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One-Carbon Metabolism in Nepalese Infant–Mother Pairs and Child Cognition at 5 Years Old

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ABSTRACT

Background: One-carbon metabolism (OCM) refers to the transfer of methyl groups central to DNA methylation and histone modification. Insufficient access to methyl donors and B-vitamin cofactors affects epigenetic maintenance and stability, and when occurring in early life may impact future health and neurodevelopment.

Objective: The objective of this study was to examine the relative associations between one-carbon metabolites in Nepalese mother–infant pairs and child cognition measured at 5 y of age.

Methods: This is a cross-sectional study from Bhaktapur, Nepal, in a population at high risk of subclinical B-vitamin deficiencies and cumulative infection burden. Venous blood samples from 500 mother–infant pairs were collected when the infants were 2 to 12 mo old, and metabolite concentrations measured by microbiological assays and GC–tandem MS. We re-enrolled 321 of these children at 5 y and assessed cognition by the Ages and Stages Questionnaire, 3rd edition, and subtests from the Developmental Neuropsychological Assessment, 2nd edition (NEPSY-II). The associations of the independent metabolites or unobserved metabolic phenotypes (identified by latent class analysis) with the cognitive outcomes were estimated by seemingly unrelated regression. We explored direct and indirect relations between the OCM pathway and the cognitive outcomes using path analysis.

Results: Infant cystathionine concentration was inversely associated with 4 cognitive outcomes (standardized βs ranging from −0.22 to −0.11, P values from <0.001 to 0.034). Infants with a metabolic phenotype indicating impaired OCM and low vitamin B-12 status had poorer cognitive outcomes compared with infants with normal OCM activity and adequate vitamin B-12 status (standardized βs ranging from −0.80 to −0.40, $P < 0.001$ and 0.05). In the path analysis, we found several OCM biomarkers were associated with affect recognition through infant plasma cystathionine.

Conclusions: Elevated plasma cystathionine during infancy reflects a metabolic phenotype of impaired OCM and low vitamin B-12 status and is associated with poorer cognitive function when the children are 5 y old. *J Nutr* 2021;151:883–891.

Keywords: one-carbon metabolism, vitamin B-12, cystathionine, child cognition, Nepal

Introduction

One-carbon metabolism (OCM) describes a network of interrelated metabolic pathways that include the methionine and folate cycles and the trans-sulfuration pathway. OCM involves the addition, transfer, or removal of one-carbon units in cellular biochemical reactions (1) and is central to multiple physiological processes, including methylation of DNA and histone modification (epigenetic maintenance), myelination, redox homeostasis, synthesis of neurotransmitters, purines, thymidine, and phospholipids (1–4). Throughout the life cycle, OCM is dependent on the dietary supply of methyl donors including folate and methionine (5), as well as cobalamin (vitamin B-12), vitamin B-6, and riboflavin, which serve as essential coenzymes (6).

Homocysteine (Hcy) represents a point of intersection between the methionine cycle and trans-sulfuration pathway. The methionine cycle, the central methylation pathway within OCM, is dependent on the presence of cobalamin as a cofactor for methionine synthase. The remethylation of Hcy to methionine intersects with the folate cycle where the flavin adenine dinucleotide (B-2 vitamer)–dependent methylenetetrahydrofolate reductase generates 5-methyltetrahydrofolate, which serves as a co-substrate for the remethylation of Hcy to methionine (1). If insufficient concentrations of cobalamin are

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available, folate becomes trapped as 5-methyltretrahydrofolate ("methyl-trap") (7), the regeneration of methionine is inhibited, and Hcy concentrations increase (8). Low concentrations of folate also disrupt remethylation, leading to accumulation of Hcy (1). In the trans-sulfuration pathway, Hcy is a substrate for the vitamin B-6–dependent enzyme cystathionine β-synthase, which catalyzes its condensation with serine to form cystathionine, which is then cleaved to form cysteine by the vitamin B-6–dependent γ-cystathionase.

Concentrations of Hcy within the cell are tightly regulated based upon the different affinities of methionine synthase (Michaelis constant (K_m) values <0.1 mM) and cystathionine β-synthase (K_m values >1 mM) for Hcy (9). Thus, at low Hcy concentrations, methionine conservation is favored, while at high Hcy concentrations, Hcy is metabolized via the trans-sulfuration pathway (10). Abnormal elevations of Hcy in plasma and urine result from increased levels of Hcy export, reflecting an imbalance between Hcy production and metabolism (9).

Renewed interest in OCM has, in part, been prompted by observations that even modest dietary inadequacies of essential methyl donors and cofactors, without typical signs of deficiency, can have consequences for DNA methylation and genetic stability (11). Evidence suggests that significant changes in DNA methylation patterns occur within the first 5 y of life (12), and early-life nutrition may impact subsequent development and health, including neurodevelopment (13). Although the underlying mechanisms linking nutrition and cognition are not well understood, epigenetic mechanisms are thought to be involved (14). Vitamin B-12 has been recognized as important for neural myelination, synaptogenesis, and neurotransmitter synthesis, with potential effects on the developing brain and ultimately cognitive functioning in childhood (15). However, the regulation of DNA methylation is likely reliant on the interaction of multiple nutrients within OCM, rather than the effect of a single nutrient alone (11).

We recently demonstrated that circulating cobalamin, total Hcy (tHcy), and methylmalonic acid (MMA) concentrations in Nepalese infants were associated with cognitive functioning when they were 5 y old (16). Further investigating the same cohort of Nepalese children, the current study aimed to expand the OCM biomarker profile and evaluate the circulating concentrations of these metabolites at infancy in relation to child cognitive performance at 5 y.

