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Infant blood concentrations of folate markers and catabolites are modified by 5,10-methylenetetrahydrofolate reductase C677T genotype and dietary folate source

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A B S T R A C T

Background: Folate intake and polymorphisms in the methylenetetrahydrofolate reductase (MTHFR) gene may affect folate metabolism in infants.
Objectives: We investigated the association between infant's MTHFR C677T genotype, the dietary folate source, and concentrations of folate markers in the blood.
Methods: We studied 110 breastfed infants (reference) and 182 infants who were randomly assigned to receive infant formulas enriched with either 78 μg folic acid or 81 μg (6S)-5-methyltetrahydrofolate (5-MTHF) per 100 g milk powder for 12 wk. The blood samples were available at the ages of <1 mo (baseline) and 16 wk. *MTHFR* genotype and concentrations of folate markers and catabolites [i.e., para-aminobenzoylglutamate (pABG)] were analyzed.
Results: At baseline, carriers of the TT genotype (vs. CC) had lower mean (SD) concentrations (all in nmol/L) of red blood cell (RBC) folate [1194 (507) vs. 1440 (521), $P = 0.033$) and plasma pABG [5.7 (4.9) vs. 12.5 (8.1), $P < 0.001$] but higher plasma 5-MTHF [33.9 (16.8) vs. 24.0 (12.6), $P < 0.001$]. Irrespective of the genotype, infant formula with 5-MTHF (vs. folic acid) caused a significant increase in RBC folate concentration [1278 (466) vs. 947 (552), $P < 0.001$]. In breastfed infants, plasma concentrations of 5-MTHF and pABG increased significantly by 7.7 (20.5) and 6.4 (10.5), respectively, from baseline to 16 wk. Infant formula that complies with the present EU legislation for folate intake increased RBC folate and plasma pABG concentrations at 16 wk ($P < 0.001$) than formula-fed infants. At 16 wk, plasma pABG concentrations remained ~50% lower in carriers of the TT (vs. the CC) genotype among all feeding groups.
Conclusions: Folate intake from infant formula according to the present EU legislation increased RBC folate and plasma pABG concentrations in infants to a greater extent than breastfeeding, particularly in carriers of the TT genotype. However, this intake did not completely abolish the between-genotype differences in pABG. Whether these differences have any clinical relevance, however, remains unclear. This trial was registered at clinicaltrials.gov as NCT02437721.

Keywords: folate catabolism, (6S)-5-methyltetrahydrofolate, infant nutrition, formula feeding, breastfeeding, 5,10-methylenetetrahydrofolate reductase, folate requirements

Introduction

Folate is required for normal fetal and infant development. In infants aged 0–6 mo, a folate intake of 9.4 μg/kg/d [1] has been considered adequate to maintain concentrations of plasma and red blood cell (RBC) folate similar to those in breastfed infants of mothers with a satisfactory folate status [2].

The serum folate concentration mirrors the recent folate intake [3], whereas the concentration of RBC folate is a long-term marker that correlates with the liver folate content [4]. The enzyme 5, 10-methylenetetrahydrofolate reductase (MTHFR) synthesizes (6S)-5-methyltetrahydrofolate (5-MTHF), the predominant folate form in blood and breastmilk [5,6]. In adult carriers of the MTHFR 677 TT genotype, the activity of the MTHFR enzyme is reduced [7],

Abbreviations: apABG, para-acetamidobenzoylglutamate; Ca, calcium; fTHF, formyltetrahydrofolate; GLM, general linear model; Hct, Hematocrit; hmTHF, 4α-hydroxy-5-methyltetrahydrofolate; LC-MS/MS, liquid chromatography tandem mass spectrometry; 5-MTHF, (6S)-5-methyltetrahydrofolate; MTHFR, 5,10-methylenetetrahydrofolate reductase; pABG, para-aminobenzoylglutamate; RBC, red blood cell; tHcy, total homocysteine; UMFA, unmetabolized folic acid.

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plasma and RBC folate concentrations are lower [8], and plasma total homocysteine (tHcy) is higher than those in carriers of the CT and CC genotypes [9], suggesting higher requirements for folate in carriers of the TT genotype. There is insufficient evidence on whether the TT genotype may affect folate utilization and catabolism and, therefore, folate requirements in infants.

Enzymatic folate degradation regulates intracellular folate concentrations and results in para-aminobenzoylglutamate (pABG) that is acetylated to para-acetamidobenzoylglutamate (apABG), the main folate catabolite in urine [10–14]. Plasma and RBC folate concentrations show moderate correlations with urinary pABG and apABG [11]. Because folate catabolism occurs in all folate-utilizing tissues, measuring the concentrations of pABG and apABG in plasma may better reflect tissue folate catabolism in response to folate intake than urinary concentrations of these catabolites.

The folate catabolic rate is upregulated during pregnancy in both rats [15] and humans [16,17] and in weanling rats during the stages of hyperplastic growth [18]. Increasing folate intake has been shown to stimulate folate catabolism in pregnant women [19], adults [20], and children [21], suggesting that folate catabolism regulates intracellular folate concentrations in response to folate intake. In infants, folate catabolism, as indicated by plasma concentrations of pABG and apABG, could be upregulated parallel to a higher rate of folate utilization for cell replication and DNA synthesis and is possibly influenced by folate intake and the MTHFR genotype.

The intake of 5-MTHF-calcium (Ca) in infants has been shown to cause higher RBC folate concentrations than equimolar intake of folic acid [22]. However, it is not known whether these 2 folate forms may differentially affect folate metabolism according to MTHFR C677T genotypes.

