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A 12-month follow-up report on safety of infant formula containing the calcium salt of (6S)-5-methyltetrahydrofolic acid or folic acid fed till the age of 4 months

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Abstract

Background/Objective: The calcium (Ca) salt of (6S)-5-methyltetrahydrofolic acid (5-MTHF) is used as a source of folate in infant foods. Infants who were fed an infant formula with 5-MTHF-Ca or folic acid for 4 months showed appropriate growth. In this 12 months follow up study, we investigated growth and safety outcomes in infants, who were fed a formula with folic acid or 5-MTHF-Ca from ≤1 month until 4 months of age.

Subjects/Methods: Body weight and length and head circumference were measured at 12 months. Separate linear regression models were applied for infants receiving infant formula with either 5-MTHF-Ca (15.8 µg/100 kcal n=120) or folic acid (15.2 µg/100 kcal, n=120) up to 4 months of age. Equivalence in growth at 12 months was used as proxy for safety of 5-MTHF-Ca. In each model, the outcomes depended on the formula group, duration of the intervention, sex of the infant, and body weight at birth. Breastfed infants (n=120) were included as an additional reference group.

Results: The estimated differences (95% confidence intervals, 95%CI) between the 5-MTHF-Ca and folic acid groups for body weight [86 (-262.15, 434.14)g], body length [-0.31 (-1.05, 0.43) cm] and head circumference [0.21 (-0.17, 0.60) cm] were within the prespecified equivalence range. The number of adverse events and results of routine hematological markers did not differ between the groups.

Conclusion: Growth measures of infants at 12 months of age are equivalent in the 5-MTHF and folic acid groups. These results confirm the long-term safety and suitability of infant formula with 5-MTHF-Ca.

Trial registration: This trial was registered at ClinicalTrials.gov (NCT02437721).

Introduction

Folate is an essential micronutrient, with important roles for growth and development. Folic acid is an oxidized synthetic water-soluble form of folate which does not exist in nature and needs to be converted into its active metabolite (6S)-5-methyltetrahydrofolic acid (5-MTHF) after absorption [1]. 5-MTHF acts as methyl donor in the one-carbon metabolism [2], which is crucial for biological pathways like nucleic acid synthesis, DNA methylation, cellular proliferation and growth [3–5]. When DNA methylation and gene expression patterns are modified during early life, these epigenetic modifications could have long-term effects on health outcomes [5;6]. In human milk, folates are excreted as 5-MTHF, the predominant physiological folate form, also present in human blood [7;8]. Higher 5-MTHF in human milk was associated with higher folate status in infants and a depletion of folate in maternal circulation [9].

The dietary folate requirement of infants in the age group 0–6 months (65 µg/d or 9.4 µg/kg body weight) is higher than that of older infants or adults [10] and is considered relevant for growth. Not achieving adequate folate intake during early postnatal life may be linked to health outcomes such as development of obesity and associated non-communicable diseases later in life [5].

Studies in adults demonstrated that 5-MTHF has a higher bioavailability than folic acid, as indicated by a more profound increase in plasma [11;12] and red blood cell (RBC) folate concentrations [13;14]. In a recent randomized controlled trial (RCT) in infants, we reported similar results with higher whole blood folate and RBC-folate levels in infants who received an infant formula with 5-MTHF-Ca compared to those who received an infant formula with folic acid [15;16]. Thus, 5-MTHF as a folate source for infant formula have higher bioavailability compared to folic acid [17]. The use of folic acid is associated with detectable amounts of unmetabolized folic acid in blood, although the impact on infant health is unclear [18;19]. Another aspect is that 5-MTHF does not depend on the conversion by the methylenetetrahydrofolate reductase (MTHFR), especially when the enzyme activity is limited due to common polymorphisms in the MTHFR gene [20].

Previously we showed that feeding infant formula containing the calcium (Ca) salt of 5-MTHF from ≤ 1 month until 4 months of age was safe, well-tolerated and associated with equivalent growth at 4 months of age when compared to a standard infant formula containing equimolar amounts of folic acid [15]. The infant's anthropometric measures (body weight, body length, and head circumference) in the two formula groups at the age of 4 months were similar to those of a breastfed infant reference group [15]. Early life exposure to different forms of folate might be associated with different long-term outcomes such as infant anthropometric measures by mechanisms related to early life epigenetic programming. Therefore, we investigated in the reported infant population [15] the infant's anthropometric measures, hematology markers and the occurrence of adverse events upto the age of 12 months, comparing the 5-MTHF-Ca to the folic acid infant formula group.