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Supplemental Figure 1 is available from the "Supplementary data" link in the online posting of the article and from the same link in the table of contents at http://academic.oup.com/jn.
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Abbreviations used: ASQ-3, the Ages and Stages Questionnaire, 3rd edition; CFI, comparative fit index; CRP, C-reactive protein; Hcy, homocysteine; K_m, Michaelis constant; MMA, methylmalonic acid; NEPSY-II, Developmental Neuropsychological Assessment, 2nd edition; OCM, one-carbon metabolism; PLP, pyridoxal 5-phosphate; RMSEA, root mean square residual; SMRM, standardized root mean square residual; tHcy, total homocysteine; WAZ, weight-for-age *z* score.

Methods

Participants

From January 2008 to February 2009, we enrolled 500 lactating women between 15 and 45 y old and their infants 2 to 12 mo of age from the Bhaktapur municipality in Nepal for a cross-sectional study on nutritional intake. We used a 2-stage cluster-sampling procedure whereby 66 neighborhoods ("toles") were randomly selected as the primary sampling unit from a total of 160. We listed all women living in these toles, and randomly selected women and infant pairs (17). The inclusion criteria for the study were that both mothers and children had no ongoing clinically assessed infections, resided in the selected clusters, were willing to provide their household information, and consented to participate. Details on the random selection for the original study have been published elsewhere (17, 18). Upon selection at baseline, mothers were asked to bring their children to Siddhi Memorial Hospital in Bhaktapur for administration of questionnaires, physical examination, anthropometric measurements, and blood collection. Information on the families and their socioeconomic situation was gathered in a household questionnaire. A detailed description of the data-collection procedures has been presented elsewhere (17).

In 2012 and 2013, ~5 y after the first study, we searched for and were able to locate 330 children of the initial 500 enrolled woman–child pairs for follow-up assessments, for whom we have cognitive data in 321 children. Details from the follow-up study have been described previously (16).

A new written consent form from the mothers was collected for the follow-up assessments. The original study obtained ethical clearance from the institutional review board at the Institute of Medicine in Kathmandu, Nepal. The new ethical approval was obtained for the follow-up study from the same Nepalese review board as well as from the Regional Committee for Medical and Health Research Ethics in Norway.

Blood collection and laboratory procedures

Approximately 3 mL of whole blood was taken from the cubital vein using polypropylene tubes with lithium heparin (Sarstedt). The samples were then centrifuged (760 × *g*, for 10 min at room temperature) and plasma was allocated into polypropylene vials (Eppendorf). Samples were stored at −20°C at the field site laboratory until they were transported with an ice pack to the central laboratory for processing in Kathmandu at the end of each day. After processing, the samples were stored at −70°C before being shipped to Bergen, Norway, on dry ice in batches from 2009 to 2012 and analyzed by Bevital in 2013–2014 following 1 freeze-thaw cycle.

Circulating concentrations of a selection of methyl donors, cofactors, and amino acids representing the main metabolic pathways (i.e., the methionine and folate cycles and the trans-sulfuration pathway) in OCM were analyzed at the Bevital Laboratory, Bergen, Norway (www.bevital.no). Plasma cobalamin and folate concentrations were determined by microbiological assays based on a colistin sulfate-resistant strain of *Lactobacillus leichmannii* (19) and a chloramphenicol-resistant strain of *Lactobacillus casei* (20), respectively. The B-6 vitamer, pyridoxal 5′-phosphate (PLP), and riboflavin were measured by LC–tandem MS (21). The functional indicators of vitamin B-12 status, tHcy and MMA, and cysteine, methionine, serine, glycine, sarcosine, and cystathionine were analyzed using GC-MS based on methyl chloroformate derivatization (22). Neopterin, a marker of cellular immune response, was measured by LC–tandem MS (21), and the inflammation marker, C-reactive protein (CRP), was measured by immune-matrix–assisted laser desorption/ionization (23). In addition to neopterin and CRP, we used the PAr index (the ratio of the B-6 vitamers 4-pyridoxic acid divided by the sum of PLP plus pyridoxal [PA:(PLP + PL)]) as a marker of vitamin B-6 catabolism during inflammation (24). The aforementioned assays were adapted to a microtiter plate format and carried out by a robotic workstation. The within-day CV was 4% for both cobalamin and folate, 3% for PLP, 6% for riboflavin, 1% for tHcy, and 2% for MMA, and ranged from 1% to 2% for cysteine, methionine, serine, glycine, sarcosine, and

cystathionine, and from 3% to 5% for neopterin and 4% to 5% for CRP.

The between-day CV was 5% for both cobalamin and folate, and ranged from 6% to 13% for PLP and riboflavin, 2% for tHcy, 3% for MMA, 2% to 6% for cysteine, methionine, serine, glycine, sarcosine, and cystathionine, and from 6% to 10% for neopterin and 7% to 9% for CRP.

Cognitive assessments at follow-up

A local pediatrician and a psychologist, both experienced in child cognitive assessments, were trained to perform the assessments for the study. The local psychologist performed the assessments under close supervision. Assessments were conducted at the study clinic in a well-lit room free from distractions. The sessions lasted for ~1 h. The cognitive assessments have been thoroughly described elsewhere (16).