We conducted an exploratory study among a subgroup of infants (median age, 20 d) who had participated in a previous randomized controlled trial [22]. We investigated the association between MTHFR C677T genotype and concentrations of folate markers (RBC folate, plasma 5-MTHF, and plasma tHcy) and plasma folate catabolites (pABG and apABG) at baseline and at the age of 16 wk after breastfeeding or feeding infant formula with equimolar amounts of either 5-MTHF-Ca or folic acid. In addition, we investigated whether there are between-genotype differences in infant weight measured between the ages of $<$1 mo and 16 wk.

Methods

Study design, settings, subjects, and intervention

The present study is an exploratory analysis of data from a previously reported double-blind randomized controlled intervention trial [22]. The trial was conducted at the Department of Neonatology, Clinical Hospital Center "Dr Dragiša Mišović-Dedinje," Belgrade, Serbia.

The participants in the formula groups were infants of parents who chose to start formula-feeding for their infant within the first 28 d of life for reasons unrelated to this study. The study inclusion criteria were as follows: apparently healthy infants who were younger than 1 mo and were born between $\geq$37 and $\leq$42 weeks of gestation, infants who had a birth weight between 2500 and 4500 g, and the parents/caregivers were able to speak Serbian. Exclusion criteria for the infants were as follows: acquired or congenital diseases that may interfere with feeding or growth of the infant, feeding of $>$10% of energy (1 bottle/d) from sources other than the formula (or breastmilk in the reference group); abnormalities in hematological, hepatic, metabolic, or renal markers or functions; use of medication and vitamin supplements except vitamin K or D supplementation or vaccination; and participation in another clinical study. The infants were also not eligible for participation if the mother had diabetes mellitus, gestational diabetes, or followed a vegan diet (because of the risk of vitamin B-12 deficiency in the child). A detailed description of the study has been published elsewhere [22]. Supplemental Fig. 1 shows the study's flow diagram.

Of the 360 infants who met the inclusion criteria, 240 were randomly allocated to feeding with an infant formula that contained folic acid (78 μg/100 g milk powder or 15.2 μg/100 kcal) or an equimolar amount of 5-MTHF-Ca (81 μg/100 g milk powder or 15.8 μg/100 kcal) [22]. The folic acid concentration added to the infant formula was according to the EU legislation [23]. The randomization list was provided by the Medical Centre of the University of Munich, Germany. A random number was allocated from a list with 6 blocks stratified by sex (numbers 1–150 for males and 151–300 for females). The random number list was forwarded directly to the center responsible for blinding the formula (SCA Full Filement Ltd). The formulas were fed until the infants were 16 weeks of age (visit 4) [22]. The 120 breastfed infants (reference group) were followed at the same time points as the formula-fed infants.

During the baseline visit at the HiPP study center in Belgrade, a medical examination was performed, anthropometric measurements were conducted, and blood samples were collected by the study nurses. Weight, height, and head circumference of infants were monitored 5 times during the study (at baseline and at visits 1–4). The mean (SD) time intervals were as follows: from baseline to visit 1 = 9 (3) d, visit 1 to visit 2 = 28 (2) d, visit 2 to visit 3 = 28 (3) d, and visit 3 to visit 4 = 28 (4) d. The mean (SD) duration of the study (baseline to visit 4) was 94 (4) d in the formula intervention groups and 93 (5) d in the reference group. The mean (SD) age of the infants was 20.0 (3.4) d at baseline, 28.8 (1.1) d at visit 1, 56.5 (2.3) d at visit 2; 84.8 (1.9) d at visit 3, and 113.1 (3.1) d at visit 4. Blood samples were collected again from all infants at the end of the intervention period (visit 4).

The present study included data from the subcohort of 292 infants where DNA samples and MTHFR C677T genotyping results were available (110 breastfed infants, 89 infants fed with formula with 5-MTHF-Ca, and 93 infants fed with formula with folic acid).

Monitoring of folate content in the formulas and compliance with the intervention

Folate content in the formulas was analytically verified at production and regularly every 1–2 mo until 18 mo (10–12 time points in total). The contents of folic acid and 5-MTHF-Ca showed 3.8%–4.0% variations over 18 mo (example is shown in Supplemental Table 1).

To monitor compliance, parents were asked to return empty or unused packages of the milk powder. In addition, parents were asked to fill in a 3-d intake protocol of feeding patterns and return the protocols during visits 1–4. Eight mothers of infants who were randomly assigned to infant formula reported occasionally breastfeeding during the first 3 study visits. Three breastfed infants were occasionally provided commercially available infant formulas.

The 3-d intake protocols from the formula-fed infants included the daily number of spoons of milk powder, volume of water used to prepare the formula, and left-over amount after the feeding sessions. The data were used to estimate the daily caloric intake (kcal/d) from the formulas (Table 1). Breastmilk or study infant formulas were the main sources of total daily caloric intake. Information regarding additional feedings with fluids as a source of calories was available but was not sufficient to calculate energy intake from these sources. Solid foods

were not reported to have been fed during the study period. This study used data from the intention-to-treat population.

The study was approved by the ethical committee at the University Hospital "Dr Dragiša Mišović-Dedinje" in Belgrade, Serbia. Written informed consent was obtained from the parents or caregivers of the infants. This study was registered at clinicaltrials.gov as NCT02437721. The safety and efficacy of the infant formulas have been reported earlier [22].

