



Title	Prevalence of childhood wheeze and modified DNA methylation at 7 years of age according to maternal folate levels during pregnancy in the Hokkaido Study
Author(s)	Miyashita, Chihiro; Araki, Atsuko; Miura, Ryu et al.
Citation	Pediatric Allergy and Immunology, 32(3), 514-523 https://doi.org/10.1111/pai.13425
Issue Date	2021-04
Doc URL	https://hdl.handle.net/2115/87340
Rights	This is the peer reviewed version of the following article: Miyashita, C, Araki, A, Miura, R, et al. Prevalence of childhood wheeze and modified DNA methylation at 7 years of age according to maternal folate levels during pregnancy in the Hokkaido Study. <i>Pediatr Allergy Immunol.</i> 2021; 32: 514- 523, which has been published in final form at https://doi.org/10.1111/pai.13425 . This article may be used for non-commercial purposes in accordance with Wiley Terms and Conditions for Use of Self-Archived Versions.
Type	journal article
File Information	10_Pediatric Allergy and Immunology, 2021.pdf



1 **Prevalence of childhood wheeze and modified DNA methylation at 7 years of age**

2 **according to maternal folate levels during pregnancy in the Hokkaido Study**

3

4 Chihiro Miyashita^a, Atsuko Araki^a, Ryu Miura^a, Yu Ait Bamai^a, Sumitaka Kobayashi^a, Sachiko

5 Itoh^a, Kumiko Ito^{a,b}, Meng-Shan Tsai^{a,c}, Reiko Kishi^{a*}

6

7 ^a Center for Environmental and Health Sciences, Hokkaido University, Sapporo, Japan

8 ^b Department of Nursing, Faculty of Health Science, Hokkaido University of Science, Sapporo,

9 Japan

10 ^c Institute of Occupational Medicine and Industrial Hygiene, National Taiwan University,

11 Taipei 10055, Taiwan

12

13 *Corresponding author:

14 Reiko Kishi

15 Center for Environmental and Health Sciences, Hokkaido University, North 12 West 7 Kita-ku,

16 Sapporo, 060-0812, Japan

17 Tel.: +81-11-706-4746, Fax: +81-(0)11-706-4725

18 E-mail: rkishi@med.hokudai.ac.jp

19

20 Running title: Maternal folate and childhood wheeze

21

22 Abbreviations

23 β , regression coefficient; CI, confidence interval; FeNO, fractional exhaled nitric oxide; GSDMB,

24 gasdermin B; IKZF3, Ikaros family zinc finger 3; IQR, inter-quartile range; ISAAC, the

25 International Study of Asthma and Allergies in Childhood; LINE-1, long interspersed nuclear

26 element-1; NTD, neural-tube defects; OR, Odds ratio; ORMDL3, orosomucoid-like 3; RSV,

27 respiratory syncytial virus; SD, standard deviation: WHO, World Health Organization

28

29 **Abstract**

30

31 Background: A high dose of folic acid during pregnancy may increase the risk of asthma,
32 wheezing, and respiratory disease in childhood. Folate acid can modify inflammation and immune
33 susceptibility of offspring with some epigenetic differentiation, including DNA methylation. This
34 study evaluated associations between maternal folate levels during pregnancy and childhood
35 wheezing; furthermore, the study assessed whether maternal folate-modified DNA methylation is
36 related to asthma. Methods: Participants in the current study were 6651 mother–child pairs who
37 had complete data on characteristics and who had completed at least one of the International Study
38 of Asthma and Allergies in Childhood questionnaires when the child was 1, 2, 4, and 7 years of
39 age. Moreover, a case-control study to assess DNA methylation at 7 years of age was conducted
40 among 136 children who experienced wheezing and a control group of 139 children with no
41 history of allergies. Results: The median of maternal serum was 16.76 nmol/L, assayed by
42 chemiluminescent immunoassay. We found significantly increased adjusted odds ratios of
43 childhood wheezing at 2 years age according to maternal folate levels, compared with the lowest
44 folate quartile (odds ratio [95% confidence interval] = highest; 1.27 [1.03, 1.56], and second, 1.27
45 [1.05, 1.55]), however, no changes were observed at 1, 4, and 7 years of age. In a case-control
46 study, no association of maternal folate levels with DNA methylation was observed. Conclusion:

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

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

51

52 **1. Introduction**

53

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

70 modifies the association between maternal folate level and childhood wheeze.

