DNA Hypomethylation at *ALOX12* Is Associated with Persistent Wheezing in Childhood

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***Rationale*: Epigenetic changes may play a role in the occurrence of asthma-related phenotypes.**

***Objectives*: To identify epigenetic marks in terms of DNA methylation of asthma-related phenotypes in childhood, and to assess the effect of prenatal exposures and genetic variation on these epigenetic marks.**

***Methods*: Data came from two cohorts embedded in the Infancia y Medio Ambiente (INMA) Project: Menorca (n = 122) and Sabadell (n = 236). Wheezing phenotypes were defined at age 4–6 years. Cytosine-guanine (CpG) dinucleotide site DNA methylation differences associated with wheezing phenotypes were screened in children of the Menorca study using the Illumina GoldenGate Panel I. Findings were validated and replicated using pyrosequencing. Information on maternal smoking and folate supplement use was obtained through questionnaires. Dichlorodiphenyldichloroethylene was measured in cord blood or maternal serum. Genotypes were extracted from genome-wide data.**

***Measurement and Main Results*: Screening identified lower DNA methylation at a CpG site in the arachidonate 12-lipoxygenase (*ALOX12*) gene in children having persistent wheezing compared with those never wheezed ($P = 0.003$). DNA hypomethylation at *ALOX12* loci was associated with higher risk of persistent wheezing in the Menorca study (odds ratio per 1% methylation decrease, 1.13; 95% confidence interval, 0.99–1.29; $P = 0.077$) and in the Sabadell study (odds ratio, 1.16; 95% confidence interval, 1.03–1.37; $P = 0.017$). Higher levels of prenatal dichlorodiphenyldichloroethylene were associated with DNA hypomethylation of *ALOX12* in the Menorca study ($P = 0.033$), but not in the Sabadell study ($P = 0.377$). *ALOX12* DNA methylation was strongly determined by underlying genetic polymorphisms.**

***Conclusions*: DNA methylation of *ALOX12* may be an epigenetic biomarker for the risk of asthma-related phenotypes.**

Keywords: *ALOX12*; asthma; dichlorodiphenyldichloroethylene; epigenetics; wheezing

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Environmental factors have been associated with the development of different asthma-related phenotypes. Epigenetic changes including DNA methylation might link environmental exposures with changes in gene expression.

What This Study Adds to the Field

Data from two independent pregnancy cohorts showed that DNA hypomethylation at *ALOX12* cytosine-guanine (CpG) dinucleotide sites, which was in part genetically determined, was associated with a higher risk of persistent wheezing in childhood. The DNA methylation status of the *ALOX12* gene could be an epigenetic biomarker of susceptibility to asthma-related phenotypes in childhood.

Developmental environment and underlying genotype can influence epigenetic variation, including DNA methylation, histone modifications, and microRNA expression, which can alter genome transcription and predetermine life-long phenotypic consequences (1–5). Asthma is a complex disease determined by a combination of genetic and nongenetic factors. Developmental environment and genetic variants have been reported to increase risk of asthma-related phenotypes in childhood (6–8), but most of the susceptibility still remains unexplained. Epigenetic mechanisms have emerged as a factor that also may contribute to the risk of asthma-related phenotypes and mediate environmental effects (9, 10).

Recent studies show that prenatal exposures linked with an increased risk of childhood asthma-related phenotypes, such as maternal diet (11), smoking (12), and stress (13), and pollutants including polycyclic aromatic hydrocarbons (14) and persistent organic pollutants (POPs), such as dichlorodiphenyldichloroethylene (DDE) (15, 16), are also associated with gene-specific and global DNA methylation changes (17–24). The identification of epigenetic marks associated with risk of asthma-related phenotypes in childhood could provide more insight into the complex pathophysiology of asthma and help develop early interventions to prevent disease.