Blood analyses

During the baseline visit and visit 4, venous blood samples were drawn into EDTA-containing tubes. Blood sampling was performed ≥3 h after the last breastfeeding or formula feeding. EDTA-blood samples were centrifuged at the HiPP study center in Belgrade, Serbia, and both the plasma and buffy coat were separated and stored at −80°C. The buffy coat was used for DNA isolation at DSM Nutritional Products Ltd. One aliquot of the EDTA whole blood (50 μL) was immediately aspirated into an Eppendorf tube, and 450 μL 1% ascorbic acid solution (w/v in bidistilled water) was added to prepare blood hemolysate for the blood folate assay. The blood hemolysate was protected from light, mixed, and incubated for 30 min at room temperature and was then stored at −80°C. Stored samples were shipped on dry ice to the Bevital AS Laboratory for analyzing folate markers. All laboratory procedures were performed according to standardized operating procedures by laboratory staff members who were blinded for group allocation and participant data.

Folate forms and catabolites were measured in EDTA plasma of the infants using a liquid chromatography tandem mass spectrometry (LC-MS/MS) method [24]. The method depends on using corresponding ^{13}C-labeled internal standards to measure 5-MTHF, unmetabolized folic acid (UMFA), formyltetrahydrofolate (fTHF), 4α-hydroxy-5-methyltetrahydrofolate (hmTHF), pABG, and apABG [24]. Before plasma sample preparation, ascorbic acid was added to the plasma samples to prevent preanalytical folate oxidation and interconversions. Acetonitrile was used to precipitate the proteins in plasma. After centrifugation, the acetonitrile layer was evaporated, and the analytes were dissolved in water and injected into LC-MS/MS. The present study focuses on 5-MTHF and pABG derivatives. The concentrations of UMFA, hmTHF, apABG, and fTHF were either very low or undetectable and, therefore, will not be discussed in detail. Plasma concentrations of tHcy were measured using gas chromatography mass spectrometry at Bevital AS Laboratory. The concentrations of whole blood folate were measured in frozen blood hemolysate (diluted 1:10 in 1% ascorbic acid) at Bevital AS Laboratory using a microbiological assay with a chloramphenicol-resistant strain of *Lactobacillus casei*. The concentrations of folate in whole blood hemolysate were used to calculate the RBC folate (nmol/L) using the following equation as reported elsewhere [25]:

$$= [(\text{Whole blood hemolysatefolate} \times 10) - \text{plasma folate}\ (1 - \text{Hct})/\ \text{Hct}]$$

The individual total plasma folate concentrations were approximated as the sum of plasma 5-MTHF and hmTHF concentrations, and hematocrit (Hct) results were used as decimal values (i.e., 0.40). The in-house quality control samples served to monitor the performance of the folate assays, and commercially available reference materials served for quality control purposes of the tHcy assay. For the microbiological assay, the between-day coefficient of variation for the in-house quality control samples was 3.1% at concentrations of 17.1 nmol/L and 3.6% at 40.9 nmol/L.

EDTA whole blood from 292 samples was used to isolate the DNA using the Qiamp DNA blood mini kit according to the manufacturer instructions (Qiagen, Cat No. 51104). For genotyping, the Applied Biosystems TaqMan SNP genotyping assay was used according to the manufacturer's instructions (Cat No. 4351379). DNA was added to the universal master-mix, and the samples were processed in the ABI 7900 instrument and measured in 96-well plates. The genotyping detected alleles of MTHFR (NCBI gene reference: NM_005957.4) single-nucleotide polymorphism MTHFR C677T (SNP rs1801133, Assay-ID: C_1202883_20).

Genotyping data were not available from 68 infants from the original cohort (10 breastfed infants, 27 infants on formula with folic acid, and 31 infants on formula with 5-MTHF-Ca). The clinical data and biomarkers did not differ significantly between infants with available DNA and those without DNA samples (data not shown), suggesting that the subcohort in the present study was representative of the original cohort.

Statistical analysis

Statistical analyses were conducted using version 28 of IBM SPSS Statistics package (SPSS Inc.). A P value of ≤0.05 was considered statistically significant.

The descriptive data are shown as mean (SD) and median [interquartile range, IQR] for continuous variables and absolute (n) and relative frequencies (%) for categorical variables. One-sample Kolmogorov-Smirnov test with Lilliefors significance correction and Q-Q plots were used to study the distribution of the continuous variables. All folate biomarkers were not normally distributed. Log_{10}-transformed plasma 5-MTHF concentrations showed normal distribution. Log_{10}-transformation of all other folate markers improved the distribution as judged from the Q-Q plots, but the 1-sample Kolmogorov-Smirnov test remained significant (P values after log_{10}-transformation were between 0.03 and <0.001). The Q-Q plots showed that the log_{10}-transformed data were more consistent with the assumption of nonnormal distribution compared with the non-transformed data. The log_{10}-transformed values were used in tests that assume normal distribution of the data.

One-factor ANOVA was used to compare log_{10}-transformed variables between independent groups (i.e., genotypes or feeding groups). For the ANOVA, the assumptions of random independent groups, normal distribution of the dependent variables, and homogeneity of the variance (i.e., $P > 0.05$ for Levene's test of equality of variances within all groups) were fulfilled. For comparisons of >2 groups, and when the ANOVA showed an overall P value of ≤0.05, the post hoc Tamhane's T2 test (due to unequal group variances as tested by Levene's test) was applied for pairwise comparisons of the subgroups.

The within-subject longitudinal changes of folate markers between baseline and visit 4 were studied by paired t test applied on the log_{10}-transformed data. In addition, we used the general linear model (GLM) Repeated Measures procedure to study the effects of the between-subjects factors (i.e., MTHFR genotype or feeding group) on the repeated measurements (i.e., the within-subjects variable) over time (repeated folate marker or body weight measurements). The interactions between within-subject and between-subject variables were studied in the same model (to study whether changes in folate markers differ by the genotype). The model for repeated measurements of infant body weight was adjusted for infant's sex, age at recruitment, and birthweight.

