

Iron status at age 6 months in Colombian infants exclusively breast-fed for 4-5 versus 6 months

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Abbreviations:

CF: Complementary feeding; EBF: Exclusive breastfeeding; Hb: Haemoglobin; Hct: Haematocrit; MCV: Mean corpuscular volume; SF: Serum ferritin; WAZ: weight for age z score; WLZ: weight for length z score; LAZ: length for age z score; WHO: World Health Organization.

Authors' contributions to the manuscript were as follows:

GAO - study concept and design, design of new CF guidelines, conducted the study, performed the statistical analyses, and wrote the manuscript, critical reading of the manuscript.

ML, MF – involved with design of new CF guidelines and trial, data interpretation and critical reading and revision of manuscript.

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Abstract

Background: The optimal age for introducing complementary feeding to breast-fed infants may differ depending on the setting. Prolonged exclusive breastfeeding (EBF) protects against infection but may increase the risk of iron deficiency (ID)/anaemia (IDA) in vulnerable infants.

Aim: To compare haemoglobin (Hb), serum ferritin (SF), anaemia (Hb<11g/l), ID (SF <12µg/l) and IDA (Hb<10.5g/dl+Hct<33%+ID) using observational analyses in 6-month old infants from Bogota, Colombia who were EBF for 4-5 versus ≥6 months; and examine predictors.

Methods: Infant feeding was recorded, anthropometry performed and blood obtained for Hb and SF at 6 months in healthy term infants (birth weight>2500g), all EBF for ≥4 months.

Results: 108 infants (54% boys) were recruited; 46% EBF for 4-5mo, 54% EBF at 6mo. Prevalence of anaemia, ID and IDA was 20%, 10% and 5%, with no significant difference between EBF4-5 and EBF6 groups. In multivariate models, anaemia/ID were predicted by greater weight gain from 0-6 mo, and anaemia also by caesarean delivery; Hb was lower in infants with higher intake of cows' milk; SF was lower in boys and those with greater weight gain. EBF4-5 versus EBF6 was not a significant predictor of any outcome.

Conclusion: Anaemia and ID were common at 6 months but were not affected by EBF for 4-5 versus 6 months, suggesting 6 months EBF is safe in this population. However, further research is required to examine effects on later iron status. The findings highlight the need to emphasise avoidance of cow's milk before 12 months. Word count 241

Key words: exclusive breastfeeding, growth, iron status, cows' milk

What is known

- The World Health Organisation (WHO) recommends exclusively breastfeeding (EBF) for 6 months
- This protects against infection but may be associated with greater risk of iron deficiency (ID) and iron deficiency anaemia (IDA)

What is new

- 20% of healthy term infants from Bogota were anaemic at 6 months, although only 5% had IDA
- EBF for 4-5 versus 6 months did not influence anaemia, ID, IDA or haemoglobin/serum ferritin
- Modifiable predictors of Hb/ anaemia were higher cow's milk intake, highlighting the need to strengthen recommendations to avoid cow's milk before 12 months and caesarean delivery, which may be related to earlier cord clamping

Introduction

The World Health Organisation (WHO) recommends that infants should be exclusively breast-fed (EBF) for 6 months, followed by the introduction of complementary foods (CF) alongside continued breastfeeding [1-3]; however in most settings, CFs are frequently introduced before 6 months of age[4]. The WHO recommendation was based on the findings of a systematic review and expert consultation which concluded that infants EBF for 6 months rather than 3-4 months had a significantly lower risk of gastrointestinal infection, together with potential benefits for the mother in terms of delayed return of menses and faster post-partum weight loss. However, 6 months EBF was also associated with an increased risk of iron deficiency (ID) and iron deficiency anaemia (IDA) in vulnerable infants studied in a RCT in Honduras [1, 3, 5]. Other observational studies not eligible for the WHO review, including several in higher income settings, have also suggested that relatively longer periods of EBF are protective against gastrointestinal, ear and respiratory infections, and against hospitalisation for infection [6-10], whilst infants with more prolonged EBF have also been reported to be at greater risk of ID and IDA later in infancy [11, 12]. The only recent RCT to compare infant iron status with 4 versus 6 months of EBF, conducted in Iceland, reported significantly higher serum ferritin (SF) concentrations at 6 months with earlier introduction of CF, although all infants in this low risk population had SF within the expected range and none were ID or had IDA [13]. Collectively, these data suggest that the optimal duration of EBF may be different depending on the setting and balance of risks and benefits.

The primary aim of our study was to compare measures of iron status (Hb, SF), Anaemia, ID and IDA at 6 months of age in infants EBF for 4-5 versus ≥ 6 months (EBF4-5 versus EBF6), from deprived areas of Bogota, Colombia where the prevalence of anaemia (defined as $Hb < 11g/dL$) measured between 6 and 11 months is high ((59.7%), ENSIN 2010[4]. The secondary aim was to determine predictors of iron status in this population.