(*Received in original form August 19, 2011; accepted in final form January 23, 2012*)

Supported by grants from the Spanish Ministry of Health (FIS-PI041436, FIS-PI041705, and FIS-PI051187); Instituto de Salud Carlos III (Red INMA G03/176 and CB06/02/0041); Ciber en Epidemiología y Salud Pública; the Generalitat de Catalunya-CIRIT (1999SGR 00241 and 2009-SGR-1502); the Spanish Ministry of Science and Innovation (SAF-2008-00357); the Seventh Framework Program of the European Commission through the ENGAGE Project; and Fundación Roger Torné. N.V. was funded by an FPI grant (BES-2009-023933) from the Spanish Ministry of Science and Innovation.

Author Contributions: E.M., M.B., and N.V. contributed equally to this work. All authors were involved in the study design and analysis, participated in the preparation and writing of the manuscript, and approved the final version. J.S. is guarantor.

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This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org

Am J Respir Crit Care Med Vol 185, Iss. 9, pp 937–943, May 1, 2012
Copyright © 2012 by the American Thoracic Society
Originally Published in Press as DOI: 10.1164/rccm.201105-0870OC on February 9, 2012
Internet address: www.atsjournals.org

In this study we aimed to identify epigenetic marks in terms of DNA methylation of asthma-related phenotypes in childhood, and to assess the effect of prenatal exposures (including maternal smoking, maternal folate supplement use, and DDE) and underlying genetic variation on these epigenetic marks. We first conducted a discovery screening using the Illumina GoldenGate Cancer Panel I array with subsequent validation by pyrosequencing using data from a pregnancy cohort embedded in the Infancia y Medio Ambiente (INMA) Project (the INMA Menorca cohort). Second, a replication study by pyrosequencing was performed using data from an independent cohort of the INMA Project (the INMA Sabadell cohort). Some of the results of these studies have been previously reported in the form of an abstract at the International Society for Environmental Epidemiology 2011 Annual Conference (25).

METHODS

Ethics Statement

Written informed consent was obtained from all participants and the studies were approved by the Clinical Research Ethical Committee of the Municipal Institute of Health Care, Barcelona, Spain.

Discovery and Validation Study

Participants in the first study were members of the INMA Menorca cohort, a population-based pregnancy cohort included in the INMA Project (26). A total of 482 children (94% of those eligible) were enrolled in 1997. For the purposes of this study, a subset of 141 children was selected for DNA methylation analysis chosen to ensure a balanced distribution of prenatal exposures, and finally 122 with complete data at age 6 years were included in the present study. This subsample was representative of the whole cohort population in terms of percentage of children with asthma-related phenotypes, and main characteristics (*see* Table E1 in the online supplement).

Wheezing was described on each interviewer-led annual questionnaire based on the validated ISAAC questionnaire (27) as "whistling or wheezing from the chest, but not noisy breathing from the nose." One or more episodes of wheezing over 12 months constituted wheezing during any given year. At age 6 years, children were assigned to four categories according to their history of wheeze: (*1*) those who had no recorded wheeze during the first 3 years of life and had no wheeze at 4 and 6 years of life (never wheeze); (*2*) those who had recorded wheeze during the first 3 years of life but no wheeze at 4 or 6 years (transient wheeze); (*3*) those who did not record wheeze during the first 3 years of life but had wheeze at 4 or 6 years (late-onset wheeze); and (*4*) those who had at least one wheeze episode in the first 3 years of life and had wheeze at 4 or 6 years of life or those doctor-diagnosed with asthma by age 6 years (persistent wheeze) (28).

