

**Adequacy of micronutrient intakes  
and status of breastfed Indonesian  
infants fed traditional complementary  
foods in Sumedang district, West Java:  
a longitudinal study**

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## Abstract

Stunting among under-five children in Indonesia is common, raising public health concerns. Inappropriate complementary feeding (CF) practices may compromise optimal growth during late infancy in Indonesia. Hence, it is not surprising that earlier biomarker studies in Indonesia have documented deficiencies of iron, zinc, vitamin A, and anaemia during infancy. Of these deficiencies, nutritional iron deficiency (ID) has been assumed to be the major factor causing the high rates of anaemia during infancy and early childhood in Indonesia.

Therefore, we conducted a prospective, longitudinal study to evaluate complementary feeding, growth, biomarkers of micronutrient status, together with potential non-nutritional confounding factors including parasitic infections, inflammation, and genetic haemoglobin (Hb) disorders among breastfed infants aged 6 to 12 months of age. The study was conducted between August 2013 and August 2014. Infants were randomly selected from all the villages (n=30) in Tanjungsari, Sukasari, and Pamulihan sub-districts in Sumedang district, West Java. We enrolled breastfed infants at 6 months of age (n=230); and followed them at 9 (n=202) and 12 months of age (n=190).

Relationships at 9 months between WHO IYCF indicators, five sentinel foods, micronutrient intakes and subsequent growth at 12 months were explored using multiple linear regression. Then we measured serum biomarkers of iron, zinc, vitamin A, and selenium status adjusted for inflammation with C-reactive protein (CRP) and  $\alpha$ -1-glycoprotein (AGP) to determine the proportion with micronutrient deficiencies at aged 6, 9 and 12 months. Next, we performed multiple regression and logistic regression analysis to examine multivariate relationships between haemoglobin and anaemia, respectively, and infant micronutrient status, household, maternal, and infant non-nutritional factors

Our results showed that stunting increased from 15.8% at 6 months to 22.6% at 12 months. Median intakes of energy, niacin, calcium, iron, and zinc from

complementary foods were consistently below WHO estimated needs. Infants consuming fortified infant foods (FIFs) at 9 months had diets with a lower dietary diversity score (2.3 vs. 3.0) and energy density, with lower median intakes of energy (250 vs. 310 kcal/d) and protein (6.5 vs. 9.1 g/d) from complementary foods than non-consumers ( $P < 0.01$ ), despite higher intakes of calcium, iron, and vitamins A, and C ( $P < 0.001$ ). Positive relations existed for the consumption of FIFs alone at 9 months with both length-for-age z-score (LAZ) and weight-for-age z-score (WAZ) at 12 months.

At 6, 9, and 12 months of age, the new BRINDA regression adjustment for inflammation yielded the highest proportion with iron deficiency (20.3, 37.8, 59.5%) and the lowest proportion with both vitamin A (26.4, 16.6, 17.3%) and zinc (16.9, 20.6, 11.0%) deficiency, respectively, compared to unadjusted estimates. For serum selenium, irrespective of adjustment, the proportion with deficiency was high (>50%) across all ages. The proportion of infants with anaemia increased from 32.9% to 38.4% at age 6 and 12 months of age, which was negatively associated with ferritin at 6 (odds ratio (OR): 0.46 [95% CI: 0.28, 0.76]) and 12 months of age (OR: 0.25 [95% CI: 0.12, 0.49]), respectively. In addition, female sex, and lower CRP concentrations were associated with higher haemoglobin concentrations at 6 months of age; whereas serum folate was a significant negative predictor of anaemia at 12 months (OR: 0.96 [95% CI: 0.92, 0.99]).

In conclusion, our findings highlight the inadequate CF practices in Indonesia. Without inflammation adjustment, iron deficiency was grossly under-estimated and vitamin A and zinc deficiency over-estimated, highlighting the importance of correcting for the influence of inflammation prior to implementing programmes to alleviate micronutrient malnutrition. Anaemia remains a persistent and severe public health problem during infancy in Sumedang district, Indonesia, of which low iron status is a major predictor at 6 and 12 months of age, although at age 12 months, folate also had a role in the anaemia. Hence, there is an urgent need to improve CF practices during the latter half of infancy in the study setting and re-evaluate the performance of iron deficiency control programs in this district.

## Preface

This longitudinal study was a collaborative project between the Department of Human Nutrition, University of Otago, Dunedin, New Zealand and the Department of Medical Nutrition, *Universitas Padjadjaran*, Bandung, Indonesia, with the funding from Meat and Livestock Australia (MLA) and a University of Otago Research Grant (UORG). The candidate alongside her supervisors, Associate Prof. Lisa Houghton and Prof. Rosalind S. Gibson, developed the concept and overall design for the longitudinal study.

The candidate, with assistance from both supervisors, was responsible for writing the Human Ethics application for the Ethics Committee in *Universitas Padjadjaran*, developing the standard operating procedures for anthropometry, collection of blood samples, and faecal specimens, and compiling and translating all the questionnaires (Appendix A, B). The candidate trained all the field doctors and coordinated the fieldwork (Appendix C). The candidate was also responsible for supervising data collection, data entry and cleaning, and compilation of the food composition table (FCT) for the complementary foods consumed by the Indonesian infants. Prof. Rosalind S. Gibson helped finalise the FCT by sharing her expertise and providing phytate values. Statistical analysis and interpretation of the data was done in consultation with Dr. Jillian J. Haszard (Biostatistician) and Associate Prof. Lisa Houghton.

Prodia Laboratory (Bandung) collected the veni-puncture blood samples and conducted the complete blood count analyses. Dr. Juergen Erhardt (VitMin Lab, Germany) conducted analyses for ferritin, sTfR, RBP, CRP, and AGP. Dr. Karl Bailey conducted analyses for serum vitamin B-12 and folate. Serum zinc and selenium were analysed by Dr. Malcolm R. Reid (Department of Chemistry, University of Otago). Serum vitamin D (as total 25-dihydroxyvitamin D) was analysed by Michelle Harper. Hb disorder analysis was performed by Dr. Pranee W. Fucharoen and Dr. Saovaros Svasti (Thalassemia Research Center, Institute of Molecular Biosciences, Mahidol University).



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## List of Abbreviations

AGP	$\alpha$ -1-glycoprotein
APPs	Acute phase proteins
BEI	Butanol extractable iodine
BF	Breastfeeding
BII	Butanol insoluble iodine
BMI	Body mass index
BMIZ	Body mass index z-score
CBC	Complete blood count
CDC	Centers for Disease Control and Prevention
CF	Complementary feeding
CI	Confidence intervals
CRP	C-reactive protein
CVs	Coefficients of variation
DHS	Demographic Health Surveys
EBF	Exclusive breastfeeding
ECFs	External correction factors
EFSA	European Food Safety Authority
ELISA	Enzyme-linked immunosorbent assay
FCT	Food Composition Table
FIFs	Fortified infant foods
G6PD	Glucose-6-phosphate dehydrogenase
GM	Geometric means
HAZ	Height-for-age z-score
Hb	Haemoglobin
ICFs	Internal correction factors
ICP-MS	Inductively coupled plasma mass spectrometry
ID	Iron deficiency
IDA	Iron deficiency anaemia
IGF-I	Insulin-like growth factor-I
IYCF	Infant and young child feeding
IZiNCG	International Zinc Nutrition Consultative Group
LAZ	Length-for-age z-score
LMICs	Low- and middle-income countries
ln	log-transformed
MAD	Minimum acceptable diet
MAR	Micronutrient adequacy ratio
MCV	Mean corpuscular volume
MCH	Mean corpuscular haemoglobin
MDD	Minimum dietary diversity

MMF	Minimum meal frequency
MNDs	Micronutrient deficiencies
NAR	Nutrient adequacy ratio
NIST	National Institute of Standards
PBI	Protein-bound iodine
OR	Odds ratio
RBC	Red blood cell
RBP	Retinol binding protein
RNIs	Recommended Nutrient Intakes
SD	Standard deviation
SES	Socio-economic status
sTfR	Soluble transferrin receptor
STH	Soil-transmitted helminthiasis
T3	Triiodothyronine
T4	Thyroxine
TBG	Thyroxine binding globulin
TE	Trace-element
TEM	Technical error of the measurement
Tg	Thyroglobulin
TI	Total iodine
TMAH	Tetra methyl ammonium hydroxide
TPOAb	Thyroid peroxidase antibody
TSH	Thyroid stimulating hormone
UIE	Urinary iodine excretion
WAZ	Weight-for-age z-score
WHO	World Health Organisation
WLZ	Weight-for-length z-score



# 1 Introduction

## 1.1 Introduction

Childhood undernutrition remains a significant problem in Indonesia, even though there have been major improvements in health over past decades. In 2010, among under-five children, mortality rates were 35 per 1000 live births, 18% were underweight, and 37% were stunted (UNICEF, 2010). Such growth faltering often begins around 4-7 months of age (Kusin et al., 1991, Kolsteren et al., 1997), and is accompanied by a high prevalence of anaemia and co-existing micronutrient deficiencies (Dijkhuizen et al., 2001b, Lind et al., 2003, Wieringa et al., 2004, Howard et al., 2007).

Despite very high national breastfeeding (BF) rates at 6 months (86%) (Yohmi et al., 2015), progress in improving infant and young child feeding (IYCF) practices in Indonesia has been slow (Ng et al., 2012). The rice-based gruels traditionally used for CF often have a low energy and nutrient density (Harper, 2006; Isabelle and Chan, 2011) leading to nutrient deficits when compared with the World Health Organisation (WHO) estimated needs (Dewey and Brown, 2003), particularly for iron, zinc, and sometimes vitamin A (Dewey, 2016). Hence, it is not surprising that earlier biomarker studies in Indonesia have documented deficiencies of iron, zinc, and vitamin A during infancy (Dijkhuizen et al., 2001b, Untoro et al., 2005, Sandjaja et al., 2013). Deficiencies of these micronutrients compromise the immune system (Gibson, 2005, Raiten et al., 2015), resulting in increases in morbidity and mortality during early childhood, while iron and zinc deficiency are also associated with an increased risk of impairments in both growth and cognition (Lozoff et al., 2006, Carter et al., 2010, King et al., 2016, Gould, 2017).

Of these three micronutrients, nutritional iron deficiency (ID) has been assumed to be the major factor causing the high rates of anaemia during infancy and early childhood in Indonesia (Kodyat et al., 1998). Increasingly, however, the contribution of many other micronutrient deficiencies besides iron to the overall burden of anaemia during early childhood is being

recognized. These include deficiencies of folate, vitamin B-12 and vitamin A, all of which have well established roles in normal haematopoiesis (Kraemer and Zimmerman, 2007). In addition, emerging evidence suggests that deficiencies of zinc, selenium, and vitamin D may also be involved in the aetiology of anaemia through several plausible mechanisms (Lander et al., 2008, Houghton et al., 2016). To date, with the exception of zinc, data on selenium, folate, and vitamin D deficiency during the CF period in Indonesia and their possible role in the aetiology of anaemia in Indonesia has not been investigated.

Additional non-nutritional factors that have the potential to contribute to anaemia during infancy in rural Indonesia include parasitic infections, inflammation, and genetic haemoglobin (Hb) disorders (Thurlow et al., 2005, Lander et al., 2008). However, in many of the earlier studies in Indonesia, these non-nutritional factors were rarely measured despite their potential to confound the interpretation of some of the serum micronutrient biomarkers, most notably ferritin, transferrin receptor, retinol or retinol binding protein (RBP), and zinc. For example, inflammation and infections due to parasites and other causes may elevate serum ferritin levels, and reduce concentrations of zinc and retinol or RBP (Raiten et al., 2015).

Consequently, the resulting prevalence estimates may not reflect the true burden of deficiency unless inflammation or infection has been taken into account. Increasingly, inflammatory biomarkers, notably C-reactive protein (CRP) and  $\alpha$ -1-glycoprotein (AGP) are analysed to provide a measure of the severity and duration of inflammation, respectively, together with the serum micronutrient biomarkers (Suchdev et al., 2016), so that the concentrations of the micronutrient biomarkers affected by the inflammatory response can be adjusted accordingly. Several approaches to adjust the concentrations of micronutrient biomarkers can be used (Thurnham et al., 2003, Beard et al., 2006, Thurnham et al., 2010, Bui et al., 2012, Engle-Stone et al., 2013, Cichon et al., 2017), of which the most recent has been developed by a collaborative research group called Biomarkers Reflecting Inflammation and Nutrition Determinants of Anaemia (BRINDA). BRINDA was formed in 2012 by the U.S

Centers for Disease Control and Prevention (CDC), National Institute for Child Health and Human Development, and Global Alliance for Improved Nutrition (Suchdev et al., 2016).

Finally, Indonesia is situated in Southeast Asia, a region with a high prevalence of genetic Hb disorders that affect the structure, function and/or production of Hb (Fucharoen and Winichagoon, 2011), some which present as mild to severe anaemia. Two genetically distinct variants common in Southeast Asia, Hb E and  $\alpha$ -thalassemia, have a carrier frequency in Indonesia that reportedly ranges from 1–33% for Hb E and 6-16% for  $\alpha$ -thalassemia, depending on ethnicity and geographic region, with  $\beta$ -thalassemia having a lower frequency (5-10%) (Ariani et al., 2017). The extent to which these genetic Hb disorders contribute to low Hb concentrations in late infancy in Indonesia is not well established. Moreover, their presence may confound the interpretation of ID as they are often associated with elevated levels of soluble transferrin receptor (sTfR) (George et al., 2012).

## 1.2 Objectives

Therefore, the overall goal of this research was to examine the growth and micronutrient status of breastfed infants during the CF period from the Sumedang district, West Java province, Indonesia. This was achieved by recruiting a cohort of breastfed infants at 6 months of age and following them at 9 and 12 months of age to accomplish the following specific objectives:

- characterise the CF practices at each age (Chapter 3);
- evaluate the adequacy of energy and nutrient intakes during CF in relation to the WHO estimated needs (Chapter 3);
- investigate relationships between WHO CF indicators, local sentinel foods, nutrient adequacy, and subsequent infant growth (Chapter 3);
- assess and compare the proportion of deficiencies of iron, zinc, vitamin A, and selenium, based on serum biomarkers among the infants at aged 6, 9, and 12 months of age with and without adjustment for inflammation (Chapter 4);

- investigate the extent to which the micronutrient biomarkers, socio-demographic factors, inflammation, helminth infections, and genetic Hb disorders are associated with infant Hb concentrations and anaemia at 6 and 12 months of age (Chapter 5).

We hope that this research might lead to the development of interventions to improve CF practices, reduce the prevalence of micronutrient deficiencies and anaemia, and enhance growth. Appropriate feeding practices during early childhood are essential because interventions after the first two years appear to have little impact on subsequent child growth (Bhutta et al., 2008). Achieving the goal set by the WHO to reduce childhood stunting by 40% by 2025 (de Onis et al., 2013) is a priority for Indonesia.

## **2 Literature Review**

### **2.1 Introduction**

Stunting and underweight are common among under-five children in Indonesia, and are often accompanied by a high prevalence of anaemia and co-existing micronutrient deficiencies (Isabelle and Chan, 2011, National Institute of Health Research and Development, 2012, National Institute of Health Research and Development, 2013, Sandjaja et al., 2013), raising public health concerns. Hence, it is important to investigate the extent to which inappropriate CF practices and micronutrient malnutrition during infancy and early childhood may be associated with this poor growth in young children in Indonesia. Therefore, this literature review will focus on the possible contribution of CF practices, anaemia, micronutrient deficiencies, and infections during the first two years on growth and other functional outcomes in under-five children in Indonesia.

### **2.2 IYCF practices in low income countries (LICs)**

Exclusive breastfeeding (EBF) in the first 6 months of life is recommended (WHO, 1998, WHO, 2009) to provide sufficient energy and nutrients to meet the requirements of full term infants (WHO, 2002, Dewey and Brown, 2003), and to protect against infections and reduce mortality (Kramer and Kakuma, 2002, Lamberti et al., 2011). However, breast milk alone is no longer sufficient to meet the energy and nutrient needs from 6 months of age (WHO, 2002).

Therefore, from 6 months of age, nutritionally adequate complementary foods together with breast milk are essential to support optimal growth and development of infants and young children up to two years of age. However, the quality of breast milk, especially the concentrations of B vitamins (except folate), vitamin A (Stoltzfus and Underwood, 1995, Muhilal et al., 1988, Dijkhuizen et al., 2001b), selenium, and iodine (Allen, 1994, Allen, 2005) are known to be affected by maternal nutritional status. Consequently, concentrations of some of these micronutrients in mature breast milk may be extremely low (Allen, 1993, Dijkhuizen et al., 2001b) in areas where there is a

high prevalence of co-existing multiple micronutrient deficiencies in women of reproductive age. Hence, extending the period of EBF for more than 6 months in such underprivileged circumstances may cause more harm to the infants.

Recommended amounts of energy and micronutrients from complementary foods alone, termed "estimated needs" have been calculated by WHO. This has been achieved by subtracting the mean amount of nutrients provided by breast milk at each age group, assuming the consumption of a low, average, and high volume of breast milk of average composition (Dewey and Brown, 2003, Brown, 2007) from the WHO estimated energy requirements (FAO/WHO/UNU, 2004) and the recommended nutrient intakes (RNIs) (WHO/FAO, 2004) for infants and young children aged 6-8 months, 9-11 months, and 12-23 months. In many LICs, intakes of energy, protein, and several micronutrients from complementary foods, including iron, zinc, calcium, vitamin A, and selected B vitamins are often lower than the corresponding estimated needs (Gibson et al., 1998, Muller and Krawinkel, 2005, Brown, 2007, Chan et al., 2007), mainly resulting from poor quality complementary diets based on cereal-based porridges with low energy/nutrient density, poor mineral bioavailability due to their high phytate-to-mineral molar ratios, and the low content of animal source foods (Gibson and Hotz, 2000, Hotz and Gibson, 2001, Yip and Ramakrishnan, 2002, Nguyen et al., 2011, Dewey, 2013).

Data from national anthropometric surveys from 39 LICs in 2001 have shown that weight-for-age z-scores (WAZ) start to falter at around 3 months of age and then decline rapidly until 12 months, while length-for-age z-scores (LAZ) often start to falter immediately after birth (Shrimpton et al., 2001). Updated analysis in 2010 using more recent anthropometric data from 54 LICs also show that WAZ falters moderately, while LAZ falters dramatically, especially after 4 months of age, until 24 months of age (Victora et al., 2010).

This same trend was also noted in a national survey in Indonesia in 2013 where growth faltering based on WAZ and LAZ or height-for-age z-scores (HAZ) increased from birth until 35 months (National Institute of Health Research and

Development, 2013). In West Java, Indonesia, a study following women from 18 weeks of pregnancy until their children were 12-15 months of age reported that WAZ and LAZ started to falter when their infants were 3-4 months of age (Schmidt et al., 2002). Moreover, a significant positive association was found between the consumption of complementary foods and linear growth, suggesting that growth faltering in the second half of infancy, was partly due to inadequate food intakes (Schmidt et al., 2002), a finding which has been reported by others (Lutter and Rivera, 2003, Caulfield et al., 2006).

## **2.3 Assessment of the adequacy of IYCF practices**

The adequacy of IYCF practices can be assessed by two methods, using either a set of population-based indicators developed by WHO, three of which focus on BF practices and five on CF practices, and/or by comparing energy and nutrient intakes from complementary foods alone against the WHO estimated needs.

### **2.3.1 Assessment of the WHO IYCF indicators**

As IYCF practices directly affect nutritional status, growth, and child survival (WHO, 1998, Dewey and Brown, 2003), WHO in 2008, developed a set of simple, valid, and reliable indicators to assess and compare IYCF practices among populations. These population-level indicators of IYCF are intended to: 1) make national/sub-national comparisons and describe trends over time; 2) identify populations at risk and make policy decisions; and 3) monitor progress in achieving goals, and evaluate the impact of interventions (WHO, 2008).

There are eight core indicators (three focused on BF and five on CF practices), and seven optional indicators. The three indicators on BF include: 1) early initiation of BF; 2) EBF under 6 months; and 3) continued BF at 1 year. The five indicators on CF include: 1) introduction of solid, semi-solid, or soft foods; 2) minimum dietary diversity (MDD); 3) minimum meal frequency (MMF), 4) minimum acceptable diet (MAD); and 5) consumption of iron-rich and iron-fortified foods (WHO, 2008). The MDD aims to assess micronutrient adequacy (i.e., diet quality), while MMF is used to assess the adequacy of the

energy intake in the diet (based on the quantity of food) (Working Group of Infant and Young Child Feeding Indicators, 2007, WHO, 2008, Ruel, 2017). The MAD represents a composite index based on both MDD and MMF designed to assess both the quality and quantity of complementary foods. The WHO has recommended that all five indicators on CF should be used together, although to date MDD is the only indicator that has been validated, and shown to reflect mean micronutrient adequacy of CF based on an extensive multi-country analysis (Ruel, 2003a, Ruel, 2003b, Arimond and Ruel, 2004, Ruel, 2017).

Data on these seven core indicators (excluding consumption of iron-rich and iron-fortified foods) from Demographic Health Surveys (DHS) conducted between 2002 and 2008 in 46 countries have been published by WHO (2010), providing baseline data at country level that allow a rapid overview and comparison across countries. The findings suggested that in general, CF practices were inadequate in most of the LICs, with <50% of breastfed children achieving MAD (range: 3.1% - 65.7%). Results on consumption of iron-rich or iron-fortified foods were not reported, as relevant data to calculate this indicator were not collected (WHO, 2010).

Data from Indonesia based on DHS 2007 indicated that 87.3% children aged 6-8 months were introduced to solid, semi-solid, or soft foods, and for children aged 6-23 months, 65%, 67%, and 42% achieved MDD, MMF, and MAD, respectively (WHO, 2010). However, the proportion achieving MDD, MMF, and MAD decreased to 48%, 62%, and 35%, respectively, when the analysis was performed only for infants aged 6-11 months (Ng et al., 2012). In the later DHS (2012) in Indonesia, the proportion in the younger age group (6-8 months) achieving MMF was higher (70%), although the proportions meeting MDD and MAD were lower (23% and 18%, respectively) (Blaney et al., 2015). Other studies conducted in Indonesia have not applied the WHO IYCF indicators (WHO, 2008), but instead have used 7 (Muslimatun and Wiradnyani, 2016) and 12 food groups (Mahmudiono et al., 2017) to calculate dietary diversity, so comparison across these studies cannot be made. However, overall, dietary

diversity among infants and children 6 to 23 months of age in Indonesia is considered low.

Despite their intended use at the population level, many researchers have applied these IYCF indicators to investigate the determinants of IYCF practices (Senarath et al., 2012, Senarath and Dibley, 2012) and explore associations between the five core CF indicators and nutritional status, growth (Arimond and Ruel, 2004, Marriott et al., 2012, Jones et al., 2014), health (Young and Krebs, 2013), and development outcomes (Gould, 2017, Larson et al., 2017, Ruel, 2017), especially among infants and young children in low- and middle-income countries (LMICs). The majority of these studies have reported a positive correlation between some CF practices and growth (Saha et al., 2008, Das et al., 2016), especially for dietary diversity and LAZ and weight-for-length z-score (WLZ) (Arimond and Ruel, 2004, Marriott et al., 2012, Jones et al., 2014, Mallard et al., 2014).

In general, reports on the consumption of iron-rich or iron-fortified foods are more limited compared to those in which the other four core CF indicators have been investigated. The main challenges of this CF indicator are associated with errors that may arise from the small amount of iron-rich/iron-fortified foods consumed and/or the fact that the responses given are based on actual intakes in the past 24 hours which do not reflect the habitual diets of the infants or young children (Ruel, 2017).

National data on recent CF practices in Indonesia are not available as the last national survey was conducted in 2012. An earlier literature review focusing on feeding practices among Indonesian infants and children aged 6-59 months from 1990 to 2010 reported that with the exception of the national surveys in 2007 and 2012, studies have been conducted in only 12 out of 33 provinces in Indonesia, and covered only 57 out of more than 500 districts/regencies (Blaney et al., 2015). Since 2010, none have covered the Sumedang districts, the settings for the research to be conducted here, although several small studies have been conducted on CF practices (Inayati et al., 2012a, Fahmida and Santika, 2016, Muslimatun and Wiradnyani, 2016).

### **2.3.2 Adequacy of energy and nutrient intakes from complementary foods in comparison with WHO estimated needs**

The WHO estimated energy requirements applied to calculate the estimated energy needs from complementary foods are based on the amount of energy needed for energy expenditure and to ensure optimal growth of healthy, well-nourished populations of infants and young children, stratified by age and gender (FAO/WHO/UNU, 2004). The estimated needs for vitamins and minerals are derived from the RNIs set by WHO/FAO (2004) with the exception of zinc, when the more recent RNI set by the International Zinc Nutrition Consultative Group (IZiNCG) is generally used (Brown et al., 2004).

These recommended energy (FAO/WHO/UNU, 2004) and nutrient (WHO/FAO, 2004) requirements are set for well-nourished and apparently-healthy populations of infants and young children; and are not appropriate for premature, small for gestational age, malnourished, or sick infants or young children. Appropriate diets for catch-up growth for such infants and young children must provide proportionally higher amounts of energy and nutrients (FAO/WHO/UNU, 2004), levels of which have been compiled by Golden (2009).

In general, studies assessing CF practices in Indonesia have reported inadequate intakes of energy and some nutrients from complementary foods. Iron, zinc, calcium, niacin, folate, and thiamine are the common problem nutrients in all three age groups (6 to 8, 9 to 11, and 12 to 23 months), especially in children from low- and middle socio-economic status (SES) households in both urban and rural areas of Indonesia (Santika et al., 2009, Fahmida et al., 2014, Fahmida and Santika, 2016). It appears that without the inclusion of fortified foods, even after inclusion of breast milk intake, it is difficult to close the gap between actual energy and nutrient intakes and the corresponding estimated needs from complementary foods (Gibson et al., 1998, Fahmida and Santika, 2016).

Unfortunately, many intervention studies in Indonesia during infancy and early childhood have been conducted without assessing the contribution of the

energy and nutrient intakes from complementary foods (Durnin et al., 2000, Dijkhuizen et al., 2001a, Lind et al., 2003, Wieringa et al., 2003, Wieringa et al., 2007), even though most of the studies assumed that the high proportion of multiple micronutrient deficiencies reported were caused by inadequate micronutrient intakes from complementary foods. In addition, these studies failed to take into account the magnitude of the gap when calculating the amount of micronutrient supplements needed. For example, although a study by Inayati et al. (2012b) measured 24-h dietary intakes of 6-59 months old children prior to the intervention, the investigators failed to adjust the dose of the supplements, resulting in intakes of iron, vitamin A, and folate that were above the Indonesian RNIs (Inayati et al., 2012b). In addition, those nutrients considered less problematic, notably niacin, folate, and thiamine, are often neglected, despite being of concern based on several Indonesian studies (Santika et al., 2009, Fahmida et al., 2014, Fahmida and Santika, 2016).

Although the use of the WHO estimated needs of energy and nutrients (except for zinc) are recommended, their application for Indonesian infants and young children has some limitations. Firstly, data on breast milk volume and composition for lactating women in Indonesia are limited. Instead, WHO uses data for breast milk volume and composition for the three age groups derived from average values from other high- and low income countries, excluding Indonesia (Dewey and Brown, 2003, Brown, 2007). However, recent data have emphasized marked variation in breast milk composition across countries (LASER Analytica, 2014). This is an important issue, as a different assumption about the nutrient composition of breast milk intake may lead to a different conclusion. Hence more accurate data on both breast milk volume and composition throughout lactation for rural women in Indonesia are urgently needed so that the calculation of the WHO estimated needs for each of the three age groups during infancy and early childhood can be adjusted accordingly.

A second limitation is that the current Indonesian Food Composition Table (FCT) has no data on phytate or zinc, and the data for folate are based on a method no longer considered accurate (*Departemen Kesehatan Republik*

Indonesia, 1995), which limits its usage. Phytate, found mainly in unrefined cereals grains, legumes, oil seeds, and nuts is a potent inhibitor of iron and zinc, and to a lesser extent calcium absorption. Therefore, one of the challenges of meeting the estimated needs for iron and zinc in predominantly plant-based complementary foods is related to their phytate content and corresponding molar ratios of phytate to iron and phytate to zinc (Gibson and Hotz, 2000, Gibson and Hotz, 2001a, Gibson et al., 2010, Dewey, 2013).

Given the necessity for phytate data, a study by Chan et al. (2007) analysed the phytate and mineral content of selected cereals and legumes habitually consumed as components of complementary diets in the eastern part of Indonesia. Based on their analysis, all the legumes, with the exception of tempe, fried tofu, and boiled long beans, had phytate: zinc molar ratios above the critical molar ratio of 18, whereas all the foods analysed, except noodles had phytate: iron molar ratios considerably higher than the critical molar ratio of 1. These critical molar ratios have the potential to inhibit zinc and non-haem iron absorption (Hurrell, 2004, Brown et al., 2004). Therefore, a more comprehensive investigation of the zinc and phytate content of Indonesian plant-based complementary foods is needed so that the quality of the Indonesian FCT can be improved.

## **2.4 Assessment of the nutritional adequacy of current IYCF practices by comparison with micronutrient biomarkers**

Globally, the most common nutritional problems (besides growth faltering) are anaemia and micronutrient deficiencies, especially vitamin A, iron, zinc, and iodine deficiency (Ramakrishnan, 2002, Bailey et al., 2015), although concerns with selenium (Mutakin et al., 2016) and vitamin D deficiencies (Sandjaja et al., 2013) are also emerging. In general, Indonesia also shares these same common nutritional problems based on the available evidence, although recently there has been some reduction in the prevalence of vitamin A deficiency in Indonesia (Sandjaja et al., 2013). However, inflammation may confound the interpretation of certain micronutrient biomarkers leading to incorrect prevalence estimates for some micronutrient deficiencies in Indonesia and elsewhere (Wieringa et al., 2002, Thurnham et al., 2003,

Thurnham et al., 2005, Thurnham et al., 2010, Thurnham et al., 2015, Larson et al., 2016, Suchdev et al., 2016).

