

Neonatal folate, homocysteine, vitamin B12 levels and *methylenetetrahydrofolate reductase* variants in childhood asthma and eczema

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Abstract

Objectives: To assess the associations of folate, homocysteine and vitamin B12 levels of children at birth and their *methylenetetrahydrofolate reductase* (*MTHFR*) variants with asthma and eczema in childhood.

Methods: This study was embedded in a population-based prospective cohort study ($n = 2,001$). Neonatal cord blood folate, homocysteine and vitamin B12 levels were measured, and *MTHFR* C677T and A1298C genotyped. Wheezing and physician-diagnosed eczema were annually obtained by questionnaire until 4 years. At 6 years, we collected information on physician-diagnosed asthma ever and self-reported eczema ever, measured fractional exhaled nitric oxide (FeNO), and interrupter resistance (Rint). Data were analysed with generalized estimating equations or logistic regression: continuous outcomes with linear regression models.

Results: Folate, homocysteine and vitamin B12 levels of children at birth were not associated with wheezing or eczema until 4 years, asthma and eczema ever, or FeNO or Rint at 6 years. In children carrying C677T mutations in *MTHFR*, higher folate levels were associated with an increased risk of eczema (repeated eczema until 4 years: OR 1.40 (95% CI 1.09–1.80) (SD change) P -interaction = 0.003, eczema ever at 6 years: OR 1.41 (0.97–2.03) P -interaction = 0.011). No interactions between *MTHFR* and child folate and homocysteine levels were observed for wheezing and asthma.

Conclusions: Folate, homocysteine and vitamin B12 levels of children at birth did not affect asthma- and eczema-related outcomes up to the age of 6 years. Further studies are warranted to establish the role of *MTHFR* variants in these associations.

Children of mothers who used folic acid-containing supplements (FACS) during pregnancy may have a higher risk of asthma (1–4). However, previous studies showed conflicting results (1–7). A study using objective measures of maternal folate showed that higher folate during pregnancy was associated with higher risks of asthma in the offspring (8). In our previous study, we found that higher maternal folate was associated with eczema, not wheeze (9). One explanation was

that higher folate, along with higher vitamin B12, and lower homocysteine levels were associated with increased DNA methylation, and hypermethylation may lead to atopic disease (10). Previous observations in mice showed that a methyl-rich diet in pregnancy leads to an allergic asthma phenotype via epigenetic mechanisms (11). Hence, methyl donors could influence programming of the foetal immune system in favour of development of allergic disease (9, 10).

Methylenetetrahydrofolate reductase (*MTHFR*) variants are genetic determinants of folate and homocysteine status. Specific *MTHFR* variants may modify the effects of folate and homocysteine on atopic disease (10). Nucleotide mutations in *MTHFR* at position 677 and 1298 lead to amino acid changes (12). *MTHFR* C677T influences folate and homocysteine levels more than *MTHFR* A1298C (12–16). *MTHFR* C677T, the well-studied functional polymorphism in *MTHFR*, has also been identified by previous genome-wide association studies on folate, homocysteine and vitamin B12 (15, 16). No other functional variants in *MTHFR* have been identified. To our knowledge, there are no reports on the associations of neonatal folate, homocysteine and vitamin B12 levels with development of asthma and eczema. The role of *MTHFR* variants in these associations is not clear. Therefore, in a population-based prospective cohort study among Caucasian children, we assessed the associations between cord blood folate, homocysteine and vitamin B12 levels, and *MTHFR* variants, with asthma- and eczema-related outcomes in childhood.

Methods

Design

This study was embedded in the Generation R Study, a population-based prospective birth cohort study in The Netherlands (17). For this study, we included a selection of Caucasian children, with cord blood available ($n = 2,001$).

Neonatal folate, homocysteine and total vitamin B12 levels

In cord blood was analysed using an immuno-electrochemoluminescence assay (Abbott Diagnostics B.V., Hoofddorp, The Netherlands) (17).

MTHFR variants

C677T (rs1801133) and A1298C (rs1801131) were genotyped. Caucasian ethnicity was defined as having principal components within 4 SD values of the CEU cluster of HapMap (18). Genotype data were extracted from an imputed genome-wide association scan (HapMap phase II release 22). The imputation quality of the two SNPs was good (rs1801133: $RSQ = 0.985$, rs1801131: $RSQ = 0.997$). The genotype frequencies of *MTHFR* C677T were 46.0% (CC), 43.6% (TC) and 10.4% (TT) (Hardy–Weinberg $P = 1.00$), and A1298C were 45.6% (AA), 43.7% (CA) and 10.7% (CC) (Hardy–Weinberg $P = 0.79$). The two *MTHFR* SNPs are in LD, but do not tag the same genetic variation (HapMap pairwise LD (phase II release 22 CEU); $D' = 1.000$, $r^2 = 0.178$).

Asthma- and eczema-related outcomes

Wheezing and physician-diagnosed eczema in the past 12 months were assessed yearly by questionnaires, until 4 years using questions from the International Study of Asthma and Allergies in Childhood (ISAAC) (19).