DNA was obtained from whole blood collected at 4 years of age using the Chemagic Magnetic Separation Module I (Chemagen Biopolymer-Technologie AG, Baesweiler, Germany) at the Barcelona-CRG Node of the Spanish Genotyping Centre. For the screening of cytosine-guanine (CpG) dinucleotide sites differently methylated according to children wheezing phenotypes, the Illumina GoldenGate Methylation Cancer Panel I array was used (Illumina, San Diego, CA). Briefly, genomic DNA (600 ng) was treated with sodium bisulfite using the EZ-96 DNA Methylation Kit (Zymo Research, Irvine, CA). Methylation levels were analyzed in two independent Sentrix Array Matrixes. Three control samples were included in the array: fully methylated genomic DNA and partially methylated genomic DNA from Jurkat cells (New England Biolabs Inc., Ipswich, MA), and unmethylated DNA obtained by whole genome amplification using GenomiPhi DNA amplification kit (GE Healthcare, Piscataway, NJ). These control samples and two additional DNA samples from the study were bisulphite converted twice and included in both arrays used in the study. Control samples were used to estimate the array discrimination threshold using the total deviation index (29). The minimal detectable methylation difference observed within array was approximately 7% and between arrays was approximately 12%. See the online supplement for additional details. Lists of CpGs with differential methylation in "Beta" values at a P value less than 0.01, a pragmatic threshold for selecting CpG sites for further study, were generated for children classified as having transient and persistent wheezing using children never wheezing as the reference group (*see* Tables E2 and E3). Additionally, results were prioritized based on differential methylation in "Beta" values above 12% (Table 1), the minimal detectable methylation difference between arrays, and on biologic functional information. Because of its relation to airway inflammation (30–32) the arachidonate 12-lipoxygenase (*ALOX12*) gene was selected as a candidate for subsequent validation and replication.

DNA methylation in the first exon of *ALOX12* was validated using the PSQ 96MA Pyrosequencing System (Biotage AB, Uppsala, Sweden). The pyrosequencing assay was designed to validate the CpG site (E85) previously identified on the array and three flanking CpG sites (CpG1, CpG3, and CpG4) located in the exon 1 of *ALOX12* (Figure 1). See the online supplement for additional details.

Study 2: Replication Study

Participants in the second study were members of the INMA Sabadell cohort, a pregnancy cohort established in Sabadell (Catalonia, Spain) between 2004 and 2006 (26). A total of 657 women were enrolled at the first trimester of pregnancy and 622 (94%) were followed until the child's birth. Overall, 236 (45%) out 519 children with available DNA extracted from whole cord blood were included in the replication study. Compared with excluded participants, mothers of children included in the present analysis had higher educational level but did not differ in other main baseline characteristics (*see* Table E1).

Information on wheeze episodes, defined as in the discovery study, was obtained from interviewer-led annual questionnaires from birth until age 4 years.

DNA samples were extracted from whole cord blood using the same protocol as in the discovery study. In addition, a subset of subjects were contacted again at 4 years and total blood was obtained for DNA

TABLE 1. LIST OF CPGS THAT SHOWED A CHANGE IN DNA METHYLATION GREATER THAN 12% AT A *P* VALUE LESS THAN 0.01 AT AGE 4 YEARS BETWEEN CHILDREN NEVER AND PERSISTENT WHEEZING AT AGE 6 YEARS (MENORCA COHORT)

							Never Wheezing ($n = 61$)	Persistent Wheezing ($n = 17$)		
Gene	Name	Chr	CpG ID	Position	Distance to TSS	CpG Island	Mean (SE)	Mean (SE)	Diff.	*P* Value
ZNF264	Zinc finger protein 264	19	ZNF264_P397_F	62394284	−397	Y	78.3 (4.6)	64.6 (5.3)	−13.7	1.4×10^{-5}
ALOX12	Arachidonate 12-lipoxygenase	17	ALOX12_E85_R	6840213	85	Y	34.4 (3.8)	21.5 (5.2)	−12.9	0.003
EPO	Erythropoietin	7	EPO_P162_R	100156197	−162	Y	15.4 (9.4)	30.8 (10.4)	15.3	0.006
PDGFB	Platelet-derived growth factor beta polypeptide	22	PDGFB_E25_R	37970911	25	Y	16.5 (19.8)	38 (20.8)	21.4	0.007

Definition of abbreviations: CpG = cytosine-guanine dinucleotide site; TSS = transcription start site.
Rank of the mixed linear regression adjusted for child sex, gestational age, maternal prepregnancy body mass index, eosinophil count at age 4 years, child body mass index z-score at age 6 years, and a random array effect.

