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# Dependence of bioavailability of folic acid and (6S)-5-methyltetrahydrofolate on baseline red blood cell folate concentrations in infants

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ABSTRACT

*Background:* Folate bioavailability may depend on folate status of an individual and the form of folate presents in foods.
*Objective:* We studied whether changes of red blood cell (RBC)-folate concentrations following dietary intervention with folic acid or the calcium salt of (6S)-5-methyltetrahydrofolate (5-MTHF-Ca) depend on baseline RBC-folate and differ by the folate form provided.
*Methods:* We studied 167 infants randomized to infant formula with either 15.2 µg folic acid or 15.8 µg 5-MTHF-Ca per 100 kcal from <1 month of age (baseline visit) until age 16 weeks (visit 4). Generalised Additive Models (GAMs) were used to study whether the changes in RBC-folate concentrations between baseline visit and visit 4 (study outcome) depend on the intervention (folic acid or 5-MTHF-Ca), length of the intervention and a smooth effect of baseline RBC-folate concentrations for each intervention group.
*Results:* The GAM base model showed that the change of RBC-folate was higher in infants with lower baseline RBC-folate concentrations. This model explained 42 % of the deviance in the data. For the group that received folic acid, this effect was estimated to be linear (effective degrees of freedom = 1). In the group receiving 5-MTHF-Ca, the effect of baseline RBC-folate on the change of RBC-folate was non-linear. The smooth effect of baseline RBC-folate on the change of RBC-folate concentrations differed between the intervention groups ($p = 0.002$). In infants with higher baseline RBC-folate concentrations, the change of RBC-folate concentration is systematically higher in the 5-MTHF-Ca group than in the folic acid group.
*Conclusion:* The bioavailability of folic acid and 5-MTHF-Ca shows physiological decline when baseline RBC-folate is high. The reduction of the bioavailability is more pronounced after folic acid intake. The results may impact infant's intake recommendations from different folate forms. The molecular mechanisms behind these results deserve further investigations.

## 1. Introduction

Folates refer to a group of chemically related cofactors that contribute to cellular methylation, amino acid metabolism, and de-novo nucleotide biosynthesis. Both folic acid and the calcium salt of (6S)-5-methyltetrahydrofolate (5-MTHF-Ca) are used as a source of folate in foods and food supplements. Repeated-dose intervention studies in adults have shown that at intake ≥400 µg/d, 5-MTHF-Ca induces a greater increase in red blood cell (RBC)-folate concentrations compared to folic acid [1–4]. Whereas, bioavailability results at daily intakes below 400 µg are inconclusive [5]. Beside the form of folate, the between study differences could be due to different intake patterns of

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folate, participant's characteristics or study duration.

In theory, intestinal folate absorption may level off upon chronic folate supplementation. Moreover, the folate forms could differ in their absorption. Human ileum and colon express two major folate transporters (influx receptors), the proton-coupled folate transporter (PCFT) and the reduced folate carrier (RFC) [6]. Provision of 400 μg/d folic acid for 16 weeks (versus no folic acid) to adults did not downregulate folate absorption or the expression of multiple folate receptors along the colon [6]. However, the participants started the intervention with rather high circulating folate concentrations due to mandatory folic acid fortification [6]. The bioavailability of folate may be lower when body stores of this nutrient are higher.

Oversupplementation of Caco-2 and HK-2 cells (intestinal and renal cell models) with folic acid causes a decrease in the protein and mRNA levels of folate receptors compared to cells incubated in a standard cell medium containing a normal amount of folic acid [7]. Whereas, folate oversupplementation in rats caused down regulation of intestinal folate uptake on the short but not on the long term [8]. In rats, experimental dietary folate deficiency caused low serum folate along with adaptive upregulation of folate uptake in the intestinal brush border membrane vesicles compared to the folate sufficient rats [9]. Feeding rats a folate deficient diet caused a physiological upregulation of mRNA and protein levels of PCFT and RFC [9]. Similar upregulation of intestinal folate receptors by insufficient dietary folate intake has been reported in mice [10]. Moreover, adaptive upregulation of renal folate reabsorption and higher expression of folate receptors have been detected in the renal brush border membrane of folate deficient rats [11]. These results suggest that folate status of the individual reflected by RBC-folate concentrations could influence intestinal folate uptake and renal elimination and thereby folate bioavailability. In addition, the different distribution of folate transporters (i.e., RFC is the main transporter in the terminal ileum and colon in human) and different binding kinetics of folic acid and 5-MTHF-Ca to PCFT and RFC [12] suggest that folate status may differentially affect folate bioavailability after supplementation of folic acid or 5-MTHF-Ca.