Information on physician-diagnosed asthma ever and self-reported eczema ever was obtained at 6 years. Fractional exhaled nitric oxide (FeNO) was measured at 6 years using the NIOX chemiluminescence analyzer (Aerocrine AB, Solna, Sweden). Lung function (interrupter resistance, MicroRint, MicroMedical, Rochester, Kent, UK) was measured during tidal breathing, with occlusion of the airway at tidal peak expiratory flow. Median values for at least 5 acceptable Rint measurements were calculated, and these were used to calculate Z-scores (20). Due to technical issues, we had to replace the MicroRint during the study period. This resulted in step-wise variation in the median, which was corrected for.

Statistical analysis

The associations of folate, homocysteine and vitamin B12 at birth with repeated wheezing and eczema in children aged 1 to 4 years were modelled per year (allowing for a time trend), and the overall effects were analysed using multiple imputation-based generalized estimating equation (MI-GEE) models allowing for correction for the within-subject dependence as a result of the repeated measurements, while physician-diagnosed asthma ever and self-reported eczema ever and FeNO and Rint at 6 years were analysed using logistic and linear regression models. FeNO was log-transformed to obtain a normal distribution. We explored effect modification by *MTHFR* variants for the associations with repeated wheezing and eczema, and asthma and eczema ever, by calculating interaction terms between *MTHFR* variants with folate and homocysteine levels. We assumed additive SNP effects. All models were adjusted for maternal age, BMI, educational level at intake, history of maternal atopy or asthma, parity, smoking, folic acid supplement use and pet keeping during pregnancy, and children's sex, gestational age, birth weight and day care attendance, based on the significance of their associations with repeated wheezing ($P < 0.05$), or a change in effect estimate of $>10\%$. We performed sensitivity analyses where we additionally adjusted for complementary measures, folate, homocysteine and total vitamin B12, as these could influence the metabolic pathway (10). The study of Lievers et al. suggested that there might be an interaction between *MTHFR* C677T and A1298C (13). In a second sensitivity analysis, we modelled the interaction between the two SNPs and the influence on our outcomes. Multiple imputations were used for all analyses of binary outcomes and covariates (21). Details are available in Table S1. Data management was performed in SPSS 20.0 (SPSS Inc., Chicago, IL, USA). Statistical modelling using MI-GEE (SAS PROC GENMOD) was performed in SAS 9.2 (SAS Institute, Cary, NC, USA).

Results

Subject characteristics

The descriptives of mothers and children were similar in the observed and multiple imputed data sets (Table 1 and Table S2).

Table 1 Descriptive characteristics of mothers and children ($n = 2,001$)

Maternal characteristics	
Age at enrolment (years)	31.8 (23.8–37.9)
BMI at enrolment (kg/m ²)	23.3 (19.4–32.0)
Smoking during pregnancy	
None (%)	76.4
First trimester only (%)	9.3
Continued (%)	14.3
Educational level	
Primary/secondary education (%)	37.2
Higher education (%)	62.8
Parity	
0 (%)	59.0
1 (%)	31.8
≥ 2 (%)	9.2
History of asthma or atopy (%)	37.8
Self-reported folic acid use during pregnancy	
None (%)	10.5
Suboptimal (first 10 weeks) (%)	33.5
Optimal (periconceptional) (%)	56.0
Pet keeping during pregnancy (%)	42.2
Child characteristics	
Sex (female %)	48.6
Gestational age at birth (weeks)	40.3 (37.6–42.1)
Birth weight (grams)	3550 (2735–4380)
Day care attendance at 1 year (%)	63.3
Child cord blood measures	
Folate (nmol/L)	21.2 (11.9–35.9)
Homocysteine (umol/L)	8.9 (5.6–14.5)
Vitamin B12 (pmol/L)	293 (138–690)
Child outcomes	
Wheezing 1st year (%)	29.8
Wheezing 2nd year (%)	18.9
Wheezing 3rd year (%)	12.2
Wheezing 4th year (%)	11.5
Physician-diagnosed eczema 1st year (%)	22.0
Physician-diagnosed eczema 2nd year (%)	13.4
Physician-diagnosed eczema 3rd year (%)	9.0
Physician-diagnosed eczema 4th year (%)	7.4
Physician-diagnosed asthma ever at 6 years (%)	7.3
Self-reported eczema ever at 6 years (%)	32.4
FeNO (ppb)*	7.1 (3.5–18.3)
Rint (kPa/l)*	0.85 (0.41–1.36)

Values are percentages for categorical variables and for continuous variables median (95% range).

*Continuous outcomes were not imputed (FeNO, $n = 1,009$; Rint, $n = 1,059$).

Folate, homocysteine and vitamin B12 levels at birth and childhood outcomes

We found an association between homocysteine and physician-diagnosed eczema at age 1 year (increase homocysteine

per standard deviation (SD): odds ratio (OR) 1.13 (95% confidence interval (CI), 1.01–1.27)). Folate, homocysteine and vitamin B12 were not associated with repeatedly measured wheezing or eczema for each year separately, and from birth until 4 years (all $P > 0.05$) (Table 2). The levels were also not associated with physician-diagnosed asthma ever and self-reported eczema ever, FeNO or Rint at 6 years (Table 3).

