Prevalence of childhood wheeze and modified DNA methylation at 7 years of age according to maternal folate levels during pregnancy in the Hokkaido Study

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Abbreviations

β, regression coefficient; CI, confidence interval; FeNO, fractional exhaled nitric oxide; GSDMB, gasdermin B; IKZF3, Ikaros family zinc finger 3; IOR, inter-quartile range; ISAAC, the International Study of Asthma and Allergies in Childhood; LINE-1, long interspersed nuclear element-1; NTD, neural-tube defects; OR, Odds ratio; ORMDL3, orosomucoid-like 3; RSV, respiratory syncytial virus; SD, standard deviation: WHO, World Health Organization

Abstract

Background: A high dose of folic acid during pregnancy may increase the risk of asthma, wheezing, and respiratory disease in childhood. Folate acid can modify inflammation and immune susceptibility of offspring with some epigenetic differentiation, including DNA methylation. This study evaluated associations between maternal folate levels during pregnancy and childhood wheezing; furthermore, the study assessed whether maternal folate-modified DNA methylation is related to asthma. Methods: Participants in the current study were 6651 mother–child pairs who had complete data on characteristics and who had completed at least one of the International Study of Asthma and Allergies in Childhood questionnaires when the child was 1, 2, 4, and 7 years of age. Moreover, a case-control study to assess DNA methylation at 7 years of age was conducted among 136 children who experienced wheezing and a control group of 139 children with no history of allergies. Results: The median of maternal serum was 16.76 nmol/L, assayed by chemiluminescent immunoassay. We found significantly increased adjusted odds ratios of childhood wheezing at 2 years age according to maternal folate levels, compared with the lowest folate quartile (odds ratio [95% confidence interval] = highest; 1.27 [1.03, 1.56], and second, 1.27 [1.05, 1.55]), however, no changes were observed at 1, 4, and 7 years of age. In a case-control study, no association of maternal folate levels with DNA methylation was observed. Conclusion:

Our results suggest that maternal folate did not affect persistent wheezing in school-aged children, or DNA methylation of gasdermin B, orosomucoid-like 3, and Ikaros family zinc finger 3 at 7 years of age.

Key words: birth cohort, case-control, childhood wheeze, DNA methylation, maternal folate

1. Introduction

The World Health Organization (WHO) and most countries recommend a healthy diet plus a folic acid supplement of 400 μg/d from preconception until the end of the first trimester of pregnancy with the goal of neural-tube defects prevention.[1] However, human studies have reported that a higher maternal folate level during pregnancy may be associated with increased prevalence of childhood wheezing, asthma, and respiratory symptoms.[2-4] Maternal folate intake can modify inflammation and immune susceptibility of offspring with an epigenetic role involving DNA methylation.[5] Folate, as a methyl donor, changes DNA methylation, i.e., by the covalent addition of a methyl group to a cytosine residue at the CpG site in the DNA sequence. In vitro experiments have suggested that epigenetic modifications also contribute to the pathogenic mechanism underlying *17q21locus*, which is a potential gene related to asthma and harbors the adjacent genes *GSDMB* (gasdermin B)/*ORMDL3* (orosomucoid-like 3) and *IKZF3* (Ikaros family zinc finger 3). Recent studies have suggested that *GSDMB/ORMDL3* and *IKZF3* have a role in IL-6 and IL-8 levels,[6] viral respiratory infections,[7] and childhood asthma.[8] In a Swedish study, differential DNA methylation among CpG including *GSDMB/ORMDL3* and *IKZF3* was associated with a risk of childhood asthma and RNA expression.[9] However, no study has evaluated whether the difference in DNA methylation in *GSDMB*/*ORMDL3* and *IKZF3*

modifies the association between maternal folate level and childhood wheeze.

Participants of this study are from part of the Hokkaido Birth Cohort Study on Environment and Children's Health. The current study evaluates the associations between maternal folate levels during pregnancy and childhood wheezing; furthermore, this study assessed whether maternal folate levels modified DNA methylation of *GSDMB*, *ORMDL3*, and *IKZF3*, which are suggested to be related to childhood asthma.

