ASSESSMENT OF COMPLEMENTARY FEEDING OF CANADIAN INFANTS

By

Wafaa A. Qasem

A Thesis submitted to the Faculty of Graduate Studies of

The University of Manitoba

In partial fulfillment of the requirements of the degree of

DOCTOR OF PHILOSOPHY

Department of Human Nutritional Sciences

University of Manitoba

Winnipeg, Manitoba

Copyright 2015 © Wafaa A. Qasem
Health Canada recommends exclusive breastfeeding (EBF) until 6 months followed by introducing iron-rich complementary foods (CFs) such as iron-fortified cereal and meat to prevent iron deficiency (ID). There is a concern that consumption of CFs with high iron dose may predispose infants to inflammation through reactive oxygen species (ROS) generation in their intestinal tract. The nutrient intake from these recommended first CFs had not been assessed in terms of meeting the daily requirements. Therefore the aim of this study was to assess if the recommended CFs are safe from a free radical and inflammatory perspective and to assess these CFs in relation to socio-demographic characteristics, feeding patterns, nutrient intake, iron status and growth. Eighty-seven EBF infants were randomly assigned to receive one of the following: iron-fortified cereal (Cer), iron-fortified cereal with fruit (Cer+Fr), meat (M). Urine and stool samples were collected before and after introduction of CFs to assess the following markers: urinary F₂-Isoprostanes, fecal ROS, fecal iron and fecal calprotectin. Blood was collected from 18 infants to measure iron parameters. Socio-demographic characteristics and feeding patterns were obtained using questionnaires. Nutrient intake was collected using 3-day dietary records. There are maternal factors that were associated with selected feeding patterns. Nutrient intake was only adequate when provided by both breast milk and CFs. Plasma ferritin decreased over time in all groups ($p = 0.04$). Infants in M group had lower fecal iron than infants in Cer and Cer+Fr groups ($p < 0.001, p = 0.014$, respectively). An increase in fecal ROS formation ($p < 0.002$) after the introduction of CFs was observed. There are maternal socio-demographic factors such as lower parity and lower BMI that...
need to be targeted in the future to optimize feeding time, type and frequency. Infants with EBF may be at risk of developing ID despite the provision of iron-rich CFs. Untargeted iron fortification may result in untoward effects including ROS generation in the infant’s intestinal tract. In future, if these findings are further confirmed in EBF and formula-fed infants, reconsidering the strategies of iron fortifications to both meet infants’ requirements and minimizing oxidative stress maybe warranted.
ACKNOWLEDGEMENTS

Special acknowledge and thanks to my advisor (Prof. James Friel) and my advisory committee members (Dr. James House, Dr. Trust Beta and Dr. Richard Keijzer), for providing me with support and guidance during my doctoral research. Also I would like to acknowledge and thank the research funding agency; Canadian Institutes of Health Research, the Children’s Hospital Research Institute of Manitoba, Manitoba Clinic pediatricians, Prof. Friel’s laboratory group, and my sponsor: Kuwait Cultural Office, Embassy of the State of Kuwait at Washington DC.
# TABLE OF CONTENTS

ABSTRACT .................................................................................................................................. II

ACKNOWLEDGEMENTS ........................................................................................................ IV

LIST OF TABLES ................................................................................................................ XIII

LIST OF FIGURES ............................................................................................................. XV

LIST OF COPYRIGHTED MATERIALS ........................................................................ XVII

ABBREVIATIONS ........................................................................................................... XVIII

CHAPTER I: OVERALL INTRODUCTION ............................................................................ 1

1.1 Introduction .................................................................................................................. 1

1.2 Objectives ................................................................................................................... 4

1.3 Hypotheses .................................................................................................................. 4

1.4 References .................................................................................................................. 5

BRIDGE TO CHAPTER II ............................................................................................... 11

CHAPTER II ..................................................................................................................... 12

MANUSCRIPT 1: LITERATURE REVIEW ..................................................................... 12

AN OVERVIEW OF IRON IN TERM BREASTFED INFANTS ........................................ 12

2.1 Abstract ...................................................................................................................... 12

2.2 Introduction ............................................................................................................... 13

2.3 The full-term infant ................................................................................................. 14
3.3 Results and discussion ........................................................................................................... 44

3.3.1 Meat as a first complementary food ............................................................................... 44

3.3.1.1 Observational studies .............................................................................................. 45
3.3.1.2 Interventional studies ............................................................................................ 46

3.3.2 Iron-fortified cereal as the first complementary food ..................................................... 51

3.3.2.1 Observational studies .............................................................................................. 52
3.3.2.2 Interventional studies ............................................................................................ 53

3.4 Conclusion .......................................................................................................................... 59

3.5 Conflict of interest ............................................................................................................ 59

3.6 Acknowledgment ............................................................................................................... 59

3.7 References ......................................................................................................................... 60

BRIDGE TO CHAPTER IV ......................................................................................................... 69

CHAPTER IV ............................................................................................................................. 70

MANUSCRIPT 3: SYSTEMATIC REVIEW ................................................................................. 70

AGE OF INTRODUCTION OF FIRST COMPLEMENTARY FEEDING FOR INFANTS: A SYSTEMATIC REVIEW ................................................................................. 70

4.1 Abstract ............................................................................................................................... 71

4.2 Introduction ........................................................................................................................ 72

4.3 Methods .............................................................................................................................. 73

4.3.1 Literature Search ........................................................................................................... 73
4.3.2 Inclusion criteria ........................................................................................................... 74
4.3.3 Exclusion criteria .......................................................................................................... 74
4.3.4 Data analysis ............................................................................................................... 74
4.4 Results .................................................................................................................... 74
  4.4.1 Iron ................................................................................................................... 81
  4.4.2 Growth ............................................................................................................. 84
4.5 Discussion ............................................................................................................... 89
4.6 Limitations ............................................................................................................. 90
4.7 Conclusion ............................................................................................................. 90
4.8 References .............................................................................................................. 91

BRIDGE TO CHAPTER V .......................................................................................... 102

CHAPTER V ................................................................................................................. 103

MANUSCRIPT 4: ......................................................................................................... 103

ASSESSMENT OF COMPLEMENTARY FEEDING OF CANADIAN INFANTS:
SOCIO-DEMOGRAPHICS, FEEDING PATTERNS, NUTRIENT INTAKE AND
GROWTH OF BREASTFED INFANTS WHO RECEIVED FIRST
COMPLEMENTARY FOODS .................................................................................... 103

5.1 Abstract ................................................................................................................ 104
5.2 Introduction ......................................................................................................... 105
5.3 Methods ................................................................................................................ 108
  5.3.1 Study design ................................................................................................... 108
  5.3.2 Ethical approval ............................................................................................. 109
  5.3.3 Study subjects ............................................................................................... 111
  5.3.4 Study foods .................................................................................................... 111
  5.3.5 Socio-demographic characteristics ............................................................... 112
5.3.6 Anthropometric measurements ................................................................. 113
5.3.7 Dietary analysis.............................................................................................. 114
5.3.8 Sample size determination ....................................................................... 115
5.3.9 Statistical analysis....................................................................................... 116

5.4 Results ............................................................................................................. 117

5.4.1 Socio-demographic characteristics ........................................................... 117
5.4.2 Feeding Patterns.......................................................................................... 119
5.4.3 Association between socio-demographic characteristics and selected feeding patterns .................................................................................................................... 119
5.4.4 Energy and selected nutrient intake from the recommended first complementary foods only ................................................................. 124
  5.4.4.1 Macronutrient intake .......................................................................... 124
  5.4.4.2 Micronutrient intake ........................................................................... 125
    5.4.4.2.1 Vitamin intake ............................................................................ 125
    5.4.4.2.2 Mineral intake ............................................................................ 127
5.4.5 Growth ...................................................................................................... 128
  5.4.5.1 Growth and nutrient intake from complementary foods .................... 131

5.5 Discussion ...................................................................................................... 135

5.5.1 Socio-demographic characteristics and feeding patterns ...................... 135
5.5.2 Feeding patterns ......................................................................................... 137
5.5.3 Nutrient intake ............................................................................................ 138
5.5.4 Growth ....................................................................................................... 142

5.6 Limitations .................................................................................................... 144

5.7 Conclusion .................................................................................................... 144
# ASSESSMENT OF COMPLEMENTARY FEEDING OF CANADIAN INFANTS: EFFECTS OF IRON RICH FOODS ON IRON STATUS AND OXIDATIVE STRESS MARKERS

## 6.1 Abstract

## 6.2 Introduction

## 6.3 Methods

### 6.3.1 Sample collection

#### 6.3.1.1 Blood sample

#### 6.3.1.2 Urine sample

#### 6.3.1.3 Fecal sample

### 6.3.2 Laboratory methods

#### 6.3.2.1 Hemoglobin

#### 6.3.2.2 Plasma ferritin

#### 6.3.2.3 Urinary creatinine

#### 6.3.2.4 Urinary F2-Isoprostanes

#### 6.3.2.5 Fecal iron

##### 6.3.2.5.1 Sample preparation

##### 6.3.2.5.2 Photometric determination of iron

##### 6.3.2.5.3 Calculation

#### 6.3.2.6 Fecal reactive oxygen species generation

##### 6.3.2.6.1 Phosphate buffer 100 mM solution preparation

---

X
7.4 References........................................................................................................................................ 207

APPENDICES....................................................................................................................................... 210

Appendix 1: Study announcement poster......................................................................................... 210

Appendix 2: Study’s questionnaire ...................................................................................................... 211

Appendix 3: Bannatyne Research Ethics Board approval letter......................................................... 214

Appendix 4: Winnipeg Regional Health Authority approval letter.................................................... 215

Appendix 5: Parent’s study handout ..................................................................................................... 216

Appendix 6: Participants consent form ................................................................................................ 217

Appendix 7: 3-day dietary record (1ST visit) ...................................................................................... 222

Appendix 8: 3-day dietary record (2nd visit) ....................................................................................... 225

Appendix 9: Urine sample collection instructions .............................................................................. 228

Appendix 10: Stool and urine sample collection instructions.............................................................. 230

Appendix 11: Additional results and figures corresponding to the study described in chapters V and VI........................................................................................................................................ 231

Appendix 12: Permissions to use copyrighted materials................................................................. 241
LIST OF TABLES

Table 1. Summary of the clinical feeding interventions with meat ................................. 48

Table 2. Summary of clinical feeding interventions of iron-fortified cereal ................... 56

Table 3. Summary of results of studies included in the systematic review ..................... 76

Table 4. Excluded studies ................................................................................................ 78

Table 5. Nutritional composition of study foods ............................................................ 113

Table 6. Baseline comparisons of the three study group’s characteristics .................... 117

Table 7. Feeding patterns among EBF infants in the three study groups ...................... 120

Table 8. Analysis of selective feeding patterns by socio-demographic characteristics .. 121

Table 9. Energy and macronutrient intake from the recommended first CFs only in the three study groups ........................................................................................................... 124

Table 10. Comparison of mean of % contribution of macronutrients towards the daily recommended DRIs from first recommended CFs only ................................................. 125

Table 11. Vitamin intake from the recommended first CFs only in the three study groups ......................................................................................................................................... 126

Table 12. Comparison of mean of % contribution of vitamins towards the daily recommended DRIs from first recommended CFs only ................................................... 126

Table 13. Selective mineral intake from the recommended first CFs only in the three study groups .................................................................................................................................................. 127

Table 14. Comparison of mean of % contribution of selective minerals towards the daily recommended DRIs from the recommended CFs only ................................................... 128
Table 15. Growth rate in z-scores in the three study groups from before and after introduction of CFs categorized according to solid feeding duration.......................... 129

Table 16. Analysis of selective feeding practices by growth rate................................... 130

Table 17. Summary of biomarker measures of infants in the three study groups ........ 176
LIST OF FIGURES

Figure 1. Iron metabolism at molecular level. .............................................................. 18
Figure 2. Study flow of the systematic review ............................................................ 75
Figure 3. Iron status analyses of developed countries .............................................. 82
Figure 4. Iron status analyses of developing countries ............................................ 83
Figure 5. Weight analyses of developed countries .................................................. 85
Figure 6. Length analyses of developed countries ................................................... 86
Figure 7. Head circumference analyses of developed countries............................. 87
Figure 8. Growth analyses of developing countries ............................................... 88
Figure 9. Study design ............................................................................................. 110
Figure 10. Correlation between total energy intake and gain in head circumference in all infants ................................................................................................................. 132
Figure 11. Correlation between carbohydrate intake and gain in head circumference in all infants ................................................................................................................. 132
Figure 12. Correlation between thiamin intake and gain in head circumference in all infants ..................................................................................................................... 133
Figure 13. Correlation between riboflavin intake and gain in head circumference in all infants ..................................................................................................................... 133
Figure 14. Correlation between iron intake and gain in head circumference in all infants. ..................................................................................................................................... 134
Figure 15. Correlation between phosphorus intake and gain in head circumference in all infants............................................................................................................................ 134

Figure 16. Plasma ferritin level in the cereal group (combined) and the meat group..... 178

Figure 17. Average creatinine corrected F2-Isoprostanes in the urinary samples of infants in the three study groups................................................................. 180

Figure 18. Fecal iron of infants in the three study groups ....................................... 182

Figure 19. Reactive oxygen species generation in fecal samples of infants in the three study groups................................................................. 183

Figure 20. Correlation between dietary iron, fecal iron and ROS production......... 185

Figure 21. Fecal calprotectin concentration of infants in the three study groups........ 186
LIST OF COPYRIGHTED MATERIALS

Figure 1. Iron metabolism at molecular level. Adapted from (Domellof 2007) ..........18
ABBREVIATIONS

ANOVA: analysis of variance
BM: breast milk
BMI: body mass index
Cer: iron fortified cereal group
Cer+Fr: iron fortified cereal with fruit group
CFs: complementary foods
CI: confidence interval
CV: coefficient of variation
DRIs: Dietary Reference Intakes
EBF: exclusive breastfeeding or exclusively breastfed
Hb: hemoglobin
IBD: inflammatory bowel disease
ID: iron deficiency
IDA: iron deficiency anemia
M: meat group
MD: mean difference
Mo: month
N: number
OD: optical density
OR: odds ratio
RCT: randomized controlled trial
ROS: reactive oxygen species
SD: standard deviation
SE: standard error
SR: systematic review
SRM: standard reference material
USA: United States of America
WHO: World Health Organization
Wks: weeks
CHAPTER I: OVERALL INTRODUCTION

1.1 Introduction

Optimal complementary feeding has been acknowledged to be critical for meeting the nutritional requirements of the breastfed infant. Indeed, introduction of nutritionally adequate complementary foods (CFs) is recognized as one of the main measures to prevent nutritional deficiency particularly iron deficiency (ID) (1, 2). The period of infancy also represents a critical window for the development of the brain, and thus of vulnerability to harm from micronutrient deficiencies mainly from lack of iron that may result in permanent neuro-cognitive developmental impairments (3, 4). There is a universal agreement that the breast milk is the optimal complete nutrition standard for infants given all its evident advantages (5). However, at about six months of age, the iron stores of the growing infant start to deplete and breast milk alone no longer meets the developmental needs of the infant (6). Moreover, prolonged exclusive breastfeeding (EBF) has been linked with the development of ID and iron deficiency anemia (IDA) (7, 8). This is due to the fact that breast milk contains relatively low amounts of iron at < 1 mg/l (9). Available evidence from Canada showed that the prevalence of IDA in eight months old breastfed infants is 15% (10). Other studies had also obtained similar results (7, 11). Accordingly, professional health organizations including Health Canada emphasized and recommended the provision of appropriate iron-containing CFs following the EBF six months period to prevent ID and IDA (5, 12).
Health Canada recommended iron-fortified cereal and meat as first iron-rich CFs in their latest statement (12). Iron fortified cereal, which is the traditional most common first CF introduced to Canadian infants (13), contains appreciable amount of iron at 25-30 mg iron/100 g dry weight (14). However, the form of this iron is non-heme electrolytic, which is absorbed at a rate of < 5% (15). Meat is the newly recommended CF by Health Canada and contains heme iron, which is better absorbed at a rate of 35% (16). Despite meat being a good source of iron, the consumption of meat was found to be the least common feeding practice among Canadian infants (13). With these absorption rates, most of the residual iron reaches the large intestine unabsorbed. Available evidence from adult iron supplementation studies demonstrates an association between the residual unabsorbed iron and the generation of reactive oxygen species (ROS) (17, 18). These findings were seen in adults receiving 120 mg/day iron (18). Infants consuming iron-fortified cereals are likely receiving the same amount of iron and probably producing ROS in their intestinal tract (19). This has led to uncertainty and a concern about the possibility of these recommended CFs to cause ROS generation.

Increased amount of ROS generation in the large intestine can lead to adverse health consequences (20). Exposure to iron has been linked with the initiation of intestinal inflammation (21, 22). In addition, oral iron supplementation has been reported as a risk factor to develop inflammatory bowel diseases including crohn’s disease and ulcerative colitis (23, 24). Furthermore, previous evidence suggests that dietary iron seems to promote carcinoma development by increasing inflammation, oxidative stress, and epithelial proliferation (25). Several case-control and prospective studies showed that dietary iron has been positively associated with a subsequent risk of colon cancer (26).
Compelling evidence had showed that anti-oxidants rich foods such as fruits prevent ROS generation thus inflammation and disease initiation (27, 28).

In addition to iron, other specific nutrients need to be derived from CF to avoid nutritional deficiencies that may result from prolonged breastfeeding for infants of this age (29). This is important since the diet of infants play an important role in short and long term health and development (30, 31). While feeding patterns yield a useful measure of an infant’s diet, it is crucial to recognize the nutrient intake underlying the pattern. Previous studies had identified some feeding patterns that were strongly associated with socio-economic factors such as maternal body mass index (BMI), duration of EBF and the type of first CFs (32, 33). The available evidence in regards to the nutrient intakes from CFs consumption and the feeding patterns is limited not only in Canada but also in North America (13, 19, 34-36).

To our knowledge, no studies have considered the iron-fortified cereal (traditional) and meat (new) from the ROS generation and inflammatory perspective. Furthermore, understanding the nutrient intakes from the traditional and from the newly recommended CFs is also part of assessing the efficiency of first CFs. This information along with feeding pattern, socio-demographic characteristics and growth can help us to determine the optimal first CF. Therefore, the aim of the present study is to assess the complementary feeding of Canadian infants by determining if the recommended CFs are safe from a free radical and inflammatory perspective, to determine whether nutrient intakes from the first recommended CFs of breastfed infants are sufficient in terms of meeting the daily recommendation; and to identify the associations between the feeding patterns, the socio-demographic characteristics and the growth of breastfed infants. The
output of this research is fundamental in advancing our knowledge of optimizing the choice of first CFs to mitigate potential free radical injury along with meeting the nutritional and developmental needs of the breastfed infants.

1.2 Objectives

The present research has 6 specific objectives:

1. To determine if infant cereal with iron increase ROS generation in the large intestine of breastfed infants.
2. To determine if infant iron-fortified cereal with an antioxidant reduces the oxidative effect of iron in the intestinal tract.
3. To identify if meat generates ROS in the large intestine of breastfed infants.
4. To identify if iron-fortified cereal and meat maintain the iron status of breastfed infants.
5. To determine whether nutrient intakes from the first recommended CFs are sufficient in meeting the daily recommendations.
6. To determine the associations between the feeding patterns, the socio-demographic characteristics and the growth of breastfed infants.

1.3 Hypotheses

The hypotheses to be tested include:

1- Consumption of infant cereals with iron will increase ROS generation in the gut.
2- Consumption of iron-fortified cereal with fruit will decrease ROS production in the gut.
3- Consumption of meat will not generate ROS.
4- Consumption of either iron fortified cereals or meat will maintain iron status during infancy.

5- Consumption of either iron-fortified cereals or meat will meet the nutritional requirements of breastfed infants.

The following manuscripts (chapter II and III) will present an overview of iron in term breastfed infants and will assess the available body of evidence surrounding breastfed infants’ feeding particularly on complementary feeding. Chapter IV (manuscript 3) will examine the effect of age of introduction of complementary feeding on the iron status and the growth of the EBF infant by performing a systematic review and meta-analyses. Chapter V (manuscript 4) will present a detailed dietary analysis of the breastfed infants from breast milk (before the introduction of CFs), from breast milk with CFs (after the introduction of CFs) and from CFs alone. In addition, chapter V will present the associations between socio-demographic factors, selective feeding patterns and growth. Chapter VI (manuscript 5) will demonstrate a detailed investigation of the effects of the provision of the recommended iron rich CFs on the intestinal health from free radical and inflammatory perspective. Thereafter, chapter VII presents the overall conclusion from the current research.

1.4 References


Available from:


36. Ponza M, Devaney B, Ziegler P, Reidy K, Squatrito C. Nutrient intakes and food
choices of infants and toddlers participating in WIC. J Am Diet Assoc. 2004 Jan;104(1
BRIDGE TO CHAPTER II

The following chapter comprises a manuscript which provides an overview of the current knowledge on the importance of iron in breastfed infants with a focus on recommendations, metabolism, requirements and its relation to oxidative stress. Wafaa Qasem was the principal manuscript author and James Friel supervised and guided the preparation of the manuscript.
2.1 Abstract

**Background:** Iron is an essential nutrient for normal growth and neurodevelopment of infants. Iron deficiency (ID) remains the most common micronutrient deficiency worldwide. There is convincing data that ID is associated with negative effects on
neurological and psychomotor development. **Objectives:** In this review we provide an overview of current knowledge of the importance of iron in normal term breastfed infants with a focus on recommendations, metabolism, iron requirements and its relation to oxidative stress. **Conclusions:** Health organizations around the world recommend the introduction of iron-rich foods or iron supplements for growing infants to prevent ID. However, there is no routine screening for ID in infancy. Multicenter trials with long-term follow up are needed to investigate the association between iron fortification/supplementation and various health outcomes including oxidative stress status.

**Key words:** iron deficiency, iron metabolism, iron recommendations, iron requirements, iron in breastfed infants

### 2.2 Introduction

The period of infancy constitutes a critical window of growth and brain development, and thus micronutrient deficiencies, during this vulnerable period may have adverse effects on neurocognitive functions (1-3). The most common micronutrient deficiency in the world is iron deficiency (ID) and results in approximately one billion cases of anemia worldwide (4). At about 6 months, infants are at risk of developing ID because of the exhaustion of their iron stores needed for rapid growth. In addition, iron concentration in breast milk is relatively low (5-7). Therefore, various organizations recommend the introduction of iron-rich foods to infants or medicinal iron supplements in order to meet their iron requirements (8-10). The aim of the following review is to update health care professionals on the current state of knowledge of iron in full-term breastfed infants with
a particular focus on the recommendations, metabolism, requirements, and its relation to oxidative stress.

2.3 The full-term infant

Full-term infants are those who are born between 37 and 42 weeks of gestation. The normal birth weight of a healthy full-term newborn ranges between 2500 g to 4000 g. Low birth weight describes a weight of less than 2500g at birth regardless of gestational age (11, 12). For the first few months of life, the length of a healthy full-term infant would increase about 3 to 5 cm/month. An infant’s head circumference will increase by 1 to 2 cm each month until 6 months of age to account for the increase in brain development. Growth is an important indicator of normal child development. The period of infancy is characterized by rapid progression of growth that is largely dependent on the infant’s endogenous stores of nutrients and exogenous sources such as human or formula milk. The breast milk intake in exclusively breastfed (EBF) infants appears to meet the energy and nutrient requirements of most infants (13, 14). An extensive body of evidence with improved epidemiological methods substantiates the advantages of breastfeeding for the infants and their mothers. These include developmental, nutritional, immunologic, health, psychological, economic, environmental and social advantages (8, 10). The data from the Maternity Experiences Survey of the Canadian Perinatal Surveillance System show that the rate of initiation of breastfeeding is 90.3% while the rate of exclusive breastfeeding at 3 months is 51.7 %. Six months after birth, the proportions of EBF infants further falls to 14.4 % (15).
2.4 Definitions and international recommendations

In 2001, a joint statement of the World Health Organization (WHO) and the United Nations Children’s Fund (16) recommended “exclusive breastfeeding for the first 6 months of age and introducing nutritionally adequate complementary food along with sustaining breastfeeding up to two years of age or beyond” (17). Exclusive breastfeeding is defined as human breast milk being the only source of food and liquid introduced to the infant. The complementary feeding period (typically 6-12 months of age) begins when food other than human breast milk is introduced to infants in addition to breastfeeding. Weaning is defined as the action of providing non-human milk to the infant regardless of the continuation of breast/bottle feeding (16).

In 2004, Health Canada like many other health organizations, revised its statement on the duration of exclusive breastfeeding to support the Global Strategy of exclusive breastfeeding by WHO (18). Other organizations that endorsed the WHO recommendations include: European Society for Pediatric Gastroenterology, Hepatology, and Nutrition (ESPGHAN) (19), United Kingdom Department of Health (20), Australia National Health and Medical Research Council (21), and New Zealand Ministry of Health (22). Although there is universal agreement that breast milk alone is the optimal first food and should be exclusively introduced for the first 6 months of life, the time of introduction of the complementary food varies among health organizations. For example, in the United States, agencies such as the US Department of Agriculture (23) and the Centers for Disease Control (CDC) (24) recommend complementary food introduction between 4 and 6 months of age. Furthermore, the statement on complementary feeding by ESPGHAN, also recommends that complementary food should not be introduced before 17 weeks nor
later than 26 weeks (25). In Canada, as with global efforts, increasing the rate and duration of breastfeeding until 6 months of age is a main public health target. This has led to less emphasis being given to complementary feeding for the older breastfed infant.

2.5 Iron

In the human body, iron is the most abundant trace element that acts as a center for a broad spectrum of functions. Its importance is derived from its redox activity, because in oxidation states iron exists mainly in ferrous (Fe$^{2+}$) and ferric (Fe$^{3+}$) forms which are interchangeable. This reaction forms part of the electron transport chain, essential in the generation of ATP during metabolism and in the reductions needed for molecule synthesis. About two thirds of iron is utilized as functional iron, which is found in hemoglobin (60%), myoglobin (5%), heme and nonheme enzymes (5%), and with transferrin (< 0.1%). The rest of the iron is stored in the two main storage proteins, ferritin (20%) and hemosiderin (10%) (26). In hemoglobin (Hb), the key function of iron is oxygen transportation, essential for cell respiration. Iron in myoglobin is required to store oxygen in muscles. As a component of tissue enzymes, iron is important in energy production and immune system functioning (27). Owing to its high presence in multiple brain regions, iron plays an important role in essential neurologic processes such as neurotransmitter synthesis and myelination (28).

At birth, full-term healthy infants have a notable iron endowment of about 75 mg/kg, and high blood volume, and Hb concentration in proportion to their body weight. During the first few months of life, they experience a physiological decline in their blood volume and Hb concentration and an active shift from fetal Hb to adult type Hb (7, 14).
2.6 Iron metabolism

In the human body, iron is balanced and regulated in order to prevent deficiency and overload. This balance is achieved through three unique mechanisms: iron storage, erythrocyte iron reutilization, and iron absorption regulation. Therefore, when the body is deficient in iron, absorption is maximum, and when the iron level is sufficient, iron absorption is limited (27, 29). The absorption of iron occurs mainly in the duodenum by an active transport process from the intestinal lumen to the enterocytes (29). If iron is required for metabolic processes, it is released through the enterocytes to the blood and transported by transferrin to the body tissues and the bone marrow. Where there is iron redundancy, it is stored in the enterocytes as ferritin and in the liver, spleen and bone marrow as hemosiderin. When the enterocytes exfoliate, iron is excreted in the feces (27). The peptide hepcidin has recently been suggested as the major iron absorption regulator (30). Hepcidin is synthesized in the hepatocytes and secreted in response to high serum iron levels, which further leads to down regulation of ferroportin expression on the basolateral membrane, and thereby resulting in the blocking of iron release through the enterocytes to the blood stream (31). Regarding iron absorption at molecular level (figure 1), the enzyme duodenal cytochrome b (Dcytb) catalyzes the reduction of \( \text{Fe}^{3+} \) to \( \text{Fe}^{2+} \), which is further transported to the enterocytes via the divalent metal transporter 1 (DMT1). Heme iron is absorbed by the transporter heme carrier protein 1 (HCP1). Recently, HCP1 has been identified as a proton-coupled folate transporter (PCFT) (32). It has been shown in animal models that PCFT/HCP1 is altered in response to the altered folate supply and that each of folate and heme substrates can affect the uptake of the other (33-34). Excess iron is stored intracellularly as ferritin. When iron is needed in the body,
Fe²⁺ is further transported by the transporter ferroportin (FPN1/IREG1) located on the basolateral membrane, which will be oxidized to Fe³⁺ by hephaestin and transported in the blood by transferrin (26). It has recently been theorized that the lactoferrin receptors that have been found in human infant intestinal cells have a role in iron absorption. This process occurs by receptor-mediated endocytosis of iron bound lactoferrin (35).

![Figure 1. Iron metabolism at molecular level. Adapted from Domellof (2007)](image)

### 2.7 Iron requirements

According to the Institute of Medicine (IOM), the amount of iron in human breast milk was used to estimate the adequate intake of iron for healthy full-term infants up to 6
months of age (36). Thus, an adequate intake of 0.27 mg/l was determined. This was calculated by multiplying the mean iron content of breast milk (0.35 mg/l) by the average daily intake of breast milk in EBF infants (0.78 l/day) (36). For infants aged 7 to 12 months, according to the IOM (36) the recommended dietary allowance is 11 mg/day. This value was calculated by adding the amount of iron lost from the urinary and intestinal tracts and from the sheds of skin epithelial cells to the amount of iron needed for increasing tissue mass, blood volume and storage iron during 7 to 12 months of age. This further reinforces the fact that iron needs to increase significantly for 6-month-old full-term infants compared with full-term infants aged less than 6 months.

2.8 Iron deficiency

Iron deficiency is a state where the iron stores progressively decline owing to prolonged negative iron balance. Subsequently, the supply of iron to the tissues is diminished leading to iron deficiency anemia (IDA). Iron deficiency is defined as a plasma ferritin concentration of < 12 µg/l (for children < 5 years) (37). Iron deficiency anemia is diagnosed clinically with signs and symptoms such as fatigue and pallor. The WHO defines anemia as an Hb level of less than 2 standard deviations (SDs) of the average Hb level for a normal population of the same gender and age group. Iron deficiency anemia is diagnosed when the hemoglobin level is < 11.0 g/dl (for children 6 months to 59 months). Other ID and IDA laboratory indicators include: serum iron, total iron binding capacity, serum transferrin, and serum transferrin receptors (37).

Untreated ID has detrimental functional outcomes. There is compelling evidence that early ID either during fetal/neonatal or toddler time periods, impairs cognitive function and retards psychomotor development in childhood, and persists to adolescence and
adulthood (38-40). Neurologic animal studies have explored the pathophysiology by which ID alters neurodevelopmental function and have found that ID results in an alteration in essential neurologic processes such as dopamine metabolism, myelination, and hippocampal function (28, 41, 42). Evidence from more than 40 human studies has linked the observed neurodevelopmental dysfunctions with these abnormal neurologic processes which occur during the ID period (43, 44). In these studies, poor behavioral and cognitive test performances were consistent findings in iron-deficient children who were < 2 years old (45). Moreover, long-term follow-up studies have reported lower achievement in verbal/quantitative learning, intelligence quotient, memory, and attention scores among formerly iron-deficient children than among children in the control group (3, 46, 47). Worldwide, ID is the most common micronutrient deficiency among infants and young children (37, 48). In developed countries, the prevalence of ID is > 8% among preschool aged children (49, 50). Breastfed infants are vulnerable to developing ID because of rapid growth, depletion of their iron endowment, and low iron content in breast milk and in some complementary foods (51). It has been shown that prolonged exclusive breastfeeding for more than 6 months is associated with increased risk of IDA (52). Other non-dietary related causes of infantile ID include intra uterine growth restriction, gestational diabetes, small for gestational age, maternal ID, preeclampsia, early clamping of umbilical cord, and prematurity (53-56). In Canada, Innis et al. found that 34% of breastfed infants living in Vancouver had ID and 7% had IDA (57). Similarly, Friel et al. showed a 33% prevalence with ID and 14% with IDA among breastfed infants living in Newfoundland (58). It was estimated that the prevalence of IDA among Aboriginal children aged 1-5 years was five times higher than among
children living in urban Canada (59, 60). Similar observations have been documented in other developed countries. For example, it was reported that 4% of Norwegian breastfed infants had low iron status (61). In the United States, the National Health and Nutrition Examination Survey (NHANES) has reported an estimated prevalence of ID±A of 7% in young children (62). In Australia, Makrides et al. found ID in 15% and IDA in 1% of 6 month-old breastfed infants (63). Thus, it is noticeable that some breastfed infants are not protected by their iron endowment. To our knowledge, iron status is not routinely examined in infancy, particularly in EBF infants.