Subjects and methods

Study design and intervention

The study design was reported in details before [15;16]. In brief, this was a randomized, double-blind, parallel-group intervention study. Healthy term singleton infants (n=360) aged < 28 d at baseline visit (BV) were enrolled at the Department of Neonatology, Clinical Hospital Center "Dr Dragisa Misović-Dedinje", Belgrade, Serbia, between June 2015 and April 2017. The formula groups consisted of 240 infants whose parents had decided not to breastfeed for reasons not related to this study. Those infants were randomized to receive either infant formula with folic acid (15.2 µg/100 kcal = 78 µg/100 g formula powder, n=120, control) or infant formula with an equimolar amount of 5-MTHF-Ca (Metafolin®, 15.8 µg/100 kcal = 81 µg/100 g formula powder, n=120, intervention).

The intervention with infant formula lasted from BV until 4 months of age (visit 4, V4). In addition, 120 breastfed infants were followed as a reference group. The reference group was not randomized and did not receive any intervention during the study. Special precautions and care were taken not to discourage breastfeeding, and not to suggest bottle-feeding to parents of a breastfed baby. All infants who completed V4 (mean (SD) of age 112 (3) d) were invited for a final follow-up assessment at age 365 (7) d (12 months follow-up visit, FUV) between June 2016 and April 2018. All outcomes reported for the

follow-up (anthropometric measurements, hematology parameters and adverse events at age 12 months) were performed according to the study protocol.

The study protocol was reviewed and approved by the ethics committee of the University Hospital “Dr Dragiša Mišović-Dedinje” in Belgrade, Serbia. The study was registered at ClinicalTrials.gov (NCT02437721) prior to the start and conducted according to the Code of Ethics of the World Medical Association (Declaration of Helsinki and ICH-GCP) for medical research involving human subjects. Written informed consent was obtained from the participants’ parent/legal guardian/next of kin.

Anthropometric measurements

Anthropometric measures of all infants were performed at the study center at BV, after 1 month (V1), 2 months (V2), 3 months (V3), 4 months (V4) and 12 months (FUV). The same calibrated digital scale (SECA 336, Hamburg, Germany) was used to measure body weight at each of the study visits. Body length was measured using a vertical, stationary headboard, and head circumference was determined with a non-stretchable insertion tape. For all anthropometric outcomes two valid measurements were recorded. The mean was calculated and used for statistical analysis.

Safety parameters and adverse events

Venous blood was collected from all infants into EDTA-containing tubes at BV, V4 and FUV. Measurements of hemoglobin, hematocrit (HCT) and mean corpuscular volume (MCV) in EDTA-whole blood samples were performed at the Clinical Chemistry Laboratory at the Institute for Laboratory Diagnostics Konzilijum, Belgrade using an automatic analyzer Sysmex, model XT-2000i according to the manufacturer`s instructions. In addition, the investigators collected information on adverse events (AEs) and serious adverse events (SAEs) at study visits 1, 2, 3, 4 and FUV. For the follow-up safety analysis we focus on the AEs and SAEs retrospectively reported by the parents and documented by the investigators during the FUV at 12 months of age. AEs and SAEs and other assessments between V1 and V4 were described earlier [15].

Statistical analysis

The main outcomes of this follow-up analysis are infant’s body weight (in g), body length and head circumference (both in cm) at the age of 12 months. To investigate if these measures are clinically equivalent in the 5-MTHF-Ca (intervention) and folic acid (control) groups at FUV, we fitted three separate normal linear regression models for infants in the per-protocol (PP) population. Residual analysis (TA-plot, SL-plot and QQ-plot) was performed to assess the model fits and the presence of major outliers (Supplementary Statistical Material, page 21, section 4.2.6). In each model, the outcome measured at 12

months was dependent on the formula group, duration of the intervention, sex of the infant, and body weight at birth.

The outcome at FUV was considered equivalent in the intervention and in the control group if the point estimate for the intervention effect and its 95% confidence interval (95% CI) were contained in the prespecified equivalence range, which was computed as -0.5*SD and +0.5*SD of the outcome for the folic acid group.

To ensure comparability of the infants showing up (assessed) at FUV and those not showing up (drop outs) at FUV we checked for difference in the joint distribution of several variables by applying hypothesis test random forest with package hypoRF [21]. More details can be found in the Supplementary Statistical Material, Section 5.