Moreover, the changes (delta) in the folate markers from baseline to visit 4 were calculated as concentrations at visit 4 minus concentrations

at baseline. The changes from baseline were expressed as mean (SD) and were compared between the 2 intervention groups using ANCOVA. ANCOVA uses a regression analysis to adjust the difference between the formula groups, with the formula as the explanatory variable and the baseline concentrations of the corresponding marker and infant's MTHFR C677T genotype as covariates. The basic assumptions that underlie ANCOVA were verified (same as for ANOVA). For each ANCOVA model, we verified that the relationship between the dependent variable (i.e., the changes of marker concentrations over time) and the covariate (i.e., baseline concentrations of the same marker) is likely to be linear and that there was no interaction between the randomization group and independent variable.

Results

Study population

Table 1 shows the main characteristics and distribution of the MTHFR677 genotypes among the infants in the present study.

Folate-related markers according to the MTHFR C677T genotype in infants at baseline

In the total group of the infants at baseline, the concentrations of RBC folate, but not of tHcy, differed between the genotypes ($P = 0.017$) (Table 2). Infants with the TT genotype had lower RBC folate concentrations than those with the CC genotype [mean (SD), 1194 (507) vs. 1440 (521) nmol/L, $P = 0.033$ according to ANOVA and post hoc Tamhane's T2 tests]. In contrast, plasma concentrations of 5-MTHF were higher in infants with the TT genotype [mean (SD) for TT 33.9 (16.8) nmol/L vs. CC 24.0 (12.6) nmol/L ($P < 0.001$) and vs. CT 25.5 (13.0) nmol/L ($P = 0.001$)] (Table 2). The mean plasma concentrations of pABG were lower in infants with the TT genotype than in those with the CC and CT genotypes; mean (SD): 5.7 (4.9) nmol/L in TT, 12.5 (8.1) nmol/L in CC, and 10.8 (6.8) nmol/L in CT ($P < 0.001$ for paired comparisons) (Table 2).

Longitudinal changes in folate markers according to feeding patterns

Among the breastfed infants, the concentrations [mean (SD)] of plasma tHcy increased between baseline and visit 4 by 1.8 (1.7) μmol/L, of plasma 5-MTHF increased by 7.7 (20.5) nmol/L, and of plasma pABG increased by 6.4 (10.5) nmol/L ($P < 0.001$ for all repeated measurements). The concentrations of RBC folate changed by −67 (728) nmol/L ($P = 0.213$ for repeated measurements) (Table 3).

In infants from both of the formula groups, the concentrations of tHcy significantly declined and those of RBC folate, plasma 5-MTHF, and plasma pABG significantly increased between baseline and visit 4 ($P < 0.001$ for all longitudinal changes) (Table 3). The concentrations of RBC folate were higher in the 5-MTHF-Ca formula group (vs. folic acid) ($P = 0.044$), whereas the plasma 5-MTHF and pABG concentrations did not differ between the 2 formula groups at visit 4 (Table 3). Infants receiving an infant formula with either 5-MTHF-Ca or folic acid showed a similar decline in plasma tHcy concentration between baseline and visit 4 [mean (SD), −0.9 (1.7) vs. −0.8 (1.9) μmol/L, respectively; $P = 0.513$ for between-group comparisons adjusted for baseline tHcy and MTHFR genotype, Table 3]. The changes in RBC folate concentrations ($P < 0.001$) between baseline and visit 4 were larger in the 5-MTHF-Ca formula group than in the folic acid group (Supplemental Fig. 2, Table 3). The changes in plasma 5-MTHF and pABG concentrations from baseline to visit 4 did not differ significantly between the formula groups (Supplemental Fig. 2, Table 3).

Folate markers at visit 4 according to the MTHFR C677T genotype and feeding patterns

Among the breastfed infants (at visit 4 or age 16 wk), the concentrations of plasma tHcy did not differ between the MTHFR genotypes. The concentrations of RBC folate [mean (SD): 1137 (438) nmol/L in TT vs. 1354 (455) nmol/L in CC] and plasma 5-MTHF [37.5 (17.8) nmol/L in TT vs. 29.9 (14.8) nmol/L in CC] were not significantly different between the breastfed infants with the TT genotype and those with the CC genotype. The mean plasma pABG concentrations in breastfed infants with the TT genotype [8.0 (5.2) nmol/L] were ~55% lower than that in those with the CC genotype [17.9 (10.0) nmol/L] and 47% lower than that in infants with the CT genotype [15.1 (11.2) nmol/L] ($P < 0.001$ for between-group comparisons and $P < 0.05$ for CC vs. TT and CT vs. TT) (Table 4).

Among infants who received infant formula with 5-MTHF-Ca (at visit 4), the MTHFR genotype was not associated with plasma tHcy or RBC folate, whereas the plasma concentrations of 5-MTHF were the highest in carriers of the TT genotype [mean (SD): 71.6 (17.9) nmol/L in TT; 53.7 (16.4) nmol/L in CT; 56.8 (23.3) nmol/L in CC; $P = 0.071$ for overall between-group comparisons and $P = 0.066$ for TT vs. CC]. The plasma concentrations of pABG were the lowest in infants with the TT genotype at visit 4 than in those with the other 2

Table 1
Main characteristics of the 292 infants who were genotyped for the MTHFR C677T polymorphism[1]