71 Participants of this study are from part of the Hokkaido Birth Cohort Study on

72 Environment and Children's Health. The current study evaluates the associations between

73 maternal folate levels during pregnancy and childhood wheezing; furthermore, this study assessed

74 whether maternal folate levels modified DNA methylation of *GSDMB*, *ORMDL3*, and *IKZF3*,

75 which are suggested to be related to childhood asthma.

76

77 **2. Materials and Methods**

78

79 2.1. Study participants and baseline study.

80

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

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

90

91 2.2. Follow-up study until the age of 7.

92

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

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

100

101 2.3. Selected participants in the prospective birth cohort study

102

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

110

111 2.4. A case-control study of children 7 years of age

112

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

123

124 2.5. Quantification of DNA Methylation

125

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

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

138

139 2.6. Maternal folate and cotinine measurements

140

141 Maternal serum folate sampled during the first-mid trimester was quantified by direct
142 chemiluminescent acridinium ester technology, which has an analytical sensitivity of 0.91 nmol/L.
143 Specimen preparations, shipping, and assays were performed in batches, depending on new
144 recruitments. We used cotinine level as a potential confounder because we have previously
145 reported the significance of the inverse association between maternal folate and cotinine levels.^{10,}

146 ¹⁶ Cotinine is the predominant metabolite of nicotine, the toxic chemical in tobacco products.

147 Cotinine can be detected in biological specimens as a biomarker of exposure to tobacco smoking.

148 Maternal plasma cotinine concentrations in the third trimester were used to quantitatively classify

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

151

152 2.7. Statistical analysis

153

154 We analyzed the association between participant characteristics and maternal folate
155 level using the Mann–Whitney U-test and rank correlation test. For the logistic regression analysis,
156 we used independent variables of maternal folate, adjusted factors, and dependent variables of
157 childhood wheezing. Adjusted odds ratios (ORs) and 95% confidence intervals (CI) of childhood
158 wheezing were evaluated according to the maternal folate level. Because a biologically relevant
159 threshold of folate on wheezing has not been elucidated, we conducted two different logistic
160 regression models. The first model used maternal folate categories of folate suboptimal (6.80–
161 13.59 nmol/L), folate optimal (≥ 13.60 nmol/L), and folate deficiency (< 6.80 nmol/L) based on
162 the WHO guideline.¹⁸ The adjusted ORs and 95% CI of wheezing for folate suboptimal and
163 optimal were calculated compared with folate deficiency (reference). The second model used four
164 categorical maternal folate levels based on the distribution of their quartiles. The adjusted ORs
165 and 95% CI of wheezing for the second, third, and highest quartile were calculated compared with
166 the lowest folate quartile (reference). For the calculation of P for trend, we handled categorical

167 values of maternal folate as ordinal variables. We selected adjusted factors, such as maternal age,
168 parity, delivery year, alcohol consumption during pregnancy, log10-transformed maternal cotinine
169 level, maternal allergic history, paternal allergic history, annual household income, and sex of the
170 child because these adjusted factors were used as potential confounders of the association between
171 maternal folate and childhood wheezing in previous studies^{3,4, 19-21}. Additionally, the selected
172 adjusted factors were significantly associated with maternal folate levels, excluding maternal
173 alcohol consumption during pregnancy (Table 1), as well as ORs of childhood wheezing (Tables
174 2 and 3), excluding delivery year in the current study.

175 For the case-control study, we used two types of maternal folate levels, including
176 category of quartile, and two categories with reference to the WHO guideline, which were optimal
177 (≥ 13.60 nmol/L) or under suboptimal (< 13.60 nmol/L) (Supplementary Table 2). In linear
178 regression analysis, we evaluated β and 95% CI for DNA methylation, including three CpG sites
179 of *IKZF3* (cg16293631, cg13432737) and *ORMDL3* (cg02305874), according to maternal folate
180 levels. For the logistic regression analysis, we evaluated adjusted ORs and 95% CI of childhood
181 wheezing at 7 years age according to DNA methylation.