All analyses were run using validated installations of the statistical software R version 4.4.2. The normal linear regression models were fitted with the add-on package stats and the ggplot2 visualization package of R was used for graphical illustrations. The Supplementary Statistical Material describes all statistical analyses in more detail and includes the analyses, plots and graphs for the modified intention-to-treat (mITT) population as well.

The per-protocol (PP) population was defined as all subjects who completed all visits without any protocol deviations. The mITT included all subjects who completed visit 1 after inclusion into the study. Further details are given in Troesch at al. 2019 [15]. Blood safety parameters were described for the PP and mITT population at FUV.

Results

Subject characteristics and disposition

In total, 360 infants were enrolled, including 240 infants that were randomly allocated either to an infant formula containing 5-MTHF-Ca or folic acid. The 120 breastfed infants served as a reference group (Supplemental Figure 1). Of the 360 initially recruited infants, 298 (82.8%) and 280 (77.8%) completed V4 and FUV, respectively and comprise the mITT population (Supplemental Table 1). Given the nature of the study, analysis of the PP population is frequently regarded as the more conservative approach in equivalence testing [22]. Therefore, we used PP analysis for the primary analysis and mITT analysis for confirmatory purposes (Supplemental Table 2 and Supplemental Table 3). The PP population consisted of 244 and 230 infants who completed V4 and FUV, respectively (**Table 1**). No difference in the number of dropouts was detected between the three study groups in the PP population. The main reasons for drop out between V4 and FUV were withdrawal of consent (n=7) and loss to follow-up (n=11).

Infants were fed the study formulas from the age of < 1 month until the age of 4 months. The longest duration in formula feeding in the PP population was 103 days and the shortest was 83 days. The FUV took place at a mean (SD) age of 365 (7) days (**Table 2**).

Comparing the joint distribution of several relevant variables for the infants assessed at FUV and those who dropped out before FUV, using hypoRF, no evidence was found for systematic bias between infants who retained in the study and those who dropped out. This suggests a non-selective drop out of participants that will not affect the outcomes. The p-values of the tests of equality in distribution at BV (p=0.99) and at V4 (p=0.84) for those assessed at FUV and those who dropped out before FUV are not significant (for percentage and density plots of included numeric variables see Supplementary Statistical Material, Section 5.2 and Supplemental Figure 2, respectively).

Anthropometric measurements

The models for the three anthropometric outcomes converged without problems and fitted the data accordingly. Log-transforming the data was not necessary as this did not lead to an improved model fit or model diagnostics (more details are available in Section 4.2 of the Supplementary Statistical Material). Infants in the 5-MTHF-Ca and folic acid group, were found to be equivalent in growth at the age of 12 months (FUV) assessed as body weight (mean (SD), 5-MTHF-Ca: 10,231 (1,137) g *vs* folic acid: 10,221 (1,077) g), body length (5-MTHF-Ca: 77.26 (2.41) cm *vs* folic acid: 77.63 (2.35) cm) and head circumference (5-MTHF-Ca: 46.40 (1.31) cm *vs* folic acid: 46.27 (1.38) cm) (**Figure 1**).

Figure 2 shows anthropometric measures at FUV plotted against birth weight for both female and male children and seem to suggest a positive relationship between birth weight and these measures, which might however depend on sex and intervention duration – other variables included in the model (Supplementary Statistical Material, Section 6.2 and 6.3). Infants in the formula groups demonstrated similar anthropometric measures when compared to the breastfed infants (**Table 2**).

Testing for clinical equivalence showed that for body weight at FUV, the estimated difference between 5-MTHF-Ca and folic acid group was 86.00 grams. With a 95% CI [-262.15, 434.14], it falls in the prespecified equivalence range [-538.39, 538.39] (p=0.626). For body length at FUV, the estimated difference between 5-MTHF-Ca and folic acid group was -0.31 cm. With a 95% CI [-1.05, 0.43], it falls in the equivalence range [-1.17, 1.17] (p=0.404). For head circumference at FUV, the estimated difference between the two formula groups was 0.21 cm. With a 95% CI [-0.17, 0.60], it falls in the equivalence range [-0.69, 0.69] (p=0.270) (**Figure 3**).

Results for the mITT population are qualitatively similar and are shown in Supplemental Figure 3 and 4 and Section 4.3 of the Supplementary Statistical Material.