Infant characteristics	Reference	Randomly assigned to infant formula	
	Breastfed $n = 110$	5-MTHF-Ca $n = 89$	Folic acid $n = 93$
Age at baseline visit, d	19.6 (2.6)	20.9 (3.8)	19.6 (3.6)
Boys, *n* (%)	50 (45.5)	53 (59.6)	51 (54.8)
Gestational age at birth, wk	39.6 (1.0)	39.8 (1.0)	39.7 (1.0)
Birthweight, g	3447 (392)	3415 (405)	3484 (400)
Weight at recruitment, g	3904 (436)	3839 (399)	3827 (410)
Length at recruitment, cm	54.0 (1.9)	53.9 (1.7)	54.0 (2.1)
Head circumference at recruitment, cm	36.2 (1.1)	36.3 (1.0)	36.0 (1.2)
Caloric intake (mean of 3 d) in formula-fed infants at baseline, kcal/d	—	489 (103)	500 (92)
Caloric intake (mean of 3 d) in formula-fed infants at visit 4, kcal/d	—	581 (101)	583 (96)
MTHFR C677T genotype, *n* (%)			
CC	40 (36.4)	30 (33.7)	30 (32.3)
CT	48 (43.6)	48 (53.9)	55 (59.1)
TT	22 (20.0)	11 (12.4)	8 (8.6)

[1] Results are shown as mean (SD) or *n* (%); 5-MTHF-Ca, (6S)-5-Methyltetrahydrofolate-calcium; MTHFR, methylenetetrahydrofolate reductase.

Table 2
Concentrations of folate markers and catabolites in blood of infants at baseline (age < 1 mo)[1]

Baseline folate markers	All genotypes ($N = 292$)	SNP rs1801133 (MTHFR C677T) genotype			
		CC ($n = 100$)	CT ($n = 151$)	TT ($n = 41$)	P-ANOVA[3]
Plasma tHcy, μmol/L	6.8 (1.9)	6.7 (1.8)	6.8 (2.1)	6.8 (1.9)	0.981
	6.3 [2.3]	6.3 [2.4]	6.2 [2.1]	6.5 [2.2]	
RBC folate,[2] nmol/L	1334 (508)	1440 (521)	1303 (490)	1194 (507)[4]	0.017
	1301 [737]	1423 [755]	1262 [725]	1094 [714]	
Plasma 5-MTHF,[2] nmol/L	26.2 (13.8)	24.0 (12.6)	25.5 (13.0)	33.9 (16.8)[5,6]	<0.001
	23.8 [15.8]	22.0 [15.8]	23.2 [15.2]	31.6 [17.2]	
Plasma pABG,[2] nmol/L	10.7 (7.3)	12.5 (8.1)	10.8 (6.8)	5.7 (4.9)[5,6]	<0.001
	8.8 [9.6]	10.6 [10.6]	9.1 [8.0]	3.8 [4.9]	
Plasma apABG, nmol/L	1.1 (0.3)	1.0 (0.3)	1.1 (0.3)	1.2 (0.4)	0.073
	1.0 [0.4]	1.0 [0.4]	1.1 [0.4]	1.1 [0.5]	
Plasma UMFA ≥ 0.50 nmol/L, n (% of total)	119 (42%)	41 (41%)	63 (43%)	15 (37%)	0.752
Birth weight, g	3449 (398)	3487 (418)	3444 (396)	3371 (350)	0.283
	3430 [398]	3495 [633]	3410 [470]	3370 [495]	
Weight gain between birth and baseline visit, g	411 (255)	431 (267)	393 (246)	426 (261)	0.468
	398 [369]	420 [378]	375 [335]	425 [415]	

[1] Data are expressed as mean (SD) and median [IQR].
[2] Baseline plasma and RBC folate concentration data in 5 infants (3 breastfed infants, 1 in the folic acid group, and 1 in the 5-MTHF-Ca group) were missing, and data on baseline pABG concentrations in 7 infants were missing.
[3] Folate marker concentrations were compared between the subgroups using 1-factor analysis ANOVA and post hoc Tamhane's T2 tests applied on the $\log_{10}$-transformed data.
[4] $P = 0.033$ for TT vs. CC.
[5] $P < 0.001$ for TT vs. CC.
[6] $P < 0.001$ for TT vs. CT.
apABG, para-acetamidobenzoylglutamate; 5-MTHF, (6S)-5-methyltetrahydrofolate; MTHFR, methylenetetrahydrofolate reductase; pABG, para-aminobenzoylglutamate; RBC, red blood cell; SNP, single-nucleotide polymorphism; tHcy, total homocysteine; UMFA, unmetabolized folic acid.

genotypes [mean (SD): 12.9 (5.5) nmol/L in TT, 21.3 (8.1) nmol/L in CT, and 21.8 (10.7) nmol/L in CC; $P < 0.004$ for overall between-group comparisons and $P < 0.05$ for CT vs. TT and for CC vs. TT] (Table 4).

Similarly, the MTHFR genotype was not associated with the concentrations of tHcy or RBC folate at visit 4 in infants who received infant formula with folic acid. In contrast, the TT genotype was associated with higher plasma 5-MTHF [mean (SD): 68.9 (21.7) nmol/L in TT, 53.9 (17.7) nmol/L in CT, and 44.9 (15.8) nmol/L in CC; $P = 0.008$ for overall between-group comparisons] and lower plasma pABG [mean (SD): 13.3 (6.9) nmol/L in TT, 18.4 (8.1) nmol/L in CT and 24.1 (11.6) nmol/L in CC; $P = 0.004$ for overall between-group comparisons, $P = 0.074$ for CT vs. CC, and $P < 0.05$ for TT vs. CC] (Table 4).

The within-subject changes in longitudinal measurements of body weight (5 measurements in total) did not show interactions with the MTHFR genotype or feeding types (Supplemental Fig. 3).