Folate and homocysteine levels, *MTHFR* variants and childhood outcomes

We found a significant interaction between *MTHFR* C677T and folate on eczema until 4 years (P -interaction = 0.003), and of *MTHFR* A1298C and homocysteine on eczema until 4 years (P -interaction = 0.033). We stratified for *MTHFR* C677T and A1298C genotype to explore the association between folate or homocysteine and eczema in children with genetic mutations (Table 4). Among children carrying the polymorphic allele of C677T in *MTHFR*, higher folate levels were associated with an increased risk of eczema until 4 years (increase folate per SD for the heterozygous mutant TC: OR 1.18 (95%, 1.02–1.37); homozygous mutant TT: OR 1.40 (95%, 1.09–1.80)). These effects were independent of homocysteine and vitamin B12. Children with wild-type *MTHFR* A1298C and higher homocysteine levels had an increased risk of eczema until 4 years (increase homocysteine SD for the homozygous wild-type AA: OR 1.19 (95%, 1.05–1.36)). This effect was independent of folate and vitamin B12 levels. We repeated the analysis for eczema ever at 6 years. We found similar results for *MTHFR* C677T, folate and eczema, but did not find a significant interaction between *MTHFR* A1298C and homocysteine on eczema ever at 6 years (Table 5). No interactions between *MTHFR* C677T and A1298C and folate or homocysteine were observed for wheezing and asthma. The two *MTHFR* SNPs did not interact in our study and adding the interaction term in our models did not influence the associations of folate or homocysteine on our outcomes (data not shown).

MTHFR C677T and A1298C may not cover all genetic variation across *MTHFR*. In Table S3, we show that 59 SNPs in and near *MTHFR* (± 10 kb), including *MTHFR* C677T and A1298C, explained only a small proportion of the variance in both folate and homocysteine levels ($R^2 = 0.028$ and 0.020, for the 59 SNPs, respectively).

Discussion

Neonatal folate, homocysteine and vitamin B12 were not associated with repeatedly measured wheezing or eczema until 4 years, or with physician-diagnosed asthma ever and self-reported eczema ever, FeNO or Rint at 6 years. In children carrying the polymorphic allele of C677T in *MTHFR*, higher folate was associated with an increased risk of eczema.

Previous studies have shown conflicting results for the associations of FACS use during pregnancy and asthma and allergy-related outcomes in children (1–7). Differences in measurement methods and the timing and amount of folate exposure between studies make it difficult to compare

Table 2 Associations of cord blood folate, homocysteine and vitamin B12 with repeatedly measured wheezing and eczema in childhood ($n = 2,001$)

Levels	OR's of wheezing (95% CI)*†					OR's of eczema (95% CI)*†				
	1st year	2nd year	3rd year	4th year	Overall	1st year	2nd year	3rd year	4th year	Overall
Folate nmol/L										
Change in SD (7.5)	1.02 (0.92–1.14)	1.00 (0.89–1.13)	1.03 (0.89–1.20)	0.92 (0.78–1.09)	1.00 (0.94–1.08)	1.06 (0.93–1.20)	1.08 (0.93–1.27)	1.00 (0.84–1.19)	0.97 (0.77–1.22)	1.05 (0.95–1.15)
Homocysteine umol/L										
Change in SD (2.9)	0.97 (0.87–1.08)	0.96 (0.84–1.09)	0.96 (0.80–1.14)	1.05 (0.89–1.25)	0.98 (0.91–1.05)	1.13 (1.01–1.27)	1.04 (0.89–1.21)	1.01 (0.83–1.22)	1.03 (0.82–1.28)	1.08 (0.99–1.17)
Vitamin B12 pmol/L										
Change in SD (188)	1.03 (0.92–1.15)	1.09 (0.98–1.22)	0.93 (0.80–1.09)	1.00 (0.86–1.16)	1.03 (0.96–1.10)	0.95 (0.83–1.09)	1.02 (0.88–1.18)	0.98 (0.83–1.17)	0.92 (0.75–1.14)	0.97 (0.89–1.07)

OR, odds ratio; CI, confidence interval; SD, standard deviation.

*Odds ratios (95% confidence intervals) were given (allowing for a time trend) for each year of age separately, and for the overall effects using multiple imputation-based generalized estimating equation models.

†Adjusted for maternal age, BMI, educational level at intake, history of maternal atopy or asthma, smoking and folic acid supplement use during pregnancy, parity and children's sex, gestational age and birth weight. Pet keeping during pregnancy and day care attendance were not significantly associated with repeatedly measured wheezing and did not alter the effect estimates of the determinants with more than 10%.

Sensitivity analyses adjusting for complementary measures showed similar results.

studies. Biological markers are objective and less prone to reporter bias. Higher maternal folate measured in blood during pregnancy have been suggested to affect the risk of preschool asthma at age 3 years (8), and eczema in the first 4 years (9). We previously reported no association between maternal folate during pregnancy and wheezing in the child up to the age of 4 years (9). To our knowledge, the associations of neonatal folate, homocysteine and vitamin B12 levels and *MTHFR* variants with asthma and eczema in childhood have not been studied before. One study of folate levels at 2 years of age found that higher levels were inversely associated with IgE, atopy and wheezing (22). A recent study showed that higher folate measured at ages 2, 4, 6 and 8 years was significantly associated with both food and aeroallergen sensitization, but not with total IgE, wheezing or asthma. This study suggested that folate status in early life may influence the risk of specific allergic sensitization rather than IgE production in general (23). No association between *MTHFR* C677T and asthma or atopy was found in 7–8 year olds (3). These studies did not explore the effects of *MTHFR* variants and biochemical measures of folate and homocysteine.