Methods

Subjects and study design

Healthy term infants with birth weight >2500g were recruited from the growth monitoring programme at Suba and Fontibon hospitals in Bogota, Colombia. Both are Baby Friendly hospitals covering a population with low socioeconomic status. Family incomes come mainly from informal and part-time jobs. Most families live in rented or shared households with public service coverage including tap water, electricity and waste disposal. Approximately 29% of families experience food insecurity (ENSIN 2010) [4].

Mothers who were EBF at 4 months were approached and invited to participate in a randomised control trial (ISRCTN57733004) of standard versus new complementary feeding guidelines, starting at 6 months [14]. All mothers were advised to EBF until 6 months. 108 mothers who met the inclusion criteria and who were willing to participate were recruited at 6 months, and gave written informed consent. A non-fasting morning blood sample (<5mL) was collected by venepuncture at 6 months. Data on iron status, anthropometry and food intake was collected from all infants; anaemic infants (defined as Hb <11g/dL according to local practice) were treated and were not included in the RCT although their data were used in this observational analysis (Figure 1). The study was approved by research ethics committees at University College London and Pontificia Universidad Javeriana, Bogota, Colombia.

Biochemical measurements: Measurements of Haemoglobin (Hb; using spectrophotometry), Haematocrit (Hct), mean cell volume (MCV; using automated flow cytometry), C-reactive protein (CRP; using two-site enzyme-linked immune-absorbent assay (ADALTIS-Dizar)) were performed within 24 hours, and samples for SF (measured using two-site enzyme linked immune-absorbent assay ELISA (DALTIS-Dizar)) were stored at <20°C prior to analysis. CRP was used as a biomarker of inflammation; infants with values >6mg/l were excluded. The following cut-offs recommended by WHO were used: Hb <11g/dL, Hct <33%,

SF<12µg/l[15], MCV < 70fl[15]. Anaemia was defined as Hb<11g/dL, and ID as SF<12µg/L. IDA was defined as Hb<11.0g/dL+Hct<33%+SF <12µg/L (WHO); results using other definitions are provided for comparison [16-18]. We also performed analyses using an altitude-adjusted value of 12.3g/dL for Hb and 37% for Hct as recommended by WHO, since Bogota lies at an altitude of 2,600m [15, 19].

Anthropometric measurements: Birth weight and length at birth were collected from medical records. At 6 months length was measured using an infantometer with fixed headboard and movable footboard, to the nearest 1mm (mean of 3 measurements). Infants were weighed naked on an electronic scale (Tanita). Results were converted to SD scores using WHO growth standard data[20].

Assessment of infant feeding: Exclusivity of breastfeeding was defined using a strict definition covering the whole of the first six months as follows: i) exclusive breastfeeding: infant has received only breast milk without any other food, liquids or supplements and ii) complementary feeding: infant has received solid and liquid foods other than breast milk including other milks such as infant formula, follow on formula and cow's milk.

At 6 months, infant feeding was recorded using a semi-quantitative food frequency questionnaire (FFQ) and a 24 hour recall. The FFQ included a table on which the researcher recorded information based on the intake of 8 selected Colombian food groups. Mothers were asked the number of times, and number of portions per day, week, or month that each food was consumed during the last month. If the infant had never consumed the food it was recorded as 'never'. Foods were then defined as being consumed daily, weekly or monthly.

A 24 hour recall was also applied. Mothers described all foods consumed by the infant in the last 24 hours. To estimate nutrient intakes, the amount of each food (as tablespoon,

cup, etc) was manually converted into grams or ml consumed per day based on standardized measures previously calculated[21], and data were entered into a spreadsheet (Excel) that included information on the nutrient content of 208 foods provided by Colombian Food Composition Tables, USDA National Nutrient Database for Standard Reference, food labels (infant food) and manufacturers' information for formula milk[22]

Daily breast milk intake was estimated using an algorithm based on published data[23], and taking into account our previous unpublished data and the information recorded in the 24 hour recall on (i) number of breastfeeds/day, (ii) duration of each breast feed, (iii) number of breastfeeds per night and (iv) infant appetite. Nutrient intakes from breast milk were estimated using Colombian Food Composition Table data and the USDA National Nutrient Database for Standard Reference; assuming 70Kcal/100ml for energy and 0.03mg/100ml for iron.

Statistics

Sample size: The target sample size for the RCT was 64 infants per group [14]. However, this sample size could not be achieved within the time-frame of the study, mainly because the prevalence of EBF in Bogota had fallen, reducing the number of eligible infants. 108 mothers who met the inclusion criteria were recruited at 6 months. For the current analyses, infants were grouped according to the duration of EBF: 4-5 months (EBF4-5) or ≥ 6 months EBF (EBF6).