The Ages and Stages Questionnaire, 3rd edition (ASQ-3), is a comprehensive developmental checklist, standardized for children aged 1–66 mo with age-appropriate questions (25). For the current study, we translated and back-translated the 60-mo questionnaire (age range: 57–66 mo). The back-translated questionnaire was compared with the original version and each item was carefully considered in terms of cultural appropriateness. No cultural adaptations were made to the questionnaire for use in the Nepalese context. The questionnaire contains 30 items [scored 0 (not yet), 5 (sometimes), or 10 (yes)] that sums up to 5 subscales and a total score, where higher scores indicate better performance. In the current study, the ASQ-3 assessments were done by the assessor directly with the child and not through parental report. The examiner used a collection of standardized material (e.g., large and small balls, pen, paper, and scissors) in the assessments, and completed the questionnaire based on observations of the child during the sessions and questions to the caregivers. Although 160 of the participants were above the upper age range of 66 mo during the assessments, we decided to perform the ASQ-3 for all children in order to secure a full assessment for the total sample.

The Developmental Neuropsychological Assessment, 2nd edition (NEPSY- II) is a comprehensive neuropsychological test battery consisting of 32 subtests in 6 functional domains for children aged 3 to 16 y (26). The following 6 age-appropriate subtests were administered for the current study: Inhibition, Statue, Visuomotor precision, Affect recognition, Geometric puzzles, and Block construction, in which the last 3 are included in the analysis for the present study based on findings of an association with vitamin B-12 status in a previous study (16). Affect recognition is a social perception test and involves the ability to recognize emotional expressions in a matching task with pictures of children. In the Geometric puzzles test, the child identifies and matches geometric shapes testing their recognition and mental rotation abilities. Block construction is a test of visuomotor and visuospatial abilities where the child produces 3-dimensional figures with blocks based on 2-dimensional drawings (26). The subtests were scored according to the instructions in the NEPSY-II manual (26). Raw scores for the Affect recognition and Block construction subtests were transformed to scaled scores according to the US norms; for the Geometric puzzles, there are no norms for this age range and we used the raw scores for the further calculations.

Statistical analysis

We present demographic information as means for continuous variables and proportions for categorical variables. Weight-for-age z scores (WAZ) were calculated based on the reference values from the WHO (27). In the analyses, we used selected one-carbon metabolites measured at baseline as predictors. Scores on the NEPSY-II subtests—Affect recognition, Block construction and Geometric puzzles—and the total ASQ-3 score were selected as cognitive outcomes based on previous findings (16). Missing data on the metabolites (16 out of 321), and similarly for the covariates, were imputed as the mean to preserve the sample size as not all missing variables were for the same cases. Due to the low number of missing data (≤5%), we assumed that these imputations would not influence the models. Predictors and outcomes that deviated clearly from normality, as assessed by quantile-quantile plots and histograms, were transformed according to Tukey's ladder of powers. Transformations included mostly log-transformations, but also square root and inverse square root according to the transformed variables fit to normality. All variables were standardized, so that interpretations of effect sizes are similar in magnitude to correlation coefficients. Seemingly unrelated regression (Stata 16: sureg) was used to accommodate for correlations between the error terms (i.e., residuals) of the 4 cognitive outcome variables. All metabolites were assessed independently. Confounders were measured at baseline and consisted of infant age, maternal age, child gender, maternal literacy, family ownership of land, and WAZ. The process by which we selected the adjustment variables has been described previously (16). For this current study analysis, we also included maternal age, maternal literacy, and family ownership of land as variables that could act as confounders.

Furthermore, we aimed to identify unobserved metabolic phenotypes among the children in the cohort. Thus, a latent class analysis (Stata 16: gsem, lclass) was implemented with all measured one-carbon metabolites as outcomes. The Bayesian information criterion was used to identify the number of latent classes, which resulted in the best model fit. The identified classes were subsequently entered in a seemingly unrelated regression model with the 4 cognitive measures as outcomes adjusted for the aforementioned confounders.

Finally, as metabolites follow predictable pathways, we implemented an explorative structural equation model path analysis (Stata 16: sem) based on our obtained results. The aim was to establish relations between the correlated pathway metabolites in line with known biochemistry and cognition. Such models, in principle estimating multiple mediators, are often referred to as path models. The comparative fit indices (CFIs), root mean square error of approximation (RMSEA), and the standardized root mean square residual (SRMR) were used as goodness-of-fit indicators to assess the fit of the model to the sample data. We considered an acceptable and good fit as indicated by an SRMR <0.08 and <0.05 and RMSEA <0.08 and <0.06 and a CFI >0.90 and >0.95 in accordance with published guidelines (28).

Results

Study participants

The final number of valid cognitive assessments was 320 since 1 child was excluded from the analysis due to the suspicion of a medical condition that questioned the validity of the assessments. One child was not tested with the NEPSY-II, and therefore we have only 319 NEPSY-II measurements. In 14 of the children we had insufficient blood samples at baseline and thus the number of children in the analysis was 306 (ASQ-3) and 305 (NEPSY-II). In 1 mother we had insufficient blood samples at baseline, and the final number of maternal analyses was 319 (ASQ-3) and 318 (NEPSY-II) (**Supplemental Figure 1**). Demographic characteristics in the total original sample and the sample at follow-up are shown in **Table 1**. Mean (SD) age at baseline was 7 (2.9) mo, whereas the children were 66.7 (3.4) mo at follow-up, equivalent to ~5.5 y. While the child characteristics are similar between the original sample and the follow-up sample, there are some differences in the level of parental education and the household characteristics, such as the number living in a joint family and ownership of land in favor of the follow-up sample. Metabolite concentrations in infants and mothers measured at baseline are shown in **Table 2**. Among the children, 45 (14.7%) had cobalamin concentrations below the cutoff of 148 pmol/L. Furthermore, 174 children (56.5%) had elevated tHcy (>10 μmol/L), 242 children (77.1%) had elevated MMA (>0.28 μmol/L), and 198 children (64.7%) had 3cB12 scores below the cutoff of -0.5, indicating low vitamin B-12 status (29). None of the children in the study had poor folate status (<10 nmol/L). For vitamin B-6, 61 (19.9%) had vitamin B-6 deficiency (PLP <20 nmol/L)

