```

Appendix 14 Duration of exclusive breastfeeding and predominantly breastfeeding between infants with normal iron status and infants with ID/ IDA

Average Duration of	Normal (n = 83)	ID/ IDA (n = 62)	*p**
EBF (months)	4.1	4.7	0.58
Predominant BF (months)	**7.7**	**9.3**	**0.04**

*Student's t-test

Appendix 15 Comparison iron status of infants from all study sites

Study sites	Normal	ID/ IDA	*p**
CMU (n = 57)	28 (49.1%)	29 (50.9%)	0.24
CTH[#] (n = 26)	15 (57.7%)	11 (42.3%)	
HPH (n = 62)	40 (64.5%)	22 (38.6%)	

*Chi's square
[#]Delayed cord clamping did not perform at this site.

Appendix 16 Comparison iron status between infants receiving weekly iron supplementation and infants who did not receive iron supplementation

Receiving iron supplementation	Normal	ID/ IDA	*p**
6-9M			
Yes (n = 61)	35 (57.4%)	26 (42.6%)	0.56
No (n = 84)	48 (57.1%)	36 (42.9%)	
9-12M			
Yes (n = 69)	41 (59.4%)	28 (40.6%)	0.37
No (n = 76)	42 (55.3%)	34 (44.7%)	

*Chi's square