Folate metabolism is influenced by multiple factors, including homocysteine and vitamin B12 (10, 24). *MTHFR* variants are genetic determinants of folate and homocysteine (10), of which *MTHFR* C677T influences folate and homocysteine more than *MTHFR* A1298C (12–16). We previously showed that maternal *MTHFR* C677T did not alter the associations of maternal folate or homocysteine and wheezing or eczema in the first 4 years (9). Interestingly, in the current study, we did observe a significant interaction between child *MTHFR* C677T and child folate levels on eczema. We can think of two hypotheses to explain this. Maternal folate levels may be associated with an increased risk of allergic outcomes in the offspring as a result of epigenetic mechanisms in utero (8, 9). Regulation of neonatal Th1/Th2 balance is under epigenetic control (25). Folate provides methyl donors for methylation of DNA (10, 26), which may determine when and where a gene is expressed, and this could play a role during neonatal immune development (25, 27). Thus, our results may be explained by increased methylation of foetal DNA as a result of high maternal folate levels. However, maternal and child folate levels were not strongly correlated (Table S4), and this favours another hypothesis: adverse effect of higher folate on the development of eczema in childhood may depend on the child's genetic mutations in *MTHFR* C677T. Mutations in *MTHFR* C677T, but not *MTHFR* A1298C, seem to cause mild hyperhomocysteinemia and decreased folate in adults (12, 13). In our study, the child's TT genotype was not associated with folate or homocysteine levels (Table S5). In healthy adults, folate has a major role in the homocysteine homeostasis by stimulating the transmethylation and remethylation of homocysteine into methionine, which may subsequently provide methyl donors for DNA methylation (28). Mutations in *MTHFR* C677T may render individuals more susceptible to DNA hypomethylation, for example, in response to low folate status (29). Also, studies in adults showed that folate supplementation reduced homocysteine

Table 3 Associations of cord blood folate, homocysteine and vitamin B12 with asthma- and eczema- related outcomes at 6 years

	Asthma (<i>n</i> = 2,001)	Eczema (<i>n</i> = 2,001)	FeNO (<i>n</i> = 1,009)	Rint (<i>n</i> = 1,059)
Levels	OR (95% CI)*,‡	OR (95% CI)*,‡	Ratio change (95% CI)†,‡	Z-score change (95% CI)†,‡
Folate nmol/L				
Change in SD (7.5)	1.02 (0.83–1.25)	1.05 (0.95–1.18)	1.02 (0.98–1.06)	–0.11 (–0.29 to 0.08)
Homocysteine umol/L				
Change in SD (2.9)	0.96 (0.77–1.19)	0.94 (0.84–1.05)	1.01 (0.97–1.05)	0.01 (–0.16 to 0.19)
Vitamin B12 pmol/L				
Change in SD (188)	0.84 (0.62–1.14)	0.97 (0.87–1.08)	1.00 (0.96–1.03)	–0.01 (–0.19 to 0.18)

OR, odds ratio; CI, confidence interval; SD, standard deviation.

*Odds ratios (95% confidence intervals) from logistic regression models.

†Ratio changes and change in standardized z-scores (95% confidence intervals) from linear regression models.

‡Adjusted for maternal age, BMI, educational level at intake, history of maternal atopy or asthma, smoking and folic acid supplement use during pregnancy, parity and children's sex, gestational age and birth weight.

Sensitivity analyses adjusting for complementary measures showed similar results.

Table 4 Associations of cord blood folate and homocysteine stratified for *MTHFR* C677T and A1298C with repeatedly measured eczema in childhood (*n* = 2,001)

<i>MTHFR</i> genotype	OR's of eczema until 4 years (95% CI)*	
	Crude	Adjusted†
C677T (rs1801133)	Folate change in SD (7.5 nmol/L)	
CC genotype (<i>n</i> = 921)	0.93 (0.82–1.06)	0.90 (0.79–1.02)
TC genotype (<i>n</i> = 873)	1.18 (1.02–1.36)	1.18 (1.02–1.37)
TT genotype (<i>n</i> = 207)	1.50 (1.17–1.91)	1.40 (1.09–1.80)
	<i>P</i> -interaction = 0.002	<i>P</i> -interaction = 0.003
<i>MTHFR</i> genotype	OR's of eczema until 4 years (95% CI)*	
	Crude	Adjusted†
A1298C (rs1801131)	Homocysteine change in SD (2.9 umol/L)	
AA genotype (<i>n</i> = 912)	1.21 (1.07–1.37)	1.19 (1.05–1.36)
CA genotype (<i>n</i> = 874)	1.04 (0.92–1.18)	1.05 (0.93–1.20)
CC genotype (<i>n</i> = 215)	0.88 (0.67–1.16)	0.88 (0.67–1.17)
	<i>P</i> -interaction = 0.026	<i>P</i> -interaction = 0.033

OR, odds ratio; CI, confidence interval; SD, standard deviation.

*Overall odds ratios (95% confidence intervals) from multiple imputation-based generalized estimating equation models.

†Adjusted for maternal age, BMI, educational level at intake, history of maternal atopy or asthma, smoking and folic acid supplement use during pregnancy, parity and children's sex, gestational age and birth weight.

Folate, homocysteine and vitamin B12 levels were in one model.

The nonsignificant crude *P*-interaction values for repeated eczema: A1298C*folate, *P*-interaction = 0.489; C677T*homocysteine, *P*-interaction = 0.064. There were no interactions with repeated wheezing: C677T*folate, *P*-interaction = 0.099; A1298C*folate, *P*-interaction = 0.498; C677T*homocysteine, *P*-interaction = 0.131; A1298C*homocysteine, *P*-interaction = 0.298.