2.9 Sources of iron for the growing infant

Hemoglobin iron and storage iron present at birth are the most important iron sources during the first few months of life for full-term infants, particularly breastfed infants (7). Another source of iron is breast milk, which contains a low amount (mean iron content = 0.35 mg/l) with a bioavailability of 45-100% (64). Ferrous sulfate is the form of iron available in cow’s milk based infant formula. Despite ferrous sulfate being well absorbable form of iron, the cow’s milk proteins available in the formula show an inhibitory effect on iron absorption (65). Iron-fortified cereals are the most common source of iron during the complementary feeding period (66). Ferric pyrophosphate and elemental iron are the two types of iron fortificants added to these cereals, which have low bioavailability (67). Depending on the type of weaning food, the infant may receive a household modified diet with low iron content or a highly absorbable heme iron-rich diet such as meat.
2.10 Iron supplementation

Iron supplementation can be delivered either by iron-fortified foods or by medicinal iron. Currently, in Canada there is no recommendation for iron supplementation for the healthy full-term infant. While in the United States, the American Academy of Pediatrics recommends iron supplementation of 1 mg/kg/day for EBF infants at 4 months of age until weaning with iron-rich foods is commenced (10). Available evidence is conflicting regarding whether iron supplementation would suffice and meet the expected beneficial outcomes for the supplemented infants. In a randomized controlled trial (RCT) conducted with Honduran and Swedish infants, two different concentrations of iron supplementation were given. It was observed that infants who received the lower iron supplementation had higher head growth and lower rates of infection than infants in the other group. Iron supplementation increased the rate of gastrointestinal infections and decreased the linear growth of the infants in the higher iron supplementation group. The prevalence of anemia was also found to be significantly lower among supplemented Honduran infant group (68). In another RCT, young infants who received iron-fortified formula of either 1 or 5 mg/l showed no significant difference in the risk of developing anemia between the two groups (69). On the other hand, some iron supplementation trials among 6- to 12-month-old infants showed improvement of serum Hb and ferritin level (58, 70, 71). In Canada, Friel et al, randomized 77 full-term breastfed infants to receive either iron supplementation or a placebo (58). They found improvements in visual acuity and psychomotor functions among infants who received iron supplementation orally. Iron status parameters were also improved among the iron supplemented group (58).
2.11 Iron nutrient interaction

Iron has been found to interact with the following nutrients: lead, zinc, copper, and calcium (72, 73). Lead poisoning has been associated with ID in children. The suggested mechanism was that the up-regulation of DMT1 during ID led to increased lead absorption. It was observed that iron supplementation of iron-deficient lead-exposed children resolved high blood lead concentrations (72, 74). Interaction between zinc and iron appears to be due to the competition between those two nutrients for the same absorptive pathway. However, the available evidence is conflicting regarding the negative effects resulting from the interaction between these two nutrients (75). Iron-deficient status has been linked to negative copper metabolism and vice versa. Iron supplementation showed no effect in improving anemia caused by copper deficiency (76). A negative effect on copper/zinc superoxide dismutase activity by iron supplementation was observed in breastfed infants (77). An interaction between calcium and iron may occur. Therefore, it is recommended to avoid calcium-rich products i.e. milk and calcium supplements, with iron-rich foods (78).

Another compound that impedes the absorption of iron is phytate. Studies have shown a positive association between the amount of phytate contained in a meal with the inhibitory effect of iron absorption by 82% (79, 80). Ascorbic acid has been shown to enhance iron absorption when provided as a food source or as a fortificant (81, 82). Animal tissues such as beef, pork, and chicken are known to provide a good source of heme iron (30-70% of the total iron) as well as having an enhancing effect on iron absorption itself (83, 84).
2.12 Iron and oxidative stress

2.12.1 Iron and reactive oxygen species

From animal and human studies, links have been suggested between excess iron in the body and an increased risk of various diseases such as cancer, vascular disease and certain neurological conditions (85-87). It has been shown that unabsorbed iron may become available to participate in Haber Weiss and Fenton type reactions that increase free radical generation in the colon and reach a level that causes mucosal injury (88). Iron mediated generation of free radicals results in DNA damage and lipid peroxidation (89). This damage is a result of oxygen exaggeration in tissues that have been transported by iron (90). Walter et al, have shown that iron supplementation results in iron accumulation in the colonocytes and hepatocytes as well as lipid peroxidation and mitochondrial dysfunction in a rat model (91). Similar findings of DNA damage and lipid peroxidation in the intestinal cells were observed by Lund et al, (92). In another rat model, it was demonstrated that the ingestion of ferrous sulfate induced acute toxicity and altered oxidative stress markers in the intestinal mucosa and in the liver (93). In a human study conducted by Lund et al, the ingestion of 19 mg of ferrous sulfate for a period of 2 weeks, increased fecal free radical generation by 40% and decreased the fecal antioxidant capacity (94). Friel et al. conducted an RCT of iron supplementation of 7.5 mg/day for 1-6 months in breastfed infants and found that the production of methanesulfinic acid (oxidative stress product) increased in the feces of iron supplemented infants compared with the feces of non-supplemented infants (0.46, 0.10 mM/g respectively) (58). Orozco et al, conducted a two phase study on 17 healthy male subjects. The subjects received either 120 mg iron, 120 mg iron with refined palm oil, or 120 mg iron with refined palm
oil combined with one of the two doses of 0.4 g or 0.8 g of Carotino-Tocotrienol-
Carotene Mixed Concentrate (CTCMC). There was an increase in the concentration of the
hydroxylated compounds in the feces of the iron and iron oil treatment groups. The
production of the hydroxylated compounds was significantly reduced in the feces of the
two iron CTCMC groups compared with the iron oil group. It was concluded that dietary
antioxidants can restore the iron-induced oxidative effects in the colon (95). Infants
receiving high doses of iron through iron-fortified complementary food or medicinal iron
may be at risk of reactive oxygen species generation and thus of intestinal inflammation.
To our knowledge, no studies have assessed the effect of iron fortification on oxidative
stress and intestinal inflammation in infants and children.

2.12.2 Iron and cancer

Experimental animal studies have substantiated the carcinogenicity of iron by showing
that iron may originate and facilitate carcinogenesis by causing free radical production
and promoting neoplasm proliferation (96). In addition, iron acts directly by activation of
transcription factors responsible for cell proliferation causing dysregulation of cell growth
(97). Evidence from cohort studies has found a positive association between high iron
stores and the risk of cancer and cancer mortality (98, 99). Mainous et al, found that
participants with normal iron intake did not have higher rates of cancer despite their high
transferrin concentration levels (100). Moreover, it has been found that a daily intake of
iron of more than 18 mg is associated with an increased risk of cancer development (101).
A causal relationship was observed in some studies between decreasing iron stores by
blood donation and a lower risk of cancer (102, 103). In the NHANES I study, the risk of
colorectal cancer development was positively associated with high dietary and body iron
stores (104). Iron overload has also been associated with colonic precancerous lesions, adenoma, and polyps (105, 106). Data similarly support the observation of excess iron and an increased risk of colorectal cancer in ulcerative colitis patients receiving therapeutic iron supplementation (107).

2.13 Conclusion

There is substantial evidence to support the negative effects of ID on neurodevelopmental outcomes in childhood. Therefore, it is of high importance to insure adequate iron intake during infancy by either complementary feeding or medicinal supplementation. Currently, there is no routine screening for ID in infants, particularly breastfed infants. Serum ferritin is the best representative indicator of iron stores. It may be necessary to develop a screening score to identify infants at risk and target them for supplementation. Recent advances in knowledge have led to greater understanding of iron metabolism, which may direct future research of iron fortification strategies. The lack of evidence in relation to iron supplementation and oxidative stress calls for further research to assess the implications of iron supplementation on the oxidative stress status of infants and children. In future, multicenter trials with long-term follow-up of infant populations are needed to investigate the association between iron fortification/supplementation and various health outcomes such as growth, iron status and intestinal inflammation.

2.14 Conflict of interest and funding

The authors have not received any funding or benefits from industry or elsewhere to conduct this study.
2.15 Acknowledgment

Special thanks to the University of Manitoba for providing us all facilities for carrying out this bibliographic search.

2.16 References


BRIDGE TO CHAPTER III

The following chapter comprises a manuscript which provides a review of the available literature on the research of the complementary foods with a focus on fortified cereal and meat and their effects on infants iron status and other health outcomes that were introduced in chapter II. This chapter further shows the limited evidence available regarding the complementary foods and confirm the existence of knowledge gap in the relation between iron, oxidative stress and inflammation that was discussed in chapter II. Wafaa Qasem was the principle manuscript author and James Friel supervised and guided the preparation of the manuscript.
CHAPTER III

MANUSCRIPT 2: LITERATURE REVIEW

THE RECOMMENDED FIRST COMPLEMENTARY FOODS: A REVIEW OF THE LITERATURE

This manuscript was published in the Journal of Pediatrics & Neonatal Care (2015).


Wafaa Qasem, James Friel

Department of Human Nutritional Sciences, University of Manitoba

Richardson Centre for Functional Foods and Nutraceuticals, 196 Innovation Drive, Winnipeg, Manitoba, Canada, R3T 2N2

Corresponding author: Wafaa Qasem

Richardson Centre for Functional Foods and Nutraceuticals
196 Innovation Drive, University of Manitoba, Winnipeg, MB, Canada, R3T 6C5

Email: qasemw@myumanitoba.ca

Co-author: James Friel

Richardson Centre for Functional Foods and Nutraceuticals
196 Innovation Drive, Winnipeg, Manitoba, Canada, R3T 2N2

Email: James.Friel@umanitoba.ca
3.1 Abstract

**Background:** During infancy, adequate nutrition is essential to allow the healthy growth and development of infants to their full potential. Nutritional deficits, particularly iron deficiency, during this critical period increase the risk of illness and long-term developmental impairment. Therefore, international organizations, including Health Canada, recommend that infants should be primarily introduced to iron-rich complementary foods (CFs), such as iron-fortified cereal and meat, to meet their iron requirements and to prevent growth retardation. **Objectives:** This review examines the available research on the recommended first CFs and their effects on various health outcomes, especially iron status. **Conclusions:** The studies on meat evaluated in this review were inconsistent in their findings regarding the improvement of iron status, although the consumption of meat had positive effects on growth and other health outcomes. Studies on fortified cereals reported an improvement in iron status and possible growth-promoting effects. Further large-scale multicenter trials are needed to support the current findings and to investigate the long-term benefits of these recommended CFs.

3.2 Introduction

During infancy, adequate nutrition is essential to allow the healthy growth and development of infants to their full potential. Nutritional deficits, particularly iron deficiency, during this critical period increase the risk of illness and long-term developmental impairment (1-3). Therefore, optimal infant feeding practices of exclusive breastfeeding (EBF) up to 6 months of age followed by the introduction of nutritionally adequate complementary foods (CFs) are highly important to ensure the disease-free and healthy development of the growing infant (4).
At about 6 months of age, full-term EBF infants are at risk of developing iron deficiency (ID) because of the large amounts of iron needed for growth and the low concentration of iron in breast milk (5-7). Thus, Health Canada recommends that infants should be primarily introduced to iron-rich foods to meet their iron requirements (8). Its statement reinforced the introduction of iron-fortified infant cereals as well as meat and meat alternatives. A survey showed that iron-fortified cereals were the most common first CFs while meats were the least common CFs introduced to Canadian infants (9). It is widely known that the non-heme iron of iron-fortified cereal has an absorption rate of <5% and whereas heme iron available from meats is better absorbed (>35%) (10). In addition to iron, there are other nutrients that need to be obtained from these first CFs to assure the normal development of infants. Thus, an understanding of the efficiency of these first foods in satisfying the developmental needs for this age group is essential. In this literature review, we provide an overview of the existing research on infant complementary feeding, with a focus on fortified cereal and meat, in relation to iron status and other health outcomes.

3.3 Results and discussion

3.3.1 Meat as a first complementary food

As a good source of bioavailable iron and zinc as well as vitamins B6 and B12, meat has been recommended as an appropriate early CF by Health Canada (11, 12). However, a Canadian survey by Friel et al. showed that <10% of 6- to 9-month old infants consume meats (13). In the United States, the Infant Feeding Practices II study found that meat was the least common first solid food introduced to infants (14). In a survey conducted in
four countries; Guatemala, Democratic Republic of Congo, Zambia, and Pakistan, Krebs et al. found that <25% of breastfed infants consumed meat regularly (15). In Germany, only 15% of infants received meat as a first CF (16). Nonetheless, there is increasing agreement regarding the health and growth advantages of meat as the first CF (15). However, the literature contains few studies of the potential benefits of the introduction of meat as a CF. Moreover, the age of the studied population was highly variable (4 months to 24 months), as was the mode of feeding (human milk vs. formula) and the outcome measures (iron status, growth, microbiome, etc.).

3.3.1.1 Observational studies

The few studies on the benefits of meat introduction have been conducted in developing and developed countries. For example, in a cross-sectional survey of 12- to 24-month old Indian children, there was a positive association between length-for-age scores and parental education and meat consumption (17). In another study, conducted on breastfed infants and toddlers in Peru, linear growth was positively associated with the intake of meats (18). In a prospective cohort study conducted in the United Kingdom, 144 full-term infants were recruited at 4 months of age and followed until they reached 24 months of age. Meat consumption of 28.3 g/day from 4 to 12 months was positively associated with weight gain ($p < 0.05$), and psychomotor development ($p < 0.02$) (19). Similar observations were reported in a Danish study, which showed an association between high protein intake and weight gain ($p = 0.03$) (20). In that study, however, in another 198 children, there was no association between iron status parameters, hemoglobin, and zinc concentrations and the intake of meat. When the relation between these parameters and the various diet groups was further explored using the chi-squared test, a significant
inverse relationship was determined between low serum iron and meat intake at 12 months of age \((p < 0.023)\) (21). In the cross-sectional study by Krebs et al., meat consumption was associated with a reduced likelihood of stunting \(\text{odds ratio} = 0.64; 95\% \text{ confidence interval, } 0.46-0.90\) (15). In a retrospective study, data from the China Food and Nutrition Surveillance System indicated that the risk of the non-consumption of animal-source food resulting in stunting and underweight was 28.2\% and 11.7\%, respectively (22). In a multi-country prospective cohort study (the Global Exploration of Human Milk) 365 breast fed infants from the United States (Cincinnati), Mexico (Mexico City) and China (Shanghai) were followed from birth until 2 years of age to examine the growth and health outcomes in relation to breast milk and CF consumption. High-protein foods (meat, eggs, legumes) were introduced earlier among Chinese infants (4.8 months) than among Mexican infants (7.0 months) and American infants (9.3 months; \(p = 0.0001\)). Infants in Shanghai had a significantly higher increase in their anthropometric measurements \(\text{weight-for-age } z\text{-score, length-for-age } z\text{-score, weight-for-length } z\text{-score, BMI-for-age } z\text{-score, head circumference-for-age } z\text{-score}\) at 1 year of age than infants in Cincinnati and Mexico City \((p < 0.001)\). However, an association between earlier high-protein food introduction and anthropometric scores at 1 year of age could not be established with the exception of a longer duration of EBF, which was associated with lower weight-for age z-score \((p < 0.05)\) (23). The authors concluded that the specific feeding practices did not explain the differences in the growth measurements.

### 3.3.1.2 Interventional studies

A number of nutritional intervention trials have targeted the improvement and diversification of complementary feeding practices through nutritional education provided...
to caregivers. For example, in Peru, conveying a message to introduce liver, eggs, and fish into the infant diet resulted in a significant reduction of stunting (24). In a recent randomized controlled trail, 85 EBF Colombian infants were randomly assigned either to a group in which caregivers received recommendations that emphasized meat consumption three times per week (new guidelines group) or to a group in which caregivers received the usual advice on solid-food introduction (control group). At 12 months of age, there was a significant increase in the hemoglobin and the hematocrit levels within the new guidelines group ($p = 0.01, p = 0.03$ respectively). However, there were no significant differences between the two groups with respect to linear growth and zinc levels (25). A clinical study in Denmark randomized 8-month old breastfed infants either to low or high meat-feeding groups for 8 weeks. Infants in the high meat-feeding group had higher serum hemoglobin concentrations than infants in the low meat-feeding group (26). A trial in the United States randomized 88 EBF infants at 4 months of age to receive either pureed meat or iron- fortified cereal. Infants in the meat group had a greater increase in head circumference than infants in the cereal group. Zinc absorption from a test meal was 16-fold higher in the meat group than in the cereal group (27). Table 1 summarizes the clinical feeding interventions in infants in which meat was the CF. Overall, the studies have suggested both the positive effects and the acceptability of meat as a first CF.
<table>
<thead>
<tr>
<th>Author/year</th>
<th>Country</th>
<th>N</th>
<th>Age (mo)</th>
<th>Duration (mo)</th>
<th>Mode of feeding</th>
<th>Intervention</th>
<th>Outcome measures</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dube et al. 2010 (28)</td>
<td>Germany</td>
<td>132</td>
<td>4-6</td>
<td>4</td>
<td>Predominantly BF</td>
<td>Low meat (15.2g/portion) vs high meat (20g/portion)</td>
<td>Iron status parameters: Hb, hematocrit, mean cell volume, mean cell Hb, ferritin, transferrin receptors zinc protoporphyrin, serum iron</td>
<td>- No sig differences founded between the groups in all iron status parameters</td>
</tr>
</tbody>
</table>
| Engelmann et al. 1998 (26) | Denmark     | 41  | 8        | 2             | Partially BF    | Low meat (10g/d) vs high meat (27g/d) | Iron status parameters, serum zinc, growth, illness | - Sig difference in the decline of Hb in the low meat group (-4.9 g/l, p<0.001)  
- No sig decline in the Hb of the high meat group  
- No sig differences in the other iron status parameters, serum zinc, growth parameters, illness |
| Jalla et al. 2002 (29) | USA         | 18  | 5-6      | 1             | EBF             | Iron fortified cereal (55 Kcal/d) vs pureed beef (65 Kcal/d) | Zinc absorption | Absorbed zinc was sig higher from beef than from cereal (p<0.001)                   |
(Table 1 continued)

<table>
<thead>
<tr>
<th>Study</th>
<th>Location</th>
<th>Sample Size</th>
<th>EBF Duration</th>
<th>Intervention</th>
<th>Outcomes</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Krebs et al. 2012 (30)</td>
<td>DR of Congo, Zambia, Guatemala, Pakistan</td>
<td>1062</td>
<td>6</td>
<td>12</td>
<td>EBF</td>
<td>Micronutrient fortified rice-soy cereal (~20g/d) vs meat (30g/d)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>- The linear growth (primary outcome) did not differ sig between the groups.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>- Sig more consumption of food groups among infants in meat group.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>- No sig differences between the groups in infectious disease occurrence.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>- No sig differences in Bayley Scales of infant development were observed.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>- ID was significantly lower in the cereal group (<em>p</em>&lt;0.001).</td>
</tr>
<tr>
<td>Krebs et al. 2012 (31)</td>
<td>USA</td>
<td>45</td>
<td>6</td>
<td>5</td>
<td>EBF</td>
<td>Zinc-iron-fortified cereal (~15g/d) vs organic whole grain iron fortified cereal (~15g/d) vs pureed meat (~71g/d)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>- Sig higher zinc intake for the meat and zinc-iron-fortified cereal groups than for iron-fortified group.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>- Sig higher total absorbed zinc amount was observed for the meat group than the other groups (<em>p</em>&lt;0.027).</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>- Sig association between exchangeable zinc pool size and both zinc intake and total absorbed zinc (<em>r</em>&lt;0.43, <em>p</em>&lt;0.01; <em>r</em>=0.54, <em>p</em>&lt;0.001 respectively).</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>- Sig higher fractional absorption of zinc from CF in the iron-fortified cereal group than in the other groups (<em>p</em>=0.003).</td>
</tr>
</tbody>
</table>
(Table 1 continued)

<table>
<thead>
<tr>
<th>Study</th>
<th>Country</th>
<th>Age (mo)</th>
<th>Weight (kg)</th>
<th>Feeding Type</th>
<th>Activity</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>Krebs et al. 2006 (27)</td>
<td>USA</td>
<td>88</td>
<td>5</td>
<td>EBF</td>
<td>Growth, iron status, zinc level, Bayley Scales of Infant Development.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2</td>
<td></td>
<td>vs pureed beef (mean±SE:80±20 Kcal/d) at 5 mo</td>
<td>- Sig higher increase in head circumference among infants in meat group (p=0.02).</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>- No sig differences in the other anthropometric measurements between the groups.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>- No sig difference in iron and zinc status between the groups.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>- Developmental scores did not differ between the groups.</td>
</tr>
</tbody>
</table>

N.B: BF = breastfed, CF = complementary food, EBF = exclusively breastfed, Hb = hemoglobin, ID = iron deficiency, mo = month, sig = significant
3.3.2 Iron-fortified cereal as the first complementary food

Because of high iron requirements of infants, iron fortification of infant cereal has become an important vehicle to meet the iron needs of the growing infant (32). However, regardless of its fortification, cereal is a low-fat and a low-energy diet and contains phytate (33). Iron-fortified cereal has been the typical first CF offered to infants in Canada and other countries (13, 34). Fruits and vegetables are also commonly introduced after cereal, despite being a poor source of iron. At the end of the infant's first year of life, protein sources are introduced gradually. This complementary feeding practice for the breastfed infant is not unique to Canada but also prevails in both developed and developing countries (34). However, the typical unfortified plant-based cereal may lead to iron and zinc deficiencies if consumed alone without supplements (35). In various African and Asian countries, the introduction of homemade plant-based cereal is a common practice owing to its affordability; however, foods such as maize porridges are low in iron, zinc, and vitamin A (33, 36). Therefore, the fortification of these low-nutrient-density foods has been adopted as a strategy to address micronutrient deficiency and malnutrition in developing countries. In developed countries, the fortification of food is the primary strategy to prevent micronutrient deficiency in infants, and improvements in the rate of anemia and growth have been documented (37). In 2003, a joint statement between the Pan American Health Organization and the WHO was published to provide consistent child-feeding guidelines (35). These addressed EBF duration and the age at which solids should be introduced, breastfeeding maintenance, responsive feeding, the preparation and storage of CFs, the amount and consistency of CFs, CF feeding frequency, the energy density of CFs, their nutrient content, the use of vitamin and
mineral supplements and fortified food for mothers and infants, and feeding throughout and after illness. Among the guidelines on CFs, it was recommended that breastfed infants living in industrialized countries consume 130 kcal/day at 6-8 months, 310 kcal/day at 9-11 months, and 580 kcal/day at 12-23 months of age. A gradual increase in food consistency, variety, and frequency with increasing infant age was also recommended, as was the daily consumption of meat, poultry, fish, eggs, fruits, and vegetables. The guidelines supported the use of fortified CFs for the infant as needed.

3.3.2.1 Observational studies

Infant feeding practices and the factors that influence them, such as maternal education, formula introduction, and breastfeeding initiation, have been investigated in a small number of studies (38-40). In developing countries, most of the observational studies examined the association between local complementary feeding practices and growth parameters, including stunting. In a cross-sectional study conducted on breastfed Malawian children, both feeding practices and the nutritional quality of CF were evaluated. The results showed that >70% of the total energy intake of 6-month old infants was from cereals and only 2-4% from meats. Intakes of iron, niacin, zinc, and calcium at all bioavailability levels were inadequate in all cases. The study reported a prevalence of at least 25% of low anthropometric scores among infants in all age groups. No correlation tests were conducted between growth, micronutrient status and complementary feeding (41). Several other observational studies have documented the relationship between feeding micronutrient-fortified CF and iron status, zinc status, anemia rate, growth, and morbidity. For example, in a cohort of 76 full-term Swedish infants, iron, zinc, and hemoglobin status were evaluated in relation to feeding with iron- and zinc-rich foods.
Iron depletion was prevalent in 26% of the infants and a low serum zinc concentration in 36%. Although there was no association between feeding pattern and iron depletion, the author suggested that the high intake of cereal as the first CF affected the bioavailability of both iron and zinc. In 200 Iranian infants, the differences in weight and length gains between EBF infants and infants who received CF were not significant. Moreover, the EBF infants had lower rates of occurrence of gastrointestinal and respiratory illnesses (43).

3.3.2.2 Interventional studies
In the studies described in the literature, iron was supplemented through different routes: orally, parenterally, as iron-fortified infant formula, and as iron-fortified cereal. Most interventions have been conducted in developing countries, where the prevalence of ID and stunting are high (44). Table 2 summarizes the clinical feeding interventions of iron-fortified cereal as a CF in infants. The trials included in the table varied in terms of population size, age group, duration, and the measured outcome variables, which in most cases were the effects of the fortified cereal on iron status parameters and growth. None of the studies showed relevant effect of iron-fortified cereal on weight and length gains or other growth parameters, although this may have been due to the short follow-up periods. Other possible explanations of the inconsistencies of the results are differences in the iron compounds used and the amount provided. Most of the interventions showed significant positive effect of iron-fortified cereal consumption on iron status parameters; however, some interventions used CFs containing multiple micronutrients, which calls into question any claim of a cause-and-effect relationship between iron consumption and iron status improvement (Table 2). Eichler et al., carried out a meta-analysis to weigh the
evidence for positive effects of fortified infant formula combined with cereal foods on infants and children (44). A clinically relevant increase in serum hemoglobin levels was determined (mean increase of 0.6 g/dl; 95% CI: 0.34-0.89) for children consuming iron-fortified formula and cereal. Similarly, iron fortification increased the mean ferritin level by 11.3 µg/l (95% CI: 3.3-19.2) compared with the control group. Iron fortification of formula and cereal was also shown to reduce the risk of anemia by 50% (risk ratio = 0.05, 95% CI: 0.33-0.75). Although the meta-analysis results were consistent with the promising effects of iron fortification on functional health outcomes, one limitation is that the data were pooled from both iron-fortified formula and cereal interventions. Another systematic review examined interventions and programs aimed at enhancing biological and clinical outcomes with CFs (45). However, rather than a meta-analysis of the effects of these foods on the outcomes, the results were presented as averaged effect sizes. The interventions included in that systematic review targeted mainly breastfed children ages 6-24 months. The strategies of the reviewed interventions included: offering education about complementary feeding, providing CFs with extra energy, the provision of both CFs and education, the use of fortified CFs, and increasing the nutrient bioavailability and energy density of CFs by simple technology. Studies that measured iron status parameters after iron-fortified complementary interventions showed an average impact increase of a mean hemoglobin of 6 g/l and a 17% reduction in the prevalence of anemia. However, the results on growth were inconsistent, with an overall mean effect size of 0.60 for weight and 0.47 for linear growth. The authors concluded that the provision of iron-fortified foods along with educational messages can substantially improve iron status, growth, and behavioral development and lower morbidity. Other reviews also combined data from
iron supplementation and CF fortifications studies in their analyses or included studies on children as well as adult women (46-49).
<table>
<thead>
<tr>
<th>Author/ year</th>
<th>Country</th>
<th>N</th>
<th>Age</th>
<th>Duration</th>
<th>Mode of feeding</th>
<th>Intervention</th>
<th>Outcome measures</th>
<th>Results</th>
</tr>
</thead>
</table>
| Faber et al. 2005 (50) | South Africa | 361 | 6-12| 6 months | Predominantly BF | Fortified maize-porridge (27.5 mg/d iron) vs unfortified maize porridge | Hb, serum retinol, zinc, ferritin, motor development, growth | - Sig intervention effect for ferritin (9.4 µg/L) and for Hb (9 g/L) among the fortified porridge group  
- Sig decrease in proportion of anemic infants from 45% to 17% in the fortified porridge group  
- Sig higher motor development score achievement (15.5 points) in fortified porridge group than the control group (14.4 points) (p=0.007)  
- No sig differences between the groups in growth parameters |
| Gibson et al. 2011 (51) | Zambia     | 743 | 6  | 12        | Predominantly BF | Fortified porridge (65% maize, 15% beans, 20% nuts), (5.36 mg/d iron) vs unfortified porridge | Iron status parameters, micronutrients deficiency, prevalence of anemia | - Sig treatment effect on serum Hb, ferritin, transferrin receptor (p<0.001), and selenium (p=0.009)  
- No treatment effect on serum zinc  
- Consumption of fortified porridge reduced the odds of anemia by 63%, elevated transferrin receptor by 79%, low ferritin by 72% and IDA by 82% |
| Javaid et al. 1991 (52) | Pakistan  | 86  | 6-12| 8        | Predominantly BF | Fortified milk cereal with ferrous fumarate (7.5mg/100g) vs fortified milk cereal with ferric pyrophosphate (7.5mg/100g) vs unfortified milk cereal | Iron status parameters, growth, morbidity | - Sig higher Hb and ferritin levels in the iron fortified cereal groups than unfortified group  
- Sig higher weight gain in the iron-fortified cereal groups  
- Sig lower incidence of malnutrition among infants in the iron-fortified cereal groups  
- Sig lower incidence of acute diarrhea in both iron fortified groups than in unfortified group (p<0.05)  
- No sig differences in incidence of infections between the groups |
(Table 2 continued)

<table>
<thead>
<tr>
<th>Study</th>
<th>Country</th>
<th>Sample Size</th>
<th>Duration</th>
<th>Type</th>
<th>Intervention Details</th>
<th>Outcomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Larney et al. 1999</td>
<td>Ghana</td>
<td>208</td>
<td>6-12</td>
<td>Partially BF</td>
<td>3 Fortified cereal-legume blend (Weaninix) groups (Weaninix only, Weaninix+multi-vitamins, Weaninix+fish powder) vs unfortified maize porridge group</td>
<td>Iron status parameters, serum zinc, erythrocyte riboflavin, vitamin A, growth, morbidity - No sig differences between the groups in growth parameters - No sig differences between the groups in iron parameters, serum zinc, erythrocyte riboflavin - Sig higher plasma retinol level among infants in the vitamins + minerals fortified porridge group - Sig improvement in all growth parameters in the combined intervention groups compared with non-intervention group ($p&lt;0.001$)</td>
</tr>
<tr>
<td>Lind et al. 2004</td>
<td>Sweden</td>
<td>300</td>
<td>6-12</td>
<td>Formula fed &amp; BF</td>
<td>Commercial milk-based porridge vs phytate-reduced porridge vs formula-based porridge</td>
<td>Growth, Bayley scales of infant development, morbidity - No sig differences between the groups in growth, development and morbidity - 77% higher risk of diarrhea among infants in the formula-based porridge group</td>
</tr>
<tr>
<td>Lind et al. 2003</td>
<td>Sweden</td>
<td>300</td>
<td>6-12</td>
<td>Formula fed &amp; BF</td>
<td>Commercial milk-based porridge vs phytate-reduced porridge vs formula-based porridge</td>
<td>Iron and zinc status parameters - Sig higher Hb level in the phytate-reduced porridge group ($p=0.012$) - Lower prevalence of anemia among phytate-reduced porridge group - No sig differences in the other iron and zinc parameters between the groups</td>
</tr>
<tr>
<td>Mamiro et al. 2004</td>
<td>Tanzania</td>
<td>137</td>
<td>6</td>
<td>Predominantly BF</td>
<td>Processed cereal (to increase E density &amp; Fe solubility &amp; to decrease phytate) (65.2% finger millet, 19.1% kidney beans, 7.7% mango puree) vs unprocessed cereal</td>
<td>Iron status parameters, Fe intake, zinc protoporphyrin, growth - No significant differences in Hb level between the groups - No sig differences in iron intake between the groups - No sig differences in zinc protoporphyrin and in growth z-scores between the groups were found</td>
</tr>
<tr>
<td>Study</td>
<td>Country</td>
<td>Sample size</td>
<td>Duration</td>
<td>Feeding Method</td>
<td>Intervention</td>
<td>Outcome Parameters</td>
</tr>
<tr>
<td>---------------------</td>
<td>-------------</td>
<td>-------------</td>
<td>----------</td>
<td>----------------</td>
<td>--------------</td>
<td>-------------------------------------------------------------------------------------</td>
</tr>
</tbody>
</table>
| Menon et al. 2007   | Haiti       | 415         | 9-24     | Predominantly BF | Fortified wheat-soy blend (sprinkles) vs unfortified wheat-soy blend | Iron status parameters | - Sig increase in Hb level among infants in the fortified wheat-soy blend group ($p<0.001$).  
- Sig decline in anemia rate in infants in the fortified wheat-soy blend group (54% to 14%, $p<0.05$) |
| Oelofse et al. 2003 | South Africa | 60          | 6        | Partially BF | Fortified porridge (0.8 mg/d iron) vs normal diet | Iron status parameters, zinc level, retinol level, growth, psychomotor development | - Sig higher retinol level among infants in the fortified porridge group ($p<0.005$)  
- No sig difference in the Hb and total iron level between the groups  
- No sig differences were detected in growth parameters and psychomotor development scores between the groups  
- No sig difference in zinc level between the groups |
| Owino et al. 2007   | Zambia      | 150         | 6        | Predominantly BF | Fortified porridge (65%maize, 15%beans, 20%nuts) vs fortified porridge + amylase vs control | Iron status parameters, growth, breast milk intake | - Sig increase in Hb level among infants in the iron-fortified groups compared to control.  
- Sig lower rate of anemia in iron-fortified groups  
- No differences in breast milk intake between the groups  
- No sig differences in weight and length z-scores between the groups |
| Pham et al. 2012    | Vietnam     | 426         | 5        | Predominantly BF | 2 fortified gruel vs unfortified gruel | Growth | - Sig higher growth parameters scores in the fortified gruel groups compared to the unfortified gruel group |
| Walter et al. 1993  | Chile       | 515         | 4        | EBF           | Iron-fortified rice cereal with electrolytic iron (55mg/100g dry cereal) vs unfortified rice cereal | Iron status parameters | - Sig differences between the groups in Hb level  
- No sig differences in ferritin level between the groups |

N.B: BF = breastfed, CF = complementary food, E = energy, EBF = exclusively breastfed, Hb = hemoglobin, mo = month, sig = significant
3.4 Conclusion

Optimal complementary feeding is a desired goal for health care providers and caregivers to ensure the optimal growth and development of infants. Micronutrients such as iron need to be derived from CFs to compensate for their low levels in breast milk. Traditional CFs (fortified cereals) provide non-heme iron, which has a low absorption rate. By contrast, meats (newly recommended) are rich in heme iron with a favorable absorption rate and contain other essential nutrients. A limited number of interventional studies on meat are available, and their findings are inconsistent with respect to its improvement of iron status. Nonetheless, the results have suggested other beneficial health outcomes of meat feeding. Based on the studies considered in this review, it can be concluded that the consumption of fortified cereals results in an improvement of iron status and possibly also growth. These findings support the current complementary feeding recommendations of the WHO and Health Canada regarding the introduction of fortified cereal and meat as first CFs for infants. Further large-scale, multicenter trials are needed to validate these conclusions and to investigate the long-term benefits of the recommended CFs.