Factors associated with plasma pABG concentrations

In general, the mean plasma concentrations of pABG differed by ~50% between the TT and CC genotypes, and these between-genotype differences were rather constant over the follow-up from baseline to visit 4 in all feeding groups (Supplemental Fig. 4 summarizes the data shown in Tables 2 and 4).

Between baseline and visit 4, the average within-person increase in plasma pABG concentrations in breastfed infants was 40%–55% in the different MTHFR genotypes ($P < 0.001$). The within-person changes of pABG from baseline to visit 4 showed no interaction with the genotype (as between-subject factor) ($P = 0.522$ for the interaction between pABG concentrations and MTHFR genotype) (Supplemental Fig. 4).

Among infants fed with infant formulas, the extent of change in plasma pABG from baseline to visit 4 (45%–81%) was larger than that in breastfed infants (Supplemental Fig. 5). The changes in plasma pABG from baseline to week 16 were significantly influenced by the source of folate (5-MTHF-Ca or folic acid vs. breastmilk) and by the MTHFR genotype (TT vs. CC or CT) ($P < 0.001$ for GLM analyses of repeated measurements with the feeding group and MTHFR genotype as between-subject factor). In addition, there was no interaction between the infant formulas and genotype in their association with pABG changes from baseline to visit 4 [$P = 0.643$ for the interaction term (intervention x genotype) in the GLM model] (Supplemental Fig. 5).

Discussion

We found that an infant TT genotype for the MTHFRC677T polymorphism was associated with lower RBC folate concentrations but higher plasma 5-MTHF concentrations, compared with the CC genotype. The T allele showed consistent dose-response associations with lower plasma concentrations of pABG (i.e., ~50% lower in TT vs. CC) at the age of 20 d and 16 wk. Plasma pABG concentrations increased in all feeding groups during the first 16 weeks of life, but the concentrations were higher in formula-fed infants (folate intake, ~80 μg/d) than in breastfed infants. In breastfed infants, the tHcy concentrations rose from the baseline to visit 4. In contrast, feeding infant formula was associated with marked increases in the concentrations of RBC folate and plasma 5-MTHF and a decline in tHcy concentration from baseline to 16 wk.

Folate markers in relation to folate intake in infants

The baseline plasma 5-MTHF concentrations in the present study were lower than plasma total folate concentrations among breastfed infants from earlier studies [26–28]. The mean RBC folate concentrations appear comparable with those reported in infants from the

Table 3
Concentrations of folate markers in 292 infants at baseline (age < 28 d) and visit 4 (age 16 wk) in breastfed infants and infants fed formula with 5-MTHF-Ca or equimolar amount of folic acid[1]

Marker	Breastfed, $n = 110$			Infant formula with 5-MTHF-Ca,[2] $n = 89$			Infant formula with folic acid,[3] $n = 93$			Pairwise-comparison of folate markers between feeding groups at visit 4[4]			Change in 5-MTHF-Ca vs. folic acid
										5-MTHF-Ca vs. breastfed	Folic acid vs. breastfed	5-MTHF-Ca vs. folic acid	
	Baseline	V4	V4-baseline[6]	Baseline	V4	V4-baseline[6]	Baseline	V4	V4-baseline[6]	*P* value	*P* value	*P* value	*P* value[7]
Plasma tHcy, µmol/L	6.8 (2.1) 6.3 [2.2]	8.7 (2.8)[5] 8.0 [3.4]	1.8 (1.7) 1.7 [1.9]	6.7 (1.8) 6.3 [2.7]	5.8 (1.1)[5] 5.6 [1.5]	−0.9 (1.7) −0.8 [2.0]	6.7 (1.9) 6.2 [2.2]	5.9 (1.1)[5] 5.7 [1.1]	−0.8 (1.9) −0.4 [2.1]	[8]	[8]	0.803	0.513
RBC folate, nmol/L	1398 (561) 1321 [779]	1329 (604) 1202 [752]	−67 (728) −80 [756]	1231 (456) 1144 [679]	2517 (536)[5] 2469 [598]	1278 (466) 1287 [585]	1355 (477) 1380 [700]	2305 (397)[5] 2371 [503]	974 (552) 959 [664]	<0.001	<0.001	0.044	<0.001
Plasma 5-MTHF, nmol/L	26.1 (15.8) 23.3 [15.5]	34.0 (17.7)[5] 31.3 [24.0]	7.7 (20.5) 8.1 [22.4]	26.3 (13.2) 25.1 [17.8]	57.0 (19.8)[5] 55.1 [25.7]	31.6 (22.1) 33.8 [31.6]	26.1 (11.9) 23.8 [14.2]	52.5 (18.5)[5] 51.2 [28.9]	25.2 (23.3) 22.9 [32.9]	<0.001	<0.001	0.495	0.054
Plasma pABG, nmol/L	8.3 (6.0) 7.3 [8.0]	14.7 (10.4)[5] 11.8 [14.7]	6.4 (10.5) 4.0 [9.1]	12.3 (8.0) 10.4 [10.7]	20.4 (9.2)[5] 20.8 [12.2]	8.2 (11.2) 8.1 [13.6]	12.0 (7.7) 9.7 [8.9]	19.7 (9.7)[5] 17.8 [12.0]	8.1 (10.5) 6.4 [13.7]	<0.001	<0.001	0.934	0.685