Table 5 Associations of cord blood folate stratified for *MTHFR* C677T with eczema ever in childhood (*n* = 2,001)

<i>MTHFR</i> genotype	OR's of eczema ever at 6 years (95% CI)*	
	Crude	Adjusted†
C677T (rs1801133)	Folate change in SD (7.5 nmol/L)	
CC genotype (<i>n</i> = 921)	0.90 (0.77–1.05)	0.88 (0.75–1.04)
TC genotype (<i>n</i> = 873)	1.16 (0.98–1.38)	1.14 (0.95–1.37)
TT genotype (<i>n</i> = 207)	1.47 (1.05–2.06)	1.41 (0.97–2.03)
	<i>P</i> -interaction = 0.009	<i>P</i> -interaction = 0.011

OR, odds ratio; CI, confidence interval; SD, standard deviation.

*Overall odds ratios (95% confidence intervals) from logistic regression models.

†Adjusted for maternal age, BMI, educational level at intake, history of maternal atopy or asthma, smoking and folic acid supplement use during pregnancy, parity and children's sex, gestational age and birth weight.

Folate, homocysteine and vitamin B12 levels were in one model.

The non-significant crude *P*-interaction values for eczema ever: A1298C*folate, *P*-interaction = 0.208; C677T* homocysteine, *P*-interaction = 0.446; A1298C*homocysteine, *P*-interaction = 0.439. There were no interactions with asthma ever: C677T*folate, *P*-interaction = 0.057; A1298C*folate, *P*-interaction = 0.983; C677T*homocysteine, *P*-interaction = 0.584; A1298C* homocysteine, *P*-interaction = 0.586.

levels in those with the TT genotype more than those with CC or TC genotype (30, 31). However, in newborns, homocysteine levels were not affected by an increased folate, suggesting that homocysteine metabolism and response to folate is different in newborns, as compared with adults (32). In addition, we found that the effect of *MTHFR* C677T on folate metabolism is also different in newborns. With this in mind, we speculate that newborns with *MTHFR* C677T variants and high folate may be particularly susceptible to increased DNA methylation as a result of a continuously

altered transmethylation and remethylation flux rate, irrespective of their homocysteine levels. This may be caused by reduced *MTHFR* activity (13) and sparing of folate in the methylenetetrahydrofolate form in those with the TT genotype as proposed by Yamada et al. (12). Unfortunately, we cannot show the effect of the two *MTHFR* SNPs on protein stability of *MTHFR* and *MTHFR* activity, as this data are not available in our cohort. Studies on global DNA methylation and of genes-specific methylation in asthma and allergic disease and the role of *MTHFR* mutations in these associations are needed.

In the current study, we found an interaction between child *MTHFR* A1298C and homocysteine on eczema until 4 years. Homocysteine converts into methionine that may provide methyl donors leading to increased DNA methylation (10), and this may depend on genetic mutations in *MTHFR* A1298C. We did not find an interaction between *MTHFR* A1298C and homocysteine on eczema ever at 6 years. We considered that this interaction might be a chance finding, because we did not find an effect for both repeated physician-diagnosed eczema until 4 years and self-reported eczema ever at 6 years.

There are some methodological issues that could have influenced our findings. Wheezing, asthma and eczema outcomes were obtained by questionnaires, and asking for symptoms and diagnosis that occurred in previous years may not produce accurate results. However, this method of assessing symptoms and diagnosis is widely accepted in epidemiological studies, and we used well-validated questionnaires (19). Assessment of biochemical measures occurred at a single point in time, at birth, while variation in levels could have been different during pregnancy. *MTHFR* C677T and A1298C may not cover all genetic variation across *MTHFR*. To the best of our knowledge, there are no other additional functional variants than *MTHFR* C677T and A1298C known that influence folate or homocysteine status. Variants in other genes may affect one carbon metabolism, the pathway that is centred around folate, but these variants are not related to both homocysteine and folate status, the pathway of our interest (14). The children with genetic data were not selected randomly, as cord blood had to be available. The numbers of missing cord blood samples were higher for lower social economic status. Therefore, we have to be cautious with the generalizability of our findings. Baseline characteristics of mothers and children were comparable in the observed and multiple imputed data sets. To reduce attrition bias, we performed the analyses after multiple imputations. We performed additional sensitivity analyses without multiple imputations of the outcomes, and the effect estimates were slightly stronger (Table S6 and S7). Neonatal folate, homocysteine and vitamin B12 levels did not affect asthma- and eczema-related outcomes. In general, the effects of common genetic variants on complex traits are small. In our study, we found that children with genetic mutations in *MTHFR* C677T and higher folate levels, and children with wild-type *MTHFR* A1298C and higher homocysteine levels had an increased risk of eczema. Gene-environment interactions might well be more impor-

tant than single environmental exposure or genetic risk factors, in particular for asthma.

In conclusion, folate, homocysteine and vitamin B12 levels of children at birth did not affect asthma- and eczema-related outcomes in childhood. We found a significant interaction between *MTHFR* C677T and folate levels, and *MTHFR* A1298C and homocysteine levels on eczema. Any adverse effects of folate and homocysteine on the development of eczema may depend on genetic mutations in *MTHFR*. These findings warrant replication in larger groups.