3.5 Conflict of interest

The authors declare they have no conflicts of interest and shall disclose any potential conflicts of interest in the future.

3.6 Acknowledgment

We thank the University of Manitoba for providing us with all facilities needed to carry out the bibliographic search that formed the basis of this review.
3.7 References


37. Lutter CK, Rivera JA. Nutritional status of infants and young children and characteristics of their diets. J Nutr. 2003 Sep;133(9):2941S-9S.


BRIDGE TO CHAPTER IV

The following chapter comprises a manuscript which presents a systematic review and a meta-analysis on the effect of age of introduction of complementary foods on EBF infants. As introduced in chapter II, the age in which solids should be commenced is uncertain and controversial. This may lead to iron deficiency in some infants. Therefore, this systematic review addresses an important and controversial topic - namely the effect of introducing solids at 4 v 6 months on iron status and growth. Wafaa Qasem is the principal author of this systematic review. She conceptualized and designed the study, carried out the analyses and the data search, and drafted the manuscript. Dr. Tanis Fenton carried out the data search, reviewed and revised the manuscript, and approved the final manuscript. Dr. James Friel conceptualized and designed the study, and coordinated and supervised data search, reviewed the manuscript, and approved the final manuscript.
CHAPTER IV

MANUSCRIPT 3: SYSTEMATIC REVIEW

AGE OF INTRODUCTION OF FIRST COMPLEMENTARY FEEDING FOR INFANTS: A SYSTEMATIC REVIEW

This manuscript has been accepted for publication in BMC Pediatrics (2015)

Wafaa Qasem, Tanis Fenton, James Friel

Corresponding author: Wafaa Qasem, MD

Department of Human Nutritional Sciences, University of Manitoba, Winnipeg, Manitoba, Canada, R3T 2N2

Richardson Centre for Functional Foods and Nutraceuticals

196 Innovation Drive, University of Manitoba, Winnipeg, MB, Canada, R3T 6C5

Co-author: Tanis Fenton, PhD

Nutrition Services, Alberta Health Services, Alberta Children’s Hospital Research Institute, Department of Community Health Sciences, University of Calgary

TRW Building, 3280 Hospital Dr NW, Calgary, Alberta, Canada, T2N 4Z6

E-mail: tfenton@ucalgary.ca

Co-author: James Friel, PhD

Department of Human Nutritional Sciences, University of Manitoba

Richardson Centre for Functional Foods and Nutraceuticals

196 Innovation drive, Winnipeg, Manitoba, Canada, R3T 2N2

E-mail: James.Friel@umanitoba.ca
4.1 Abstract

Background: With the recommendation of exclusive breastfeeding to six months of age for all full-term infants, it is not clear what the health implications may be. Eventually breast milk alone may not meet nutrition needs for the growing infant leaving them at risk for deficiencies. Objective: to investigate the relation between moderate (4 months) versus late (6 months) introduction of complementary foods to the full-term breastfed infant related to iron status and growth. Methods: an electronic search of electronic and gray-literature for randomized control trials (RCTs) related to the timing of introduction of complementary foods was conducted. Iron status and growth data from the relevant RCTs were analyzed using RevMan 5.2.11. Results: Limited data met the inclusion criteria: four RCTs and one observational study. Our meta-analysis showed that there was a significantly higher hemoglobin among the 4 months solid fed infants versus 6 months solid fed infants living in developing countries [mean difference [MD]: 5 g/l; 95% CI: 1.54, 8.6 g/l; \( p = 0.005 \)], and serum ferritin levels in both developed and developing countries [MD: 26 \( \mu g/l \); 95% CI: -0.10, 52.10 \( \mu g/l \), \( p = 0.05 \)], [MD: 18.90 \( \mu g/l \); 95% CI: 0.74, 37.06 \( \mu g/l \), \( p = 0.04 \)]. Limitations: short follow-up period and small sample size of the included studies were the major limitations. Conclusions: RCT evidence suggests the rate of iron deficiency anemia in breastfed infants could be positively altered by introduction of solids at 4 months.

What's known on this subject

Current infant feeding recommendation is exclusive breastfeeding for the first six months of life followed by introduction of adequate complementary foods. However, the age in
which solids should be commenced is uncertain and controversial. This may lead to iron deficiency in some infants.

**What this study adds**

This study shows the available evidence that is related to the age of introduction of solids which may form the basis of complementary feeding guidelines. This study assesses the effect of earlier introduction of solids on the iron status and growth of exclusively breastfed infants.

**4.2 Introduction**

The World Health Organization current infant feeding recommendation is exclusive breastfeeding for the first six months of life followed by introduction of adequate complementary foods. This recommendation is for infants living in developing and developed countries, including Canada (1, 2). Although there is almost universal agreement that breast milk alone is the optimal first food, the age range in which solids should be commenced is uncertain leading to what has been called “weanling’s dilemma” (3).

The importance of the period of complementary feeding is that it accompanies a critical window of vulnerability. During this time period growth faltering is highly evident (4). Another concern during this period is micronutrient deficiencies, mostly prevalent because infants have higher nutrient demands relative to increased energy requirements. Deficiencies of certain micronutrients, such as iron results in possible irreversible negative effects on brain development as well as other detrimental psychological outcomes (5). There is general but not universal agreement that iron stores of infants start to deplete at about six months of age which leave the infants at high risk of iron
deficiency and iron deficiency anemia. This is especially true among the exclusively breastfed (6, 7). The estimated prevalence of iron deficiency anemia among Canadian children aged 1-5 years is 5% and was found to be five times higher among Aboriginal children (8, 9). Therefore, it is highly important to determine the ideal age to introduce iron rich complementary foods. Thus, our objective was to evaluate the current scientific evidence and to investigate the relation between moderate versus later introduction of complementary foods to the full-term breastfed infant with respect to iron status primarily, and growth secondarily, for infants in developing and developed countries. This review includes any relevant studies that targeted exclusively breastfed infants within the age range 4-6 months.

4.3 Methods

Our review was conducted according to the PRISMA guidelines (10).

4.3.1 Literature Search

Electronic searches of the MEDLINE and CINHAL databases were used to study the timing of introduction of complementary foods; the searches were completed by two authors (WQ, TRF) in May 2014. Medical subject headings and text words used to search were as follows: complementary feeding, infant food, solid(s), weaning, timing of introduction, micronutrient, iron, developmental outcomes, iron supplementation, random allocation, cohort studies, follow up studies, prospective studies, cross over studies, cross sectional studies. To decrease the chance of publication bias influencing the results, TRF conducted a gray literature search to include studies that may not be included in
bibliographic retrieval systems. Google, Current Controlled Trials, NIH Clinical Research Trials, ISRCTN, Cochrane Register of Clinical Trials were searched up to May 2014.

4.3.2 Inclusion criteria

Inclusion criteria included: randomized control trials (RCTs) and observational studies focusing on introduction of CF at 4 months versus at 6 months of age all conducted on healthy full-term exclusively breastfed infants.

4.3.3 Exclusion criteria

Studies were excluded if they included formula fed, preterm, or low birth weight infants or used medicinal iron supplementation. Studies in which infants were introduced to solid foods at ages younger than 4 or greater than 6 months of age were also excluded.

4.3.4 Data analysis

Weighted mean difference meta-analysis of iron status data extracted from the relevant RCTs was carried out using Review Manager software (RevMan Version 5.2.11, The Cochrane Collaboration) (11) to assess the effect of age of introduction of solids on iron status and linear growth. The analyses were stratified by whether the study was conducted in developing versus developed countries and by study design, that is, randomized controlled trials versus observational studies.

4.4 Results

A total of 923 study citations were found related to age of complementary feeding (Figure 2). Twenty-five RCTs were found but only three of them met the inclusion criteria, one in developed (with 2 publications), two in developing countries (Table 3). Forty-seven observational studies examining the age of introduction of CF were located.
Only one of the observational studies (in a developing country) met the inclusion criteria (Table 3). Table 4 lists the excluded studies and the reasons behind their exclusion.

![Study flow diagram](image)

**Figure 2. Study flow of the systematic review**
<table>
<thead>
<tr>
<th>Study</th>
<th>Study design</th>
<th>Country</th>
<th>N</th>
<th>Outcomes related to age of CF introduction</th>
<th>Results from CF introduction at</th>
<th>Conclusion/ Main findings related to age of introduction of solids</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cohen et al. 1994 (12)</td>
<td>RCT</td>
<td>Honduras</td>
<td>141</td>
<td>Growth</td>
<td>6 mo: 1092 (356)</td>
<td>&gt;0.05 No sig differences in weight and length gain were found between the groups.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Wt gain (g)</td>
<td>3.9 (1.2)</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Length gain (cm)</td>
<td>1051 (315)</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Dewey et al. 1998 (13)</td>
<td>RCT</td>
<td>Honduras</td>
<td>164</td>
<td>Fe status</td>
<td>6 mo: 104 (10)</td>
<td>&lt;0.05 Infants who received CF at 4 months had sig higher iron status parameters than EBF infants</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Hb (g/L)</td>
<td>0.33 (0.027)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Ht</td>
<td>48.4 (44.2)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Ferritin (µ/L)</td>
<td>109 (10)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>67.3 (64.5)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Jonsdottir et al. 2012 (14)</td>
<td>RCT</td>
<td>Iceland</td>
<td>100</td>
<td>Growth</td>
<td>6 mo: -0.01 (0.42)</td>
<td>0.9 No sig differences were found between the groups in growth. Sig positive effect of earlier CF introduction on iron stores</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Wt gain (z score)</td>
<td>0.04 (0.51)</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Length gain (z score)</td>
<td>0.06 (0.48)</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Gain in HC (z score)</td>
<td>-0.02 (0.31)</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.06 (0.40)</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-0.08 (1.04)</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.02 (0.89)</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.94 (0.77)</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>113.7 (7.3)</td>
<td>0.91</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>113.9 (6.1)</td>
<td>0.91</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>70.0 (77.3)</td>
<td>0.91</td>
</tr>
<tr>
<td>Wells et al. 2012 (15)</td>
<td>RCT</td>
<td>Iceland</td>
<td>100</td>
<td>Growth</td>
<td>6 mo: 0.36 (0.99)</td>
<td>0.7 No significant differences were found between the groups in growth and body composition.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Wt (z score)</td>
<td>0.77 (0.84)</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Length (z score)</td>
<td>-0.10 (1.04)</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>BMI (z score)</td>
<td>1.02 (0.89)</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>HC (z score)</td>
<td>0.36 (0.99)</td>
<td>0.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.77 (0.84)</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Body composition</td>
<td>4.96 (1.18)</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Lean mass (kg)</td>
<td>3.04 (1.12)</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Fat mass (kg)</td>
<td>5.13 (0.92)</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2.71 (0.96)</td>
<td>0.1</td>
</tr>
<tr>
<td>Khadivzadeh and Parsai 2004 (16)</td>
<td>Islamic republic of Iran</td>
<td>200 Growth Wt (g)</td>
<td>7719 (763)</td>
<td>7762 (843)</td>
<td>0.95</td>
<td>There were no significant differences in wt and length between infants fed solids at 4 months and infants fed solids at 6 mo of age.</td>
</tr>
<tr>
<td>-------------------------------</td>
<td>-------------------------</td>
<td>------------------</td>
<td>-------------</td>
<td>-------------</td>
<td>------</td>
<td>---------------------------------------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Length (cm)</td>
<td>66.5 (3.0)</td>
<td>66.6 (3.1)</td>
<td>0.86</td>
<td>N.B: BMI = body mass index, CF = complementary feeding, EBF = exclusively breastfeeding, HC = head circumference, Ht: hematocrit, mo = month, Observ.= observational, Wt = weight, data are presented as mean (SD). Jonsdottir et al. 2012 and Wells et al. 2012 were two articles published from a single RCT.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Wt gain (g)</td>
<td>922 (500)</td>
<td>1015 (419)</td>
<td>0.86</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Length gain (cm)</td>
<td>3.6 (1.3)</td>
<td>3.5 (1.1)</td>
<td>0.70</td>
<td></td>
</tr>
</tbody>
</table>
**Table 4. Excluded studies**

<table>
<thead>
<tr>
<th>Study (design)</th>
<th>Reason behind exclusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adu-Afarwuah et al. 2007 (17) (RCT)</td>
<td>Age of introduction of solids &gt; 6 mo</td>
</tr>
<tr>
<td>Bisimwa et al. 2012 (18) (RCT)</td>
<td>Age of introduction of solids &gt; 6 mo</td>
</tr>
<tr>
<td>Fewtrell et al. 2012 (19) (RCT)</td>
<td>Age of introduction of solids &gt; 6 mo</td>
</tr>
<tr>
<td>Gibson et al. 2011 (20) (RCT)</td>
<td>Age of introduction of solids &gt; 6 mo</td>
</tr>
<tr>
<td>Hambidge et al. 2004 (21) (RCT)</td>
<td>Age of introduction of solids &gt; 6 mo</td>
</tr>
<tr>
<td>Krebs et al. 2011 (22) (RCT)</td>
<td>Age of introduction of solids &gt; 6 mo</td>
</tr>
<tr>
<td>Ly et al. 2006 (23) (RCT)</td>
<td>No EBF group (no control group)</td>
</tr>
<tr>
<td>Martin-Calama et al. 1997 (24) (RCT)</td>
<td>Age of introduction of solids &lt; 4 mo</td>
</tr>
<tr>
<td>Mehta et al. 1998 (25) (RCT)</td>
<td>Age of introduction of solids &lt; 4 mo</td>
</tr>
<tr>
<td>Mosley et al. 2001 (26) (RCT)</td>
<td>Preterm infants</td>
</tr>
<tr>
<td>Nicoll et al. 1982 (27) (RCT)</td>
<td>Newborn infants</td>
</tr>
<tr>
<td>Ojofeitimi and Elegbe 1982 (28) (RCT)</td>
<td>Newborn infants</td>
</tr>
<tr>
<td>Phuka et al. 2008 (29) (RCT)</td>
<td>Age of introduction of solids &gt; 6 mo</td>
</tr>
<tr>
<td>Rivera et al. 2004 (30) (RCT)</td>
<td>Age of introduction of solids non specified</td>
</tr>
<tr>
<td>Roy 2006 (31) (RCT)</td>
<td>Age of introduction of solids &gt; 6 mo. Malnourished infants</td>
</tr>
<tr>
<td>Sachdev et al. 1991 (32) (RCT)</td>
<td>Water supplementation. Infants age &lt;4 mo</td>
</tr>
<tr>
<td>Saleem 2010 (33) (RCT)</td>
<td>Age of introduction of solids &gt; 6 mo</td>
</tr>
<tr>
<td>Sarker 2009 (34) (RCT)</td>
<td>Age of introduction of solids &gt; 6 mo. No EBF group</td>
</tr>
<tr>
<td>Schutzman et al. 1986 (35) (RCT)</td>
<td>Newborn infants</td>
</tr>
<tr>
<td>Simondon et al. 1996 (36) (RCT)</td>
<td>No EBF group</td>
</tr>
<tr>
<td>Ziegler et al. 2009 (37) (RCT)</td>
<td>Non EBF</td>
</tr>
<tr>
<td>Ahmed et al. 1993 (38)</td>
<td>Age of introduction of solids &lt; 4 mo</td>
</tr>
</tbody>
</table>
(Table 4 continued)

<table>
<thead>
<tr>
<th>Study</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Armar-Klemesu et al. 1991 (39)</td>
<td>Age of introduction of solids non specified</td>
</tr>
<tr>
<td>Arvas et al. 2000 (40)</td>
<td>Medicinal iron supplementation</td>
</tr>
<tr>
<td>Baker et al. 2004 (41)</td>
<td>Age of introduction of solids &lt; 4 mo</td>
</tr>
<tr>
<td>Baird et al. 2008 (42)</td>
<td>Mixed feeding (formula + BM)</td>
</tr>
<tr>
<td>Calvo et al. 1992 (43)</td>
<td>Age of introduction of solids was at 6 mo for both groups</td>
</tr>
<tr>
<td>Castro et al. 2009 (44)</td>
<td>Mixed feeding (formula + BM), no data on postnatal birth wt and conditions</td>
</tr>
<tr>
<td>Chantry et al. 2007 (45)</td>
<td>Non EBF (other foods introduced)</td>
</tr>
<tr>
<td>Domellöf et al. 2001 (46)</td>
<td>Age of introduction of solids &gt; 6 mo, medicinal iron supplementation</td>
</tr>
<tr>
<td>Dube et al. 2010 (47)</td>
<td>No analysis on early vs late introduction of solids among the groups</td>
</tr>
<tr>
<td>Durá Travé &amp; Diaz Velaz 2002 (48)</td>
<td>Early weaned group had mixed feeding (formula + BM)</td>
</tr>
<tr>
<td>Eissa et al. 1990 (49)</td>
<td>Age of introduction of solids non specified</td>
</tr>
<tr>
<td>Filipiak et al. 2007 (50)</td>
<td>Mixed feeding (formula + BM), no EBF group</td>
</tr>
<tr>
<td>Forsyth et al. 1993 (51)</td>
<td>Age of introduction of solids &lt; 4 mo</td>
</tr>
<tr>
<td>Freeman et al. 1998 (52)</td>
<td>Mixed feeding (formula + BM)</td>
</tr>
<tr>
<td>Gray 1996 (53)</td>
<td>Mixed feeding (formula + BM)</td>
</tr>
<tr>
<td>Haschke &amp; van’t Hof 2000 (54)</td>
<td>Age of introduction of solids &lt; 4 mo</td>
</tr>
<tr>
<td>Heinig et al. 1993 (55)</td>
<td>Mixed feeding (formula + BM), age of introduction of solids = or &gt; 6 months</td>
</tr>
<tr>
<td>Hokama 1993 (56)</td>
<td>No analysis on association between age of introduction of solids and iron parameters</td>
</tr>
<tr>
<td>Kajosaari &amp; Saarinen 1983 (57)</td>
<td>Age of introduction of solids &lt; 4 mo</td>
</tr>
<tr>
<td>Kajosaari 1991 (58)</td>
<td>Age of introduction of solids &lt; 4 mo</td>
</tr>
<tr>
<td>Kikafunda et al. 2009 (59)</td>
<td>Age of introduction of solids &gt; 6 mo</td>
</tr>
<tr>
<td>Kramer et al. 2011 (60)</td>
<td>Age of introduction of solids at 1, 2, 3 mo</td>
</tr>
<tr>
<td>Lartey et al. 1999 (61)</td>
<td>Age of introduction of solids &gt; 6 mo</td>
</tr>
</tbody>
</table>
(Table 4 continued)

<table>
<thead>
<tr>
<th>Reference</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Marlin et al. 1980(63)</td>
<td>Age of introduction of solids &lt; 4 mo</td>
</tr>
<tr>
<td>Marquis et al. 1997 (64)</td>
<td>Infants age group 12-15 mo</td>
</tr>
<tr>
<td>Messiah et al. 2012 (65)</td>
<td>Non specific information on how exclusive breastfeeding in BF and in CF groups</td>
</tr>
<tr>
<td>Nielsen et al. 1998 (66)</td>
<td>No analysis on association between age of introduction of solids among EBF and growth</td>
</tr>
<tr>
<td>Piwoz et al. 1996 (67)</td>
<td>Age of introduction of solids &lt; 4 mo</td>
</tr>
<tr>
<td>Popkin et al. 1990 (68)</td>
<td>Age of introduction of solids non specified</td>
</tr>
<tr>
<td>Quigley et al. 2009 (69)</td>
<td>No analysis on the type of milk received by CF group</td>
</tr>
<tr>
<td>Rowland et al. 1988 (70)</td>
<td>Age of introduction of solids non specified</td>
</tr>
<tr>
<td>Saarinen &amp; Siimes 1978 (71)</td>
<td>Age of introduction of solids &lt; 4 mo. Mixed feeding (formula + BM)</td>
</tr>
<tr>
<td>Saarinen 1978 (72)</td>
<td>Age of introduction of solids non specified</td>
</tr>
<tr>
<td>Salmenpera et al. 1985 (73)</td>
<td>Age of introduction of solids &lt; 4 mo</td>
</tr>
<tr>
<td>Simondon &amp; Simondon 1997 (74)</td>
<td>Age of introduction of solids &lt; 4 mo</td>
</tr>
<tr>
<td>Sloan et al. 2008 (75)</td>
<td>Age of introduction of solids &lt; 4 mo</td>
</tr>
<tr>
<td>Victora et al. 1998 (76)</td>
<td>Age of introduction of solids &lt; 4 mo, low birth weight infants included in the analysis</td>
</tr>
<tr>
<td>Wilson et al. 1998 (77)</td>
<td>Age of introduction of solids &lt; 4 mo</td>
</tr>
<tr>
<td>Wilson et al. 2006 (78)</td>
<td>Age of introduction of solids &lt; 4 mo</td>
</tr>
<tr>
<td>Zhou et al. 2012 (79)</td>
<td>Age of introduction of solids &gt; 6 mo</td>
</tr>
</tbody>
</table>

N.B: CF= complementary feeding, EBF= exclusively breastfeeding, mo= month.
4.4.1 Iron

A total of two RCTs had assessed iron status outcomes (Table 3). Meta-analysis (figure 3.1) suggested that introduction of solids at 4 months of age, compared with introduction of solids at 6 months did not improve hemoglobin status of breastfed infants in developed countries [mean difference [MD]: 0.20 g/l; 95% CI: -2.44, 2.84 g/l; \( p = 0.88 \)], while in developing countries (Fig. 4.1), significant improvement was detected [MD: 5 g/l; 95% CI: 1.54, 8.6 g/l; \( p = 0.005 \)]. Plasma ferritin concentration was improved with introduction of solids at 4 months of age of infants living in both developed and developing countries [MD: 26 μg/l; 95% CI: -0.10, 52.10 μg/l, \( p = 0.05 \)], [MD: 18.90 μg/l; 95% CI: 0.74, 37.06 μg/l, \( p = 0.04 \)] (Figures 3.2 & 4.2). The included observational study did not include iron parameters in their assessment.
Plasma Hb concentration (g/l), developed countries

<table>
<thead>
<tr>
<th>Study or Subgroup</th>
<th>Solids at 4 mo Mean</th>
<th>SD</th>
<th>Total</th>
<th>Solids at 6 mo Mean</th>
<th>SD</th>
<th>Total</th>
<th>Weight</th>
<th>IV, Fixed, 95% CI</th>
<th>Mean Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jonsdottir 2012</td>
<td>113.9</td>
<td>6.1</td>
<td>50</td>
<td>113.7</td>
<td>7.3</td>
<td>50</td>
<td>100.0%</td>
<td>0.20 [-2.44, 2.84]</td>
<td></td>
</tr>
<tr>
<td>Total (95% CI)</td>
<td></td>
<td></td>
<td>50</td>
<td></td>
<td></td>
<td>50</td>
<td>100.0%</td>
<td>0.20 [-2.44, 2.84]</td>
<td></td>
</tr>
</tbody>
</table>

Heterogeneity: Not applicable
Test for overall effect: Z = 0.15 (P = 0.88)

Favours [solids at 6 mo] Favours [solids at 4 mo]

Plasma ferritin concentration (µg/l), developed countries

<table>
<thead>
<tr>
<th>Study or Subgroup</th>
<th>Solids at 4 mo Mean</th>
<th>SD</th>
<th>Total</th>
<th>Solids at 6 mo Mean</th>
<th>SD</th>
<th>Total</th>
<th>Weight</th>
<th>IV, Fixed, 95% CI</th>
<th>Mean Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jonsdottir 2012</td>
<td>70</td>
<td>77.3</td>
<td>50</td>
<td>44</td>
<td>53.8</td>
<td>50</td>
<td>100.0%</td>
<td>26.00 [-0.10, 52.10]</td>
<td></td>
</tr>
<tr>
<td>Total (95% CI)</td>
<td></td>
<td></td>
<td>50</td>
<td></td>
<td></td>
<td>50</td>
<td>100.0%</td>
<td>26.00 [-0.10, 52.10]</td>
<td></td>
</tr>
</tbody>
</table>

Heterogeneity: Not applicable
Test for overall effect: Z = 1.95 (P = 0.05)

Favours [solids at 6 mo] Favours [solids at 4 mo]

Figure 3. Iron status analyses of developed countries

82
**Plasma Hb concentration (g/l), developing countries**

<table>
<thead>
<tr>
<th>Study or Subgroup</th>
<th>Mean</th>
<th>SD</th>
<th>Total</th>
<th>Mean</th>
<th>SD</th>
<th>Total</th>
<th>Weight</th>
<th>IV, Fixed, 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dewey 1998</td>
<td>109</td>
<td>10</td>
<td>89</td>
<td>104</td>
<td>10</td>
<td>50</td>
<td>100.0%</td>
<td>5.00 [1.54, 8.46]</td>
</tr>
<tr>
<td>Total (95% CI)</td>
<td>89</td>
<td></td>
<td>50</td>
<td>100.0%</td>
<td>5.00 [1.54, 8.46]</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Heterogeneity: Not applicable

Test for overall effect: Z = 2.83 (P = 0.005)

**Plasma ferritin concentration (µg/l), developing countries**

<table>
<thead>
<tr>
<th>Study or Subgroup</th>
<th>Mean</th>
<th>SD</th>
<th>Total</th>
<th>Mean</th>
<th>SD</th>
<th>Total</th>
<th>Weight</th>
<th>IV, Fixed, 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dewey 1998</td>
<td>67.3</td>
<td>64.5</td>
<td>89</td>
<td>48.4</td>
<td>44.2</td>
<td>50</td>
<td>100.0%</td>
<td>18.90 [0.74, 37.06]</td>
</tr>
<tr>
<td>Total (95% CI)</td>
<td>89</td>
<td></td>
<td>50</td>
<td>100.0%</td>
<td>18.90 [0.74, 37.06]</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Heterogeneity: Not applicable

Test for overall effect: Z = 2.04 (P = 0.04)

**Figure 4. Iron status analyses of developing countries**
4.4.2 Growth

Growth was assessed in the included studies mainly by differences between the groups in weight and length. Three (12, 14, 15) of the included four interventional studies reported the impact of solid introduction (Table 3). The meta-analyses showed a nonsignificant effect of earlier solids introduction in accelerating growth in both developing and developed countries (Figures 5, 6, 7 and 8). In addition, the study by Wells et al. (Table 3) showed nonsignificant differences between the two groups in body composition (lean mass \( p = 0.4 \), fat mass \( p = 0.14 \)).

There was no association between the effect of early introduction of complementary foods with a difference in weight and/or length in the study conducted in developing country \( (p = 0.95, p = 0.86, \text{respectively}) \) (16).
Figure 5. Weight analyses of developed countries
Length z-score, developed countries

<table>
<thead>
<tr>
<th>Study or Subgroup</th>
<th>Solids at 4 mo Mean</th>
<th>SD</th>
<th>Total</th>
<th>Solids at 6 mo Mean</th>
<th>SD</th>
<th>Total</th>
<th>Mean Difference</th>
<th>Weight</th>
<th>IV, Fixed, 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wells 2012</td>
<td>0.6</td>
<td>0.92</td>
<td>50</td>
<td>0.77</td>
<td>0.64</td>
<td>50</td>
<td>-0.17</td>
<td>100.0%</td>
<td>[-0.52, 0.18]</td>
</tr>
</tbody>
</table>

Total (95% CI) 50 50 100.0% -0.17 [-0.52, 0.18]

Heterogeneity: Not applicable

Test for overall effect: Z = 0.96 (P = 0.33)

Gain in length z-score, developed countries

<table>
<thead>
<tr>
<th>Study or Subgroup</th>
<th>Solids at 4 months Mean</th>
<th>SD</th>
<th>Total</th>
<th>Solids at 6 months Mean</th>
<th>SD</th>
<th>Total</th>
<th>Mean Difference</th>
<th>Weight</th>
<th>IV, Fixed, 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jonsdotir 2012</td>
<td>0.03</td>
<td>0.5</td>
<td>50</td>
<td>0.04</td>
<td>0.51</td>
<td>50</td>
<td>-0.01</td>
<td>100.0%</td>
<td>[-0.21, 0.19]</td>
</tr>
</tbody>
</table>

Total (95% CI) 50 50 100.0% -0.01 [-0.21, 0.19]

Heterogeneity: Not applicable

Test for overall effect: Z = 0.10 (P = 0.92)

Figure 6. Length analyses of developed countries
### Head circumference z-score, developed countries

<table>
<thead>
<tr>
<th>Study or Subgroup</th>
<th>Solids at 4 mo</th>
<th>Solids at 6 mo</th>
<th>Mean Difference</th>
<th>Mean Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
<td>Total</td>
<td>Mean</td>
</tr>
<tr>
<td>Wells 2012</td>
<td>0.94</td>
<td>0.77</td>
<td>50</td>
<td>1.02</td>
</tr>
<tr>
<td>Total (95% CI)</td>
<td>50</td>
<td></td>
<td>50</td>
<td>100.0%</td>
</tr>
</tbody>
</table>

Heterogeneity: Not applicable
Test for overall effect: Z = 0.48 (P = 0.63)

---

### Gain in head circumference z-score, developed countries

<table>
<thead>
<tr>
<th>Study or Subgroup</th>
<th>Solids at 4 mo</th>
<th>Solids at 6 mo</th>
<th>Mean Difference</th>
<th>Mean Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
<td>Total</td>
<td>Mean</td>
</tr>
<tr>
<td>Jonsdottir 2012</td>
<td>0.06</td>
<td>0.4</td>
<td>50</td>
<td>0.06</td>
</tr>
<tr>
<td>Total (95% CI)</td>
<td>50</td>
<td></td>
<td>50</td>
<td>100.0%</td>
</tr>
</tbody>
</table>

Heterogeneity: Not applicable
Test for overall effect: Z = 0.00 (P = 1.00)

---

**Figure 7. Head circumference analyses of developed countries**
Figure 8. Growth analyses of developing countries
4.5 Discussion

In this meta-analysis, we found that infants in developing countries who were introduced to solid foods at 4 months of age had a clinically relevant increase in hemoglobin and ferritin levels compared with hemoglobin and ferritin in exclusively breastfed infants at 6 months of age. The data from developed countries showed a significant increase in ferritin level only resulting from the earlier introduction of solids. In regards to growth, our meta-analysis indicated that there was no significant impact of earlier introduction of solids on weight, length and head circumference in both developed and developing countries.