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

185

186 **3. Results**

187

188 3.1. Association of maternal folate levels and characteristics

189

190 In our current study, a total of 6651 participants who responded to at least one of the
191 ISSAC questionnaires, when their child was 1, 2, 4, and 7 years of age, were included (Figure 1).
192 The median (inter-quartile range [IQR]) folate level in the maternal serum was 16.76 nmol/L
193 (13.36–21.97 nmol/L). Participants were grouped according to the WHO guidelines for folate
194 deficiency as follows: folate deficient: n = 32 (0.48%); suboptimal: n = 1739 (26.15%); and
195 optimal: n = 4880 (73.37%). Associations of maternal folate levels and characteristics are shown
196 in Table 1.

197

198 3.2. Prospective birth cohort study of children 1, 2, 4, and 7 years of age

199

200 We found that the adjusted ORs of childhood wheezing at 2 years of age according to
201 optimal maternal folate levels were increased [OR 95% CI = 3.60 (0.83, 15.53), $P = 0.086$],
202 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

212

213 For the case-control study, mean \pm 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

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

224

225 **4. Discussion**

226

227 In this prospective birth cohort study, higher maternal folate was associated with
228 increased prevalence of childhood wheezing at 2 years of age, but not at 1, 4, and 7 years of age.

229 In the case-control study, no significant association was observed between maternal folate and
230 DNA methylation in the child's saliva. This study provided novel evidence that maternal folate
231 does not affect persistent wheezing in school-aged children, and that maternal folate cannot
232 modify DNA methylation at the three CpG sites of *IKZF3* and *ORMDL3* of 7-year old children.

233 No association among children 1 year of age may be caused by a protective effect via transitional
234 immunity from the mother. Our results suggest that children around 2 years of age may be
235 vulnerable to maternal folate, because the transitional immunity begins to attenuate, and the
236 child's immune function and respiratory structure is still immature.²² Symptoms of childhood
237 wheezing and asthma consisted of a combination of three phenotypes: transient early wheezes,
238 non-atopic wheezes, and IgE-associated wheezes,²³ which were induced by multiple triggers,
239 including a child's maturation status, genetic, pre/post-environment, and infections.²⁴

240 Predominant phenotypes among infants were transient early wheezing and non-atopic wheezing.²³

241 In fact, the prevalence of childhood wheezing at 2 years of age in the current study was higher
242 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 Netherlands²² 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

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

316

317 **5. Conclusion**

318

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

331

332 **Funding**

333 The authors acknowledge all the participants and staff in this study.

334 This work was supported by a Grant-in-Aid for Health Science Research from the Japanese

335 Ministry of Health, Labor and Welfare (No. 201624002B; 17932352), a Grant-in-Aid for

336 Scientific Research from the Japanese Ministry of Education, Culture, Sports, Science and

337 Technology (No. 25860433; 16H02645; 16K19245; 19H01071).

338

339 **Key Message**

340 In this prospective birth cohort study, higher maternal folate was associated with increased

341 prevalence of childhood wheezing at 2 years of age, which appeared to be caused by insufficient

342 maternal folate among our participants. There was no association between maternal folate and

343 wheezing at 7 years of age, suggesting that maternal folate does not affect persistent wheezing in

344 school-aged children. Moreover, this study is the first that detected individual DNA methylation

345 at three CpG sites of *IKZF3* (cg16293631, cg13432737) and *ORMDL3* (cg02305874) related to

346 asthma at 7 years of age by pyrosequencing and these findings did not differ according to maternal

347 folate level during pregnancy.