2. Materials and Methods

2.1. Study participants and baseline study.

At the first trimester, participants completed the baseline questionnaire on maternal and paternal characteristics. Maternal peripheral blood samples were taken during the first-mid and third trimester in a non-fasting state during the participants' prenatal checkups. Medical birth records from the delivery hospital were collected for birth weight, height, sex, and other medical conditions.[10, 11]

The institutional ethics board for epidemiological studies at Hokkaido University Graduate School of Medicine and Hokkaido University Center for Environmental and Health Sciences approved the study protocol (approval number 69). In this study, informed consent was obtained from all study participants before enrollment.

2.2. Follow-up study until the age of 7.

Data was collected regarding wheezing symptoms of children at 1, 2, 4, and 7 years of age using a modified section of the Japanese version of the International Study of Asthma and

Allergies in Childhood (ISAAC) Phase Three questionnaire.[12] We defined childhood wheezing as a positive answer to the question "Has your child had wheezing or whistling in the chest in the past 12 months?," based on each ISAAC questionnaire administered when the child was 1, 2, 4, and 7 years of age (Figure 1). Moreover, we collected information on each child's history of infectious diseases.[13]

2.3. Selected participants in the prospective birth cohort study

A total of 19,176 pregnant women with maternal folate levels measured during pregnancy were assessed; we excluded 844 participants who had uncertain folate levels (Figure 1). At delivery, 13,899 participants, whose baseline questionnaire and maternal cotinine level data were available, had given live births. Of the 13,899 participants, we excluded 5,254 who had missing data on characteristics. Eventually, complete data on the pregnancy until delivery were obtained from 8,645 participants. Of these, we included 6,651 mothers who completed questionnaires on their children at age 1, 2, 4, or 7 years until December 2019 (Figure 1).

2.4. A case-control study of children 7 years of age

For the case-control study, we selected all 314 children who experienced wheezing and randomly selected 374 children without any allergic symptoms, such as wheezing, eczema, and rhino-conjunctivitis (extraction rate 24%) for controls based on responses from the ISAAC questionnaire filled out by children 7 years of age collected until September 2015. We sent a self-collection kit (The Oragene® OG-300 DNA Self-Collection kit (DNA Genotek Inc., Ottawa, Ontario, Canada)) to 688 children (cases and controls) via mail. Children provided their salivary samples (2 mL) at home, and a total of 278 self-collection kits were returned by the participants (response rate 40.4%). Of the 278 samples, three samples that had insufficient sample volumes for extracting DNA were excluded. Eventually, we used 275 salivary samples (cases: n = 136, controls: n = 139) to analyze DNA methylation (Figure 1).

2.5. Quantification of DNA Methylation

Genomic DNA was extracted from saliva using a Maxwell 16 DNA Purification Kit (Promega, Madison, WI, USA). The DNA (500 ng) was then subjected to bisulfite conversion using Epitect Plus Bisulfite Kit (Qiagen, Venlo, The Netherlands). Bisulfite pyro sequence was performed as described previously.[14, 15] We analyzed DNA methylation in genes related to asthma for five of the CpG sites of *IKZF3* (cg16293631, cg13432737), *ORMDL3* (cg02305874,

cg14647739), and *GSDMB* (cg12360886). Pyrosequencing was performed using Pyromark Q24 system (Qiagen) and data were analyzed using the Pyro Q-CpG Software (Qiagen). Eventually, we completed the quantification of DNA methylation only three CpG sites of *ORMDL3* (cg02305874), and *IKZF3* (cg16293631, cg13432737). Other PCR primers of two CpG sites including *GSDMB* (cg12360886) and *ORMDL3* (cg14647739) did not work. Conditions of primers are described in Supplementary Table 1. Average methylation levels of each CpG site that were analyzed in duplicate were used in statistical analyses.

2.6. Maternal folate and cotinine measurements

Maternal serum folate sampled during the first-mid trimester was quantified by direct chemiluminescent acridinium ester technology, which has an analytical sensitivity of 0.91 nmol/L. Specimen preparations, shipping, and assays were performed in batches, depending on new recruitments. We used cotinine level as a potential confounder because we have previously reported the significance of the inverse association between maternal folate and cotinine levels.[10, 16] Cotinine is the predominant metabolite of nicotine, the toxic chemical in tobacco products. Cotinine can be detected in biological specimens as a biomarker of exposure to tobacco smoking. Maternal plasma cotinine concentrations in the third trimester were used to quantitatively classify

maternal smoking status. The details of serum folate and plasma cotinine measurements are described in our previous report.[17]