We tested the hypothesis that folate bioavailability upon repeated-dose supplementation is lower when baseline RBC-folate (as a tissue folate marker) is higher. In addition, we hypothesized that baseline RBC-folate may affect the differential bioavailability of folic acid and 5-MTHF-Ca. In a 12-weeks randomized controlled trial (RCT) [13], we demonstrated that feeding infants with infant formula containing 5-MTHF-Ca (15.8 μg/100 kcal) causes on average a higher increase in infant's RBC-folate concentration compared to a formula with an equimolar amount of folic acid (15.2 μg/100 kcal) [14]. In the present study, we evaluated data from the same RCT to study whether the comparative bioavailability of 5-MTHF-Ca and folic acid depends on infant's baseline RBC-folate concentrations.

## 2. Subjects and methods

### 2.1. Study design and intervention

The study was conducted between June 2015 and April 2017 at the Department of Neonatology, Clinical Hospital Center "Dr. Dragiša Mišović-Dedinje", Belgrade, Serbia. The study design and methods have been published before [13,14]. Briefly, 240 full term normal birth weight infants [age <1 month; mean (SD) of age 20.5 (3.6) days] were randomized to an infant formula that contained either folic acid (78 μg/100g milk powder or 15.2 μg/100 kcal) or an equimolar amount of 5-MTHF-Ca (81 μg/100g milk powder or 15.8 μg/100 kcal). The formulas were fed from the baseline visit until the infants were 16 weeks of age (visit 4). Blood samples were collected from the infants at baseline visit and at the end of the intervention (visit 4). Body weight was measured at the study center at five independent visits. Additional information related to the study design is described in the **Supplementary Data File 1**. The present study included a subgroup of 167 infants who attended baseline visit and visit 4 and had blood samples for measuring folate concentrations at both visits. Blood collection was not possible from 13 infants at baseline visit (9 in the 5-MTHF-Ca group and 4 in the folic acid group) and from 9 infants at visit 4 (3 in the 5-MTHF-Ca group and 6 in the folic acid group) (Supplementary Fig. 1. Study Flow Diagram).

The study is registered at ClinicalTrials.gov (NCT02437721). Ethical approval was obtained from the ethical committee at the University Hospital "Dr. Dragiša Mišović-Dedinje" in Belgrade, Serbia (approval number 18–14977/15). The study was 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 to the infants.

### 2.2. Assessment of dietary folate intake

A standardized scoop was provided along with the powdered formula, and parents received clear instructions on formula preparation. For each meal, three scoops of formula powder weighing approximately 13 g were added to 90 ml of water to give a final volume of 100 ml. Parents were asked to fill in a 3-day intake diary for the infant to obtain information on feeding patterns prior to each of the study visits. The diaries included details on the daily number of infant formula scoops, the volume of water used to prepare it and the amount leftover in the bottle after the feeding sessions. Parents had to return the completed diaries and the empty or unused packages of the infant formula at each of the study visits. The 3-day intake diaries were used to estimate mean daily folate intake (in μg) and caloric intake (kcal) based on the actual volumes of the formula that were consumed per day (Table 1). Infant formulas were the primary source of total daily caloric intake during the study. Eight mothers (7 in the folic acid group and 1 in the 5-MTHF-Ca group) reported occasionally breastfeeding at the first 3 study visits. No additional feeding with external foods was reported during the intervention.

### 2.3. Measurement of folate concentrations

Venous blood was drawn into tubes containing EDTA-$K^+$ at baseline visit and visit 4 at least 3 h after the last formula intake. EDTA blood samples for folate measurements were centrifuged and plasma was separated. An aliquot of the EDTA-whole blood was diluted 1:10 in 1 % ascorbic acid to prepare blood hemolysates (50 μl whole blood and 450 μl ascorbic acid solution) that were used to measure RBC-folate. All samples were protected from light and stored at −80 °C until analysis. The concentrations of folate were measured in blood hemolysate at Bevital AS (Bergen, Norway) using an established microbiological assay with *Lactobacillus casei* [15]. Folate forms were measured in EDTA-plasma using a validated liquid chromatography tandem mass spectrometry (LC-MS/MS) method at Bevital AS (Bergen, Norway) [16]. In the present study, we considered the sum of plasma 5-MTHF and 4-alpha-hydroxy-5-methyltetrahydrofolate (hmTHF) as a marker for total plasma folate.