To our knowledge, this is the first systematic review that weighs the evidence of the effect of complementary food introduction at 4 versus 6 months of age on iron status and growth. Other reviews have examined the effect of iron fortified food on iron status and anemia rate however, in different age ranges (80). In the systematic review by Dewey and Adu-Afaruah, the authors gave a general overview of existing studies that looked at the effects of complementary foods on various biochemical and functional outcomes, however, they did not conduct an evaluation of effects of solids introduction at 4 versus 6 months (81). The effects on growth in our review is in line with the finding of the systematic review of Kramer and Kakuma in which WHO recommendation was largely based. The authors found nonsignificant differences in linear growth in their comparison of effects of the introduction of solids between before 4 months and at 6 months (82). A previous review showed significant growth improvements with provision of solid foods; (83, 84) however, this finding could be due to studies, conducted on moderately malnourished infants.
Further evidence is needed to confirm the optimal timing of introduction of solids for the exclusively breastfed infants. In future studies, perhaps multi-central with long-term follow up, with special attention to hematological results would be warranted to clarify this important issue.

4.6 Limitations

The included studies had short follow-up periods in which to assess the impact of complementary food introduction on outcomes. At this point the longer-term outcomes are not known. Small sample size is another limitation. Finally, pooled data analyses could not be performed for all the outcomes due to the differences in the outcome measures assessed in the individual studies.

4.7 Conclusion

Encouraging exclusive breastfeeding is a desirable goal for the health care professionals and there is consistent evidence to support breastfeeding. We are concerned that the generalization of the age of introduction of solid recommendation at six months may not be optimum for all breastfed healthy infants. From this review iron status of healthy full-term infants could be positively altered by an earlier introduction of complementary food leading to maintenance of infant iron stores. Furthermore, there may be value in considering the changing of the current statement regarding solid introduction from a fixed time (6 months) to a range of time and leave the decision of solid commencement for individual infants to health care professionals and parents. Larger randomized controlled trials perhaps with a multi-center design in developed and developing countries
are needed to investigate the differences between the outcomes of introduction of solids before and at six months of age.

**4.8 References**


45. Chantry CJ, Howard CR, Auinger P. Full breastfeeding duration and risk for iron

supplementation of breast-fed Honduran and Swedish infants from 4 to 9 months of age. J


48. Dura Trave T, Diaz Velaz L. [Prevalence of iron deficiency in healthy 12-month-old

49. Eissa AM, El-sherbini AF, Khashaba AA, El-bakry M, Abou Amer ER. Breast
feeding and infant growth, a follow-up study in Menoufia, Egypt. Popul Sci. 1990
Jul;9:69-75.

food introduction in relation to eczema: results from a four-year prospective birth cohort

51. Forsyth JS, Ogston SA, Clark A, Florey CD, Howie PW. Relation between early
introduction of solid food to infants and their weight and illnesses during the first two

52. Freeman VE, Mulder J, van't Hof MA, Hoey HM, Gibney MJ. A longitudinal study of

53. Gray SJ. Ecology of weaning among nomadic Turkana pastoralists of Kenya:


61. Lartey A, Manu A, Brown KH, Peerson JM, Dewey KG. A randomized, community-based trial of the effects of improved, centrally processed complementary foods on


BRIDGE TO CHAPTER V

The previous chapters showed that the available evidence is limited in regards to the assessment of the optimal time and type of complementary foods. Therefore, chapter V comprises a manuscript that demonstrates the assessment of the recommended first complementary foods in terms of meeting the requirements of the EBF infants by determining their nutrient intakes. This manuscript also illustrates the associations between socio-demographic factors, selected feeding patterns, nutrient intakes and growth of the EBF infants. Wafaa Qasem was the principal author of this manuscript. Wafaa Qasem helped in the data collection, coordinated and supervised data collection, carried out the analyses, drafted the manuscript. Sarah Jorgensen, Sandra Castillo San Juan, and Chenxi Cai helped in the data collection. Dr. Trust Beta, helped in the study design and the data collection methods, and currently reviewing the manuscript. Dr. James Friel was the principal investigator of this study. He conceptualized and designed the study, designed the data collection methods, and coordinated and supervised data collection, reviewed the manuscript, and approved the final manuscript as submitted.
CHAPTER V

MANUSCRIPT 4:

ASSESSMENT OF COMPLEMENTARY FEEDING OF CANADIAN INFANTS:
SOCIO-DEMOGRAPHICS, FEEDING PATTERNS, NUTRIENT INTAKE AND
GROWTH OF BREASTFED INFANTS WHO RECEIVED FIRST
COMPLEMENTARY FOODS

This manuscript is currently under internal review and will be submitted to the journal
Pediatrics for review for publication (2015)

Wafaa Qasem1,2, Sarah Jorgensen2, Sandra Castillo San Juan1,2, Chenxi Cai1,2, Trust
Beta2,3,4, James Friel1,2,3

1Department of Human Nutritional Sciences, University of Manitoba, Winnipeg, MB, Canada
2Richardson Centre for Functional Foods and Nutraceuticals, 196 Innovation Drive,
University of Manitoba, Winnipeg, MB, Canada
3Children’s Hospital Research Institute of Manitoba, Winnipeg, MB, Canada
4Department of Food Science, University of Manitoba, Winnipeg, MB, Canada

Corresponding author: Wafaa Qasem, Richardson Centre for Functional Foods and
Nutraceuticals, 196 Innovation Drive, University of Manitoba, Winnipeg, MB, Canada

Email: [qasemw@myumanitoba.ca]

Key words: complementary feeding, breastfed infants, socio-demographics, feeding
patterns, nutrient intake, growth.


Funding source: All phases of this clinical trial were supported by the Canadian Institutes of Health Research (CIHR).

Conflict of interest: The authors have no conflicts of interest to disclose.

Clinical trial registration: The study was registered in the ClinicalTrial.gov registry (Assessment of Complementary Feeding of Canadian Infants, NCT01790542).

What’s known on this subject?
Health Canada recommends EBF followed by the introduction of iron rich foods such as infant cereal (traditional) and meat (newly recommended). No studies had assessed the effect of these recommended foods on the breastfed infant’s overall health and in terms of meeting the daily recommendations.

What this study adds?
This study assessed and compared the effect of the recommended first complementary foods (CFs) on multiple health outcomes including growth and macronutrient and micronutrient intakes before and after the introduction of CF. In addition, socio-demographic factors and feeding patterns were also evaluated.

5.1 Abstract

Background: Health Canada recommends EBF up to 6 months of age followed by the introduction of iron-rich foods such as iron-fortified cereal and meat. Limited number of studies have assessed the effect of first complementary foods (CFs) on infant health.

Objectives: This study aimed to assess the complementary feeding of breastfed infants in relation to socio-demographic characteristics, feeding patterns, nutrient intake and growth. Methods: Eighty-seven EBF were randomly assigned to receive one of the following CFs: iron-fortified cereal (Cer), iron-fortified cereal with fruit (Cer+Fr), meat
Details of socio-demographic characteristics and infant feeding patterns were obtained using parent-completed questionnaires. Infant dietary intake data were collected using 3-day dietary records. Infants’ growth was determined using standardized measurements. Results: Only 27.3% of mothers adhered to the Health Canada recommendation of EBF for up to 6 months. The rate of later introduction of CFs was associated with maternal factors: lower parity (odds ratio (OR) = 0.2, CI: 0.09, 0.9), receiving multi-vitamins during breastfeeding (OR = 0.3, CI: 0.1, 1.9), working prior to delivery (OR = 0.09, CI: 0.01, 0.5). Macronutrient and micronutrient intake was adequate and met the recommendation when provided by both breast milk and CFs. Higher weight-gain z-scores were significantly associated with earlier introduction of CFs (OR = 3.0, CI: 1.0, 9.3, $p < 0.001$). Gain in head circumference was positively correlated with iron intake among all the breastfed infants ($r = 0.31, p = 0.01$). Conclusions: Maternal factors were associated with selected feeding patterns. In future, targeting those factors may be warranted. Recommending the three types of CFs as the first such foods together with breast milk appears to satisfy the high requirements of growing infants.

5.2 Introduction

The World Health Organization (WHO) and various agencies, including Health Canada, have established official recommendations that support exclusive breastfeeding (EBF) for infants up to 6 months of age (1, 2). As energy requirements steadily increase at around 6 months, the total calories as well as specific micronutrients and macronutrients supplied by breast milk become inadequate (3). Feeding by breast milk should therefore then be accompanied by the first complementary foods (CFs) to avoid nutritional deficiencies and inadequate growth (4, 5). The latest recommendation of Health Canada, “Nutrition for
Healthy Term Infants: Recommendations from Birth to Six Months”, clearly states the need to introduce iron-rich foods such as iron-fortified cereal, meat, and meat alternatives, two to three times per day to ensure that the infant’s nutritional requirements are met (2). Other specific nutrients have to be derived from complementary feeding to compensate for the nutritional deficiencies of breast milk for infants of this age. This is important given that the diet of infants play a major role in short- and long-term health and development (6-9).

Although feeding patterns provide a useful measure of an infant’s diet, it is highly important to understand the nutrient intake within those patterns. It has been shown that infant feeding patterns are associated with nutrient intake and eating habits (10, 11). Some studies have identified feeding patterns that were strongly associated with socio-demographic variables, such as maternal pre-pregnancy body mass index (BMI), educational level, breastfeeding duration, and type of first CFs (12-14). In addition, compared with formula feeding, longer duration of breastfeeding has been associated with later introduction of solid foods and slower weight gain during the second half of infancy (15-17). Moreover, earlier introduction of solid foods (before 4 months) has been linked to childhood obesity (18), however, a recent systematic review concluded that the overall relationship was weak (19). Other evidence has linked feeding patterns with various child health outcomes, such as IQ and overall food allergy (20, 21).

The term "complementary feeding” refers to energy- and nutrient- containing semi-solids or solids fed to infants in addition to breast milk or formula (22). Typically, the transition from a fully milk to a mixed diet generally occurs between 6 and 24 months of age (23). The most important factor that determines the required CF type and amount is whether
the infant received EBF or was formula fed, as this clearly affects the nutritional intake and nutrient utilization (24). At about 6 months, the addition of CFs is critical, because breast milk alone may not provide adequate amounts of macronutrients, such as protein and some micronutrients, such as zinc and vitamin D (2, 5). However, introducing CFs before 6 months is a concern because it may displace breast milk, which is the major source of nutrients for growing infants (22, 25). Only one study in Canada has reported the CF intake of infants during the first year of life (26), independent of breast milk and formula intake, in which the average daily intake of nutrients from all types of CFs were compared with daily recommendations (Dietary Reference Intakes (DRIs), for macronutrients and micronutrients (27-29).

Ensuring normal growth and development is a priority during infancy. Therefore, the type of complementary feeding and feeding patterns have great importance during this period. Of particular interest is the timing of the introduction of solids, which is important because it may affect growth among breastfed infants. Breast milk contains a lower quantity of protein than formula. In one study, infant formula with a low protein content showed growth effects similar to those with breastfeeding, which suggests an association between protein intake and growth (30). There is increasing agreement about the growth advantages of meat as the first CF (31). The available evidence from randomized controlled trials (RCTs) has indicated non-significant differences in growth parameters between infants introduced to solids at 4 months compared with 6 months (32-34). However, data from RCTs comparing the growth effects of different types of first CFs are very limited (35).
It is necessary to understand energy and nutrient intake when assessing the efficiency of the first recommended CFs. Along with feeding patterns, socio-demographic characteristics and growth, this information can help us determine the optimal first CFs. The aims of the present study were therefore as follows: 1) to determine whether nutrient intake from the first recommended CF of breastfed infants is sufficient in terms of meeting daily recommendations; and 2) to identify any associations among feeding patterns, socio-demographic characteristics and growth.

5.3 Methods

5.3.1 Study design

The study was an RCT of the daily intake of one of the following foods over a period of 2-4 weeks: iron-fortified rice cereal, iron-fortified rice cereal with fruit, meat (beef) (figure 9). Exclusively breastfed infants were recruited throughout the city of Winnipeg between December 2012 and May 2014. The study was advertised in local newspapers, a study poster, and websites; mothers were also recruited by word of mouth. In addition, breastfeeding mothers were approached by pediatricians working in private clinics or in Winnipeg Regional Health Authority hospitals and by public health nurses. The study data were gathered on the first and second visits. The first visits were conducted at the age of 4-6 months and before introducing the study food. The second visits were conducted 2-4 weeks after introducing the study foods. At both visits, urine and stool samples and dietary records were collected, and anthropometric measurements were obtained. During the first visit, there was the additional task of completing a
questionnaire to obtain baseline information (Appendix 1). Both visits took place in the participating mother’s homes.

Visits to the research clinic at the Children’s Hospital Research Institute of Manitoba were conducted for the infants of mothers and caregivers who agreed to supply blood for iron status analyses (Chapter VI). Mothers and care providers of infants in all the groups were encouraged to continue breastfeeding during the intervention period. With the help of the PhD student, a research assistant was responsible for the recruitment, initial contact, distribution of CFs, first and second home or/clinic visits, the collection of baseline data, collection of samples, conducting anthropometric measurements, and monitoring participant compliance with the study food.

5.3.2 Ethical approval

This study was approved by the Bannatyne Campus Research Ethics Board, University of Manitoba (Protocol no. H2011:166) and the Winnipeg Regional Health Authority Research Review Committee (Ref. No.2012-006), (Appendices 2 and 3). The study was registered in the ClinicalTrial.gov registry (Identifier No. NCT01790542). A brief handout explaining eligibility for the study and its objectives was given to the breastfeeding mothers; they were informed that if they wished to participate in the study, they should contact the research team (Appendix 4). Written informed consent was obtained from all the mothers before participation (Appendix 5).
Figure 9. Study design
5.3.3 Study subjects

We enrolled 87 EBF infants aged 4-6 months from City of Winnipeg area in this study. Infants who fulfilled the following inclusion criteria were considered eligible for participation: full term (>37 gestational weeks), birth weight of > 2500 g, absence of congenital anomalies and medical conditions, exclusively breastfed, willingness of mothers to provide urine and stool samples and to complete the dietary records. Infants were excluded if they had already started on solid foods or consumed more than 200 ml of iron-fortified formula.

5.3.4 Study foods

The three study foods were as follows: iron-fortified rice cereal (Milupa GmbH, Danone, Friedrichsdorf, Germany), iron-fortified cereal with raspberry (Milupa GmbH, Danone, Friedrichsdorf, Germany), pureed beef (Heinz®, Pittsburgh, PA, USA). The nutritional compositions of the study foods are presented in table 5. The nutritional content of the study foods was obtained from the product label as the percentage of the daily value and it was converted to the equivalent amount in scientific units using the DRIs (28, 29, 36). The nutritional content of the pureed meat jar was calculated using the nutritional software FoodFocus© version 4.1. Iron fortified rice cereal was selected because it was found to be the most common first complementary food choice among Canadian infants. Iron fortified rice cereal with fruit (raspberry) was selected because of the following: another commonly consumed infant cereal, contains single fruit extract (raspberry powder) ingredient rather than multiple fruits extract which allows the isolation of the other fruits effects on the measurable endpoints, raspberry is a rich source of phenolic phytochemicals (total phenolic content 359-512 mg/100g fruit, total flavonoid compound
63-103 mg/100g, total anthocyanin content 2.6-57.6 mg/100g) and exhibits high antioxidant activities; it contains anthocyanins, proanthocyanidin-like tannins and ellagitannins (37, 39). The iron fortified rice cereal with raspberry contains raspberry powder in an amount of 1.8% of the total ingredients. The present study aimed to assess the newly recommended CF by Health Canada which is meat. Pureed beef infant food was selected for the following reasons: it contains single meat product (beef), red meat is a good source of heme iron, and the product is mostly readily available on shelf of Canadian grocery stores.

At the first visit, the mothers were provided with sufficient amount of the study foods for 4 weeks. The decision on when to start giving the study foods was left to the parents and they were asked to report the initial feeding date. We made follow-up telephone calls to the parents to monitor compliance and to check about the occurrence of any adverse events with feeding. In addition, parents were also asked to complete two 3-day dietary records (Appendices 6 and 7) before introducing the study food and at the end of the feeding duration.

5.3.5 Socio-demographic characteristics

Detailed background socio-demographic data such as, age, sex, family composition and educational level were collected by questionnaire (Appendix 1) at the initial visit. In addition, data were obtained about infant feeding practices such as, breastfeeding and timing of introduction of CFs and the use of nutrient supplements.
Table 5. Nutritional composition of study foods

<table>
<thead>
<tr>
<th>Energy &amp; nutrients</th>
<th>Iron fortified rice cereal Per 28 g (7-8 tbs)</th>
<th>Iron fortified rice cereal and fruit Per 28 g (7-8 tbs)</th>
<th>Meat Per 100 g (1 jar)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Energy (Kcal)</td>
<td>120</td>
<td>120</td>
<td>140.9</td>
</tr>
<tr>
<td>Protein (g)</td>
<td>4.0</td>
<td>3.0</td>
<td>12.0</td>
</tr>
<tr>
<td>Carbohydrate (g)</td>
<td>21.0</td>
<td>21.0</td>
<td>0.3</td>
</tr>
<tr>
<td>Fibre (g)</td>
<td>0.0</td>
<td>0.0</td>
<td>0.3</td>
</tr>
<tr>
<td>Sugar (g)</td>
<td>3.0</td>
<td>4.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Fat (g)</td>
<td>2.5</td>
<td>2.5</td>
<td>10.5</td>
</tr>
<tr>
<td>Cholesterol (mg)</td>
<td>ND</td>
<td>ND</td>
<td>29.4</td>
</tr>
<tr>
<td>Vitamin A (µg)</td>
<td>0.0</td>
<td>0.0</td>
<td>5.1</td>
</tr>
<tr>
<td>Vitamin B12 (µg)</td>
<td>0.35</td>
<td>0.17</td>
<td>1.4</td>
</tr>
<tr>
<td>Vitamin B6 (mg)</td>
<td>ND</td>
<td>ND</td>
<td>0.1</td>
</tr>
<tr>
<td>Thiamin (mg)</td>
<td>0.36</td>
<td>0.27</td>
<td>0.1</td>
</tr>
<tr>
<td>Riboflavin (mg)</td>
<td>0.38</td>
<td>0.38</td>
<td>0.2</td>
</tr>
<tr>
<td>Niacin (NE)</td>
<td>0.20</td>
<td>0.20</td>
<td>3.8</td>
</tr>
<tr>
<td>β Carotene (µg)</td>
<td>ND</td>
<td>ND</td>
<td>55.3</td>
</tr>
<tr>
<td>Vitamin C (mg)</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Vitamin D (IU)</td>
<td>ND</td>
<td>ND</td>
<td>10.1</td>
</tr>
<tr>
<td>Vitamin E (IU)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Sodium (mg)</td>
<td>20.0</td>
<td>20.0</td>
<td>38.5</td>
</tr>
<tr>
<td>Potassium (mg)</td>
<td>ND</td>
<td>ND</td>
<td>190.5</td>
</tr>
<tr>
<td>Calcium (mg)</td>
<td>30.3</td>
<td>20.0</td>
<td>4.1</td>
</tr>
<tr>
<td>Iron (mg)</td>
<td>8.80</td>
<td>8.80</td>
<td>1.3</td>
</tr>
<tr>
<td>Zinc (mg)</td>
<td>0.09</td>
<td>0.07</td>
<td>ND</td>
</tr>
<tr>
<td>Copper (µg)</td>
<td>13.2</td>
<td>13.2</td>
<td>ND</td>
</tr>
<tr>
<td>Magnesium (µg)</td>
<td>11.2</td>
<td>15.0</td>
<td>ND</td>
</tr>
<tr>
<td>Phosphorus (mg)</td>
<td>41.2</td>
<td>41.2</td>
<td>92.2</td>
</tr>
<tr>
<td>Iodine (µg)</td>
<td>13.0</td>
<td>13.0</td>
<td>ND</td>
</tr>
<tr>
<td>Manganese (mg)</td>
<td>0.04</td>
<td>0.00</td>
<td>ND</td>
</tr>
<tr>
<td>Selenium (µg)</td>
<td>ND</td>
<td>ND</td>
<td>2.7</td>
</tr>
</tbody>
</table>

N.B: IU = international unit, NE = niacin equivalent, ND = no data provided, tbs = tablespoon

5.3.6 Anthropometric measurements

Anthropometric measurements were converted to z-scores and used to determine various indicators of infant growth such as, length-for-age and weight-for-age z-scores, which was done according to WHO standards (40, 41). Length, weight and head circumference were measured at both the first and second visits. Recumbent length was measured using an infantometer (417 Seca, Seca GmbH & Co, Hamburg, Germany), accurate to 0.1 cm. The infants were weighed naked on a calibrated electronic scale (BabyChecker™Scale,
Medela Inc. Breastfeeding U.S, IL, USA) accurate to 120 g. Using a flexible non-stretchable tape, the head circumference of the infants was measured to the nearest 0.1 cm. We took three measurements for each variable and averaged the values. All the techniques used in the measurements and the WHO growth charts employed to monitor infants' growth were done in accordance with “A health professional's guide for using the new WHO growth charts” by Dieticians of Canada, Canadian Paediatric Society, The College of Family Physicians of Canada, and Community Health Nurses of Canada (42).

5.3.7 Dietary analysis
Nutrient intake from first CFs was assessed using a 3-day dietary record (Appendices 6 and 7) at two time points; the record was to be completed and submitted to the research team at the first visit before the introduction of solids and at the second visit (last 3 days of feeding). These time points were chosen to assure exclusive breastfeeding and compliance with the study foods. Mothers were instructed to record their infant's food and beverage intake on 3 consecutive days. Common household measures were used to describe the food portion size. To further assure compliance with the use of study foods, mothers were also asked to record the recipes of any homemade foods and brand names of products consumed. Dietary intake data from the food records were entered into the nutritional analysis software FoodFocus© version 4.1. That program contains details relating to over 6000 foods utilizing food composition tables obtained from the Canadian Nutrient File 2010, and from the USDA database. The FoodFocus© program was compared with other food composition software in the International Survey for the National Nutrient Databank Conference. Each infant’s mean daily macronutrient and
micronutrient intake from the first CFs was generated by the FoodFocus© database.

Macronutrient and micronutrient intake was compared with DRIs provided by Health Canada. When other foods were consumed, these foods were excluded and were not entered into the FoodFocus© program. The nutrient intake from vitamin D supplement was not included in our analysis.

Breast milk volume intake was estimated for each recorded feeding. For each minute, we used an estimated amount of 12.5 ml, up to 125 ml for full feeding lasting 10 minutes or longer (43, 44).

5.3.8 Sample size determination

The sample size of each feeding group was determined by power analysis for reactive oxygen species generation (ROS) (primary outcome, Chapter VI). One study (45) found mean ROS generation in adults (0.27 ± 0.03 mg/ml) to be 10% lower in those who received anti-oxidant supplements. To observe a similar difference in infants, the required sample size is 25 infants in each feeding group (i.e N = 75) at a significant level (α) of 0.05, with power of 95% (β = 5%). We initially aimed at a sample size of 120 (40 infants in each group) to accommodate drop-off and non-compliance exclusion. The following formula was used to determine the sample size:

\[
\text{Power} = \text{Prob} \left( F < F_{\text{crit}}, r_1, r_2, \text{NC} \right), \text{where } F \text{ is distributed as the non-central } F (\text{NC, } -r_1, -r_2) \text{ and } F_{\text{crit}} \text{ is the quantile } (1-\alpha) \text{ of the } F \text{ distribution } r_1 \text{ and } r_2 \text{ degrees of freedom.}
\]

\[
n = \text{number per group}
\]

\[
r = \text{number of groups}
\]

\[
V1 = r - 1
\]
\[ V2 = r (n-1) \]

\[ NC \text{ (non centrality parameter)} = \frac{n_{\text{CSS}}}{\sigma^2} \]

\[ \text{CSS (corrected sum of squares)} = \sum_{g=1}^{G} (\mu_g - \mu)^2 \]

\[ \mu_g = \text{mean of the gth group} \]

\[ \mu = \text{overall mean} \]

\[ \sigma^2 = \text{estimated mean square error} \]

**5.3.9 Statistical analysis**

Statistical analyses were performed using the statistical software package IBM SPSS Statistics for Windows version 22.0 (IBM Corp. Armonk, NY, USA). Variables were assessed for normality. The data are presented as means ± standard error (SE) unless otherwise stated. To compare the feeding group means for two different time points (before and after introducing CFs), repeated measures analysis of variance (ANOVA) test was used. For pair-wise comparisons, the post hoc Bonferroni test was used. Pearson's product-moment correlation coefficient was used to determine the strength of the relationships between normally distributed variables, whereas Spearman’s rank correlation coefficient test was used to measure the association between non-normally distributed variables. Where appropriate, pearson's chi-squared (\( \chi^2 \)) test and odds ratios, with 95% confidence intervals, were calculated to estimate the strength of association between exposures (certain levels of socio-demographic and other baseline variables, growth and selected feeding patterns). Statistical significant difference was determined at \( p < 0.05 \).
5.4 Results

In all, 90 infants were assessed for eligibility, and 3 infants met the exclusion criteria.

Eighty-seven infants were enrolled in the study between December 2012 and May 2014 and they were allocated as follows: 25 in Cer group, 28 in Cer+Fr group and 34 in M group. A total of 82 infants completed the study and 5 were withdrawn (did not receive the allocated intervention) due to the following: mothers wish to discontinue compliance with the study food (cereal or meat), they had moved to a different province, no response to the study team calls.

5.4.1 Socio-demographic characteristics

Table 6 presents a baseline comparison of the socio-demographic characteristics among the three study groups. There were no statistical significant differences among the groups except for maternal age, and vitamin supplementation during pregnancy, in mothers of the meat group.

| Table 6. Baseline comparisons of the three study group’s characteristics |
|--------------------------------------------------|----------------|----------------|----------------|----------------|
| Variable                                         | Cer (n = 22)   | Cer+Fr (n = 28) | M (n = 32)     | All groups (n = 82) |
| Males (%)                                        | 44.0           | 48.3           | 54.8           | 49.4           |
| Birth weight (g) mean±SD                         | 3437±475       | 3693±494       | 3544±519       | 3563±503       |
| Length at birth (cm) mean±SD                     | 52±2.8         | 52.5±2.6       | 51.7±2.7       | 52±2.7         |
| HC at birth (cm) mean±SD                         | 34.1±2.0       | 34±1.9         | 35.5±1.8       | 34.7±1.9       |
| Gestation (wks) mean±SD                          | 39.8±1.3       | 39.8±1.7       | 39.4±1.0       | 39.7±1.4       |
(Table 6 continued)

<table>
<thead>
<tr>
<th>Parity (%)</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>32</td>
<td>27.6</td>
<td>35.5</td>
<td>31.8</td>
</tr>
<tr>
<td>2</td>
<td>36</td>
<td>41.4</td>
<td>51.6</td>
<td>43.5</td>
</tr>
<tr>
<td>≥3</td>
<td>32</td>
<td>30.8</td>
<td>12.9</td>
<td>24.8</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Parity mean±SD</th>
<th>2.0±0.8</th>
<th>2.3±1.5</th>
<th>1.8±0.9</th>
<th>2±1.1</th>
</tr>
</thead>
</table>

| Mean Maternal age (years) | 31.1±4.4 | 31.9±4.1 | 34.5±3.7* | 32.6±4.3 |

<table>
<thead>
<tr>
<th>Mode of delivery (%)</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>83.3</td>
<td>92.3</td>
<td>83.3</td>
<td>86.3</td>
</tr>
<tr>
<td>Caesarian</td>
<td>12.5</td>
<td>3.8</td>
<td>16.7</td>
<td>11.3</td>
</tr>
<tr>
<td>Other</td>
<td>4.2</td>
<td>3.8</td>
<td>0.0</td>
<td>2.5</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Antenatal-postnatal complication (%)</th>
<th>Yes</th>
<th>24</th>
<th>20.7</th>
<th>29</th>
<th>24.7</th>
</tr>
</thead>
<tbody>
<tr>
<td>No</td>
<td>76</td>
<td>79.3</td>
<td>71</td>
<td>75.3</td>
<td></td>
</tr>
</tbody>
</table>

| Fe supplementation during pregnancy (%) | 20 | 88 | 89.7 | 96.8 | 91.8 |

| Vitamin supplementation during pregnancy (%) | 48 | 62.1 | 79.3* | 63.9 |

<table>
<thead>
<tr>
<th>Vitamin supplementation during BF (%)</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Married</td>
<td>88</td>
<td>93.1</td>
<td>93.5</td>
<td>91.8</td>
</tr>
<tr>
<td>Single</td>
<td>4.0</td>
<td>6.9</td>
<td>0.0</td>
<td>3.5</td>
</tr>
<tr>
<td>Divorced</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Common-law</td>
<td>8.0</td>
<td>0.0</td>
<td>6.5</td>
<td>4.7</td>
</tr>
</tbody>
</table>

| Maternal pre-pregnancy weight (Kg) mean±SD | 67±14.7 | 66.6±12.1 | 69.8±14.7 | 67.9±13.8 |

| Maternal height (cm) mean±SD | 165±6.3 | 166.3±6.9 | 166.5±7.5 | 166±6.9 |

| Maternal pre-pregnancy BMI mean±SD | 24.6±4.4 | 24.1±3.9 | 25.3±5.3 | 24.7±4.6 |

| Duration of previous BF (months) mean±SD | 14.9±8.9 | 12.3±7.5 | 13.4±6.2 | 13.5±7.4 |

<table>
<thead>
<tr>
<th>Maternal education (%)</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary &amp; secondary</td>
<td>16</td>
<td>10.7</td>
<td>9.7</td>
<td>11.9</td>
</tr>
<tr>
<td>Post secondary</td>
<td>84</td>
<td>89.3</td>
<td>90.3</td>
<td>88.1</td>
</tr>
</tbody>
</table>

| Pre-delivery working mothers (%) | 76 | 85.7 | 96.8 | 86.9 |

| Paternal weight (Kg) mean±SD | 88.4±14.9 | 92±18.8 | 87.9±11.7 | 89.5±15.2 |

| Paternal height (cm) mean±SD | 179.5±8.0 | 180±7.1 | 180±7.9 | 180±7.6 |

| Paternal BMI mean±SD | 25.1±8.7 | 28.3±5.3 | 27.0±4.2 | 26.9±6.1 |

<table>
<thead>
<tr>
<th>Paternal education (%)</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary &amp; secondary</td>
<td>16.0</td>
<td>3.8</td>
<td>3.2</td>
<td>7.3</td>
</tr>
<tr>
<td>Post secondary</td>
<td>84.0</td>
<td>96.2</td>
<td>96.8</td>
<td>92.7</td>
</tr>
</tbody>
</table>

| No. of household occupants mean±SD | 3.9±0.8 | 4.3±1.5 | 3.9±1.0 | 4.0±1.2 |

| No. of household children mean±SD | 1.8±0.7 | 2.4±1.5 | 1.9±0.9 | 2.0±1.1 |

| Maternal smoking (%) | 4.0 | 0.0 | 3.2 | 2.4 |

| Infant Vitamin D supplementation (%) | 96.0 | 100.0 | 90.3 | 95.3 |

N.B. BF = breastfeeding, HC = head circumference, wks = weeks, ∗: p < 0.05 by ANOVA
5.4.2 Feeding Patterns

The feeding patterns of the infants in the three study groups appear in table 7. A statistically significant decrease in the number of breast milk feeds per day and in the amount of breast milk intake ($p = 0.001$, $p < 0.001$ respectively) occurred after the introduction of CFs in all (within groups) the groups. However, there was no significant difference between the feeding groups regarding the number of breast milk feeds per day and in the amount of breast milk intake.