2.7. Statistical analysis

We analyzed the association between participant characteristics and maternal folate level using the Mann–Whitney U-test and rank correlation test. For the logistic regression analysis, we used independent variables of maternal folate, adjusted factors, and dependent variables of childhood wheezing. Adjusted odds ratios (ORs) and 95% confidence intervals (CI) of childhood wheezing were evaluated according to the maternal folate level. Because a biologically relevant threshold of folate on wheezing has not been elucidated, we conducted two different logistic regression models. The first model used maternal folate categories of folate suboptimal (6.80–13.59 nmol/L), folate optimal (≥ 13.60 nmol/L), and folate deficiency (< 6.80 nmol/L) based on the WHO guideline.[18] The adjusted ORs and 95% CI of wheezing for folate suboptimal and optimal were calculated compared with folate deficiency (reference). The second model used four categorical maternal folate levels based on the distribution of their quartiles. The adjusted ORs and 95% CI of wheezing for the second, third, and highest quartile were calculated compared with the lowest folate quartile (reference). For the calculation of P for trend, we handled categorical

values of maternal folate as ordinal variables. We selected adjusted factors, such as maternal age, parity, delivery year, alcohol consumption during pregnancy, log10-transformed maternal cotinine level, maternal allergic history, paternal allergic history, annual household income, and sex of the child because these adjusted factors were used as potential confounders of the association between maternal folate and childhood wheezing in previous studies [3,4, 19-21]. Additionally, the selected adjusted factors were significantly associated with maternal folate levels, excluding maternal alcohol consumption during pregnancy (Table 1), as well as ORs of childhood wheezing (Tables 2 and 3), excluding delivery year in the current study.

For the case-control study, we used two types of maternal folate levels, including category of quartile, and two categories with reference to the WHO guideline, which were optimal (≥ 13.60 nmol/L) or under suboptimal (< 13.60 nmol/L) (Supplementary Table 2). In linear regression analysis, we evaluated β and 95% CI for DNA methylation, including three CpG sites of *IKZF3* (cg16293631, cg13432737) and *ORMDL3* (cg02305874), according to maternal folate levels. For the logistic regression analysis, we evaluated adjusted ORs and 95% CI of childhood wheezing at 7 years age according to DNA methylation.

P values of less than 0.05 were considered statistically significant. All statistical analyses were performed with SPSS software for Windows (version 21.0J; IBM, Armonk, NY, USA).

3. Results

3.1. Association of maternal folate levels and characteristics

In our current study, a total of 6651 participants who responded to at least one of the ISSAC questionnaires, when their child was 1, 2, 4, and 7 years of age, were included (Figure 1). The median (inter-quartile range [IQR]) folate level in the maternal serum was 16.76 nmol/L (13.36–21.97 nmol/L). Participants were grouped according to the WHO guidelines for folate deficiency as follows: folate deficient: n = 32 (0.48%); suboptimal: n = 1739 (26.15%); and optimal: n = 4880 (73.37%). Associations of maternal folate levels and characteristics are shown in Table 1.

3.2. Prospective birth cohort study of children 1, 2, 4, and 7 years of age

We found that the adjusted ORs of childhood wheezing at 2 years of age according to optimal maternal folate levels were increased [OR 95% CI = 3.60 (0.83, 15.53), $P = 0.086$], compared to those with deficient maternal folate levels (based on WHO guidelines), but the

203 difference was not statistically significant (Table 2). We found that the adjusted ORs of childhood
204 wheezing at 2 years of age according to maternal folate levels of the second quartiles [OR (95%
205 CI) = 1.27 (1.05, 1.55)] and highest quartiles [OR (95% CI) = 1.27 (1.03, 1.56)] were significantly
206 increased, compared to those of the lowest quartile, but not at 1, 4, and 7 years of age (Table 3).
207 Additionally, we found that higher maternal folate trended towards an increased OR of childhood
208 wheezing at 2 years of age, but this increase was not statistically significant (P for trend = 0.069)
209 (Table 3).
210
211 3.3. Case-control study at 7 years of age
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213 For the case-control study, mean ± standard deviation (SD) of DNA methylation at three
214 CpG sites: *ORMDL3* (cg02305874) and *IKZF3* (cg16293631, cg13432737) are illustrated in
215 Supplementary Table 3. Comparison of characteristics among 136 case children who experienced
216 wheezing and the 139 control children who had no allergies showed that only the frequencies of
217 maternal and paternal allergic history were significantly different for the case versus control
218 children (Supplementary Table 4). We found no significant adjusted β for DNA methylation
219 according to maternal folate levels (Table 4). We found that adjusted OR for childhood wheezing
220 at 7 years of age was significantly decreased with hypomethylation of *IKZF3* (cg16293631), but

not of *ORMDL3* (cg02305874) or *IKZF3* (cg13432737) (Supplementary Table 5). We found no significant adjusted OR for childhood wheezing at 7 years age was associated with maternal folate in the case-control study (Supplementary Table 5).