TABLE 1 Demographic characteristics of Nepalese children and their parentsduring infancy and at 5 y[1]

	Total sample at follow-up ($n = 321$)	Total original sample ($n = 500$)
Child characteristics at baseline		
Boys, *n* (%)	179 (55.9)	277 (55.4)
Age, mo	7.0 ± 2.9	6.9 ± 3.0
Birth weight, g	2872 ± 475.8	2891.5 ± 491.8
Exclusively breastfed at 6 mo, *n* (%)	43 (13.6)	73 (15)
Growth status, *z* score		
Weight-for-age	−0.3 ± 1.0	−0.3 ± 1.0
Weight-for-length	0.0 ± 1.0	0.0 ± 1.1
Length-for-age	−0.4 ± 1.3	−0.5 ± 1.3
Iron status		
Mean plasma ferritin, μg/L	54.1 ± 70.5	57.8 ± 93.9
Inflammation		
hsCRP,[2] μg/mL	4.4 ± 8.4	4.3 ± 10.7
PAr[3]	0.2 ± 0.1	0.1 ± 0.1
Neopterin, nmol/L	26.7 ± 16.2	26.4 ± 15.3
Family situation at baseline		
Maternal characteristics		
Age, y	26.1 ± 4.2	25.8 ± 4.2
Less than grade 10 of schooling, *n* (%)	147 (48.2)	244 (52.6)
10th grade and higher, *n* (%)	158 (51.8)	220 (47.4)
Mothers who work, *n* (%)	80 (26.2)	122 (26.3)
Paternal characteristics, *n* (%)		
Less than grade 10 of schooling	94 (30.3)	165 (35.6)
10th grade and higher	216 (69.7)	298 (64.4)
Fathers who work	281 (93.1)	431 (93.5)
Household characteristics, *n* (%)		
Joint family	170 (53.8)	243 (49.19)
Own land	182 (57.6)	270 (54.7)
Follow-up at 5 y		
Age at testing, mo	66.7 ± 3.4	NA
Number of completed tests	321	NA
Total ASQ-3, *n*	321	NA
Total NEPSY-II, *n*	320	NA

[1] Values are frequencies (%) or means ± SDs. ASQ-3, Ages and Stages Questionnaire, 3rd edition; hsCRP, highly sensitive C-reactive protein; NA, not applicable; NEPSY-II, Developmental Neuropsychological Assessment, 2nd edition.
[2] $n = 294$.
[3] PAr index is the ratio of 4-pyridoxic acid (PA) divided by the sum of pyridoxal 5′-phosphate (PLP) plus pyridoxal (PL) [PA:(PLP + PL)] and reflects vitamin B-6 catabolism during inflammation.

TABLE 2 Plasma metabolite concentrations in Nepalese infants and their mothers[1]

	Values
Infants[2]	
Cobalamin, pmol/L	263 ± 133 (10–908)
Folate, nmol/L	72 ± 34 (10–151)
Total homocysteine, μmol/L	12 ± 5 (4–35)
Methylmalonic acid, μmol/L	0.7 ± 0.7 (0.1–4.2)
Total cysteine, μmol/L	198 ± 23 (119–296)
Methionine, μmol/L	26 ± 7 (9–68)
Serine, μmol/L	142 ± 23 (75–255)
Glycine, μmol/L	214 ± 45 (87–400)
Sarcosine, μmol/L	2.1 ± 1.2 (0.3–17.4)
Cystathionine, μmol/L	0.4 ± 0.2 (0.1–1.2)
Riboflavin, nmol/L	21 ± 20 (6–6229)
Flavin mononucleotide, nmol/L	7.9 ± 5.5 (2–42)
Pyridoxal 5′-phosphate (PLP), nmol/L	42 ± 39 (10–322)
Pyridoxal (PL), nmol/L	37 ± 42 (6–333)
Mothers[3]	
Cobalamin, pmol/L	289 ± 115 (101–862)
Folate, nmol/L	20 ± 15 (2–98)
Total homocysteine, μmol/L	10 ± 6 (4–50)
Methylmalonic acid, μmol/L	0.5 ± 0.5 (0.1–3.6)
Total cysteine, μmol/L	227 ± 29 (112–334)
Methionine, μmol/L	24 ± 5 (14–45)
Serine, μmol/L	133 ± 29 (72–297)
Glycine, μmol/L	354 ± 115 (130–817)
Sarcosine, μmol/L	1.6 ± 0.7 (0.6–6.8)
Cystathionine, μmol/L	0.2 ± 0.1 (0.1–0.7)

[1] Values are means ± SDs (range).
[2] $n = 307$.
[3] $n = 321$.

and 80 (26.1%) had suboptimal status (plasma PLP = 20–30 nmol/L).