Data Analyses were performed using SPSS version 18. In the primary analyses, indicators of iron status and growth were compared between the EBF4-5 and EBF6 groups using t-test, Mann-Whitney or chi-square test. Secondary analyses were performed to explore predictors of iron status at 6 months and to adjust for potential confounders. Correlations between potential predictors of iron status were first examined and significant factors included in multivariate regression models. Gestational age, gender, birth weight $\leq / > 3$ kg, and mode of

delivery were included in all models as these variables have been reported to be significant predictors of iron status in other studies. Other potential predictors considered were anthropometric measures (LAZ 6mo, WLZ 0-6 mo, WAZ 0-6 mo), intake of complementary foods (such as meat, fruit, vegetable, milk (infant formula, cows milk) egg, legumes) and estimated energy, protein, fat, carbohydrate, iron and zinc intakes from breast milk and complementary foods. Logistic regression was used to determine predictors of anaemia and ID. It was not possible to examine predictors of IDA due to the small number of affected infants. Linear regression models were used to examine predictors of Hb/SF. SF concentrations and cow's milk consumption were not normally distributed and were therefore log transformed for analysis.

Results

Figure 1 shows the study design. Infants attended for the screening blood test at 6 months and outcomes reported in this paper were also measured at 6 months, within one week of the blood test. Fifty six (52%) of the infants were EBF at 6 months (EBF6) and 52 (48%) were exclusively breast-fed for 4-5 months (EBF4-5 infants). Nineteen of the EBF4-5 infants received CF between 4 and 5 months and 33 received CF between 5 and 6 months. 3 infants received a small amount of CF (fruit juice) on the day before the study visit; it was considered that this could not have altered their iron status and therefore they were included in the EBF6 group. There was no significant difference between groups in gestational age, delivery mode, birth weight or socio-economic characteristics (Table 1). EBF6 mothers were significantly older whilst a significantly higher proportion of the EBF4-5 group were boys.

Anaemia and Iron status

The proportion of infants with Hb<11g/dL in this population was high – 20% of all infants - but it was not significantly different between groups (11 (21.2%) for EBF4-5 and 11 (19.6%) for EBF6). Adjusting for altitude resulted in an increase in the proportion of infants with anaemia

to 67.3% and 69.6% respectively, but still with no significant difference between groups. Four (8.2%) of EBF4-5 and 6 (12.8%) of EBF6 infants had ID (SF < 12µg/L; Table 2). The number and proportion of infants with IDA was 5.1%. The prevalence of IDA did not differ significantly between groups and was similar when alternative definitions were used and when Hb was adjusted for altitude (Table 2). There were no significant differences in Hb, Hct, MCV or SF between EBF4-5 versus EBF6 groups (Table 2); nor when further sub-divided into those EBF for 4, 5 and ≥6 months (see supplemental digital content Table S1).

Growth

There was no significant difference in WAZ, WLZ, or LAZ at 6 months, or in the change in WAZ or LAZ from 0-6 months between EBF4-5 and EBF6 infants (See supplemental digital content Table S2). The proportion of EBF4-5 and EBF6 infants with LAZ < -2 SD at 6 months was not significantly different although a greater proportion of boys had LAZ < -2SD (12 (21.4%) versus 1 (2.0%) girl, $p=0.002$). There was no significant difference between EBF4-5 and EBF6 groups in the proportion with WAZ < -2SD (2 (3.8%) versus 2 (3.6%), $p=0.9$).

Breastfeeding practices

EBF6 infants had a significantly higher mean number of breastfeeds/day (7 (SD2) versus 6 (SD2), $p=0.02$) and higher estimated breast milk intake/day (830 ml (143) versus 714 ml (136), $p<0.001$) than EBF4-5 infants. The estimated average breast milk intake was 94ml/kg body weight in EBF4-5mo and 109 ml/kg body weight in EBF6 infants at 6 months of age. There was no significant difference in the number of night-time breastfeeds (EBF4-5 2 (1) versus EBF6 2 (1)).