4. Discussion

In this prospective birth cohort study, higher maternal folate was associated with increased prevalence of childhood wheezing at 2 years of age, but not at 1, 4, and 7 years of age. In the case-control study, no significant association was observed between maternal folate and DNA methylation in the child's saliva. This study provided novel evidence that maternal folate does not affect persistent wheezing in school-aged children, and that maternal folate cannot modify DNA methylation at the three CpG sites of *IKZF3* and *ORMDL3* of 7-year old children. No association among children 1 year of age may be caused by a protective effect via transitional immunity from the mother. Our results suggest that children around 2 years of age may be vulnerable to maternal folate, because the transitional immunity begins to attenuate, and the child's immune function and respiratory structure is still immature.[22] Symptoms of childhood wheezing and asthma consisted of a combination of three phenotypes: transient early wheezes, non-atopic wheezes, and IgE-associated wheezes,[23] which were induced by multiple triggers, including a child's maturation status, genetic, pre/post-environment, and infections.[24] Predominant phenotypes among infants were transient early wheezing and non-atopic wheezing.[23] In fact, the prevalence of childhood wheezing at 2 years of age in the current study was higher than that observed for children at other ages (Table 1). Comparing the prevalence of childhood

243 wheezing with/without an infant's infections until 2 years of age, the prevalence of childhood
244 wheezing was approximately 2–3 fold higher with an infant's infections in the current study
245 (Supplementary Table 6). Among school-aged children, phenotypes, such as IgE-associated
246 wheezes, became more predominant, whereas other transient early wheezes and non-atopic
247 wheezes reduced, as the child matured and progressed; overall, stable allergic symptoms, such as
248 persistent wheezing appeared over time.[23] Therefore, our study suggests that maternal folate does
249 not affect persistent wheezing in school-aged children.

250 Median values of detected maternal serum folate levels in four previous human studies
251 were reported to be 21.5 nmol/L (9.5 ng/mL) in South Korea,[19] 21.2 nmol/L (9.36 ng/mL) in the
252 Netherlands[22] and 51.2 nmol/L (22.6 ng/mL)[3] and 43.5 nmol/L (19.2 ng/mL)[4] in the United States
253 of America. Although previous studies had various sample sizes and different timing of maternal
254 sample collection (first, second, or third trimester) with an outcome range of 1 to 7 years of age,
255 these studies reported diminished or insignificant association between maternal folate levels and
256 risk of wheezing, asthma, and respiratory symptoms in childhood. Additionally, more than 80%
257 of participants had optimal folate of maternal intracellular folate concentration, which is
258 recommended as a better biomarker for folate status than serum levels,[1] showing decreased
259 asthma risk at 6–7 years of age in a dose-dependent manner.[25] Inconsistently, in a Norwegian
260 study, higher maternal folate (median 8.7 nmol/L) levels increased the prevalence of respiratory

261 tract infections up to 18 months, asthma at 3 and 7 years of age.[20] The median maternal folate in
262 the current study was 16.7 nmol/L (7.40 ng/mL), which was lower than that in previous studies
263 reporting a protective or null effect [3,4,19,21] and higher than that in the Norwegian study reporting
264 an adverse effect.[20] These observations indicate that the adverse effect among the population is
265 reasonably due to lower maternal folate levels. Therefore, among the participants in our study, the
266 increased prevalence of wheezing at age 2 years associated with higher folate could be caused by
267 insufficient maternal folate levels. As a potential explanation, a nonlinear inverted-U shaped
268 relationship between folate and fractional exhaled nitric oxide (FeNO) was observed among
269 children, with an FeNO peak at a folate concentration of approximately 13 ng/mL, and a low
270 FeNO level on either side of the peak.[26] FeNO is a marker of eosinophilic airway inflammation
271 that is positively correlated with respiratory symptoms, including wheezing and asthma.[26] The
272 IQR of the folate level in this study (5.9–9.7 ng/ml) was comparable to the range of increasing
273 FeNO with higher folate levels, and an improvement of the folate status past the inverted U-
274 shaped peak may result in decreasing FeNO with higher folate levels. Considering that there was
275 no association between maternal folate level and persistent wheezing at age 7 years, and the
276 benefit of folate supplementation at preventing neural tube defects, our results do not support the
277 recommendation to reduce maternal folate intake.