5.4.3 Association between socio-demographic characteristics and selected feeding patterns

Table 8 summarizes the relation between selected feeding patterns and socio-demographic characteristics. The rate of later introduction of solids was significantly associated with lower parity (odds ratio (OR) for lower parity = 0.2, confidence interval (CI): 0.09, 0.9), mothers who received multi-vitamins during breastfeeding (OR for vitamin supplementation during breastfeeding = 0.3, CI: 0.1, 1.9), and mothers who worked during the year preceding delivery (OR for having worked during the previous 12 months = 0.09, CI: 0.01, 0.5). Households with a greater number of occupants were significantly associated with earlier introduction of solid foods (OR = 0.2, CI: 0.05, 0.7). There was a significant association between lower maternal pre-pregnancy BMI and lower number of CFs introduced to the infants (OR = 3.3, CI: 1.0, 10.7).
<table>
<thead>
<tr>
<th>Variable</th>
<th>Cer (n=22)</th>
<th>Cer+Fr (n=28)</th>
<th>M (n=32)</th>
<th>All groups (n=82)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Infants EBF until 6 months (%)</strong></td>
<td>18.2</td>
<td>25.9</td>
<td>34.4</td>
<td>27.3</td>
</tr>
<tr>
<td><strong>Age of introduction of CF (months) mean±SD</strong></td>
<td>5.2±0.7</td>
<td>5.5±0.6</td>
<td>5.4±0.6</td>
<td>5.4±0.6</td>
</tr>
<tr>
<td><strong>Number of CF per day mean±SD</strong></td>
<td>2.1±0.7</td>
<td>1.9±0.6</td>
<td>2.2±1.0</td>
<td>2.0±0.8</td>
</tr>
<tr>
<td><strong>CF intake per day (g) mean±SD</strong></td>
<td>51±35.7</td>
<td>67.5±55.8</td>
<td>41.3±29.6</td>
<td>53.3±43</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Variable</th>
<th>Cer (n=22)</th>
<th>Cer+Fr (n=28)</th>
<th>M (n=32)</th>
<th>All groups (n=82)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Number of BM feeds per day mean±SD</strong></td>
<td>8.9±2.3</td>
<td>8.0±2.7*</td>
<td>8.3±2.0</td>
<td>7.4±2.0*</td>
</tr>
<tr>
<td><strong>BM intake per day (ml) mean±SD</strong></td>
<td>890±186</td>
<td>760±257*</td>
<td>930±216</td>
<td>827±186*</td>
</tr>
</tbody>
</table>

N.B: BM = breast milk, CF = complementary feeding, EBF = exclusively breastfed, *significant difference (p < 0.05) (within a group) over time by repeated measures ANOVA test.
### Table 8. Analysis of selective feeding patterns by socio-demographic characteristics

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Age of introduction of solids</th>
<th>Frequency of CF per day</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% at 4-5 mo</td>
<td>% at 5-6 mo</td>
</tr>
<tr>
<td><strong>Gender</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>54.2</td>
<td>43.4</td>
</tr>
<tr>
<td>Female</td>
<td>45.8</td>
<td>56.6</td>
</tr>
<tr>
<td><strong>Gestation (wks)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>36-40</td>
<td>62.5</td>
<td>79.2</td>
</tr>
<tr>
<td>≥41.1</td>
<td>37.5</td>
<td>20.8</td>
</tr>
<tr>
<td><strong>Parity</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-2</td>
<td>62.5</td>
<td>84.9</td>
</tr>
<tr>
<td>≥3</td>
<td>37.5</td>
<td>15.1</td>
</tr>
<tr>
<td><strong>Maternal age</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25-34</td>
<td>79.2</td>
<td>62.3</td>
</tr>
<tr>
<td>≥35</td>
<td>20.8</td>
<td>37.7</td>
</tr>
<tr>
<td><strong>Mode of delivery</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>86.4</td>
<td>84.3</td>
</tr>
<tr>
<td>Cesarean/other</td>
<td>13.6</td>
<td>15.7</td>
</tr>
<tr>
<td><strong>Antenatal/postnatal complications</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>29.2</td>
<td>22.6</td>
</tr>
<tr>
<td>No</td>
<td>70.8</td>
<td>77.4</td>
</tr>
<tr>
<td><strong>Fe supplementation during pregnancy</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>29.2</td>
<td>22.6</td>
</tr>
<tr>
<td>No</td>
<td>70.8</td>
<td>77.4</td>
</tr>
</tbody>
</table>
(Table 8 continued)

<table>
<thead>
<tr>
<th>Vitamin supplementation during pregnancy</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Yes</td>
<td>95.8</td>
<td>92.5</td>
<td>1.8</td>
<td>(0.1, 17.7)</td>
<td>86.4</td>
<td>94.0</td>
</tr>
<tr>
<td>No</td>
<td>4.2</td>
<td>7.5</td>
<td></td>
<td></td>
<td>13.6</td>
<td>6.0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Vitamin supplementation during BF*</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Yes</td>
<td>47.8</td>
<td>73.1</td>
<td>0.3*</td>
<td>(0.1, 1.9)</td>
<td>52.4</td>
<td>69.4</td>
</tr>
<tr>
<td>No</td>
<td>52.2</td>
<td>26.9</td>
<td></td>
<td></td>
<td>47.6</td>
<td>30.6</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Marital status</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Married/common-law</td>
<td>100.0</td>
<td>94.3</td>
<td>NA</td>
<td>NA</td>
<td>95.5</td>
<td>98.0</td>
</tr>
<tr>
<td>Single</td>
<td>0.0</td>
<td>5.7</td>
<td></td>
<td></td>
<td>4.5</td>
<td>2.0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Maternal pre-pregnancy BMI*</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>≤24.99</td>
<td>60.9</td>
<td>56.9</td>
<td>1.1</td>
<td>(0.4, 3.2)</td>
<td>75.0</td>
<td>46.9</td>
</tr>
<tr>
<td>≥25</td>
<td>39.1</td>
<td>34.1</td>
<td></td>
<td></td>
<td>25.0</td>
<td>53.1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Previous BF duration</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>≤6 mo</td>
<td>21.1</td>
<td>17.2</td>
<td>1.2</td>
<td>(0.2, 5.5)</td>
<td>26.7</td>
<td>9.7</td>
</tr>
<tr>
<td>≥6.1 mo</td>
<td>78.9</td>
<td>82.8</td>
<td></td>
<td></td>
<td>73.3</td>
<td>90.3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Maternal education</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary &amp; secondary</td>
<td>8.7</td>
<td>9.4</td>
<td>0.9</td>
<td>(0.1, 5.0)</td>
<td>13.6</td>
<td>8.2</td>
</tr>
<tr>
<td>Post secondary</td>
<td>91.3</td>
<td>90.6</td>
<td></td>
<td></td>
<td>86.4</td>
<td>91.8</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Mother worked during past 12 mo*</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Yes</td>
<td>70.8</td>
<td>96.2</td>
<td>0.09*</td>
<td>(0.01, 0.5)</td>
<td>81.8</td>
<td>86.0</td>
</tr>
<tr>
<td>No</td>
<td>29.2</td>
<td>3.8</td>
<td></td>
<td></td>
<td>18.2</td>
<td>14.0</td>
</tr>
</tbody>
</table>
(Table 8 continued)

| Paternal BMI | ≤24.99 | 34.5  | 24.0  | 2.4  | (0.8, 6.9) | 28.6 | 25.5 | 1.1 | (0.3, 3.6) |
| Paternal education | | | | | | | | | |
| Primary & secondary | 0.0 | 7.8 | NA | NA | 4.8 | 6.1 | 0.7 | (0.07, 7.8) |
| Post secondary | 100.0 | 92.2 | | | 95.2 | 93.9 | | |
| Household occupants* | ≤3 | 12.5 | 41.5 | 0.2* | (0.05, 0.7) | 27.3 | 34.0 | 0.7 | (0.2, 2.1) |
| ≥4 | 87.5 | 58.5 | | | 72.7 | 66.0 | | |
| Household children | 1-2 | 66.7 | 83.0 | 0.4 | (0.1, 1.2) | 59.1 | 80.0 | 0.3 | (0.1, 1.0) |
| ≥3 | 33.3 | 17.0 | | | 40.9 | 20.0 | | |
| Maternal smoking | Yes | 0.0 | 1.9 | NA | NA | 0.0 | 2.0 | NA | NA |
| No | 100.0 | 98.1 | | | 100.0 | 98.0 | | |

BF = breastfeeding, BMI = body mass index, CF = complementary feeding, CI = confidence interval, mo = month, NA = not applicable, OR = odds ratio, wks = weeks, *significant difference (p < 0.05) by pearson's chi-squared ($\chi^2$) test.
5.4.4 Energy and selected nutrient intake from the recommended first complementary foods only

5.4.4.1 Macronutrient intake

The energy and the macronutrient intake from the recommended first CFs alone of the three study groups appear in table 9. There was a statistically significant difference in energy intake from CFs between infants in M group compared with the cereal groups. Complementary food intake provided additional energy and macronutrients to the infants in the three groups (table 10). The carbohydrate intake of 1.6% (contribution) of the DRI was significantly lower in M group and differed from the carbohydrate contributions in the two cereal groups.

Table 9. Energy and macronutrient intake from the recommended first CFs only in the three study groups

<table>
<thead>
<tr>
<th>Nutrients (mean±SE)</th>
<th>Feeding group</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cer (n=22)</td>
<td>Cer+Fr (n=28)</td>
<td>M (n=32)</td>
<td>All groups (n=82)</td>
</tr>
<tr>
<td>Energy (Kcal)</td>
<td>223±33.5</td>
<td>294±47</td>
<td>62.8±8.9*</td>
<td>189±22.5</td>
</tr>
<tr>
<td>Energy (KJ)</td>
<td>932±42.0</td>
<td>1266±198</td>
<td>262±37.0*</td>
<td>804±95.6</td>
</tr>
<tr>
<td>Carbohydrate (g)</td>
<td>39.0±5.8</td>
<td>50.9±8.3</td>
<td>1.4±0.87*</td>
<td>29.4±4.2</td>
</tr>
<tr>
<td>Protein (g)</td>
<td>7.4±1.1</td>
<td>7.4±1.1</td>
<td>7.0±0.1</td>
<td>9.8±0.2</td>
</tr>
<tr>
<td>Fat (g)</td>
<td>4.7±0.69</td>
<td>6.4±0.98</td>
<td>4.2±0.58</td>
<td>5.1±0.45</td>
</tr>
</tbody>
</table>

N.B: * = p < 0.05
Table 10. Comparison of mean of % contribution of macronutrients towards the daily recommended DRIs from first recommended CFs only

<table>
<thead>
<tr>
<th>Nutrients (mean±SE)</th>
<th>Feeding group</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Cer (n=22)</td>
<td>Cer+Fr (n=28)</td>
<td>M (n=32)</td>
</tr>
<tr>
<td>Food energy (%)</td>
<td>41.6±6.2</td>
<td>52.2±8.6</td>
<td>12.5±1.7*</td>
<td>29.9±2.9</td>
</tr>
<tr>
<td>Carbohydrate (%)</td>
<td>66.9±9.5</td>
<td>84.8±13.8</td>
<td>1.0±0.4*</td>
<td>49.1±7.0</td>
</tr>
<tr>
<td>Protein (%)</td>
<td>82.6±12.1</td>
<td>81.5±12.8</td>
<td>50.9±7.7</td>
<td>70.6±6.5</td>
</tr>
<tr>
<td>Fat (%)</td>
<td>15.1±2.2</td>
<td>20.5±3.1</td>
<td>13.8±1.8</td>
<td>16.5±1.4</td>
</tr>
</tbody>
</table>

N.B: DRIs for 0-6 mo, * = p < 0.05

5.4.4.2 Micronutrient intake

5.4.4.2.1 Vitamin intake

Table 11 shows the vitamin intake from the recommended first CFs in the three study groups. The two infant cereals did not provide vitamin A. Vitamin C was not provided by any of the CFs. There was a statistically significant higher intake of niacin in M group than in the cereal groups. Riboflavin and thiamin intake was significantly lower in M group than in the two cereal groups. The percentage contributions of vitamins with respect to the daily DRIs for the three CFs appear in table 12. The three recommended foods satisfied the daily recommended DRIs for vitamin B₁₂ for infants aged 0-6 months. For riboflavin and thiamin, the intake in the two cereal groups exceeded the daily recommendation unlike the intake in M group. Niacin intake did not attain the recommended daily value in any of the groups, however, meat provided over 50% of the recommended daily amount.
### Table 11. Vitamin intake from the recommended first CFs only in the three study groups

<table>
<thead>
<tr>
<th>Nutrients</th>
<th>Feeding group</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cer (n=22)</td>
<td>Cer+Fr (n=28)</td>
<td>M (n=32)</td>
<td>All groups (n=82)</td>
<td></td>
</tr>
<tr>
<td>Vitamin A (µg)</td>
<td>0.0</td>
<td>0.0</td>
<td>2.1±0.30*</td>
<td>0.82±0.1</td>
<td></td>
</tr>
<tr>
<td>Vitamin D (IU)</td>
<td>ND</td>
<td>ND</td>
<td>4.1±0.56</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>Thiamin (mg)</td>
<td>0.66±0.10</td>
<td>0.65±0.10</td>
<td>0.02±0.00*</td>
<td>0.4±0.05</td>
<td></td>
</tr>
<tr>
<td>Riboflavin (mg)</td>
<td>0.70±0.10</td>
<td>0.92±0.14</td>
<td>0.08±0.01*</td>
<td>0.5±0.07</td>
<td></td>
</tr>
<tr>
<td>Niacin (NE)</td>
<td>0.43±0.03</td>
<td>0.54±0.08</td>
<td>1.6±0.210*</td>
<td>0.9±0.01</td>
<td></td>
</tr>
<tr>
<td>Vitamin B12 (µg)</td>
<td>0.65±0.09</td>
<td>0.46±0.07</td>
<td>0.57±0.80</td>
<td>0.5±0.04</td>
<td></td>
</tr>
<tr>
<td>Vitamin C (mg)</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td></td>
</tr>
</tbody>
</table>

N.B: NA = not applicable, ND = no data provided, * = p < 0.05

### Table 12. Comparison of mean of % contribution of vitamins towards the daily recommended DRIs from first recommended CFs only

<table>
<thead>
<tr>
<th>Nutrients</th>
<th>Feeding group</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cer (n=22)</td>
<td>Cer+Fr (n=28)</td>
<td>M (n=32)</td>
<td>All groups (n=82)</td>
<td></td>
</tr>
<tr>
<td>Vitamin A (%)</td>
<td>0.0±0.0</td>
<td>0.0±0.0</td>
<td>0.38±0.11*</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>Vitamin D (%)</td>
<td>ND</td>
<td>ND</td>
<td>1.0±0.14</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>Thiamin (%)</td>
<td>330.5±51.0</td>
<td>327.6±53.4</td>
<td>15.2±2.5*</td>
<td>214±29.5</td>
<td></td>
</tr>
<tr>
<td>Riboflavin (%)</td>
<td>233.0±35.8</td>
<td>308.0±50.0</td>
<td>26.1±3.5*</td>
<td>183.6±24.9</td>
<td></td>
</tr>
<tr>
<td>Niacin (%)</td>
<td>14.3±1.8</td>
<td>18.3±2.8</td>
<td>53.5±7.2*</td>
<td>30.0±3.5</td>
<td></td>
</tr>
<tr>
<td>Vitamin B12 (%)</td>
<td>164.8±24.5</td>
<td>115.5±19.2</td>
<td>146.3±19.9</td>
<td>140.7±12.1</td>
<td></td>
</tr>
<tr>
<td>Vitamin C (%)</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td></td>
</tr>
</tbody>
</table>

N.B: DRI for 0-6 mo, NA = not applicable, ND = no data provided, * = p < 0.05
5.4.4.2.2 Mineral intake

Overall, the intake of sodium, calcium, and phosphorus was significantly lower in M group (table 13). There was a statistically significant higher intake of iron in the two cereal groups than in M group. Table 14 presents the percentage contribution of selective minerals to the daily recommended DRIs from the three CFs only. Iron intake exceeded the daily recommendation for infants aged 0-6 months in the three groups. The intake of phosphorus in the cereal and fruit group fulfilled the required daily amount.

Table 13. Selective mineral intake from the recommended first CFs only in the three study groups

<table>
<thead>
<tr>
<th>Nutrients (mean±SE)</th>
<th>Feeding group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cer (n=22)</td>
</tr>
<tr>
<td>Sodium (mg)</td>
<td>51.6±8.30</td>
</tr>
<tr>
<td>Calcium (mg)</td>
<td>56.8±8.50</td>
</tr>
<tr>
<td>Iron (mg)</td>
<td>16.0±2.50</td>
</tr>
<tr>
<td>Zinc (mg)</td>
<td>0.15±0.03</td>
</tr>
<tr>
<td>Magnesium (mg)</td>
<td>1.40±0.58</td>
</tr>
<tr>
<td>Phosphorus (mg)</td>
<td>78.6±7.40</td>
</tr>
</tbody>
</table>

N.B: NA = not applicable, ND = no data provided, * = p < 0.05
Table 14. Comparison of mean of % contribution of selective minerals towards the daily recommended DRIs from the recommended CFs only

<table>
<thead>
<tr>
<th>Nutrients</th>
<th>Feeding group</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cer (n=22)</td>
<td>Cer+Fr (n=28)</td>
<td>M (n=32)</td>
<td>All groups (n=82)</td>
</tr>
<tr>
<td>Sodium (%)</td>
<td>44.1±7.2</td>
<td>41.0±6.5</td>
<td>15.9±2.6*</td>
<td>32.7±3.5</td>
</tr>
<tr>
<td>Calcium (%)</td>
<td>28.5±4.2</td>
<td>25.0±3.8</td>
<td>1.9±0.82*</td>
<td>17.5±2.3</td>
</tr>
<tr>
<td>Iron (%)</td>
<td>3963±499</td>
<td>4622±437</td>
<td>217±30.3*</td>
<td>2823±315</td>
</tr>
<tr>
<td>Zinc (%)</td>
<td>9.6±1.2</td>
<td>10.2±1.8</td>
<td>ND</td>
<td>NA</td>
</tr>
<tr>
<td>Magnesium (%)</td>
<td>1.2±0.38</td>
<td>1.3±0.19</td>
<td>ND</td>
<td>NA</td>
</tr>
<tr>
<td>Phosphorus (%)</td>
<td>78.8±11.4</td>
<td>104.1±16.0</td>
<td>40.4±5.5*</td>
<td>73.6±7.4</td>
</tr>
</tbody>
</table>

N.B: DRI for 0-6 mo, NA = not applicable, ND = no data provided, * = p < 0.05

5.4.5 Growth

Table 15 compares the gain in weight, length and head circumference z-scores from before and after introduction of the study foods stratified by feeding duration. There were no significant differences in growth rates between male and female infants. No significant differences were recorded in growth rates between the three groups except for gain in head circumference of infants who were fed meat for 2-3 weeks. Table 16 summarizes the association between selected feeding patterns and the growth rate of all the infants. As evident in that table, higher weight gain was significantly associated with earlier age of introduction of the study foods (OR = 3.0, CI: 1.0 to 9.3). There was no significant correlation between the total estimated amount of breast milk and gain in weight, length and head circumference (r = 0.12, p = 0.3; r = -0.02, p = 0.85; r = 0.1, p = 0.4 respectively). Similarly, the amount of CF intake did not correlate with any of the growth
variables (weight gain; $r = 0.09$, $p = 0.4$, gain in length; $r = 0.22$, $p = 0.07$, gain in head circumference; $r = -0.007$, $p = 0.9$).

Table 15. Growth rate in $z$-scores in the three study groups from before and after introduction of CFs categorized according to solid feeding duration

<table>
<thead>
<tr>
<th>Growth variable (mean±SE)</th>
<th>Cer (n=8)</th>
<th>Cer+Fr (n=13)</th>
<th>M (n=18)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Feeding duration 2-3 wks</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight gain</td>
<td>-0.1±0.18</td>
<td>-0.06±0.13</td>
<td>-0.16±0.11</td>
</tr>
<tr>
<td>Gain in length</td>
<td>0.31±0.4</td>
<td>-0.3±0.25</td>
<td>-0.01±0.1</td>
</tr>
<tr>
<td>Gain in HC</td>
<td>0.5±0.39</td>
<td>0.59±0.39</td>
<td>-0.4±0.15*</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Growth variable (mean±SE)</th>
<th>Cer (n=13)</th>
<th>Cer+Fr (n=15)</th>
<th>M (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Feeding duration 3-4 wks</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight gain</td>
<td>0.15±0.08</td>
<td>0.14±0.1</td>
<td>0.003±0.1</td>
</tr>
<tr>
<td>Gain in length</td>
<td>-0.14±0.21</td>
<td>-0.23±0.25</td>
<td>-0.22±0.22</td>
</tr>
<tr>
<td>Gain in HC</td>
<td>-0.16±0.21</td>
<td>0.23±0.17</td>
<td>-0.34±0.21</td>
</tr>
</tbody>
</table>

N.B: HC = head circumference, wks = weeks, $* = p < 0.05$
Table 16. Analysis of selective feeding practices by growth rate

<table>
<thead>
<tr>
<th>Growth variable</th>
<th>Age of introduction of solids</th>
<th></th>
<th></th>
<th>Frequency of CF per day</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% at 4-5 mo</td>
<td>% at 5-6 mo</td>
<td>OR (95% CI)</td>
<td>% of 1 CF</td>
<td>% of ≥2 CF</td>
<td>OR (95% CI)</td>
</tr>
<tr>
<td>Weight gain*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Higher z-score (0.0 to 2.5)</td>
<td>71.4</td>
<td>44.7</td>
<td>3.0* (1.0, 9.3)</td>
<td>55.0</td>
<td>53.3</td>
<td>1.0 (0.3, 3.0)</td>
</tr>
<tr>
<td>Lower z-score (-0.01 to -2.5)</td>
<td>28.6</td>
<td>55.3</td>
<td></td>
<td>45.0</td>
<td>46.7</td>
<td></td>
</tr>
<tr>
<td>Gain in length</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Higher z-score (0.0 to 2.5)</td>
<td>52.4</td>
<td>45.7</td>
<td>1.3 (0.4, 3.6)</td>
<td>45.0</td>
<td>48.9</td>
<td>0.8 (0.2, 2.4)</td>
</tr>
<tr>
<td>Lower z-score (-0.01 to -2.5)</td>
<td>47.6</td>
<td>54.3</td>
<td></td>
<td>55.0</td>
<td>51.1</td>
<td></td>
</tr>
<tr>
<td>Gain in HC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Higher z-score (0.0 to 2.5)</td>
<td>65.0</td>
<td>43.5</td>
<td>2.4 (0.8, 7.1)</td>
<td>55.0</td>
<td>51.2</td>
<td>1.1 (0.4, 3.3)</td>
</tr>
<tr>
<td>Lower z-score (-0.01 to -2.5)</td>
<td>35.0</td>
<td>56.5</td>
<td></td>
<td>45.0</td>
<td>48.8</td>
<td></td>
</tr>
</tbody>
</table>

N.B: HC = head circumference, CF = complementary feeding, mo = month, *significant difference (p < 0.05) by pearson's chi-squared ($\chi^2$) test
5.4.5.1 Growth and nutrient intake from complementary foods

Overall, the macronutrient and micronutrient intake from the CFs did not correlate with weight gain nor with gain in length. Total energy (figure 10) and carbohydrate intake (figure 11) were significantly correlated with gain in head circumference ($r = 0.3, p = 0.01, r = 0.3, p = 0.011$ respectively). Significant positive correlations were observed between thiamin, riboflavin, iron and phosphorus intake (figures 12, 13, 14, 15) and gain in head circumference ($r = 0.3, p = 0.02; r = 0.28, p = 0.02; r = 0.31, p = 0.01; r = 0.3, p = 0.03$ respectively). When each feeding group analysis was performed separately, no significant correlations were observed between the macronutrient and micronutrient intake of the infants in each group and the growth variables.
Figure 10. Correlation between total energy intake and gain in head circumference in all infants. Spearman’s correlation = 0.3 ($p = 0.01$, significant at 0.05 level).

Figure 11. Correlation between carbohydrate intake and gain in head circumference in all infants. Spearman’s correlation = 0.3 ($p = 0.01$, significant at 0.05 level).
Figure 12. Correlation between thiamin intake and gain in head circumference in all infants. Spearman’s correlation = 0.3 ($p = 0.02$, significant at 0.05 level).

Figure 13. Correlation between riboflavin intake and gain in head circumference in all infants. Spearman’s correlation = 0.28 ($p = 0.02$, significant at 0.05 level).
Figure 14. Correlation between iron intake and gain in head circumference in all infants. Spearman’s correlation = 0.31 ($p = 0.01$, significant at 0.05 level).

Figure 15. Correlation between phosphorus intake and gain in head circumference in all infants. Spearman’s correlation = 0.3 ($p = 0.03$, significant at 0.05 level).
5.5 Discussion

5.5.1 Socio-demographic characteristics and feeding patterns

It is highly important to determine the socio-demographic factors that are associated with the feeding patterns of breastfed infants. This will allow a focus on those factors, which will in turn help in the future promotion of optimal feeding practices. Although we found similar baseline socio-demographic characteristics among the groups, our results showed that a number of socio-demographic factors were associated with selective feeding patterns of the infants. The rates of later introduction of solid foods were associated with the following maternal factors: mothers with lower parity (OR for lower parity = 0.2, CI: 0.09, 0.9), mothers who received multi-vitamins during breastfeeding (OR for vitamin supplementation during breastfeeding = 0.3, CI: 0.1, 1.9), and mothers who worked during the year preceding delivery (OR for having worked during the previous 12 months = 0.09, CI: 0.01, 0.5). Our data indicate that the factor of households with a higher number of occupants was associated with earlier introduction of solid foods (OR = 0.2, CI: 0.05, 0.7). The results show that there was a relation between lower maternal pre-pregnancy BMI and lower number of CFs given to the infants (OR = 3.3, CI: 1.0, 10.7).

To our knowledge, few studies conducted in North America have examined the relationship between socio-demographic characteristics and infant feeding patterns. In the US Infant Feeding Practices Study II (IFPS II), the association between maternal education level and feeding practices was examined among 2400 mothers (46). Undesirable feeding practices of complementary feeding were more likely to be found
among lower-educated mothers. For example, introduction of CFs before 4 months of age was significantly higher among women with high school education or less compared with women with college education (35.8%, \( p < 0.001 \)). Moreover, similar to our findings, the IFPS II found no association between maternal education and the numbers of times of receiving CFs per day (\( p = 0.26 \)) (46). It has also been found that noncompliance with child and maternal health recommendations was inversely associated with socioeconomic status (47). The Feeding Infants and Toddlers (FIT) study, was a large cross-sectional examination of 3022 infants and toddlers conducted in the United States. It evaluated infant and toddler feeding in relation to multiple factors such as, nutrient intake, feeding practices and socio-demographic characteristics (48). One particular analysis within the FIT study assessed the maternal and child characteristics associated with the adherence to American Academy of Pediatrics feeding guidelines and specific feeding patterns (49). The FIT study found that later introduction of solids or exclusive breastfeeding up to 6 months was associated with higher maternal level of education (OR = 3.2) and being married (OR = 2.0). We did not find similar results in the present study owing to the small sample size compared with that of the FIT study. With regard to household occupants, the FIT study observed a negative association (OR, 0.6; \( p < 0.5 \)) between having additional children (occupants) younger than age 18 years and continuing breastfeeding until 12 months of age. Moreover, The FIT study also reported that mothers who introduced CF between the age of 4 and 6 months had more college education (OR = 2.0), lived in the western region of the United States (OR = 2.1), had a history of previous breastfeeding (OR = 1.7), were of higher age (OR = 1.05), and their current infant was the firstborn (OR = 1.4) (49). With respect to maternal pre-pregnancy BMI, it was found that
higher maternal BMI was strongly associated with earlier introduction of CFs in a cohort of breastfed infants (50). Although our study was not powered to detect such a difference, we observed that lower maternal pre-pregnancy BMI was associated with undesirable lower CF intake (OR = 3.3, CI: 1.0, 10.7).

5.5.2 Feeding patterns

Feeding patterns during infancy affect infant growth and may influence eating habits, preferences, and overall health later in life (51). By leaving the decision about the timing of the introduction of the study foods to the parents, we were able to determine whether the parents followed the EBF guideline up to 6 months of age. The present study found that among the total study subjects, only 27.3% of the infants continued with EBF until 6 months. One Canadian investigation determined that over 83% of infants were started on cereal at 3 months of age (26). The larger FIT study of 3022 infants and toddlers reported that 76% of infants were exclusively or partially breastfed at birth and that this proportion declined to 30% at 6 months (52). In 2008, the second FIT study survey revealed that there was a significant increase in the breastfeeding rate to 42.5% accompanied by delay in introduction of solid foods compared with the 2002 survey (53). Our results showed that the average age of introduction of the study CFs for all the infants was 5.4 months. In the FIT study, about two thirds of infants were given CFs at the age of 4-6 months (52). Health Canada recommends that iron-rich foods should be introduced two to three times a day to breastfed infants to ensure adequate iron intake (2). We found that the daily average number of CFs introduced to the infants was 2 times as recommended (table 7). In regards to our finding of the significant decrease in the number of breast feeds per day and in the estimated volume of milk after introduction of the three study foods (table 7).
would seem that the breastfed infants were able to regulate their total energy intake by decreasing the amount of breast milk consumed after the CFs were introduced. In confirmation of our findings, one RCT showed that infants who received CFs consumed less breast milk than those who received EBF, when measured using a stable isotope method (34). Another RCT conducted in a developing country found that mean breast milk intake was similar at baseline between two groups of breastfed infants, however, it decreased significantly later in the group receiving CFs than in the group with continued exclusive breastfeeding (33). Further more, a one longitudinal study reported that breastfed infants who had more than six daily feeds consumed less CF than breastfed infants who had fewer than six breast milk feeds (54).