Metabolites and cognitive outcomes

Table 3 shows the metabolite-by-metabolite associations with the 4 cognitive outcomes. There were strong inverse associations between infant tHcy, MMA, serine, sarcosine, and cystathionine concentrations and the Affect recognition subtest at 5 y (standardized βs from −0.22 to −0.14, P values from <0.001 to 0.009). Maternal folate ($\beta = 0.11$, $P = 0.045$) and infant PLP ($\beta = 0.14$, $P = 0.017$) concentration was positively associated, while infant cystathionine concentration was negatively associated ($\beta = -0.15$, $P = 0.011$) with the Block construction subtest. Both infant and maternal cobalamin concentration ($\beta = 0.15$ and 0.22 and $P = 0.011$ and <0.001, respectively) as well as infant tHcy ($\beta = -0.12$, $P = 0.043$) and cystathionine ($\beta = -0.16$, $P = 0.006$) and maternal MMA ($\beta = -0.12$, $P = 0.031$) concentration predicted the Geometric puzzles subtest. For the total ASQ-3 score there was an inverse association between both infant and maternal sarcosine concentration (βs of −0.13 for both, $P = 0.009$ and 0.014, respectively) and infant cystathionine ($\beta = -0.11$, $P = 0.034$).

Infant metabolic phenotypes as identified by latent class analysis

Figure 1 shows 4 metabolic phenotypes in children identified through latent class analyses and the associations with the cognitive outcomes. There was a probability of 25%, 11%, 51%, and 13% that the children belonged to class 1, 2, 3, and 4, respectively. We set the reference group to class 1 where the marginal means of the metabolites reflect a cobalamin-replete profile with normal OCM activity. Compared with the children in class 1, the children in class 4 represent a metabolic phenotype reflecting low cobalamin status and impaired OCM (i.e., elevated MMA, tHcy, and cystathionine concentrations) and performed significantly lower on all the cognitive tests (βs ranging from −0.80 to −0.40) (Figure 1).

A path model examining mediation by infant cystathionine

We examined the associations between maternal cobalamin concentration, 4 cognitive outcomes, and infant one-carbon metabolite concentrations in path analysis models (**Figure 2**). In these models, all upstream metabolites were indirectly associated with infant cystathionine, the strongest single

TABLE 3 Maternal and infant plasma metabolite-by-metabolite associations with child cognitive outcomes at 5 y of age[1]

	Affect recognition		Block construction		Geometric puzzles		Total ASQ-3 score	
ID	β^2	*P*	β^2	*P*	β^2	*P*	β^2	*P*
Cobalamin								
I	0.09	0.112	0.10	0.064	0.15	0.011*	0.07	0.166
M	0.04	0.449	0.07	0.210	0.22	<0.001*	0.05	0.393
Folate								
I	−0.05	0.402	−0.05	0.596	0.01	0.437	0.03	0.575
M	0.05	0.381	0.11	0.045*	−0.01	0.888	0.03	0.581
Total homocysteine								
I	−0.16	0.006*	−0.10	0.075	−0.12	0.043*	−0.10	0.061
M	−0.07	0.201	0.01	0.370	0.01	0.891	−0.08	0.148
Methylmalonic acid								
I	−0.15	0.008*	−0.09	0.111	−0.10	0.085	−0.10	0.068
M	−0.01	0.961	−0.03	0.539	−0.12	0.031*	−0.04	0.435
Total cysteine								
I	0.09	0.113	−0.04	0.492	0.02	0.730	0.09	0.113
M	0.04	0.454	0.09	0.118	0.02	0.671	−.001	0.792
Methionine								
I	−0.05	0.393	0.02	0.685	0.03	0.647	−0.02	0.688
M	0.09	0.095	0.05	0.391	−0.07	0.215	−0.04	0.446
Serine								
I	−0.14	0.009*	−0.09	0.110	−0.05	0.361	−0.08	0.149
M	0.06	0.238	−0.04	0.441	−0.06	0.263	−0.04	0.427
Glycine								
I	−0.02	0.705	0.01	0.942	0.01	0.976	−0.05	0.367
M	0.01	0.873	−0.01	0.874	−0.04	0.433	0.02	0.683
Sarcosine								
I	−0.16	0.004*	−0.10	0.083	−0.05	0.354	−0.13	0.009*
M	0.02	0.774	−0.04	0.508	−0.06	0.315	−0.13	0.014*
Cystathionine								
I	−0.22	<0.001*	−0.15	0.011*	−0.16	0.006*	−0.11	0.034*
M	0.06	0.239	0.01	0.936	−0.03	0.560	−0.04	0.455
Infant only								
Riboflavin	0.01	0.458	0.04	0.419	−0.05	0.418	0.04	0.402
Flavin mononucleotide	−0.02	0.683	−0.04	0.514	−0.06	0.257	−0.03	0.577
Total vitamin B-2[3]	−0.01	0.810	−0.03	0.612	−0.07	0.198	0.01	0.816
Pyridoxal 5′-phosphate	0.02	0.822	0.14	0.017*	0.03	0.661	0.07	0.223
Pyridoxal	0.02	0.769	0.09	0.130	0.08	0.169	−0.06	0.298
Total vitamin B-6[4]	0.01	0.913	0.11	0.057	0.07	0.271	0.06	0.264
HK:XA	−0.01	0.938	−0.10	0.079	−0.09	0.118	−0.04	0.440