278 In the case-control study, three CpG sites were not associated with maternal folate (Table

279 4). A previous systematic review specifies the necessity of detecting modified DNA methylation
280 change by assessing maternal folate levels.[27] This study is the first to report that individual DNA
281 methylation was detected at three CpG sites of *IKZF3* and *ORMDL3*-related asthma at 7 years of
282 age by pyrosequencing; and these findings did not differ according to maternal folate during
283 pregnancy. Only one study has described methylation beta-values of CpG sites related to asthma,
284 including *IKZF3* and *ORMDL3* using the Infinium 450 K assay; the results showed that asthmatic
285 children had lower methylation than the control children.[9] In this study, the same trend was
286 observed, i.e., inverse association between childhood wheezing and DNA methylation of *IKZF3*
287 (Supplementary Table 5). DNA hyper-methylation of long interspersed nucleotide element –1
288 (*LINE–1*) from the buccal cells of children 6–17 years age was associated with maternal intake of
289 methyl-donating nutrients, including folate, but not associated with quality of life measures
290 among children with asthma.[5] *LINE–1* is considered an indicator of global methylation; therefore,
291 dietary nutrients can affect not only *LINE–1,* but also a wide range differentiation DNA
292 methylation among several genes. No association between maternal folate and DNA methylation
293 at three CpG sites was observed; however, maternal folate and dietary nutrients during pregnancy
294 can modify DNA methylation. Further study is needed to examine the modified effects of global
295 epigenetic differentiation including the epigenome wide association study on the association
296 between folate levels and childhood wheezing and asthma.

297 The advantages of this study included the large-scale prospective birth cohort, which
298 helped to minimize bias in the data. Furthermore, the biological assessment of folate levels to
299 detect maternal folate in serum during pregnancy was performed. In addition, assessment of
300 wheezing symptoms using the ISAAC questionnaire, which is an international standard
301 questionnaire for the assessment of children's allergies, was undertaken. This study also had some
302 limitations. First, DNA methylation for only three CpG sites was analyzed, and therefore, global
303 epigenetic differentiation was not covered. Furthermore, the modified effect of DNA methylation
304 at age 2 was not evaluated, despite its significant association with maternal folate; however, DNA
305 methylation of children at 7 years of age is meaningful because allergic symptoms in school-aged
306 children often become stable and are generally predictive of future symptoms. Second,
307 categorizing maternal folate according to the WHO guidelines and using the small sample size of
308 the deficient group as the reference may lead to unstable statistical results. However, we
309 confirmed that the trend of results remained the same using the optimal folate group as reference.
310 Finally, in this study, maternal serum folate levels were measured at a single sampling point and
311 were not based on intracellular folate concentration; the estimated half-life of folate was assessed
312 at approximately 6 h.[18] This limitation can cause the misclassification of maternal folate levels.
313 However, a serum folate assay was commonly conducted in large epidemiological studies.[3, 20]
314 Moreover, studies in Japan have reported that maternal diets were generally well-maintained

during pregnancy,[28] thereby providing relatively stable maternal folate levels.

5. Conclusion

In this prospective birth cohort study, higher maternal folate was associated with increased prevalence of childhood wheezing at 2 years of age, which appeared to be caused by insufficient maternal folate among our participants. There was no association between maternal folate and wheezing at 7 years of age, suggesting that maternal folate does not affect persistent wheezing in school-aged children. Moreover, this study is the first to detect individual DNA methylation at three CpG sites, *IKZF3* (cg16293631, cg13432737) and *ORMDL3* (cg02305874), related to asthma at 7 years of age by pyrosequencing; furthermore these findings did not differ according to maternal folate levels during pregnancy. Future research is needed to elucidate the mechanism of wheezing and asthma development. Studies that examine the mediation of epigenomic changes that provide appropriate maternal folate status for the prevention of childhood wheezing and other respiratory symptoms among pre-school and school-aged children are warranted.