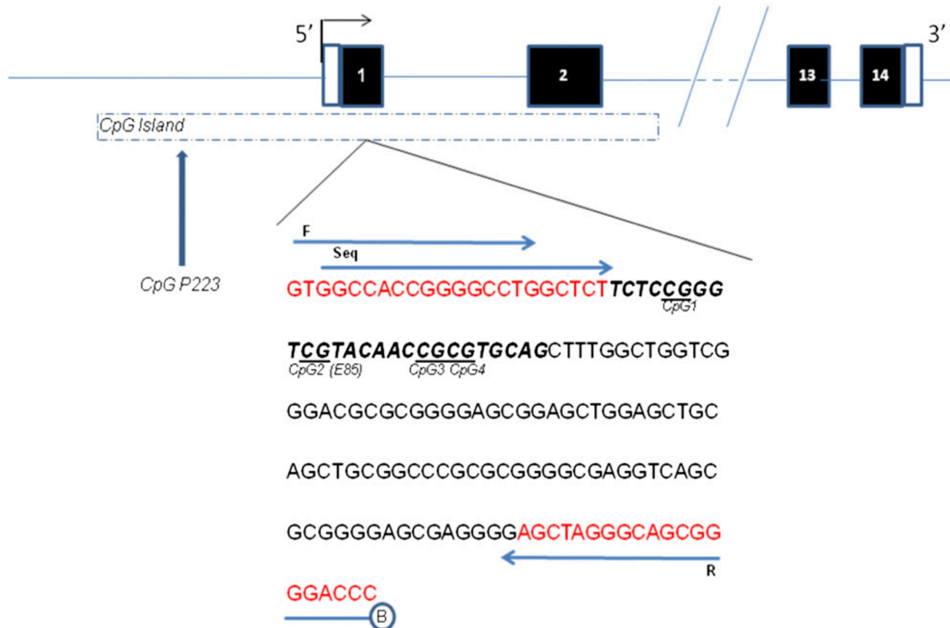

Figure 1. Location of cytosine-guanine (CpG) dinucleotide sites analyzed in *ALOX12* gene. The CpGs in *ALOX12* gene included in the array were CpG P223 and CpG E85, located in the promoter region and in exon 1, respectively, both in a 1.6-kb CpG island that spans from the promoter region up to the second exon of the gene. CpG E85 plus three flanking CpGs (CpG1, CpG3, and CpG4) in exon 1 were validated by pyrosequencing. Location of the polymerase chain reaction and the sequencing primers used in the pyrosequencing assay (*arrows*) and the pyrosequenced fragment (*in bold italics*) are shown.

extraction (n = 19). The same pyrosequencing assay described previously was used to determine DNA methylation levels in the four CpG sites located in the exon 1 of *ALOX12*.

Assessment of Prenatal Exposures

DDE was measured by gas chromatography in cord blood serum of children participating in the Menorca study and in maternal blood serum extracted during the first trimester of pregnancy of women participating in the Sabadell study (33). Information on maternal smoking habits and use of folate supplements during pregnancy was obtained through questionnaires in both studies.

Genetic Variation in *ALOX12*

Genotypes in single-nucleotide polymorphisms (SNPs) in *ALOX12* gene and surrounding region (5 kb upstream and downstream) were extracted from genome-wide genotypic data. Genotyping was performed using the Human Omni1 array (Illumina). See the online supplement for additional details. Genotyping data was available in 174 children participating in the Menorca study (85 of them with methylation data) and in 396 children participating in the Sabadell study (204 of them with methylation data).

Statistical Analysis

In the discovery round, to identify CpGs that showed significant differences in methylation levels among children having had transient or persistent wheezing by using children never wheeze as the reference group, data were analyzed by modeling the methylation at each individual CpG site as quantified by the Illumina "Beta" value by mixed linear regression models. Wheezing phenotypes and potential covariates (including child sex, gestational age, maternal prepregnancy body mass index, child z-score body mass index at age 6 yr, and percentage of eosinophils at age 4 yr) were included as fixed coefficients and array effects as random coefficients. Results were summarized as means and standard errors.

The association between wheezing phenotypes and DNA methylation levels at *ALOX12* CpG sites obtained by pyrosequencing was also analyzed using linear mixed models. Here, individual random effects were specified to take into account the correlation between replicates from the same individual and the information of the working plate. Similar statistical models were performed to analyze the association between prenatal exposures (i.e., maternal smoking and use of folate supplements, and prenatal DDE levels) and genetic polymorphisms with DNA methylation levels of *ALOX12*.