5.5.3 Nutrient intake

The dietary assessment revealed nutrient adequate first CFs that complied with the daily recommendations, with few exceptions that will be discussed below. Though the reported mean nutrient intakes were for 0-6 months of age, it is important to note that the corresponding recommendations for this age were based on breast milk intake only (28, 29, 36, 55). The current data and previous data consistently show that breast milk continues to make a major nutritional contribution to the breastfed infant’s diet (56). In the present study, the macronutrient intake from breast milk alone and from breast milk with solid food met the recommendations for carbohydrates (60 g/day). However, when infants in M group were introduced to solid food, their carbohydrate intake was significantly lower than in the two cereal groups, though it remained within the recommended daily amount. This difference was predominantly due to the composition of meat which has less carbohydrate than cereal, and resulted in the significant difference in
energy intake between M group and the two cereal groups. The provision of all three types of foods led to a significant increase in protein intake in all the groups. Although infants in M group had significantly lower energy intake, their protein intake was comparable with that of the two cereal groups. Fat intake before and after introduction of the study foods exceeded the recommended total fat intake of 31 g/day. Fat intake did not increase significantly after the introduction of solid foods due to the displacement of breast milk intake by the introduction of CFs (24). Moreover, breast milk has a relatively high fat content compared with most CFs (56). Breast milk provides a considerable quantity of micronutrients (56), but the present study found that the estimated amount of breast milk alone did not satisfy the recommended daily amount of vitamin A, vitamin D, thiamin, zinc and magnesium for infants aged 0-6 months. However, after introduction of the three study foods, breast milk and CF together resulted in fulfillment of the micronutrient requirements of infants in that age group. The iron intake of all the infants in the three study groups was adequate (tables 13 & 14). In a Honduran RCT of 139 breastfed infants, the nutrient intake from either breast milk (n = 50) or from CF in addition to breast milk (n = 89) was assessed. It was found that iron intake from breast milk alone was inadequate, whereas the iron intake from breast milk together with CF was adequate and it significantly differed from the EBF infant group (0.17 mg/day, 4.3 mg/day, \( p < 0.001 \)) (32). In a recent cross-sectional survey of 21 infants aged 0-6 months living in Baltimore, USA, it was found that the mean macronutrient and micronutrient intake was adequate for this age group (57). Comparable studies have also determined that infants exceeded the recommended macronutrient and the micronutrient levels (58, 59).
The WHO has estimated that the requirements from CFs alone for children living in developing countries is 269 Kcal/day for infants aged 6-8 months. This was calculated by subtracting the amount of energy and nutrients provided by breast milk from age-specific estimates of children’s average nutrient and energy needs (56). For infants living in developed countries, Dewey has estimated the required energy and nutrient intake from CFs directly using DRIs and WHO guidelines (60). Other studies have estimated energy and protein intake, which were believed to represent the normal “usual intake” of American infants (61). Based on the recommended intakes from CFs alone (28, 29, 36, 55, 60), the estimated energy intake from the groups was considered adequate except for meat which provided 22% (62.8 Kcal/day) of the recommendation of CFs. To our knowledge, only one Canadian survey has thoroughly examined infants' nutrient intake from CFs alone (26). In that study, it was estimated that CFs contributed a daily mean of 118 Kcal and 197 Kcal to the total diet at 4 and 6 months respectively, that accounted for 41.9% and 70%, respectively, of the recommended daily intake from CFs. Dewey et al. (60) found that CFs provided an estimated energy of 129 Kcal/day for infants aged 6-8 months; that compares with the findings of 223 Kcal, 294 Kcal and 62.8 Kcal from iron-fortified cereal, iron-fortified cereal with fruit and meat respectively, provided for infants aged 4-6 months in the present study. Similar to our findings lower carbohydrate intake from CFs alone was also observed in the previous Canadian study: the mean carbohydrate intake for infant aged 4 and 6 months was 22 g/day and 37 g/day respectively (26). Our study found no significant differences in protein intake from CFs between the three feeding groups, which met the recommended protein intake from CFs. The Darling study of breastfed (n = 73) and formula-fed (n = 46) American infants observed no significant
differences in energy and protein intake from CFs between the two groups (62). The mean protein intake from CFs was 2.1 g/day in breastfed and 2.3 g/day in formula-fed infants aged 6 months, that was lower than the recommended amount of protein from CFs of 7 g/day. Our finding of lower fat contribution from iron-fortified cereal and meat is in line with the previous Canadian study for infants aged 4-6 months (26). The fat intake from CFs for the ages of 4 and 6 months was 2 g/day and 3 g/day respectively (26).

With regard to vitamins in the present study, consumption of the two iron-fortified cereals provided the recommended amount of thiamin, riboflavin, and vitamin B₁₂; but it did not fulfill the required amount of vitamin A, vitamin C, and niacin. Meat consumption provided 2.1 µg/day of vitamin A. However, consumption of meat did not meet the required amounts of all other vitamins. Compared with the results of the previous Canadian study, we found that the vitamin intake from the CFs was adequate for infants aged 6 months, except for vitamins C and D (26). It should be noted that the calculated nutrient intakes in the previous Canadian study were derived from the consumption of different types of CFs. Similar findings were obtained in the Darling study which was conducted on 46 breastfed infants in the United States, the average vitamin content provided by CFs was generally satisfactory for infants aged 6-8 months for such vitamins as thiamin, riboflavin, and niacin (62). According to the findings of the present study, mean iron intake from meat did not satisfy the desired amount from CFs alone (table 14), however, the amount of iron intake from the two cereal groups was two- to threefold greater than required. This was due to the fact that only iron-fortified cereal contains appreciable amount of iron (8.8 mg per 28 g of cereal, table 5); canned meat would provide about 2-10% of the daily required iron per 100 ml container (table 5). Our finding
of low intake of zinc (from cereals only) and calcium from the study CFs alone is in agreement with the results of the Darling study. The mean intake of zinc and calcium was 0.4 mg and 73 mg, respectively, which met only 36% and 52% of the recommended level (62). The previous Canadian study determined that the mean iron intake of 6-month old infants accounted for 81% of the recommendation for CFs alone (26). In a recent Icelandic RCT, 100 breast fed infants were randomized to receive CFs at either 4 or 6 months of age. Analysis of the nutrient intake from CFs alone at 5.25 months for the infants who received CFs revealed that the intake of iron, calcium, vitamin D and vitamin C fell below the recommended daily intake for infants aged 6-11 months (63). Although it has been reported that infant cereal is the most important complementary source of all minerals (64), our data show that consumption of cereals alone was not able to meet the recommendations for CFs for sodium, magnesium and phosphorus.

5.5.4 Growth

In the present study, no significant differences were observed between the three feeding groups in terms of weight gain and length gain when infants were fed CFs for either 2-3 weeks or 3-4 weeks duration. However, we found that feeding meat for 2-3 weeks resulted in a significant difference in gain in head circumference compared with infants who received cereal over the same duration (table 15). When we conducted an analysis of growth rate by selective feeding practices, we found that infants with higher weight-gain z-scores were 3 times more likely to have their solid feeding started earlier than infants with lower weight gain z-scores (OR = 3.0, CI: 1.0, 9.3, p < 0.001). Among infants in all groups, total energy and carbohydrate intake was positively correlated with a gain in head circumference (figures 11 & 12). The intake of thiamin, riboflavin, iron and phosphorus
was also positively correlated with a gain in head circumference (figures 13, 14, 15 and 16). The available evidence from North America on the effects of different types of complementary feeding on growth in breastfed infants is limited. In a recent RCT conducted in the United States, the linear growth and weight gain of breastfed infants fed either iron-fortified cereal, zinc- and iron-fortified cereal or meat were not significantly different between the three feeding groups (35). A number of studies have examined the effect of age of CF introduction on growth. Our meta-analysis (Chapter IV), found no significant effect of earlier solid food introduction on the growth of EBF infants in both developed and developing countries. Kramer and Kakuma conducted a large Cochrane review on the effect of duration of exclusive breastfeeding and concluded that neither RCTs nor observational studies conducted in developed and developing countries found inadequacy in terms of gain in weight, length and head circumference for infants who continued to receive EBF for 6 months (65). In agreement with our findings, a significant positive association between earlier CF introduction (before 16 weeks) and infant weight gain was observed in a large cohort of Danish infants (50). In an RCT conducted in Denver, USA, breastfed infants were randomized to receive either commercial beef or iron-fortified cereal (66). Infants in the meat group showed a significant higher gain in head circumference than those in the cereal group. With regard to the association between growth and nutrient intake, a study conducted in the United Kingdom found no significant association between energy intake and weight gain among 299 breastfed infants which is inline with our findings (43). In a longitudinal study, modest meat intake of < 28.3 g/day was significantly associated with gain in weight during infancy, and with psycho-motor development at 24 months of age (67). Finally, a recent systematic review of RCTs
suggested that the evidence from developing and developed countries is inconclusive regarding the relationship between the effect of micronutrient-fortified cereal and infant growth (68).

5.6 Limitations

One advantage of the present study over previous cross-sectional investigations is the RCT design, which is the gold standard in nutrition research (69). Another strength of our study is the use of analysis of repeated measures before and after the introduction of CFs, that provided important information about nutrient intake within individuals. Although the present study design offers a high level of evidence and important findings, our study sample is limited by including only breastfed infants that has been previously linked with specific demographic factors such as higher educated mothers, thus we have to interpret our findings with caution. In addition, there is also a possibility of underrepresentation of parents from minority groups, and those from lower socio-economic status. One important limitation to note is that we may have under- or over-estimated the volume of breast milk intake by using an approximate volume of milk consumed per minute of feeding (125 ml/minute). Although there is a possibility of recall bias through using the 3-day dietary record, the consumption of other foods was limited by the provision of the study foods and due to the fact that during this time period there is less possibility of daily variability in intake (70).

5.7 Conclusion

Longer duration of breastfeeding and delayed introduction of solid foods are both positive trends and desirable goals for health-care professionals. Several important findings
emerge from the present study. Our data appear to indicate that the exclusive breastfeeding mothers did not heed the Health Canada message given the average age of introduction of solids of 5.4 months. Information about the transition to CFs should be conveyed to breastfeeding mothers in an easily understandable manner that emphasize the type, time and the frequency of CFs. Intake of both macronutrients and micronutrients is adequate if the sources are both breast milk and CFs, however, EBF infants may be at risk of micronutrient deficiency especially if breast milk intake decreases over time. A lower consumption of specific vitamins and minerals through introducing meat is only a concern if no other types of CFs are consumed. Given the small stomach capacity of infants in this age group, and the high demand of nutrients required for growth, both iron-fortified cereal and meat could be offered as the first CFs since each food type will complement the other as well as breast milk. With regard to growth, our findings substantiate the relation between earlier CF introduction and gain in weight. Although our study has a small sample size compared with previous epidemiological studies of infant feeding practices, we found that breastfed infant feeding patterns were related to caregivers' socio-demographic characteristics. The overall findings presented here yield important insights to the possible content of future recommendations. These are needed to improve infant feeding patterns and indicate the most beneficial type of first CFs for infants, which is of paramount importance for overall health later in life. In future, special attention need to be devoted to educating the health-care providers who advise breastfeeding mothers on the type, time and frequency of first complementary feeding. More RCTs and observational studies with larger sample sizes need to be conducted in
Canada to support building a definitive evidence base for the optimal complementary feeding of EBF infants.

5.8 References


BRIDGE TO CHAPTER VI

Manuscript V showed that the consumption of iron-fortified cereal appears more efficient than consumption of meat in satisfying the recommendation from complementary foods only for macro and micronutrients. The following chapter consists of a manuscript which presents another assessment and a comparison of the safety of the recommended first complementary foods from oxidative stress status and inflammatory perspective in the clinical trial described in chapter V. Wafaa Qasem was the principal author of the manuscript and the coordinator of the clinical trial. Wafaa Qasem drafted the manuscript, helped in the data collection and in the analyses. Dr. Meghan Azad helped in the data analysis, and reviewed the manuscript. Dr. Zakir Hossain helped in the data analysis. Sarah Jorgensen, Sandra Castillo San Juan, and Chenxi Cai helped in the data collection. Dr. Ehsan Khafipour helped in the data analysis and will review the manuscript. Dr. L.Jackson Roberts helped in the data analysis. Dr. Trust Beta, helped in the study design and the data collection methods, and she will review the manuscript. Dr. James Friel was the principal investigator of this clinical trial. He conceptualized and designed the study, designed the data collection methods, and coordinated and supervised data collection, reviewed the manuscript, and approved the final manuscript.
CHAPTER VI

MANUSCRIPT 5

ASSESSMENT OF COMPLEMENTARY FEEDING OF CANADIAN INFANTS:
EFFECTS OF IRON RICH FOODS ON IRON STATUS AND OXIDATIVE
STRESS MARKERS

This manuscript is currently under internal review and will be submitted to the journal Pediatr...
**6.1 Abstract**

**Background:** Health Canada recommends exclusive breastfeeding (EBF) until six months followed by introducing iron-rich complementary foods (CFs). There is a concern that consumption of these CFs with high iron dose may predispose infants to inflammation through reactive oxygen species (ROS) generation in their intestinal tract. The aim of this study was to assess if the recommended CFs were safe from a free radical and inflammatory perspective and to assess if these CFs maintain the iron status of the breastfed infants. **Methods:** 87 EBF infants were randomly assigned to receive one of the following: iron-fortified cereal (Cer), iron-fortified cereal with fruit (Cer+Fr), meat (M). Blood, urine and stool samples were collected to assess iron status, ROS generation, and inflammatory markers. **Results:** There was a significant decrease in plasma ferritin over time in all groups (\( p = 0.04 \)). A significant increase in fecal ROS formation (\( p < 0.002 \))
after the introduction of CFs was observed, with meat group having the lowest concentration. **Conclusions:** EBF infants may be at risk of developing iron deficiency despite the provision of any type of iron rich CFs. Untargeted iron fortification of infant cereals may result in untoward effects including ROS generation in the intestinal tract.

6.2 Introduction

By 6 months of age, breastfed infants become dependent on complementary foods (CFs) as a source of iron to prevent iron deficiency (ID), as a result of declining of the iron stores present at birth and low levels of iron in breast milk (1-3). Health Canada recommends the introduction of traditional iron-fortified cereal and meat as first CFs in order to meet the iron requirements of the growing infants and to prevent ID and iron deficiency anemia (IDA) during infancy which can have devastating effects on neurodevelopment (4, 5). In North America, iron is provided most commonly through iron-fortified cereals (6, 7). These cereals contain non-heme electrolytic iron, which is absorbed at a rate of < 5% (8, 9). Meat provides the more readily absorbed heme iron with an absorption rate of 35% (10). However, we found that meat was among the least common CFs introduced to Canadian infants (6).

Available evidence suggests an association between excessive iron exposure in the intestinal tract and initiation of inflammatory process by reactive oxygen species (ROS) generation (11, 12). With such a high amount of iron in fortified cereals (25-30 mg of iron/100g dry weight) and low absorption rates, a concern has been raised about the possibility of the iron in these CFs causing ROS generation and inflammation to the large intestine of the infants (13, 14). Cumulative evidence indicates that anti-oxidant rich foods such as fruits prevent ROS generation thus inflammation (15-17). A study
conducted on adults who received high iron dosage of 120 mg/day for 2 separate 7-days periods, has implicated iron as a stimulator of oxidative stress and inflammation (14) which was ameliorated with a concurrent administration of an antioxidant supplement. Infants introduced to iron-fortified cereals are receiving an equivalent dose of iron per kilogram body weight and may be producing ROS, leaving them prone to inflammation (11, 12). To date, no studies have considered the iron-fortified cereal and meat from the ROS generation and inflammatory perspective. Therefore, the aim of this study was to assess and determine the safety of the traditional (iron-fortified cereal) and the newly recommended (meat) first CFs in regards to ROS generation and inflammation, to determine if the presence of antioxidant (fruit) in the iron-fortified cereal reduce the oxidative effect of iron in the intestinal tract and to assess the efficacy of these foods in maintaining the iron status of the breastfed infants.

6.3 Methods

The study design, ethical approval, study subjects, study foods, dietary analysis, sample size determination and statistical analysis were previously reported in chapter V. In brief, 87 full term healthy EBF infants were randomly assigned to one of three study foods: iron-fortified cereal (Cer), iron-fortified cereal with fruit (Cer+Fr), meat (M). Random allocation sequence for the three feeding groups was generated using computer generated random numbers. This was done by the statistician of the Children’s Hospital Research Institute of Manitoba, Winnipeg, Manitoba using SAS/STAT® software (SAS Institute Inc., Cary, NC, USA). The caregivers of the participating infants were blinded to the allocation in this trial. These foods were the first CFs introduced to the infants for a period of 2-4 weeks. Parents decided when to begin complementary feeding and reported
this date. The study consisted of two visits in which blood (optional), urine, stool, dietary records and growth measurements were collected. In addition, a questionnaire was completed at the first visit to obtain socio-demographic characteristics and infant feeding patterns. Detailed descriptions of the method sections of the socio-demographic characteristics and the anthropometric measurements were reported in chapter V. Blood samples of 18 infants were collected to measure plasma ferritin and hemoglobin concentrations. Plasma ferritin concentration is the method of choice and the most specific test that reflects total body iron stores (18). Hence, ID was defined as plasma ferritin $< 12 \mu g/l$. Iron deficiency anemia was defined as hemoglobin level of $< 110$ g/l in combination with plasma ferritin level of $< 12 \mu g/l$ (18).

6.3.1 Sample collection

6.3.1.1 Blood sample

Blood samples of 0.5 ml were drawn by the research nurse at the Children’s Hospital Research Institute of Manitoba, Winnipeg, Manitoba. The blood samples were obtained using BD Microtainer® contact activated lancets (Becton Dickinson and company®, Franklin Lakes, New Jersey, USA). This allowed a free flow of blood with minimal squeeze of the finger. The blood samples were collected in separate BD Microtainer® MAP containing 1.0 mg of dipotassium ethylene diaminetetraacetic acid (K$_2$EDTA) (Becton Dickinson and company®, Franklin Lakes, New Jersey, USA). Samples were placed in a cooler with ice packs and transferred to the laboratory at Richardson Centre for Functional Foods and Nutraceuticals (RCFFN), University of Manitoba, Winnipeg. Within one hour of blood collection, the samples were centrifuged at 3000 rpm for 20
minutes to separate blood cells from plasma. The separated aliquots were stored at -80°C until analysis.

6.3.1.2 Urine sample

Urine samples were collected by the infants caregivers using the pediatric urine collector (Kendall pediatric urine collector, Covidien©, Mansfield, Massachusetts, USA) provided by the study team along with instructions (Appendix 8). Samples were placed in cooler and transferred to RCFFN and separated in aliquots, stored in 1.5 ml tubes (Eppendorf® Safe-Lock tube™, Hamburg, Germany) at -80°C until analysis.

6.3.1.3 Fecal sample

Fecal samples were collected from soiled diapers. Instructions on sample collection along with plastic collection tubes were sent to the caregivers (Appendix 9). Samples were transported using a cooler with ice packs to the RCFFN by the study team, separated in labeled 1.5 ml tubes (Eppendorf® Safe-Lock tube™, Hamburg, Germany) and stored at -80°C until analysis.

6.3.2 Laboratory methods

6.3.2.1 Hemoglobin

Hemoglobin was analyzed using the HemoCue meter Hb 201+ (HemoCue™, Angelholm, Sweden). The HemoCue portable photometer measures hemoglobin according to a modified azidemethemoglobin method first described by Vanzetti et al (19), which correlates with the international reference method for hemoglobin determination (International Committee for Standardization in Hematology Method) (20, 21).

Basically, the sodium deoxycholate disintegrate the erythrocyte membranes, followed by
the conversion of the hemoglobin iron from the ferrous form to the ferric form by nitrite resulting in methemoglobin, which binds to azide to form azidemethemoglobin. At the finger-prick blood sample collection, 10 µl of the initial blood flow was applied directly on the HemoCue Hb microcuvette (HemoCue™, Angelholm, Sweden) that contains the appropriate reagents and inserted into the HemoCue photometer for reading at two wavelengths (570 and 880 nm) to correct for turbidity. All the results were provided within 15-60 seconds. The HemoCue analyzer was a factory calibrated device against the cyanomethaemoglobin method and did not require recalibration (18).

6.3.2.2 Plasma ferritin

Plasma ferritin concentrations were determined using the quantitative colorimetric enzyme-linked immunoassay (ELISA) Spectro Ferritin following the manufacturer’s guidelines (Ramco Laboratories Inc, Stafford, Texas, USA). Basically, in a two-stage reaction, the enzyme immunoassay allows the quantification of plasma ferritin. Stage one consisted of the binding of human serum ferritin to a solid phase antihuman ferritin, and the simultaneous binding of the purified antihuman ferritin conjugated with alkaline phosphatase to the insoluble immune-complex. Stage two consisted of a reaction of alkaline phosphatase with a substrate solution (phenylphosphate disodium and 4-amino-antipyrine). The addition of potassium ferricyanide allowed a color to develop (optical density 490-510nm), of which was directly proportional to the ferritin concentration in the sample. The kit contained the following reagents:

-Six pre-diluted ferritin calibrator solutions containing 0.3 ml human spleen ferritin calibrated to concentrations of 6, 20, 60, 200, 600, and 2000 ng/ml against WHO reference material.
- Solid phase antihuman ferritin: 96 wells coated with rabbit antihuman spleen ferritin.

- Sample dilution buffer (20 ml).

- Conjugated antihuman ferritin (23 ml).

- Substrate solution (23 ml).

- Potassium ferricyanide (15 ml).

The required number of wells were prepared and placed on the well holder and shook dry. In an amount of 10 \( \mu l \) of each calibrator solution and sample in duplicate were added to the prepared wells using micropipette (Research\textsuperscript{®} Plus, Eppendorf Canada, Mississauga, Ontario, Canada). Two hundred microliter of the conjugated antihuman ferritin was added into all wells. On the mini-shaker (MS1 IKA\textsuperscript{®}, Works Inc., North Carolina, USA) set at 180-200 rpm, the plate was incubated for 2 hours at room temperature. Following incubation, each well was filled with deionized water and washed three times. At final wash, the plate was taped against absorbent material for 30 seconds to drain. Two hundred microliter of the substrate solution was added into each well, followed by incubation for 30 minutes at room temperature. To develop the color, one hundred microliter of the 0.24% potassium ferricyanide was added to each well and mixed for 1 minute at the mini-shaker. A blank was prepared with two hundred microliter of the substrate solution and one hundred microliter of the potassium ferricyanide. Absorbance was read at 500 nm (Cary\textsuperscript{®} 50 MPR Microplate Reader Varian, Agilent Technologies Canada, Mississauga, Ontario, Canada). A calibration curve was constructed by plotting the absorbance of each calibrator on the Y-axis and the corresponding ferritin concentration in ng/ml on the X-axis of the log-log graph paper provided in the kit. A curve was draw using a line drawn point to point. The average absorbance value for each
control and infant sample was calculated and the location of the average absorbance value on the Y-axis was determined. These points were followed horizontally until it intersected the calibration curve. These points of intersection were followed with the curve vertically until it intersects the X-axis. The X-axis values were the serum ferritin concentration of the controls or the infant samples. The sensitivity of the assay reported by the manufacturer had a minimum detectable concentration that can be distinguished from zero standard of 0.59 ng/ml. The highest intra-assay and the inter-assay coefficient of variation (CV) values reported were 9.6% and 8.7% respectively.

6.3.2.3 Urinary creatinine

The renal system has the capacity to regulate water reabsorption over one order of magnitude range; hence, a biomarker urinary concentration is dependent on its excretion rate and on the urinary flow rate. Owing to the constant amount of daily excreted creatinine and to the effects of water reabsorption on the urinary solute concentration, urinary biomarkers are expressed as a ratio to the urinary creatinine (22). Therefore, urinary creatinine concentrations were measured to correct F₂-Isoprostanes levels. Urinary creatinine concentrations were determined using the Creatinine Parameter Assay kit (R&D Systems® Inc., Minneapolis, MN, USA) and following the manufacture’s guidelines. The principle of this assay was based on Jaffe reaction, a red-orange color is formed by the interaction of creatinine and alkaline picrate solution. The alkaline picrate solution was added to the diluted samples, which were all added to a microplate followed by incubation at room temperature for 30 minutes. Concentrations of creatinine in the samples were reflected by the intensity of the color. The samples were compared to the standard curve. The kit contained the following material:
- Two 96 well microplates.
- Creatinine standard (2 mL of creatinine solution at 100 mg/dl).
- Picric acid reagent (25 ml of a 0.13% of picric acid solution).
- NaOH (5 ml of 1N sodium hydroxide).

Initially, the standard curve was prepared as follows: 200 μl of the 100 mg/dl standard was added using pipette (Research®Plus, Eppendorf Canada, Mississauga, Ontario, Canada) in a tube along with 800 μl of distal water to yield the 20 mg/dl standard. This standard solution was used to obtain a dilution series. In the remaining tubes 500 μl of distal water was added. Before each transfer, the tubes were mixed thoroughly. The distilled water was used as the zero standard (0 mg/dl) and the 20 mg/dl as the high standard.

All samples, standards and controls were prepared in duplicate. Samples were brought to room temperature initially and centrifuged at 10000 rpm for five minutes. This was followed by sample dilution in ratio of 1:10 (10 μl sample + 190 μl distilled water). Fifty microliter of each control, sample, or standard were pipetted to each well. Alkaline Picrate Solution was prepared by adding 125 ml picric acid to 2.5 ml of NaOH and mixed well. One hundred microliter of alkaline picrate solution was added to each well and incubated in room temperature for 30 minutes. The microplate reader was set to 490 nm (Cary® 50 MPR Microplate Reader Varian, Agilent Technologies Canada, Mississauga, Ontario, Canada), to obtain the optical density (OD) of each well. The averages of the duplicates for the standards and samples were calculated and the average of the zero standard OD was subtracted from these averages. The standard curve was created using Microsoft Excel software (Microsoft Corporation, Washington, USA). The mean
absorbance of each standard was plotted on the Y-axis and the concentration on the X-axis. The concentration of the samples were calculated using the formula: \( y = mx \pm c \)

Since samples have been diluted, the results were multiplied by the dilution factor 10. The highest intra-assay and the inter-assay CV values reported were 3.5 % and 5.5% respectively.

6.3.2.4 Urinary F2-Isoprostanes

F2-Isoprostanes are prostaglandin like compounds produced as a result of free radical lipid peroxidation of arachidonic acid (23). Levels of urinary F2-Isoprostanes were determined by the liquid chromatography-mass spectrometry (LC/MS/MS) assay method developed by Davies et al. (24). The precision and the accuracy of this assay were ±5.0% and 80% respectively. The analysis was conducted in Dr. L. Jackson Roberts II laboratory, Vanderbilt University, Nashville, TN, USA.

6.3.2.5 Fecal iron

The aim of this assay was to quantify the amount of iron in fecal sample using the commercially available spectrophotometrically-based assay Feren-B kit (Bioanalytic, Umrich, Freiburg, Germany). This method was adapted from Orozco et al. (14). The kit contained the following material:

- Iron buffer reagent.
- Reducing agent.
- Color reagent.
- Standard (140 µg/dl Fe\(^{3+}\)).

The analytic steps were as follows:
6.3.2.5.1 Sample preparation

Samples were digested with an acid mixture prepared as follows: 100 ml of 6 M 37% hydrochloric acid (HCL) (Thermo Fisher Scientific Inc, Massachusetts, USA) was added to 100 ml 20% trichloroacetic acid (Thermo Fisher Scientific Inc, Massachusetts, USA). 50 ml of HCL was added to make up to 100 ml (50 ml HCL + 50 ml dd H2O), the 20 ml ddH2O was first added then the acid (HCL) followed by the full amount to reach the 100 ml. Twenty grams of trichloroacetic acid were weighed (Sartorius CP, Sartorius AG, Goettingen, Germany) and added to 100 ml dd H2O. In 15 ml glass tube, 300-400 mg of each fecal sample were placed and weighed. This step was followed by adding 5 ml of the acid mixture to the each sample and mixed using vortex mixer (Analog Vortex Mixer, VWR®, Pennsylvania, USA). Samples were incubated at 65ºC for 20 hours. After incubation, vortex mixer mixed samples and 1 ml of each sample supernatant was transferred into 1.5 ml micro-centrifuge tube (Eppendorf® Safe-Lock tube™, Hamburg, Germany) and centrifuged at 3000 rpm for 3 minutes. A 1:10 dilution of each sample was prepared by adding 100 µl of each supernatant to 900 µl of Millipore water.

6.3.2.5.2 Photometric determination of iron

One package of the reducing agent was added and dissolved in the bottle of the iron buffer reagent to yield solution R1. Blank was prepared as follows: 500 µl of R1 solution, 100 µl Millipore water, and 20 µl of the color reagent solution were added in 1.5 ml micro-tube and mixed for 1 minute using vortex mixer (Analog Vortex Mixer, VWR®, Pennsylvania, USA). The blank was transferred into UV-cuvette (Sigma Aldrich Co. Missouri, USA) and placed into the spectrophotometer (Cary® 50 MPR
spectrophotometer Varian, Agilent Technologies Canada, Mississauga, Ontario, Canada) and absorbance was adjusted to 590 nm, and zeroed. In a 1.5 ml microtube, 100 µl of each diluted sample was added to 500 µl of R1 solution and vortex, followed by adding 20 µl of the color reagent solution, and vortex for an additional minute. The samples were transferred into UV-cuvette and read at 590 nm absorbance. Standard solution was prepared by adding 500 µl of R1 solution to 100 µl of the iron standard (140µg/dl Fe³⁺) into 1.5 ml microtube and mixed, followed by adding 20 µl of color reagent solution and vortexed. Standard was vortex again after 1 minute. The standard was transferred into UV-cuvette and the absorbance was read at 590 nm.

6.3.2.5.3 Calculation

Iron content in fecal samples was calculated as follows:

\[
\frac{(\text{Absorbance} \times 10.52 \times 5 \times 1000)}{\text{(Sample weight (mg) x 10)}} = \mu g \text{ Fe/g feces}
\]

Iron content in the standard was calculated as follows:

Absorbance x 1062 = µg/dl

6.3.2.6 Fecal reactive oxygen species generation

The aim was to quantify and separate the end products of the hydroxyl radical attack on salicylic acid, particularly, 2,5 dihydroxybenzoic acid, 2,3 dihydroxy benzoic acid, and catechol. This was a high performance liquid chromatography (HPLC) method that was validated by Orozco et al. and adapted from Orozco et al. and Owen et al. (14, 26). The following analytic steps were performed:
6.3.2.6.1 Phosphate buffer 100 mM solution preparation

In a 500 glass beaker (Pyrex® beaker, Corning Incorporated Life Sciences, Massachusetts, USA), 6.81 g (0.2 M) of KH$_2$PO$_4$ (Thermo Fisher Scientific Inc, Massachusetts, USA) was added to 250 ml ddH$_2$O, mixed to dissolve and transferred to 100 ml flask (Pyrex® Erlenmeyer flask, Corning Incorporated Life Sciences, Massachusetts, USA). Similarly, 3.48 g (0.2 M) of K$_2$HPO$_4$ (Sigma Aldrich Co. Missouri, USA) was added to 100 ml ddH$_2$O, mixed to dissolve and transferred to 100 ml flask. From the prepared K$_2$HPO$_4$, 94.5 ml was collected using measuring cylinder (Pyrex® Graduated Cylinder, Corning Incorporated Life Sciences, Massachusetts, USA) and pipette (Research®Plus, Eppendorf Canada, Mississauga, Ontario, Canada) and added to 600 ml ddH$_2$O. From the prepared KH$_2$PO$_4$, 205.5 ml was collected using measuring cylinder and pipette and added to the mixture. The buffer solution pH was adjusted 6.5 (Accumet® Basic, Thermo Fisher Scientific Inc, Massachusetts, USA).

6.3.2.6.2 Incubation buffer solution preparation

The incubation buffer was composed of 100 mM phosphate buffer, 500 mM ethylenediaminetetraacetic acid (EDTA) (Sigma Aldrich Co. Missouri, USA), 50 μM FeCl$_3$H$_2$O (Thermo Fisher Scientific Inc, Massachusetts, USA), and 2 mM salicylic acid (Sigma Aldrich Co. Missouri, USA). In a 500 ml volumetric flask (Pyrex® volumetric flask, Corning Incorporated Life Sciences, Massachusetts, USA), 73.06 mg of EDTA, 6.7 mg of FeCl$_3$H$_2$O, and 138.12 mg of salicylic acid were added to 500 ml of the phosphate buffer and mixed to dissolve. The incubation buffer was adjusted for 6.5 pH value (Accumet® Basic, Thermo Fisher Scientific Inc, Massachusetts, USA).
6.3.2.6.3 Calibration curve dilutions

Initially, 50 milliliters of 5mM of stock solutions of salicylic acid, 2,5 dihydroxybenzoic acid, 2,3 dihydroxybenzoic acid, and catechol were prepared (Sigma Aldrich Co. Missouri, USA). This step was followed by the dilution of the stock solutions to yield concentrations of 0.2 mM, 0.4 mM, 0.6 mM, 0.8 mM, 1 mM, and 2 mM to construct the calibration curve.