Therefore, in this section the magnitude of anaemia and micronutrient deficiencies among infants and young children in Indonesia will be discussed with emphasis on the role of inflammation in generating misleading estimates of the prevalence of micronutrient deficiencies, namely for vitamin A, iron, zinc, and selenium. For available data in Indonesia, associations between nutrient adequacy based on intakes from CFs in relation to the WHO estimated needs and anaemia and micronutrient status will be examined, taking into account the multiple nutritional and non-nutritional aetiological factors associated with anaemia, when available.

However, evaluation of studies conducted in Indonesia in the past 20 years regarding this topic (Table 2.1) has revealed some concerns. Small sample sizes and self-selected recruitment strategies for participants who are not representative nationally have limited the generalisation of the results to the national level. In addition, studies have used different cut-offs to define deficiency states, especially for vitamin A and iron, making the results difficult to compare across studies. In addition, the effect of inflammation on prevalence estimates of deficiency has been considered only in some (Dijkhuizen et al., 2001b, Wieringa et al., 2002, Wieringa et al., 2004, Untoro et al., 2005, Sandjaja et al., 2013), but not all studies (Table 2.1). Moreover, frequently no details about whether the blood samples taken were fasting or non-fasting, time of the last meal before blood collection, and time of the blood collection are provided, even though all these factors are known to influence serum/plasma zinc concentrations (Brown et al., 2004).

#### **2.4.1 Prevalence of anaemia and micronutrient deficiencies in Indonesia**

Anaemia is a widespread problem in many LICs, and persists during early childhood and infancy. Almost 30% of preschool children in Indonesia are anaemic based on the latest national data (National Institute of Health Research and Development, 2013), a wide range exists among infancy,

varying from 22% to 82% depending on geographic region and age (Table 2.1).

Nutritional ID has been assumed to be the major cause of anaemia in Indonesia (Kodyat et al., 1998) based on findings that more than 20% of infants and young children have ID or IDA, irrespective of geographic region or age (Dijkhuizen et al., 2001a, Wieringa et al., 2002, Lind et al., 2003, Untoro et al., 2005). Despite Indonesia having both a national micronutrient fortification programme for wheat flour since 2003 and an iron/folic acid supplementation programme for pregnant women, in general there has been no significant decrease in the prevalence of anaemia in pregnant women and infants and young children (National Institute of Health Research and Development, 2012).

In contrast to ID, the prevalence of vitamin A deficiency among infants and young children has been reduced dramatically from more than 50% in early 2000 (Dijkhuizen et al., 2001b, Wieringa et al., 2002, Wieringa et al., 2003) to around 21% in 2005 (Untoro et al., 2005), to <2% in 2013 (Sandjaja et al., 2013). This dramatic fall is probably due to the introduction in 1978 of a national vitamin A capsule supplementation program together with improvements in both the coverage and compliance to the programme in the community. Thus, in general, the prevalence of vitamin A deficiency is no longer of public health concern among infants and young children in Indonesia, as it has fallen below the “trigger” level of 20% (WHO, 2011c).

The prevalence of zinc deficiency in recent studies (Wieringa et al., 2004, Untoro et al., 2005) was below the level (i.e., 20%) said to be of public health concern for infants and young children (Hotz, 2007). This trend may be associated with the high level of zinc fortificant (30 µg Zn/g) which has been added to wheat flour since 2003 (Kimura, 2013), combined with the level of zinc in the rice in Indonesia, which is high and comparable to that reported in Japan and China (Herawati et al., 2000). Until recently, there has been no zinc supplementation programme in Indonesia to prevent zinc deficiency among infants and young children.

**Table 2.1 Studies assessing anaemia/micronutrient status and outcomes after micronutrient interventions in under 5 year-old children in Indonesia (from year 1997 onwards)**

Authors, year	Province	Design, duration	Samples (age, n)	Groups (n)	Proportion		Inflammation adjustment	Outcomes
					Micronutrient deficiency	Anaemia/IDA		
Hadi et al., 1999	Central Java	RCT, double masked, 24 mos; Single dose every 4 mos	6-48 mos, n = 1,405	(n = 2): Vit A 206 000 IU (≥12 mos) or 103 000 IU (<12 mos) Placebo	Vit A <sup>b</sup> : 67% (n = ?)	NR	NR	Vit A suppl. significantly improved linear growth in those who had low intake of vit A (<400 RE/day); and no respiratory infection.
Beckett et al., 2000	West Java	RCT, single-blind, 12 mos; Twice daily, 6 days/wk	Cohorts: 12-mos, n = 53; 18-mos, n = 83	(n = 3) <sup>a</sup> : C.milk+iron S.milk+iron S.milk	NR	NR	NR	C.milk+iron group showed significantly greater weight gain at 2, 8, and 12 mos, esp. the females and younger cohort.
Jahari et al., 2000	West Java	RCT, single-blind, 12 mos; Twice daily, 6 days/wk	Cohorts: 12-mos, n = 53; 18-mos, n = 83	(n = 3) <sup>a</sup> : C.milk+iron S.milk+iron S.milk	NR	NR	NR	C.milk+iron group walked at an earlier age, had significantly higher Bayley scores and were more active.
Pollit et al., 2000b	West Java	RCT, single-blind, 12 mos; Twice daily, 6 days/wk	Cohorts: 12-mos, n = 53; 18-mos, n = 83	(n = 3) <sup>a</sup> : C.milk+iron S.milk+iron S.milk <sup>a</sup>	NR	NR	NR	C.milk+iron group had significantly higher Bayley scores and were more socially, cognitively, and emotionally mature.

Authors, year	Province	Design, duration	Samples (age, n)	Groups (n)	Proportion		Inflammation adjustment	Outcomes
					Micronutrient deficiency	Anaemia/IDA		
Harahap et al., 2000	West Java	RCT, single-blind, 12 mos; Twice daily, 6 days/wk	Anaemic, n = 18; Non-anaemic, n=18	(n = 3) <sup>a</sup> : C.milk+iron S.milk+iron S.milk	NR	NR	NR	Anaemic children showed significantly faster motor development and greater physical activity after intervention.
Dijkhuizen et al., 2001a	West Java	RCT, double-blind, 6 mos Daily	4 mos, n = 478	(n = 4) <sup>c</sup> : Iron Zinc Iron + zinc Placebo	Zinc <sup>d</sup> : 24% (n = 87)	Anaemia: 66%, IDA <sup>e</sup> : 30%, (n = 87)	None for the prevalence. CRP was used as a covariate.	Iron, zinc, iron+zinc suppl. significantly reduced the prevalence of anaemia, IDA, and zinc deficiency. No differences in growth among 4 groups.
Dijkhuizen et al., 2001b	West Java	Cross-sectional survey	2.4-10.5 mos, n = 155		Vit A <sup>b</sup> : 54%; Zinc <sup>d</sup> : 17% (n = 155)	Anaemia: 57%, IDA <sup>f</sup> : 20% (n = 155)	Children with elevated CRP and/or ferritin were excluded (n = 17).	
Semba et al., 2001	West Java	RCT, double-blind, 13.5 mos; Single dose at 6, 10, 14 wk of age	1.5 mos, n = 467	(n = 3): Vit A 7.5 mg RE Vit A15 mg RE Placebo	NR	NR	NR	Vit A suppl. (irrespective of dose) had no impact on linear or ponderal growth or infectious disease morbidity.

Authors, year	Province	Design, duration	Samples (age, n)	Groups (n)	Proportion		Inflammation adjustment	Outcomes
					Micronutrient deficiency	Anaemia/IDA		
Wieringa et al., 2002	West Java	RCT, double-blind, 6 mos; Daily	4 mos, n = 418	(n = 6) <sup>g</sup> : Iron Zinc $\beta$ -carotene Iron+zinc Zinc+ $\beta$ -carotene Placebo	Before adjustment: Vit A <sup>b</sup> : 58% (n = 256); Zinc <sup>d</sup> : 13% (n = 418); After adjustment: Vit A was overestimated by >16%.	Before adjustment: Anaemia: 50%; IDA <sup>e</sup> : 22% (n = 418); After adjustment: IDA was underestimated by >15%.	Adjusted with elevated CRP, ACT, AGP.	
de Pee et al., 2002	West/Central / East Java	Cross-sectional, multistage cluster	3-5 mos, n = 990		NR	Anaemia: 71% (n = 990)	NR	
Wieringa et al., 2003	West Java	RCT, double-blind, 6 months; Daily	4 mos, n = 387	(n = 6) <sup>g</sup> : Iron Zinc $\beta$ -carotene Iron+zinc Zinc+ $\beta$ -carotene Placebo	Vit A <sup>b</sup> : 53% Zinc <sup>d</sup> : 11% (n = 43).	Anaemia: 49%; IDA <sup>f</sup> : 29% (n = 43).	NR	Iron group had significantly lower plasma retinol and a significantly higher prevalence of vit A deficiency.
Lind et al., 2003	Central Java	RCT, double-blind, 6 mos; Daily	6 mos, n = 680	(n = 4) <sup>c</sup> : Iron Zinc Iron+zinc Placebo	ID <sup>h</sup> : 15%; Zinc <sup>d</sup> : 78% (n = 549).	Anaemia: 41%; IDA <sup>e</sup> : 8% (n = 549).	NR	Iron group had significantly higher Hb and serum ferritin compared with iron+zinc group. Zinc group had significantly higher serum zinc compared with placebo group.

Authors, year	Province	Design, duration	Samples (age, n)	Groups (n)	Proportion		Inflammation adjustment	Outcomes
					Micronutrient deficiency	Anaemia/IDA		
Lind et al., 2004	Central Java	RCT, double-blind, 6 mos; Daily	6 mos, n = 680	(n = 4) <sup>c</sup> : Iron Zinc Iron+zinc Placebo	NR	NR	NR	Zinc suppl. significantly improved growth, and iron suppl. significantly improved growth and psychomotor development, but iron+zinc had no effect on growth or development.
Lind et al., 2008	Central Java	RCT, double-blind, 6 mos; Daily	6 mos, Iron-suppl.: iron replete (IR), n = 80; non-IR, n = 220; Non-iron-suppl.: IR, n = 74 non-IR, n = 232	(n = 2) <sup>c</sup> : Iron suppl.: - Iron - Zinc Non-iron-suppl.: - Iron + zinc - Placebo	NR	NR	NR	Iron suppl. of non-IR infants increased Hb and serum ferritin, but did not affect serum zinc or WAZ. Iron suppl. of IR infants significantly reduced WAZ. No difference in morbidity between groups.
Pangaribuan et al., 2003	Central Java	Prospective cohort study, 4 mos	1-5 ys, n = 400	(n = 2): Recipients of vit A No-recipients	Semi-urban: - ID <sup>h</sup> : 20%; - Vit A <sup>b</sup> : 27% (n = 148); Rural: - ID <sup>h</sup> : 2%; - Vit A <sup>b</sup> : 22% (n = 157).	Semi-urban: - Anaemia: 30% (n = 148); Rural: - Anaemia: 22% (n = 157).	None for the prevalence. CRP and AGP were used as covariates.	Vit A suppl. significantly reduced the proportion of children with a low concentration of retinol.

Authors, year	Province	Design, duration	Samples (age, n)	Groups (n)	Proportion		Inflammation adjustment	Outcomes
					Micronutrient deficiency	Anaemia/IDA		
Sankaranarayanan et al., 2004	Central Java	RCT, double-blind, 6 months; Daily	6-12 mos, Without inflammation, n = 44; With inflammation, n = 46	(n = 3): Iron Iron + MMN Placebo	Without inflammation: - Zinc <sup>d</sup> : 51%; With inflammation: - Zinc <sup>d</sup> : 69%.	Without inflammation: - IDA <sup>f</sup> : 37%; With inflammation: - IDA <sup>f</sup> : 31%.	Categorisation.	Ferritin increased significantly in infants administered iron alone independently of inflammation status at baseline or at the end of the study. No effect on morbidity.
Wieringa et al., 2004	West Java	Cross sectional study	3-10 mos, n = 60		Vit A <sup>b</sup> : 48%; Zinc <sup>c</sup> : 17% (n = 52).	Anaemia: 44%; IDA <sup>f</sup> : 17% (n = 52).	Children with elevated CRP were excluded (n = 7).	
Untoro et al., 2005	Central Java	RCT, double-blind, 6 mos	6-12 mos, n = 284	(n = 4): Daily iron Daily iron+MMN, Weekly MMN Placebo	ID <sup>h</sup> : 34%; Vit A <sup>b</sup> : 21%; Zinc <sup>d</sup> : 11% (n = 260).	Anaemia: 58% (n = 260).	Children with elevated CRP were excluded (n = 15).	Daily iron and daily iron+MMN significantly increased Hb and plasma ferritin. No significant differences in plasma zinc, retinol, growth, and morbidity among 4 groups.
Howard et al., 2007	7 provinces	Nutritional surveillance, multistage cluster	6-59 mos, n = 85,229		NR	Anaemia: 56% (n = 85,229).	NR	
Fahmida et al., 2007	West Nusa Tenggara	RCT, double-blind, 6 mos; Daily	3-6 mos, n = 800; subset for biochemical, n = 212	(n = 4) <sup>k</sup> : Zinc, Zinc+iron, Zinc+iron+vit A, Placebo	NR	Anaemia: 82% (n = 610)	NR	Zinc+iron and zinc+iron+vit A improved zinc and iron status, while zinc alone decreased Hb and iron status. Linear growth was improved among initially-

Authors, year	Province	Design, duration	Samples (age, n)	Groups (n)	Proportion		Inflammation adjustment	Outcomes
					Micronutrient deficiency	Anaemia/IDA		
Semba et al., 2008	Urban slum areas	Nutritional surveillance, multistage cluster	6-59 mos, n = 32,873		NR	Anaemia: 59% (n = 32,873).	NR	stunted subjects in zinc+iron and zinc+iron+vif.A groups.  Weight gain (g/kg body weight/day) was highest in INE+MMN, followed by INE group. In both MMN intervention groups (INE+MMN, non-INE+MMN), supplements significantly increased Hb. Weight gain (g/kg body weight/day) was higher in daily RUF compared to weekly RUF, and they achieved significantly higher WHZ.
Inayati et al., 2012b	North Sumatra	Quasi experimental; Weekly INE, Monthly non-INE, Daily MMN	6-59 mos, WHZ $\geq -1.5$ to $< -1.0$ , n = 251	(n = 4): Intensive nutrition education (INE) INE+MMN non-INE non-INE+MMN	NR	Anaemia: 61% (n = 180).	NR	
Purwestri et al., 2012	North Sumatra	Quasi experimental	6-59 mos, WHZ $\geq -2$ to $< -1.5$ , n = 99	(n = 2): Daily ready-to-use food (RUF) Weekly RUF	NR	NR	NR	
Sandjaja et al., 2015	West Java	Cross-sectional study, poor households, random sample	6-11 mos, n = 343; 12-59 mos, n = 491		6-11 mos: - Vit A <sup>b</sup> : 18% (n = 318); 12-59 mos: - Vit A <sup>b</sup> : 10-15% (n = 469)	NR	NR	

Authors, year	Province	Design, duration	Samples (age, n)	Groups (n)	Proportion		Inflammation adjustment	Outcomes
					Micronutrient deficiency	Anaemia/IDA		
Sandjaja et al., 2013	National	Cross-sectional study, multistage cluster	0.5-1.9 ys, urban (n = 1142) & rural (n = 1249); 2.0-4.9 ys, urban (n = 959) & rural (n = 1089)		2.0-4.9 ys: Vit A <sup>b</sup> : urban 0%, rural 2%; ID <sup>h</sup> : urban 10%, rural 15%; Vit D (<50 nmol/L): urban 35%, rural 43%	0.5-1.9 ys, Anaemia: urban 54%, rural 57%; 2.0-4.9 ys, Anaemia: urban 11%, rural 17%	Children with elevated CRP and/or AGP were excluded (n = ?).	
Barkley et al., 2015	13 provinces	Indonesia Family Life Survey	0-59 mos: year 1997/8, n = 1,867 year 2000, n = 2,346 year 2007/8, n = 2,963		NR	Anaemia: 1997/8: 46% 2000: 55% 2007/8: 31%	NR	

Note: NR, not reported; RE, retinol equivalent; APP, acute phase response; A, anaemia; IDA, iron deficiency anaemia; ID, iron deficiency; CRP, C-reactive protein; ACT,  $\alpha$ 1-antichymotrypsin; AGP,  $\alpha$ 1-acid glycoprotein; WHZ, weight-for-height z-score  
MMN, multimicronutrients (all include zinc); C.milk, condensed milk; S.milk, skimmed milk

<sup>a</sup>E: condensed milk + iron (1171 kJ + 12 mg iron), M: skimmed milk + iron (209 kJ + 12 mg iron), S: skimmed milk + placebo (104 kJ)

<sup>b</sup>Serum/plasma retinol <0.70  $\mu$ mol/L

<sup>c</sup>Iron (10 mg/d), zinc (10 mg/d), iron + zinc (10 mg/d each)

<sup>d</sup>Serum/plasma zinc <10.7  $\mu$ mol/L

<sup>e</sup>Hb <110 g/L & plasma ferritin <12  $\mu$ g/L

<sup>f</sup>Hb <110 g/L & plasma ferritin <15  $\mu$ g/L

<sup>g</sup>Iron (10 mg/d), zinc (10 mg/d),  $\beta$ -carotene (2.4 mg/d), iron + zinc (10 mg/d each), zinc +  $\beta$ -carotene (10 & 2.4 mg/d)

<sup>h</sup>Serum/plasma ferritin <12  $\mu$ g/L

<sup>i</sup>Iron (10 mg/d), daily iron + MMN

<sup>j</sup>Iron (10 mg/d), daily iron + MMN, weekly MMN (twice dose)

<sup>k</sup>Zinc (10 mg/d), zinc + iron (10 mg/d each), zinc + iron + vit A (10 mg/d each + 1,000 IU vit A)

<sup>l</sup>Serum/plasma zinc <9.95  $\mu$ mol/L

Vitamin D deficiency among infants and young children has not been widely explored in Indonesia. The first study that measured a biomarker of vitamin D status (serum 25-dihydroxyvitamin D) was conducted in 2013 (Sandjaja, 2013), and reported a prevalence of deficiency of 35% and 43% among children aged 2.0-4.9 years living in urban and rural areas, respectively, based on a cut-off point of <50 nmol/L. Selenium deficiency among infants and young children in Indonesia has gained even less attention and to my knowledge no prevalence data has been reported.

#### **2.4.2 Effect of inflammation on selected micronutrient biomarkers**

Micronutrient biomarkers in serum are generally used to assess the status of four micronutrients: iron, zinc, and vitamin A, and selenium. However, the presence of inflammation or infection may confound their assessment (Brown et al., 1993, Brown, 1998, Thurnham et al., 2003, Raiten et al., 2015). Consequently, the resulting prevalence estimates of deficiency may not reflect the true burden unless inflammation or infection has been taken into account, as noted earlier.

Many of the studies in Indonesia (Table 2.1) have not taken into account the existence of inflammation as a possible confounding variable when measuring these micronutrient biomarkers during infancy and early childhood (Hadi et al., 1999, Lind et al., 2003, National Institute of Health Research and Development, 2012, Sandjaja et al., 2015). This is unfortunate because during the frequent episodes of inflammation that often occur during infancy and early childhood, pro-inflammatory cytokines stimulate the hepatic synthesis of acute phase proteins (APPs), some of which result in elevated concentrations of certain micronutrients in serum (e.g ferritin, a positive APP) and others decrease (e.g. retinol and RBP, zinc, and selenoprotein P, negative APPs). The fall in retinol or RBP arises from a decrease in hepatic synthesis of RBP mRNA, which interrupts the release of retinol-RBP from the liver, lowering serum RBP concentrations (Rosales et al., 1996, Stephensen, 2001). Whereas the decrease in plasma zinc and possibly selenium concentrations arise from redistribution of zinc and selenium from the circulating plasma to the sites of

inflammation (Galloway et al., 2000, King et al., 2016), although such a reduction in plasma selenium has not been consistent.

As a consequence, during the acute phase response, the increase in serum ferritin concentrations independent of iron status results in an under-estimate of the prevalence of ID (Galloway et al., 2000, Tomkins, 2003, Northrop-Clewes, 2008), whereas any decreases in serum retinol, RBP, zinc, or selenoprotein P (reflected by plasma selenium) will result in an over-estimate of the corresponding prevalence of micronutrient deficiency, irrespective of their true burden of deficiency, when no adjustment for inflammation or infection is performed. To correct for the effect of inflammation on selected micronutrient biomarkers, WHO (2014) recommends two inflammatory biomarkers, CRP and AGP, because the magnitude of these two inflammatory biomarkers provides an assessment of both the severity and duration of inflammation (Raiten et al., 2015).

Some studies in Indonesia have excluded children with elevated CRP and/or AGP from their statistical analysis (Dijkhuizen et al., 2001b, Wieringa et al., 2004, Untoro et al., 2005, Sandjaja et al., 2013). However, this practice may reduce the sample size and bias the results, as reported in other studies (Grant et al., 2012, Larson et al., 2016), especially in areas such as Indonesia where inflammation/infection persist throughout the whole year. In these areas, conducting the survey in a season with low rates of inflammation, as suggested by WHO (2011b), is not feasible because the prevalence of inflammation tends to be reasonably constant.

To avoid the problems mentioned above, several approaches have been developed to adjust certain biomarkers for the effect of inflammation. The most common approaches include the use of internal (Thurnham et al., 2010, Thurnham et al., 2015) or external correction factors (Thurnham et al., 2003, Thurnham et al., 2010). Recently, the BRINDA regression approach has been developed and used specifically for adjusting serum RBP and ferritin (Stoltzfus and Klemm, 2017; Larson et al., 2017; Larson et al., 2016, Suchdev et al., 2016). More recently, the effect of inflammation has also been investigated for adjusting serum zinc (Karakochuk et al., 2017). Without such adjustments,

prevalence estimates for these micronutrient deficiencies will be incorrect in settings with a high burden of inflammation, as noted earlier. ID may be grossly under-estimated, and vitamin A and zinc deficiency over-estimated, leading to false public health priorities and for some nutrients, needless implementation of programmes. Whether plasma selenium, considered as a negative acute-phase reactant (Maehira et al., 2002, Sattar et al., 1997), should be adjusted for inflammation requires more investigation, as to date the use of this approach for this biomarker has not been conducted.

#### **2.4.3 Role of co-existing nutritional and non-nutritional factors on micronutrient status of infants and young children**

There has been very limited investigation in Indonesia examining the role of both nutritional and non-nutritional factors on micronutrient status during the CF period. Non-nutritional factors may include parasitic infections, inflammation, the presence of environmental enteropathy, genetic Hb disorders, and gender, each of which have the potential to exacerbate one or more micronutrient deficiency states arising from nutrient inadequacies in the complementary foods (Thurlow et al., 2005, Lander et al., 2008).

In addition to iron, deficiencies of several other micronutrients have been associated with low Hb concentrations and anaemia during the CF period (Fishman et al., 2000). Of these, most notable are folate, vitamin B12, and vitamin A deficiency (Kraemer and Zimmerman, 2007), although emerging evidence suggests that zinc, selenium, and vitamin D may also have a role in Hb concentrations through several plausible mechanisms (Lander et al., 2008, Houghton et al., 2016). For example, deficiency of either folate or vitamin B-12 impairs the production of 5,10-methylene tetrahydrofolate which is essential for the synthesis of DNA (Scott, 2007). Interference with DNA synthesis leads to a failure of the red cell precursors to divide normally (Bailey and Gregory, 1999), resulting in megaloblastic anaemia. In terms of vitamin A, improvements in Hb and anaemia status have been reported among vitamin A deficient-infants receiving a vitamin A supplement (Zimmermann et al., 2006, Fahmida et al., 2007). Three mechanisms whereby vitamin A deficiency may affect Hb have been proposed, including lowering resistance to

infection, and consequently inducing secondary anaemia due to infection; altering absorption, storage, release or transport of iron to the bone marrow; and/or a direct effect of vitamin A on modulating erythropoiesis (Bloem, 1995, Semba and Bloem, 2002, Zimmermann et al., 2006, Michelazzo et al., 2013).

Some studies have also shown that zinc status is a strong predictor of Hb (Gibson et al., 2008, Houghton et al., 2016). Plausible mechanisms include the activity of several zinc-dependent enzyme systems in HB synthesis (Prasad, 1985), the role of zinc in stabilizing red cell membranes (O'Dell, 2000), and in stimulating erythropoiesis through its association with plasma insulin-like growth factor-I (IGF-I). Together with erythropoietin, IGF-I is an important factor that stimulates haematopoiesis (Muta et al., 1993, Vihervuori et al., 1996). Moreover, zinc deficiency significantly decreased expression of IGF-I (Ninh et al., 1995). In a study of 38 anaemic women in the second trimester of pregnancy, who were divided into 3 groups (receiving supplements of iron, zinc, or iron + zinc for 8 weeks) significant positive correlations with IGF-I and Hb and red blood cell (RBC) were reported in the two groups receiving either zinc alone or iron + zinc supplements (Nishiyama et al., 1999).

A link between vitamin D status, ID, and iron deficiency anaemia (IDA) has been observed among young Asian children (Yoon et al., 2012, Jin et al., 2013, Sharma et al., 2015) and between vitamin D deficiency and low Hb levels in healthy adults (Sim et al., 2010, Lee et al., 2015). The mechanism is uncertain but may be due to the direct stimulation by vitamin D of erythroid precursors in the bone marrow (Yoon et al., 2012) and/or an interaction between 1,25-dihydroxvitamin D<sub>3</sub> and myeloid zinc finger-1 which may also play role in haematopoiesis and myeloid cell differentiation (Piszczałowski et al., 2014).

More recently, a positive association between selenium concentrations and Hb has been reported among children in Brazil and Vietnam (Nhien et al., 2008, Lander et al., 2014), although to date no investigation has been undertaken in Indonesia. The precise mechanism is uncertain but selenium may act as a potent antioxidant in erythrocytes (Chow and Chen, 1980) as

well as having a role in optimal immune function (McKenzie et al., 1998, Mattmiller et al., 2013).

Of the non-nutritional factors, exposure to infective eggs of soil-transmitted helminths is likely when infants begin to crawl in and around the home and eat solid foods. Some intestinal parasitic infections (e.g., the hookworm and *Trichuris trichiura*) induce anaemia through blood loss, whereas for others (e.g., *Ascaris lumbricoides* and *Giardia intestinalis*) malabsorption of micronutrients is a major factor contributing to micronutrient deficiencies and in some cases anaemia (Crompton, 1999, Hotez et al., 2006, Balarajan et al., 2011). In some rural settings in Indonesia where access to an improved sanitation system is limited, intestinal parasitic infections such as *Trichuris trichiura*, *Necator americanus*, and *Ascaris lumbricoides* may also lower Hb (Nurdia et al., 2001), although whether they have such a role during infancy is less certain.

An additional factor with the potential to exacerbate low Hb concentrations is the inflammatory response induced by parasitic and other infections when pro-inflammatory cytokines stimulate an increase in circulating hepcidin levels, reducing iron absorption independent of iron status (Ganz, 2015). As a consequence, functional ID develops, and subsequently, possibly IDA.

Indonesia is situated in Southeast Asia, a region with a high prevalence of genetic Hb disorders that affect the structure, function and/or production of Hb (Fucharoen and Winichagoon, 2011). Of the genetic Hb disorders known to occur in many Asian countries, the carrier frequency in Indonesia for  $\beta$ -thalassemia, Hb E, and  $\alpha$ -thalassemia reportedly ranges from 5–10%, 1–33%, and 6–16% respectively, depending on the ethnic population (Ariani et al., 2017). Some of these genetic Hb disorders present as mild to severe anaemia (Fucharoen and Winichagoon, 2011, Williams and Weatherall, 2012), although the extent to which they contribute to low Hb concentrations in Indonesia in later infancy is not well established.

Several studies have reported a positive relationship between female sex and concentrations of Hb and/or ferritin in Indonesia (Wieringa et al., 2007) and elsewhere (Soh et al., 2004, Foote et al., 2013). Differences in growth rate may account for these sex-related trends in Hb and ferritin. Female infants usually have lower body weights at each age (Williams and Weatherall, 2012), and thus potentially lower iron requirements than males, as postulated in an earlier study conducted in the second half of infancy in Southeast Asia where males were at higher risk of ID (Wieringa et al., 2007).

## **2.5 Assessment of the nutritional adequacy of current IYCF practices in relation to functional outcomes**

The transition from EBF to CF plus BF is a very vulnerable period during infancy which can lead to nutritional deficiencies that can have far reaching consequences on the growth, development, and health of the children (Netting and Makrides, 2017). There is growing evidence that inadequate intakes of nutrients during CF can be associated with negative impacts on health. This period is the time of peak incidence of growth faltering, micronutrient deficiencies, and infectious diseases, especially in LICs (Dewey and Adu-Afarwuah, 2008). Some but not all studies investigating the adequacy of energy and nutrient intakes during CF have reported positive relationships with selected functional outcomes, notably growth, appetite, cognitive and motor development, and morbidity; these are discussed below.

### **2.5.1 Ponderal and linear growth**

In LMICs, the nutritional adequacy of CF has focused primarily on the prevention of growth faltering. In these settings, the most common problems compromising the nutritional adequacy of complementary foods are delayed CF, inadequacies in the amount of complementary food consumed arising from mothers/caregivers offering too little food, or poor appetite, and/or providing foods with a low energy and nutrient density (Gibson, Hotz, and Perlas, 2004, Dewey, 2016, Makrides, 2017). Indeed, positive associations between the inadequate consumption of good quality complementary foods and growth faltering has been reported in some studies in Indonesia (Schmidt

et al., 2002, Sekiyama et al., 2012) as well as elsewhere in Asia (Adair and Guilkey, 1997, Christian et al., 2015).

Several reviews have been conducted in the past 10 years to explore the impact of CF interventions on ponderal and linear growth of children <2 years old in LMICs (Walker and Black, 2007, Dewey and Adu-Afarwuah, 2008, Lassi et al., 2013, Heidkamp, 2017) and for children 3 months – 5 years old in broader settings (Kristjansson et al., 2015). The average effect size on length of these efficacy studies has been small, ranging from 0.12 SD (range -0.02, 0.45) (Dewey and Adu-Afarwuah, 2008) to 0.21 SD (0.01, 0.41) (Imdad et al., 2011), and 0.23 SD (-0.00, 0.45) (Lassi et al., 2013). A comparable small average effect size has also been reported for weight, with a range 0.26 to 0.35 in the three review papers cited above.