[1]*Significant at $P \leq 0.05$; ID indicates if infant or mother metabolite associations. ASQ-3, Ages and Stages Questionnaire, 3rd edition; HK:XA, 3-hydroxykynurenine to xanthurenic acid ratio; I, infant; M, mother.
[2]Standardized βs estimated using seemingly unrelated regression, adjusted for residual correlation between the outcomes and for infant age, child gender, weight-for-age *z*-score, maternal age, maternal literacy and family ownership of land.
[3]Riboflavin and flavin mononucleotide combined.
[4]Pyridoxal 5′-phosphate and pyridoxal.

predictor identified in prior analyses. Of the cognitive outcomes, Affect recognition was the only outcome for which a significant mediation from infant cystathionine concentration was observed (**Table 4**). For the other outcomes, there were no such significant associations. The path analyses revealed indirect associations from maternal cobalamin, to infant cobalamin, infant tHcy, and infant serine, and PLP mediated by cystathionine, which was finally associated with Affect recognition. The associations between maternal cobalamin and infant cobalamin on Affect recognition were entirely indirect, as was the association between infant tHcy and Affect recognition. Infant tHcy was the one-carbon metabolite that was most strongly indirectly associated with the cognitive outcome, followed by maternal cobalamin. The direct association of the model's main mediator, infant cystathionine concentration, was $\beta = -0.25$ (95% CI: −0.42, −0.07; $P = 0.005$), larger than any of the individual indirect associations. In the path analysis, there was no significant association between infant folate concentration and the Affect recognition score (Table 4).

Discussion

Among the one-carbon metabolites measured during infancy, plasma cystathionine concentration was the strongest predictor of the cognitive outcomes in Nepalese children at 5 y. A metabolic phenotype, which included elevated plasma cystathionine, reflecting impaired OCM and low vitamin B-12 status, was associated with the poorest outcomes in the

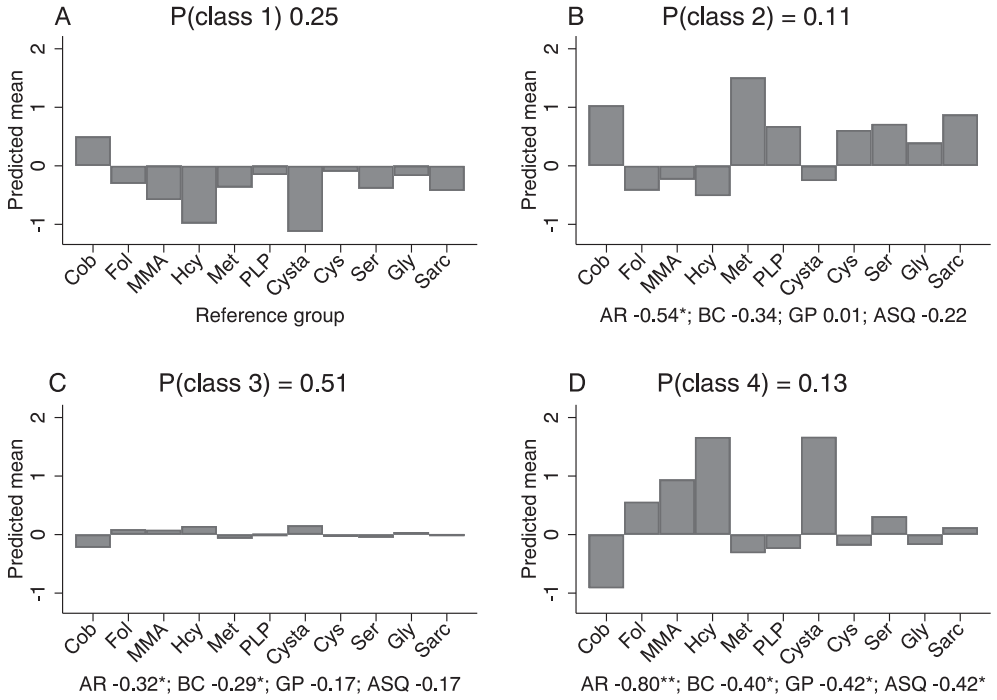

FIGURE 1 Infant one-carbon metabolic phenotypes (A, B, C and D) identified by latent class analysis and their associations with child cognitive outcomes at 5 y in Nepalese mother–child pairs. The predicted marginal mean on the *y*-axes refers to all metabolites on a standardized scale where correlated errors were allowed. The probability of class membership, P(class), is indicated in the headers. The standardized βs were estimated through seemingly unrelated regression, adjusted for infant age, child gender, weight-for-age *z* score, maternal age, maternal literacy, and family ownership of land. The βs comparing each class to class 1 (A) are given in the subtitles for each neurodevelopmental outcome. $^{*}P < 0.05$, $^{**}P < 0.001$. AR, Affect recognition; ASQ, total Ages and Stages Questionnaire, 3rd edition; BC, Block construction; Cob, cobalamin; Cys, cysteine; Cysta, cystathionine; Fol, folate; Gly, glycine; GP, Geometric puzzles; Hcy, total homocysteine; Met, methionine; MMA, methylmalonic acid; PLP, pyridoxal 5′-phosphate; Sarc, sarcosine; Ser, serine.

children. Path analyses demonstrated indirect associations between maternal cobalamin concentration, as well as infant plasma cobalamin, tHcy, serine, and vitamin B-6 (PLP) concentrations via infant plasma cystathionine with affect recognition abilities.