[1] Data are expressed as mean (SD) and median [IQR].
[2] 81 µg/100 g of milk powder or 15.8 µg/100 kcal of 5-MTHF-Ca.
[3] 78 µg/100 g of milk powder or 15.2 µg/100 kcal of folic acid.
[4] Comparisons of the concentrations of the biomarkers between the independent feeding groups at visit 4 (age 16 wk). The between-group differences were tested using ANOVA, followed by a post hoc Tamhane's T2 test for pairwise comparisons when ANOVA was significant ($P < 0.05$). ANOVA used $\log_{10}$-transformed data (without adjustment for baseline concentrations).
[5] Significantly different from baseline ($P < 0.001$) using paired *t* test applied on the $\log_{10}$-transformed data.
[6] The change of the biomarker between baseline and visit 4 was calculated as concentrations at visit 4 minus those at baseline.
[7] Changes between baseline and visit 4 (16 weeks of age) were calculated and then compared between the 2 formula groups using ANCOVA, including baseline concentrations of the corresponding folate biomarker and infant's MTHFR C677T genotype as covariates.
[8] Plasma tHcy was not compared between breastfed infants and formula-fed infants at visit 4 because of expected differences in vitamin B-12 status that were not analyzed here; Ca, calcium; 5-MTHF, (6S)-5-methyltetrahydrofolate; MTHFR, methylenetetrahydrofolate reductase; pABG, para-aminobenzoylglutamate; RBC, red blood cell; tHcy, total homocysteine.

Table 4
Concentrations of folate biomarkers and catabolites in 110 infants at visit 4 (age 16 wk) among the feeding types according to MTHFR C677T genotypes[1]

Variable	MTHFR C677T genotype			*P* value[2]
	CC	CT	TT	
Breastfed infants, n	40	48	22	
Plasma tHcy, μmol/L	8.7 (2.1)	8.8 (3.5)	8.5 (2.4)	0.848
	8.8 [3.2]	7.9 [3.4]	7.8 [3.5]	
RBC folate, nmol/L	1354 (455)	1396 (759)	1137 (438)	0.252
	1282 [702]	1183 [949]	1163 [577]	
Plasma 5-MTHF, nmol/L	29.9 (14.8)	35.9 (19.5)	37.5 (17.8)	0.191
	29.5 [17.5]	32.3 [23.9]	34.3 [21.7]	
Plasma pABG, nmol/L	17.9 (10.0)	15.1 (11.2)	8.0 (5.2)[3,4]	<0.001
	16.6 [16.6]	11.8 [13.9]	6.7 [7.7]	
Fed on infant formula with 5-MTHF-Ca, n	30	48	11	
Plasma tHcy, μmol/L	5.7 (1.1)	5.8 (1.2)	5.8 (1.1)	0.870
	5.6 [1.2]	5.6 [1.6]	5.8 [1.7]	
RBC folate, nmol/L	2726 (632)	2449 (476)	2246 (269)	0.102
	2787 [711]	2382 [492]	2329 [389]	
Plasma 5-MTHF, nmol/L	56.8 (23.3)	53.7 (16.4)	71.6 (17.9)[5]	0.071
	58.3 [26.9]	50.6 [23.6]	74.2 [31.2]	
Plasma pABG, nmol/L	21.8 (10.7)	21.3 (8.1)	12.9 (5.5)[3,4]	0.004
	21.7 [16.4]	21.3 [9.1]	12.2 [7.1]	
Fed on infant formula with folic acid, n	30	55	8	
Plasma tHcy, μmol/L	5.9 (1.2)	6.0 (1.2)	5.6 (0.6)	0.790
	5.5 [1.1]	5.9 [1.2]	5.5 [1.2]	
RBC folate, nmol/L	2342 (405)	2328 (397)	2038 (304)	0.176
	2360 [673]	2389 [405]	1986 [568]	
Plasma 5-MTHF, nmol/L	44.9 (15.8)	53.9 (17.7)	68.9 (21.7)[3]	0.008
	43.2 [20.1]	52.6 [27.3]	68.2 [24.9]	
Plasma pABG, nmol/L	24.1 (11.6)	18.4 (8.1)[6]	13.3 (6.9)[3]	0.004
	23.6 [13.6]	17.4 [11.2]	10.8 [9.1]	

[1] Data are expressed as mean (SD) and median [IQR].
[2] Folate markers were compared between the genotypes using ANOVA and post hoc Tamhane's T2 applied on the $\log_{10}$-transformed data.
[3] Different from CC ($P < 0.05$).
[4] Different from CT ($P < 0.05$).
[5] $P = 0.066$ compared with CC.
[6] $P = 0.074$ compared with CC.
Ca, calcium; 5-MTHF, (6S)-5-methyltetrahydrofolate; MTHFR, methylenetetrahydrofolate reductase; pABG, para-aminobenzoylglutamate; RBC, red blood cell; tHcy, total homocysteine.

United States (1133 nmol/L [26]) and Japan (972 nmol/L [29]). Irrespective of maternal exposure to folic acid during pregnancy and lactation, the concentrations of plasma and RBC folate in infants are generally higher than those in children and adults; thus, age-specific reference intervals for folate markers should be established. The plasma folate concentration declined faster in infants after a dose of 30 μg/kg folic acid than in adults [30], suggesting a higher rate of utilization or turnover in infants. Concentrations of plasma 5-MTHF and RBC folate decline in the first 4–6 wk after birth [26,27,31]. This decline could be because of uptake into tissues [32], utilization, expanding blood volume, urinary loss of folate [33], or changes in bulk transfer of folate via the placenta vs. postnatal nutrition. The guidelines for the amount of folate in infant formula are based on the folate composition in the breastmilk of well-nourished women [2]. However, these studies did not consider the infant MTHFR genotype. The concentrations of folate in breastmilk varied between different studies (i.e., 180–224 nmol/L) [34,35], which may be partly explained by using different analytical methods. The intake of micronutrients that are needed to maintain low homocysteine (folate and/or vitamin B-12) could be low in breastfed infants, thus explaining the raise in plasma tHcy between baseline and visit 4. We did not measure vitamin B-12 concentration, but insufficient intake of vitamin B-12 could also affect plasma tHcy concentrations in the first 4 mo after birth [36]. However, infant formula contains the recommended folate and vitamin B-12 concentrations.