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Author contributions

RV, JK, AS, LD, EH, HM, HT, ES, AH, VJ and JJ: design, analysis, and writing.

Conflict of interest

The authors declare no conflict of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

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Table S2. Descriptive characteristics of mothers and children in observed and multiple imputed dataset.

Table S3. Variance in folate and homocysteine explained by *MTHFR* SNPs (± 10 kb).

Table S4. Relation of maternal folate levels during pregnancy and child cord blood levels ($n = 1,554$).

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Table S6. Sensitivity analysis without multiple imputations of the outcomes for the associations of cord blood folate and homocysteine stratified for *MTHFR* C677T and A1298C with repeatedly measured eczema in childhood ($n = 1,643$).

Table S7. Sensitivity analysis without multiple imputations of the outcome for the association of cord blood folate stratified for *MTHFR* C677T with eczema ever in childhood ($n = 1,445$).

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Online Supplement

**Neonatal folate, homocysteine, vitamin B12 levels and
methylenetetrahydrofolate reductase variants in childhood asthma
and eczema**

Short title: Folate, homocysteine, asthma and eczema

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Supplementary Table 1. Details of multiple imputations modelling.

Software used and key setting: SPSS 20.0 software (SPSS Inc., Chicago, IL, USA) – Imputation Method determined by SPSS (automatic option) with 10 iterations.
Number of imputed datasets created: 10
Variables included in the imputation procedure: <u>Imputed asthma-related variables:</u> wheezing year 1 (binary: no, yes) wheezing year 2 (binary: no, yes) wheezing year 3 (binary: no, yes) wheezing year 4 (binary: no, yes) eczema year 1 (combination of 2 binary questions: eczema 1-6 months, eczema 7-12 months)* eczema year 2 (binary: no, yes) eczema year 3 (binary: no, yes) eczema year 4 (binary: no, yes) asthma ever birth till year 6 (binary: no, yes) eczema ever birth till year 6 (binary: no, yes) age mother at intake (continuous) bmi mother at intake (continuous) maternal prenatal smoking (nominal: no, first trimester, continued smoking) maternal education (nominal: primary, secondary, higher) number of deliveries (nominal: 0, 1 >=2) maternal history (combination of 4 binary questions: house dust, hay fever, eczema, asthma)* pets keeping during pregnancy (combination of 3 binary questions: cat, dog and bird)* gender child (binary: male, female) gestational age (continuous) birth weight (continuous) daycare attendance (binary: no, yes) folic acid supplement use during pregnancy (nominal: none, suboptimal, optimal) <u>Predictor variables:</u> wheezing year 6 (binary: no, yes) respiratory tract infections severity year 1 (binary: no, yes) respiratory tract infections severity year 2 (binary: no, yes) respiratory tract infections severity year 3 (binary: no, yes) respiratory tract infections severity year 4 (binary: no, yes) respiratory tract infections severity year 6 (binary: no, yes) shortness of breath year 1 (binary: no, yes) shortness of breath year 2 (binary: no, yes) shortness of breath year 3 (binary: no, yes) shortness of breath year 4 (binary: no, yes) cough year 1 (binary: no, yes) cough year 2 (binary: no, yes) cough year 3 (binary: no, yes) cough year 4 (binary: no, yes) cough year 6 (binary: no, yes) phlegm year 1 (binary: no, yes) phlegm year 2 (binary: no, yes) phlegm year 3 (binary: no, yes) phlegm year 4 ((binary: no, yes) weight year 1 (continuous) height year 1 (continuous) weight year 2 (continuous) height year 2 (continuous) weight year 3 (continuous) height year 3 (continuous) weight year 4 (continuous) height year 4 (continuous) weight - year 6 (continuous)

height - year 6 (continuous)
breastfeeding ever (binary: no, yes)
paternal house dust mite allergy (binary: no, yes)
paternal hay fever (binary: no, yes)
paternal eczema (binary: no, yes)
paternal asthma (binary: no, yes)
maternal prenatal alcohol (binary: no, yes)
paternal education (nominal: primary, secondary, higher)
paternal prenatal smoking (binary: no, yes)
bmi father at intake (continuous)
age father at intake (continuous)
maternal prenatal stress (continuous)
parenting stress score (continuous)
cows allergy (binary: no, yes)
income household (binary: < €2000,-, > €2000,-)
postnatal environmental smoke year 2 (binary: no, yes)
Treatment of binary/categorical variables: Logistic/multinomial models
Statistical interactions included in imputation models: None

*Each binary variable was imputed first and combination of variables was done after MI procedure.

Supplementary Table 2. Descriptive characteristics of mothers and children in observed and multiple imputed dataset.