The concentrations of RBC-folate (nmol/L) were calculated from the concentrations of folate in whole blood hemolysate after correcting for individual total plasma folate and hematocrit level as reported before [13,14]. Concentrations of whole blood folate (not corrected for hematocrit and plasma folate) [13] and those of RBC-folate [14] were reported earlier.

### 2.4. Statistical analyses

The outcome of the present study is the change of RBC-folate concentrations between baseline visit and visit 4 (as a surrogate marker of bioavailability) calculated as concentrations at visit 4 minus concentrations at baseline visit. The baseline RBC-folate concentration is

**Table 1**
Age, bodyweight and daily folate intake of infants who received infant formula that contained either 5-MTHF-Ca or folic acid from the age of <1 month (baseline visit) to the age of 16 weeks (visit 4).

| Variables | Infant formula with 5-MTHF-Ca | Infant formula with folic acid |
|---|---|---|
| Female sex of the infant, N/ total | 34/82 | 39/85 |
| **Baseline visit** | | |
| Age, days | 20.9 (3.6) | 19.4 (3.5) |
| Weight, g | 3848 (401) | 3805 (391) |
| **Visit 1** | | |
| Age, days | 29.2 (1.1) | 28.6 (1.0) |
| Weight, g | 4170 (415) | 4194 (391) |
| 3-days mean daily caloric intake, kcal/day[a] | 492 (102) | 500 (92) |
| Folate intake, µg/day[b] | 77.7 (16.1); [39.3–116.4] | 76.2 (14.0); [43.7–106.4] |
| **Visit 2** | | |
| Age, days | 57.0 (2.9) | 56.5 (1.9) |
| Weight, g | 5214 (481) | 5249 (478) |
| 3-days mean daily caloric intake, kcal/day | 582 (103) | 586 (93) |
| Folate intake, µg/day | 91.9 (16.2); [58.9–138.7] | 88.8 (14.2); [51.9–136.3] |
| **Visit 3** | | |
| Age, days | 84.9 (1.7) | 84.7 (1.6) |
| Weight, g | 6043 (550) | 6090 (565) |
| 3-days mean daily caloric intake, kcal/day | 616 (102) | 610 (92) |
| Folate intake, µg/day[e] | 97.4 (16.1); [67.6–156.6] | 92.7 (14.0); [54.3–121.0] [c] |
| **Visit 4** | | |
| Age, days | 113.3 (3.8) | 113.0 (2.8) |
| Weight, g | 6758 (626) | 6792 (622) |
| 3-days mean daily caloric intake, kcal/day | 648 (108) | 621 (80) |
| Folate intake, µg/day | 102.4 (17.0); [51.9–149.2] | 94.3 (12.1); [63.4–127.5] [d, e] |

Data are mean (SD). Data on folate intake is provided also as [min. – max.].
N = 85 infants received formula with folic acid and N = 82 received formula with 5-MTHF-Ca and had available data on RBC-folate concentrations both at baseline and at visit 4. Data on dietary intake were missing from 3 to 5 infants at various study visits.
5-MTHF-Ca, calcium salt of (6S)-5-methyltetrahydrofolate.
[a] Data on intake were collected using 3-day diaries that were filled by the parents and returned to the study center during each study visit. The diaries reported the number of scoops of the milk powder used to prepare the individual volumes of milk over the day and the leftover amounts.
[b] The infant formulas contained either folic acid (78 µg/100g milk powder or 15.2 µg per 100 kcal) or equimolar amounts of 5-MTHF-Ca (81 µg/100g milk powder or 15.8 µg/100 kcal). Folate intake was estimated from the amount of milk actually consumed per day and the µg folate per g milk powder that was used to prepare the milk. The folate contents in the milk powder of both infant formulas were analytically verified over the whole duration of the study. To convert folate intake from folic acid or 5-MTHF-Ca into dietary folate equivalent (DFE), the intake is multiplied by 1.7.
[c] p = 0.045 between 5-MTHF-Ca and folic acid groups (Mann-Whitney test).
[d] p = 0.001 between 5-MTHF-Ca and folic acid groups (Mann-Whitney test).
[e] Occasional breastfeeding was reported by 7 women in the folic acid group and 1 in the 5-MTHF-Ca group.

considered a predictor of the response of RBC-folate to the intervention. We first inspected the data by conducting a graphical analysis. The difference in RBC-folate concentrations between visit 4 and the baseline visit were plotted against baseline RBC-folate concentrations and two separate loess curves for the folic acid and 5-MTHF-Ca groups were added.