Safety assessment

For all infants a total number of 6 AEs and 6 SAEs were reported retrospectively at FUV for the time between 4 months (V4) and 12 months of age (FUV). The 6 AEs were reported in 5 infants (folic acid: n=0, 5-MTHF-Ca: n=3, breastfed: n=3) and included overweight (n=2), respiratory tract infection (n=1),

gastrointestinal disorder (n=1), varicella (n=1) and common cold (n=1). They were of mild severity, and none was judged as related to the intervention. The 6 SAEs (folic acid: n=4, 5-MTHF-Ca: n=1, breastfed: n=1) included bronchiolitis acuta (n=2), acute serious otitis media (n=1), transient anemia (n=1), enteritis rotaviralis (n=1) and gastroenteritis and colitis origine non specificata (n=1), and affected 4 infants. None of these SAEs were related to the intervention and all infants recovered completely.

The majority of infants in the infant formula groups had normal levels of hemoglobin, hematocrit and mean corpuscular volume at FUV within the reference range for this age group (**Table 3**). The hematology markers were also similar between the breastfed infants and the formula fed infants, except for the hematocrit levels, where only 52.9% of the breastfed infants had hematocrit levels within the normal reference range.

Discussion

5-MTHF is the predominant form of folate in human milk [7;8]. For many years, folic acid has been used as the sole source of folate in infant formula. In 2020, the Scientific Committee on Food of the European Commission approved 5-MTHF-Ca as a source of folate for infant and follow-on formula [23]. A randomized, double-blind, controlled intervention trial in Serbian infants did not show adverse effects of the use of 5-MTHF-Ca in infant formula and demonstrated equivalence in weight gain compared to infants fed an infant formula with folic acid until 4 months of age [15].

In this follow-up assessment of the same infant cohort at the age of 12 months, we report here that infants receiving 5-MTHF-Ca (intervention) or folic acid (control) formula until 4 months of age show equivalence of anthropometric measures (body weight, body length and head circumference). In addition, the anthropometric outcomes of the formula fed groups were similar to those of a reference group of breastfed infants.

Folates play an important role for infant growth and development due to their involvement in the biosynthesis of purines and pyrimidines, the building blocks of DNA and RNA and consequently cell growth [3–5]. Maternal supplementation with folic acid during pregnancy is associated with higher placenta and birth weight and a lower risk of low birth weight or premature birth [24;25]. Folic acid supplementation of premature infants was associated with a stronger increase in length and weight gain, when compared to the non-supplemented group of premature infants [26]. However, another study comparing a low-folate cow's milk diet with a folic acid enriched diet reported that infants supplemented with folic acid only showed a greater growth (weight and length) at 6 months of age, with no difference in growth between the folic acid and control group in the second half of the first year of life [27]. This could suggest that effects of folate supplementation on growth (weight and length) may be particularly marked during phases of very rapid growth. Along this line, a study among Brazilian toddlers (6–24 months of age) used 50 µg/day folic acid supplementation for ~ 3 months and found that there was no effect on linear growth compared to the placebo group [28]. Another randomized controlled trial in Indian toddlers (6 to 35 months), supplementing twice the RDA of folic acid and/or vitamin B12 for 6 months,

reported that increases in anthropometric measures were confined to wasted, underweight, and stunted children in the supplemented groups. Higher dose supplementation may affect child growth only if growth is restricted due to deficiencies [29]. Above findings corroborate that in healthy study populations like ours, infants born after 37 weeks of gestation with normal birth weight (2500 to 4500 g), dietary folate intakes within the present adequate intake recommendations during the first half year of life should have no growth altering effects.

The reported AEs / SAEs were all in the range expected for this young population of infants and did not differ between the formula groups. Given the low number of AEs / SAEs occurring between V4 and FUV, the small difference (0 versus 3 AEs; 4 versus 1 SAEs) between the folic acid and the 5-MTHF-Ca group does not indicate unexpected effects of the intervention, also considering that three AEs and one SAE were reported in the reference group. The two cases of overweight infants occurred in the 5-MTHF-Ca formula and breastfed group, respectively. All observed AEs / SAEs were considered “not related” to feeding of the formula with either folic acid or 5-MTHF-Ca. The hematology parameters determined at 12 months of age in our infant cohort were mostly within the normal ranges for hemoglobin, hematocrit and MCV. The lower relative frequency of the hematocrit value within the normal ranges in the intervention group, which was even more pronounced in the reference group, was not considered as clinically significant by the pediatricians of the study team. In a cohort of Canadian infants, a hematocrit reference interval of 0.265 to 0.4 L/L for the first year of life was reported [30], which is in line with our findings. Therefore, the hematology markers did not suggest any issues related to the safety of the infant formula with 5-MTHF-Ca.