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Key Message

In this prospective birth cohort study, higher maternal folate was associated with increased prevalence of childhood wheezing at 2 years of age, which appeared to be caused by insufficient maternal folate among our participants. There was no association between maternal folate and wheezing at 7 years of age, suggesting that maternal folate does not affect persistent wheezing in school-aged children. Moreover, this study is the first that detected individual DNA methylation at three CpG sites of *IKZF3* (cg16293631, cg13432737) and *ORMDL3* (cg02305874) related to asthma at 7 years of age by pyrosequencing and these findings did not differ according to maternal folate level during pregnancy.

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420 without nausea and vomiting. J Nutr Sci. 2016; 5.

422 Table 1: Characteristics and associations with maternal folate levels (n = 6651).

	Categories	Number (%)	Mean ± SD or median (IQR)	Maternal folate levels (median (IQR)	r
Mother					
Age (years)			30.6 ± 4.5		0.149**
Parity	Primipara	2909 (43.7)		16.76 (13.59, 21.97)***	
	Multipara	3742 (56.3)		16.99 (13.59, 22.88)	
Pre-pregnancy weight (kg)			53.0 ± 8.7		–0.021
Height (cm)			158.2 ± 5.3		–0.012
Delivery year (2003–2012)			2007 (2005, 2010)		0.178***
Educational level	≤ 12	3066 (46.1)		15.86 (12.91, 20.61)***	
	> 12	3585 (53.9)		17.67 (14.04, 23.10)	

Allergic history	Never	4348 (65.4)		16.65 (13.36, 21.74)*	
	Ever	2303 (34.6)		16.99 (13.59, 22.20)	
Alcohol consumption during the first trimester	No	5844 (87.9)		16.76 (13.36, 21.97)	
	Yes	807 (12.1)		16.76 (13.82, 21.29)	
Folic acid supplements use during first trimester	No	5091 (76.5)		15.63 (12.91, 19.48)***	
	Yes	1560 (23.5)		24.01 (18.12, 31.71)	
Plasma cotinine level (nmol/L) during third trimester			0.27 (0.06–0.90)		–0.202***
Father					
Age (years)			32.1 ± 5.3		0.114**
Educational level	≤ 12	3197 (48.1)		16.08 (12.91, 20.84)***	

	> 12	3454 (51.9)	17.44 (14.04, 23.33)	
Allergic history	Never	5098 (76.7)	16.53 (13.36, 21.74)**	
	Ever	1553 (23.3)	17.21 (13.82, 22.42)	
Annual household income (million Japanese Yen)	< 5	4487 (67.5)	16.31 (13.14, 21.29)***	
	≥ 5	2164 (32.5)	17.89 (14.04, 23.56)	
Child				
Sex	Boy	3322 (49.9)	16.76 (13.36–21.97)	
	Girl	3329 (50.1)	16.53 (13.36–21.74)	
Gestational age (weeks)			38.9 ± 1.3	–0.028*
Birth weight (g)			3071.7 ± 386.1	–0.016

1 year with wheezing	No	5322 (87.9)	16.76 (13.59–22.20)
	Yes	732 (12.1)	16.53 (13.59–21.97)
2 years with wheezing	No	4368 (80.1)	16.99 (13.59–22.20)
	Yes	1087 (19.9)	16.99 (13.59–22.20)
4 years with wheezing	No	3631 (81.2)	17.21 (13.82–22.88)
	Yes	838 (18.8)	16.76 (13.76–22.42)
7 years with wheezing	No	3544 (88.4)	17.44 (13.82–22.88)
	Yes	466 (11.6)	16.76 (13.82–22.48)

SD, standard deviation. IQR, inter-quartile range. r, Spearman's rank correlation coefficient. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ by Mann–Whitney U-test and Spearman's rank correlation test. For the Spearman's rank correlation test, maternal folate levels were significantly correlated with continuous variables of maternal age, delivery year, cotinine level, paternal age, and gestational age. For the Mann–Whitney U-test, maternal folate levels were significantly different according to categorical

variables of maternal parity, folic acid supplements use, educational level, allergic history, and annual household income.