The only study showing a significant impact on length and weight was conducted on young Indian children consuming micronutrient fortified compared to unfortified cow's milk (Sazawal et al., 2010). In the other studies in which only small improvements in length and weight have been reported, the interventions have supplied complementary food or a food product with extra energy with added micronutrients such as lipid-based supplements (Dewey and Arimond, 2012, Arimond et al., 2015, Mangani et al., 2015). Meta-analysis from nine randomized controlled trials in LMICs showed evidence (classified as moderate), for a positive effect of food supplementation on linear growth, with an average increase of 0.27 cm over 6 months compared with those who were not supplemented (95% CI 0.07 to 0.48, 1463 participants) (Kristjansson et al., 2015).

However, comparison across these studies is difficult because of the differences in the methods used to enhance the adequacy of the complementary diets, including their nutrient composition, and level and form of micronutrient fortificant doses (Imdad et al., 2011, Lassi et al., 2013, Kristjansson et al., 2015, Heidkamp, 2017). In some cases, processed fortified complementary foods, fortified cow's milk, or home fortification using micronutrient powders, have been used as acceptable methods of delivering

those micronutrients known to be at risk in the complementary foods, with no differences in energy provided to the intervention and control groups (Imdad et al., 2011, Lassi et al., 2013, Heidkamp, 2017). Place of provision of the food supplementations may be an additional factor for the lack of positive effect on health. When supplementary feeding has been given at home, food was often distributed to other family members. Therefore, children given food at home benefited from only 36% of the energy in the supplement, compared with 85% when it was given at day-care/feeding centers (Kristjansson et al., 2015).

All reviews have shown the potential of CF interventions to improve the nutritional status of children in LMICs (Dewey and Adu-Afarwuah, 2008, Lassi et al., 2013, Kristjansson et al., 2015, Heidkamp, 2017). However, in general, the reviews conclude that there is no single universal intervention because the various interventions for CF to improve growth opportunities and constraints across settings and countries make generalisation almost impossible. For example, the responses to interventions are most likely also affected by pre-natal factors, as well as characteristics of the target population (age, degree of nutritional deficiency, food security), infection, appetite among the infants, together with the quality of the intervention (Dewey and Adu-Afarwuah, 2008, Lassi et al., 2013, Heidkamp, 2017).

Therefore, a more critical evaluation of the studies on CF in Indonesia is required. Mixed results on linear growth have been reported for the few Indonesian intervention studies in which selected daily micronutrient supplements have been provided to infants aged 3-6 months for 6 months. For example, a randomised controlled trial of infants who were supplemented with iron and zinc showed no improvement in linear or ponderal growth (Dijkhuizen et al., 2001a), whereas in another study in which iron+zinc and iron+zinc+vitamin A supplements were provided, a positive effect on linear growth was reported in all supplemented groups compared with placebo (Fahmida et al., 2007). In addition, a study which provided iron and zinc supplementation for Indonesian infants, also for 6 months, showed that single supplementation of iron or zinc significantly improved growth, but combined

iron and zinc showed no effect when compared with placebo (Lind et al., 2004). It is possible that such discrepancies on the effects on growth may be associated with lower LAZ and WAZ at baseline. Certainly, other investigators have reported a larger effect among more stunted/wasted children at baseline (Brown, 1998, Umeta et al., 2000, Dewey and Adu-Afarwuah, 2008).

Another factor that must be considered is the selection of the micronutrients for the intervention, and whether they are nutrients that have a direct and/or an indirect role on linear growth. The indirect nutrients affect growth through their effects on appetite, immune competence, and physiological function. Protein, calcium, phosphorous, and iodine have been suggested to have a direct role on growth, iron and riboflavin have indirect roles, whereas zinc and vitamin A have both important direct and indirect roles on growth (Gibson and Hotz, 2001b).

Zinc has been postulated as an essential micronutrient to promote normal growth, as it has critical roles in multiple metabolic pathways, including DNA transcription and gene expression, signal transduction pathways, and endocrine function (King et al., 2016). Therefore, zinc deficiency has been suggested to contribute to impaired growth among infants and young children in LICs (Walker and Black, 2007). However, more research is needed to examine the magnitude of the effect of interventions using micronutrients on growth, especially in Indonesia where previous studies have only investigated the effect of iron, zinc, and vitamin A on growth.

### **2.5.2 Poor appetite**

Inadequate intakes of some micronutrients, namely iron and zinc, may have indirect impacts on growth, induced in part by impairing appetite, which in turn results in a reduction in energy intake, especially from non-breast milk-sources (Prasad, 1985, Brown et al., 1995, Shay and Mangian, 2000, Gibson and Hotz, 2001b). The mechanisms involved have been explored through both animal and human studies, but to date uncertainties remain (Topaloglu et al., 2001, Akarsu et al., 2007).

Human iron supplementation studies have shown improvements in appetite together with an improvement in iron status (Lawless et al., 1994, Kanani and Poojara, 2000, Topaloglu et al., 2001, Stoltzfus et al., 2004). For example, in a 12-month iron supplementation study of 538 children aged 6-59 months in Zanzibar with or without mebendazole, improvements in appetite in both groups were reported (Stoltzfus et al., 2004). Another study, in which iron supplements were given to 24 children aged 8-36 months for 15 weeks, showed an improvement in appetite in the iron supplemented group, but failed to demonstrate an association between leptin level and appetite. These findings suggest that another mechanism may play a role to improve appetite (Topaloglu et al., 2001). Other investigators have explored the role of ghrelin in children as lower ghrelin levels in children with IDA can lead to loss of appetite and a reduced desire to eat diverse foods (Akarsu et al., 2007). Further, a significant positive correlation between iron status and levels of ghrelin (Isguven et al., 2007, Dogan et al., 2013) has been reported. Nevertheless, despite these studies, evidence that iron specifically influences food intake/appetite in humans, especially among infants and young children, is still uncertain.

Some studies have shown a relationship between zinc deficiency and poor appetite. Zinc may stimulate dietary intake, possibly by affecting taste acuity (Hambidge et al., 1972a, Gibson, 2005) or influencing hormonal or neuroendocrine transmitters that affect appetite, such as leptin, ghrelin, and insulin (Shay and Mangian, 2000, Arsenault et al., 2007, Breij et al., 2016).

Studies among adults who have zinc deficiency have reported that zinc supplementation increased circulating leptin levels and improved appetite (Mantzoros et al., 1998, Chen et al., 2000), but results among children have been inconsistent. For example, poor appetite and impaired taste acuity in 7 out of 10 children aged 4-16 years were associated with low hair zinc concentrations. Following zinc supplementation, taste acuity was improved (Hambidge et al., 1972b). In a 12 week zinc supplementation study of 300 preschool children in Iran, improvements in appetite as indicated by greater intakes of energy, carbohydrate, protein, and fat were reported in those

receiving the zinc supplement compared to the placebo (Khademian et al., 2014). In contrast, in a zinc supplementation study of 142 children (aged 6-8 months) in northern Peru, no effect on dietary intakes, or on concentrations of plasma leptin, ghrelin, or linear growth was reported in the group receiving 3 mg Zn/day for 6 months compared to the placebo (Arsenault et al., 2007). Differences in baseline plasma zinc concentrations indicative of the absence of zinc deficiency, and the smaller zinc supplementation dosage in the study in northern Peru may be some of the reasons for such discrepancies. In addition, the lack of a more sensitive measure to assess human zinc deficiency than plasma/serum zinc may also contribute to the inconsistent results (King et al., 2016).

### **2.5.3 Cognitive and motor development**

The first two years of life is also a crucial time for brain development. A high energy intake promotes creation of new synapses; and helps to maintain the established synapses and myelination, which are important for both cognitive and motor development (Gould, 2017). In addition to macronutrients (Grantham-McGregor and Baker-Henningham, 2005), other key nutrients needed for infant development include iron (Walter, 2003, Ayala et al., 2008, Madan et al., 2011, Algarin et al., 2017), iodine (Bougma et al., 2013, Rohner et al., 2014), and zinc (Black et al., 2004a, King et al., 2016).

To date, most of the available evidence has focussed on iron and development. Positive correlations between ID/IDA and impaired cognitive and motor development in children have been reported (Grantham-McGregor and Ani, 2001, Lozoff, 2007). For example, an early intervention study on 24 children with IDA (aged 9-26 months) reported higher scores for the Bayley Mental Developmental Scores in those children receiving intermuscular iron for 6.8 days on average compared with the control group (Osiki and Honig, 1978). Numerous intervention studies on the effect of ID on cognition have been reported in the last few decades in humans (Osendarp et al., 2010, Madan et al., 2011, Lozoff, 2011). Some of intervention studies have hypothesised that developmental alterations caused by ID are rapidly reversible with iron therapy (Osiki and Honig, 1978, Idjradinata and Pollitt,

1993). However, a recent Cochrane review has shown no convincing evidence of positive effects of iron treatment on cognitive and motoric development for children <3 years old within 30 days after treatment; and the effects of longer treatment remain unclear (Wang et al., 2013). In addition, another review has concluded that ID has long-lasting cognitive and motor effects (Lozoff et al., 2006). For example, a follow-up study undertaken 10 years after an iron intervention showed that 48 children (11-14 years) who had been treated for severe ID in infancy still had lower achievements in arithmetic, writing, and motor function compared with the 114 children who had adequate iron status in the initial intervention during infancy (Lozoff et al., 2000).

Although the exact mechanism for the role of iron in development has not been clearly defined because of the complexity of the neuro-behavioural system (Lozoff et al., 2006, Hermoso et al., 2011), iron has been suggested to play an important role in dopamine system functioning. ID has also altered the function of other neurotransmitters, as well as myelination, dendritogenesis, neurometabolism in the hippocampus and striatum, gene and protein profiles, which together have been associated with cognitive and motor development (Lozoff, 2011). However, the dopaminergic alterations vary with timing and severity of ID (Beard, 1999, Beard and Connor, 2003, Beard, Wiesinger, and Connor, 2003, Lozoff et al., 2006), with the associated risks being higher in infancy, highlighting the need to prevent ID at this time, and to find interventions which have the capacity to lessen the long-term adverse effects (Lozoff, 2011).

Iodine has also been recognised to play an important role in both cognitive and motor development (de Escobar et al., 2004, Zimmermann, 2012, Rohner et al., 2014). The first trimester of pregnancy is the most critical period for brain development as T3 (the active hormone interacting with nuclear thyroid receptors) is produced by the fetus from maternal T4. During this period fundamental processes occur in the development of the fetal central nervous system (Andersson et al., 2007, Zimmermann, 2012). In addition to the fetal stage, iodine deficiency leading to low concentrations of thyroid hormone

during early infancy, are also associated with irreversible brain damage, including mental retardation and neurologic abnormalities (de Escobar et al., 2004). The key factors influencing the magnitude of the neurologic complications are the timing and severity of the iodine deficiency (Rohner et al., 2014).

A systematic review indicates that iodine repletion in moderately iodine-deficient school-age children has clear benefits of improving cognitive and motor functions (Zimmermann, 2007). A more recent systematic review and meta-analysis also show a substantial impact of iodine deficiency on cognitive development, with children under 5 years with moderate iodine deficiency (based on different indicators of iodine: urinary iodine excretion, T3, T4 TSH, TPOAb-, Tg, TBG, BII, TI, PBI, BEI) had lower IQ points (6.9 to 10.2) compared with iodine replete children (Bougma et al., 2013). However, long-term data on the effect of early iodine supplementation on infant development is still limited (Zimmermann, 2012). Impact of the intervention may be stronger if the intervention takes place earlier when the brain as well as language and cognition develop rapidly and would therefore be more affected by iodine deficiency (Bougma et al., 2013).

With the exception of iron and iodine, a limited number of randomized controlled trials have investigated whether a causal relationship exists between deficiencies of other key nutrients during the CF period and cognitive and motor development outcomes later in life (Gould, 2017). Results of zinc supplementation trials among children designed to assess cognitive development have been mixed, and overall have not shown a significant effect (Hamadani et al., 2001, Black et al., 2004b, Locks et al., 2017).

Studies of the effect of macronutrients on cognitive and motor development during infancy are also limited, mostly because the earlier studies used a combination of macronutrients and micronutrients, which make it difficult to distinguish the effect of each nutrient on cognitive and motor functions (Grantham-McGregor and Baker-Henningham, 2005, Mennella et al., 2016). A recent meta-analysis of nutrition interventions on mental development of

children under two in LMICs has shown a small effect. Postnatal supplements consisting of multiple micronutrients yielded an effect size (95% CI) of 0.082 (-0.012, 0.18) and single micronutrients 0.058 (-0.0015, 0.12), suggesting that multiple micronutrients tend to have greater impact on cognitive development (Larson and Yousafzai, 2017).

Previous reviews of the efficacy of CF interventions in LICs have located only 4 studies that included data on motor development, of which only 3 were conducted during the first two years of life. A lipid-based micronutrient fortified food or micronutrients alone both improved gross motor development in young children in Ghana. In this study, children who received the micronutrient treatments were more likely to walk independently by 12 months compared with children in the non-intervention group (Adu-Afarwuah et al., 2007), in contrast to the findings in South Africa where no improvement in motor development was observed in the children receiving the micronutrients (Oelofse et al., 2003). A positive finding was also reported in a study of toddlers in Indonesia who were provided with high energy plus iron (Group 1) compared to a low energy plus iron (Group 2) or low energy plus placebo (Group 3) for 12 months (Pollitt et al., 2000a). In this study, Group 1 had higher scores in both the Bayley Scale of Mental Development (Pollitt et al., 2000b) and the Bayley Scale of Motor Development (Jahari et al., 2000). However, lack of evidence has restricted the compilation of public health recommendation/guidelines on specific nutrients to improve optimal cognitive and motor development in children.

#### **2.5.4 Morbidity**

Early introduction of CF before 6 months of age is related to increased risk of morbidity in LICs (Khadivzadeh and Parsai, 2004, Kalanda et al., 2006), and should be avoided. In the available trials in LICs that have examined the effect of CF interventions, almost none have shown significant effects on morbidity outcomes. An exception is the systematic review of Lassi et al. (2013) which suggested a benefit on respiratory infections (relative risk (RR): 0.67 [95%CI: 0.49, 0.91]), based on the data from three randomised controlled

trials conducted in Ghana (Adu-Afarwuah et al., 2007), Vietnam (Schroeder et al., 2002), and Brazil (Vitolo et al., 2005 as cited by Lassi et al., 2013).

Supplementation with multi-micronutrients or iron during infancy in different geographical areas have also shown no significant effect on morbidity (Dewey et al., 2002, Lind et al., 2004, Lopez de Romana et al., 2005, Untoro et al., 2005). Surprisingly, these results differ with some other studies, in which zinc alone (Bhutta et al., 1999, Roy et al., 1999) has shown reductions in morbidity among children <5 years. It is possible that such discrepancies may arise because of the differing zinc status of the children at baseline. Providing zinc supplements to young children who are zinc deficient at baseline may result in a greater impact on reducing infectious disease morbidity and mortality than for children with no evidence of zinc deficiency (Penny, 2013).

There are only two randomised controlled trials conducted in Indonesia which have examined the effects of zinc supplementation on morbidity, specifically diarrhoea (Hidayat et al., 1998, Lind et al., 2004). The RCT in 1998 was conducted on 1185 children aged <3 years. The treatment group received 4-5 mg zinc acetate per kg body weight/day for 12 months and the control group received placebo syrup. There was 11% reduction in the risk of continued diarrhoea (Hidayat et al., 1998) in the treatment group. A more recent study in Indonesia was conducted on 680 infants who were assigned into 4 groups, receiving daily supplementation with 10 mg iron, 10 mg zinc, 10 mg iron + 10 mg zinc, and placebo from 6 to 12 months old. This study found no significant effect of any of the daily supplementation regimens on diarrhoea (Lind et al., 2004). However, only a small proportion of intervention studies have included data on morbidity outcomes; and most of these studies have not been designed or powered to detect differences in morbidity, which may have contributed to the lack of effect on morbidity (Dewey and Adu-Afarwuah, 2008).

## 2.6 Conclusion

Under five children are a very vulnerable group in LMICs, especially in Indonesia, where there is a high prevalence of stunting, anaemia, and co-existing micronutrient deficiencies (Isabelle and Chan, 2011, National Institute of Health Research and Development, 2013, National Institute of Health Research and Development, 2012, Sandjaja et al., 2013). The incidence of these adverse health effects increases after 6 months of age when the transition period from BF to CF occurs. Effective strategies during the CF period are urgently required to address these public health concerns, because interventions after the first two years of life appear to have little impact on subsequent child growth and development (Bhutta et al., 2008).

In general, data on CF practices during infancy and early childhood and their association with poor growth and micronutrient malnutrition in young children in Indonesia have been very limited, especially when focussed on selected functional outcomes. To date, the major mineral deficits identified in complementary foods in Indonesia have been iron, zinc, and calcium, all known to directly and indirectly affect growth (Gibson and Hotz, 2001b). Numerous authors (Walker and Black, 2007, Dewey and Adu-Afarwuah, 2008, Lassi et al., 2013, Black, 2017, Gould, 2017) have suggested that all interventions should be designed to be context specific; and include baseline measurements to determine the level of micronutrient deficiency with appropriate adjustment for inflammation, where necessary, to avoid misleading conclusions. In addition, studies should ensure that the sample size has enough power to detect significant outcomes on morbidity, cognitive and motor development, in addition to growth. Studies to reduce ID and IDA and their long-lasting negative effects on cognitive and motor development are urgently required.



### **3 Relationship between WHO Infant and Young Child Feeding (IYCF) Indicators & Nutrient Density Adequacy and Growth in Infants age 6, 9, and 12 months in Sumedang District, West Java Province, Indonesia**

#### **3.1 Introduction**

Childhood undernutrition remains a significant problem in Indonesia, even though there have been major improvements in health over past decades. In 2013, among under-five children, mortality rates were 32 per 1000 live births (*Badan Pusat Statistik et al., 2013*), 20% were underweight, and 37% were stunted (National Institute of Health Research and Development, 2013). Such growth faltering often begins around 4-7 months of age (Kusin et al., 1991, Kolsteren et al., 1997), and is accompanied by a high prevalence of anaemia and co-existing micronutrient deficiencies (Dijkhuizen et al., 2001b, Lind et al., 2004).

Progress in improving CF practices in Indonesia has been slow (Ng et al., 2012). The rice-based gruels traditionally used for CF often have low energy and nutrient density (Isabelle and Chan, 2011) leading to nutrient deficits when compared with estimated needs (Dewey and Brown, 2003), particularly for calcium, iron, and zinc (Dewey, 2016). Such deficits may be exacerbated both by the displacement of breast milk if CF is introduced prior to 6 months of age, and poor appetite induced by infection (Dewey and Mayers, 2011).

The WHO has set a global target to reduce childhood stunting by 40% by 2025 (de Onis et al., 2013). Achieving this goal is a priority for Indonesia. Guiding principles for the CF of the breastfed child have been developed (PAHO/WHO, 2003) but whether they are widely practiced in Indonesia is uncertain. Therefore, in an initial survey, we studied a group of breastfed infants from the Sumedang district, West Java province, Indonesia, a district with rates of stunting (41.1%) and underweight (14.6%) comparable to the national prevalence in Indonesia (National Institute of Health Research and Development, 2013). Our objectives were to: a) characterise and evaluate

prevailing CF practices, b) assess the adequacy of energy and nutrient intakes during CF, and c) investigate relationships between WHO CF indicators, local sentinel food groups, nutrient adequacy, and subsequent infant growth in a cohort of breastfed infants at 6, 9, and 12 months of age. In view of the increasing use of fortified infant foods (FIFs) by mothers in Asia (Huffman et al., 2014, Pries et al., 2016a), we also examined these relationships with the consumption of FIFs. We envisaged that our study might lead to the development of interventions to improve CF practices and reduce risk of infection. Appropriate feeding practices during early childhood are essential because interventions after the first two years appear to have little impact on subsequent child growth (Bhutta et al., 2008).

## **3.2 Methods**

### **3.2.1 Study design and participants**

This study was a prospective, longitudinal study designed to evaluate nutritional status and growth of breastfed infants. The study was conducted between August 2013 and August 2014. Infants were randomly selected from all the villages (n=30) in Tanjungsari, Sukasari, and Pamulihan sub-districts in Sumedang district, West Java, and enrolled at 6 months of age based on local birth registry data collected by village midwives. Sumedang district is located around 50 km from Bandung City (capital of West Java), with an area of 152,220 hectares and a population of 1.1 million. The climate is tropical with rainfall during most months of the year, and a short dry season. Approximately 22% of the area is used for paddy plantation (*BAPPEDA Sumedang, 2014*). The majority of the population of Sumedang district is Muslim, and most inhabitants are of Sundanese ethnicity.

Infants were eligible for the study if they were apparently healthy. Infants who had been breastfed for less than four months, were premature (<37 weeks of gestation), and/or of very low birth weight (<1500 gram) were excluded. In addition, infants with evidence of diseases such as cancer, active tuberculosis, severe anaemia (Hb <90 g/L), and severe acute malnutrition (mid-upper arm circumference <115 mm) were also ineligible. Of the 275

infants (6 months + 4 weeks), 253 were eligible, and caregivers of 230 infants (i.e., 90.9%) consented to participate in the study.

Of the infants, 202 and 190 were followed-up at ages 9 (+4 weeks) and 12 months (+ 4 weeks), respectively, resulting in 190 infants who completed the 6-month study (i.e., 82.6% completion). Ethical approval was obtained from the Human Ethics Committees of *Universitas Padjadjaran*, Indonesia, and the University of Otago Human Ethics Committee, New Zealand, and complied with the Helsinki Declaration as revised in 1983. Informed written consent to participate in the study was given by the parents or primary guardians of the infants. Participants were free to withdraw from the study at any time.

### **3.2.2 Socio-demographic and health status**

All questionnaires were developed in English and translated into Bahasa Indonesia, back translated to English, and then pre-tested prior to administration to parents/caregivers by trained field doctors/nutritionists during a home visit. Data on socio-demographic, health, morbidity status and vaccination history, IYCF practices, sanitation, and hygiene were collected.

### **3.2.3 Anthropometric measurements**

Weight and recumbent length were measured using standardised techniques (WHO, 2004) and calibrated equipment on the infants at ages 6, 9, and 12 months. Weight was recorded to the nearest 10 gram using an electronic scale (Seca 334, Seca GmbH & Co. KG., Hamburg, Germany) and length to the nearest mm using an infantometer (Seca 417, Seca GmbH & Co. KG., Hamburg, Germany). The measurements were recorded in duplicate, or triplicate if the difference between first and second measurement was more than the recommended range (i.e. 100 gram for weight, 5 mm for length) (de Onis et al., 2004). All anthropometric measurements were made by trained field doctors on infants nude or wearing a weighed dry diaper (weight 30-50 gram).

Measurement quality was achieved by calculating the technical error of the measurement (TEM) for each of the anthropometric measurements

performed by three trained field doctors using standardised WHO procedures (WHO, 2004) on 20 (non-study) infants aged 6 months. Both the inter- and intra-examiner TEMs achieved were acceptable, with a coefficient of reliability >0.95 (Gibson, 2005). Maternal height (to nearest mm) and weight (to nearest 100 gram) were also measured at baseline using a stadiometer (Seca 213, Seca GmbH & Co. KG., Hamburg, Germany) and a bioelectrical impedance analyser (TANITA SC-240, TANITA Corporation, Tokyo, Japan), respectively, from which body mass index (BMI) was calculated. LAZ, WLZ, and WAZ were calculated using the new WHO Child Growth Standards (2006) and WHO AnthroPlus 3.2.2.

### **3.2.4 Assessment of complementary food intakes**

Food intakes of infants were assessed via two-day in-home weighed food records completed by trained community cadres from 06.30 until 18.30 or from breakfast until dinner on non-consecutive days within a one-week period. Weighed food records were collected using calibrated dietary scales (Camry Electronic Kitchen Scale EK3131, Camry Electronic Ltd, Guangdong, China) accurate to  $\pm 1$  gram on three occasions when the infants were 6 (+4 weeks), 9 (+4 weeks), and 12 (+4 weeks) months of age. Weighed ingredients from recipe data were collected for mixed dishes prepared in the home (Gibson and Ferguson, 2008). In cases where the foods were bought from street vendors or from a restaurant, five recipes for each food type were compiled from Indonesian websites and used to calculate the average energy and nutrient content of each of these purchased foods. To gain information on food intake during the night, parents or caregivers were asked to record all foods and liquids consumed by the child from 18.30 (or after dinner) in household measures on the previous evening until 06.30 (or before breakfast) on the next day. Cadres were trained to convert the household measures into gram equivalents.

### **3.2.5 Compilation of WHO IYCF indicators**

All infants at age 6, and 9 months of age were breastfed, and all at 12 months with the exception of four. These non-breastfed infants (i.e., 4) were not included in the compilation of the IYCF indicators because the sample size

was so small. Data from the food records were used to compile the five core population-level CF indicators developed by WHO (2008, 2010). They included: 1) introduction of solid, semi-solid or soft foods; 2) minimum dietary diversity (MDD); 3) minimum meal frequency (MMF); 4) minimum acceptable diet (MAD); 5) consumption of iron-rich (i.e., flesh foods) or iron-fortified food.

To compile MDD and MAD, the foods consumed by the infants at each study visit were classified into seven major food groups (FGs) based on the WHO specification (WHO, 2008) with no minimum quantity of consumption defined: 1) grains, roots, and tubers; 2) legumes and nuts; 3) dairy products (milk, yogurt, cheese); 4) eggs; 5) flesh foods (meat, fish, poultry, and liver/organ meats); 6) vitamin A-rich fruits and vegetables; and 7) other fruits and vegetables. In addition, the proportion of infants at each age consuming four WHO nutrient-dense sentinel food group indicators (dairy products, flesh foods, eggs, and animal-source foods), and a local sentinel food termed "fortified infant foods (FIFs)" was also calculated. The animal-source foods comprised dairy products, flesh foods, and eggs. The FIFs group comprised infant formulae, fortified infant cereals, and rusks, many of which also contained significant amounts of dried milk powder. All food groups contained in a mixed dish were counted separately. Clear broths from simmered dishes and soups were not included in the compilation of MDD and MMF.

Dietary diversity at each age was calculated by summing the number of the seven food groups (WHO, 2010) consumed in the in-home food records. MDD was defined as consumption of four or more food groups for at least once out of the two days. Meal frequency at each age was calculated by summing the number of meals (breakfast, lunch, dinner) and snacks other than trivial amounts (<10 gram) consumed in the two-day in-home food records. MMF was defined as having consumed 2 or more solid, semisolid, or soft meals per day for each of the two food record days at 6 months and 3 or more meals per day at 9 and 12 months of age (WHO, 2008). MAD was defined as meeting the requirements for both MDD and MMF on the same day at each age. Consumption of iron-rich or iron-fortified foods was defined as having

consumed a flesh food or an iron-fortified food or commercially processed iron-fortified complementary food specially designed for infants and young children in a 24-h period, at least once out of two days. In addition, the proportion of infants at each age consuming branded/commercially produced foods or beverages intended for the general population, and eaten as “snacks” at least once out of two days was calculated.

### **3.2.6 Compilation of an Indonesian complementary FCT**

An Indonesian FCT for all the foods and beverages consumed by infants during the period of the study was created using energy and nutrient composition values from the following sources: Sustainable Micronutrient Interventions to Control Deficiencies and Improve Nutritional Status and General Health in Asia Project (SMILING, 2013); FAO/INFOODS Food Composition Databases for Asia (FAO/INFOODS, 2016), augmented where necessary with nutrient values from the U.S. Department of Agriculture (USDA, 2005), and ProPan 2.0 (ProPAN, 2012).

Nutrient values from recipes were also calculated for 54 mixed dishes taking into account both yield and retention factors, depending on the cooking method (Bognar, 2002). Phytate values for the plant-based complementary foods were compiled from the literature (Ferguson et al., 1988, Bunch and Murphy, 1997, Chan et al., 2007); and adjusted to take into account differences in their moisture content. In cases where phytate values were not available for commercially processed complementary foods, then values for comparable processed complementary foods were imputed. This local FCT was then used with the digitized 24-hr dietary records to calculate the energy and nutrient intakes of the infants on each of the two record days.

### **3.2.7 Assessment of energy and nutrient intakes and adequacy of complementary foods**

Data for children who were no longer being breastfed at aged 12 months were omitted from this analysis (n=4). The median intakes (first and third quartiles) of energy and nutrients (per day) from complementary foods at each age were calculated using the complementary FCT. Median daily

intakes were then compared with the corresponding estimated energy and nutrient needs from complementary foods. The latter were calculated by subtracting the estimated contribution of energy or nutrient intakes from breast milk (assuming an average volume and composition of breast milk (WHO, 1998) from the corresponding energy requirements (FAO/WHO/UNU, 2004) or the WHO/FAO (2004) RNIs, with the exception of zinc. For the latter, the RNIs of Brown et al. (2004) assuming a bioavailability equivalent to a mixed diet were used. Intakes of energy were compared with the estimated energy needs unadjusted and adjusted for the body weights of the infants (i.e., kcal/kg/day) (FAO/WHO, 2004). In addition, the proportion of infants at each age meeting their estimated energy and nutrient needs based on their average intakes over two days was also calculated, and expressed as nutrient adequacy ratios (NARs). The latter were calculated as percentages by dividing the actual average daily intakes from complementary foods for each nutrient by the corresponding estimated needs. These percentages were capped at 100% and averaged to create an overall micronutrient adequacy ratio (MAR) for individuals at each age group (Madden et al, 1976). Median (interquartile range) of micronutrient densities per 100 kcal of complementary foods was also calculated. Usual nutrient intakes were not estimated to determine the prevalence of nutrient adequacy because all the infants were breastfed and intakes of breast milk were not measured in this study. Instead, nutrient intakes from complementary foods alone were calculated and compared to the WHO estimated needs, assuming an average intake of breast milk (WHO, 1998).

### **3.2.8 Statistical analysis**

All data were transferred into Stata® 12 (StataCorp LP, Texas, USA), where descriptive and comparative statistics were calculated. Selected characteristics of the infants, mothers, and households are presented as percentages (%) for categorical variables and as means with standard deviation (SD) for continuous variables, where applicable. Maternal education was categorized as primary school or less, secondary school, and college/university. A wealth index was calculated using principal component analysis based on asset variables recommended for use in the Indonesian

Demographic Health Survey (DHS) (Badan Pusat Statistik et al., 2013), following the DHS Wealth Index guidelines (Rutstein & Johnson, 2004). The following variables were included: home ownership, clock, television, hand-phone, fridge, bicycle, motor cycle, car, electricity, and radio ownership, floor type, access to water, ownership of garden, water source, toilet facility, and floor/roof type. This continuous index was then divided into quintiles from the lowest to highest household wealth.