In summary, the formula groups were comparable regarding the hematology markers at 12 months of age and the incidence of reported adverse events. Neither the AEs / SAEs nor the hematology data raise any safety concerns up to the age of one year in the assessed population and for the chosen folate dose given during the intervention period.

A limitation of the present follow-up study concerns the lack of data regarding dietary intake beyond the end of the intervention phase (4 months of age). We have no information on the type of formula used after the study formula, or on food intake after weaning. Although our data showed equivalent body weight, body length and head circumference, we cannot attribute these results to the study product intervention only. Furthermore, blood samples were taken only to determine hemoglobin, HCT and MCV. No folate markers like RBC folate were measured at follow up, which could have allowed for estimating the folate intake for the months between V4 and FUV. Since we found that both formula groups had higher plasma folate levels than breastfed infants and the 5-MTHF-Ca group had higher RBC-folate levels than the folic acid group at V4 [16], such blood measurements could have allowed for observing if the between-group differences in folate concentrations either continued or disappeared after stopping the intervention. In addition, we do not have information if parents who became aware of the study purpose have started using supplemental folate for their infants after the study intervention.

In conclusion, the administration of the infant formula with 5-MTHF-Ca or folic acid until 4 months of age supports adequate growth and development in infants until 12 months of age similar to breastfed infants and does not raise any safety concerns.

Declarations

Data availability statement

Individual data will not be shared. Aggregated data beyond what is published in this paper can be shared with researchers upon reasonable proposal.

Ethical approval

The study protocol was reviewed and approved by the ethics committee of the University Hospital “Dr Dragiša Mišović-Dedinje” in Belgrade, Serbia.

Conflict of interest

Ines Warnke is employee of dsm-firmenich, Christina Hecht is employee of HiPP GmbH & Co. Vertrieb KG, Barbara Troesch was employee of DSM Nutritional Products Ltd. at the time of study conduction, Anja Wittke was employed at HiPP GmbH & Co Vertrieb KG at the time of manuscript drafting, Matteo Tanadini and Luisa Barbanti was employed by Zurich Data Scientists GmbH at the time of data analysis. Rima Obeid and Berthold Koletzko received remuneration from DSM and HiPP as speakers for medical education or for acting as scientific reviewers. Berthold Koletzko is the Else Kröner Senior Professor of Paediatrics at LMU – University of Munich, financially supported by the charitable Else Kröner-Fresenius-Foundation, LMU Medical Faculty and LMU University Hospitals.

Author contributions

Conceptualization: Barbara Troesch and Berthold Koletzko. Data curation / visualization: Rima Obeid and Luisa Barbanti. Methodology and formal analysis: Luisa Barbanti and Matteo Tanadini. Funding acquisition: Berthold Koletzko and Ines Warnke. Project administration: Barbara Troesch, Christina Hecht and Berthold Koletzko. Supervision: Barbara Troesch and Berthold Koletzko. Writing original draft: Ines Warnke, Anja Wittke, Barbara Troesch and Luisa Barbanti. Writing review & editing: all authors.

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Tables

Tables 1 to 3 are available in the Supplementary Files section.