6.3.2.6.4 Sample incubation and analysis

Samples were thawed over night at 4°C. One hundred milligrams of each sample were weighed (Sartorius CP, Sartorius AG, Goettingen, Germany) and placed in 50 ml conical flask (Pyrex® Erlenmeyer flask, Corning Incorporated Life Sciences, Massachusetts, USA) containing 10 ml of the incubation buffer. Samples were homogenized using pipette (Research® Plus, Eppendorf Canada, Mississauga, Ontario, Canada) followed by incubation at 37°C for 21 hours (Branstead Lab-Line, Thermo Fisher Scientific Inc, Massachusetts, USA). Following incubation, 2 milliliters of each sample was transferred into 2 ml microtube (Eppendorf® Safe-Lock tube™, Hamburg, Germany) and centrifuged for 5 minutes at 10000 rpm. Using 1 ml syringe (NoRM-JECT®, Henke Sass Wolf, Tuttlingen, Germany), 2 ml of each sample was filter-sterilized with 0.2 µm filter (CORNING® Incorporated, Corning NY, USA). After filter sterilization, 20 µl of each sample was injected using 10 µl microliter syringe (Microliter Syringe™ Hamilton Company, Nevada, USA) directly onto the HPLC column (ODS Hypersil 200 x 2.1 Thermo Fisher Scientific Inc, Massachusetts, USA) (HPLC Shimadzu USA Manufacturing Inc, Oregon, USA). For the chromatographic separation of individual
compounds, the mobile phase comprised of 2% acetic acid glacial in water (solvent A) and methanol (Thermo Fisher Scientific Inc, Massachusetts, USA) making up solvent B utilizing the following gradient: 95% A/5% B for 2 min, 75% A/25% B for 8 minutes, 60% A/40% B for 10 minutes, 50% A/50% B for 10 minutes and 0% A/100% B for 10 minutes. For the first 5.5 minutes, the UV/VIS detector was adjusted at 278 nm and changed to 301 nm until completion of the run. The optimal flow rate was 0.5 ml/minute.

Data handling and instrument control were conducted using the software ChemStation® (Agilent ChemStation, Agilent Technologies, California, USA).

6.3.2.7 Fecal calprotectin

Calprotectin is a calcium binding protein which is released from inflammatory cells at the site of intestinal inflammation and excreted into the feces (27). It is used as a non-specific marker of intestinal inflammation and was shown to be elevated in adults and children with inflammatory bowel disease (28, 29). It was shown to correlate with the gold standard test (excretion of fecal ¹¹¹indium) of intestinal inflammation (30).

Fecal calprotectin concentrations were determined using the commercially available Calprotectin ELISA kit (BUHLMANN Calprotectin ELISA, ALPCO, Salem, NH, USA) and following the manufacture’s guidelines. Basically, the test allowed for the selective quantification of calprotectin-antigen by sandwich ELISA. The kit contained the following materials:

- Extraction buffer.
- Wash buffer concentrate.
- Incubation buffer.
- Calibrators A to E.
- Control low and high (human serum).
- Enzyme label.
- TMB (tetramethylbenzidine) substrate.
- Stop solution.
- Microtiter plate.
- Plate sealer.

The analytic steps were as follows:

6.3.2.7.1 Calprotectin extraction

Empty glass tubes were labeled and weighed. Fecal samples (between 50-100 mg) were added to the pre-weighted tubes and weighed. In each tube, 49 volumes of the extraction buffer (volume provided by the manufacturer’s manual) were added according to the weight of the sample. The samples were vortexed (Analog Vortex Mixer, VWR®, Pennsylvania, USA) and homogenized for 30 minutes. After homogenization, 1 ml of each homogenate was transferred into 2 ml Eppendorf tube and centrifuged at 3000 rpm for 5 minutes (Eppendorf® Safe-Lock tube™, Hamburg, Germany). Following centrifuging, supernatants were transferred into fresh tubes.

6.3.2.7.2 Calprotectin ELISA

The fecal extracts were diluted by 1:50 with the incubation buffer (2 μl fecal extract + 98 μl incubation buffer) and mixed and left to equilibrate for 5 minutes at room temperature. The plate was prepared with the strips required to test the calibrators, controls and the diluted samples. Then the coated well were washed twice using 300 μl of the wash buffer. The wells were emptied and tapped against blotting paper. Between each washing step, an
incubation time of 20 seconds was allowed. One hundred microliter of the incubation buffer, calibrators, controls, and diluted samples were pipetted (Research® Plus, Eppendorf Canada, Mississauga, Ontario, Canada) in duplicates into the wells. The plate was covered with the plate sealer and incubated for 30 minutes at room temperature on a rotator set at 500 rpm (MS1 IKA®, Works Inc., North Carolina, USA). After incubation, the wells were emptied and washed using 300 µl of the wash buffer for 3 times. This was followed by adding 100 µl of the Enzyme label into all wells. The plate was covered with a plate sealer and incubated for 30 minutes on a plate rotator set at 500 rpm at room temperature. Following the incubation, the wells were emptied and washed 5 times using 300 µl of the wash buffer. One hundred microliter of the TMB substrate solution was pipetted in each well. The plate was sealed and covered with foil and incubated for 15 minutes on a rotator set at 500 rpm at room temperature. This was followed by adding 100 µl of stop solution into all wells. The plate was placed in the microtiter plate reader (Cary® 50 MPR Microplate Reader Varian, Agilent Technologies Canada, Mississauga, Ontario, Canada) and the absorbance was read at 450 nm.

6.4 Results

Detailed baseline characteristics of the participants were presented in chapter V. A total of 90 infants were assessed for eligibility, and 3 infants met the exclusion criteria. Eighty-seven infants were enrolled in the study between December 2012 and May 2014 and received allocated intervention as follows: 25 in Cer group, 28 in Cer+Fr group and 34 in M group. A total number of 82 infants completed the study and 5 were withdrawn due to the following: mothers no more wanted to comply with the study food (cereal or meat),
moved to a different province, no response to the study team calls. Due to insufficient sample volumes, the number of infant samples analyzed for each outcome varied thus, were set to the minimum number required to detect significant differences between the feeding groups for each outcome. The sample numbers were as follows: 25 urine samples for F2-Isoprostanes analysis, 77 stool samples for fecal iron analysis, 66 stool samples for fecal ROS analysis, and 43 stool samples for fecal calprotectin analysis. A total of 18 mothers who agreed to obtain blood samples from their infants for iron status parameters. Table 17 summarizes the biomarker measures of infants in the three study groups.
Table 17. Summary of biomarker measures of infants in the three study groups

<table>
<thead>
<tr>
<th>Biomarker (mean±SE)</th>
<th>Cer Before</th>
<th>% Change</th>
<th>Cer+Fr Before</th>
<th>% Change</th>
<th>M Before</th>
<th>% Change</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemoglobin (g/l) ¶ (n=18)</td>
<td>Cer + Cer+Fr before</td>
<td>120.1±1.3</td>
<td>126.0±2.6</td>
<td>124.5±2.6</td>
<td>1.1%</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cer + Cer+Fr after</td>
<td>117.7±1.7</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum ferritin (µg/l) ¶ (n=18)</td>
<td>Cer + Cer+Fr before</td>
<td>43.8±8.9</td>
<td>99.6±37.2</td>
<td>76.8±31.2</td>
<td>22.8%</td>
<td>0.04a</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cer + Cer+Fr after</td>
<td>37.1±6.6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urinary F2-Isoprostane (ng/mg) (n=25)</td>
<td>0.45±0.81</td>
<td>0.35±0.47</td>
<td>0.41±0.08</td>
<td>17.1%</td>
<td>0.48±0.14</td>
<td>0.68±0.12</td>
<td>41.6%</td>
</tr>
<tr>
<td>Fecal iron (Fe g/feces) (n=77)</td>
<td>3.9±0.37</td>
<td>4.7±0.34</td>
<td>20.5%</td>
<td>2.9±0.25</td>
<td>3.7±0.25</td>
<td>21.6%</td>
<td>&lt;0.05ab</td>
</tr>
<tr>
<td>Fecal ROS (mmol/l) (n=66)</td>
<td>0.024±0.007</td>
<td>0.014±0.003</td>
<td>121%</td>
<td>0.023±0.004</td>
<td>0.028±0.003</td>
<td>21.7%</td>
<td>0.002a</td>
</tr>
<tr>
<td>Fecal calprotectin (µg/g feces) (n=43)</td>
<td>111.0±12</td>
<td>154.5±23</td>
<td>64.8%</td>
<td>108.1±11</td>
<td>131.9±18</td>
<td>22%</td>
<td>0.004a</td>
</tr>
</tbody>
</table>

*a values superscript letters are significantly different over time (within groups) by repeated measures ANOVA (p < 0.05)

*b values superscript letters are significantly different between the groups by repeated measures ANOVA (p < 0.05)

¶ values were combined for cereal groups
6.4.1 Hemoglobin concentration

A total of 18 infants whom parents agreed to give blood samples. Due to small number of blood samples, hemoglobin concentration results from both cereal groups were combined to perform the statistical analysis. There was no statistical significant difference between the groups in hemoglobin concentration before and after the introduction of the study foods (table 23).

6.4.2 Plasma ferritin

Iron status was assessed for a subsample of 18 infants whose parents agreed to provide the optional blood sample. Due to small number of blood samples, ferritin results from both cereal groups were combined for statistical analysis. Mean results are presented in table 23 and figure 16. Repeated measures ANOVA within groups detected a significant effect of time for plasma ferritin level in the iron-fortified cereal and iron fortified cereal with fruit combined group and in the meat group ($p = 0.04$). A total of two infants had low ferritin levels (< 15 µg/l) and non had low hemoglobin (< 110 g/l).
6.4.3 Urinary creatinine

A total of 25 urine samples were analyzed for creatinine. The values of the calculated creatinine (µg/dl) were converted to g/l and used for the determination of the corrected
urinary F₂-Isoprostanes. No significant differences in urinary creatinine concentration were observed between the three study groups nor at the two time points ($p = 0.17$, $p = 0.32$, respectively).

6.4.4 Urinary F₂-Isoprostanes

The values of the urinary F₂-Isoprostanes were creatinine corrected by the following equation:

$$\text{Concentration of F₂-Isoprostanes ng/ml} = \frac{\text{F₂-Isoprostanes ng/mg creatinine}}{\text{Concentration of creatinine g/l}}$$

Repeated measures ANOVA demonstrates that the feeding effect of the three foods on the excretion of the urinary F₂-Isoprostanes was not significant ($p = 0.8$). Urinary F₂-Isoprostanes tended to increase after the introduction of CFs (figure 17), but these elevations were not significant ($p = 0.3$).
Figure 17. Average creatinine corrected F2-Isoprostanes in the urinary samples of infants in the three study groups.
6.4.5 Fecal iron

Seventy-seven fecal samples were analyzed for residual iron (figure 18). At baseline, although there was a slight variation in the amount of residual iron between the meat group compared with the cereal groups, this difference was not significant ($p = 0.075$). After the introduction of the three study foods, there was a statistically significant increase in the amount of the residual iron in all three feeding groups ($p < 0.001$, by repeated measures ANOVA). There was also a significant feeding group effect ($p < 0.001$) with lower iron levels in the meat group ($p < 0.001$ vs. Cer and $p = 0.014$ vs. Cer+Fr).

6.4.6 Fecal reactive oxygen species generation

In total, 66 fecal samples were analyzed for formation of hydroxylated products, indicating the production of ROS (Figure 19). Overall there was a 55% increase in ROS following introduction of CFs ($p < 0.002$, by repeated measures ANOVA; $p = 0.003$ by paired t-test). The generation of ROS in the meat group was lower in comparison with the two cereal groups; however, this difference did not reach significance ($p = 0.28$).
Figure 18. Fecal iron of infants in the three study groups

N.B. a: statistical significant increase over time (within groups) by repeated measures ANOVA

b: statistical significant different between the groups by repeated measure ANOVA
Figure 19. Reactive oxygen species generation in fecal samples of infants in the three study groups

N.B: *: statistical significant difference over time (within groups) by repeated measures ANOVA.
6.4.7 Associations between fecal iron, ROS generation and dietary iron

To determine if there was an association between dietary iron, residual fecal iron, and ROS production, multiple correlation regression analysis was performed. As shown in figure 20, the overall correlation between both ROS generation and the amount of the residual iron with dietary iron is positively significant ($r = 0.22, p = 0.04$).

6.4.8 Fecal calprotectin

A total of 43 fecal samples were analyzed to determine calprotectin concentration. Repeated measures ANOVA detected an overall significant increase in the fecal calprotectin concentration after the introduction of CFs ($p = 0.004$) (figure 21). However, no significant difference was observed between the three study groups ($p = 0.9$).
Figure 20. Correlation between dietary iron, fecal iron and ROS production

\[ r = 0.22, \ p = 0.04, \] by multiple regression analysis
Figure 21. Fecal calprotectin concentration of infants in the three study groups
N.B: *a*: statistical significant difference over time (within groups) by repeated measures ANOVA.


6.5 Discussion

This is the first study to investigate the effect of different first CFs on oxidative stress, inflammation in healthy EBF infants. Important findings emerge from the present study. The iron status of the breastfed infants was deteriorating regardless of the introduction of iron-rich foods and two infants developed ID (ferritin < 12 µg/l). Residual fecal iron was lower in the meat group compared to the iron-fortified cereal groups. The urinary oxidative stress marker F₂-Isoprostane did not differ significantly between the groups. However, the fecal oxidative stress marker of ROS production differed significantly over time with meat group having the lowest levels. ROS production was positively correlated with residual fecal iron and dietary iron intake ($r = 0.22$, $p = 0.04$). Both ROS generation and fecal iron, were lower in the iron-fortified cereal with fruit than in iron-fortified cereal only. Although the fecal calprotectin concentration increased after the introduction of the CFs, it did not differ significantly with different CF regimens.

6.5.1 Iron status

In the recent analysis of a cross-sectional survey of the Canadian TARGet kids research group, the relation between the total duration of breastfeeding and ID was investigated in 1647 healthy children, revealed that serum ferritin concentration decreased by 24.0 µg/l (% 4.8) for every additional month of breastfeeding ($p = 0.001$) (31). A secondary analysis had also indicated a significant association between the breastfeeding duration and the odds of ID (Odds ratio (OR): 1.02, CI: 1.004-1.05). Similarly, the current study observed a decrease by 17.1 µg/l in plasma ferritin among infants in all groups for a feeding duration of 2-4 weeks (table 23). The decline in iron stores in all the CF groups
despite the higher intake of dietary iron in the two cereal groups compared with the meat group underline the variations in bioavailability of iron from the non-heme versus heme sources. In a previous Canadian RCT of 77 breastfed infants, it was found that low ferritin was associated with EBF for 6 months compared to EBF and iron supplementation between 1 to 6 months (33%, 7% respectively) (32). Our finding of a negligible effect of CFs in restoring iron status of breastfed infants is also in agreement with the finding of an RCT conducted on 41 healthy breastfed American infants who received different CFs of either iron-fortified cereal, or iron- and zinc-fortified cereal or meat. It was reported that after the feeding period, 27% of all infants had low ferritin, and 36% had mild anemia with no difference observed between the feeding groups (33). The consistency of these results necessitate the need of implementing screening tools for ID in EBF and perhaps iron supplementation since CFs alone may not restore iron status (34).

6.5.2 Oxidative stress and inflammation

To our knowledge, no previous studies have evaluated the effect of different iron fortified CF regimens on the oxidative stress status of EBF infants. A previous study conducted on 3 healthy adults who received daily 120 mg of oral iron for 7 days, showed that 2 of the subjects had a two-fold increase in urinary F2-Isoprostanes from the baseline (12). F2-Isoprostanes have been measured in adult populations with various health conditions and mainly in children with type 1 diabetes (35-40). There are few studies that reported normal F2-Isoprostanes levels in healthy children (41, 42). In one of these studies, 342 children at risk of developing type one diabetes were followed up and their urinary F2-Isoprostanes values were measured (42). It was indicated that urinary F2-Isoprostanes levels were highest among 9 months old infants and that there was an inverse association
between age and the concentration of urinary F$_2$-Isoprostanes (β coefficient: -0.14, $p = 0.0001$). Even though in the present study the levels of urinary F$_2$-Isoprostanes increased after the addition of the CFs, these values were in the normal range of urinary F$_2$-Isoprostanes according to the Denver study (42). Another explanation for our finding of insignificant differences between the groups in F$_2$-Isoprostanes levels is the small number of urine samples analyzed over all and in each feeding group (n = 25).

We aimed to present evidence for any association between high iron intake by CF and intestinal disease by relating the iron to the increased ROS generation in the stool. Our results are in keeping with those reported in a previous study conducted by Orozco et al. on 17 healthy men who received daily 120 mg of iron for 2 cycles of 7 days supplementation (14). From that study, it was reported that after iron supplementation, there was a significant increase in fecal ROS production by 36% ($p = 0.026$) (14).

Similarly, in the present study, after the provision of iron-rich foods, ROS generation increased significantly by 55% ($p = 0.003$). With regard to the residual iron, in Orozco et al. study, the mean fecal iron was $1.8 \pm 0.34 \mu g/g$ feces during the baseline and washout period, and increased significantly to $4.4 \pm 0.47 \mu g/g$ feces during iron supplementation ($p < 0.0001$) with 145% increase rate. Compared to our findings, we also found that the fecal iron was $3.6 \pm 0.46 \mu g/g$ feces before the introduction of all study foods, and raised significantly to $4.6 \pm 0.21 \mu g/g$ (27.7%, $p < 0.0001$) with highest amount in infants received iron-fortified cereal only ($5.6 \pm 0.38 \mu g/g$). Although the iron dose received from the three CFs (average daily CF iron intake (chapter V) mean ± SE, Cer: $16.3 \pm 2.5$, Cer+Fr: $21.5 \pm 3.4$, M: $0.86 \pm 0.08$) was lower than the iron dose received in the adult
study, we observed higher ROS production and lower fecal iron when compared to the adult study. One explanation for this observation is the differences between the adult and infant intestinal iron absorption. Previous studies have suggested that the dietary regulator of iron absorption at the age between 6-9 months is immature and remains under developmental changes (2, 43). Another possible cause is the individual variation in the iron stores which might had influenced the absorption rate thus affecting the amount of intestinal residual iron and ROS production (44). We had demonstrated such variation existed among the breastfed infants as assessed by plasma ferritin concentrations (table 23). Moreover, an important factor is the phytate content of the iron-fortified cereals that might had interfered with iron absorption (45, 46). A previous study found that the phytate effect on the minerals bioavailability in 36 CFs, the phytate: iron molar ratio (a ratio used to predict the inhibitory effect of phytate on the bioavailability of iron) was higher than the recommended level of < 1 among 32 of the assessed CFs (46). We also aimed to assess another common choice of first CF among Canadian infants which was the commercially available iron-fortified cereal mixed with fruit (raspberry). Our findings did not support our hypothesis of antioxidant’s potential effect of the fruit contained in the iron-fortified cereal in ameliorating the oxidative stress generation in the intestinal tract. This may be due to the low content of raspberry powder (1.8%) of the iron-fortified cereal with fruit, which was insufficient to eliminate the iron oxidative effects. It seems like that the average daily iron intake from Cer+Fr of 21.5 ± 3.4 mg (67.5g of cereal/day) was enough to increase the production of ROS. This observation is in accordance with the finding of the study Lund et al. in which a lower daily dose (19 mg) of ferrous sulfate was
provided to 18 adult subjects for 14 days and a significant association between iron supplementation and free radical production \( (p < 0.001) \) was reported (11). 

In the present study variability was observed in the baseline of both residual fecal iron and ROS generation before the introduction of CFs. Although all of the included infants were EBF, variations in the breast milk composition including iron or other genetic factors such as single nucleotide polymorphisms (SNPs) in the iron transporters responsible for iron homeostasis in the secretory mammary epithelial cells, may likely attributed to these observations (47-49).

There are no existing studies that have measured calprotectin concentration in response to iron supplementation or iron fortification. Our study showed a significant feeding effect with the addition of CFs, however, with no significant effect of the type of CF. Fecal calprotectin concentration has been used to evaluate the degree of inflammation in various systemic and gastrointestinal conditions (30,50). For example, it was found that the values of fecal calprotectin were elevated in adult and children populations with different GI infections, however, these values were lower than in inflammatory bowel disease patients (51-53). These variations in calprotectin concentration by the degree of inflammation may explain why we did not find significant feeding effect on intestinal inflammation. It seems like that calprotectin is a chronic inflammatory phase reactant rather than acute phase reactant, and owing to our short duration of feeding, calprotectin did not cause high degree of intestinal inflammation. High fecal calprotectin levels are normal in young infants as reported previously (54, 55). Although the present study found that fecal calprotectin concentrations have increased after the introduction of the study foods, these elevations remained within the suggested normal values of calprotectin for
this age group (54, 56). In a previous study conducted on 74 (39 EBF and 35 formula fed) full-term Italian infants, it was reported that the median fecal calprotectin level was higher in EBF infants than in formula fed infant (555 µg/g feces range: 122.5-2000 µg/g feces; 206.6 µg/g feces range: 31.2-797.6 µg/g feces, \( p < 0.001 \)) (55). It was concluded that there was a feeding effect on calprotectin concentration, which might be due to the effect of human milk bioactive molecules, which clearly contribute to the development of the gastrointestinal system (54). Increased intestinal permeability and immature adaptive immunity in infancy are also possible causes (54, 57). Another study also indicated that calprotectin is higher in EBF infants than in mixed-fed infants (58). A previous study compared the concentration of fecal calprotectin of healthy full-term infants to preterm infants, and found that the values were significantly higher among healthy infants than in preterm infants (235 µg/g feces, range: 172-2880 µg/g feces; 150 µg/g feces, range: 81-221 µg/g feces; \( p < 0.001 \)) (59).

6.6 Conclusion

The present RCT highlights clinically important findings to inform the infant feeding guideline updates regarding the optimal first CFs. Our findings suggest that EBF infants are at risk of developing ID despite the provision of iron-rich CFs. Therefore, labeling EBF infants as at risk for ID and routinely screen them may be warranted. The results of this study support the conclusion that untargeted iron fortification may result in untoward effects including ROS generation in the intestinal tract of breastfed infants receiving high iron-fortified cereals (60). Thus, it is maybe noteworthy revising the guidelines of the first complementary feeding when these findings are further confirmed. Future research is
required to determine the association between ROS generation in the intestinal tract with other inflammatory markers and various health outcomes.

6.7 References


CHAPTER VII: OVERALL CONCLUSION

7.1 Summary and implications

The results of the present research have important implications for infant feeding, particularly related to the introduction of first complementary foods. Currently, the most common first CF provided to Canadian infants is single grain iron-fortified cereal (1). The recent Health Canada statement guideline “Nutrition for Healthy Term Infants: Recommendations from Birth to Six Months” supports exclusive breastfeeding until six months of age; and reinforced the role of exclusive breastfeeding as a risk factor for the development of iron deficiency (2). Therefore, the guidelines recognized the necessity of introducing iron rich foods and the suggestions were that iron-fortified cereals or meats should be introduced at about six months of age. Evidence in regards to the timing, the type, the nutritional efficiency and the safety of CF is limited (3-5). The duration of exclusive breastfeeding remains a primary factor in the development of iron deficiency in infancy and a primary target of feeding guidelines (6). The meta-analysis of RCTs from current research demonstrates that moderate timing (at four months of age) for the introduction of CFs positively influenced the iron stores of the exclusively breastfed infants living in developed countries [serum ferritin, MD: 26 μg/L; 95% CI: -0.10, 52.10 μg/L, \( p = 0.05 \)]. Therefore, results suggest that introducing CFs between four to six months is necessary for some infants with a higher risk of iron deficiency especially for those who had been exclusively breastfed. Data from chapter VI showed that independent of the type of CF, the iron status of these healthy breastfed infants was declining.
Therefore, this observation has implications for iron deficiency screening between the age of four to six months. With respect to the relationship of socio-demographic factors and feeding patterns, although our study was not powered to detect these relationships, we were able to find some maternal factors that were associated with the timing of the introduction of CFs and with complementary feeding times per day. We also found that among the participating mothers, only 27.3% of them adhered to Health Canada guidelines in regards to EBF until six months. The average timing of introduction of CFs in our study was 5.4 months. A previous Canadian survey showed that the average timing of introduction of solids was 4.8 months (3). These socio-demographic and feeding patterns findings call for time for a national representative survey to confirm and update these findings and when more socio-demographic maternal factors are identified, the next step would be targeting these factors to improve the feeding practices of the infants. Of interest, data from chapter V suggests that the estimated amount of breast milk alone did not satisfy the recommended daily amount for infants aged 0 to 6 months of vitamin A, vitamin D, thiamin, zinc and magnesium. However, after the introduction of the three study foods, breast milk and CF together met the micronutrients requirements of infants in that age group. In terms of the nutrient intakes from CF only, consumption of iron-fortified cereals (average daily consumption of 51 g/day for Cer and 67.5 g/day for Cer+Fr) satisfied most of the macronutrient and micronutrient daily recommendations for infants aged 0-6 months. In comparison, consumption of meat (average daily consumption of 41.3 g/day) did not meet the daily recommendation from CF only for total energy, all macronutrients (except protein) and most of the vitamins and minerals. These findings are of significance; further supporting the Health Canada recommendation
of iron-fortified cereal as first choice CF rather than meat, from a nutrient intake perspective. Nevertheless, in terms of a free radical perspective (chapter VI), the consumption of iron-fortified cereal led to the highest elevation in fecal ROS production ($p = 0.002$) after the provision of CF raising a concern of the possibility of intestinal inflammation. Thus, these findings contradict the Health Canada recommendation that iron-fortified cereal be the first CF from a safety perspective (2). We were able to provide more evidence on the associations between ROS generation, fecal iron and dietary iron as shown in chapter VI ($r = 0.22, p = 0.04$), which confirms the reports from other researchers (7, 8). However, the data presented in chapter VI showed no significant effect of iron-fortified cereals on the systemic oxidation reflected in urinary F2-Isoprostanes. This suggests that while ROS may be produced in the intestine, systemic oxidation may not be occurring in the infants. Further more, a protective effect of the fruit in the cereal on the oxidation response was not significantly achieved; hence, our hypothesis that the addition of fruit would ameliorate the negative effects of iron fortification was not supported. More research is needed to determine the amount required, perhaps from fresh fruits, necessary to achieve a consistent suppressing effect on ROS generation. According to our results shown in chapter VI, fecal calprotectin concentrations were elevated in response to the addition of CFs. However, the number of infants analyzed for fecal calprotectin were too small to draw conclusions concerning the relationship to intestinal inflammation. In addition, this finding supports the conclusion that high calprotectin concentration is a normal phenomenon in infants (9, 10) and provides valuable insight surrounding the normal development of fecal calprotectin levels in infants fed CFs.
7.2 Limitations and future recommendations

Findings from the current body of research yield valuable insights into the complementary feeding of breastfed infants. However, considering limitations in the current research, the following suggested future directions would provide additional knowledge regarding iron status, feeding practices, oxidative stress and intestinal inflammation.

The current study is in agreement with previous Canadian studies in terms of the risk of developing iron deficiency among breastfed infants (11-14). To our knowledge, the iron status is not routinely examined in infancy. Based on these consistent findings, we are concerned about the possibility that the iron status of our infants may be deteriorating, which can lead to ID and IDA. Therefore, we suggest future studies to develop ID risk screening score to tackle this problem.

Another limitation of the current study was that a few number of mothers reported that their infants received other foods or formula. In addition, the variability between the infants on the time of the introduction of CFs was another limitation. These limitations could have impaired our ability to detect differences between the groups. Although the present study was sufficient in sample size to detect differences in fecal ROS generation, we were not able to assay all analytes in all infants, therefore, some comparisons were underpowered to observe differences between the groups. Hence, future studies studying the relationship of iron fortification of CF to the inflammatory biomarkers should power their sample size needed to investigate this relationship. In addition, we suggest that other systemic inflammatory biomarkers such as, TNF-α, interleukin 1 (IL-1), IL-6, IL-8, IL-12, and C-reactive protein that are related to intestinal inflammation may be worth
considering in future studies. Infants varied in their number of times of stool passage per
day, however, the majority of them passed stool one time every 24 hours. Since we did
not perform 24 hours stool sample collection, the residual iron may not reflect the total
excretion of iron in some infants, which explains the variation in ROS production.
After evaluating the effects of iron fortification on the apparently healthy EBF infants, an
important next step would be to assess the effects of iron fortification on infants receiving
formula, which is fortified at a level of 4-8 mg/l ferrous sulfate (15, 16) and to assess this
effect in addition to iron-fortified cereals. In addition, there are other populations who
receive iron supplementation such as pregnant women with iron deficiency that require
further research to assess the implications of iron supplementation on the oxidative status
of women in this vulnerable period.
The present study provided an overview of the feeding patterns and the related socio-
demographic variables among EBF infants. However, the current study was not powered
to detect these associations and it did not include other infant population (formula fed)
from this perspective. Therefore, future studies should use a larger more representative
sample in order to identify these associations and target the influencing socio-
demographic factors.
It is important to acknowledge that we used the duration of feeding to estimate the
volume of breast milk, thus we cannot exclude a lower estimated amount of breast milk in
our study. Therefore, in the future, using a test weighing technique (infant weighing
before and after feeds) that is a reliable and a consistent measure of breast milk intake,
would provide more precise nutrient intake from breast milk (17). Although, the dietary
records were checked for completeness by the research assistant, potential bias such as,
reporting bias might exist from the three-day dietary record dietary assessment method used in the current study. A more accurate dietary assessment method such as the three-day weighed intake may be worth considering in future interventions. This method, however, would place a greater burden on the parents (18).

A major strength in the current study is the use of a diet controlled randomized design, which is considered the gold standard for nutritional intervention (19). The precise CF given allowed more isolation of the effect of the iron on the endpoint measures and reduced possible confounding influences from other foods that might have been given. Results from chapter VI revealed variations in the iron status parameters of the breastfed infants. This observation has important implications as SNPs in the iron transporters in this population may account for these discrepancies in the iron status of the breastfed infants. Therefore, future studies should incorporate measures of genetic variants of iron transporters into the study to identify any existing SNPs.

In the future, multicenter trials should ultimately use a larger, more diverse infant population, including a broad range of baseline and genetic characteristics that would increase the generalizability of the study results.

7.3 Final conclusion

The assessment of complementary feeding of Canadian infants study provide a foundation of building more understanding of the effects of complementary feeding consumption on overall health in this age group. From the current study we conclude that, if an interaction between the iron fortified cereal and the free radical production in the intestine is further confirmed, then reviewing the guidelines to meet the iron requirements while also diminishing negative effects on the intestinal tract will be warranted.
Meanwhile, the key messages until these findings are confirmed are the following: the dietary advice provided to the caregivers should stress the importance of meeting the nutrient requirements of the infants, emphasis should be placed on the provision of pureed meat as first CF, limiting the frequency and the amount of iron-fortified cereal consumption and including fruits in the infant diet in case of consumption of iron-fortified cereal.

7.4 References


Appendix 1: Study announcement poster

*We will provide one month’s supply of baby food for all participants*

If you are interested, or have any questions, please contact:

Dr. James Friel

Dr. Wafaa Qasem

Find us on Facebook! /AssessmentofComplementaryFeeding

Are You Breastfeeding? Do You Plan to Breastfeed?

If so, please consider participating in our Clinical Study: Assessment of Complementary Feeding of Canadian Infants

This study will investigate the changes that occur in your baby’s gut when solid foods are introduced after breastfeeding.
Appendix 2: Study’s questionnaire

Assessment of Complementary Feeding of Canadian Infants
Dr. James Friel, Principal Investigator
University of Manitoba
Department of Human Nutritional Sciences

1st Visit, Date: ________________

Mother’s Name ___________________ Infant’s Name: ___________________

Address: ________________________

Telephone # ______________________ Other/Emergency Contact: ____________

DOB Baby ________________________ Gestation ________ Sex ____________________

Baby’s Birth Weight _______________ Length ___________________ H.C. __________

Mother’s Age ___________ Para _______ Gravida _______ Delivery? c/s vaginal

Allergies: ________________________

Complications @ birth or major health concerns? ________________________________

Did you take Fe or Vitamins during Pregnancy? _____________ Now? ______________

Have you Breast fed before? ____________ How Long ______________________

Pediatrician _________________ How did you hear about the study? _________________

Mother’s Marital Status: M __ S __ D __ CL ________________

Mother’s Height _________________ Mother’s pre-pregnant weight ________________

Father’s Height _________________ Father’s weight ________________

Mother’s Education/Occupation ________________________________

Father’s Education/Occupation ________________________________

# of persons in household __________ # of children in household __________

Do you smoke? No____ Yes_____ # cigarettes /day _______ #Smokers in house_______

Version 3, November 25, 2013
Feeding Group A B C Amount Given: 

Dietary Record Obtained? Yes No # of times Baby feeds daily 

Blood Work Done Stool collected Urine collected 

# of stools per day: Color/consistency Cream/ointment? 

Is baby receiving any vitamins/minerals at present? Type and amount 

Is Baby receiving any form of Fe than study food? If yes, what? 