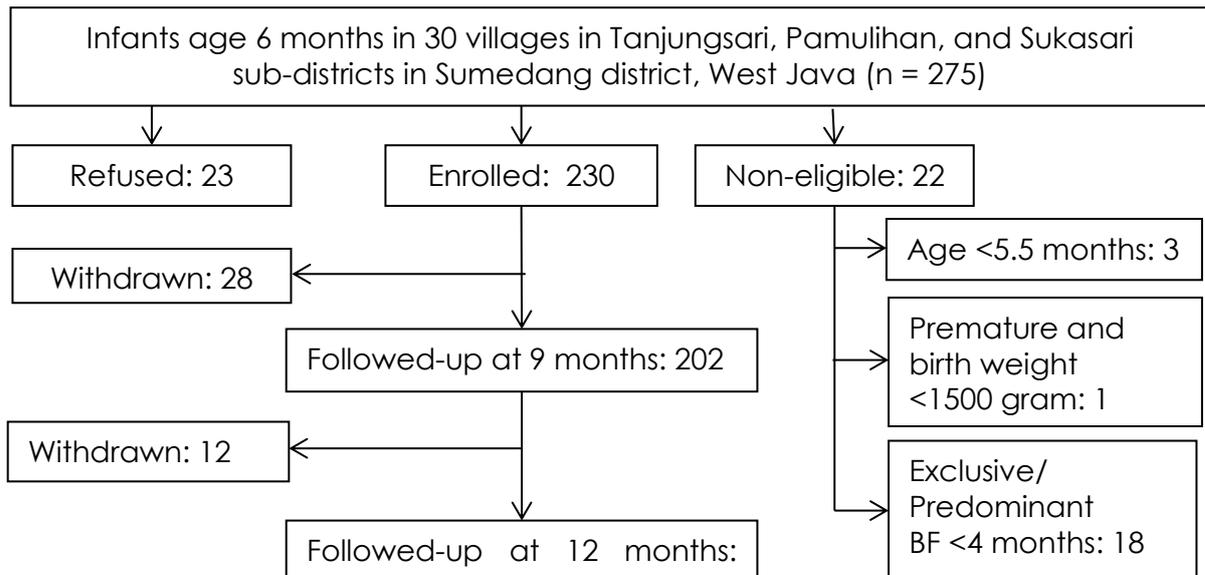
The five core IYCF indicators and the four "sentinel food groups" were compiled as reported by WHO (2008, 2010). Corellation between energy intakes from complementary and meal frequency at 6, 9, and 12 months was tested using Spearman's correlation coefficient. Energy and nutrient intakes (per day) from complementary foods at each age and for those infants consuming and not consuming FIFs at 9 months were calculated as the median (first and third quartiles) for consistency because some nutrients were not normally distributed. Wilcoxon rank-sum test was used to determine the differences.

Multiple linear regression was used to determine association with WLZ, WAZ, and WHZ at 12 months (excluding the four infants who were no longer being breastfed) as dependent variables and the following independent variables at 9 months: WHO IYCF indicators (MDD, MMF, MAD, and consumption of iron-rich foods); consumption of the "sentinel food groups": dairy products (FG 3), flesh foods (FG 4), eggs (FG5), and animal-source foods, and FIFs at 9 months; intakes of selected growth-limiting nutrients (per day, per kg body weight); and nutrient adequacy ratios. All analyses using independent variables at 9 months of age were controlled for WLZ, WAZ, and WHZ at the same time as the observations for the independent variables. Fully adjusted analyses included the following covariates: maternal height, sex, wealth index (quintiles), and maternal education. Model assumptions were checked using residual plots, and Levene's test was additionally used to assess homogeneity of variance and heteroscedasticity. Residuals for these models were checked and showed a fairly random pattern. A 2-sided  $P < 0.05$  level of significance was used in all cases.

### 3.3 Results

#### 3.3.1 Socio-demographic and health status

A total of 275 children were approached at aged 6 months, of whom 230 enrolled in the study (83.6%); 23 (8.4%) refused, and 22 (8.0%) did not meet the inclusion criteria. Of the 230 children, 190 completed the study (completion rate, 82.6%), with the main reason of withdrawal being the repeated blood measurements (Figure 3.1). Average age of mothers was 27.5 (SD 7.2) years and average height of mothers was 150.2 (SD 5.2) cm; nearly 16% had a height less than 145 cm classified as short stature. Of the mothers, 7.3% were underweight (BMI <18.5) and 36.4% overweight or obese (BMI >25). More than half of mothers (53.7%) finished secondary school, 40.6% had primary education or less, and only a small proportion (6.1%) attended college/university (Table 3.1).



**Figure 3.1** Flow of respondents in the longitudinal study

Almost all mothers were housewives (91.7%) and nearly two thirds of the fathers (~60%) did not have a regular income. Most households had an improved water source (79.4%), defined as drinking water source that by nature of its construction or through active intervention is protected from outside contamination, in particular with faecal matter. Most households also had access to an improved sanitation facility, consisting of flush toilet, piped sewer system, septic tank, and flush/pour flush to pit latrine (84.7%).

The average age of infants was 6.5 (SD 0.4) months, of whom 53.5% were female. The birth order of nearly 80% of the infants was either first or second. Almost all of the infants had a birth weight of 2500-4500 gram (96.8%); only 2.7% had a birth weight ranging from 1500 and 2500 gram, as measured within 1-3 days by village midwives.

**Table 3.1 Maternal, socio-demographic status and infant birth characteristics at baseline**

<b>Maternal characteristics</b>	<b>n</b>	
Age, years, mean (SD)	226	27.5 (7.2)
Height, cm, mean (SD)	225	150.2 (5.2)
Height <145 cm, %	35/226	15.5%
Body Mass Index (kg/m <sup>2</sup> ), mean (SD)	220	23.7 (4.0)
Underweight	16/220	7.3%
Normal	124/220	56.4%
Overweight	67/220	30.5%
Obese	13/220	5.9%
Education, %		
Primary school or less	93/229	40.6%
Secondary school	122/229	53.3%
College/university	14/229	6.1%
Number of pregnancies, %		
1	89/229	38.9%
2	82/229	35.8%
3 or more	58/229	25.3%
<b>Household characteristics</b>		
Father's education		
Primary school or less	109/229	46.7%
Secondary school	108/229	47.2%
College/university	12/229	5.2%
Father's occupation		
Regular wage earner	22/228	9.6%
Business or trade owner	69/228	30.3%
Manual labour	100/228	43.9%
Farmer	26/228	11.4%
Unemployed	11/228	4.8%
Improved drinking-water source	181/228	79.4%
Improved sanitation facility	193/228	84.7%
<b>Infant characteristics</b>		
Female	123/230	53.5%
Birth weight, 2500 - 4500 gram	211/218	96.8%

\*Classification of BMI for Asian population (WHO, 2004): < 18.5 kg/m<sup>2</sup> underweight; 18.5–22.9 kg/m<sup>2</sup> increasing but acceptable risk (normal); 23–27.4 kg/m<sup>2</sup> increased risk (overweight); and 27.5 kg/m<sup>2</sup> or higher high risk (obese)

### 3.3.2 Anthropometric status

The mean LAZ and WAZ scores for the infants were negative at each age, and were progressively lower at 12 months compared to 6 months of age. As a result, the proportion of stunting and underweight increased significantly from 15.7% and 3.9% at 6 months, 19.3% and 5.4% at 9 months, to 22.6% and 10.5%, respectively at 12 months of age (Table 3.2). In contrast, the proportion of wasting was much lower at each age. All anthropometric values of the infants were within +3 SD from the population mean as recommended by WHO (1995).

**Table 3.2 Anthropometric characteristics of infants at 6, 9, and 12 months**

	6 months	9 months	12 months
LAZ, mean (SD)*	-1.02 (0.97)	-1.16 (0.97)	-1.26 (1.00)
Proportion of LAZ <-2 SD	36/230 (15.7%)	39/202 (19.3%)	43/190 (22.6%)
WLZ, mean (SD)*	0.45 (1.03)	0.17 (1.03)	-0.08 (1.08)
Proportion of WLZ <-2 SD	4/230 (1.7%)	4/202 (2.0%)	6/190 (3.2%)
WAZ, mean (SD)*	-0.34 (1.00)	-0.52 (1.02)	-0.66 (1.08)
Proportion of WAZ <-2 SD	9/230 (3.9%)	11/202 (5.4%)	20/190 (10.5%)

LAZ, length-for-age z-score; WLZ, weight-for-length z-score; WAZ, weight-for-age z-score  
SD, standard deviation

\* P-value <0.05

### 3.3.3 IYCF practices

Most of the infants continued to be breastfed from 6 months to 9 months of age, but at 12 months, four were no longer being breastfed. In relation to the core CF indicators, almost all of the infants at aged 6 months (99.5%) had been introduced to solid, semi-solid, or soft foods, whereas achievements for minimum dietary diversity and minimum acceptable diet at aged 6 months were very low (both 2.8%), despite 93% achieving minimum meal frequency at this age (Table 3.3). Indeed, nearly two-thirds (i.e., 63.6%) of the infants were consuming only 0-2 food groups at 6 months (data not shown). At 9 and 12 months of age, however, the proportion of infants who consumed a minimally diverse diet (i.e.,  $\geq 4$  food groups) and a minimum acceptable diet was higher, although still only ~60% at 12 months.

**Table 3.3 Number and percentage of infants meeting five core WHO complementary feeding indicators and consuming four sentinel food groups at 6, 9, and 12 months**

WHO IYCF Indicator, n (%)	6 months n=218	9 months n=193	12 months n=186
Introduction of solid, semi-solid, or soft foods	217 (99.5)	NA	NA
Minimum dietary diversity ( $\geq 4$ food groups)	6 (2.8)	78 (40.4)	112 (60.2)
Minimum meal frequency	202 (92.7)	191 (99.0)	186 (100)
Minimum acceptable diet	6 (2.8)	78 (40.4)	112 (60.2)
Consumption of iron-rich/iron fortified infant foods	201 (92.2)	145 (75.1)	150 (80.1)
<b>Sentinel food groups, n (%)</b>			
Dairy products	9 (4.1)	3 (1.6)	9 (4.8)
Flesh foods	5 (2.3)	105 (54.4)	140 (75.3)
Eggs	7 (3.2)	69 (35.8)	106 (57.0)
Animal-source foods	19 (8.7)	132 (68.4)	161 (86.6)
Fortified infant foods	200 (91.7)	69 (35.8)	23 (12.4)

Consumption of iron-rich/iron-fortified foods was very high at 6 months (91.3%), of which the major sources were fortified infant products; and 100% at 9 months, of which the sources were a combination of fortified infant products and flesh foods, notably meat balls and sausages. However, consumption of iron-rich/iron-fortified foods decreased at 12 months of age (80.1%), following the reduction in the consumption of fortified infant cereals. In contrast, consumption of animal-source foods increased progressively from 6 months (8.7%) to 86.6% at 12 months. The major flesh foods consumed were meatballs containing 1.6 mg Fe/100 gram and sausages (1.9 mg/100 gram). Consumption of dairy products was very limited at all age groups (i.e., <6%) (Table 3.3).

### 3.3.4 Commercially produced foods

In contrast to fortified infant products, the consumption of snack products progressively increased from 6.5% at 6 months, to 50.9% at 9 months and 69.1% at 12 months, providing 14.0%, 22.5%, and 18.3% of the total energy intake at 6,9, and 12 months, respectively.

### 3.3.5 Adequacy of energy and nutrient intakes

Energy intakes from complementary food were positively correlated with meal frequency at 6 ( $r = 0.67$ ,  $P < 0.001$ ), 9 ( $r = 0.42$ ,  $P < 0.001$ ), and 12 months ( $r =$

0.39,  $P < 0.001$ ). Energy densities (kcal/g) of the complementary foods were positively correlated with the amount of complementary food consumed (g/d) at each age (6 months,  $r = 0.35$ ,  $P < 0.001$ ; 9 months  $r = 0.64$ ,  $P < 0.001$ ; 12 months,  $r = 0.51$ ,  $P < 0.001$ ), and increased with age from a median of 1.0 kcal/g at 6 months, 1.3 kcal/g at 9 months and 1.7 kcal/g at 12 months.

**Table 3.4 Estimated need and median (IQR) energy and nutrient intakes from complementary food at 6, 9, and 12 month**

Nutrient	6 months		9 months		12 months	
	Need	Intake <i>n</i> = 215	Need	Intake <i>n</i> = 192	Need	Intake <i>n</i> = 186
Energy (kcal) <sup>a</sup>	202	127 (95-166)	307	275 (218-378)	548	396 (302-492)
Energy (kcal/kg) <sup>b</sup>	177		251		340	
Protein (g)	4.4	3.7 (2.8-5.1)	5.6	7.9 (5.6-11.9)	6.9	12.4 (8.7-16.9)
Niacin (mg) <sup>c</sup>	1.6	0.6 (0.3-1.0)	3.7	1.5 (0.9-1.9)	5.7	2.0 (1.3-2.7)
Riboflavin (mg)	0.09	0.09 (0.06-0.12)	0.22	0.15 (0.09-0.30)	0.33	0.26 (0.17-0.42)
Thiamin (mg)	0	0.08 (0.06-0.11)	0	0.14 (0.07-0.19)	0	0.18 (0.12-0.25)
Vitamin C (mg)	1	15 (10-21)	10	10 (4-20)	10	13 (5-22)
Vitamin A ( $\mu$ g RAE)	44	77 (56-115)	75	53 (26-105)	87	57 (26-99)
Calcium (mg)	161	83 (55-116)	293	109 (69-144)	402	122 (85-170)
Iron (mg) <sup>d</sup>	9.1	3.1 (1.9-4.2)	9.1	2.4 (1.5-3.4)	5.6	2.7 (1.9-3.7)
Zinc (mg) <sup>e</sup>	2.5	1.2 (0.8-1.7)	2.6	1.5 (1.0-2.1)	1.7	2.0 (1.5-2.7)

<sup>a,b</sup> FAO/WHO, 2004

<sup>c</sup> Does not include contribution of niacin from tryptophan

<sup>d</sup> Assuming medium bioavailability (10%) (WHO/FAO,2004)

<sup>e</sup> Assuming mixed or refined vegetarian diets (Brown et al., 2004)

There were also consistent deficits in several micronutrients, with median intakes (per day) of iron and zinc, and seemingly niacin below the estimated needs from complementary foods at all three ages. However whether shortfalls in niacin actually existed is uncertain as the contribution of niacin from tryptophan was not included in the data presented in Table 3.4. Median intakes of protein, riboflavin, vitamin A, and calcium fell below the estimated needs at 6 months, but met or exceeded them at 9 and 12 months of age. Nutrient densities (i.e., intakes per 100 kcal) were also examined relative to the desired nutrient densities. However, these results are not included here because the low energy intakes shown in Table 3.4 limit the interpretation of the nutrient densities.

### 3.3.6 Intakes of energy and selected nutrients in relation to consumption of FIFs

Differences existed in median intakes of energy and selected nutrients from complementary foods among infants consuming fortified vs. non-fortified infant foods at aged 9 months (Table 3.5). Median energy density, intakes of energy and protein, together with dietary diversity score (2.3 vs. 3.0;  $P < 0.001$ ) for infants consuming fortified foods were significantly lower ( $P < 0.01$ ), whereas their median intakes of vitamin C, vitamin A, calcium, and iron (but not zinc) were higher ( $P < 0.001$ ) compared to those consuming non-fortified infant foods. In addition, the MAR for infants at aged 9 months also differed according to whether infants were consuming ( $n=69$ ) or not consuming ( $n=123$ ) FIFs (66+13% vs. 60+13%;  $P=0.05$ ), respectively (data not shown).

**Table 3.5 Median (IQR) of energy density, intakes of energy and nutrients of infants consuming and not consuming infant fortified foods at 9 months**

	9 months		P-value
	Median (IQR)		
	Fortified (n = 69)	Non-fortified (n = 123)	
Energy density (kcal/g)	1.2 (1.0-1.5)	1.5 (1.0-1.9)	0.006
Energy (kcal)	250 (206-293)	310 (237-400)	<0.001
Protein (g)	6.5 (5.2-8.7)	9.1 (6.2-12.7)	<0.001
Niacin (mg) <sup>a</sup>	1.2 (0.8-1.8)	1.5 (0.9-2.0)	0.313
Riboflavin (mg)	0.14 (0.09-0.22)	0.16 (0.08-0.36)	0.493
Thiamine (mg)	0.14 (0.08-0.18)	0.14 (0.06-0.20)	0.815
Vitamin C (mg)	16 (9-27)	7 (3-14)	<0.001
Vitamin A ( $\mu$ g RAE)	76 (50-144)	42 (16-84)	<0.001
Calcium (mg)	128 (87-168)	106 (53-133)	<0.001
Iron (mg)	2.7 (2.1-3.8)	2.2 (1.2-3.0)	<0.001
Zinc (mg)	1.4 (1.2-2.0)	1.6 (0.8-2.1)	0.692

<sup>a</sup> Does not include contribution of niacin from tryptophan

### 3.3.7 Inter-relationships between nutrient adequacy, CF indicators, sentinel food groups and subsequent growth

Inter-relationships were not examined for the data at 6 months because so few infants met the MDD indicator (Table 3.3). Table 3.6 shows the relation of three WHO IYCF indicators (excluding MMF) and four sentinel food groups at aged 9 months with LAZ, WLZ, and WAZ at aged 12 months.

**Table 3.6 WHO complementary feeding indicators and sentinel food indicators at aged 9 months in relation to LAZ, WLZ, and WAZ at 12 months**

Core WHO indicators at 9 mos	$\beta$	Growth at 12 months	
		95% Conf. Interval	P-value
<b>LAZ</b>			
Minimum dietary diversity	0.01	-0.18, 0.20	0.91
Minimum acceptable diet	0.01	-0.18, 0.20	0.91
Iron-rich/iron fortified infant foods	0.22	0.01, 0.44	0.04
<b>WLZ</b>			
Minimum dietary diversity	0.05	-0.13, 0.24	0.58
Minimum acceptable diet	0.05	-0.13, 0.24	0.58
Iron-rich/iron fortified infant foods	-0.22	-0.42, 0.00	0.05
<b>WAZ</b>			
Minimum dietary diversity	-0.01	-0.13, 0.10	0.83
Minimum acceptable diet	-0.01	-0.13, 0.10	0.83
Iron-rich/iron fortified infant foods	-0.03	-0.15, 0.10	0.70
<b>Sentinel food indicators at 9 mos</b>			
<b>LAZ</b>			
Flesh foods	0.07	-0.12, 0.26	0.45
Eggs	0.02	-0.18, 0.21	0.88
Animal-source foods	0.10	-0.10, 0.30	0.32
Fortified infant foods	0.29	0.09, 0.48	0.04
<b>WLZ</b>			
Flesh foods	-0.11	-0.30, 0.07	0.24
Eggs	-0.05	-0.24, 0.14	0.59
Animal-source foods	-0.11	-0.31, 0.09	0.27
Fortified infant foods	-0.09	-0.29, 0.11	0.37
<b>WAZ</b>			
Flesh foods	-0.07	-0.18, 0.04	0.22
Eggs	-0.05	-0.16, 0.07	0.47
Animal-source foods	-0.06	-0.18, 0.06	0.32
Fortified infant foods	0.14	0.02, 0.26	0.02

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

\*Analysis was not conducted for dairy products as there were less than 15 observations in the category and all, except 2 children had minimum meal frequency. Model is adjusted for growth at baseline (LAZ, WLZ, WAZ at 6 months), sex, mother's height, wealth index (quintile), mother's education, and mother's height.

Of the three, consumption of iron-rich/iron-fortified infant foods at 9 months was the only indicator that was positively and significantly associated with linear growth at 12 months; ( $\beta = 0.22$ ; 95% CI: 0.01, 0.44; P=0.04) in fully adjusted models. Of the four sentinel foods also investigated at 9 months, FIFs alone were significantly related to both a greater LAZ at 12 months ( $\beta = 0.29$ ; 95% CI: 0.09, 0.48; P=0.04) and a greater WAZ ( $\beta = 0.14$ ; 95% CI: 0.02, 0.26;

P=0.02). No significant associations were observed with growth at 12 months for the average daily intakes at 9 months of the critical growth-limiting nutrients (protein, zinc, iron, calcium, and riboflavin (per day; per kg body weight) or for the NARs for the infants.

### **3.4 Discussion**

The increase in the proportion of stunting from 6 to 12 months of age observed here among infants living in Sumedang district, West Java is not unexpected. A similar pattern has been reported in the 2013 Indonesian National Health Report (National Institute of Health Research and Development, 2013), as well as in other LICs (Marriott, 2012, Senarath and Dibley, 2012). Despite the high compliance to BF practices, the transition from EBF to CF in this study was not in accordance with the WHO Guiding Principles for Breastfed Children (PAHO/WHO, 2003), and may have been a major factor contributing to the increase in the proportion of stunting and underweight observed here. Overall, the complementary diets were inadequate in both quantity and quality.

#### **3.4.1 Compliance to WHO CF indicators**

At 6 months, the proportion of infants who received complementary diets with a minimum dietary diversity and had a minimum acceptable diet was very low, despite most of the infants achieving the minimum meal frequency (Table 3.3), a finding in contrast to reports elsewhere in Indonesia (Ng et al., 2012) and other parts of the region (Marriott et al., 2010). The poor dietary diversity at aged 6 months arose because most of the infants consumed almost exclusively commercially-produced infant cereals fortified with micronutrients and cow's milk powder for their main meals; intakes of other food groups at this age such as fruits, vegetables, and flesh foods were negligible. Hence, it is not surprising that the food group containing cereals grains, roots and tubers provided the greatest proportion of energy, as well as calcium, iron, and zinc at this age. At 9 and 12 months of age, consumption of these infant fortified foods declined and other food groups (legumes/nuts,

eggs, and flesh foods) replaced the cereals as the major sources of protein, calcium, iron, and zinc.

Most of the earlier reports from Indonesia and elsewhere in the region come from DHS which are based on maternal 24-hr recalls instead of two-day in-home food records used here. Maternal recalls may lead to recall bias resulting in over- or under-reports of certain food items consumed, especially snack foods. Discrepancies in food group classifications may also occur. For example, when caregivers report instant chicken porridge, it may be classified as a flesh food, even though the product only contains chicken flavouring. In addition, the age range of infants studied is often wider (e.g., 6-11 months) (Marriot et al., 2010, Ng et al., 2012) so infants are more likely to consume diets with higher dietary diversity than the six-month-old infants studied here.

#### **3.4.2 Commercially produced foods**

As noted earlier, a high proportion of children consumed commercially-produced FIFs at 6 months, and commercially-produced 'snack foods' at 12 months, a trend also reported among children in Nepal (Pries et al., 2016a) and Cambodia (Pries et al., 2016b). It is possible that the progressive substitution of FIFs by commercially produced "snack" foods with limited nutritional value at 9 and 12 months may have contributed in part to the progressive increase in the proportion of stunted and underweight infants in this area. Given the high proportion of consumption of snack foods (Cogswell et al., 2015, Dasgupta et al., 2015, Pries et al., 2016a, Pries et al., 2016b), a strong national quality control mechanism in Indonesia is required. Regulation for processed FIFs in Indonesia exists (*Menteri Kesehatan Republik Indonesia, 2007*) and states that the products should contain 15-22 gram of protein/100 gram (with at least 70% casein). However, compliance to the regulations in Indonesia is questionable even for international companies as monitoring is very sporadic.

Discouraging the consumption of commercially-produced snack foods by infants in Indonesia may have certain health advantages because the high consumption of sugar and salt during infancy increases the risk of overweight and obesity, as well as degenerative diseases (Dasgupta et al., 2015, Sahoo

et al., 2015). Care must also be taken in choosing FIFs as many of these products also contain additional sugar and salt (Cogswell et al., 2015).

### **3.4.3 Adequacy of energy and nutrient intakes from complementary foods**

Despite the higher average meal frequency in this population compared to the WHO recommendation (WHO, 2008) and the positive correlation between energy intakes and meal frequency, deficits in energy intakes were apparent at all ages when compared to absolute energy requirements. It should be noted that these estimates may be over-estimated given the small physiological size of the infants and their inability to experience full catch-up growth (Table 3.4). According to WHO/FAO/UNU, 2004, a 14.5% increase in energy requirements is needed (i.e., 1.83 g/kg/day) for infants aged 6-9 months to gain twice their normal growth rate. Of note is the greater deficit in energy intakes for infants consuming FIFs at 9 months compared to those who did not (Table 3.5). The failure of these complementary diets to meet the estimated needs for several micronutrients, despite almost all achieving a MMF (Table 3.3), is also of concern. Such persistent shortfalls in micronutrient intakes were exacerbated by the low energy intakes.

Micronutrient density adequacies were not calculated because the low energy intakes reported here distort the nutrient density data. Instead we calculated NARs and found a moderate and significant correlation between NARs and dietary diversity score for the “non-consumers” of infant fortified foods, but not for the “consumers”, as reported by others (Working Group of Infant and Young Child Feeding Indicators, 2006, Mallard et al., 2016). This finding arises because the single food group --FIFs-- supplied ~ 50% of the total intake of iron, zinc, and calcium at 9 months. These findings highlight that even in this underprivileged rural setting, consumption of expensive FIFs limits the usefulness of dietary diversity score as a proxy for dietary quality and subsequent growth.

Nevertheless, despite the lower energy density and lower energy intakes of the infants consuming FIFs at 9 months, their intakes of protein and several micronutrients were significantly higher than for the non-consumers ( $P < 0.001$ ) attributed, at least in part, to their fortification with micronutrients and

powdered cow's milk (Table 3.6). The effect of the FIFs on growth is discussed below.

#### **3.4.4 Inter-relationships between nutrient adequacy, CF indicators, sentinel food groups and subsequent growth**

Unlike several earlier studies (Zongrone et al., 2012, Mallard et al., 2014, Menon et al., 2015), we found no associations between either dietary diversity or a minimum acceptable diet and subsequent growth. This finding is not unexpected; earlier reports on the effects of the WHO CF indicators on growth have been inconsistent (Marriott et al., 2010, Marriott et al., 2012, Jones et al., 2014). Jones et al. (2014) has highlighted that the lack of sensitivity and specificity of the WHO core CF indicators may contribute to the inconsistent associations observed with growth. In most studies, the indicators are derived from questionnaire data based on maternal self-reports, which without additional quantitative data on energy and nutrient intakes, may lead to biased results.

In our study, intake of iron-rich/iron fortified foods at 9 months were positively associated with a greater LAZ (Table 3.6) but tended to be negatively related to WLZ. This inconsistent relationship may have arisen because flesh foods were included with iron-fortified foods, as recommended by WHO (2010). When the effects of these two sentinel foods on growth were examined separately (Table 3.6), flesh foods had no significant effect on linear growth whereas the intake of FIFs alone were associated with greater LAZ and WAZ at 12 months (Table 3.6). Lack of a positive effect of flesh foods on linear growth is plausible given that the major items consumed in this food group at aged 9 months were meat balls and sausages, which were predominantly cereal-based with very little meat content. Indeed, the consumption of meat balls and sausages may have been responsible for the tendency for a negative association to exist between iron-rich/iron fortified foods and WLZ.

Failure to observe an association between animal-source foods and linear growth was unexpected. Earlier studies have reported a positive effect of animal-source foods on linear growth, some of which has been associated

specifically with flesh foods (Dror and Allen, 2011, Hambidge et al., 2011, Krebs et al., 2011) or dairy products (Hoppe et al., 2006, Michaelsen, 2013). Early introduction of animal source-foods, specifically meat, has been recommended in view of the potential of meat for increasing intakes of high quality animal protein, bioavailable zinc (Krebs et al., 2006) and iron (Hambidge et al., 2011), vitamin B-12, and riboflavin (Dror and Allen, 2011). In this population, the lack of association may be due to the low quality of flesh foods consumed and very low proportion of children consuming dairy products (<5%).

The strong positive outcome of infant fortified foods on linear and to a lesser extent ponderal growth reported here was also unexpected because so few studies of micronutrient fortified complementary foods have promoted growth (Faber et al., 2005, Dewey and Adu-Afarwuch, 2008, CIGNIS Study team, 2010, De-Regil et al., 2011, Jack et al., 2012). However, in most of these studies no additional sources of macronutrients (energy, protein or fat) were included in the products (Dewey, 2016), whereas here most of the specialized infant foods consumed by almost all of the Sumedang infants at 6 months (91.5%) and about a third (35.3%) at 9 months were fortified, not only with micronutrients, but also with cow's milk powder.

Dairy products have been linked with improvements in linear growth because of their content of high quality protein, lactose, potassium, magnesium, phosphorus, and an insulin-like growth factor-1, all of which are growth-promoting substances (Hoppe et al., 2006, Michaelsen, 2013). Both observational and randomized controlled trials have reported enhanced linear and ponderal growth in childhood following the consumption of dairy products, specifically cow's milk (Hoppe et al., 2006, Michaelsen, 2013).

Interestingly, the energy density the diets of those infants consuming FIFs was lower than for the non-consumers (1.2 kcal/g vs. 1.5 kcal/g;  $P < 0.006$ ). Likewise, intakes of energy and protein (per day) of these consumers were significantly lower than for the non-consumers (Table 3.5). These findings probably arose because caregivers prepared porridges from these expensive fortified infant

products with a lower dry matter content in an effort to make them more long lasting. Hence, despite almost all infants achieving MMF, this practice may have been responsible, at least in part, for the deficits in some of the micronutrients observed when compared with the estimated needs (Table 3.4). Whether there were also shortfalls in energy is uncertain; estimates for absolute energy requirements shown in Table 3.4 could be over-estimated because of the small size of these infants together with their inability to experience full catch-up growth because of the challenges of overcoming inter-generational growth stunting (Dewey, 2001, Kimmons et al., 2005).