Figures

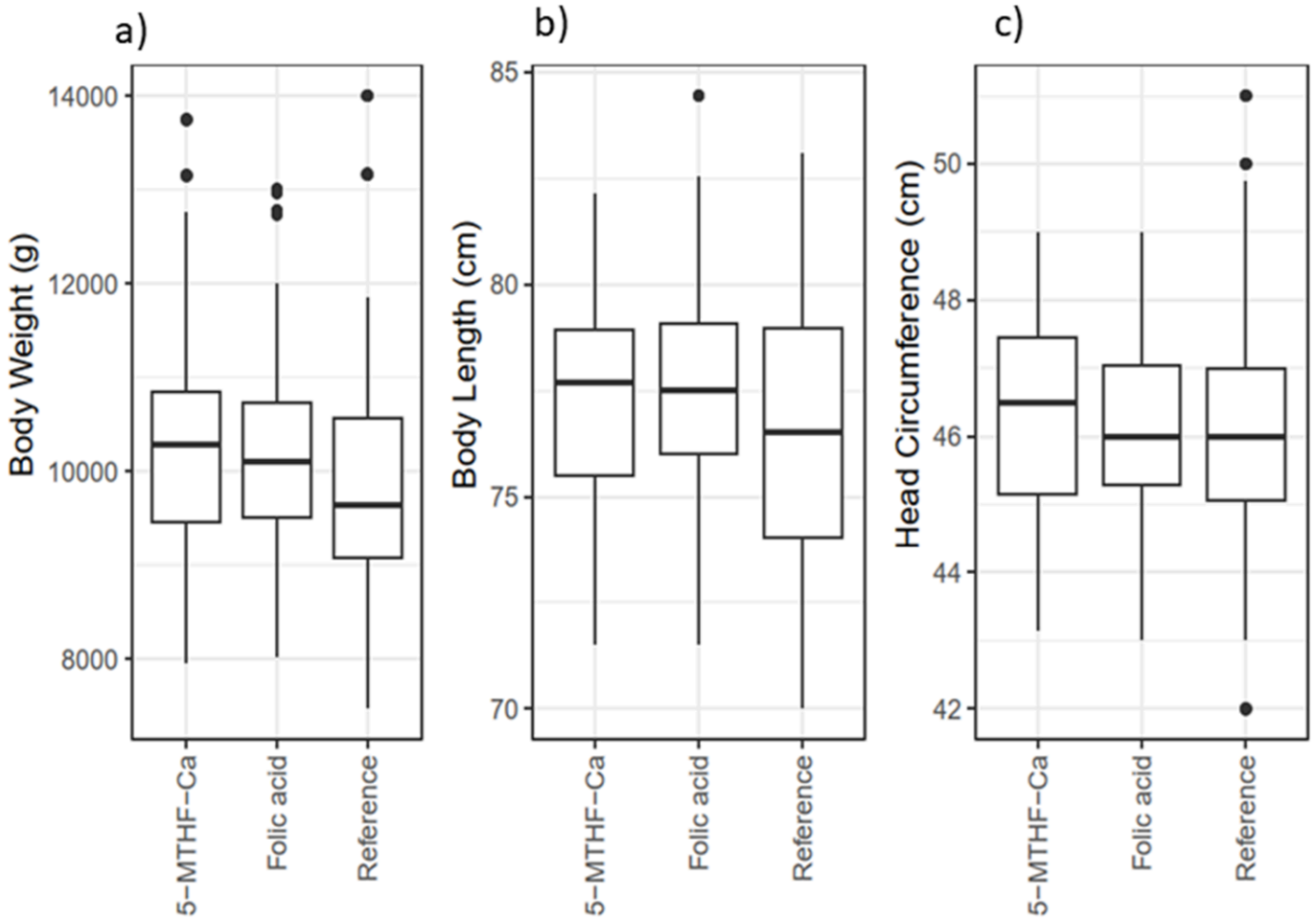


Figure 1

Anthropometric measures of infants in the intervention groups (5-MTHF-Ca, n=66 and folic acid, n=79) and the reference group (breastfed, n=85) at 12 months follow-up visit (FUV) in the per-protocol (PP) population (n=230). The figures show box-plots of: a) body weight (g); b) body length (cm); and c) head circumference (cm) with the medians, the 25th and 75th percentiles, and maximum and minimum values.