The graphical analysis suggested that the relation between baseline RBC-folate concentrations on the one hand and the difference in RBC-folate concentrations between visit 4 and the baseline visit on the other hand is not linear and that the relation between these two variables appears to differ between the two intervention groups. We chose Generalised Additive Models (GAMs) to model this possibly non-linear relation between the outcome and the baseline RBC-folate. Two GAMs were fitted to the data of the change of RBC-folate according to baseline RBC-folate in the intervention groups (for further details, see Section 3.2 of the **Supplementary Data File 2**). The first model (base model) explains the change in RBC-folate concentrations (outcome) depending on the intervention group, number of days the intervention lasted and a smooth effect of the baseline RBC-folate concentrations for each intervention group. The smooth effect is a way of modeling the relationship between the outcome of interest and the predictor in a flexible, smooth way, rather than with a straight line. In addition, a second GAM model (the extended model) that included the change of RBC-folate between baseline visit and visit 4 (outcome), baseline RBC-folate (predictor), and number of days on the formula incorporated also a set of predefined covariates (i.e., age and body weight of the infant at baseline visit and sex of the infant) (**Section 3.3 of the Supplementary Data File 2**). Comparison of the base model and the extended model was performed by comparing formally nested models using a likelihood-ratio test to decide which GAM model should be preferred (**Section 3.4 Model comparison in Supplementary Data File** 2). P-values below 0.05 were considered as statistically significant.

We also fitted a linear mixed-effects model to estimate the average increase of RBC-folate concentrations between baseline visit and visit 4 in the two intervention groups, while controlling for age (in days) at the visit and including a random effect per infant (**Section 4.2 Modelling in the Supplementary Data File 2**).

All analyses were run using the statistical software R version 4.3.3 [17]. The Generalised Additive Models were fitted with the add-on package mgcv [18,19]. The linear mixed model was fitted with the add-on package lme4 [20]. A more detailed description of the statistical analyses is reported in Supplementary Data File 2.

## 3. Results

### 3.1. Infants characteristics and changes in RBC-folate

Of the 240 infants randomized in the original RCT [13], plasma and RBC-folate concentrations were available from 167 infants both at the baseline visit and at visit 4; n = 82 (34 females) in the 5-MTHF-Ca group and n = 85 (39 females) in the folic acid group (Supplementary Fig. 1). The main characteristics of the infants, including body weight and folate intake during the visits at the study center are shown in Table 1. Concentrations of RBC-folate increased from baseline visit to visit 4 in both intervention groups. The mean raise of RBC-folate was significantly higher in the 5-MTHF-Ca group compared to the folic acid group (Table 2).

**Table 2**
Concentrations of RBC-folate in infants who received infant formula that contained either folic acid or 5-MTHF-Ca from the age of <1 month (baseline visit) to 16 weeks (visit 4).

| RBC-folate, nmol/L | Infant formula with 5-MTHF-Ca[a], N = 82 | Infant formula with folic acid[a], N = 85 | p (Mann-Whitney test) |
|---|---|---|---|
| Baseline visit (age <1 month) | 1220 (447) | 1337 (478) | 0.117 |
| Visit 4 (age 16 weeks) | 2498 (514) | 2311 (400) | <0.001 |
| Changes of RBC-folate from baseline visit to visit 4[b] | 1278 (466) | 974 (552) | <0.001 |

Data are shown as mean (SD).
5-MTHF-Ca, calcium salt of (6S)-5-methyltetrahydrofolate; RBC-folate, red blood cell folate.
[a] The infant formulas contained either folic acid (15.2 µg/100 kcal) or an equimolar amount of 5-MTHF-Ca (15.8 µg/100 kcal).
[b] Changes in the concentrations were calculated as concentrations at visit 4 minus those at baseline visit.

### 3.2. Results of the Generalised Additive Models

When modeling the change in RBC-folate (base model), the different estimated effect of the intervention (folic acid or 5-MTHF-Ca) on the change in RBC-folate concentrations indicated that infants receiving 5-MTHF-Ca would have on average 231 nmol/L larger difference in RBC-folate concentrations between baseline visit and visit 4 than the infants in the folic acid group with the same baseline RBC-folate concentrations ($p = 0.0005$, Section 3.2 **Modelling (base model) in the Supplementary Data File 2**).

For lower baseline RBC-folate concentrations, the two intervention groups showed similar changes of RBC-folate concentration between baseline visit and visit 4 (Fig. 1). For higher baseline RBC-folate concentrations, the difference of RBC-folate concentration between baseline visit and visit 4 is systematically higher in the 5-MTHF-Ca group compared to the folic acid group (Fig. 1).