348

349 **References**

- 350 1. World Health Organization (WHO) – 2012. Guideline: daily iron and folic acid
351 supplementation in pregnant women. ISBN-13: 978-924-150199-6.
352 <https://www.ncbi.nlm.nih.gov/books/NBK132263/>
- 353 2. Veeranki SP, Gebretsadik T, Dorris SL, et al. Association of folic acid supplementation
354 during pregnancy and infant bronchiolitis. *AmJ Epidemiol* 2014;179:938-946.
- 355 3. Roy A, Kocak M, Hartman TJ et al. Association of prenatal folate status with early
356 childhood wheeze and atopic dermatitis. *Pediatr Allerg Immunol* 2018;29:144-150.
- 357 4. Vereen S, Gebretsadik T, Johnson N et al. Association between maternal 2nd trimester
358 plasma folate levels and infant bronchiolitis'. *Matern Child Health J* 2018;23:164-172.
- 359 5. Montrose L, Ward TJ, Semmens EO et al. Dietary intake is associated with respiratory
360 health outcomes and DNA methylation in children with asthma. *Allergy Asthma Clin*
361 *Immunol.* 2017;13:12.
- 362 6. Wu XL, Li R, Zhang HW et al. Methylation status of ORMDL3 regulates cytokine
363 production and p-ERK/MMP9 pathway expression. *Exp Cell Res.* 2018;372:43-51.
- 364 7. Bouzigon E, Corda E, Aschard H, et al. Effect of 17q21 variants and smoking exposure
365 in early-onset asthma. *New Engl J Med* 2008;359:1985-1994.
- 366 8. Breslow DK, Collins SR, Bodenmiller B et al. Orm family proteins mediate sphingolipid

367 homeostasis. *Nature*. 2010;463:1048-1053.

368 9. Acevedo N, Reinius LE, Greco D et al. Risk of childhood asthma is associated with
369 CpG-site polymorphisms, regional DNA methylation and mRNA levels at the
370 GSDMB/ORMDL3 locus', *Human Mol Gen* 2015;24:875-890.

371 10. Kishi R, Kobayashi S, Ikeno T, et al. Ten years of progress in the Hokkaido birth cohort
372 study on environment and children's health: cohort profile--updated 2013. *Environ*
373 *Health Prev Med*. 2013;18:429-450.

374 11. Kishi R, Araki A, Minatoya M, et al. The Hokkaido birth cohort study on environment
375 and children's health: cohort profile--updated 2017 *Environ Health Prev Med*.2017;22:46

376 12. Asher MI, Keil U, Anderson HR et al. International study of asthma and allergies in
377 childhood (Isaac) - rationale and methods. *Eur Respir J*. 1995;8:483-491.

378 13. Goudarzi H, Konno S, Kimura H, et al. Contrasting associations of maternal smoking
379 and pre-pregnancy BMI with wheeze and eczema in children. *Sci Total Environ*
380 2018;639: 1601-1609.

381 14. Murphy SK, Adigun A, Huang Z et al. Gender-specific methylation differences in
382 relation to prenatal exposure to cigarette smoke. *Gene*. 2012;494:36-43.

383 15. Bollati V, Baccarelli A, Hou L, et al. Changes in DNA methylation patterns in subjects
384 exposed to low-dose benzene. *Cancer Res* 2007;67:876-880.

- 385 16. Yila TA, Araki A, Sasaki S, et al. Predictors of folate status among pregnant Japanese
386 women: the Hokkaido study on environment and children's health, 2002-2012. *Br J Nutr*
387 2016;115:2227-2235.
- 388 17. Sasaki S, Braimoh TS, Yila TA, et al. Self-reported tobacco smoke exposure and plasma
389 cotinine levels during pregnancy--a validation study in Northern Japan. *Sci Total*
390 *Environ* 2011;412-413:114-118.
- 391 18. Sauberlich HE. Folate (FA, pteroylmonoglutamic acid, folacin). In: *Laboratory tests for*
392 *the assessment of nutritional status*, 2nd ed., [I Wolinsky, editor]. Washington, DC: CRC
393 Press; 1999. 103–134 p
- 394 19. Kim JH, Jeong KS, Ha EH, et al. Relationship between prenatal and postnatal exposures
395 to folate and risks of allergic and respiratory diseases in early childhood. *Pediatr*
396 *Pulmonol* 2015;50:155-163.
- 397 20. Parr CL, Magnus MC, Karlstad Ø, et al. Maternal folate intake during pregnancy and
398 childhood asthma in a population-based cohort. *Am J Respir Crit Care Med*
399 2017;195:221-228.
- 400 21. van der Valk RJ, Kiefte-de Jong JC, Sonnenschein-van der Voort AM, et al. Neonatal
401 folate, homocysteine, vitamin B12 levels and methylenetetrahydrofolate reductase
402 variants in childhood asthma and eczema. *Allergy* 2013;68:788-795.