	Observed dataset	Multiple imputed dataset
	N=2,001	N=2,001
Maternal characteristics		
Age at enrolment (years)	31.8 (23.8-37.9)	31.8 (23.8-37.9)
Missing*	0.0	-
BMI at enrolment (kg/m ²)	23.3 (19.4-32.0)	23.3 (19.4-32.0)
Missing*	0.3	-
Smoking during pregnancy		
None (%)	76.3	76.4
First trimester only (%)	9.2	9.3
Continued (%)	14.5	14.3
Missing*	8.0	-
Educational level		
Primary/secondary education (%)	37.0	37.2
Higher education (%)	63.0	62.8
Missing*	1.2	-
Parity		
0 (%)	59.0	59.0
1 (%)	31.8	31.8
≥2 (%)	9.2	9.2
Missing*	0.0	-
History of asthma or atopy (%)	38.4	37.8
Missing*	12.0	-
Self reported folic acid use during pregnancy		
None (%)	9.6	10.5
Suboptimal (first 10 weeks) (%)	33.2	33.5
Optimal (periconceptional) (%)	57.2	56.0
Missing*	17.7	-
Pets keeping during pregnancy (%)	44.7	42.2
Missing*	9.3	-
Child characteristics		
Sex (female %)	48.6	48.6
Missing*	0.0	-
Gestational age at birth (weeks)	40.3 (37.6-42.1)	40.3 (37.6-42.1)
Missing*	0.0	-
Birth weight (grams)	3550 (2735-4380)	3550 (2735-4380)

<i>Missing</i> *	0.1	-
Day care attendance at 1 year (%)	68.1	63.3
<i>Missing</i> *	29.4	-
Child cord blood measures		
Folate (nmol/L)	21.2 (11.9-35.9)	21.2 (11.9-35.9)
<i>Missing</i> *	-	-
Homocysteine (umol/L)	8.9 (5.6-14.5)	8.9 (5.6-14.5)
<i>Missing</i> *	-	-
Vitamin B12 (pmol/L)	293 (138-690)	293 (138-690)
<i>Missing</i> *	-	-
Child outcomes		
Wheezing 1 st year (%)	28.8	29.8
<i>Missing</i> *	23.1	-
Wheezing 2 nd year (%)	17.1	18.9
<i>Missing</i> *	23.3	-
Wheezing 3 rd year (%)	10.5	12.2
<i>Missing</i> *	29.3	-
Wheezing 4 th year (%)	9.7	11.5
<i>Missing</i> *	27.6	-
Physician-diagnosed eczema 1 st year (%)	25.9	22.0
<i>Missing</i> *	43.0	-
Physician-diagnosed eczema 2 nd year (%)	12.9	13.4
<i>Missing</i> *	24.0	-
Physician-diagnosed eczema 3 rd year (%)	8.0	9.0
<i>Missing</i> *	30.3	-
Physician-diagnosed eczema 4 th year (%)	6.1	7.4
<i>Missing</i> *	30.0	-
Physician-diagnosed asthma ever at 6 years (%)	6.2	7.3
<i>Missing</i> *	27.6	-
Self-reported eczema ever at 6 years (%)	31.7	32.4
<i>Missing</i> *	27.8	-
FeNO (ppb)	7.1 (3.5-18.3)	7.1 (3.5-18.3)
<i>Missing</i> *	49.6	49.6
Rint (kPa/l)	0.85 (0.41-1.36)	0.85 (0.41-1.36)
<i>Missing</i> *	47.1	47.1

Values are percentages for categorical variables and for continuous variables median (95%

range). * Percentage of valid missing in the total group (N=2,001).

Supplementary Table 3. Variance in folate and homocysteine explained by *MTHFR* SNPs (-/+ 10kb).

SNPs (<i>MTHFR</i>)	Position (build 36)	Folate explained (R square)	Homocysteine explained (R square)
rs6696752	11758522	0.000	0.000
rs12132479	11759037	0.001	0.001
rs6699881	11759215	0.000	0.000
rs7538516	11759269	0.000	0.000
rs4846039	11759455	0.000	0.000
rs4846040	11759635	0.000	0.000
rs6541001	11759954	0.000	0.001
rs10779765	11760598	0.000	0.000
rs10864540	11760686	0.000	0.000
rs12134663	11761233	0.001	0.001
rs6697244	11761435	0.000	0.000
rs4846044	11762585	0.000	0.001
rs4845882	11765754	0.000	0.000
rs4846047	11766219	0.000	0.000
rs4846048	11768839	0.000	0.000
rs2184226	11770023	0.001	0.001
rs3737967	11770036	0.001	0.000
rs1537516	11770448	0.000	0.001
rs1537514	11770655	0.000	0.001
rs4846049	11772952	0.001	0.001
rs2274976	11773514	0.001	0.000
rs3818762	11773590	0.001	0.002
rs2274974	11773906	0.000	0.001
rs13306556	11774697	0.000	0.000
rs1476413	11774887	0.001	0.002
rs17375901	11775103	0.000	0.001
rs1801131	11777063	0.002	0.001
rs12121543	11777258	0.001	0.003
rs1994798	11777342	0.000	0.000
rs2066462	11777483	0.000	0.001
rs6541003	11778454	0.000	0.000
rs1801133	11778965	0.001	0.000
rs17421462	11779434	0.001	0.001
rs1572151	11780298	0.001	0.001
rs17421511	11780375	0.002	0.001

rs4846052	11780538	0.000	0.000
rs17421560	11780911	0.000	0.001
rs2066471	11783045	0.002	0.001
rs13306567	11783052	0.000	0.001
rs7533315	11783270	0.000	0.000
rs17037390	11783430	0.000	0.000
rs17037396	11784634	0.000	0.001
rs17037397	11784750	0.001	0.000
rs9651118	11784801	0.001	0.000
rs7525338	11784919	0.003	0.001
rs17367504	11785365	0.000	0.000
rs2066470	11785644	0.000	0.001
rs7553194	11786736	0.000	0.000
rs3753582	11788129	0.000	0.000
rs13306561	11788391	0.000	0.000
rs13306560	11788770	0.000	0.001
rs3737965	11789038	0.001	0.000
rs3737964	11789631	0.000	0.000
rs4846054	11791817	0.000	0.000
rs4846056	11792165	0.000	0.000
rs17037425	11792970	0.000	0.000
rs6687229	11795243	0.000	0.000
rs12123964	11795301	0.002	0.001
rs12404124	11796456	0.000	0.000

The 59 *MTHFR* SNPs (-/+ 10kb) together explained only a small proportion of the variance in folate (R Square=0.028) and homocysteine (R Square=0.020).