Overall, infants in the group that received infant's formula with 5-MTHF-Ca had 16 % higher RBC-folate concentrations compared to the folic acid group after 92 days, which is the median number of days the study infant formulas was fed (**Section 4.2.1 Interpretation of the coefficient in Supplementary Data File 2**).

Both the base model and the model with covariates converged without problems and fitted the data well as shown by 42 % and 45 % of the deviance explained by these models, respectively. A likelihood-ratio test that compares the base model and the extended model showed a p-value of 0.02, suggesting a marginally better fit of the extended model with the covariates (age, sex and body weight). However, the Akaike's Information Criterion (AIC) of the model with the covariates is only slightly better (−4 points) than those of the base model, while the Bayesian Information Criterion (BIC) suggests that the base model is better (−5 points) than the model with the covariates **(Section 3.4 Model comparison in Supplementary Data File 2**). Considering that the model fits are essentially the same (42 % versus 45 % of the deviance explained), even though one model contains 3 more variables, we proceeded according to the principle of parsimony and focused on the base model. Conclusions drawn in this paper are not affected by the choice of focusing on the base model, which is in agreement with selecting the more parsimonious model.

The base model has shown that the smoothers differ in shape for the effect of baseline RBC-folate concentrations on the difference in RBC-folate between the baseline visit and visit 4. The estimated degrees of freedom (edf) from the GAMs indicate whether the effect of the baseline RBC-folate concentrations on the response of RBC-folate levels to the intervention is linear. The edf was practically 1 indicating a linear effect of baseline RBC-folate on the change of RBC-folate in the group that received folic acid. In the group that received 5-MTHF-Ca, the effect was non-linear (edf = 1.64), thus confirming the need to use a GAM model able to capture this more complex relationship. The smooth effect of the baseline RBC-folate concentrations on the change of RBC-folate was found to be significantly different between the two interventions ($p = 0.002$) [Section 3.2 **Modelling (base model) in Supplementary Data File 2]** .

In the base model, the coefficient for the duration of the intervention was not significant ($p = 0.28$), implying that the number of days during which the infant formula was consumed (range: 78 days–118 days) had no effect on the results. Due to the study design where visits were planned at fixed time points, the variation in the number of days the infant formula was consumed was small and had no effect on the relation between baseline RBC-folate and the change of RBC-folate.

A sensitivity analyses included data on polymorphism in the methylenetetrahydrofolate reductase gene MTHFRC677T (available from a subgroup of the infants) in the linear mixed-effects model. The results showed strong evidence that the only interaction needed in the model is the one between infants formula type (intervention) and age of child