#### **3.4.5 Dietary diversity and gut microbiota**

We failed to demonstrate a relationship between dietary diversity and subsequent growth in this study, as noted earlier. Nevertheless, the benefits of dietary diversity on the maturity of gut microbiota is well recognized. Gut microbiota have important roles in metabolism, immunity, and development of the host (Erkosar et al., 2013, Backhead et al., 2015). Studies have suggested that more diverse diet leads to more diverse gut microbiota which are more adaptable to the changes of the environment (Heiman et al., 2016, Sonnenburg et al., 2016), and may have the potential to prevent growth faltering (Gough et al., 2015). In contrast, low microbial diversity in early life has been linked to some diseases, such as eczema (Forno et al., 2008; Ismail et al., 2012), asthma (Abrahamsson et al., 2014), and type 1 diabetes (Giorgio et al., 2010; Kostic et al., 2015). The first year of life has been designated as a window of opportunity for microbial modulation, and comprises a phase that coincides with the complementary feeding period (Laursen et al., 2017; Kostic et al., 2015). Introduction of complementary foods with a high protein and dietary fibre content may provide selective advantages for specific microbes to establish in the gut, which will increase diversity (Laursen et al., 2016). However, the best combination of complementary foods to improve microbiota maturity is still unknown and needs further research (Blanton et al., 2016, Heiman et al., 2016).

### 3.4.6 Strengths and limitations

We recognize that our study has strengths and limitations. Our findings were based on a prospective cohort study that followed the same infants at 6, 9, and 12 months, so we can define the incidence and investigate potential causes of impaired growth. Moreover, our anthropometric findings are reliable as we used standardised and calibrated equipment, with acceptable inter- and intra-examiner TEMs. In addition, our WHO CF indicators were compiled mainly from weighed food records on 2 non-consecutive days instead of a single 24-hr recall from maternal self-report. We also compiled a food composition table specific for the study setting that included adjustments for the mandatory wheat flour micronutrient fortification levels in Indonesia. Nevertheless, we restricted our evaluation of nutrient adequacy to 8 key micronutrients, omitted niacin data expressed as niacin equivalents, and excluded vitamin B-6, vitamin B-12, and folate, all micronutrients that have been included elsewhere (Working Group of Infant and Young Child Feeding Indicators, 2006, 2007) because of the paucity of reliable Indonesian nutrient composition values for these micronutrients.

However, we acknowledge that our comparison of nutrient intakes with the WHO estimated needs is based on assumed values for breast milk volume and composition and not measurements undertaken in our population. Hence, the deficits for vitamin A, niacin, and riboflavin reported here may be even greater if breast milk concentrations were compromised by poor maternal status (WHO, 1998). Finally, we chose to use the WHO/FAO RNIs, except for zinc rather than the most recent values compiled by European Food Safety Authority (EFSA, 2013) to facilitate comparison of our data with published reports in other LICs. In addition, EFSA recommendations for breast fed infants aged 6 months assumed an average volume of breast milk of 800 mL/day compared to the lower value (i.e., 593 mL/day) used by WHO (2002).

### 3.4.7 Implications for programmes

Our results indicate that the intakes of selected micronutrients and possibly energy from complementary foods must be increased to meet the needs of these Sumedang infants across all three ages. This should not be achieved,

however, by recommending any further increase in the consumption of specially FIFs, despite their seemingly positive effect on linear growth. Mothers seem to over-rely on this single food group for CF for infants at 6 and to a lesser extent at 9 months of age, thus reducing dietary diversity, and because of their expense, prepare them with a low dry matter content than recommended. Instead, caregivers should be advised to prepare FIFs with the recommended dry matter content. In addition, caregivers should be encouraged to increase the consumption during the CF period of appropriate and affordable animal-source foods (i.e., dairy products, flesh foods, and eggs), fruits and vegetables to ensure the WHO indicators for MDD and MAD are achieved. In this way infants will also become exposed to a variety of textures and flavours, thus facilitating the development of healthy food preferences (Birch and Doub, 2014) and the maturity of gut microbiota. Finally, our results emphasize the use of a local sentinel food group (FIFs) as a practical indicator for predicting subsequent infant growth in this setting.

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## **4 Interpreting biomarkers of iron, vitamin A, zinc, and selenium status after accounting for inflammation in a cohort of Indonesian infants**

### **4.1 Introduction**

Micronutrient deficiencies (MNDs) are widespread in many low-income countries (Ramakrishnan, 2002), especially among infants and children under 5 years of age. The most common micronutrient deficiencies are vitamin A, iron, and zinc (Ramakrishnan, 2002, Bailey et al., 2015), although selenium deficiency is an increasing concern (Mutakin et al., 2016). Deficiency of all four of these micronutrients compromise the immune system (Gibson, 2005, Raiten et al., 2015), resulting in increases in morbidity and mortality during early childhood, while iron and zinc deficiencies are also associated with an increased risk of impairments in both growth and cognition (King et al., 2016).

Blood biomarkers are used to assess micronutrient status, however, the presence of inflammation or infection confounds the assessment of these four micronutrients (Brown et al., 1993, Brown, 1998, Thurnham et al., 2003, Raiten et al., 2015), elevating serum ferritin levels, and reducing concentrations of serum zinc, RBP, and selenium (Raiten et al., 2015). Consequently, the resulting deficiency prevalence estimates may not reflect the true burden unless inflammation or infection has been taken into account.

During the acute phase response initiated by inflammatory cytokines, the hepatic synthesis of several proteins, termed APPs, increases (Raiten et al., 2015). Some of these APPs respond to the acute phase response by increases in circulating plasma levels (i.e., positive APPs) such as ferritin, and others (e.g., RBP, zinc, and selenoprotein P) by decreases (i.e., negative APPs). Of the APPs, some, notably CRP and AGP are most commonly used as inflammatory biomarkers (WHO, 2014), each providing a measure of the severity and duration of inflammation, respectively (Thurnham D.I. and McCabe G.P., 2012, Raiten et al., 2015, Suchdev et al., 2016).

In the past, estimates of micronutrient deficiencies have often been based on excluding participants with an elevated CRP and/or AGP concentration (Thurnham et al., 2010). However, such an approach may reduce the sample size and/or introduce a sampling bias in low-income settings where the burden of inflammation is often high (Raiten et al., 2015). As a result, several approaches to adjust the concentrations of micronutrient biomarkers affected by the inflammatory response have been developed (Thurnham et al., 2003, Beard et al., 2006, Thurnham et al., 2010, Bui et al., 2012, Engle-Stone et al., 2013, Cichon et al., 2017). For example, Thurnham and co-workers (Thurnham et al., 2003, Thurnham et al., 2010) adjusted for four levels of inflammation based on elevated serum CRP and/or AGP concentrations by applying calculated internal or external correction factors. The internal correction factors were generated from study population data whereas the external correction factors were generated from a meta-analysis (Thurnham et al., 2015). Recently a new approach involving regression modelling has been recommended by the BRINDA Project, a collaborative research group of CDC, National Institute for Child Health and Human Development, and Global Alliance for Improved Nutrition. A major advantage of the new regression approach is that the inflammatory biomarkers (CRP and AGP) are treated as continuous variables so that greater corrections can be applied when the inflammatory biomarkers indicate severe inflammation (Raiten et al., 2015). This approach is in contrast to the method of Thurnham and co-workers which applies specific cut-offs for elevated levels of serum CRP and AGP.

Therefore, the aims of this study were to: a) characterise biomarkers for iron, zinc, vitamin A, and selenium status for a cohort of infants aged 6, 9, and 12 months of age from Sumedang District, Indonesia after adjustment for inflammation using the new standardised BRINDA approach; b) determine the proportion of micronutrient deficiencies after the BRINDA adjustment for inflammation, and c) determine trends in biomarker levels and proportion of deficiencies between the ages of 6 months to 12 months and report sex differences at each age, d) compare these proportion estimates with those based on the unadjusted and Thurnham adjusted micronutrient

concentrations for infants at each age. Proportion estimates for ID were based on serum ferritin or sTfR, whereas for deficiencies of zinc, vitamin A, and selenium, serum concentrations of zinc, RBP, and selenium, respectively were used.

## **4.2 Methods**

### **4.2.1 Study design and participants**

This study was a prospective, longitudinal study designed to evaluate micronutrient status and growth of breastfed Indonesian infants and conducted between August 2013 and August 2014. Infants were enrolled at 6 months and followed-up at 9 and 12 months of age. Infants were randomly selected from all thirty villages in three sub-districts of Sumedang district, West Java, using local birth registry data.

Pre-tested structured questionnaires were used to collect information on the socio-demographic, health, and morbidity status of the participants. Information collected on health status of the infants included their vaccination and hospitalisation history, deworming and vitamin A supplementation in the past 6 months, and maternal reports of fever, diarrhoea, vomiting, and cough in the last 2 weeks prior to the examination. The protocol was approved by the Human Ethics Committees of *Universitas Padjadjaran*, Indonesia, and the University of Otago, New Zealand.

### **4.2.2 Blood collection and processing**

Experienced phlebotomists collected morning non-fasting venepuncture blood samples from the infants. Blood was drawn into an evacuated tube containing EDTA as an anticoagulant (Becton Dickinson, Franklin Lakes, NJ, USA) and into a trace-element-(TE)-free evacuated tube (Becton Dickinson, Franklin Lakes, NJ, USA) following the IZINCG procedures (Brown et al., 2004). The presence of symptoms of infection were also recorded at this time. All blood samples were labelled, and both the time of blood collection and last meal recorded prior to transfer to a chilled container (-4°C). Blood samples were transported within 6-8 hours after collection to the base laboratory,

where serum was separated using TE-free techniques (Brown et al., 2004). Aliquots of serum were frozen at -20°C, and later shipped on dry ice to the Department of Human Nutrition, University of Otago, New Zealand and to the laboratory of Dr J Erhardt in Germany for analysis.

#### 4.2.3 Biomarker analyses

Hb was analysed in a local laboratory (Prodia Laboratory) using an automated counter (Sysmex XN 1000, Sysmex Corporation, Kobe, Japan). Serum was analysed in duplicate in the laboratory of Dr J Erhardt for ferritin, sTfR, RBP, CRP, and AGP by a combined sandwich enzyme-linked immunosorbent assay (ELISA) technique (Erhardt et al., 2004). The inter-assay coefficients of variation (CVs) of a control sample on 26 ELISA plates for each of the proteins assayed by the sandwich ELISA were 3.0% for ferritin, 3.3% for sTfR, 3.3% for RBP, 6.7% for CRP, and 9.2% for AGP.

Serum zinc and selenium were analysed by inductively coupled plasma mass spectrometry (ICP-MS) (Agilent 7500ce ICP-MS, Agilent Technologies, Tokyo, Japan, CV <3%) at the University of Otago. Frozen samples were thawed to room temperature and vortexed. A 100uL aliquot was diluted 1:20 with a diluent containing 0.5%v/v tetra methyl ammonium hydroxide (TMAH, 25%v/v Merck Synthesis), 0.05%v/v H4EDTA Ajax Finechem, 2% v/v n butanol (BDH HiPerSolV), 0.05% Triton X-100 and 20ug/L germanium as a reference element. Instrument calibration used solutions prepared from a National Institute of Standards (NIST) traceable multi-element standard (High Purity Standards SC, USA) with matrix matching on a pooled sample. Measurements on multiple isotopes of the analytes were made to confirm removal of any interferences by the collision and reaction gases used during the detection of zinc and selenium, respectively. Repeated analysis of the pooled sample (n=38) and the UTAK control sample (n=41) indicated the analytical variation to be less than 3% CV for both zinc and selenium with the mean results for the UTAK control within 3% of both verified values.

Thresholds for defining suboptimal biomarkers were as follows: Hb <110 g/L (WHO, 2011a); serum ferritin <12 µg/L (WHO, 2011b); sTfR >8.3 mg/L (Erhardt et

al., 2004); RBP <0.83  $\mu\text{mol/L}$  (Engle-Stone et al., 2011); serum zinc <9.9  $\mu\text{mol/L}$  (Hess et al., 2007); and serum selenium  $\leq 0.82$   $\mu\text{mol/L}$  (Thomson, 2004). Inflammation was assessed by serum CRP >5 mg/L and AGP >1 g/L, respectively (Thurnham et al., 2010). Values for ferritin, sTfR, RBP, zinc, and selenium were adjusted for inflammation using both CRP and AGP.

#### 4.2.4 Statistical analyses

Descriptive statistics were calculated for selected health, morbidity, and biomarker variables at ages 6, 9 and 12 months. All continuous biomarker variables were plotted and visually assessed for normality. Ferritin, sTfR, RBP, zinc, selenium, CRP, and AGP were log transformed to reflect better regression diagnostics. Mean (SD) or geometric means (GM) (95% CI) were calculated, where appropriate, for Hb, and unadjusted and adjusted concentrations for serum ferritin, sTfR, RBP, zinc, and selenium at each age. Serum ferritin and RBP values were adjusted for subclinical inflammation using 3 approaches: 1) internal correction factors (ICFs) calculated from the study population data using Thurnham's method (Thurnham et al., 2010, Thurnham et al., 2015); 2) external correction factors (ECFs) derived from the meta-analysis of 32 studies for iron and 15 studies for vitamin A by Thurnham and co-workers (Thurnham et al., 2010, Thurnham et al., 2003); and 3) the recent BRINDA regression approach (Larson et al., 2016, Suchdev et al., 2016). Values for serum sTfR, selenium, and zinc were adjusted for inflammation using both internal correction factors (ICFs) derived according to Thurnham et al. (2010) and the BRINDA regression approach. Before serum zinc was adjusted for inflammation, concentrations were adjusted for both time of day and the time since the last meal prior to the blood collection (Brown et al., 2004, Arsenault et al., 2011, King et al., 2016).

For the BRINDA regression approach, linear regression analysis for each biomarker was run with the biomarker as the dependent variable; and CRP and AGP as the independent variables. The slope (regression coefficient) of CRP and AGP was used to adjust for the effect of inflammation. The equation used for the BRINDA regression approach was:  $\exp[\text{unadjusted } \ln \text{ biomarkers} - (\text{regression coefficient for CRP}) * (\text{CRP} - (\text{maximum of lowest decile for CRP})) -$

*(regression coefficient for CRP)\*(AGP - (maximum of lowest decile for AGP))*].

A reference concentration (maximum of lowest decile) for serum CRP and AGP was used to avoid over-adjusting the micronutrient biomarkers among individuals with low levels of inflammation. All models were checked to ensure all assumptions were met by examining the plot of residuals for homogeneity of variance and normality.

Mixed effects regression models were used to detect trends in biomarker concentrations by age and by adjustment method, with participant identification as a random effect, while generalised estimating equations were used to detect changes in the proportion of micronutrient deficiencies at 6, 9, to 12 months. The paired t-test and the McNemar test were used to detect differences in biomarker concentrations and the proportion of deficiencies before and after adjustment using the regression approach, respectively. Sex differences in the proportion with micronutrient deficiencies were examined using the chi-square test. Statistical analyses were conducted using Stata® 12 (StataCorp LP, Texas, USA). A P-value <0.05 was used to assess statistical significance.

### **4.3 Results**

There were 230, 202, and 190 respondents at 6, 9, and 12 months. Blood was collected from all respondents, but the amount was not enough for conducting a CBC test for 2 and 3 respondents at 6 and 9 months, respectively. We could not collect enough blood samples for sTfR, ferritin, RBP, CRP, and AGP examinations for 16, 6, and 5 respondents at 6, 9, and 12 months (respectively). A further reduction was observed as 36 respondents at all age groups had not enough blood samples for zinc and selenium examinations. The proportion of male and female in this study is quite constant at all age groups, with 45-46% male participants (Table 4.1).

#### **4.3.1 Health characteristics**

More than two thirds of the infants at 12 months had received vitamin A supplements since the last visit, and almost 90% had been completely

vaccinated by 12 months of age. The proportion of infants who had ever been hospitalised was low at all ages, ranging from 0.5% to 5.1% depending on the age of the infants. In contrast, the percentage of infants with fever and/or cough ranged from 43% at 6 months to 51% at 12 months, whereas those experiencing vomiting and diarrhoea was lower (11-18%). The proportions of infants with inflammation (based on CRP >5 mg/L or AGP >1 g/L) were similar (approximately 20%) at all three ages. The proportion of any inflammation based on elevated CRP (>5 mg/L) and/or AGP (>1 g/L) concentrations was slightly higher at approximately 25% at 6, 9, and 12 months. In general, based on the Thurnham approach, more infants were classified in the early convalescence stage of inflammation at all three ages (Table 4.1).

#### **4.3.2 Assessment of micronutrient biomarkers unadjusted and adjusted for inflammation**

Table 4.2 also presents the GM (95% CI) unadjusted and adjusted for inflammation, where appropriate, for Hb, sTfR, ferritin, RBP, serum zinc, and selenium concentrations for the three age groups. GM of Hb at all ages were similar, about 110 g/L. There were significant age-related trends in GM values ( $P<0.001$ ) for serum sTfR, ferritin, RBP, and selenium, but not zinc, irrespective of the adjustment method applied.

All adjusted GM values for serum ferritin and sTfR were lower than those based on the unadjusted values ( $P<0.001$ ), with the BRINDA regression approach producing the lowest GM values at all three ages. In contrast, all adjusted GM values for RBP and zinc were higher at all three ages compared with the unadjusted values ( $P<0.001$ ). For RBP and zinc, the highest GM values were derived with the BRINDA regression approach. GM values for adjusted serum selenium concentrations were significantly lower at 6 and 9 months, but higher at 12 months ( $P<0.001$ ) (Table 4.2).

The proportion of anaemia based on unadjusted Hb <110 g/L ranged from 33 to 42%, with the highest proportion at 9 months. For ID, the proportion of deficiency varied depending on the biomarker applied, the adjustment

approach used, and the age group. The BRINDA regression approach generated the highest proportion of ID based on low serum ferritin, with increases ranging from 5-20% compared to the unadjusted values.

**Table 4.1 Health and morbidity characteristics**

	6 months		9 months		12 months	
	n	%	n	%	n	%
Maternal age (year)	226	27.5 (7.2)				
Sex (male)	107/230	46.5%	93/202	46.0%	86/190	45.3%
Anthropometric status						
Stunting (LAZ)	36/230	15.7%	39/202	19.3%	43/190	22.6%
Wasting (WLZ)	4/230	1.7%	4/202	2.0%	6/190	3.2%
Underweight (WAZ)	9/230	3.9%	11/202	5.4%	20/190	10.5%
Vit A supplementation <sup>‡</sup>	N/A		101/189	53.4%	132/185	71.4%
Complete vaccination <sup>§</sup>	N/A		N/A		165/185	89.2%
Hospitalisation						
1-2 times	11/217	5.1%	1/189	0.5%	1/185	0.5%
3 or more times	0/217	0.0%	0/189	0.0%	0/185	0.0%
Morbidity in the last 2 weeks						
Fever	93/217	42.9%	96/189	50.8%	82/185	44.3%
Vomiting	32/217	14.7%	30/189	15.9%	20/185	10.8%
Diarrhoea	39/217	18.0%	20/189	10.6%	21/185	11.4%
Cough	103/217	47.5%	93/189	49.2%	88/185	47.6%
CRP >5 mg/L	41/212	19.3%	38/193	19.7%	30/185	16.2%
AGP >1 g/L	35/212	16.5%	43/193	22.3%	41/185	22.2%
Any inflammation <sup>  </sup>	54/212	25.5%	51/193	26.4%	49/185	26.5%
Stage of inflammation <sup>¶</sup>						
No inflammation	158/212	74.5%	142/193	73.6%	136/185	73.5%
Incubation	19/212	9.0%	8/193	4.1%	8/185	4.3%
Early convalescence	22/212	10.4%	30/193	15.5%	22/185	11.9%
Late convalescence	13/212	6.1%	13/193	6.7%	19/185	10.3%

LAZ, Length-for-age z-score; WLZ, Weight-for-length z-score; WAZ, Weight-for-age z-score; CRP, C-reactive protein; AGP,  $\alpha$ -1-glycoprotein.

<sup>‡</sup>In the last 9 months and since the last examination (for 12 months)

<sup>§</sup>At least 1 Bacillus Calmette-Guerin (BCG), 1 Polio, 1 Diphtheria, Pertussis, Tetanus (DPT), 1 Hepatitis B, and 1 Measles (taken from immunisation card)

<sup>||</sup>Any inflammation (CRP >5 mg/L or AGP >1 g/L)

<sup>¶</sup>Stage of inflammation: No inflammation (CRP <5 mg/L and AGP <1 g/L); Incubation (CRP >5 mg/L and AGP <1 g/L); Early convalescence (CRP >5 mg/L and AGP >1 g/L); Late convalescence (CRP  $\leq$ 5 mg/L and AGP >1 g/L)

**Table 4.2 Geometric means (95% CI) values of haemoglobin and micronutrient biomarkers**

	6 months		9 months		12 months		P-trend
	n	GM (95% CI)	n	GM (95% CI)	n	GM (95% CI)	
<b>Hb, g/L</b>	228	112 (111-114)	199	111 (109-112)	190	11.1 (109-113)	
<b>Soluble transferrin receptor (sTfR), mg/L</b>							
No adjustment	212	6.3 (5.9-6.7)	193	7.0 (6.6-7.5)	185	7.5 (7.0-8.0)	
ICFs‡	212	6.2 (5.8-6.6)	193	7.0 (6.6-7.5)	185	7.9 (7.4-8.5)	P<0.001
Regression	212	5.9 (5.6-6.3)	193	6.9 (6.4-7.3)	185	7.4 (6.9-7.9)	P<0.001
Mean percent difference between no adjusted and regression method (95% CI)		-6.1% (-5.2%,-7.0%)		-2.7% (-2.2%, -3.2%)		-1.2% (-0.5%, -1.9%)	
<b>Ferritin, µg/L</b>							
No adjustment	212	32.6 (28.9-36.8)	193	20.9 (18.5-23.6)	185	14.5 (13.6-17.5)	
ICFs‡	212	30.4 (27.0-34.2)	193	16.2 (14.9-18.5)	185	12.3 (11.1-13.8)	P<0.001
ECFs§	212	29.3 (26.0-33.0)	193	18.1 (16.2-20.3)	185	13.6 (12.1-15.2)	P<0.001
Regression	212	25.4 (22.7-28.5)	193	13.4 (12.0-14.9)	185	8.8 (8.0-9.8)	P<0.001
Mean percent difference between no adjusted and regression method (95% CI)		-28.4% (-23.7%,-33.2%)		-56.3% (-48.0%,-65.2%)		-74.9% (-64.2%,-86.4%)	
<b>Retinol binding protein (RBP), µmol/L</b>							
No adjustment	212	0.91 (0.90-0.96)	193	0.94 (0.91-0.97)	185	0.98 (0.94-1.01)	
ICFs‡	212	0.96 (0.93-0.98)	193	0.99 (0.96-1.02)	185	1.02 (0.98-1.05)	P<0.001
ECFs§	212	0.95 (0.92-0.98)	193	0.99 (0.96-1.03)	185	1.02 (0.99-1.06)	P<0.001
Regression	212	1.01 (0.99-1.04)	193	1.08 (1.05-1.11)	185	1.07 (1.04-1.10)	P<0.001
Mean percent difference between no adjusted and regression method (95% CI)		10.4% (9.4%,11.4%)		12.8% (11.4%,14.2%)		8.7% (7.7%,9.8%)	
<b>Zinc, µmol/L</b>							
No adjustment	194	11.3 (11.0-11.7)	166	10.8 (10.5-11.0)	154	11.5 (11.2-11.7)	
Time adjustment¶	194	11.5 (11.1-11.9)	166	10.5 (10.3-10.8)	154	11.3 (11.0-11.6)	
ICFs‡	189	11.7 (11.4-12.1)	165	10.8 (10.6-11.1)	154	11.4 (11.1-11.7)	P=0.751
Regression	189	12.1 (11.7-12.5)	165	11.3 (11.1-11.5)	154	11.7 (11.4-12.0)	P=0.054
Mean percent difference between no adjusted and regression method (95% CI)		6.2% (5.7%,6.7%)		4.6% (3.8%,5.5%)		2.0% (1.5%,2.4%)	
<b>Selenium, µmol/L</b>							
No adjustment	194	0.75 (0.73-0.77)	166	0.80 (0.78-0.82)	154	0.80 (0.78-0.82)	
ICFs‡	189	0.75 (0.73-0.77)	165	0.79 (0.77-0.81)	154	0.80 (0.78-0.82)	P<0.001
Regression	189	0.74 (0.72-0.75)	165	0.78 (0.76-0.80)	154	0.82 (0.80-0.84)	P<0.001
Mean percent difference between no adjusted and regression method (95% CI)		-2.4% (-1.6%; -3.1%)		-1.6% (-1.3%; -1.8%)		1.8% (1.5%; 2.1%)	

‡ICFs: Study generated/Internal Correction Factors

§ECFs: Thurnham External Correction Factors

|| Adjusted for inflammation = exp[unadjusted ln biomarkers - (regression coefficient for CRP)\*(CRP - (maximum of lowest decile for CRP)) - (regression coefficient for AGP)\*(AGP - (maximum of lowest decile for AGP))]

¶ Adjusted for time of the day and interval since the last meal = exp [unadjusted ln biomarkers + (regression coefficient for time of day\*(time of day-8) - (regression coefficient for interval since previous meal\*interval since previous meal))]

**Table 4.3 Proportion of micronutrient deficiencies with and without adjustments**

	6 months		9 months		12 months		P-trend
	n	%	n	%	n	%	
<b>Anaemia (Hb), &lt;110 g/L</b>	75/228	32.9%	84/199	42.2%	73/190	38.4%	
<b>Soluble transferrin receptor (sTfR), &gt;8.3 mg/L</b>							
No adjustment	46/212	21.7%	64/193	33.2%	74/185	40.0%	
ICFs‡	46/212	21.7%	65/193	33.7%	84/185	45.4%	
Regression	36/212	17.0%	54/193	28.0%	71/185	38.4%	P<0.001
No adj vs reg		P=0.002		P=0.002		P=0.453	
<b>Ferritin, &lt;12 µg/L</b>							
No adjustment	35/212	16.5%	51/193	26.4%	83/185	44.9%	
ICFs‡	36/212	17.0%	66/193	34.2%	101/185	54.6%	
ECFs§	38/212	17.9%	57/193	29.5%	93/185	50.3%	
Regression	45/212	21.2%	87/193	45.1%	120/185	64.9%	P<0.001
No adj vs reg		P=0.006		P<0.001		P<0.001	
<b>Retinol binding protein (RBP), &lt;0.83 µmol/L</b>							
No adjustment	73/212	34.4%	55/193	28.5%	45/185	24.3%	
ICFs‡	60/212	28.3%	43/193	22.3%	35/185	18.9%	
ECFs§	60/212	28.3%	43/193	22.3%	33/185	17.8%	
Regression	37/212	17.5%	16/193	8.3%	23/185	12.4%	P=0.059
No adj vs reg		P<0.001		P<0.001		P<0.001	
<b>Zinc, &lt;9.9 µmol/L</b>							
No adjustment	49/194	25.3%	39/166	23.5%	20/154	13.0%	
Time adjustment¶	41/194	21.1%	45/166	27.1%	25/154	16.2%	
ICFs‡	32/189	16.9%	38/165	23.0%	21/154	13.6%	
Regression	27/198	14.3%	27/165	16.4%	16/154	10.4%	P=0.287
No adj vs reg		P<0.001		P<0.001		P=0.219	
<b>Selenium, ≤0.82 µmol/L</b>							
No adjustment	135/194	69.6%	93/166	56.0%	87/154	56.5%	
ICFs‡	132/189	69.8%	98/165	59.4%	83/154	53.9%	
Regression	138/189	73.0%	97/165	58.8%	81/154	52.6%	P<0.001
No adj vs reg		P=0.039		P=0.219		P=0.031	

‡ICFs: Study generated/Internal Correction Factors

§ECFs: Thurnham External Correction Factors

|| Adjusted for inflammation =  $\exp[\text{unadjusted ln biomarkers} - (\text{regression coefficient for CRP}) * (\text{CRP} - (\text{maximum of lowest decile for CRP})) - (\text{regression coefficient for AGP}) * (\text{AGP} - (\text{maximum of lowest decile for AGP}))]$

¶ Adjusted for time of the day and interval since the last meal =  $\exp [\text{unadjusted ln biomarkers} - (\text{regression coefficient for CRP}) * (\text{CRP} - (\text{maximum of lowest decile for CRP})) - (\text{regression coefficient for AGP}) * (\text{AGP} - (\text{maximum of lowest decile for AGP})) + (\text{regression coefficient for time of day}) * (\text{time of day} - 8) + (\text{regression coefficient for interval since previous meal}) * (\text{interval since previous meal})]$

In contrast, the proportion of ID based on adjusted elevated sTfR levels was lower across all three age groups compared to those based on adjusted serum ferritin <12 µg/g, with only slight changes in the proportion after adjusting for inflammation (Table 4.3).

**Table 4.4 Proportion of micronutrient deficiencies with adjustments stratified by sex**

	6 months		9 months		12 months	
	n	%	n	%	n	%
<b>sTfR, &gt;8.3 mg/L</b>						
Regression <sup>  </sup>	36/212	17.0%	54/193	28.0%	71/185	38.4%
Boys	25/96	26.0%	28/90	31.1%	37/84	44.0%
Girls	11/116	9.5%	26/103	25.2%	34/101	33.7%
		P<0.001		P=0.365		P=0.148
<b>Ferritin, &lt;12 µg/L</b>						
Regression <sup>  </sup>	45/212	21.2%	87/193	45.1%	120/185	64.9%
Boys	29/96	30.2%	45/90	50.0%	60/84	71.4%
Girls	16/116	13.8%	42/103	40.8%	60/101	59.4%
		P=0.004		P=0.199		P=0.088
<b>Retinol binding protein (RBP), &lt;0.83 µmol/L</b>						
Regression <sup>  </sup>	37/212	17.5%	16/193	8.3%	23/185	12.4%
Boys	22/96	22.9%	9/90	10.0%	14/84	16.7%
Girls	15/116	12.9%	7/103	6.8%	9/101	8.9%
		P=0.057		P=0.421		P=0.111
<b>Zinc, &lt;9.9 µmol/L</b>						
Regression <sup>  </sup>	27/198	14.3%	27/165	16.4%	16/154	10.4%
Boys	15/86	17.4%	14/84	16.7%	7/68	10.3%
Girls	12/103	11.7%	13/81	16.0%	9/86	10.5%
		P=0.888		P=0.915		P=0.972
<b>Selenium, ≤0.82 µmol/L</b>						
Regression <sup>  </sup>	138/189	73.0%	97/165	58.8%	81/154	52.6%
Boys	63/86	73.3%	52/84	61.9%	31/68	45.6%
Girls	75/103	72.8%	45/81	55.6%	50/86	58.1%
		P=0.944		P=0.407		P=0.121

<sup>||</sup> Adjusted for inflammation = exp[unadjusted ln biomarkers - (regression coefficient for CRP)\*(CRP - (maximum of lowest decile for CRP)) - (regression coefficient for AGP)\*(AGP - (maximum of lowest decile for AGP))]

For vitamin A deficiency, the proportion decreased at 6, 9, and 12 months irrespective of the inflammation adjustment approach used, with the lowest proportion after regression adjustment, with decreases ranging from 12-20% compared with the proportion based on unadjusted RBP. The proportion for zinc deficiency also decreased after applying Thurnham inflammation adjustments but only at 6 and 9 months, whereas after using the regression adjustment, time of day, and the interval since the last meal, the proportion decreased across all ages by 3 to 11%. In contrast, changes in the proportion of selenium deficiency were less marked and inconsistent after adjustment, increasing slightly at 6 (P<0.05) and 9 months (P=0.219), but decreasing at 12 months after applying the regression approach (P<0.05) (Table 4.3).