430 Table 2: OR for childhood wheezing, according to maternal folate levels (based on the WHO guidelines) during pregnancy

	Childhood wheezing at 1 year of age		Childhood wheezing at 2 years of age		Childhood wheezing at 4 years of age		Childhood wheezing at 7 years of age	
	OR 95%CI	$P_{for\ trend}$	OR 95%CI	$P_{for\ trend}$	OR 95%CI	$P_{for\ trend}$	OR 95%CI	$P_{for\ trend}$
Maternal folate levels based on WHO guidelines								
Deficient	Reference	0.648	Reference	0.151	Reference	0.866	Reference	0.818
Suboptimal	0.76 (0.28, 2.05)		3.32 (0.77, 14.38)		0.83 (0.30, 2.32)		0.78 (0.22, 2.80)	
Optimal	0.81 (0.30, 2.16)		3.60 (0.83, 15.53)†		0.85 (0.31, 2.36)		0.82 (0.23, 2.89)	
Adjusted factors								
Maternal age (continuous)	0.98 (0.96, 0.99)*		0.97 (0.95, 0.98)**		0.97 (0.95, 0.99)*		0.97 (0.95, 0.99)*	
Multipara (reference: primipara)	1.99 (1.67, 2.36)**		1.69 (1.47, 1.96)**		0.97 (0.83, 1.13)		1.02 (0.83, 1.24)	
Delivery year (order: from 2003 to 2012)	1.02 (0.99, 1.06)		1.00 (0.97, 1.03)		1.00 (0.97, 1.03)		0.99 (0.95, 1.03)	
Annual household income > 12 (reference: ≤ 12)	0.97 (0.82, 1.16)		1.10 (0.95, 1.27)		0.99 (0.84, 1.17)		0.76 (0.61, 0.95)*	
Female child (reference: male child)	0.62 (0.53, 0.72)**		0.69 (0.61, 0.79)**		0.77 (0.66, 0.90)*		0.69 (0.57, 0.84)**	

Maternal plasma cotinine level during third trimester (continuous)	1.13 (1.05, 1.23)*	1.17 (1.09, 1.25)**	1.11 (1.02, 1.21)*	1.07 (0.96, 1.19)
Maternal alcohol consumption during first trimester (reference: no)	1.19 (0.94, 1.49)	1.12 (0.92, 1.37)	1.27 (1.02, 1.59)*	1.06 (0.80, 1.42)
Maternal allergy history (reference: no)	1.25 (1.07, 1.47)*	1.30 (1.13, 1.50)**	1.60 (1.37, 1.87)**	1.94 (1.59, 2.36)**
Paternal allergy history (reference: no)	1.26 (1.06, 1.51)*	1.35 (1.16, 1.58)**	1.35 (1.14, 1.60)**	1.44 (1.17, 1.79)**

OR: odds ratio, was calculated by adjustments to each independent variable including maternal folate and adjusted factors (maternal age, parity, annual household income, delivery year, cotinine levels during third trimester (log10), maternal allergic history, paternal allergic history, and sex of the child) in the logistic regression analysis. Folate levels according to the WHO guidelines (Sauberlich 1999): Deficient (< 6.80 nmol/L); Suboptimal (6.80–13.59 nmol/L); Optimal (≥ 13.60 nmol/L). † $P < 0.01$, *$P < 0.05$, **$P < 0.01$

436 Table 3: OR for childhood wheezing, according to quartile maternal folate levels during pregnancy

	Childhood wheezing at 1 year of age		Childhood wheezing at 2 years of age		Childhood wheezing at 4 years of age		Childhood wheezing at 7 years of age	
	OR 95%CI	$P_{for\ trend}$	OR 95%CI	$P_{for\ trend}$	OR 95%CI	$P_{for\ trend}$	OR 95%CI	$P_{for\ trend}$
Maternal folate levels of quartiles								
First quartile	Reference	0.486	Reference	0.069	Reference	0.744	Reference	0.827
Second quartile	1.08 (0.87, 1.35)		1.27 (1.05, 1.55)*		1.09 (0.88, 1.36)		1.22 (0.92, 1.62)	
Third quartile	1.00 (0.79, 1.26)		1.17 (0.96, 1.43)		0.95 (0.75, 1.19)		1.02 (0.76, 1.38)	
Fourth quartile	1.13 (0.89, 1.42)		1.27 (1.03, 1.56)*		1.01 (0.81, 1.27)		1.04 (0.77, 1.40)	
Adjusted factors								
Maternal age (continuous)	0.98 (0.96, 0.99)*		0.97 (0.95, 0.98)**		0.97 (0.95, 0.99)*		0.97 (0.95, 0.99)*	
Multipara (reference: primipara)	2.00 (1.68, 2.37)**		1.70 (1.47, 1.96)**		0.96 (0.82, 1.13)		1.01 (0.82, 1.23)	
Delivery year (order: from 2003 to 2012)	1.02 (0.99, 1.05)		1.00 (0.97, 1.03)		1.00 (0.97, 1.03)		0.99 (0.95, 1.03)	
Annual household income > 12 (reference: ≤ 12)	0.97 (0.82, 1.16)		1.09 (0.94, 1.27)		0.99 (0.84, 1.17)		0.77 (0.62, 0.96)*	
Female child (reference: male child)	0.62 (0.53, 0.72)**		0.69 (0.61, 0.80)**		0.77 (0.66, 0.90)*		0.69 (0.57, 0.84)**	