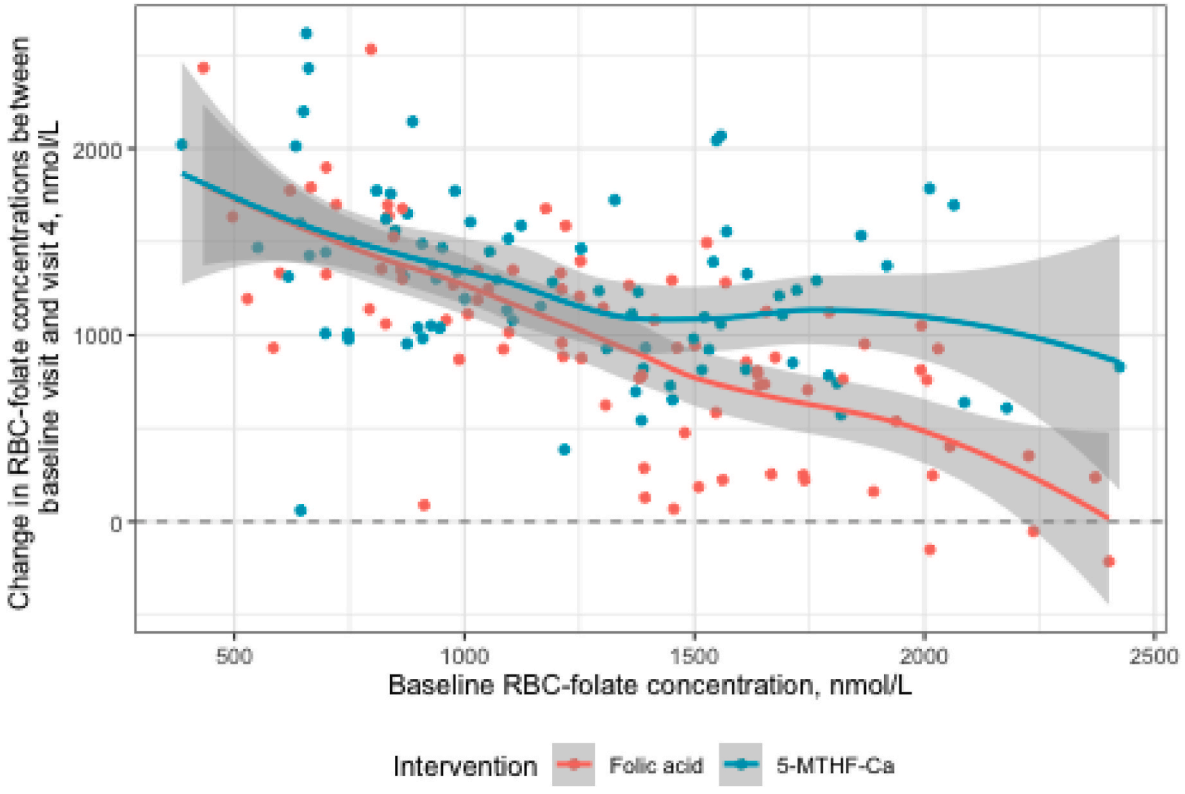


**Fig. 1.** This figure displays the difference in RBC-folate concentrations between visit 4 and the baseline visit against the baseline concentrations of RBC-folate for the folic acid group (red curve) and the 5-MTHF-Ca group (blue curve). The plotted curves are LOESS curves that do not control for additional co-variables. This figure suggests that especially for the higher range of baseline RBC-folate concentrations, the change in the RBC-folate concentrations between baseline and visit 4 is higher in the 5-MTHF-Ca group versus the folic acid group (**Supplementary Data File 2 shows the formal analysis and the smooth effects estimated form the models**).

(estimate 0.0019, 95 % confidence interval [0.0006, 0.0031]). Otherwise, there was no evidence that gene-specific interactions are needed in the model (**Section 4.3 Subgroup analysis in Supplementary Data File 2**). Thus, the MTHFR genotype had no influence on the changes of RBC-folate in this dataset and this variable was not included as a covariate in the GAM modelling.

## 4. Discussion

The present study demonstrates that infant formulas containing either folic acid or 5-MTHF-Ca clearly differ in their effects on the changes of RBC-folate concentrations over time, depending on the baseline RBC-folate concentrations. In the folic acid group, the difference of RBC-folate concentrations between baseline visit and visit 4 showed a gradual decline with increasing baseline RBC-folate concentrations (a linear dose-response association). The change in RBC-folate concentrations between baseline visit and visit 4 is larger in the 5-MTHF-Ca group than in the folic acid group at lower baseline RBC-folate concentrations, but the change flattens out at higher baseline RBC-folate concentrations. Although the bioavailability was generally lower in infants with higher baseline RBC-folate concentrations when compared to infants with lower baseline RBC-folate concentrations, the decline in the bioavailability was smaller in infants from the 5-MTHF-Ca group compared to infants form the folic acid group.

To the best of our knowledge, this is the first demonstration of a differential and folate-status dependent bioavailability of folic acid and 5-MTHF-Ca in infants. We hypothesize an adaptive regulation mechanism that influences intestinal folate absorption and/or renal reabsorption. Our results of an inverse relationship between baseline folate status and bioavailability are in line with available evidence from *in vivo* animal studies [9–11] and *in vitro* studies [7]. Differential bioavailability at the same intake levels of these two folate forms is likely to be due to absorption or metabolism specific for the folate chemical structure.

Natural folate is absorbed primarily in the duodenum and jejunum [21]. The transport of folic acid across the intestinal epithelium to the circulation could be influenced by its binding affinity to folate transporters and may involve metabolic conversions to THF [22]. The PCFT is the primary transporter of folates across the apical brush-border membrane in the proximal small intestine under physiological folate intake levels [12] such as in the present study. Folic acid has a high affinity for PCFT only at low pH (5.5) [12]. The narrow optimal pH for binding folic acid to PCFT could be a limiting factor for folic acid absorption, thus explaining the generally lower bioavailability of folic acid compared to 5-MTHF-Ca salt (Fig. 2).