Sex-related differences for the proportion of ID, irrespective of the biomarker used, were apparent at 6 months only, with boys having a higher proportion than girls ( $P<0.05$ ). No sex-related difference existed for vitamin A, zinc, and selenium (Table 4.4).

## 4.4 Discussion

Our findings highlight a high proportion of iron and selenium deficiency among these Indonesian infants compared to a lower proportion of vitamin A and zinc deficiency, especially at 12 months of age. Furthermore, our study has confirmed that correcting biomarkers of iron, vitamin A, and zinc for inflammation, irrespective of the method used, markedly changed the proportion estimates for deficiency for these Indonesian infants in all three age groups. The regression adjustment approach presented here significantly increased the proportion of ID (based on adjusted low serum ferritin values), while simultaneously decreasing the proportion of vitamin A and zinc deficiency. The proportion of ID was significantly greater in boys than girls at 6 months. Adjusting for inflammation also had a significant effect on the proportion of selenium deficiency, but the direction of the effect varied across age groups.

### 4.4.1 Iron status and ID

GM values of serum ferritin among these Indonesian infants at 6 and 9 months (even after adjustment for inflammation using regression approach) were much higher than those reported for infants of a comparable age in Indonesia (Wieringa et al., 2002, Fahmida et al., 2007) and elsewhere in Asia (Wieringa et al., 2007, Lander et al., 2008). The high consumption of infant foods fortified with multiple micronutrients (including iron) at age 6 months in this population was probably a major factor contributing to these higher adjusted serum ferritin concentrations. However, by 12 months, serum ferritin concentrations had significantly declined ( $P<0.001$ ) (Table 4.2).

This downward trend has been reported earlier (Osiki, 1993, Domellof et al., 2014), and associated with a gradual depletion of iron stores for growth which

fail to be replaced by an adequate supply of absorbable iron from the diet. In this study, the decline was probably exacerbated by the progressive fall after 6 months of age in intake of commercial infant foods fortified with iron (see section 3.3.3), which were not entirely replaced by wheat flour products fortified with iron. In Indonesia wheat flour is fortified with 50 mg Fe/kg nationally (*Menteri Kesehatan Republik Indonesia, 2003*). However, the form of the iron fortificant is uncertain, and may be poorly available. WHO recommends using ferrous fumarate/sulphate (WHO/FAO/UNICEF/GAIN/MI/FFI, 2009) as the fortificant rather than electrolytic iron to improve bioavailability. Indeed, in view of the high proportion of low adjusted ferritin levels among these infants at 12 months of age, it appears that their intake of bioavailable dietary iron was not sufficient to prevent a significant rise in the proportion of ID (Table 4.3).

Another factor, besides inflammation, that may have contributed to the elevated serum ferritin concentrations at 6 months of age is vitamin A deficiency. Mobilization of iron is impaired in vitamin A deficiency resulting in an accumulation of iron in the liver and spleen, reflected by increased levels of serum ferritin, even though Hb synthesis is reduced (International Vitamin A Consultative Group, 1998). In studies of children with co-existing iron and vitamin A deficiency, the absence of low serum ferritin concentrations can potentially confound the detection of ID (Bloem et al., 1989, Thurlow et al., 2005). Hence, in this Indonesian study, it is possible that vitamin A deficiency may have also had a role in the high adjusted serum ferritin concentrations, particularly at 6 months of age. It has been suggested that zinc deficiency may also facilitate the accumulation of iron, sequestered mainly as ferritin, thus increasing serum ferritin concentrations (Niles et al., 2008), although this finding remains to be confirmed in human studies.

#### **4.4.2 Vitamin A status and deficiency**

In the study presented here, serum RBP was used as a proxy for serum retinol concentrations to identify vitamin A deficiency. Use of serum RBP is recommended because it is more stable than retinol (Fujita et al., 2009), the assay is cheaper and easier, and RBP concentrations correlate closely with

serum retinol provided individuals are not obese and have normal kidney function (Tanumihardjo et al., 2016). RBP is not consistently 100% saturated with retinol so the RBP-retinol molar ratio is not always ~1:1. However, we were not able to measure serum retinol concentrations in a subset to establish a specific cut-off for serum RBP in our population as recommended by Tanumihardjo et al. (2016). Instead we adopted a cut-off of <0.83  $\mu\text{mol}$  RBP/L which was reported to correspond to serum retinol concentrations of <0.70  $\mu\text{mol}$ /L in a study of Cameroon children (Engle-Stone et al., 2011).

The proportion of vitamin A deficiency adjusted for inflammation in this population was lower than that reported in an earlier study of Indonesian infants (Dijkhuizen et al., 2001b), and no longer at a level considered a severe public health problem (i.e. >20%) (WHO, 1996). Since 1978, a vitamin A supplementation program has been initiated in Indonesia for both children under 5 years and post-partum mothers. Indeed, the Indonesian Basic Health Surveys (*Riskesdas*) have reported the national coverage of high-dose vitamin A supplements among children under five years as ~ 70% in 2007, 2010, and 2013, a level very comparable to that reported here at 12 months (71.4%) (Table 4.1). Hence, the high uptake of vitamin A supplements in later infancy is likely to be responsible in part for the lower proportion of vitamin A deficiency among the infants here compared to the earlier reports (Dijkhuizen et al., 2001b).

The coverage for vitamin A supplements among postpartum women in Indonesia is lower than for children under 5 years of age; only 52.2% in 2010 (National Institute of Health Research and Development, 2010). Nevertheless, for some of the Indonesian mothers, adequate levels of vitamin A from breast milk may have also contributed to the relatively low proportion of vitamin A deficiency reported here in later infancy (Allen, 2005).

The relatively low proportion of zinc deficiency noted here may have also played a role in the vitamin A status of the infants. A positive and linear association between zinc and vitamin A concentrations has been observed in previous studies (Munoz et al., 2000, Lander et al., 2014). This association is not

unexpected as zinc deficiency is known to depress the synthesis of RBP in the liver, thus reducing concentrations of RBP and hence lowering serum retinol. Studies have shown improvements in serum RBP concentrations following zinc supplementation (Udomkesmalee et al., 1992, Rahman et al., 2002).

#### **4.4.3 Zinc status and deficiency**

The low proportion of zinc deficiency reported here is not unexpected as several studies examining the zinc status of Indonesian infants have also observed comparable findings (Wieringa et al., 2002, Wieringa et al., 2003) in contrast to the higher prevalence observed in many other low resource settings (Engle-Stone et al., 2014). The prevalence of zinc deficiency was no longer at a level said to be indicative of a public health problem (i.e., >20%) (Hotz, 2007). Several factors may have played a role in these unexpected findings. For example, the concentration of zinc in soil and thus rice grown in parts of Indonesia is quite high, with levels reportedly about 24 µg/g (Herawati et al., 2000). These values are higher than those reported for rice in North East Thailand where the prevalence of zinc deficiency among school-aged children was much higher (Thurlow et al., 2006).

The high consumption of infant foods fortified with zinc at 6 months of age, followed by the consumption of zinc-fortified wheat flour products (30 µg/g) at 12 months may be additional factors contributing to the relatively low proportion of zinc deficiency observed here. Indeed, the wheat flour fortification standard in Indonesia is higher than other countries with mandatory fortification programmes (30 µg/g vs. 15 µg/g in South Africa and 16 µg/g in Mexico and UK) (Kimura, 2013). Nonetheless, despite evidence of absorption of the zinc fortificant in wheat flour products, few studies have shown any impact of zinc fortification on serum zinc or zinc-related health outcomes (Hess and Brown, 2009), perhaps because the zinc fortificant levels used were all lower than those in Indonesia.

#### **4.4.4 Selenium status and deficiency**

Despite the increasing concern about selenium deficiency, to our knowledge there are no data on the selenium status of infants in Indonesia. It is

noteworthy that 72.5% of the infants at 6 months had selenium deficiency. Moreover, although the proportion of selenium deficiency fell progressively with age, it was still high at aged 9 (58.8%) and 12 (52.6%) months. Indeed, overall, GM serum selenium concentrations at 12 months of age were at the lower end of international levels (Muntau et al., 2002).

Low selenium status has been reported among infants and young children in Mongolia (Lander et al., 2008) and in the South Island of New Zealand where soil selenium concentrations are known to be low (McLachlan et al., 2004), and affect the levels of selenium in locally grown plant foods (Zhao et al., 2005). A recent study has reported very low levels of selenium in a species of rice (0.011 µg/g) grown in Sumedang district (Holik et al., 2013), the major cereal staple of this district. Hence, lactating Sumedang mothers consuming low-selenium-rice without other dietary sources of selenium-rich foods may have low selenium intakes, and thus at risk of low selenium status. Maternal selenium status strongly affects the concentration of selenium secreted in breast milk (Allen, 2005), so that low breast milk selenium concentrations may be a major factor contributing to the very high proportion of selenium deficiency among the Sumedang breastfed infants, especially at 6 months of age. The progressive fall in the proportion of selenium deficiency in later infancy may be associated with the gradual increase in the consumption of selenium-rich foods such as fish and chicken liver in place of breast milk.

#### **4.4.5 Influence of inflammation on the interpretation of the micronutrient biomarkers**

In this study about 25% of the infants across all age groups had elevated CRP and/or AGP concentrations indicative of inflammation. Therefore, exclusion of these children from the analyses would reduce the sample size and may bias the results, as reported in other studies (Grant et al., 2012, Larson et al., 2016). Conducting the survey in a season with low rates of inflammation, as suggested by WHO (2011b) was not feasible here where the proportion of inflammation was reasonably constant during the 1 year study period (Table 4.1).

During the acute phase response induced by inflammation or infection, concentrations of serum ferritin, a positive APP secreted by the liver during inflammation, increase independent of iron status as noted earlier (Galloway et al., 2000, Tomkins, 2003, Northrop-Clewes, 2008). During the acute phase response, pro-inflammatory cytokines stimulate an increase in circulating hepcidin, which in turn reduces intestinal absorption of iron and its release from body stores (Wessling-Resnick, 2010, Ganz and Nemeth, 2012). As a consequence, concentrations of iron in the serum fall, whereas serum ferritin rises. This fall in serum iron limits the access to iron by invading pathological microorganisms for growth and reproduction, as well as reducing free radical production (Galloway et al., 2000). The impact of inflammation on serum levels of sTfR is less certain. The sTfR, unlike ferritin, is not an APP, and is said to be less influenced by the inflammatory response (Thurnham D.I. and McCabe G.P., 2012). In addition, it has been suggested that reduction of erythropoietin production and suppression of erythropoiesis by cytokines may prevent elevation of sTfR during the acute phase response (Beguin, 2003, Northrop-Clewes, 2008). Certainly the decrease in the GM of sTfR was much less here after inflammation adjustment than the reductions reported for sTfR in malarial endemic areas (Stoltzfus et al., 2000, Grant et al., 2012).

In contrast to ferritin, circulating levels of zinc, selenium, and RBP fall in response to inflammation (Galloway et al., 2000), irrespective of the true burden of micronutrient deficiencies in children. This fall in serum zinc and selenium is caused largely by redistribution of these micronutrients to the sites of inflammation. For zinc, uptake into the liver occurs for cytokine-induced hepatic metallothionein synthesis (King et al., 2016), whereas the selenoproteins (mainly glutathione peroxidase and thioredoxin reductase) are redistributed to the sites of inflammation (Galloway et al., 2000) because of their redox-regulating properties (Mattmiller et al., 2013). Serum levels of RBP fall in response to inflammation as a result of decreased synthesis of RBP mRNA in the liver, which interrupts the release of retinol-RBP (Rosales et al., 1996, Stephensen, 2001).

In this study we used two positive APPs to examine the impact of inflammation on the serum micronutrient biomarker concentrations: serum CRP and AGP. CRP is used as an early marker of inflammation or infection, rising within 24 h post-inflammation and normalizing rapidly, whereas AGP responds later, increasing after 48 hr and remaining elevated during the recovery period (Thurnham D.I. and McCabe G.P., 2012, Bresnahan and Tanumihardjo, 2014). Therefore by using CRP and AGP in combination we were able to capture the three phases of inflammation as well as the healthy state in our population of apparently healthy infants living in a setting with high prevalence of sub-clinical inflammation (Thurnham et al., 2005).

Not surprisingly, because all the adjustments used both CRP and AGP, the proportion estimates for ID were consistently lower and those for vitamin A and zinc deficiency consistently higher irrespective of the approach used. However, the effect of correcting for inflammation on the proportion of selenium deficiency was limited and varied with age, an outcome that was not unexpected, and consistent with earlier reports (Wieringa et al., 2002, Engle-Stone et al., 2013, Larson et al., 2016).

Applying adjustments based on internal or external correction factors for serum ferritin and RBP based on Thurnham's method resulted in comparable proportion estimates for deficiencies of iron and vitamin A, as reported earlier (Grant et al., 2012, Larson et al., 2016). However, there are some concerns regarding the use of the cut-offs for CRP (>5 mg/L) and AGP (>1 g/L) applied in the Thurnham approach irrespective of the magnitude and stage of the inflammatory response. Some individuals in apparently healthy subgroups may still have sub-clinical inflammation (based on these cut-offs) resulting in elevated ferritin and lower RBP concentrations in the reference subgroups. Hence, the true proportion of iron and vitamin A deficiency may still be under or overestimated (Bui et al., 2012). Some findings support the suggestion that using very low CRP cut-offs may result in more accurate adjustments for inflammation (Beard et al., 2006, Bui et al., 2012).

Marked differences were found between the proportion of iron, vitamin A, zinc, and selenium deficiency prior to and after adjustment using the regression approach. While the proportion of ID based on ferritin was increased significantly as expected, the proportion of both vitamin A and zinc was decreased across all age groups ( $P < 0.05$ ). Selenium was an exception, because the proportion of deficiency decreased significantly ( $P < 0.05$ ) at 12 months but increased at 6 and 9 months. Reasons for this inconsistency are unclear.

The regression approach can also be used to evaluate potential confounders of the effect of inflammation on the biomarkers (e.g., parasite infection) (Suchdev et al., 2016) as well as covariates (e.g., sex, SES) (Raiten et al., 2015). However, adjusting for other factors in the same models when we are adjusting for inflammation should be reconsidered, as it may mask the true condition and can lead into a wrong conclusion. The results of this study show that the proportion of ID was higher among boys than girls at 6 months, although there was no difference for vitamin A, zinc, or selenium deficiency. Although recommendations for the daily iron requirement do not differ by sex during infancy (U.S. Institute of Medicine, 2000), male infants are generally larger than females (even after adjustment for gestational age) (Storms and Van Howe, 2004), and hence probably require higher iron intakes after the EBF period has finished (Domellof et al., 2014). Our results suggest that sex should be taken into account as one of possible predictors for biomarker deficiency, especially for ferritin, sTfR, and RBP.

#### **4.4.6 Strengths and limitation of the study**

Our data are based on a longitudinal study designed to describe trends in micronutrient biomarker concentrations of infants at age 6, 9, and 12 months. Our study was restricted to infants from Sumedang district who were randomly selected from all the villages in the three sub-districts using local birth registry data. Hence it was not designed to evaluate the micronutrient status of a representative sample of infants from West Java, Indonesia. Nonetheless, the proportion of stunting in our Sumedang infants at 9 months was comparable to that reported for 6-11 month old infants in the West Java Province (19.3 vs.

21.5%), the most populous province in Indonesia. However our Sumedang infants had a higher proportion of anaemia based on a cut-off of 110 g/dL across all three age groups than national prevalence data reported for infants at 12-59 months (33-42% vs. 28.1 %) (National Institute of Health Research and Development, 2013). There is some concern that this WHO recommended cut-off may be too high for infants (Domellof et al., 2002) leading to an overestimate of the anaemia proportion in our study population.

We measured biomarkers for four micronutrients, concentrations of which were all known to be influenced by inflammation and infection to varying degrees. Moreover, we applied the new BRINDA regression approach to adjust these biomarkers for the presence of inflammation using serum CRP and AGP as recommended by WHO (2014). Our results have highlighted marked changes in the proportion of deficiency of these micronutrients by age, and in some cases sex, after the adjustment for inflammation, confirming the importance of taking age, sex, and inflammation into account when comparing such proportion estimates across countries. In addition, for serum zinc, concentrations were adjusted simultaneously for the influence of inflammation, time of day, and time since the last meal in an effort to reduce the variance in serum zinc, as recommended by IZiNCG (King et al., 2016).

Nonetheless our proportion estimates were based on cut-offs for the micronutrient biomarkers, some of which are ill defined during infancy, and inconsistent across studies making comparisons difficult (Raghavan et al., 2016). For example, our cut-off for RBP was drawn from the results of a national population-based study in the Cameroon (Engle-Stone et al., 2011) rather than a specific cut-off developed for our infant population. Likewise the cut-off used for serum zinc was developed for all children <10 years (Brown et al., 2004), despite the marked age-related changes in serum zinc concentrations during infancy (Hotz et al., 2003).

A further limitation is the cut-offs for the reference values for CRP and AGP defined by the maximum of the lowest decile. These reference values were

used to avoid over-adjusting the micronutrient biomarkers among individuals with low levels of inflammation, but are somehow arbitrary, as we have no proof that their use only excludes infants without inflammation. Nevertheless, applying different cut-offs for both biomarkers and the reference values for the APPs would not change our main conclusion.

Both the biomarkers' GMs and the proportion of deficiency changed in the expected direction after applying the regression approach. A notable exception was for the GM for selenium and its associated proportion of deficiency at aged 6 and 9 months, for reasons that are uncertain. It is possible that the discrepancy might be associated with the stage of inflammation, as a higher proportion of children at aged 6 and 9 months were among the incubation and early convalescence stages at these times, based on elevated CRP and/or CRP+AGP levels. Finally, it is well recognized that concentrations of some micronutrient biomarkers, notably serum zinc and ferritin, are subject to day-to-day variations, so that theoretically more than one independent measurement is required to obtain an accurate value (Borel et al., 1991). However, the age of our respondents precluded the collection of repeated blood samples.

In conclusion, our results emphasize the necessity for adjusting for inflammation when interpreting biomarkers of iron, vitamin A, zinc, and possibly selenium status. Without such adjustments, proportion estimates for these micronutrient deficiencies will be incorrect in settings with a high burden of inflammation. ID may be grossly under-estimated, and vitamin A and zinc deficiency over-estimated, and possibly at levels designated as public health problems that require urgent intervention, even though the true proportion estimates are much lower. However, further work is needed to validate the proposed approaches with a particular focus on assessing the influence of varying degrees of inflammation (i.e., recurrent acute infections and low-grade chronic inflammation) on each affected nutrient biomarker.

Researchers are urged to collaborate with governments and health practitioners to ensure reliable proportion estimates, develop effective policies and guidelines designed to support, implement, and evaluate

programmes for improving micronutrient status and associated health outcomes. Only in this way can countries with limited resources such as Indonesia prioritize those programmes capable of delivering the greatest improvements in the nutritional status and quality of life of their children in the future.

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## 5 Predictors of haemoglobin and anaemia in a cohort of Indonesian infants

### 5.1 Introduction

The prevalence of anaemia during infancy in Indonesia is high, varying from 22% to 82% depending on geographic region and age (de Pee et al., 2002, Lind et al., 2003, Pangaribuan et al., 2003, Fahmida et al., 2007, Howard et al., 2007). In Indonesia, no official nationally representative data for anaemia in infants exist, although in children aged 12-59 months, the anaemia prevalence based on the latest national survey, was 28.1% (National Institute of Health Research and Development, 2013). In Indonesia, nutritional ID has been assumed to be the major cause of anaemia (Kodyat et al., 1998), despite limited data on biomarkers of iron status in some of studies conducted in Indonesia.

Increasingly, several other micronutrients besides iron are reported to have a role in Hb concentrations (Kraemer and Zimmerman, 2007). Nevertheless, only a few of these Indonesian studies have included other micronutrients besides iron, most notably RBP and zinc (Dijkhuizen et al., 2001b, Untoro et al., 2005, Fahmida et al., 2007). Three mechanisms have been suggested whereby vitamin A may affect Hb, including lowering resistance to infection, and consequently inducing secondary anaemia due to infection; altering absorption, storage, release or transport of iron to the bone marrow; and/or a direct effect on modulating erythropoiesis (Bloem, 1995, Semba and Bloem, 2002, Zimmermann et al., 2006, Michelazzo et al., 2013).

Some studies have also shown that zinc status is a strong predictor of Hb (Gibson et al., 2008, Siyame et al., 2013, Houghton et al., 2016), in some cases independent of selenium status (Gibson et al., 2008, Siyame et al., 2013), whereas in others the relationship has been dependent on selenium status (Houghton, et al., 2016). Several plausible mechanisms have been proposed to explain the relationships of these micronutrients with Hb. Several zinc-dependent enzyme systems, specifically aminolevulinic acid dehydrase

(Garnica, 1981), thymidine kinase, and DNA polymerase (Spivak et al., 1992) are important in Hb synthesis. Zinc is also known to be involved in erythropoiesis through zinc-finger transcription factor, namely GATA-binding protein-1 (Labbaye et al., 1995). In addition, zinc plays a role in stabilizing red cell membranes (Dash et al., 1974) through the zinc dependent enzyme copper-zinc superoxide dismutase that protects against oxidative stress and contributes to cell integrity (Powell, 2000, O'Dell, 2000). The association with low selenium status may arise from the reduction of the activity of the selenoenzyme glutathione peroxidase that protects Hb against oxidation. When the activity of glutathione peroxidase is reduced, the lifespan of erythrocytes is shortened (Nagababu et al., 2003). In addition glutathione peroxidase activity also has a role in the regulation, release and transfer of zinc from metallothionein to copper-zinc superoxide dismutase (Maret, 2000) so reduction in the activity of glutathione peroxidase may compromise zinc status, and thus have an indirect negative impact on Hb.

Zinc supplementation studies of infants in Indonesia have shown mixed results, with some reporting a higher prevalence of anaemia and IDA after supplementation with zinc alone or iron+zinc supplements for 6 months compared with the corresponding prevalence among infants receiving iron alone (Dijkhuizen et al., 2001a, Lind et al., 2003, Wieringa et al., 2007). In contrast, others have observed that a combination of iron+zinc or multimicronutrient supplements were an effective intervention to control ID and anaemia among infants in Indonesia (Untoro et al., 2005, Zlotkin et al., 2003, Berger et al., 2006). However, to date, the role of selenium status, and its relation to Hb and zinc status in the Indonesian population has never been investigated.

Vitamin B-12 and folate status have well established roles in haematopoiesis (Kraemer and Zimmerman, 2007), but deficiencies of these vitamins during infancy in several LICs, including in Indonesia is uncertain (Molloy, et al., 2008). More recently, a positive association between selenium concentrations and Hb has been reported among children in Brazil (Nhien et al., 2008, Lander et

al., 2014), although to date no investigation has been undertaken in Indonesia. The precise mechanism is uncertain but selenium may act as a potent antioxidant in erythrocytes (Chow and Chen, 1980). Likewise, data on vitamin D status among infants in Indonesia are not available, even though a link between vitamin D status and ID and IDA has been observed earlier among young Asian children (Yoon et al., 2012, Jin et al., 2013, Sharma et al., 2015). One possible mechanism is that vitamin D directly stimulates erythroid precursors in the bone marrow (Yoon et al., 2012). An interaction between 1,25-dihydroxyvitamin D<sub>3</sub> and myeloid zinc finger-1 may also play role in haematopoiesis and myeloid cell differentiation (Piszczatowski et al., 2014).

In addition to micronutrient status, certain non-nutritional factors, such as soil-transmitted helminthiasis (STH) and genetic Hb disorders have been associated with Hb concentrations and anaemia (Balarajan et al., 2011). Exposure to infective eggs of soil transmitted helminths is likely when infants begin to crawl in and around the home and eat solid foods (Crompton, 1999). Hookworm and *T. trichiura* induce anaemia through blood loss, whereas *A. lumbricoides* and *G. intestinalis* induce anaemia through malabsorption of iron (Balarajan et al., 2011). Of the genetic Hb disorders known to occur in many Asian countries, the carrier frequency in Indonesia for  $\beta$ -thalassemia, Hb E, and  $\alpha$ -thalassemia reportedly ranges from 5–10%, 1–33%, and 6–16% respectively, depending on the ethnic population (Ariani et al., 2017).

In Chapter 4, the influence of inflammation on the interpretation of micronutrient biomarkers in a cohort of breastfed infants from Sumedang district, West Java, Indonesia was investigated. In this chapter the role of the status of seven micronutrients (iron, zinc, selenium, vitamin A, folate, vitamin B12, and vitamin D), together with two non-nutritional factors, STH and genetic Hb disorders as potential predictors of Hb and anaemia among Indonesian infants at 6,9 and 12 months of age was determined. In addition, we also explored whether the relationship between biomarkers indicative of micronutrient deficiency and Hb was moderated by ID.

## 5.2 Methods

### 5.2.1 Study design and participants

Details of the study design and characteristics of the participants have been described in Chapter 3. The sample size of 200 healthy breastfed infants was based on an estimated prevalence of stunting (LAZ  $<-2SD$ ) with a 95% confidence interval precision of 7% at the most. This sample size permitted several variables with at least a minimum of 10 respondents per variable to be included in the multiple regression analyses model (Schumacker and Lomax, 2010). Ethical approval was obtained from the Human Ethics Committees of University of Otago, New Zealand (H14/022) and the *Universitas Padjadjaran*, Indonesia (No 132/UN6C2.1.2/KEPK/PN/2014). All the parents of the respondents provided written informed consent.

### 5.2.2 Assessment of socio-demographic and health status

Pre-tested structured questionnaires collected information on socio-demographic and health status, IYCF practices, and infant morbidity status. Additional information collected from the mothers included history of smoking in the home, parity, the age at which CF began, and measurements of maternal height and weight at the first visit.

### 5.2.3 Stool collection and assessment of parasite status

Labelled plastic stool collection containers with an attached scoop were distributed to caregivers, along with both oral and written information about the collection procedures. Caregivers were instructed to keep the stool samples in a cool place and to bring them to the health facility on the examination day for immediate transfer to a chilled container for transport to the Parasite Laboratory, Faculty of Medicine, *Universitas Padjadjaran*, Indonesia. Replacement containers and instructions were provided if the caregivers failed to return a stool sample for their child to the health facility.

All stool samples were submitted for parasite analysis within 4-6 hours of collection. The Kato Katz method (Montresor et al., 1998) was used for microscopic detection of the absence/presence of soil transmitted helminths

and the number of eggs of each of the following species: *Ascaris lumbricoides*, *Trichuris trichiura*, *Necator americanus*, and *Ancylostoma duodenale*. No examination was performed for protozoa.

#### **5.2.4 Blood collection and processing**

Experienced phlebotomists collected morning, non-fasting, venepuncture blood samples from the infants using a 21G butterfly paediatric needle. Blood was drawn into an evacuated tube containing EDTA as an anticoagulant (Becton Dickinson, Franklin Lakes, NJ, USA) and into a TE-free evacuated tube (Becton Dickinson, Franklin Lakes, NJ, USA) following IZiNCG procedures for serum zinc (Brown et al., 2004). Presence of symptoms of infection were also recorded at this time. All blood samples were labelled with identification numbers, time of blood collection and time since the last meal, and transferred immediately to a chilled container (-4°C), prior to transport to the base laboratory within 6-8 hours after collection. Here an aliquot of EDTA anticoagulated whole blood was hemolyzed by a 1:10 dilution in 1% ascorbic acid, and frozen for assay of whole blood folate, and the remainder transported to a commercial laboratory (Prodia Laboratory) for a complete blood count (CBC). Maternal finger-prick blood samples were collected at baseline for assay of Hb via HemoCue (HemoCue AB, Kuvettgatan, Angelholm, Sweden).

Serum was separated using TE-free techniques and aliquoted into TE-free polyethylene storage vials for storage at -20°C. Red blood cells were washed 3 times, aliquoted into cyrovials for storage at -8°C, prior to analysis of genetic Hb disorders. Frozen aliquots were shipped on dry ice to the Department of Human Nutrition, University of Otago, New Zealand and to the laboratory of Dr J Erhardt in Germany for analyses.

#### **5.2.5 Biomarker analyses**

Biomarker analysis for ferritin, sTfR, RBP, CRP, AGP, zinc, and selenium has been described in Chapter 4. Serum B-12 was determined by an automated electrochemiluminescence immunoassay (ECLIA) using a commercial kit (Vitamin B-12 Elecsys reagent kit, Roche Diagnostics, GmbH, Mannheim, Germany) on

an auto-analyser (Roche Elecsys 2010 Immunoassay Analyser, Roche Diagnostics, Rotkreuz, Switzerland). Serum folate was analysed by microbiological (O'Broin and Kelleher, 1992, Molloy and Scott, 1997) in a 96-well plate (Costar 3596, Corning Inc, Corning, NY) by using chloramphenicol-resistant *Lactobacillus rhamnosus* (ATCC 7469). Calibration curves were produced with 11 concentration points from 0 to 0.1 pmol/well by using 5-methyltetrahydrofolate [(6S)-5-methyl-5,6,7,8-tetrahydropteroyl-L-glutamic acid, sodium salt; Merck Eprova]. Serum was diluted 1 in 75 by adding 40  $\mu$ L of serum to 2.96 ml of 0.5% sodium ascorbate solution. Turbidity was measured by absorbance at 590 nm on a Varioskan LUX multimode microplate reader (Thermo Scientific, Waltham, MA), and a cubic spline (Quartic Excel Macro, Interactive Design Services Pty, Sydney, Australia) was applied to the calibration curve. A high, medium and low pooled quality control serum control was included on each plate (expressed as average ( $\pm$  2SD); "High" 45.5nmol/L (11.5); "Medium" 26.35 nmol/L (5.2); and "Low" 15.0 nmol/L (3.8)). Our analysed values for the high, medium, and low controls used in this study were found to be (expressed as average (%RSD)) 49.7 nmol/L (9.7%), 30.3 nmol/L (7.3%), and 15.3 nmol/L (9.7%), respectively.