- 403 22. Goenka A, Kollmann TR. Development of immunity in early life. *J Infect* 2015; 71:
404 S112-S20.
- 405 23. Stein RT, Holberg CJ, Morgan WJ, et al. Peak flow variability, methacholine
406 responsiveness and atopy as markers for detecting different wheezing phenotypes in
407 childhood. *Thorax* 1007;52:946-952.
- 408 24. Brand PL, Baraldi E, Bisgaard H, et al. Definition, assessment and treatment of
409 wheezing disorders in preschool children: an evidence-based approach. *Eur Respir J*
410 2008; 32:1096-110.
- 411 25. Magdelijns FJ, Mommers M, Penders J, et al. Folic acid use in pregnancy and the
412 development of atopy, asthma, and lung function in childhood. *Pediatrics*
413 2011;128:e135-e144.
- 414 26. Lin JH, Matsui W, Aloe C, et al. Relationships between folate and inflammatory features
415 of asthma. *J Allergy Clin Immunol* 2013;131:918-920.
- 416 27. Brown SB, Reeves KW, Bertone-Johnson ER. Maternal folate exposure in pregnancy
417 and childhood asthma and allergy: a systematic review. *Nutr Rev* 2014;72:55-64.
- 418 28. Jwa SC, Ogawa K, Kobayashi M et al. Validation of a food-frequency questionnaire for
419 assessing vitamin intake of Japanese women in early and late pregnancy with and
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)***	

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

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)

423 SD, standard deviation. IQR, inter-quartile range. r , Spearman's rank correlation coefficient. $*P < 0.05$,
424 $**P < 0.01$, and $***P < 0.001$ by Mann–Whitney U-test and Spearman's rank correlation test. For the
425 Spearman's rank correlation test, maternal folate levels were significantly correlated with continuous
426 variables of maternal age, delivery year, cotinine level, paternal age, and gestational age. For the
427 Mann–Whitney U-test, maternal folate levels were significantly different according to categorical

428 variables of maternal parity, folic acid supplements use, educational level, allergic history, and annual

429 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	P for trend	OR	P for trend	OR	P for trend	OR	P for trend
	95%CI		95%CI		95%CI		95%CI	
Maternal folate levels based on WHO guidelines								
Deficient	Reference	0.64 8	Reference	0.15 1	Reference	0.86 6	Reference	0.81 8
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)**

431

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

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

434 logistic regression analysis. Folate levels according to the WHO guidelines (Sauberlich 1999): Deficient (< 6.80 nmol/L); Suboptimal (6.80–13.59 nmol/L);

435 Optimal (\geq 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	0.48 6	Reference	0.06 9	Reference	0.74 4	Reference	0.82 7
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	1.13 (1.05, 1.23)*	1.17 (1.09, 1.26)**	1.11 (1.02, 1.20)*	1.07 (0.96, 1.19)
(continuous)				
Maternal alcohol consumption				
during the first trimester	1.19 (0.94, 1.49)	1.12 (0.92, 1.37)	1.28 (1.02, 1.59)*	1.06 (0.79, 1.42)
(reference: no)				
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.

	<i>ORMDL3</i> (cg02305874)	<i>IKZF3</i> (cg13432737)	<i>IKZF3</i> (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
-------------	-------	-------	-------

444 β ; regression coefficient calculated using linear regression was adjusted for maternal age, parity, annual household income, delivery year, cotinine levels

445 during third trimester (log10), maternal allergic history, paternal allergic history, and sex of the child.

446 Folate levels according to WHO guidelines (Sauberlich 1999): Under suboptimal (< 13.59 nmol/L); Optimal (\geq 13.60 nmol/L). Folate levels of quartiles: First

447 quartile (\leq 13.82 nmol/L); Second quartile (14.04–17.21 nmol/L); Third quartile (17.44–22.42 nmol/L); Fourth quartile (\geq 22.65 nmol/L). CI, confidence

448 interval

449

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