Complementary feeding

There was no significant difference between EBF4-5 and EBF6 in estimated total energy intake (630 (SD83) Kcal/day versus 600 (94) Kcal/day, (95% CI -7 to 62), $p=0.12$). CFs in the

Dietary records were available for 51 infants in the EBF4-5 group (18 EBF for 4 mo, 33 EBF for 5 mo). CF provided an estimated 128 ((98) Kcal/day, 20% of total energy intake), 4.6 (3.9) g/day protein (39.3% of intake), and 1.09 (1.1) mg/d of iron (80.4 % of intake). 13.7% (7/51) of EBF4-5 infants received infant formula, 17.6% (9/51) follow on formula and 39% (20/51) (non-fortified) cow's milk, with a reported weekly frequency of consumption of 1.0 (2.9), 1.0 (2.6) and 2.0 (3.7) times/week and mean daily intake of 137ml (119), 149ml (139) and 85ml (112), respectively. The main sources of iron in EBF4-5 infants were infant formula (1.03mg/day), received by 14% (7/51); infant cereal (0.78 mg/day), received by 23.5% (12/51); follow on formula (0.76mg/day) received by 17.6% (9/51), and red meat (0.73mg/day) received by 17.6% (9/51). EBF4-5 infants had significantly higher estimated total iron intake than EBF6 infants (1.35mg/d (1.1) versus 0.32mg/d (0.07), $p<0.001$), of which 1.1 (1.1) mg/day came from complementary foods, while EBF6 infants had significantly higher iron intake from breast milk (0.32mg (0.06) versus 0.27 (0.06), $p<0.001$).

Predictors of anaemia and ID

Logistic regression models included (i) EBF4-5 v EBF6; (ii) independent variables found to be associated with anaemia or ID in univariate analyses in this study (for anaemia; sex ($r=0.193$, $p=0.045$), change in WLZ 0 to 6mo ($r=-0.259$, $p=0.007$), mode of delivery ($r=-0.203$, $p=0.036$) and LAZ at 6mo ($r=-0.228$, $p=0.019$): for ID; change in WAZ 0 to 6mo (-0.217 , $p=0.031$)): (iii) other variables reported to be related to these outcomes in previous studies (for anaemia; gestational age and birthweight $\leq/>3$ kg: for ID; sex, mode of delivery, gestational age, birthweight $\leq/>3$ kg). The significant predictors of anaemia were mode of delivery (higher risk in infants delivered by caesarean section compared to vaginal delivery, $\beta=1.29$, OR 3.7 (95% CI 1.16 to 11.4), $p=0.027$) and change in WLZ from 0 to 6 months ($\beta=0.47$, OR 1.6 (95% CI 1.14 to 2.24), $p=0.007$). ID was predicted by change in WAZ from 0 to 6 months ($\beta=0.59$,

OR 2.6 (95% CI 1.13 to 5.97), $p=0.02$). Anaemia and ID in this cohort were not predicted by the age of introduction of CF.

Predictors of Hb and SF

In univariate analyses, Hb at 6 months was significantly and negatively correlated with daily intake of protein ($r=-0.29$, $p=0.003$), fat ($r=-0.23$, $p=0.02$), zinc ($r=-0.25$, $p=0.01$), cow's milk ml/day ($r=-0.58$, $p=0.01$); and with positive change in WLZ from 0 to 6 mo ($r=-0.21$, $p=0.03$). SF was negatively correlated with weight at 6mo ($r=-0.23$, $p=0.02$), and with change in WAZ from 0 to 6mo ($r=-0.295$, $p=0.003$). There were no significant correlations between indicators of iron status and other dietary variables. Stepwise multiple regression models were used to identify predictors of Hb and SF. Intake of cow's milk ml/day was a negative predictor of Hb. SF was negatively predicted by the change in WAZ from 0 to 6mo and positively by female sex (Table S3).

Discussion

In this population of infants living in poor socioeconomic conditions in Bogota, Colombia, where 59% of infants are anaemic and 24% ID at 1 year of age despite existing supplementation policies (ENSIN 2010)[4], we found a high prevalence of anaemia and ID even at 6 months. 20% of the infants had Hb < 11g/dl, the cut-off used routinely in Bogota, and 10.4% had ID, although only 25% of the anaemia was attributable to ID. Indicators of iron status (Hb, SF) and the prevalence of anaemia, ID or IDA were not influenced by whether complementary foods had been introduced alongside breastfeeding from 4 months or delayed until 6 months. Our findings suggest that EBF for 6 months is safe, even in this high-risk population, in terms of the prevalence of ID and IDA at 6 months of age, although effects on iron status beyond this age need to be determined.