Serum vitamin D (as 25-dihydroxyvitamin D) was analysed using isotope-dilution liquid chromatography tandem mass spectrometry, using an API 3200 instrument (Applied Biosystems) connected to a Dionex Ultimate 3000 HPLC system (Polak et al., 2014). Repeated analysis of a pooled sample (n=9) and low, medium, and high serum control (UTAK Laboratories Inc., Valencia, CA) indicated a CV<5%.

Values for ferritin, sTfR, RBP, zinc, and selenium were adjusted for inflammation using both CRP and AGP following the BRINDA regression approach, as described earlier (Suchdev et al., 2016). The following interpretive criteria for assessing proportion of suboptimal biochemical indices were used: Hb <110 g/L (WHO, 2011a); serum vitamin B-12 <150 pmol/L (de Benoist, 2008); serum folate <10 nmol/L (WHO, 2012); serum ferritin <12  $\mu$ g/L (WHO, 2011b); serum vitamin D <50 nmol/L (WHO, 2003, Gallagher and Sai, 2010); total body iron <0 mg/kg (Cook et al., 2003); sTfR >8.3 mg/L; RBP <0.83  $\mu$ mol/L (Erhardt et al.,

2004); serum zinc  $<9.9 \mu\text{mol/L}$  (Brown et al, 2004); and serum selenium  $\leq 0.82 \mu\text{mol/L}$  (Thomson, 2004). Total body iron (mg/kg) was calculated using the formula:  $-\left[\log(\text{sTfR}/\text{ferritin ratio}) - 2.8229\right]/0.1207$  (Engle-Stone et al., 2013, Cook et al., 2003). IDA was defined as low Hb ( $<110 \text{ g/L}$  in the presence of elevated sTfR ( $>8.3 \text{ mg/L}$ ), low serum ferritin ( $<12 \mu\text{g/L}$ ), or low total body iron ( $<0 \text{ g/dL}$ ) and iron depletion as elevated sTfR or low serum ferritin/total body iron and a Hb  $\geq 110 \text{ g/L}$ .

### 5.2.6 Genetic Hb disorders analysis

Hb separation and quantification was performed by automated cation exchange high-performance liquid chromatography (VARIANT IITM,  $30 \mu\text{g Zn/g}$   $\alpha$ -thalassemia short program, Bio-Rad Laboratories, Hercules, CA) (Fucharoen et al., 1998).

### 5.2.7 Statistical analyses

Data were transferred into Stata® 12 (StataCorp LP, Texas, USA), and descriptive and comparative statistics calculated. All continuous variables were plotted and distributions visually assessed. Ferritin and sTfR were log-transformed before analysis. The mean (SD) or GM (95% CI) for haematology, micronutrient biomarkers, and growth indicators were calculated, where appropriate at 6, 9, and 12 months of age. The proportions (95% CI) of anaemia, single, and co-existing micronutrient deficiencies were also calculated, where appropriate at 6, 9, and 12 months of age. Differences in the mean haematological indices and iron biomarkers between infants with and without suspected  $\alpha$ -thalassemia were tested using independent t-test.

Univariate regression analysis was performed to examine the relation between Hb and all predictors; using the same respondents at age 6, 9, and 12 months to allow for age-related hypotheses. Sensitivity analyses were carried out including all available respondents. Household, mother, and infant predictors that showed a significance level of  $P < 0.250$  at any time point were included in a multivariate analysis of the associations between all biomarkers and Hb. Of the three biomarkers of iron status (total body iron, ferritin, sTfR) investigated, only one was included in the model, the choice determined by the highest

amount of variance explained ( $R^2$ ) with Hb. Only data from the full 6 and 12 month samples were used to generate the multivariate model to reduce the number of statistical models created, and to enable a biomarker of vitamin D, not assessed at 9 months, to be included in the model.

To test the hypothesis that the relationship between biomarkers and Hb is moderated by ID, an interaction term was included in the multivariate models between each biomarker and ID (with ferritin removed from the model). Stratified models were then run to find potential moderation. Missing values were imputed and sensitivity analyses undertaken. Imputation was performed using multivariate normal regression with an iterative Markov Chain Monte Carlo method for 20 imputations. Model assumptions were checked using residual plots.

## 5.3 Results

### 5.3.1 Socio-demographic characteristics

Of the mothers, 53% had attended secondary school, but few (6%) had completed tertiary education (Table 5.1). Mothers were aged 27.5 years on average; 72.8% were exposed to smoking in the home, 30.7% were overweight and 6% obese, and about one third were primiparous. Maternal GM concentrations of Hb was 13.2 g/dL (95% CI, 13.0-13.4). Of the infants, 27.6% commenced CF at age 4 months; 55.3% were female. Only one case (1/166, 0.6%) of *Ascaris lumbricoides* infection for an infant aged 9 months was found. Additional health characteristics of the infants have been reported in Chapter 3.

### 5.3.2 Hb and micronutrient status of infants

Of the biomarkers presented in

The proportion of infants with anaemia was highest at aged 9 months (42.2% (95% CI, 35.3-49.1)), whereas the proportion with IDA or ID was highest at 12 months of age, regardless of the iron indicators applied (

Table 5.3). Of the other micronutrient biomarkers examined, the highest proportion below the cut-off indicative of risk of deficiency was for serum

selenium at all three ages, followed by serum zinc, RBP, and vitamin B-12. No infants had folate deficiency, irrespective of age; and very few had vitamin D deficiency. Nonetheless, only 17.2% at aged 6 months and 10.0% at aged 12 months had no evidence of any micronutrient deficiencies. Instead, nearly 50% of the infants (42-43%) had one micronutrient deficiency, generally iron, and about one third (28-31%) had two micronutrient deficiencies during the study.

Table 5.2, GM for serum ferritin decreased whereas sTfR increased from 6 to 12 months of age. Small fluctuations in the GM for vitamin B12, folate, vitamin D, RBP, zinc, selenium, and MMA were apparent across the three age groups. Mean BMI z-score (BMIZ) for infants decreased from 0.30 (SD 1.11) at aged 6 months to 0.10 (SD 1.08) at aged 12 months.

**Table 5.1 Socio-demographic characteristics**

	n	6 months
<b>Household and maternal factors</b>		
Education	228	
No/primary school, %		40.8
Secondary school, %		53.1
Tertiary school, %		6.1
Maternal age (years), mean (SD)	225	27.5 (7.2)
Smoking at home, %	217	72.8
Body mass index (BMI)*	218	
Underweight, %		7.3
Normal, %		56.0
Overweight, %		30.7
Obese, %		6.0
Height <145 cm, %	223	15.3
Haemoglobin (g/L), geometric mean (95% CI)	217	132 (130, 134)
Parity	223	
1, %		39.0
2, %		35.9
3 or more, %		25.1
<b>Infant factors</b>		
Female, %	228	55.3
Age of introduction of complementary feeding	214	
4 months, %		27.6
6 months, %		72.4

\*Classification of BMI for Asian population (WHO, 2004): < 18.5 kg/m<sup>2</sup> underweight; 18.5–22.9 kg/m<sup>2</sup> increasing but acceptable risk (normal); 23–27.4 kg/m<sup>2</sup> increased risk (overweight); and 27.5 kg/m<sup>2</sup> or higher high risk (obese)

The proportion of infants with anaemia was highest at aged 9 months (42.2% (95% CI, 35.3-49.1)), whereas the proportion with IDA or ID was highest at 12 months of age, regardless of the iron indicators applied (Table 5.3). Of the other micronutrient biomarkers examined, the highest proportion below the cut-off indicative of risk of deficiency was for serum selenium at all three ages, followed by serum zinc, RBP, and vitamin B-12. No infants had folate deficiency, irrespective of age; and very few had vitamin D deficiency. Nonetheless, only 17.2% at aged 6 months and 10.0% at aged 12 months had no evidence of any micronutrient deficiencies. Instead, nearly 50% of the infants (42-43%) had one micronutrient deficiency, generally iron, and about one third (28-31%) had two micronutrient deficiencies during the study.

**Table 5.2 Infant haemoglobin, BMI z-score, and micronutrient concentrations at 6, 9, and 12 months of age**

	6 months		9 months		12 months	
	n	Mean (SD)	n	Mean (SD)	n	Mean (SD)
BMI z-score,	228	0.30 (1.11)	199	0.21 (1.05)	190	0.10 (1.08)
Haemoglobin, g/L	228	113 (10.9)	199	111 (11.4)	190	111 (12.2)
sTfR, mg/L	212	6.5 (2.9)	193	7.6 (3.7)	185	8.2 (4.0)
Ferritin, µg/L, GM (95% CI)	212	25.4 (22.7-28.5)	193	13.4 (12.0-14.9)	185	8.8 (8.0-9.8)
Total body iron, mg/kg	212	3.78 (4.08)	193	0.94 (3.81)	185	-0.84 (3.79)
Inflammation*, %	212	25.5%	193	26.4%	185	26.5%
Vitamin B12, pmol/L	167	373 (193)	163	411 (224)	143	368 (189)
Folate, nmol/L	177	50.5 (16.6)	162	52.1 (16.2)	146	54.0 (14.0)
Vitamin D, nmol/L	178	95.5 (21.0)		NA	153	87.9 (19.6)
RBP, µmol/L	212	1.04 (0.22)	193	1.10 (0.23)	185	1.09 (0.22)
Zinc, µmol/L	189	12.4 (3.3)	165	11.4 (1.5)	154	11.8 (1.7)
Selenium, µmol/L	189	0.75 (0.12)	165	0.79 (0.13)	119	0.83 (0.13)

SD, standard deviation; GM (95% CI), geometric mean (95% confidence interval); BMI, body mass index; sTfR, serum transferrin receptor; RBP, retinol binding protein

\*Inflammation (CRP >5 mg/L and/or AGP >1 g/L)

**Table 5.3 Proportion of infants with anaemia and micronutrient deficiencies at 6, 9, and 12 months of age**

	6 months	9 months	12 months
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	n	Pr (95% CI)	n	Pr (95% CI)	n	Pr (95% CI)
Anaemia, Hb <110 g/L	228	32.9 (26.8, 39.0)	199	42.2 (35.3, 49.1)	190	38.4 (31.5, 45.4)
<b>Iron deficiency anaemia</b>						
sTfR, >8.3 mg/L	212	12.6 (7.6, 17.5)	193	20.7 (14.1, 27.3)	185	22.5 (15.8, 29.2)
Ferritin, <12 µg/L	212	13.6 (8.5, 18.6)	193	30.3 (23.3, 37.4)	185	33.5 (26.5, 40.5)
Total body iron, <0 mg/kg	212	13.1 (8.1, 18.1)	193	28.1 (21.1, 35.1)	185	32.8 (25.7, 39.8)
<b>Iron deficiency</b>						
sTfR, >8.3 mg/L	212	17.0 (11.9, 22.1)	193	28.0 (21.6, 34.3)	185	38.4 (31.3, 45.4)
Ferritin, <12 µg/L	212	21.2 (15.7, 26.8)	193	45.1 (38.0, 52.1)	185	64.9 (58.0, 71.8)
Total body iron, <0 mg/kg	212	15.6 (10.7, 20.5)	193	38.9 (32.0, 45.8)	185	62.2 (55.1, 69.2)
Vitamin B12, <150 pmol/L	167	15.6 (10.0, 21.1)	163	12.3 (7.2, 17.3)	143	14.0 (8.3, 19.7)
Folate, <6.8 nmol/L	177		0.0 162		0.0 146	0.0
Vitamin D, <50 nmol/L	178	1.1 (0.0, 2.7)			- 153	3.3 (0.4, 6.1)
RBP, <0.83 µmol/L	212	17.5 (12.3, 22.6)	193	8.3 (4.4, 12.2)	185	12.4 (7.7, 17.2)
Zinc, <9.9 µmol/L	189	14.3 (9.3, 19.3)	165	16.4 (10.7, 22.0)	154	10.4 (5.5, 15.2)
Selenium, <0.82 µmol/L	189	73.0 (66.7, 79.4)	165	58.8 (51.2, 66.3)	119	52.6 (44.7, 60.5)

Pr, Proportion; sTfR, serum transferrin receptor; RBP, retinol binding protein

Iron deficiency anaemia: Hb <110 g/L plus sTfR >8.3 mg/L or ferritin <12 µg/L or total body iron <0 mg/kg

Iron deficiency: Hb ≥110 g/L plus sTfR >8.3 mg/L or ferritin <12 µg/L or total body iron <0 mg/kg

### 5.3.3 Assessment of haematological indices and iron biomarkers in infants with and without suspected $\alpha$ -thalassemia

Infants with suspected  $\alpha$ -thalassemia (n=17) had lower GM values for mean cell volume, mean cell Hb, red cell distribution width and ferritin but higher sTfR values than their counterparts without evidence of suspected  $\alpha$ -thalassemia, although Hb concentrations were not significantly different (Table 5.4).

### 5.3.4 Predictors of haemoglobin and anaemia

At 6 months of age, CRP and maternal height were associated with Hb and anaemia in the univariate models but these relationships were no longer significant at 12 months of age (Table 5.5, Table 5.6). Ferritin, sex, and RBP were consistently associated with Hb and anaemia across the ages.

When adjusted for other potential predictors it was noted that ferritin, female sex, and lower CRP concentrations were associated with Hb level at 6 months of age, whereas at 12 months ferritin was the only significant and positive predictor for Hb (Table 5.7). Both multivariate models (with and without imputation) showed the same trends. The final regression models without

imputations for both infants aged 6 and 12 months explained ~40% of the variance in Hb.

**Table 5.4 Geometric mean (95% CI) values for hematology and iron biomarker**

	<b>Suspected <math>\alpha</math>-thalassemia (n=17)</b>	<b>Normal (n=56)</b>	<b>P value*</b>
Haemoglobin, g/L	118 (115, 122)	118 (115, 120)	0.908
MCV, fL	65.9 (64.1, 67.8)	73.8 (72.9, 74.8)	<b>&lt;0.001</b>
MCH, pg	20.4 (19.6, 21.2)	23.7 (23.3, 24.2)	<b>&lt;0.001</b>
RDW, %	37.3 (35.9, 38.7)	39.0 (38.2, 39.8)	<b>0.045</b>
sTfR, mg/L	7.4 (6.0, 9.1)	5.4 (4.8, 6.0)	<b>0.007</b>
Ferritin, $\mu$ g/L	8.7 (6.4, 11.9)	19.5 (17.8, 21.4)	<b>&lt;0.001</b>

MCV, mean corpuscular volume; MCH, mean corpuscular haemoglobin; RDW, red cell distribution width; sTfR, serum transferrin receptor

\*Independent t-test to compare between suspected  $\alpha$ -thalassemia and normal group; ferritin and sTfR were log-transformed

When anaemia was the outcome, the potential predictors based on multivariate model were similar to those reported for Hb, with the exception of CRP, but the trend remained the same (Table 5.8). Serum folate was a significant predictor in the multivariate model for anaemia at 12 months, and although higher serum folate tended to offer a protective effect against low Hb, this was not statistically significant in the Hb model.

Potential moderation by ID was detected for the relationship between vitamin D (as 25-dihydroxyvitaminD) and Hb at age 12 months (interaction P=0.029). Those infants who were iron sufficient at 12 months (n=53) showed positive (though non-significant) associations with Hb (vitamin D: 0.14 (-0.01, 0.29)); while infants who were iron deficient (n=105) showed negative associations (vitamin D: -0.12 (-0.25, 0.00)) (Table 5.9).

**Table 5.5 Univariate associations with haemoglobin with same respondents at 6, 9, 12 months**

	n	6 months Mean g/L difference in Hb per unit increase (95% CI)	P-value	9 months Mean g/L difference in Hb per unit increase (95% CI)	P-value	12 months Mean g/L difference in Hb per unit increase (95% CI)	P-value
<b>Household and maternal factors</b>							
Wealth index (quintiles)	183	-0.11 (-1.31, 1.10)	0.860	0.95 (-0.21, 2.1)	<b>0.108</b>	0.55 (-0.74, 1.84)	0.403
Education	188						
No/primary school		Reference		Reference		Reference	
Secondary school		1.93 (-1.34, 5.20)	<b>0.246</b>	0.46 (-3.05, 3.97)	0.796	-0.05 (-3.79, 3.70)	0.98
Tertiary school		5.04 (-1.34, 11.43)	<b>0.121</b>	6.57 (1.29, 11.85)	<b>0.015</b>	4.47 (-3.20, 12.15)	0.252
Maternal age, years	185	-0.08 (-0.28, 0.12)	0.431	0.06 (-0.14, 0.27)	0.542	0.11 (-0.13, 0.34)	0.362
Smoking at home	179	-0.86 (-4.28, 2.55)	0.619	1.58 (-1.98, 5.15)	0.382	0.06 (-3.94, 4.05)	0.977
Maternal BMI, kg/m <sup>2</sup>	180	0.05 (-0.39, 0.48)	0.828	0.16 (-0.30, 0.63)	0.486	0.00 (-0.46, 0.45)	0.988
Maternal height, cm	185	-0.44 (-0.72, -0.17)	<b>0.002</b>	-0.36 (-0.68, -0.03)	<b>0.031</b>	-0.20 (-0.54, 0.14)	<b>0.246</b>
Maternal haemoglobin, g/L	178	0.09 (-0.02, 0.2)	<b>0.128</b>	0.09 (-0.02, 0.20)	<b>0.099</b>	0.06 (-0.06, 0.19)	0.337
Parity	183	-0.56 (-1.87, 0.75)	0.399	0.84 (-0.48, 2.16)	<b>0.212</b>	0.87 (-0.50, 2.23)	<b>0.211</b>
ln(CRP), ln(mg/L)	166	-1 (-1.77, -0.23)	<b>0.011</b>	-0.77 (-1.75, 0.21)	<b>0.125</b>	-0.46 (-1.53, 0.60)	0.392
Infant sex (female compared to male)	188	7.88 (4.96, 10.79)	<b>&lt;0.001</b>	5.83 (2.58, 9.08)	<b>0.001</b>	5.49 (1.95, 9.03)	<b>0.003</b>
CF introduced at <6 months	175	-2.37 (-5.67, 0.93)	<b>0.159</b>	-0.97 (-4.60, 2.67)	0.6	-3.48 (-7.32, 0.36)	<b>0.075</b>
BMI z-score	188	-0.02 (-1.36, 1.32)	0.977	0.87 (-0.76, 2.49)	0.292	-0.33 (-2.10, 1.44)	0.713
<b>Biomarkers</b>							
Folate, nmol/L	91	0.10 (-0.02, 0.22)	<b>0.112</b>	-0.02 (-0.18, 0.13)	0.759	0.03 (-0.15, 0.21)	0.719
B12, pmol/L	84	0.00 (-0.01, 0.01)	0.53	0.01 (-0.00, 0.02)	<b>0.243</b>	0.00 (-0.02, 0.02)	0.825
Vitamin D, nmol/L	117	0.04 (-0.03, 0.12)	0.28	NA	NA	0.05 (-0.03, 0.13)	0.225
ln(Ferritin), ln(µg/L)	166	4.74 (2.83, 6.65)	<b>&lt;0.001</b>	7.10 (4.97, 9.22)	<b>&lt;0.001</b>	8.34 (5.80, 10.89)	<b>&lt;0.001</b>
RBP, µmol/L	166	6.23 (-0.36, 12.81)	<b>0.064</b>	14.36 (5.91, 22.8)	<b>0.001</b>	15.47 (7.83, 23.12)	<b>&lt;0.001</b>
Zinc, µmol/L	103	0.10 (-0.41, 0.61)	0.696	-0.44 (-2.10, 1.23)	0.606	-0.31 (-1.35, 0.72)	0.551
Selenium, µmol/L	103	-1.66 (-18.61, 15.29)	0.846	4.16 (-14.16, 22.49)	0.653	-7.82 (-22.49, 6.84)	0.292

BMI, body mass index; ln, log-transformed; CRP, C-Reactive Protein; RBP, retinol binding protein; CF, complementary feeding

**Table 5.6 Univariate associations with anaemia with same respondents at 6, 9, 12 months**

	N	6 months		9 months		12 months	
		Odds ratio (95% CI)	P-value	Odds ratio (95% CI)	P-value	Odds ratio (95% CI)	P-value
<b>Household and mother's factors</b>							
Wealth index (quintiles)	183	0.92 (0.74, 1.15)	0.479	0.90 (0.73, 1.11)	0.332	1.01 (0.82, 1.25)	0.930
Maternal education	188						
No/primary school		Reference		Reference		Reference	
Secondary school		0.70 (0.37, 1.31)	0.262	1.13 (0.62, 2.06)	0.688	1.15 (0.62, 2.11)	0.663
Tertiary school		0.28 (0.06, 1.35)	<b>0.112</b>	0.24 (0.05, 1.14)	<b>0.073</b>	0.49 (0.12, 1.91)	0.302
Age, years	185	1.00 (0.96, 1.04)	0.937	1.00 (0.96, 1.04)	0.896	0.99 (0.95, 1.03)	0.663
Smoking at home	179	1.47 (0.71, 3.04)	0.304	0.87 (0.45, 1.69)	0.685	0.99 (0.50, 1.94)	0.969
Maternal body mass index (BMI), kg/cm <sup>2</sup>	180	0.99 (0.92, 1.07)	0.842	0.97 (0.90, 1.05)	0.473	1.02 (0.95, 1.10)	0.611
Maternal height, cm	185	3.05 (1.04, 1.17)	<b>0.002</b>	1.07 (1.01, 1.13)	<b>0.023</b>	1.06 (1.00, 1.13)	<b>0.040</b>
Maternal haemoglobin, g/L	178	0.99 (0.99, 1.01)	0.443	0.99 (0.97, 1.01)	0.348	0.98 (0.95, 1.00)	<b>0.029</b>
Parity	183	0.94 (0.71, 1.26)	0.695	0.80 (0.60, 1.07)	<b>0.131</b>	0.83 (0.63, 1.11)	<b>0.215</b>
ln(CRP), ln(mg/L)	166	1.22 (1.01, 1.47)	<b>0.038</b>	1.12 (0.94, 1.33)	<b>0.208</b>	1.04 (0.88, 1.23)	0.635
Infant sex (female compared to male)	188	0.24 (0.13, 0.46)	<b>&lt;0.001</b>	0.50 (0.28, 0.90)	<b>0.021</b>	0.45 (0.25, 0.82)	<b>0.009</b>
CF introduced at < 6 months	175	1.48 (0.71, 3.08)	0.297	1.07 (0.55, 2.10)	0.845	1.86 (0.91, 3.79)	<b>0.090</b>
BMI z-score	188	0.98 (0.75, 1.28)	0.874	0.80 (0.60, 1.06)	<b>0.124</b>	1.04 (0.79, 1.37)	0.769
<b>Biomarkers</b>							
Folate, nmol/L	91	0.98 (0.96, 1.01)	0.264	1.01 (0.98, 1.04)	0.532	0.96 (0.93, 1.00)	<b>0.030</b>
B12, pmol/L	84	1.00 (1.00, 1.00)	0.861	1.00 (1.00, 1.00)	0.383	1.00 (1.00, 1.00)	0.429
Vitamin D, nmol/L	117	0.99 (0.98, 1.01)	0.464	NA	NA	0.99 (0.97, 1.01)	<b>0.225</b>
ln(Ferritin), ln(µg/L)	166	0.41 (0.26, 0.65)	<b>&lt;0.001</b>	0.27 (0.16, 0.47)	<b>&lt;0.001</b>	0.36 (0.08, 1.68)	<b>0.192</b>
RBP, µmol/L	166	0.36 (0.08, 1.68)	<b>0.192</b>	0.13 (0.03, 0.56)	<b>0.007</b>	0.21 (0.05, 0.90)	<b>0.036</b>
Zinc, µmol/L	103	0.99 (0.86, 1.13)	0.854	1.18 (0.90, 1.55)	<b>0.225</b>	0.99 (0.80, 1.23)	0.950

Selenium,  $\mu\text{mol/L}$  103 4.30 (0.12, 158.78) 0.429 0.11 (0.00, 2.53) **0.165** 2.13 (0.10, 47.20) 0.633

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In, log-transformed; CRP, C-reactive protein; RBP, retinol binding protein; CF, complementary feeding

**Table 5.7 Multivariate associations with infant haemoglobin at 6 and 12 months of age**

Variable, unit	Mean g/L difference in Hb per unit higher (95% CI)	P-value
<b>6 months (n = 180)</b>		
Serum folate, nmol/L	0.10 (-0.01, 0.20)	0.068
Vitamin D, nmol/L	0.01 (-0.05, 0.08)	0.722
ln(Ferritin), ln(µg/L)	4.45 (2.32, 6.58)	<b>&lt;0.001</b>
RBP, µmol/L	4.88 (-2.22, 11.99)	0.177
Zinc, µmol/L	0.01 (-0.48, 0.50)	0.977
Selenium, µmol/L	-1.26 (-15.91, 13.39)	0.865
B12, pmol/L	0.00 (-0.01, 0.01)	0.650
Wealth index (quintiles)	-0.5 (-1.69, 0.70)	0.416
Maternal education		
No/primary school	Reference	
Secondary school	2.02 (-1.61, 5.64)	0.273
Tertiary school	5.53 (-0.66, 11.72)	0.080
Maternal height, cm	-0.18 (-0.47, 0.11)	0.216
Maternal haemoglobin, g/L	0.09 (-0.01, 0.18)	0.082
ln(CRP), ln(mg/L)	-1.32 (-2.07, -0.58)	<b>0.001</b>
Sex (female compared to male)	3.93 (0.71, 7.15)	<b>0.017</b>
CF introduced at <6 months	1.71 (-1.29, 4.72)	0.262
Parity	-0.44 (-2.02, 1.15)	0.587
<b>12 months (n = 158)</b>		
Serum folate, nmol/L	0.13 (-0.04, 0.30)	0.122
Vitamin D, nmol/L	-0.03 (-0.12, 0.06)	0.505
ln(Ferritin), ln(µg/L)	7.24 (4.64, 9.85)	<b>&lt;0.001</b>
RBP, µmol/L	7.61 (-0.27, 15.49)	0.058
Zinc, µmol/L	0.39 (-0.90, 1.69)	0.544
Selenium, µmol/L	2.21 (-15.57, 19.99)	0.804
B12, pmol/L	0.02 (0.00, 0.03)	0.062
Wealth index (quintiles)	0.34 (-1.05, 1.74)	0.626
Maternal education		
No/primary school	Reference	
Secondary school	-1.20 (-5.34, 2.94)	0.567
Tertiary school	-0.24 (-9.00, 8.53)	0.958
Maternal height, cm	-0.12 (-0.51, 0.27)	0.552
Maternal haemoglobin, g/L	-0.01 (-0.14, 0.12)	0.924
ln(CRP), ln(mg/L)	-0.76 (-1.88, 0.35)	0.177
Sex (female compared to male)	2.55 (-0.98, 6.08)	0.156
CF introduced at <6 months	3.42 (-0.23, 7.07)	0.066
Parity	-0.23 (-1.93, 1.48)	0.795

ln, log-transformed; RBP, retinol binding protein; CRP, C-Reactive Protein; CF, complementary feeding

**Table 5.8 Multivariate associations with infant anaemia at 6 and 12 months of age**

Variable, unit	Odds ratio (95% CI)	P-value
<b>6 months (n = 180)</b>		
Serum folate, nmol/L	0.99 (0.96, 1.02)	0.440
Vitamin D, nmol/L	1.00 (0.98, 1.02)	0.845
ln(Ferritin), ln(µg/L)	0.46 (0.28, 0.76)	<b>0.003</b>
RBP, µmol/L	0.43 (0.06, 2.98)	0.392
Zinc, µmol/L	1.05 (0.91, 1.21)	0.496
Selenium, µmol/L	0.31 (0.01, 7.72)	0.474
B12, pmol/L	1.00 (1.00, 1.00)	0.483
Wealth index (quintiles)	0.93 (0.69, 1.26)	0.655
Maternal education		
No/primary school	Reference	
Secondary school	0.71 (0.29, 1.70)	0.436
Tertiary school	0.28 (0.04, 1.92)	0.195
Maternal height, cm	1.07 (0.99, 1.15)	0.085
Maternal haemoglobin, g/L	0.99 (0.97, 1.02)	0.601
ln(CRP), ln(mg/L)	1.25 (0.99, 1.57)	0.062
Sex (female compared to male)	0.41 (0.19, 0.88)	<b>0.022</b>
CF introduced at <6 months	0.74 (0.31, 1.80)	0.504
Parity	0.77 (0.52, 1.14)	0.189
BMI z-score	0.72 (0.51, 1.01)	0.057
<b>12 months (n = 158)</b>		
Serum folate, nmol/L	0.96 (0.92, 0.99)	<b>0.019</b>
Vitamin D, nmol/L	1.00 (0.98, 1.02)	0.808
ln(Ferritin), ln(µg/L)	0.25 (0.12, 0.49)	<b>&lt;0.001</b>
RBP, µmol/L	0.42 (0.06, 3.20)	0.403
Zinc, µmol/L	0.97 (0.70, 1.33)	0.828
Selenium, µmol/L	0.38 (0.01, 16.83)	0.615
B12, pmol/L	1.00 (0.99, 1.00)	0.325
Wealth index (quintiles)	1.07 (0.77, 1.48)	0.689
Maternal education		
No/primary school	Reference	
Secondary school	1.03 (0.40, 2.66)	0.947
Tertiary school	0.56 (0.08, 4.03)	0.564
Maternal height, cm	1.06 (0.97, 1.15)	0.191
Maternal haemoglobin, g/L	0.99 (0.96, 1.02)	0.360
ln(CRP), ln(mg/L)	1.10 (0.88, 1.38)	0.397
Sex (female compared to male)	0.75 (0.33, 1.72)	0.502
CF introduced at <6 months	0.44 (0.17, 1.12)	0.082
Parity	0.83 (0.55, 1.26)	0.387
BMI z-score	0.79 (0.55, 1.13)	0.196

BMI, body mass index; ln, log-transformed; RBP, retinol binding protein; CRP, C-reactive protein CF, complementary feeding

**Table 5.9 Interaction between iron sufficiency/deficiency and biomarkers**

Variable	Iron sufficiency	Iron deficiency	P-value (Interaction)
	Mean g/L difference in Hb per unit increase (95% CI)	Mean g/L difference in Hb per unit increase (95% CI)	
<b>6 months (n=180)</b>	n=139	n=41	
Serum folate, nmol/L	0.09 (-0.01, 0.18)	0.12 (-0.38, 0.62)	0.271
Vitamin D, nmol/L	0.02 (-0.06, 0.10)	-0.08 (-0.31, 0.16)	0.219
RBP, $\mu$ mol/L	7.16 (-0.32, 14.63)	10.64 (-17.96, 39.23)	0.249
Zinc, $\mu$ mol/L	0.06 (-0.58, 0.71)	0.10 (-1.71, 1.92)	0.405
Selenium, $\mu$ mol/L	-0.35 (-14.03, 13.32)	-5.40 (-46.82, 36.03)	0.676
Vitamin B12, pmol/L	0.00 (-0.01, 0.01)	0.01 (-0.03, 0.06)	0.895
<b>12 months (n=158)</b>	n=53	n=105	
Serum folate, nmol/L	<b>0.30 (0.05, 0.56)</b>	0.08 (-0.14, 0.30)	0.722
Vitamin D, nmol/L	0.14 (-0.01, 0.29)	-0.12 (-0.25, 0.01)	<b>0.029</b>
RBP, $\mu$ mol/L	9.36 (-3.01, 21.73)	3.86 (-9.07, 16.80)	0.921
Zinc, $\mu$ mol/L	0.57 (-1.59, 2.73)	-0.20 (-2.37, 1.98)	0.351
Selenium, $\mu$ mol/L	-2.85 (127.24, 21.53)	8.53 (-17.90, 34.97)	0.785
Vitamin B12, pmol/L	<b>0.03 (0.01, 0.05)</b>	0.01 (-0.02, 0.03)	0.172

RBP, retinol binding protein

## 5.4 Discussion

One of the most notable findings of this study was the high proportion of anaemia among these breastfed Sumedang infants approaching levels indicative of a severe public health problem at 9 and 12 months of age (WHO, 2011a). Of the seven micronutrient biomarkers investigated, serum ferritin was the only strong positive predictor of Hb at both 6 and 12 months of age, highlighting the importance of an adequate iron status for these infants at this time. The non-nutritional factors shown to be positive predictors of Hb for the infants were low concentrations of CRP and being female, these two factors significantly associated with Hb for the infants at 6 months of age but only being female at 12 months.