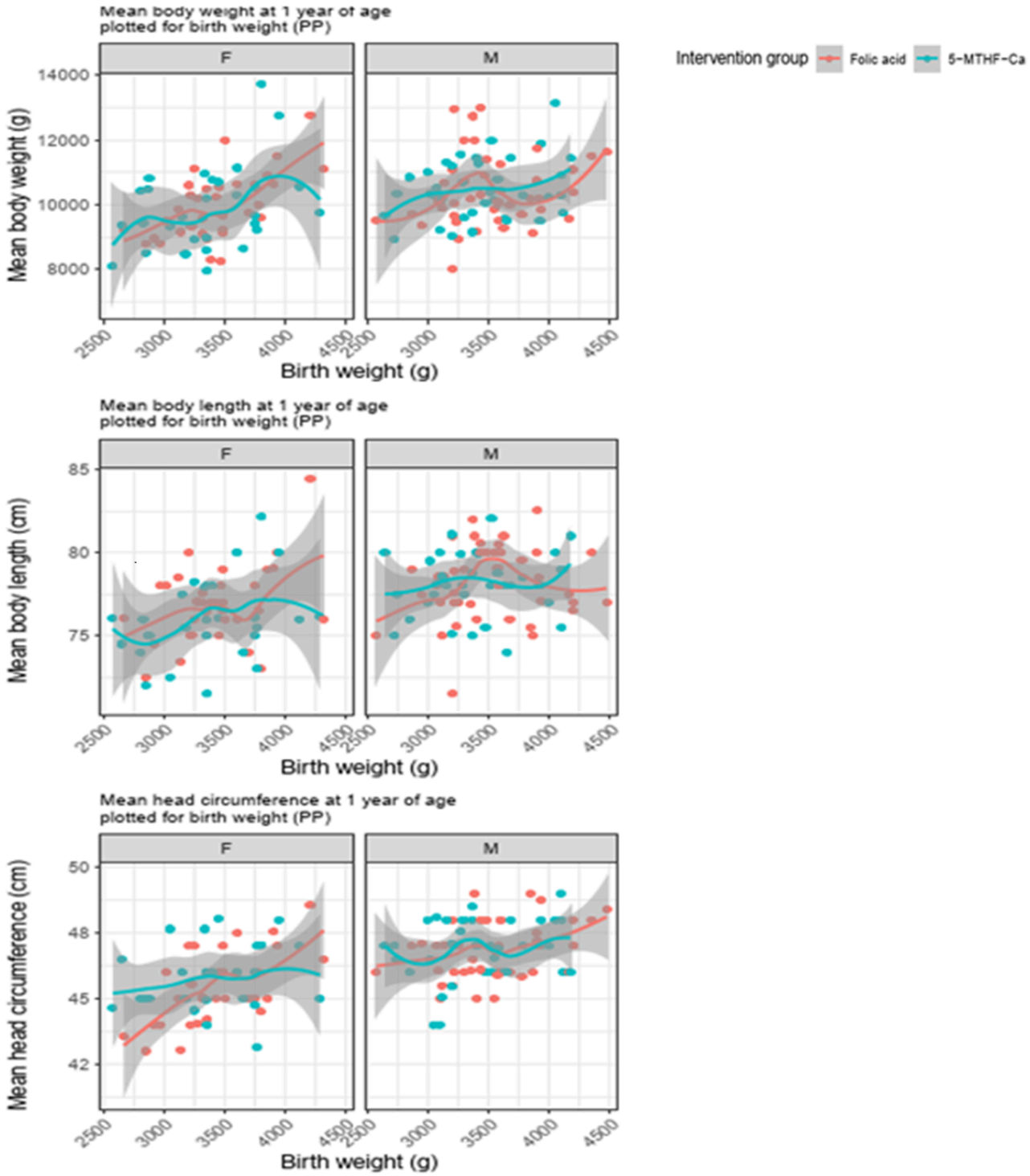


Figure 2

Descriptive graphs displaying the three outcomes mean body weight (g), mean body length (cm) and mean head circumference (cm) plotted against birth weight (g) for female (F) and male (M) infants according to the intervention (5-MTHF-Ca in blue and folic acid in red) at follow-up visit (FUV, age: 12 months). The individual data points are presented in the figure for the per-protocol (PP) population. A LOESS curve with confidence bands is added to each plot via the geom smooth function. All these

variables are considered in the modelling when assessing equivalence of the three responses (see Section 4 of the Supplementary Statistical Material for more details). Results were available from 145 infants who received infant's formula with 5-MTHF-Ca (n= 66) and folic acid (n= 79).