The optimal duration of EBF has been a topic of much discussion, particularly in relation to short-term health effects on infection risk, allergy risk and ID. More prolonged EBF has beneficial effects on the risk of gastrointestinal and respiratory tract infections, including risk of hospitalisation for infection, even for infants in higher income countries. However, it is uncertain whether 6 months EBF is adequate in terms of providing sufficient iron to ensure adequate iron status for all infants, particularly those with poor iron stores at birth. This is of concern given irreversible adverse effects of ID and IDA on brain development [24, 25]. A RCT in Honduras using iron-fortified commercial baby foods reported significantly lower Hb in infants EBF for 6 months without iron supplements (10.4 (0.1)) than in infants who received CF (10.9 (0.1)) [5]. In another trial, Icelandic infants randomised to receive CF from 4 months had significantly higher SF at 6 months (70.0 (73.3) µg/L) than infants EBF for 6 months (44.0 (53.8) µg/L), although both groups in this low-risk population had SF within the normal range[13]. Observational studies have also reported that more prolonged EBF is associated with an increased risk of anaemia later in infancy. In a retrospective analysis of data from a study comparing German infants who were fully BF for 4 months with those fed iron-fortified

formula, the prevalence of ID/IDA in BF infants was 19%/4% and 21%/2% at 7 and 10 months respectively; formula-fed infants had normal iron status at both ages. At 7 months, infants from the breast milk group also had lower Hb (11.4 g/dl versus 12.1g/dl) compared with formula fed infants [12] Similar results were found in a cross sectional survey of infants aged 6-24 months in the US (NHANES III), in which the proportion with a reported history of anaemia was significantly higher in infants fully BF for 6months (10%) than in those fully BF for 4-5 months (2.3%, $p=0.007$) [11].

In our study, infants in the EBF4-5 group received an estimated 1.35mg/day of iron per day with 1.09mg/day coming from CFs, while the EBF6 group received only 0.32mg/day from breast milk. Neither group met recommended intakes for iron (DRI EAR 6.9mg/day, RDA 11mg/day from 6-8 months[26], WHO RDA 9.3mg/d[15]), although it should be noted that 6 months is the lower end of the range for these recommendations, and occurs at around the time that infant iron stores from birth are likely to be exhausted and an alternative supply required. Nevertheless, it is clear that to achieve the recommended intakes in a short period of time would require a significant amount of either iron-rich or iron-fortified CF, or supplementation. The main sources of iron in the EBF4-5 group were infant formula, infant cereals, follow on formula, and red meat, with the latter contributing approximately 10% of total iron intake. However, the small amount of these CFs consumed may have displaced breast milk without contributing significant amounts of bioavailable iron, which may explain the lack of effect on iron status. Furthermore, 63% of the EBF4-5 group started CF during the 5th month which may have blunted differences in iron intake and consequently iron status.

Consistent with previous studies in slightly older infants, we found that cow's milk consumption was a negative predictor of Hb. Soh [27] reported that cow's milk consumption >500ml/day in infants less than 1 year of age was negatively related to Hb levels, probably due to displacement of iron-rich foods. Similar results were reported in the ALSPAC study, where

infants were categorised according to the type of milk received, with the cow's milk group divided by intake (<600 and >600ml); a higher proportion of anaemic infants were identified in the cow's milk (28%) and breast milk (32%) groups compared with those receiving formula [28]. The estimated mean daily intake from cow's milk in the 6 month old infants in our study was much lower at 85ml/day, and it seems more likely that the adverse association with Hb may be due to blood loss related to gastrointestinal bleeding[29]. Surprisingly, almost 39% of the infants in our EBF4-5 group were receiving cow's milk, despite that fact that it is not recommended for infants less than 12 months in Colombia. This is clearly an area which requires further attention in terms of educational messages. Indeed, we previously reported that the new CF guidelines tested in this trial, which included advice to avoid cow's milk as the main drink, resulted in reduced cow's milk intake at 12 months and higher Hb[14].

Anaemia was common in our population despite the fact that all were born at term, with birth weight above 2.5kg and EBF for at least 4 months. However, the prevalence of IDA was lower at 5%. The cause of anaemia not attributable to ID is unclear, although our results are very similar to those reported by Yang et al [18] in fully breast-fed Mexican infants at 6 months of age. Potential explanations include deficiencies of other nutrients such as vitamin A or B12; both of which are plausible in this population [4]. Haemoglobinopathies and chronic infection may also be a factor in other settings but are not likely explanations in our study population. Both anaemia and ID were predicted by relatively greater weight gain from birth to 6 months, consistent with other studies that have reported a higher risk of iron deficiency in infants with the most rapid growth[17,18]. Anaemia was also associated with the mode of delivery, with a higher risk in infants born by caesarean session. This may be due to earlier umbilical cord clamping following surgical delivery resulting in lower Hb and infant iron stores at birth. Delayed umbilical cord clamping has been shown to improve infant iron status in RCTs in both Mexico and Sweden [30, 31]. ID was also higher in boys, consistent with several published studies suggesting that male infants are at greater risk.