When the pH level increases from the proximal to the distal parts of the small intestine [24], 5-MTHF continues to be absorbed. The absorption of folic acid via PCFT is likely to take place only in the upper part of the duodenum where the pH is closest to PCFTs pH optimum (5.5) (Fig. 2). Moreover, a fast increase of intestinal pH after the meal and the rather fast gastrointestinal motility in infants partly due to feeding with fluids could shorten the passage time available for folic acid to be absorbed. The affinity of 5-MTHF to PCFT tolerates a broader pH range and thus, the absorption may take place over a larger part of the

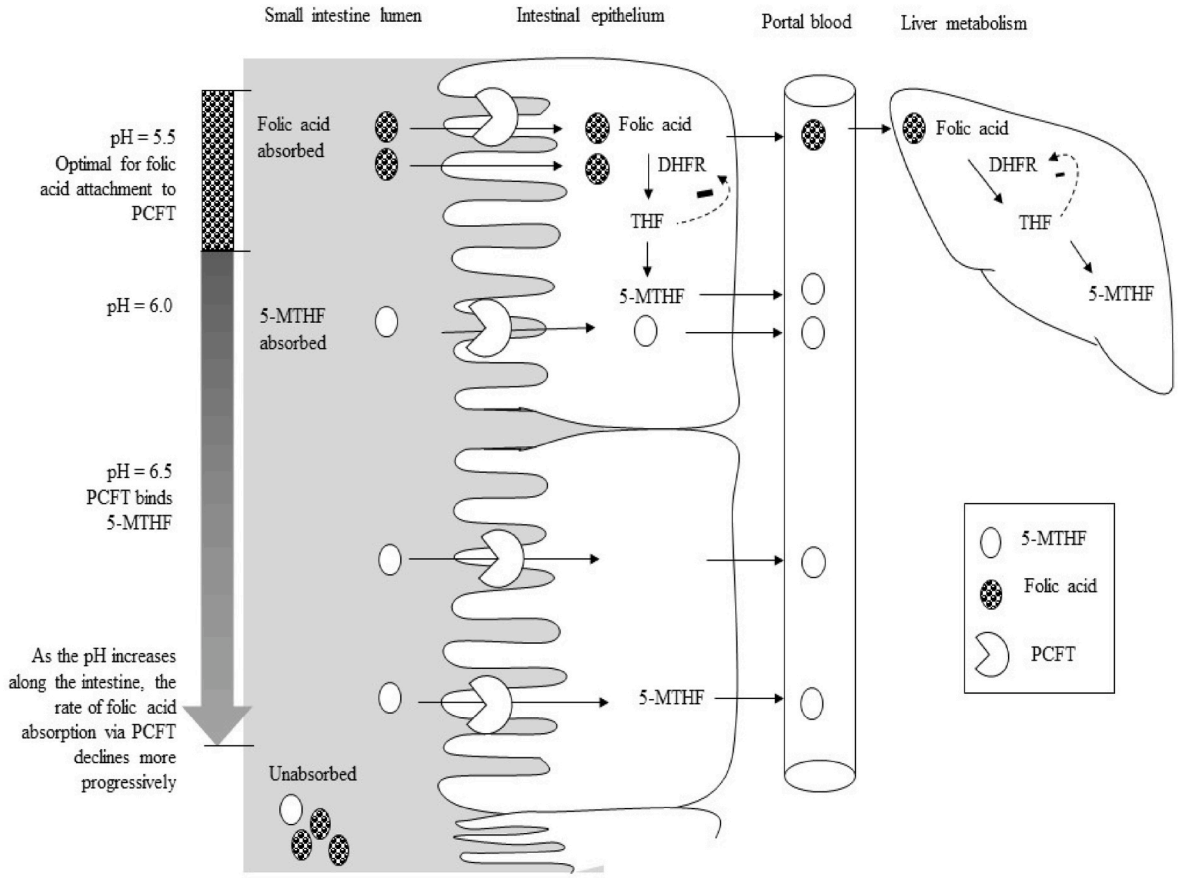


**Fig. 2.** Possible mechanisms that may explain higher bioavailability of (6S)-5-methyltetrahydrofolate-Ca (5-MTHF-Ca) compared to folic acid. First, the distribution and kinetics of folate-binding receptors such as the proton coupled folate transporter (PCFT), and reduced folate carrier (RFC) may vary along the intestine. For example, in the more distal part of the intestine, the rate of folic acid absorption declines compared to that of 5-MTHF when the pH increases above the optimal pH needed for folic acid (pH = 5.5) to attach to PCFT. Second, when tissue folate stores are high [i.e., tissue tetrahydrofolate (THF)], a reduction of additional dietary folic acid via dihydrofolate reductase (DHFR) may become a rate-limiting step [23]. Thus, the conversion of additional input from dietary folic acid is slowed down and folic acid is not efficiently converted to THF and further to 5-MTHF. This could cause the bioavailability of folic acid to progressively decline at high folate status.

small intestine resulting in higher net absorption from 5-MTHF-Ca than from folic acid.