MTHFR polymorphism and folate markers in infants

The concentrations of RBC folate in infants aged <1 mo (i.e., those in this study) can be considered to reflect fetal folate supply during late gestation, given the slow kinetics of this marker. In contrast, plasma folate is sensitive to recent changes in folate intake or homeostasis. In a study among Canadian newborns [37], the cord RBC folate concentration (mean: 2689 nmol/L) was >2-fold higher than that in our study (mean: 1334 nmol/L). In the Canadian study, the TT genotype was associated with ~23% lower serum folate and 14% higher RBC folate concentrations than the CC genotype [37]. Unlike in the Canadian study, the TT genotype in our study was associated with 44% higher plasma 5-MTHF and 23% lower RBC folate concentrations compared with the CC genotype (Table 2). High maternal folate intake during pregnancy may saturate fetal tissues with folate. Postnatal tissue expansion and the increase of blood volume may have caused a short-term decline in serum folate concentrations in the Canadian newborns.

Higher plasma 5-MTHF concentrations in infants with the TT genotype (vs. CC) are not in line with those reported in studies conducted

in adults [9,38]. In theory, higher plasma 5-MTHF in infants could represent a flexible reservoir of folate that can be taken up by the brain, rather than being stored in tissues such as the erythrocytes.

Modification of folate catabolism by folate intake and MTHFR genotype; implications for folate requirements

We found that pABG is the main folate breakdown compound in plasma (Supplemental Fig. 6). Studies in adults suggested that pABG could reflect the overall turnover of the tissue folates [14]. The dose-response and coherent inverse association between the number of T alleles and plasma pABG concentrations suggests that the folate utilization/turnover rate is ≥50% lower in carriers of the T allele, although the infant formula contained 15.2 μg folic acid or 15.8 μg 5-MTHF-Ca /100 kcal according to the present EU legislations [22]. The magnitudes of between-genotype differences in plasma pABG were constant and appeared independent of the age of the infant, feeding patterns, and folate forms in infant formula. Irrespective of the genotypes, intake of 15.2 μg folic acid /100 kcal or 15.8 μg 5-MTHF-Ca/100 kcal was associated with a markedly higher plasma pABG concentration compared with breastfed infants, suggesting that folate content in breastmilk was lower than that in the infant formula. Our results suggest that folate requirements could be ~50% higher in infant carriers of the TT genotype (vs. CC).

Plasma pABG is affected by hyperplastic growth [18], folate intake [21], and MTHFR genotype (i.e., present study). Plasma pABG in breastfed infants increased by an average of 40% in all genotypes between <1 mo and 16 wk. The extent of pABG increase among formula-fed infants was higher than that in breastfed infants (Supplemental Figs. 4 and 5). In the present study, the MTHFR genotype was not associated with infant weight. However, it is possible that between-genotype differences at the molecular level such as on the level of DNA methylation could exist.

The limitations of the present study include its exploratory nature. The study may have been underpowered to detect potentially existing differences, and type I errors (false-positive results) may be higher when testing multiple and unplanned outcomes. Although the results need to be confirmed in other populations, the coherent genotype differences in pABG at 2 time points strongly suggest that these results are not because of chance. In addition, we did not measure urine folate catabolites. The renal excretion of folate may vary with the age of the infant, intervention, or MTHFR genotype. However, compared with urinary pABG, plasma pABG could be more sensitive to recent changes in folate catabolism because it originates from intracellular folate. Finally, breastfed infants were not randomly assigned, and breastfeeding could be associated with possible differences in maternal characteristics and variations in individual intakes of micro- and macronutrients. Therefore, comparisons of breastfed- and formula-fed infants could be subject to inherent bias.

In conclusion, folate turnover as indicated by plasma pABG markedly increased from the age of <1 mo to 16 wk, thereby showing a high catabolic rate of tissue folates. The infant's MTHFR677TT genotype was associated with ≥50% lower plasma pABG than the CC genotype. The associations between the genotype and pABG remained significant also in infants achieving an intake of 15.2 μg folic acid/100 kcal or 15.8 μg 5-MTHF-Ca/100 kcal according to the current EU legislations. The data suggests reduced folate utilization/turnover in carriers of the TT genotype, which could impact folate intake requirements in infants aged 0–6 mo. The results need to be confirmed in populations not exposed to folic acid fortification. Moreover, the association between infant's MTHFR genotype and folate-dependent molecular processes such as DNA methylation deserves more investigation.

Author contributions

RO performed data analyses, wrote the manuscript, and is responsible for the final content. IW, AW, IB, BT, RS, CH, JD, and BK participated in acquisition of the data and provided critical revisions and input to important intellectual content of the manuscript. IB provided input to the selection of single-nucleotide polymorphism during the planning phase and was responsible for genotyping analysis. BK was responsible for the study design and protocol and serves as the principal investigator of the original trial. All authors have read and approved the final manuscript.

Conflict of interest

IW, IB, BT, and RS are employees of DSM Nutritional Products Ltd. AW and CH are employees of HiPP GmbH & Co. RO and BK received remuneration from DSM and HiPP as speaker for medical education and for providing scientific advice. JD reports no conflicts of interest.

Data availability

The data that support the study are available from the authors upon request for research purposes, with adherence to the EU General Data Protection Regulation and the protection of personal rights of study subjects.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ajcnut.2022.09.002.

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