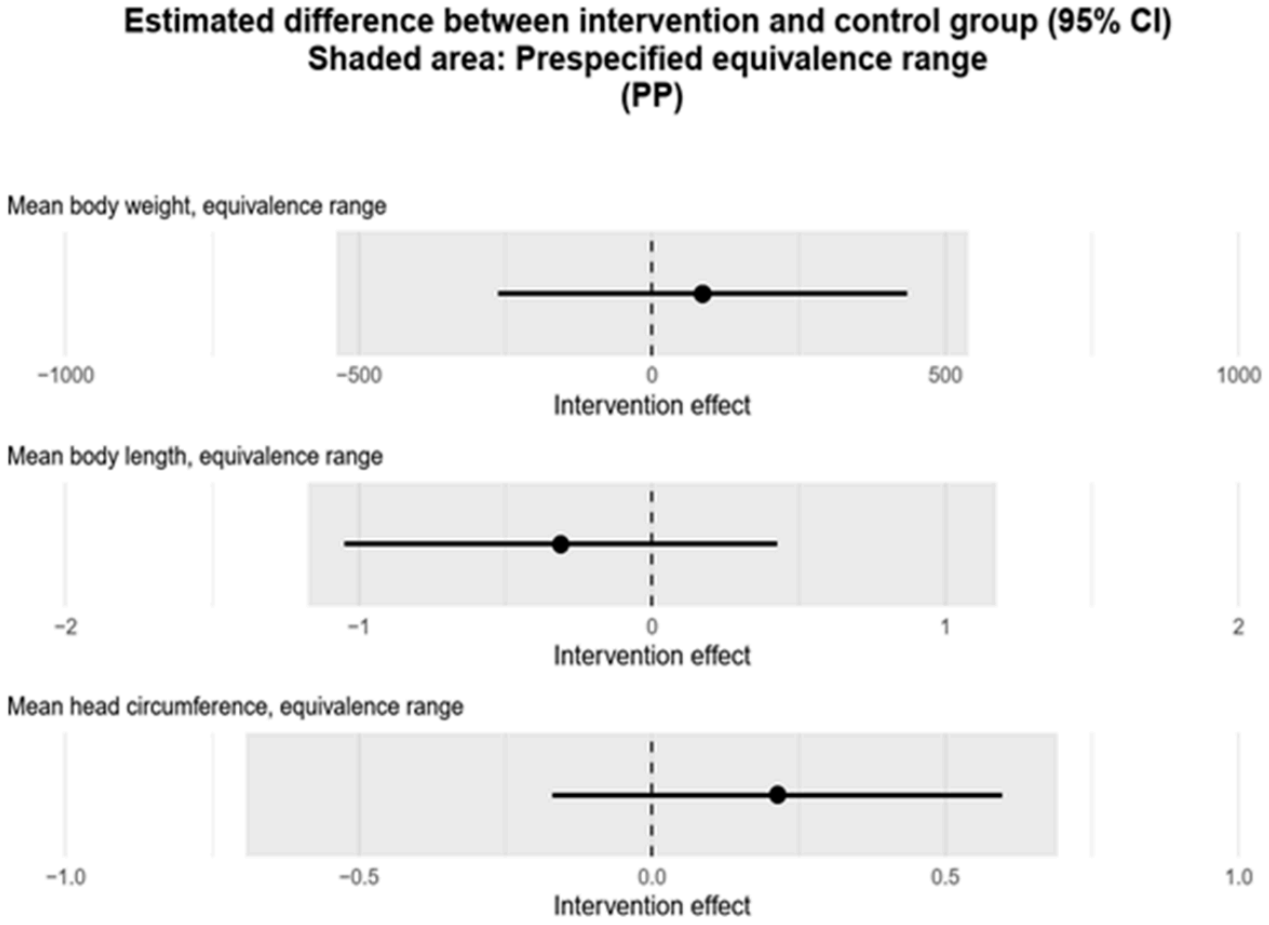


Figure 3

Graphical presentation of prespecified equivalence ranges (ER, grey shading) and calculated 95% confidence intervals (95%CI) for (from top to bottom) body weight (86.00 g; 95%CI [-262.15, 434.14]; ER [-538.39, 538.39], p=0.626), body length (-0.31 cm; 95%CI [-1.05, 0.43]; ER [-1.17, 1.17], p=0.404) and head circumference (0.21 cm; 95%CI [-0.17, 0.60]; ER [-0.69, 0.69], p=0.270) at the follow-up visit (FUV) for the per-protocol (PP) population. Details of the formal analysis are shown in the Supplementary Statistical Material.

Anthropometric outcomes at FUV were considered equivalent for the infant formula groups if the point estimate for the intervention effect and its 95%CI are contained in the prespecified equivalence range, which is computed as -0.5*SD and +0.5*SD of the outcome for the control (folic acid) group. The control

group received 15.2 μg folic acid per 100 kcal reconstituted infant formula; the intervention group received 15.8 μg 5-MTHF-Ca per 100 kcal starting in the first 28 days of life until 4 months of age.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryFiguresEJCN.docx
- 3Statisticalanalysis20260508.pdf
- Table1EJCN.docx
- Table2EJCN.docx
- Table3EJCN.docx