A direct association between cystathionine and cognition is not established in the literature. However, the structural equation modeling suggested that, among these Nepalese children, elevated cystathionine may both be directly associated with, and a key mediator of, suboptimal concentrations of infant cobalamin, tHcy, serine, PLP, and maternal cobalamin with respect to the NEPSY-II Affect recognition subtest score. The Affect recognition subtest is a test of social perception that involves the identification of emotions in others through pictures of children. Development of the ability to recognize emotions in others is vital for appropriate social functioning (30). The Affect recognition subtest also involves other factors underlying cognitive capacities, however, and it would be premature to conclude that there is a link between OCM and social perception skills as a specific domain based on the current findings. It is also plausible that suboptimal vitamin B-12 status in itself, rather than broader OCM impairment, could impact determinants of normal growth, development, and cognitive functioning among infants and young children, such as neural myelination, nucleic acid, and neurotransmitter and protein synthesis (15, 31). In addition, elevated circulating Hcy and cystathionine may have potentially toxic effects detrimental to neurodevelopment (9).

Vitamin B-12 status has been linked to cognitive function and neurodevelopment previously (32, 33, 34). Among 18-mo-old toddlers from poor rural China, circulating cobalamin concentrations were positively associated with cognitive and fine-motor scores (32). These findings add to previous work underscoring the importance of vitamin B-12 status to neurodevelopment in young children (35), including findings from the current sample where cobalamin, tHcy, and MMA concentrations were found to be associated with the cognitive outcomes (16). With the exception of tHcy and MMA, which are established functional indicators of vitamin B-12 status that may be more sensitive than plasma cobalamin in instances of suboptimal status, there is a paucity of research evaluating neurodevelopment in the context of other circulating one-carbon metabolites. This is despite evidence suggesting that even modest reductions in availability of essential OCM methyl donors and cofactors can have consequences for DNA methylation and genetic stability (11).

The underlying assumption of studies investigating mediation is that the causal relations are known and that the path is correctly specified by the investigator. As a cause–effect relation between cystathionine and cognition is not established, the current analysis should be considered hypothesis-generating. We also assessed the one-carbon metabolites as defining features of unobserved groups using latent class analysis, a data-driven analytic approach where causal relations do not need to be set by the investigator. Notably, the identified metabolic phenotype associated with the poorest cognitive outcomes among children in the present study broadly indicated an imbalance in OCM. Specifically, the metabolic profile of these children reflected low cobalamin status, potential methyl trap response, and accumulation and subsequent metabolism of

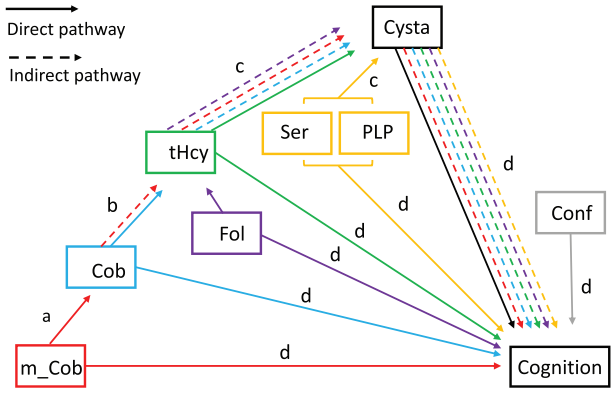

FIGURE 2 Path analysis on direct and indirect pathways from maternal cobalamin concentration and infant metabolite concentrations to child cognition at 5 y in Nepalese mother–child pairs. The Comparative Fit Index of the model was 0.93, the root mean square error of approximation was 0.76, and the standard root mean square residual was 0.046, suggesting an acceptable fit to the model. Direct and indirect pathways: a, to Cob; b, to tHcy; c, to Cysta; d, to Cognition; red, from m_Cob; blue, from Cob; purple, from Fol; green, from tHcy; yellow, from Ser and PLP; black, from Cysta; gray, from Conf. Cob, infant cobalamin; Conf, confounders; Cysta, infant cystathionine; Fol, infant folate; m_Cob, maternal cobalamin; PLP, infant pyridoxal 5′-phosphate; Ser, infant serine; tHcy, infant total homocysteine.

Hcy via the trans-sulfuration pathway (indicated by elevated cystathionine). Although the underlying mechanisms are not clear, our observations support the theory that it is likely an interaction between multiple metabolites within OCM rather than the effect of a single nutrient alone (11) that explains the associations with the cognitive outcomes.

A growing body of research has increasingly recognized the importance of early-life nutrition for normal development and health, including neurodevelopment (13). It is possible that in these Nepalese children, cystathionine may represent a sensitive, functional indicator of suboptimal vitamin B-12 status.

Previously, circulating cystathionine has been proposed as a functional marker of vitamin B-6 status (36), as elevated plasma cystathionine has been consistently observed in dietary vitamin B-6 restriction studies (37–39). In the present study, deficiency or suboptimal vitamin B-6 status was observed in ∼45% of the infants, and it is conceivable that this could contribute to the elevated infant cystathionine concentrations we observed. However, SEM analyses suggest that in this population it was maternal and infant cobalamin status (and to a lesser extent folate and vitamin B-6 status) in the presence of elevated infant tHcy concentrations that had the largest overall effect on elevated infant cystathionine concentrations. Support for our observation can be drawn from earlier work by Stabler et al. (40), where cystathionine evaluation was proposed in the differential diagnosis of elevated circulating tHcy concentrations in patients with overt cobalamin or folate deficiency. In addition, due to its positioning in the trans-sulfuration pathway, the elevated cystathionine may also serve as an indicator of broader OCM impairment. The relevance of this should not be understated, particularly when considering infants and children at risk of undernutrition who are exposed to significant cumulative infection burden throughout early life. Children from this population have a high burden of respiratory and gastrointestinal infections (41). This additional inflammatory pressure potentially amplifies the burden of malnutrition both through the increased demand for nutrients and attenuated nutrient absorption (42, 43). Moreover, chronic inflammation also attenuates the activity of the vitamin B-12–dependent methionine synthase, which regenerates methionine from Hcy thereby disrupting OCM activity (44). Thus, marginalized children might face more severe consequences of inadequate vitamin B-12 nutrition than those from more affluent societies.