Using an adjusted Hb cut-off of 12.3g/dl, given the high altitude of Bogota, the proportion of anaemic infants increased almost three fold, which seems implausible for a group of apparently healthy infants. To our knowledge, there are no published data on associations between Hb at 6 months and later brain development at different altitudes. However, in a large clinical trial in young children treated for WHO-defined severe pneumonia across 8 sites in 7 low-resource countries [32], children at high altitude (>2000m) presented with significantly more severe hypoxemia and cyanosis than children at low altitude and took longer to recover from hypoxemia. Importantly, children with anaemia (defined as Hb<11g/dl) at high altitude had a significantly increased risk of poor outcome. This suggests that the combination of anaemia and high altitude is of particular concern and that a higher cut-off for Hb may well be appropriate in these settings.

The major strength of our study is the availability of dietary intake data which allowed us to examine associations with iron status. However, the study had a number of limitations including the observational design and the relatively small sample size which meant that we were not able to examine predictors of IDA in logistic regression models. In addition, we were not able to measure other markers of iron status, or to examine the prevalence of ID/IDA later in infancy, since anaemic infants were treated and were not enrolled in the subsequent RCT. Data on the timing of cord clamping were not available thus we cannot establish that this was responsible for the association between mode of delivery and risk of anaemia.

Conclusion

Healthy term, normal birth weight infants from Bogota, all EBF for at least 4 months, had a high prevalence of anaemia at 6 months; 20% of the infants had Hb< 11g/dl and 10% were ID, although only 25% of anaemia was attributable to ID, suggesting that other factors such as Vitamin A or vitamin B12 may be important. Indicators of iron status and prevalence of anaemia, ID and IDA were not influenced by the introduction of CF between 4-5 and 6 months

alongside breastfeeding. This suggests that 6 months EBF is safe in terms of iron status at age 6 months in this population, although effects on later iron status were not investigated.

Anaemia and ID were more common in faster growing infants, and anaemia was more common in infants delivered by Caesarean section. The latter finding may relate to the timing of clamping of the umbilical cord, but this information was not available in our study and further studies exploring time of cord clamping and other possible risk factors in infants born by CS are warranted. The intake of cow's milk was negatively associated with Hb, highlighting the need to strengthen the recommendation to avoid cow's milk in the first year of life and to make sure this message is delivered before 6 months. Further research is required to define the most 'appropriate' infant Hb cut-offs for anaemia in infants living at high altitudes, preferably relating proposed cut-offs to clinical outcomes such as cognitive development and morbidity from respiratory infection, and to identify other factors contributing to anaemia in this population.

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Figure 1 legend. Flow diagram of study

Table legends.

Table 1. Baseline characteristics of mothers and infants in the study according to the duration of EBF

Table 2. Anaemia, ID, IDA and measures of iron status at 6 months in infants EBF for 4-5mo versus 6mo

Table 3. Predictors of Anaemia and ID at 6 months in infants EBF for 4-5mo versus 6mo

Supplemental digital content legend

Table S1. Exclusive breastfeeding for 4mo, 5mo or 6mo and iron status

Table S2. Comparison of anthropometric measurements at 6 months between EBF 4-6mo and EBF 6mo

Table S3. Predictors of Anaemia and ID at 6 months in infants EBF for 4-5mo versus 6mo