Maternal plasma cotinine level during third trimester (continuous)	1.13 (1.05, 1.23)*	1.17 (1.09, 1.26)**	1.11 (1.02, 1.20)*	1.07 (0.96, 1.19)
Maternal alcohol consumption during the first trimester (reference: no)	1.19 (0.94, 1.49)	1.12 (0.92, 1.37)	1.28 (1.02, 1.59)*	1.06 (0.79, 1.42)
Maternal allergy history (reference: no)	1.25 (1.06, 1.47)*	1.30 (1.13, 1.49)**	1.59 (1.36, 1.86)**	1.93 (1.58, 2.35)**
Paternal allergy history (reference: no)	1.27 (1.06, 1.52)*	1.35 (1.15, 1.57)**	1.35 (1.14, 1.60)**	1.45 (1.17, 1.80)**

437

438 OR: odds ratio, was calculated by adjustments to each independent variable, including maternal folate and adjusted factors (maternal age, parity, annual

439 household income, delivery year, cotinine levels during third trimester (log10), maternal allergic history, paternal allergic history, and sex of the child) in the

440 logistic regression analysis. P for trend indicates order throughout quartile maternal folate levels. Folate levels of quartiles: First quartile (≤ 13.82 nmol/L);

441 Second quartile (14.04–17.21 nmol/L); Third quartile (17.44–22.42 nmol/L); Fourth quartile (≥ 22.65 nmol/L). Maternal plasma cotinine level during the

442 third trimester was continuous: nmol/L of log10 translated. CI, confidence interval. †P < 0.01, *P < 0.05, **P < 0.01

443 Table 4: Beta values for DNA methylation of children at 7 years of age, according to maternal folate levels during pregnancy in the case-control study.

	ORMDL3 (cg02305874)	*IKZF3* (cg13432737)	*IKZF3* (cg16293631)
	β 95% CI	β 95% CI	β 95% CI
Folate levels of WHO guideline			
Under suboptimal	Reference	Reference	Reference
Optimal	–0.22 (–1.05, 0.61)	0.03 (–0.50, 0.57)	0.03 (–0.50, 0.56)
Folate levels of quartiles			
First quartile	Reference	Reference	Reference
Second quartile	–0.72 (–1.68, 0.25)	–0.08 (–0.68, 0.52)	–0.03 (–0.62, 0.56)
Third quartile	0.48 (–0.52, 1.47)	0.35 (–0.30, 1.00)	0.36 (–0.28, 1.00)
Fourth quartile	0.01 (–1.20, 1.23)	0.30 (–0.51, 1.10)	–0.36 (–1.15, 0.44)

P for trend	0.452	0.959	0.975

β; regression coefficient calculated using linear regression was adjusted for maternal age, parity, annual household income, delivery year, cotinine levels during third trimester (log10), maternal allergic history, paternal allergic history, and sex of the child.

Folate levels according to WHO guidelines (Sauberlich 1999): Under suboptimal (< 13.59 nmol/L); Optimal (≥ 13.60 nmol/L). Folate levels of quartiles: First quartile (≤ 13.82 nmol/L); Second quartile (14.04–17.21 nmol/L); Third quartile (17.44–22.42 nmol/L); Fourth quartile (≥ 22.65 nmol/L). CI, confidence interval

Figure 1: Flowchart for the selection of participants up to 7 years of age and selected participants in the case-control study on childhood wheezing and DNA methylation at 7 years of age.