The initial primary objective of the source project was to estimate nutritional status in a sample of 500 randomly selected mother–infant dyads representative of Nepal's semi-urban population. Measuring cognition at 5 y of age was decided after the original sample had been recruited and, for this objective, we were only able to re-enroll two-thirds of the original study participants, which reduces the external validity of the observed findings. The observed differences in the SES indicators between the original and follow-up sample were small, however, so the effect on generalizability is assumed to be minimal. Furthermore, we do not believe that the observed metabolic and cognitive associations reported here are likely to have been affected. There are many key variables that are important to consider when investigating the associations between early OCM concentration and later cognition, such as maternal mental health and maternal cognitive abilities, which are known to predict cognitive development in children. A limitation to the current study is that we do not have this information available. We have, however, included variables in the models on maternal education and ownership of land reflecting the socioeconomic situation of the families in this population and, to a certain degree, the child home environment, which is known to be of importance for child development. Moreover, there could be deficiencies in other limiting nutrients, repeated infections, and poor dietary quality (43), which may explain the changes in the cognitive outcomes in these children (i.e., residual confounding).

The fact that this is a population-based study where we endeavored to enroll mother and infant pairs free of chronic or acute illnesses that could impact circulating biomarker concentrations is a notable strength of the study. Other strengths include the extensive experience of study staff involved in testing and collecting blood samples from a pediatric population and optimized processing, storage, and transport of samples at less than −70°C. Moreover, a strength of the study is that, through re-enrolling the children at 5 y and collecting data on their cognitive development, we were able to assess the long-term associations between early OCM and cognition. The ASQ-3 was translated and back-translated specifically for the study. Due to low levels of education and limited experience with completing questionnaires among the caregivers in the study population we used the ASQ-3 as an observational assessment tool and not as a parental report as done previously by others (45). We acknowledge the ASQ-3 was not originally designed or intended for use as an observational assessment tool, but findings both from the current field site (46) and others (47, 48) indicate the validity of this approach in collecting data that are sensitive to child development. A limitation to the study is the lack of quality-control measures when assessing cognition. It is important to note, however, that the assessor received thorough training and supervision from experienced staff in the field before and during the study assessments. Both findings from previous work (16) and the current analyses show that the cognitive measures are sensitive to changes in both maternal and infant metabolite concentrations, supporting the reliability of the cognitive assessment and consistency of the findings. Due to slower than anticipated participant enrollment, 160 children

TABLE 4 Path analysis on direct and indirect pathways from maternal cobalamin concentration and infant metabolite concentrations to affect recognition in Nepalese 5-y-olds[1]

			β[2]		
Path	Outcome	Predictor	Total	Direct	Indirect
a	Infant cobalamin	Maternal cobalamin	0.40**	0.40**	NA
b	Infant total homocysteine	Maternal cobalamin	−0.29**	−0.14*	−0.15**
		Infant cobalamin	−0.38**	−0.38**	NA
		Infant folate	0.21**	0.21**	NA
c	Infant cystathionine	Infant total homocysteine	0.75**	0.75**	NA
		Maternal cobalamin	−0.35**	−0.12*	−0.23**
		Infant cobalamin	−0.32**	−0.03	−0.29**
		Infant folate	0.14*	−0.02	−0.16**
		Infant serine	0.08*	0.08*	NA
		Infant pyridoxal 5′-phosphate	0.10*	0.10*	NA
d	Child affect recognition	Infant cystathionine	−0.25*	−0.25*	NA
		Infant total homocysteine	−0.13*	0.05	−0.18*
		Maternal cobalamin	0.03	−0.05	0.08*
		Infant cobalamin	0.08	0.03	0.06*
		Infant folate	−0.04	−0.01	−0.02
		Infant serine	−0.12*	−0.10	−0.02*
		Infant pyridoxal 5′-phosphate	−0.02	0.01	−0.03
		Infant age	0.12*	0.12*	NA
		Infant male	−0.20	−0.20	NA
		Infant weight-for-age *z* score	−0.01	−0.01	NA
		Maternal age	0.11	0.11	NA
		Maternal literacy	0.10	0.10	NA
		Family ownership of land	−0.08	−0.08	NA

[1] * $P < 0.05$, ** $P < 0.001$. NA, not applicable.
[2] Standardized β-coefficients.

were above the upper age range of the ASQ-3 at the time of assessment. This did not lead to a ceiling effect in the scores, and hence we decided not to omit these 160 children and use all data that were available for the present study analyses.

In conclusion, in the present study, a metabolic phenotype indicative of impaired OCM and low vitamin B-12 status in infancy was associated with the poorest cognitive performance when the children were 5 y old. Of the circulating OCM metabolites measured during infancy, plasma cystathionine concentration was the strongest determinant of the cognitive outcomes, demonstrating both direct and mediating associations. Our observations suggest that cystathionine may represent a sensitive, functional indicator of suboptimal vitamin B-12 status and underscore the need to investigate OCM metabolites collectively, particularly among populations with increased risk of subclinical dietary deficiencies and cumulative infection burden.

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