Figure 1. Flow Diagram of study

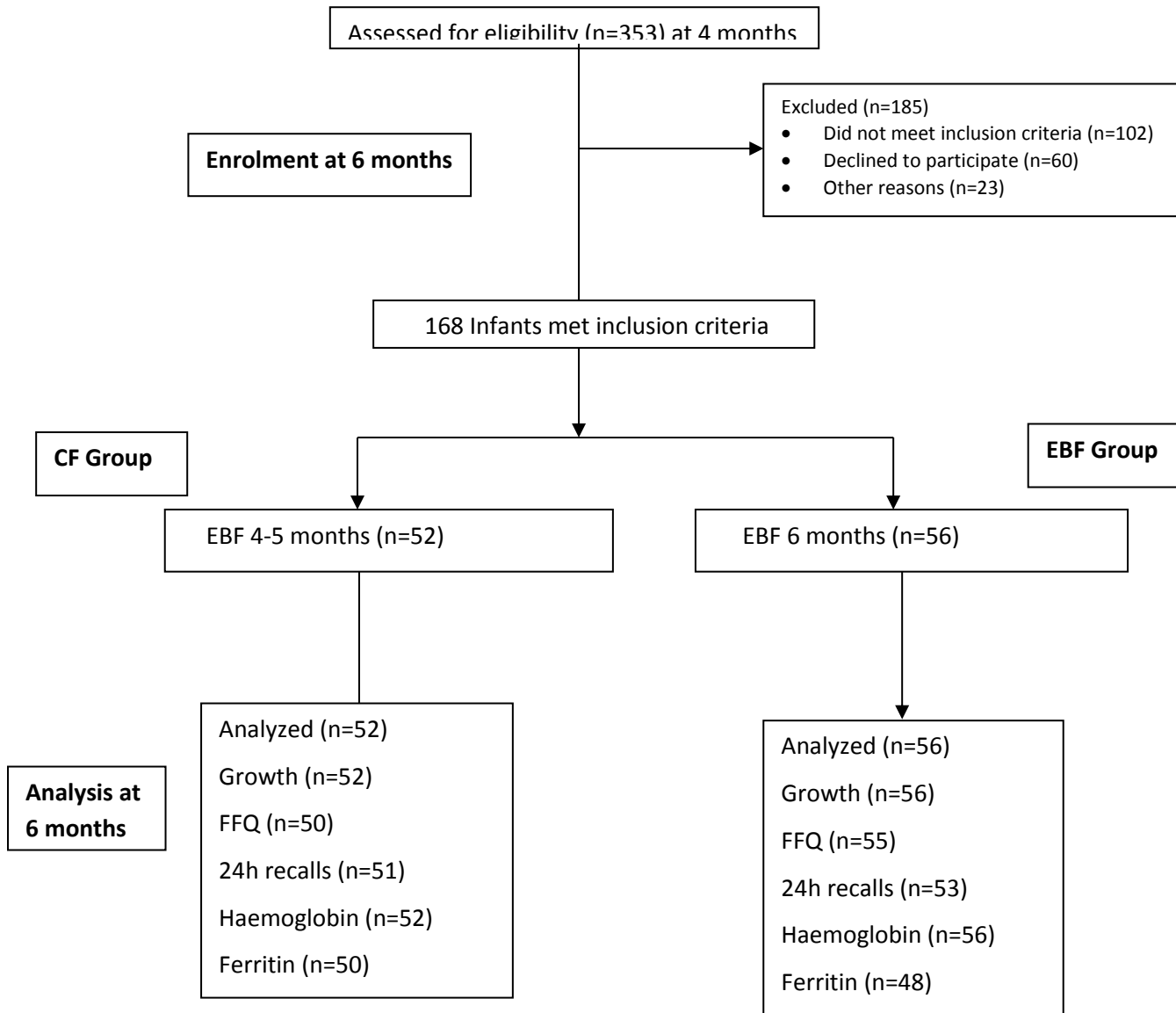


Table 1. Baseline characteristics of mothers and infants in the study according to the duration of EBF

| Variable | EBF4-5 (n=52) | EBF6 (n=56) | p value |
|-----------------------------------|--------------------------|--------------------|----------------|
| Female n (%) | 18 (35) | 32 (57) | 0.02* |
| Male n (%) | 34 (65) | 24 (43) | |
| Gestational age Wks (SD) | 39.1 (0.94) | 39.1 (1.1) | 0.92 |
| Birth weight g (SD) | 3106 (340) | 3063 (336) | 0.51 |
| Birth Length cm (SD) | 50.0 (1.7) | 49.9 (1.9) | 0.82 |
| Head circumference cm (SD) | 34.0 (1.4) | 33.8 (1.1) | 0.41 |
| WAZ at birth | -0.45 (0.71) | -0.50(0.72) | 0.75 |
| WLZ at birth | -0.92(1.21) | -1.04 (0.90) | 0.56 |
| LAZ at birth | 0.20 (0.96) | 0.25 (1.0) | 0.83 |
| HCZ at birth | -0.15 (1.1) | -0.27 (0.89) | 0.62 |
| Mother's age years (SD) | 22.5 (5.8) | 25.0 (6.5) | 0.04 |
| Father's age years (SD) | 26.6 (8.4) | 27.7 (8.3) | 0.52 |
| Family size n (SD) | 5.5 (2.1) | 5.0 (1.5) | 0.17 |
| # children n (SD) | 1.5 (0.6) | 1.6 (0.8) | 0.40 |
| # rooms in the house n (SD) | 2.4 (1.0) | 2.4 (1.2) | 0.94 |
| Mother finished high school n (%) | 30 (59) | 29 (53) | 0.12* |
| Birth weight < 3Kg | 21 (40) | 25 (45) | 0.66 |
| <i>Mode of delivery</i> | | | |
| Caesarean n (%) | 14 (27) | 17 (31) | |
| Vaginal n (%) | 38 (73) | 38 (69) | 0.65* |

EBF= Exclusive breastfeeding; LAZ= length for age z score; WAZ= weight for age z score, WLZ= weight for length z score; HCZ= head for age z score. Wks= weeks. P values from Student's t-test unless indicated; *p<0.05 by Pearson chi square; SD= Standard Deviation