Health of Baby since birth 

Visits to Doctor for illness 

Hospitalization? Yes No Details 

Weight 1) 2) 3) 

Length 1) 2) 3) 

Head C: 1) 2) 3) 

Thoughts/Comments about first foods, cereal vs. meat, current guidelines? 


Version 3, November 25, 2013
Assessment of Complementary Feeding of Canadian Infants
2nd visit questionnaire

Date:______________ Subject #:___________ Feeding Group: A B C
Feeding Start Date: _______________ Feeding End Date: _______________
Total Duration of Feeding: _______________

Comments (did baby like the food? Was there a change in stool consistency, color, or frequency? Any discomfort or adverse events?)

Was the baby given any other food/formula during the feeding duration noted above? (list):

__________________________________________________________

Total # boxes/jars consumed: __________________________________

Weight: 1)_________ 2)_________ 3)_________
Length: 1)_________ 2)_________ 3)_________
Head C. 1)_________ 2)_________ 3)_________

Additional Contact for Follow-up/results of Study (email):

__________________________________________________________

Version 3, November 25, 2013
Appendix 3: Bannatyne Research Ethics Board approval letter

Principal Investigator: Dr. J. Friel

Protocol Title: Assessment of Complementary Feeding of Canadian Infants

The following is/are approved for use:

- Annual Approval

The above was approved by Dr. John Arnett, Ph.D., C. Psych., Chair, Health Research Ethics Board, Bannatyne Campus, University of Manitoba on behalf of the committee per your submission dated May 23, 2012. The Research Ethics Board is organized and operates according to Health Canada/ICH Good Clinical Practices, Tri-Council Policy Statement, and all applicable laws and regulations of Manitoba. The membership of this Research Ethics Board complies with the membership requirements for Research Ethics Boards defined in Division 5 of the Food and Drug Regulations of Canada.

This approval is valid until the expiry date only. A study status report must be submitted annually and must accompany your request for re-approval. Any significant changes of the protocol and informed consent form should be reported to the Chair for consideration in advance of implementation of such changes. The REB must be notified regarding discontinuation or study closure.

This approval is for the ethics of human use only. For the logistics of performing the study, approval must be sought from the relevant institution, if required.

Sincerely yours,

John Arnett, Ph.D., C. Psych.
Chair, Health Research Ethics Board
Bannatyne Campus

Please quote the above Ethics Reference Number on all correspondence.
Inquiries should be directed to the REB Secretary  Telephone: (204) 789-3255 / Fax: (204) 789-3414

www.unmanitoba.ca/medicine/ethics
Appendix 4: Winnipeg Regional Health Authority approval letter

April 10, 2012

Dr. James Friel
University of Manitoba
Room 203 Richardson Center
for Functional Foods and
Neurochemicals
Winnipeg, MB R3T 2N2

Dear Dr. Friel:

Re: “Assessment of Complementary Feeding of Canadian Infants” —
WRHA Reference No: 2012-096

We are pleased to inform you that your research access request for the above-named study has been approved by the Winnipeg Regional Health Authority (WRHA) Research Review Committee.

Your research access is also approved pending confirmation that the following conditions are met or agreed to:

- You, your co-investigators, and your research assistants comply with the relevant privacy legislation as indicated below.

  - The Personal Health Information Act
  - The Freedom of Information and Protection of Privacy Act
  - The Personal Health Information Act and The Freedom of Information and Protection of Privacy Act

- You complete and return the attached Confidentiality Agreement(s) to Kelly Clarke, Concordia Hip & Knee Institute, WRHA, 200 – 1155 Concordia Avenue, Winnipeg, MB R2K 2A6;
- You submit to our attention any significant changes in your proposal prior to implementation or any significant changes during the course of the study;
- You submit a summary of the final results of the study to the WRHA and provide us with a copy of any publications arising from the study;
- It is an expected courtesy that WRHA will be given a minimum of five working days advance notice of publication or presentation of results with policy implications, in order to be prepared for public response;
- You agree to be accountable for appropriate storage and elimination of material;
- You agree to acknowledge the WRHA and/or affiliated organizations in any peer-reviewed publications of the results of this study.

Thank you for selecting the Winnipeg Regional Health Authority as the site to conduct your research. Please let us know should you encounter any site-related difficulties during the course of your study.

We extend best wishes for successful completion of your study.

Yours Sincerely,

Dr. Michael Moffatt, M.D., MSc, FRCPC
Executive Director, Division of Research and Applied Learning
Chair, Research Review Committee
Winnipeg Regional Health Authority

cc: Ms. Arlene Wagle, WRHA
Dr. John Amell, Chair, HREB
Appendix 5: Parent’s study handout

Assessment of Complementary feeding of Canadian Infants

We would like to invite you to participate in an important research study that will help us to understand the best way to meet the dietary iron needs of Canadian infants and how this affects their intestinal health.

“As a global public health recommendation, the world health organization recommends that infants should be exclusively breastfed for the first six months of life. Thereafter, infants should receive nutritionally adequate & safe solid foods along with breast milk until 2 years of age and beyond...First complementary foods should be iron-rich. (We) recommend meat, meat alternatives, and iron-fortified cereal as an infant’s first complementary foods.” (Health Canada)

This Clinical Trial is being conducted to study the best foods to feed infants once they are ready to begin eating solid foods as well as receiving mother’s milk. The purpose of this study is to find out what effects infant cereals and meat have on your infant’s ability to meet iron needs. The University of Manitoba is initiating this study with researcher and member of the Health Canada professional advisory board on infant health Dr. James Friel as the principle investigator. The research will help us to understand how our current recommendations affect infant iron stores and help to shape future recommendations.

Acceptance criteria:
- You have been exclusively breastfeeding your infant and plan to start feeding solid foods
- Your baby was born at or more than 37 gestational weeks
- Your baby’s birth weight was more than 2500 g (5.5 pounds)
- Your baby has not been diagnosed with any health concerns

Why you should participate:
The results of this study will help families know what foods are best to feed their babies.

There will be 2 visits to your home. In case of blood withdrawal (optional), visits will take place at Health Sciences Centre.

<table>
<thead>
<tr>
<th>When visits take place</th>
<th>What we request</th>
</tr>
</thead>
</table>
| Before introduction of solids (baby’s age ~ 4-6 months) | 1) Dietary record for 3 days  
2) Urine  
3) Stool  
4) Breast Milk sample  
5) Drops of blood drawn from your baby’s finger (optional) |
| 3 weeks after feeding start | Same samples as first visit (above) |

For more information and to participate please contact the research principal investigator

Dr. James Friel
Sarah Jorgensen
Dr. Wafaa Qasem
Appendix 6: Participants consent form

Assessment of Complimentary Feeding of Canadian Infants

UNIVERSITY OF MANITOBA Faculty of Human Ecology
Human Nutritional Sciences

RESEARCH PARTICIPANT INFORMATION AND CONSENT FORM

Title of Study: Assessment of Complimentary Feeding of Canadian Infants
Protocol #: H2011:168
Principal Investigator: James K. Friel
Room 203
Richardson Center for Functional Foods and Nutraceuticals
106 Innovation Drive
University of Manitoba
Winnipeg, MB R3T 2N2

Co-Investigator: Trust Beta, Wafaa Qasem

Sponsor: CIHR

You are being asked to allow your child to participate in a human research study. Please take your time to review this consent form and discuss any questions you may have with the study staff. You may take your time to make your decision about your child participating in this study and you may discuss it with your regular doctor, friends and family before you make your decision. This consent form may contain words that you do not understand. Please ask the study doctor or study staff to explain any words or information that you do not clearly understand.

The study doctor and institution are receiving financial support to conduct this study.

PURPOSE OF STUDY

This Clinical Trial is being conducted to study the best solid food to feed infants once they are ready to begin solid foods as well as mother’s milk (complementary feeding). You are being asked to allow your child to take part in this study because you have decided to exclusively breast-feed your infant, and are ready to begin introducing solids. A total of 120 participants will participate in this study.

The purpose of this study is to find out what effects (good and bad) infant cereals and meat has on your infant’s ability to meet iron needs and reduce inflammation.

October 25, 2013 Version 5

1 Participant’s initials ______
Assessment of Complimentary Feeding of Canadian Infants

This research is being done because we do not know which of these commonly used infant foods are better.

Study procedures

In this study, your child will be "randomized" into one of three study groups described below. "Randomized" means that your child is put into a group by chance, like flipping a coin. Your child will have an equal chance of being placed in any group. A computer will do the randomization. The three groups are: traditional rice cereal with iron; traditional rice cereal with iron and fruit; beef.

If you allow your child to take part in this study, your child will have the following tests and procedures: We will ask you to bring your child to our clinic, or we will conduct a visit to your home during the exclusive breastfeeding period, before introducing solids. The 2nd visit will take place approx. 3 weeks after you had introduced solids. For both visits, we will ask you to prepare a stool sample, a small urine sample and a dietary record of what foods your child is eating. We will provide the collection containers for the samples beforehand. We also ask for an (optional) blood sample from your child, a few drops of blood obtained by a finger poke at each visit. You are also being asked to provide samples of breast milk to be analyzed in addition to the samples you provide from your infant. We will ask you for 2 frozen breast milk samples: before feeding and after feeding, and one fresh breast milk sample pumped at either the 1st or 2nd visit. We will ask you questions about your infant and health and background information on you the parents/guardians. We will also weigh and measure the length of your infant at each visit. We anticipate each visit to last no longer than 60 minutes.

We will store the samples in a locked site until five years after the study termination and then they will be discarded. No identifying information will leave the study site. All stored samples and data will be coded by letter-number in order to link to a master list held by the PI and no one else.

The researcher may decide to take your child off this study if that would be in the participant's medical best interest, funding is stopped, or failure to adhere to the diet as proscribed.

Your child can stop participating at any time. However, if you decide to stop your child from participating in the study, we encourage you to talk to the study staff and your regular doctor first.

Study results will be presented at the end of the study for the entire group.

Risks and Discomforts

While on the study, there is minimal risk from food consumption for your infant. All foods provided are commonly consumed first foods. The potential risks associated with drawing blood from your infant's finger include minimal discomfort and/or bruising and infection. Your infant's health may not improve or may worsen while participating in this study.

Benefits

By allowing your child to participate in this study, you will be providing information to the study doctors that will show the effects of solid food introduction and their ability to resist.

October 25, 2013 Version 5 2 Participant's initials ______
Assessment of Complimentary Feeding of Canadian Infants

Inflammation for the infant. There may or may not be direct medical benefit to your infant from participating in this study. We hope the information learned from this study will benefit all breast-fed infants when their parents choose a first food in the future. All food for this study will be provided free of charge to participants.

Costs

All clinic and professional fees, diagnostic and laboratory tests, which will be performed as part of this study, are provided at no cost to you. There will be no cost for the study treatment that you will receive.

Payment for participation

You will be given ten dollars for parking per completed study visit to a maximum of thirty dollars upon termination of your participation in this research study.

Alternatives

Instead of allowing your child to be in this study, you may request to feed your infant whatever food you choose at whatever time. Choosing not to participate will have no effect on the care and treatment your infant receives while in hospital or at home.

You do not have to have your child participate in this study to receive information on best feeding practices for your infant. Please talk to your regular doctor about all your infant feeding options.

Confidentiality

Information gathered in this research study may be published or presented in public forums, however your name and other identifying information will not be used or revealed. Medical records that contain your identity will be treated as confidential in accordance with the Personal Health Information Act of Manitoba. Despite efforts to keep your personal information confidential, absolute confidentiality cannot be guaranteed. Your personal information may be disclosed if required by law. All study documents related to you will bear only your assigned subject code to be used for data storage and entry.

Organizations that may inspect and/or copy your research and medical records for quality assurance and data analysis include groups such as the Health Protection Branch. Only study personnel will have access to your records on site.

The University of Manitoba Health Research Ethics Board may review research-related records for quality assurance purposes.

All records will be kept in a locked secure area and only those persons identified will have access to these records. If any of your research records need to be copied to any of the above, your name and all identifying information will be removed. No information revealing any personal information such as your name, address or telephone number will leave the University of Manitoba.

October 25, 2013 Version 5

Participant's Initials _____
Assessment of Complimentary Feeding of Canadian Infants

Your Family Physician (GP) will be notified about your participation in this study.

Voluntary Participation/Withdrawal From the Study

You decision to allow your child to participate is voluntary. You may refuse to allow your child to participate or you may withdraw your child from the study at any time. Your decision will not affect your child’s other medical care at this site. If your study doctor feels that it is in your best interest to withdraw your child from the study, your study doctor will remove your child without your consent.

We will tell you about any new information that may affect your child’s health, welfare, or your willingness to allow your child to stay in this study.

Medical Care for Injury Related to the Study

You are not waiving any of your legal rights by signing this consent form or releasing the investigators or the sponsor from their legal and professional responsibilities.

Questions

You are free to ask any questions that you may have about your child’s treatment and your child’s rights as a research participant. If any questions come up during or after the study or if you have a research-related injury, contact the study doctor and the study staff:

Dr. James Friel

For questions about your child’s rights as a research participant, you may contact The University of Manitoba Health Research Ethics Board at (204) 789-3389.

Do not sign this consent form unless you have had a chance to ask questions and have received satisfactory answers to all of your questions.

Statement of Consent

I have read this consent form. I have had the opportunity to discuss this research study with James Friel and/or study staff. I have had my questions answered by them in language I understand. The risks and benefits have been explained to me. I believe that I have not been unduly influenced by any study team member to allow my child to participate in the research study by any statement or implied statements. Any relationship (such as employee, student or family member) I may have with the study team has not affected my decision to allow my child to participate. I understand that I will be given a copy of this consent form after signing it. I understand that my participation in this clinical trial is voluntary and that I may choose to withdraw my child at any time. I freely agree to allow my child to participate in this research study.

October 25, 2013 Version 5

Participant’s initials _______
Assessment of Complimentary Feeding of Canadian Infants

I understand that information regarding my personal identity or that of my child will be kept confidential, but that confidentiality is not guaranteed.

By signing this consent form, I have not waived any of the legal rights that I have for my child as a participant in a research study.

I agree to being contacted in relation to this study. Yes ☐ No ☐

Legal guardian’s printed name: ____________________________________________

Legal guardian’s signature_________________________ Date ________________
(day/month/year)

To be completed by study staff:

_____________________________________________________________________

I, the undersigned, have fully explained the relevant details of this research study to the participant named above and believe that the participant has understood and has knowingly given their consent

Printed Name: ________________________________ Date ________________

Signature: ________________________________
(day/month/year)

Role in the study:

Witness signature_________________________ Date ________________
(day/month/year)

Witness printed name: ________________________________

October 25, 2013 Version 5

Participant’s initials ______
Appendix 7: 3-day dietary record (1^{ST} visit)

Day 1 Feeding Record, date: ________

Name: ___________  Age: ________

<table>
<thead>
<tr>
<th>Feeds</th>
<th>Feed type/amount</th>
<th>Feeding time</th>
<th>Feeding duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other comments</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Version 1 Sep 12, 2012
Day 2 Feeding Record, date: __________

Name: __________
Age: __________

<table>
<thead>
<tr>
<th>Feeds</th>
<th>Feed type/amount</th>
<th>Feeding time</th>
<th>Feeding duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other comments</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Version 1 Sep 12, 2012
# Day 3 Feeding Record

Date: ________

Name: ______________ Age: __________

<table>
<thead>
<tr>
<th>Feeds</th>
<th>Feed type/amount</th>
<th>Feeding time</th>
<th>Feeding duration (if breastfeeds)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other comments</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Version 1 Sep 12, 2012
Appendix 8: 3-day dietary record (2nd visit)

2nd Dietary Record, After/During introduction of Solids

DAY 1, Date ____________
Your name, baby’s name ______________________

<table>
<thead>
<tr>
<th>#</th>
<th>Time</th>
<th>Feeding Type (Breast milk = BM, vitamin, supplement, or solid food, please specify)</th>
<th>Duration (BM) or amount (solid food)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Other Comments:
2nd Dietary Record, After/During introduction of Solids

DAY 2, Date ____________
Your name, baby’s name ______________

<table>
<thead>
<tr>
<th>#</th>
<th>Time</th>
<th>Feeding Type (Breast milk = BM, vitamin, supplement, or solid food, please specify)</th>
<th>Duration (BM) or amount (solid food)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Other Comments:
2nd Dietary Record, After/During introduction of Solids

DAY 3, Date ____________________________
Your name, baby’s name ____________________________

<table>
<thead>
<tr>
<th>#</th>
<th>Time</th>
<th>Feeding Type <em>(Breast milk = BM, vitamin, supplement, or solid food, please specify)</em></th>
<th>Duration (BM) or amount (solid food)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Other Comments:
Appendix 9: Urine sample collection instructions

Urine Sample Instructions

Provided to you are four (4) pediatric urine collectors. We hope that this is sufficient for both samples (before feeding and after feeding). If you have trouble and need more urine collectors, please let us know and we will happily provide more!

For Both Male and Female babies:

1) Make sure the genital area is clean and dry.
2) The collector is best applied directly before nursing, because babies often urinate within minutes after nursing.
3) To avoid spilling, it is best to keep baby upright, in a “standing” position after nursing. This allows the pressure from baby’s abdomen to gently press on the bladder. Also, if baby is left lying down, the urine often spills out.
4) Run a little water in the sink after nursing, let baby touch the water and hear the sound (yes, this works with babies too!)
5) You will have the most success if you dedicate a few minutes after nursing to keeping baby upright and (ideally) naked so you can see when they urinate.
6) Immediately after collecting the sample, pour it into the screw-cap tube. If it looks like you got very little (just a few drops) this is ok – place the entire urine collector inside the tube. Try again with another collector later or another day.

For Male Babies:
Remove the adhesive backing, place the opening over the penis, and gently press down around all sides of the adhesive portion. Try to gently pull out the collection bag so it is not lying directly against the skin, creating space. The bag should be pointing down.

For Female Babies:
Remove the adhesive backing, and place the oval opening over the vulva (see image). Gently press around all sides of the urine collector to avoid spilling (this is more important w/ female babies). Make sure the bag is pointing down, and fold/pull the bag gently to create space for the urine to enter.

Note: The adhesive is not very strong, which is good because it will not harm baby upon removal. However, this means it is more likely to pop off and spill!

Additional Tips:
If you know your baby often urinates right before a bath, this is a good time to try and collect a sample. Some mothers have even been able to “catch” some urine directly into a tube right before a bath.

Some participants who had difficulty with the above methods were successful using a training potty to collect the samples. If you have this available to you, as long as the potty is clean and dry before attempting to collect a sample, you can attempt to collect urine this way.

Good Luck! 😊
Bag pointing down
toward feet
+ pulled/folded out
and open

Female Babies:
Cover the vulva, avoid placing hole too
close to the anus (to avoid contamination)
or too high that the urine spills out the bottom

Adhesive portion

Adhesive portion

Narrow end of opening can be used to pour
urine sample into collection tube
Appendix 10: Stool and urine sample collection instructions

Sample Collection Instructions

Thank you for providing us with a sample of your infant’s stool and urine for our study! While you have likely experienced your baby’s urine and stool on a daily basis, this time we ask that you COLLECT these bodily fluids for us, and in a sterile manner. This may take a few attempts, and we have included the necessary equipment you need for multiple tries. To reduce the contamination of the samples as much as possible, please follow the following guidelines:

- Please wear the gloves provided (This eliminates introduction of contaminants that may be present on your hands)
- Before applying the urine collector, please clean your baby’s genital area with the alcohol swabs provided
- Open sterile screw-cap tubes only just before transferring urine and stool into them
- Once you have collected the urine and stool and they are in the sterile screw-cap tubes (with blue lids), please place them in the freezer immediately

Below are more detailed instructions for each of the samples that we request:

Urine

The urine collector has gentle adhesive tape around the opening. Make sure the baby’s genital area is cleaned with the alcohol swabs provided and dry, to aid in proper adhesion to the skin. We have had the most success collecting urine just after breastfeeding. You can then apply the urine collector and sit the baby upright, which will encourage urination through the gentle pressure of the baby’s abdominal weight upon the bladder. Also, giving the baby a small sip of water or even turning on the tap for the baby to hear the running water is often a successful trick. When the baby passes urine it will be collected in the urine collector, and you can then gently peel it off the skin and pour the urine into the screw-top tube.

For obvious reasons, moms with baby boys have an easier time collecting urine. Know that this can be a little tricky with baby girls and note that we have included two urine collectors for you!

Stool

The key to collecting stool is to try to remove it from the diaper and place it in the collection tube as soon as possible. This prevents the stool, which is almost always runny in breastfed infants, from absorbing too much into the diaper.
Appendix 11: Additional results and figures corresponding to the study described in chapters V and VI

**Comparison of the mean % contribution of energy and macronutrients to the recommended daily DRIs before introduction of solids**

**Comparison of the mean % contribution of energy and macronutrients to the recommended daily DRIs after introduction of solids**

Figure 22. Comparison of mean % contribution of energy and macronutrients towards the daily recommended DRIs from before and after introduction of CFs
Figure 23. Comparison of mean % contribution of vitamins towards the daily recommended DRIs from before and after introduction of CFs
Figure 24. Comparison of mean of % contribution of selective minerals towards the daily recommended DRIs from before and after introduction of CFs
Figure 25. Comparison of mean % contribution of energy and macronutrients towards the daily recommended DRIs from CFs only

Comparison of the mean % contribution of energy and macronutrients to the recommended daily DRIs from CFs only

Food energy
CHO
Protein
Fat

Figure 26. Comparison of mean % contribution of selective vitamins towards the daily recommended DRIs from CFs only

Comparison of the mean % contribution of selective vitamins to the recommended daily DRIs from CFs only

Vitamin A
Thiamin
Riboflavin
Niacin
Vitamin B12
Vitamin C

Figure 26. Comparison of mean % contribution of selective vitamins towards the recommended daily DRIs from CFs only
Figure 27. Comparison of mean of % contribution of selective minerals towards the recommended daily DRIs from CFs only

Additional detailed methods and results corresponding to fecal iron determination

Coefficient of variation

To measure the variability of the values of this method, the coefficient of variation was determined. It is the measure of dispersion of a probability distribution and defined as the ratio of the standard deviation to the mean.

Three adult fecal samples were used to determine the $CV$ for this test. From each adult sample, ten samples were prepared, incubated, and the iron values were calculated to determine whether the ten sub-samples from each adult sample have the same iron concentration based on the measured $CV$.

Table 18 shows the results of the analysis of the adult fecal samples.
Table 18. Fecal iron in the three adult samples

<table>
<thead>
<tr>
<th>Sample</th>
<th>N</th>
<th>Minimum</th>
<th>Maximum</th>
<th>Mean</th>
<th>SE</th>
<th>SD</th>
<th>CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adult 1</td>
<td>10</td>
<td>2.3</td>
<td>2.9</td>
<td>2.6</td>
<td>0.05</td>
<td>0.18</td>
<td>0.07%</td>
</tr>
<tr>
<td>Adult 2</td>
<td>10</td>
<td>4.0</td>
<td>5.2</td>
<td>4.5</td>
<td>0.14</td>
<td>0.45</td>
<td>0.10%</td>
</tr>
<tr>
<td>Adult 3</td>
<td>10</td>
<td>4.0</td>
<td>4.7</td>
<td>4.3</td>
<td>0.06</td>
<td>0.21</td>
<td>0.048%</td>
</tr>
</tbody>
</table>

N.B. $CV$=coefficient of variation, N=number, SE=standard error, SD=standard deviation

The values of $CV$ were less than 1.0, which is indicative of lower variance (low spread of the data) and higher precision.

**Standard reference material**

The standard reference material (SRM) of bovine liver, non-fat milk powder and formula were analyzed for their iron content and compared to the manufacturer’s reference. Following the manufacturer’s instruction for drying the SRM, 10 to 15 grams (wet) of each SRM was incubated in a vacuum oven (Thermo Electron Precision Mechanical Incubator 30M, Thermo Fischer Scientific Inc, Waltham, MA, USA) at $60^\circ$C for 24 hours. After incubation, each SRM sample was weighed consequently every 2 hours until the weight of the SRM was stable. Following drying of the SRM, a total of five samples of each SRM was prepared, incubated and analyzed for their iron content following the aforementioned iron method steps.

As shown in table 19, the non-fat milk powder was the closest value to the manufacturer’s reference value; therefore, the non-fat milk powder was used to determine the method’s repeatability.
Table 19. Iron content in standard reference materials

<table>
<thead>
<tr>
<th>Sample</th>
<th>Iron content µg/g (mean±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine liver</td>
<td>2.8±1.1</td>
</tr>
<tr>
<td>Non-fat milk powder</td>
<td>3.1±1.2</td>
</tr>
<tr>
<td>Formula</td>
<td>5.0±0.6</td>
</tr>
</tbody>
</table>

Certified values; bovine liver: 194±20.0 µg/g, non-fat milk powder: 1.78±0.1µg/g, formula: 177±3.3 µg/g

**Method repeatability**

To assess repeatability of the method, the following samples were prepared, incubated and analyzed for iron content and compared to the corresponding previous results: five samples of the standard non-fat milk powder, three samples of infants stool with low iron content, three samples of infants stool with middle iron content, and three samples of infants stool with high iron content. The steps of the analysis of the samples for the residual iron is equivalent to the fecal iron method.

The results of the method repeatability are illustrated in table 20. The results obtained were comparable to the previous results, which support the repeatability of this method.

Table 20. Iron content in non-fat milk powder and infants stool samples

<table>
<thead>
<tr>
<th>Sample</th>
<th>Previous results Fe µg/g sample</th>
<th>Re-test results Fe µg/g sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-fat milk powder (mean±SD)</td>
<td>3.1±1.3</td>
<td>3.1±1.2</td>
</tr>
<tr>
<td>Low Fe content stool</td>
<td>1.9</td>
<td>2.1</td>
</tr>
<tr>
<td></td>
<td>1.9</td>
<td>1.9</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>1.9</td>
</tr>
<tr>
<td>Middle Fe content stool</td>
<td>3.6</td>
<td>3.2</td>
</tr>
<tr>
<td></td>
<td>3.8</td>
<td>3.2</td>
</tr>
<tr>
<td></td>
<td>3.6</td>
<td>3.9</td>
</tr>
<tr>
<td>High Fe content stool</td>
<td>7.9</td>
<td>7.3</td>
</tr>
<tr>
<td></td>
<td>7.5</td>
<td>8.3</td>
</tr>
<tr>
<td></td>
<td>7.5</td>
<td>7.9</td>
</tr>
</tbody>
</table>
Standard curves

A primary standard curve was constructed from the readily available standard of the kit. Starting from the highest concentration (140 µg/dl Fe³⁺), five series of ten-fold dilutions were prepared. Then the standards were read at 590 nm (Cary® 50 MPR spectrophotometer Varian, Agilent Technologies Canada, Mississauga, Ontario, Canada). Another five series (ten-fold dilutions) of the standard were prepared and treated under the same conditions of the fecal iron method (identically to the samples; digested and incubated). Then the treated standards were also read at 590 nm. The standard curve (of the treated standards) was constructed and compared to the primary standard curve (figure 28). Table 21 shows the comparison between the absorption of the standards (primary vs treated). The concentrations of the treated standards were comparable to the concentration of the primary standards.

Calibration curve for non-fat milk powder

![Calibration curve](image)

\[ y = 0.0233x + 0.1708 \]

\[ R^2 = 0.93357 \]
Figure 28. Comparisons of standard curves

Table 21. Comparison between the absorptions of the primary standard and the treated standards

<table>
<thead>
<tr>
<th>Standard concentration (µg/µl)</th>
<th>Standard absorption</th>
<th>Treated standard absorption</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.014</td>
<td>0.16</td>
<td>0.15</td>
</tr>
<tr>
<td>0.14</td>
<td>0.165</td>
<td>0.16</td>
</tr>
<tr>
<td>1.4</td>
<td>0.22</td>
<td>0.22</td>
</tr>
<tr>
<td>2.8</td>
<td>0.25</td>
<td>0.24</td>
</tr>
<tr>
<td>5.6</td>
<td>0.29</td>
<td>0.27</td>
</tr>
</tbody>
</table>

y = 0.0209x + 0.1664

$R^2 = 0.87209$
For screening EBF infants, we suggest the following example (table 22) of how risk factors may be included in an iron status assessment score for breastfed infants. A future study may develop and validate a scoring system that would predict the need to check the iron profile of four to six months old infants.

Table 22. Example of risk factors in an iron status assessment score

<table>
<thead>
<tr>
<th>Variable</th>
<th>Iron status assessment score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gestational age</td>
<td>&lt; 36 weeks</td>
</tr>
<tr>
<td></td>
<td>&gt; 36 weeks</td>
</tr>
<tr>
<td>Birth weight</td>
<td>&lt; 2500 g</td>
</tr>
<tr>
<td></td>
<td>&gt; 2500 g</td>
</tr>
<tr>
<td>Maternal iron status</td>
<td>Iron deficient</td>
</tr>
<tr>
<td></td>
<td>Non-iron deficient</td>
</tr>
<tr>
<td>Anemia during pregnancy</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>No</td>
</tr>
<tr>
<td>Gestational diabetes</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>No</td>
</tr>
<tr>
<td>Feeding method since birth</td>
<td>Breastfeeding only</td>
</tr>
<tr>
<td></td>
<td>Breastfeeding + Formula</td>
</tr>
<tr>
<td>Estimated age of introduction of solids</td>
<td>At 6 months</td>
</tr>
<tr>
<td></td>
<td>Between 4-6 months</td>
</tr>
<tr>
<td>Expected duration of EBF</td>
<td>6 months</td>
</tr>
<tr>
<td></td>
<td>&lt; 6 months</td>
</tr>
<tr>
<td>Introduction of cow’s milk</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>No</td>
</tr>
<tr>
<td>Low-iron formula</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>No</td>
</tr>
</tbody>
</table>
Appendix 12: Permissions to use copyrighted materials

Title: Iron requirements, absorption and metabolism in infancy and childhood
Author: Domellof, Magnus
Publication: Current Opinion in Clinical Nutrition and Metabolic Care
Publisher: Lippincott Williams & Wilkins
Date: Jan 1, 2007
Copyright © 2007, (C) 2007 Lippincott Williams & Wilkins, Inc.

Order Completed
Thank you very much for your order.

This is a License Agreement between Wafaa Qasem ("You") and Wolters Kluwer Health ("Wolters Kluwer Health"). The license consists of your order details, the terms and conditions provided by Wolters Kluwer Health, and the payment terms and conditions.

Get the printable license.

| License Number   | 3580920025380 |
| License date     | Mar 02, 2015  |
| Licensed content publisher | Wolters Kluwer Health |
| Licensed content publication | Current Opinion in Clinical Nutrition and Metabolic Care |
| Licensed content title | Iron requirements, absorption and metabolism in infancy and childhood. |
| Licensed content author | Domellof, Magnus |
| Licensed content date | Jan 1, 2007 |
| Volume number    | 10           |
| Issue Number     | 3            |
| Type of Use      | Dissertation/Thesis |
| Requestor type   | Individual   |
| Portion          | Figures/table/illustration |
| Number of figures/tables/illustrations used | 1 |
| Author of this Wolters Kluwer article | No |
| Title of your thesis / dissertation | Assessment of Complementary feeding of Canadian Infants |
| Expected completion date | May 2015 |
| Estimated size (pages) | 270 |
| Total            | 0.00 USD     |