Our finding that ID (based on low serum ferritin) was significantly associated with lower Hb concentrations during later infancy is not unexpected, and has been reported in several earlier studies in Indonesia (Lind et al., 2004, Fahmida et al., 2007, Wieringa et al., 2007) and elsewhere (Oski, 1993, Habib et al.,

2016). However, very few of these earlier studies also investigated the potential and simultaneous role of non-nutritional factors studied here -- helminth parasitic infections, genetic Hb disorders, and inflammation-- on Hb concentrations. Of these factors, only inflammation based on CRP was a significant and negative predictor of Hb at 6 months, as noted earlier. This finding is attributed to a reduction in iron absorption and its release from macrophages induced by high levels of circulating hepcidin, stimulated by pro-inflammatory cytokines. As a consequence, the supply of iron for RBC production is restricted, so Hb declines (Weiss, 2009). This response is said to be a vital defence strategy of the body to deprive iron and make it unavailable for any pathogens (Lynch, 2007).

Unlike ferritin, none of the other six micronutrients known to have a role in normal haematopoiesis were significantly associated with Hb, despite the presence of deficiencies of vitamin A, B12, zinc, and most notably selenium among these infants. Indeed, of the infants, only 17.2% at age 6 months and 10.0% at age 12 months had no micronutrient deficiencies, with approximately one third (28-31%) having two micronutrient deficiencies during the second half of infancy. This null finding for vitamin A may be related in part to the improved coverage of vitamin A supplements in Indonesia for infants and lactating mothers compared to earlier studies when positive associations between vitamin A status and Hb during infancy were observed. The positive effect of serum folate on anaemia at 12 months is not unexpected. Folate deficiency diminishes and compromises the cell's ability to synthesise purines and pyrimidines and thus DNA (Scott, 2007), leads to a failure of the red cell precursors to divide normally (Bailey and Gregory, 1999), and results in megaloblastic anaemia. Previous studies show that iron and folic acid supplementation significantly improve Hb (Olney et al., 2006) and reduce anaemia (Kapil et al., 2013).

Suspected  $\alpha$ -thalassemia was not included as a covariate in our multiple regression model because only 17 cases were identified and there was no significant difference in Hb concentrations between those infants with or without suspected  $\alpha$ -thalassemia. Likewise, the number of helminth parasite

cases was low, thus association between helminth parasites and Hb cannot be established in this study.

Instead, dietary factors probably contributed to the marked age-related decline in iron status biomarkers in the second half of infancy ( Table 5.3). The proportion of infants with anaemia was highest at aged 9 months (42.2% (95% CI, 35.3-49.1)), whereas the proportion with IDA or ID was highest at 12 months of age, regardless of the iron indicators applied ( Table 5.3). Of the other micronutrient biomarkers examined, the highest proportion below the cut-off indicative of risk of deficiency was for serum selenium at all three ages, followed by serum zinc, RBP, and vitamin B-12. No infants had folate deficiency, irrespective of age; and very few had vitamin D deficiency. Nonetheless, only 17.2% at aged 6 months and 10.0% at aged 12 months had no evidence of any micronutrient deficiencies. Instead, nearly 50% of the infants (42-43%) had one micronutrient deficiency, generally iron, and about one third (28-31%) had two micronutrient deficiencies during the study.

Table 5.2), as well as the positive association between serum ferritin and Hb, noted at 6 and 12 months. Certainly, intakes of total iron from complementary foods at all three ages (Diana et al., 2017) did not meet the WHO estimated needs from complementary foods, even though almost all of the infants (91.7%) consumed FIFs at 6 months of age (Diana et al., 2017), and wheat flour products are fortified with iron (50 mg Fe/kg) in Indonesia (*Menteri Kesehatan Republik Indonesia, 2003*). Reasons for the reported dietary iron deficits include the low dry matter content of FIFs at 6 months emphasized in Chapter 3, together with low intakes of iron-fortified wheat flour-based foods, micronutrient powders containing iron, or readily available haem iron from animal source foods at 9 and 12 months of age (Diana et al., 2017).

The positive relationship between female sex and Hb concentrations observed here is consistent with earlier reports in which female infants in Indonesia (Wieringa et al., 2007) and elsewhere (Soh et al., 2004, Foote et al., 2013) had higher Hb and serum ferritin concentrations than males in the

second half of infancy, and a lower prevalence of both anaemia and IDA. Differences in growth rate may account for these sex-related trends in Hb and ferritin. Certainly, our female infants had consistently lower body weights at each age, and thus potentially lower iron requirements than males, as postulated in the earlier Southeast Asian study (Wieringa et al., 2007).

The attenuation of the associations between the non-nutritional factors (sex and inflammation) and Hb for the infants by age 12 months may be linked to the marked depletion in their iron stores at this time compared to the levels when CF first began to replace breast milk (i.e. ~ at 6 months of age). Of the infants at 12 months of age, 65% had depleted iron stores (based on ferritin <12 µg/L) compared to 21% at aged 6 months. In a cross-sectional survey of young New Zealand children aged 6-24 months (Soh et al., 2004), iron stores (measured by serum ferritin) became a stronger predictor of anaemia in 12 month old infants than their counterparts of aged 6 months. When iron stores are low, iron becomes the single strongest predictor of Hb, adjusted for many other nutritional and non-nutritional factors.

Exploratory analysis suggested that iron deficiency moderated the relationship between vitamin D and Hb. This moderation effect in infants has never been reported before. However, some studies exploring the relationship between vitamin D and Hb show that respondents with vitamin D deficiency have a lower Hb level (Sim et al., 2010, Lee et al., 2015). One study reported an odds ratio for the likelihood of iron-deficient infants to have subnormal vitamin D levels of 4.1 (Jin et al., 2013). As the hydroxylation of vitamin D is dependent on iron (ferredoxin reductase and ferredoxin), its deficiency might disturb vitamin D activation (Azizi-Soleiman et al., 2016).

Our study has several strengths, including a longitudinal design and a comprehensive range of variables known to be associated with low Hb concentrations during infancy. We examined seven micronutrients, adjusted for inflammation where appropriate, and measured non-nutritional factors including inflammation, selected genetic Hb disorders, and helminth infections. Nevertheless, we did not investigate pathogenic protozoan

infections that can pose a significant health threat in later infancy. The method used to detect genetic Hb disorders was unable to identify  $\alpha$ -thalassemia, especially among the anaemic infants. Other biomarkers related to Hb and iron absorption, such as hepcidin and glucose-6-phosphate dehydrogenase (G6PD) were also not investigated.

In conclusion, our results emphasize that anaemia remains a persistent and severe public health problem during infancy in Sumedang district, Indonesia. Moreover, despite the introduction of public health programs aimed to improve iron status during later infancy in Indonesia, low iron status remains a major predictor of low Hb concentrations at this time among infants in Sumedang district. Hence there is an urgent need to re-evaluate the performance of both the anaemia and ID control programmes in the district.

## **6 Summary, Conclusions, and Recommendations**

### **6.1 Summary and Conclusions**

Children under five are a very vulnerable group in LMICs, especially in Indonesia, where there is a high prevalence of stunting, anaemia, and co-existing micronutrient deficiencies (Isabelle and Chan, 2011, National Institute of Health Research and Development, 2012, National Institute of Health Research and Development, 2013). Despite very high national breastfeeding (BF) rates at 6 months (86%) (Yohmi et al., 2015), progress in improving CF practices in Indonesia has been slow (Ng et al., 2012). The rice-based gruels traditionally used for CF often have a low energy and nutrient density (Harper, 2006; Isabelle and Chan, 2011) leading to nutrient deficits when compared with the WHO estimated needs (Dewey and Brown, 2003), particularly for iron, zinc, and sometimes vitamin A (Dewey, 2016).

Hence, it is not surprising that earlier biomarker studies in Indonesia have documented deficiencies of iron, zinc, and vitamin A during infancy (Dijkhuizen et al., 2001b, Untoro et al., 2005, Fahmida et al., 2007). Of these three micronutrients, nutritional iron deficiency (ID) has been assumed to be

the major factor causing the high rates of anaemia during infancy and early childhood in Indonesia (Kodyat et al., 1998). Increasingly however, the contribution of many other micronutrient deficiencies besides iron to the overall burden of anaemia during early childhood is being recognized. These include deficiencies of folate, vitamin B-12 and vitamin A, all of which have well established roles in normal haematopoiesis (Kraemer and Zimmerman, 2007). In addition, emerging evidence suggests that deficiencies of zinc, selenium, and vitamin D may also be involved in the aetiology of anaemia through several plausible mechanisms (Lander et al., 2008, Houghton et al., 2016). To date, apart from zinc, data on selenium and vitamin D deficiency during the CF period in Indonesia and their possible role in the aetiology of anaemia in Indonesia has not been investigated.

Additional non-nutritional factors that have the potential to contribute to anaemia during infancy in rural Indonesia include parasitic infections, inflammation, and genetic haemoglobin (Hb) disorders (Thurlow et al., 2005, Lander et al., 2008). However, in many of the earlier studies in Indonesia, these non-nutritional factors were not measured despite their potential to confound the interpretation of some of the serum micronutrient biomarkers, most notably ferritin, transferrin receptor, retinol or retinol binding protein (RBP), and zinc. Consequently, the resulting proportion estimates may not reflect the true burden of deficiency unless inflammation or infection has been considered.

In general, data on CF practices and micronutrient malnutrition during infancy and early childhood and their association with poor growth and micronutrient malnutrition in young children in Indonesia have been very limited, especially when focussed on selected functional outcomes. Numerous authors (Walker and Black, 2007, Dewey and Adu-Afarwuah, 2008, Lassi et al., 2013, Black, 2017, Gould, 2017) have suggested that all interventions should be designed to be context specific; and include baseline measurements to determine the level of micronutrient deficiency with appropriate adjustment for inflammation, where necessary, to avoid misleading conclusions.

We hope that this research reported here might lead to the development of interventions to improve CF practices, reduce the proportion of micronutrient deficiencies, anaemia and morbidity and enhance growth. Effective strategies during the CF period are urgently required to address these public health concerns, because interventions after the first two years of life appear to have little impact on subsequent child growth and development (Bhutta et al., 2008). Achieving the goal set by the WHO to reduce childhood stunting by 40% by 2025 (de Onis et al., 2013) is a priority for Indonesia.

Therefore, the overall goal of this research was to examine the growth and micronutrient status of breastfed infants during the CF period from the Sumedang district, West Java province, Indonesia. This was achieved by recruiting a cohort of breastfed infants at 6 months of age and following them at 9 and 12 months of age to accomplish the following specific objectives: a) characterise the CF practices at each age (Chapter 3); b) evaluate the adequacy of energy and nutrient intakes during CF in relation to the WHO estimated needs (Chapter 3); c) investigate relationships between WHO CF indicators, local sentinel foods, nutrient adequacy, and subsequent infant growth (Chapter 3); d) assess and compare the proportion of deficiencies of iron, zinc, vitamin A, and selenium, based on serum biomarkers among the infants at aged 6, 9, and 12 months of age with and without adjustment for inflammation (Chapter 4); e) investigate the extent to which the micronutrient biomarkers, socio-demographic factors, inflammation, helminth infections, and genetic Hb disorders are associated with infant Hb concentrations and anaemia at 6 and 12 months of age (Chapter 5).

The results showed increase in the proportion of stunting from 6 to 12 months of age among infants living in Sumedang district, West Java. Despite the high compliance to BF practices, the transition from EBF to CF in this study was not in accordance with the WHO Guiding Principles for Breastfed Children (PAHO/WHO, 2003), and may have been a major factor contributing to the increase in the proportion of stunting and underweight observed here. Fortified infant foods predicted subsequent linear growth in this rural setting, although their consumption compromised the use of dietary diversity score as

an indicator of dietary quality. Fortification of infant foods with powdered cow's milk together with micronutrients may have been responsible for their positive effect on subsequent linear growth. However, the complementary diets were inadequate in both quantity and quality (Chapter 3).

We also found a high proportion of iron and selenium deficiency among these Indonesian infants although a lower proportion of vitamin A and zinc deficiency, especially at 12 months of age. Furthermore, our study has confirmed that correcting biomarkers of iron, vitamin A, and zinc for inflammation, irrespective of the method used, markedly changed the proportion estimates for deficiency for these Indonesian infants in all three age groups. The regression adjustment approach presented here significantly increased the proportion of ID (based on adjusted low serum ferritin values), while simultaneously decreasing the proportion of vitamin A and zinc deficiency (Chapter 4).

One of the most notable findings of this study was the high proportion of anaemia among these breastfed Sumedang infants approaching levels indicative of a severe public health problem (i.e., >40%) at 9 and 12 months of age (WHO, 2011a). Of the seven micronutrient biomarkers investigated, serum ferritin was the only strong positive predictor of Hb at both 6 and 12 months of age, highlighting the importance of an adequate iron status for these infants at this time. The non-nutritional factors shown to be positive predictors of Hb for the infants were low concentrations of CRP and being female. Both of these two factors were significantly associated with Hb level for the infants at 6 months of age, whereas only being female was significant at 12 months.

Hence, overall, our findings suggest that the CF practices of infants in Sumedang district, West Java were inadequate in both quantity and quality. FIFs may improve growth, but because they are also associated with a lower dietary diversity score during infancy, their consumption among infants is of concern. Of the micronutrient deficiencies examined, ID was the strongest predictor for both Hb and anaemia at 6 and 12 months of age, emphasizing the importance of adjusting ferritin for inflammation, as without such

adjustment, the proportion of ID is significantly higher, which is misleading compared to the actual burden of deficiency.

## 6.2 Recommendations

- We recommend that mothers or caregivers are trained to prepare FIFs with the recommended dry matter content to enhance their energy and nutrient density. In addition, mothers/caregivers should be encouraged to increase the consumption of appropriate and affordable animal-source foods (i.e., dairy products, flesh foods, and eggs), as well as fruits and vegetables during the CF period to ensure the WHO indicators for MDD and MAD are achieved. In this way infants will also become exposed to a variety of textures and flavours, thus facilitating the development of both healthy food preferences and healthy gut microbiota.
- However, we do not recommend any further increase in the consumption of FIFs, despite their seemingly positive effect on linear growth in view of the safety concerns surrounding high doses of iron fortificants and the importance of exposing infants to a range of foods and textures to develop healthy food preferences.
- We emphasize the necessity for adjusting for inflammation when interpreting biomarkers of iron, vitamin A, zinc, and possibly selenium status. Without such adjustments, proportion estimates for these micronutrient deficiencies will be incorrect in settings with a high burden of inflammation.
- Our results show that anaemia remains a persistent and severe public health problem during infancy in Sumedang district, Indonesia. Despite the introduction of public health programs aimed to improve iron status during later infancy in Indonesia, low iron status remains a major predictor of low Hb concentrations at this time among infants in Sumedang district. Therefore, we recommend an urgent re-evaluation of the efficacy of the performance of both the anaemia and ID control programmes in the district.

- A printed report of the results together with individual consultation with a field doctor was provided after every follow-up to develop a strong rapport between research team and participants. In addition, any positive results would encourage parents to continue good feeding/caring practices, while negative results would encourage parents to seek support from existing health systems. Therefore, we recommend the provision of oral and written feedback to parents who participated in the study, immediately after obtaining results for anthropometry assessment and complete blood count examination.
- The study was conducted in Sumedang district, West Java province, a district with rates of stunting (41.1%) and underweight (14.6%) comparable to the national prevalence in Indonesia (National Institute of Health Research and Development, 2013). Although the coverage of our study area prevents our results from being generalisable to the country, nevertheless this study area represents the agricultural community which is the largest community in Indonesia (around 33.5% of Indonesian population in 2015).
- We recognise the need for long-term and more sustainable solutions to improve nutritional status of under 5 children in our study area. Therefore, we plan to develop and evaluate a tailor-made nutrition education program for school children and for parents/caregivers by engaging grandmothers. Grandmothers play a pivotal role in advising mothers and changing community perceptions of feeding practices, while school children play an important role in modifying their own behavior toward foods both currently and when they become parents
- Finally, researchers are urged to collaborate with governments and health practitioners to ensure proportion estimate of micronutrient deficiencies are reliable, develop effective policies and guidelines designed to support, implement, and evaluate programmes for improving micronutrient status and associated health outcomes. Only in this way can countries with limited resources such as Indonesia prioritize programmes that are capable of delivering the

greatest improvements in the nutritional status and quality of life for their children in the future.

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## Appendices

### Appendix A Questionnaires

1. Name of interviewer : \_\_\_\_\_
2. Date of interview (dd/mm/yyyy) : \_\_\_\_\_
3. Mother's name : \_\_\_\_\_
4. Mother's identification number : \_\_\_\_\_
5. Infant's name : \_\_\_\_\_
6. Infant's identification number : \_\_\_\_\_
7. Date of birth (dd/mm/yyyy) : \_\_\_\_\_
  
8. Infant's age 6 months (+ 4 weeks) :  Yes  No
9. Gestational age > 37 weeks :  Yes  No
10. Birth weight >1500 gram :  Yes  No
11. Exclusively/predominantly breastfed for at least 4 months:  
 Yes  No
12. Mother's (parents') informed consent :  Yes  No

**Note: if the answer of questions #8-12 is NO, the infant cannot be enrolled to this study**

13. At the time of screening, does the baby have a severe illness (active tuberculosis, severe anaemia (haemoglobin < 70 g/L), acute malnutrition (mid-upper arm circumference < 11.5 cm) :  Yes  No

**Note: if the answer of question #13 is YES, the infant cannot be enrolled to this study**

- 
14. Gender :  Male  Female
  15. Infant's birth order:  
 First infant  Second infant  Third or subsequent
  16. Number of pregnancy(ies):  
 1  2  3  4  5+
  17. Number of live birth(s):  
 1  2  3  4  5+
  18. Please provide under five children's age (in year):

	1	2	3	4	5	NA
First	<input type="checkbox"/>					
Second	<input type="checkbox"/>					
Third	<input type="checkbox"/>					
Fourth	<input type="checkbox"/>					
Fifth	<input type="checkbox"/>					

19. Village name : \_\_\_\_\_

20. Mother's recent job?  
 Civil servant    Working in private company    Entrepreneur  
 Farmer    Manual worker    Other: \_\_\_\_
21. Mother's last formal education?  
 Elementary school    Junior high school    Senior high school  
 University    Don't know
22. Father's recent job?  
 Civil servant    Working in private company    Entrepreneur  
 Farmer    Manual worker    Other: \_\_\_\_
23. Father's last formal education?  
 Elementary school    Junior high school    Senior high school  
 University    Don't know
24. Number of adult(s) family member ( $\geq 18$  y.o) in the house: \_\_\_\_
25. Number of child(ren)  $\leq 5$  y.o in the house : \_\_\_\_
26. Number of member of child(ren) 6-12 y.o in the house : \_\_\_\_
27. Number of member of adolescent(s) (13-17 y.o) in the house : \_\_\_\_
28. Total member of the family in the house : \_\_\_\_
29. Do you have a private house? :  Yes    No
30. Do you have this item at home?

	Yes	No
Watch	<input type="checkbox"/>	<input type="checkbox"/>
Television	<input type="checkbox"/>	<input type="checkbox"/>
Mobile-phone or phone	<input type="checkbox"/>	<input type="checkbox"/>
Refrigerator	<input type="checkbox"/>	<input type="checkbox"/>
Bicycle	<input type="checkbox"/>	<input type="checkbox"/>
Motorcycle	<input type="checkbox"/>	<input type="checkbox"/>
Car	<input type="checkbox"/>	<input type="checkbox"/>
Electricity	<input type="checkbox"/>	<input type="checkbox"/>
Radio	<input type="checkbox"/>	<input type="checkbox"/>

31. Do you own a garden or farmyard at home? :  Yes    No
32. Do your food sources come from your own garden/farmyard?  
 Yes    No
33. If yes, please specify the food : \_\_\_\_
34. Does your family have livestock (cow, buffalo, fish, poultry)?  
 Yes    No
35. If yes, please specify the livestock : \_\_\_\_
36. Has your infant been hospitalised? :  Yes    No
37. If yes, please specify the reason(s) for hospitalization : \_\_\_\_
38. Has your infant been vomiting in the last 2 weeks? :  Yes    No
39. Has your infant had fever in the last 2 weeks? :  Yes    No
40. Has your infant had diarrhea in the last 2 weeks? :  Yes    No
41. Has your child had cough in the last 2 weeks? :  Yes    No
42. If your child had cough in the last 2 weeks, did he/she breathe faster than usual with short, rapid breaths or have difficulty breathing?

- ( ) Yes ( ) No
43. Has your infant been refusing food in the last 2 weeks?  
( ) Yes ( ) No
44. Has your infant been immunised? : ( ) Yes ( ) No
45. Can mother show the infant's immunisation card?: ( ) Yes ( ) No

**If yes, please take picture of the immunisation card!**

46. Has your infant received BCG vaccination? : ( ) Yes ( ) No
47. Has your infant received Polio vaccination? : ( ) Yes ( ) No
48. Has your infant received DPT vaccination? : ( ) Yes ( ) No
49. Has your infant received Measle vaccination? : ( ) Yes ( ) No
50. Has your infant received Hepatitis B vaccinations?: ( ) Yes ( ) No
51. Has your infant received worm medicine in the past 6 months?  
( ) Yes ( ) No
52. What type of toilet facilities do members of your household use?  
( ) Private with septic tank ( ) Private with no septic tank  
( ) Shared/public ( ) Pit  
( ) River/stream/creek ( ) Yard/bush/forest
53. What is the main source of drinking water for your household?  
( ) Piped water inside the house ( ) Piped water inside the yard  
( ) Piped water to the public tap ( ) Open well inside the house  
( ) Open well in house yard ( ) Open public well  
( ) Protected well inside the house ( ) Protected well in houseyard  
( ) Protected public well ( ) Spring water  
( ) Water from rivers ( ) Water from lake  
( ) Rain water ( ) Bottled water
54. Do make your water safer for consumption? : ( ) Yes ( ) No
55. If yes, what strategy do you usually do to make your water safer to be consumed?  
( ) Boil ( ) Add bleach or chlorine ( ) Solar disinfection  
( ) Strain it through a cloth ( ) Let it stand and settle  
( ) Use water filter (ceramic or sand or composite) ( ) Other: \_\_\_\_
56. Does anyone smoke/tobacco in the house? : ( ) Yes ( ) No
57. Do you use an open fire for cooking in the house?: ( ) Yes ( ) No
58. How long after birth did you breastfed your infant?  
( ) Soon/within first hour after birth ( ) More than one hour
59. During the first three days after birth, did you give your infant yellow liquid form your breasts? : ( ) Yes ( ) No
60. During the first three days after birth, did you give your infant other food or drink?  
[ ] Honey [ ] Milk (other than breastmilk) [ ] Infant's formula  
[ ] Butter [ ] Mineral water [ ] Fruit juice  
[ ] Water with sugar and/or salt (exclude ORS) [ ] Coffee or tea  
[ ] Nothing [ ] Other: \_\_\_\_
61. Did your infant consume breastmilk during the day and night?  
( ) Yes ( ) No
62. If no, how long have you been breastfeeding your infants (in months)?
63. Have you introduced complementary foods to your infant?  
( ) Yes ( ) No

64. Has your infant received vitamin A supplementation in the last 6 months?  
 Yes                       No                       Don't know
65. Has your infant had any multivitamin/minerals supplements in the last 1 month?  
 Yes                       No                       Don't know
66. Has your infant received sprinkles (TABURIA) in the last 6 months? :  
 Yes                       No                       Don't know
67. What kind of roads are by the house? :  Paved                       Unpaved
68. What are the walls of the house made of?
69.  Concrete/brick                       Wood                       Bamboo                       Other: \_\_\_\_
70. What is the material of the floor of the house?
71.  Earth or dirt                       Bamboo                       Wood
72.  Concrete/brick                       Marble/granite/ceramic                       Other: \_\_\_\_
73. What is the construction material of the roof of the house?  
 Brick/Concrete                       Wood                       Asbestos/zinc
74.  Leaves                       Other: \_\_\_\_
75. Is there enough light inside the house during daylight?
76.  Yes                       No
77. Are there any ventilation/windows in the house which can support good air change? :  Yes                       No
78. Are the ventilation/windows opened during the visit?  
 Yes                       No
79. Are there any mechanical equipment to circulate/move the air inside the house? :  Yes                       No
-

## Appendix B Measurement and Blood Collection

1. Name of measurer : \_\_\_\_
2. Date of measurement (dd/mm/yyyy) : \_\_\_\_
3. Mother's name : \_\_\_\_
4. Mother's identification number : \_\_\_\_
5. Infant's name : \_\_\_\_
6. Infant's identification number : \_\_\_\_
7. Infant's weight (kg)  
First: \_\_\_\_ Second: \_\_\_\_ Third: \_\_\_\_
8. Infant's length (cm)  
First: \_\_\_\_ Second: \_\_\_\_ Third: \_\_\_\_
9. Mid-upper arm circumference (cm)  
First: \_\_\_\_ Second: \_\_\_\_ Third: \_\_\_\_
10. Head circumference (cm)  
First: \_\_\_\_ Second: \_\_\_\_ Third: \_\_\_\_
11. Mother's height (cm)  
First: \_\_\_\_ Second: \_\_\_\_ Third: \_\_\_\_
12. Mother's weight (cm)  
First: \_\_\_\_ Second: \_\_\_\_ Third: \_\_\_\_
13. Father's height (cm)  
First: \_\_\_\_ Second: \_\_\_\_ Third: \_\_\_\_
14. Mother's haemoglobin (g/L) : \_\_\_\_
15. Time of blood collection (hh/mm) : \_\_\_\_
16. Time of last meal before blood collection (hh/mm) : \_\_\_\_
17. Does your infant feel sick today?  
( ) Yes ( ) No
18. If yes, please specify : \_\_\_\_

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**Thank you for your time, your responses are very important to us.**

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## Appendix C Lesson learnt from the data collection process

- Conducting trainings on study protocols and standard operational procedures (SOPs) for research assistants and cadres were very important to make sure that the data collection process went as planned.



- Conducting a pilot study under supervision would help research assistants and cadres to find solutions for common problems. Some of the most common problems:

1. Difficulty in finding the respondents' address as it only consisted of the name of the village without name of the street and number. Solution: Getting help from local people to find the address and arrange the travel route before the home visit to save time.



2. Respondents and cadres called the same food different names. Solution: Providing pictures of food with names to make sure that the food listed in the weighed record was correct.



3. We collected a substantial amount of data, including questionnaires, anthropometric measurements, weighed record, blood, urine, and faeces samples, with a high possibility of omitting a variable. Solution: We made a record card with checklist boxes and assigned a coordinator to check the completeness of the record card for every respondent.



4. Blood collection was one of the most difficult components of the study as some parents refused it as they felt that they could not see the immediate results. Solution: We provided blood type and complete blood count examination to parents who participated in the study.



Nama	: Ryon		
Umur	: .....		
Alamat	: .....		
<b>GOLONGAN DARAH :</b> / Rh :			
			
Anti - A	Anti - B	Anti - AB	Anti - Rh

5. Handling, labelling, and mapping samples were very important. Solution: We worked together with the laboratory personnel to revise the SOP for handling, labelling, and mapping the samples.



- Although the pilot study helped us with troubleshooting, we still found some new problems during the data collection process. Having discussion time and learning as a team made it easier to solve the problems faster. Providing feedback for research assistants and cadres helped the team to perform better. A happy team makes for a great team.