Table 2. Anaemia, ID, IDA and measures of iron status at 6 months in infants EBF for 4-5mo versus 6mo

| Variable | EBF4-5 | | EBF6 | | Mean Difference | |
|---------------------------------------|-----------|-------------|-----------|-------------|-----------------------|-----------|
| | n | Mean (SD) | n | Mean (SD) | (95% CI) | p value* |
| Hb (g/dL) | 52 | 11.7 (1.0) | 56 | 11.7 (1.0) | -0.04 (-0.43 to 0.35) | 0.84 |
| Hct (%) | 52 | 34.6 (2.4) | 56 | 34.9 (2.5) | -0.35 (-1.28 to 0.59) | 0.47 |
| MCV (fL) | 51 | 70.6 (4.0) | 55 | 71.0 (3.9) | -0.37 (-1.89 to 1.16) | 0.64 |
| Ferritin (µg/L) | 50 | 35.7 (30.3) | 48 | 40.6 (34.6) | -4.95 (-18.0 to 8.09) | 0.45 |
| | n (%) | | n (%) | | Total n (%) | p value** |
| Anaemia (Hb<11g/dL) | 11 (21.2) | | 11 (19.6) | | 22 (20.4) | 0.85 |
| Anaemia (Hb<12.3g/dL) | 35 (67.3) | | 39 (69.6) | | 74 (68.5) | 0.79 |
| Iron Deficiency (SF<12µg/L) | 4 (8.2) | | 6 (12.8) | | 10 (10.4) | 0.46 |
| IDA ¹ (WHO,2001) | 2 (4.0) | | 3 (6.3) | | 5 (5.1) | 0.61 |
| IDA ² (Domellof ,2002) | 2 (4.1) | | 2 (4.3) | | 4 (4.2) | 0.96 |
| IDA ³ (Thorisdottir, 2003) | 2 (4.1) | | 3 (6.4) | | 5 (5.2) | 0.61 |
| IDA ⁴ (Yang, 2009) | 2 (4.0) | | 3 (6.3) | | 5 (5.1) | 0.61 |
| IDA adjusted for altitude | | | | | | |
| IDA ¹ (WHO,2001) | 2 (4.0) | | 3 (6.3) | | 5 (5.1) | 0.61 |
| IDA ² (Domellof ,2002) | 2 (4.1) | | 3 (6.4) | | 5 (5.2) | 0.61 |
| IDA ³ (Thorisdottir, 2003) | 3 (6.1) | | 4 (8.7) | | 7 (7.4) | 0.63 |
| IDA ⁴ (Yang, 2009) | 3 (6.0) | | 6 (12.5) | | 9 (9.2) | 0.26 |

Hb= haemoglobin; Hct= haematocrit; MCV= mean corpuscular volume. SF= serum ferritin (log transformed)

EBF= exclusive breastfeeding.

SD= Standard Deviation, *P<0.05 (T test), 95% CI (confidence interval), **P<0.05 (Pearson chi square)

1. WHO (2001) 6 to 59 months: definition Hb<11.0g/dL+Hct<33%+SF <12µg/L

2. Domellof (2002) 6 months: definition Hb10.5 g/dL + SF <9µg/L + MCV <71fL

3. Thorisdottir (2003) 6 to 24 months: definition: Hb<10.5g/dL + SF <10.5 µg/L+ MCV<74fL

4. Yang (2009) 6 months: definition Hb<10.5g/dL+SF<12µg/L

Table 3. Predictors of Anaemia and ID at 6 months in infants EBF for 4-5mo versus 6mo

| <i>Variables</i> | Beta | OR | 95% CI | P value | R² adjusted |
|-------------------------|-------------|-----------|---------------|----------------|---|
| Anaemia | | | | | 0.24 |
| Change in WLZ 0 to 6 mo | 0.47 | 1.6 | 1.14 to 2.24 | 0.007 | |
| Delivery by Caesarean | 1.29 | 3.7 | 1.16 to 11.4 | 0.027 | |
| ID | | | | | 0.16 |
| Change in WAZ 0 to 6 mo | 0.59 | 2.6 | 1.13 to 5.97 | 0.02 | |

Logistic regression models; only significant predictors are shown in the table.

Anaemia was defined as Hb as: Hb<11g/dL. Variables entered into model were study variables: WLZ from 0 to 6mo, mode of delivery (0= vaginal; 1=Caesarean), mode of feeding (0=EBF 4-5, 1=EBF ≥6mo). Other variables: Birth weight (birth weight less than 3kg=1) sex (1=male, 0= female). Nagelkerke R Square R=0.24

ID was defined as SF<12µg/L. Variables entered into model were study variables: WAZ from 0 to 6mo, mode of feeding (0=EBF 4-5, 1=EBF ≥6mo). Other variables: mode of delivery (0= vaginal; 1=Caesarean); Birth weight (birth weight less than 3kg=1) sex (1=male, 0= female), gestational age (number of weeks). Nagelkerke R Square R=0.16