Supplementary Table 4. Relation of maternal folate levels during pregnancy and child cord blood levels (N=1,554).

		<u>Child folate</u>		
<u>Mother folate</u>		T1	T2	T3
	T1	51.2%	34.9%	13.9%
	T2	31.1%	35.9%	33.0%
	T3	17.3%	32.0%	50.7%

Abbreviations: T1=first tertile; T2=second tertile; T3=third tertile (highest folate levels). Values are percentages of children that remain or move to another tertile compared to the tertile of maternal folate during pregnancy. Maternal and child folate levels were not strongly correlated ($r = 0.365$).

Supplementary Table 5. Associations of *MTHFR* C677T and A1298C and cord blood folate and homocysteine (N=2,001).

<i>MTHFR</i> genotype C677T (rs1801133)	β (95% CI)	
	Folate	Homocysteine
CC genotype (n=921)	Reference	Reference
TC genotype (n=873)	0.36 (-0.34 - 1.06), $P = 0.311$	0.04 (-0.23 - 0.31), $P = 0.768$
TT genotype (n=207)	0.78 (-0.35 - 1.92), $P = 0.175$	0.23 (-0.21 - 0.67), $P = 0.302$

<i>MTHFR</i> genotype A1298C (rs1801131)	β (95% CI)	
	Folate	Homocysteine
AA genotype (n=912)	Reference	Reference
CA genotype (n=874)	-0.88 (-1.58 - -0.19), $P = 0.013$	0.09 (-0.18 - 0.37), $P = 0.499$
CC genotype (n=215)	-0.65 (-1.76 - 0.47), $P = 0.256$	0.41 (-0.03 - 0.84), $P = 0.065$

Abbreviations: CI=confidence interval. β = Changes in folate/homocysteine (95% confidence intervals) from linear regression models.

Supplementary Table 6. Sensitivity analysis without multiple imputations of the outcomes for the associations of cord blood folate and homocysteine stratified for *MTHFR* C677T and A1298C with repeatedly measured eczema in childhood (N=1,643).

<i>MTHFR</i> genotype C677T (rs1801133)	OR's of eczema until 4 years (95% CI) ^a	
	Crude	Adjusted*
Folate change in SD (7.5 nmol/L)		
CC genotype (n=757)	0.86 (0.72-1.02)	0.80 (0.70-0.93)
TC genotype (n=709)	1.23 (1.06-1.44)	1.23 (1.07-1.41)
TT genotype (n=177)	1.51 (1.08-2.12)	1.45 (1.09-1.92)
	<i>P</i> -interaction = 0.003	<i>P</i> -interaction = 0.0002
<i>MTHFR</i> genotype A1298C (rs1801131)	OR's of eczema until 4 years (95% CI) ^a	
	Crude	Adjusted*
Homocysteine change in SD (2.9 umol/L)		
AA genotype (n=752)	1.30 (1.09-1.54)	1.31 (1.14-1.49)
CA genotype (n=716)	1.08 (0.92-1.28)	1.09 (0.96-1.23)
CC genotype (n=175)	0.82 (0.61-1.09)	0.63 (0.46-0.85)
	<i>P</i> -interaction = 0.031	<i>P</i> -interaction = 0.009

Abbreviations: OR=odds ratio; CI=confidence interval; SD=standard deviation. ^aOverall odds ratios (95% confidence intervals) from multiple imputation-based generalized estimating equation models. *Adjusted for maternal age, BMI, educational level at intake, history of maternal atopy or asthma, smoking and folic acid supplement use during pregnancy, parity, and children's sex, gestational age and birth weight. Folate, homocysteine and vitamin B12 levels were in one model.

Supplementary Table 7. Sensitivity analysis without multiple imputations of the outcome for the association of cord blood folate stratified for *MTHFR* C677T with eczema ever in childhood (N=1,445).

<i>MTHFR</i> genotype C677T (rs1801133)	OR's of eczema ever at 6 years (95% CI) ^a	
	Crude	Adjusted*
Folate change in SD (7.5 nmol/L)		
CC genotype (n=671)	0.90 (0.76-1.07)	0.88 (0.73-1.06)
TC genotype (n=619)	1.19 (1.00-1.42)	1.15 (0.96-1.39)
TT genotype (n=155)	1.49 (1.05-2.12)	1.37 (0.92-2.06)
	<i>P</i> -interaction = 0.015	<i>P</i> -interaction = 0.027

Abbreviations: OR=odds ratio; CI=confidence interval; SD=standard deviation. ^aOverall odds ratios (95% confidence intervals) from logistic regression models. *Adjusted for maternal age, BMI, educational level at intake, history of maternal atopy or asthma, smoking and folic acid supplement use during pregnancy, parity, and children's sex, gestational age and birth weight. Folate, homocysteine and vitamin B12 levels were in one model.