RCF is a major folate transporter in the colon, but it has a very low affinity for folic acid [12], suggesting insignificant contribution of RCF to intestinal absorption of folic acid. In addition, the ability of the mucosal dihydrofolate reductase (DHFR) to reduce folic acid to THF which is converted to MTHF may be limited when tissue folate is high [23], which may explain the progressive decline of the change in RBC-folate at high baseline RBC-folate in the folic acid group. It needs to be considered, that all infants received physiological amounts of both folate forms. 5-MTHF is the predominant form in food, including human milk, while folic acid is the oxidized form not present otherwise in nature. Considering the observed differences in bioavailability and that 5-MTHF-Ca is mimicking the natural dietary folate supply, the methyl folate form may replace folic acid in infant formula.

The folate intake in our study (average 76 μg/day at visit 1 and 100 μg/day at visit 4) did not allow reliable dose-response analyses between the intake and differential bioavailability of folic acid and 5-MTHF. A study among preterm infants found no dose-response association between the folic acid dose (25 μg, 50 μg or 75 μg folic acid per day for a maximum of 1 month) and RBC-folate concentrations [baseline RBC-folate concentrations mean (SD) = 923 (461) nmol/L] [25]. Thus, folate bioavailability appears to generally level off in infants with high baseline blood folate. In a study among adults, supplementing 400 μg folic acid per day for 16 weeks did not have major influence on protein expression of intestinal PCFT and RFC in human studies [6]. Therefore, in the range of dietary intakes, folic acid intake has no major effect on folate receptors, while similar data are not available for 5-MTHF intake. Studying the amount of folate taken up by cultured hepatocytes according to folate concentrations and the folate form in the cell culture medium may be used to investigate a dose-response relationship.

The generalizability of the results to adult populations with larger variations in baseline RBC-folate concentrations remains unclear. If the results can be extrapolated and given a higher rate of folate catabolism during pregnancy [26,27] and early life (in weanling rats [28]), our results suggest that whenever the folate stores are depleted, folate requirements can be fulfilled to a similar degree from folic acid or 5-MTHF. However, a 16 % higher bioavailability of 5-MTHF versus folic acid may be advantageous in term of boosting intracellular folate concentrations and reducing the risk of neural tube defects in women who are not folate deficient but seek to increase their blood folate to a desirable level. Our study did not investigate clinical endpoints such as anemia or developmental outcomes. However, the data suggests that infants who might have folate deficiency may benefit from both folic acid and 5-MTHF-Ca to the same extent.

This study has limitations such as its explorative nature. Moreover, dynamic changes in blood hemoglobin (increase of hemoglobin A and decline of hemoglobin F) [29] could introduce measurement errors in RBC-folate concentrations. This could be especially in place if folic acid and 5-MTHF-Ca (due to higher bioavailability) have differential effects on the developing hematopoietic system. In addition, future studies may control for additional factors that may influence folate absorption such as diarrhea, stool consistency or antibiotic use.

In conclusion, the results of the present study show a higher bioavailability of folate (either as folic acid or 5-MTHF-Ca) at low baseline RBC-folate concentrations and a decline in the bioavailability at high baseline RBC-folate concentrations. We demonstrated that the bioavailability of folic acid is lower than that of 5-MTHF-Ca in infants with high baseline RBC-folate concentrations. Potential effects on developmental end points in the infants, and generalizability of the results to adult population and pregnant women should be investigated.

## CRediT authorship contribution statement

**Rima Obeid:** Writing – original draft, Visualization, Supervision, Conceptualization. **Ines Warnke:** Visualization, Supervision, Resources, Project administration. **Christina Hecht:** Writing – review & editing, Visualization, Supervision, Project administration. **Barbara Troesch:** Investigation, Data curation. **Luisa Barbanti:** Visualization, Validation, Formal analysis. **Matteo Tanadini:** Visualization, Validation, Supervision, Methodology, Formal analysis. **Berthold Koletzko:** Writing – review & editing, Supervision, Methodology, Investigation, Conceptualization.

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## Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: **Ines Warnke** is employer of dsm-firmenich, **Barbara Troesch** was employer of DSM Nutritional Products Ltd. at the time of conducting the study, **Christina Hecht** is employee of HiPP GmbH & Co. Vertrieb KG. **Matteo Tanadini** and **Luisa Barbanti** are employed by Zurich Data Scientists GmbH. **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. **Ines Warnke, Barbara Troesch** and **Christina Hecht** had no role in data collection, analysis, interpretation of the results, or drafting of the manuscript.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.hnm.2025.200335.

## Data availability

Individual data described in the manuscript will not be made available because they constitute internal documents of the study. Detailed data analyses are shown in the Supplementary Data File.

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