Multivariable logistic regression models were performed to evaluate the association between asthma-related phenotypes (dependent variable) and methylation status of the selected candidate gene (independent variable). Odds ratios (OR) are presented for a decrease in 1% of DNA methylation levels.

Mixed linear models were used to evaluate the association between genetic polymorphism and DNA methylation at *ALOX12* CpG sites. Multivariable logistic regression models were used to explore the association between genetic polymorphisms in *ALOX12* and risk of wheezing phenotypes.

Analyses were conducted using STATA 10.1 statistical software (Stata Corporation, College Station, TX) and R statistical package version 2.9.1 (R Foundation for Statistical Computing, Vienna, Austria).

RESULTS

Discovery Study: Illumina GoldenGate Panel I in the Menorca Cohort

Among the 122 subjects included in the discovery round, 61 (50%) reported never wheezing at age 6 years; 41 (34%) were classified as having transient wheezing; 3 (2%) as having late-onset wheezing; and 17 (14%) as having persistent wheezing. Main characteristics of study population are shown in Table E1.

Mixed linear regression models showed differences at a *P* value less than 0.01 in DNA methylation levels at age 4 years in 54 CpG sites comparing children having persistent wheezing with those who never wheezed at age 6 years (*see* Table E2). Among these CpGs, four showed a difference above 12% (Table 1). Because of its biologic function the CpG located in *ALOX12* gene was selected for subsequent validation and replication of DNA methylation differences between children persistent wheezing and those who never wheezed.

Overall, no CpG site showed DNA methylation differences above 12% between children classified as having transient wheezing compared with those never wheezing (*see* Table E3).

Validation and Independent Replication Study

In the Menorca study, pyrosequencing results featured lower methylation levels at *ALOX12* CpG sites than the Illumina Golden Gate, but the overall pattern of group differences with lower methylation level at age 4 years in children having persistent wheezing at age 6 years compared with children never wheezing was found (Table 2).

TABLE 2. DNA METHYLATION LEVELS (%) IN *ALOX12* CPG SITES OBTAINED USING PYROSEQUENCING ASSAYS BY CHILD WHEEZING PHENOTYPES

	Menorca Cohort*				Sabadell Cohort†			
CpG	Never/Persistent N	Never Wheezing Mean (SE)	Persistent Wheezing Mean (SE)	*P* Value	Never/Persistent N	Never Wheezing Mean (SE)	Persistent Wheezing Mean (SE)	*P* Value
CpG1	60/17	14.7 (0.9)	12.2 (2.3)	0.114	109/37	5.5 (0.1)	4.9 (0.2)	0.168
CpG2 (E85)	60/17	17.3 (1.6)	14.6 (4.1)	0.212	109/37	6.3 (0.2)	5.1 (0.3)	0.028
CpG3	59/17	21.2 (1.8)	18.1 (4.7)	0.177	107/34	9.6 (0.3)	8.2 (0.6)	0.078
CpG4	59/14	12.3 (0.9)	10.6 (2.7)	0.333	108/33	4.4 (0.1)	3.3 (0.2)	0.019

Definition of abbreviation: CpG = cytosine-guanine dinucleotide site.

*DNA methylation levels in peripheral blood at age 4 years by wheezing phenotypes at age 6 years estimated using mixed linear regression models adjusted for child's sex, gestational age, eosinophil count, maternal prepregnancy body mass index, and child body mass index z score at age 6 years.

†DNA methylation levels in cord blood by wheezing phenotypes at age 4 years estimated using mixed linear regression models adjusted for child's sex, gestational age, and maternal prepregnancy body mass index.

In the Sabadell study, similar to other *ALOX12* CpGs DNA methylation levels in *ALOX12* CpG2 (E85) site in cord blood were overall lower compared with levels detected in blood at 4 years of age (*see* Table E4), but still the methylation levels of each CpG site measured at these different ages showed a strong correlation ranging from 0.70–0.99. DNA hypomethylation at *ALOX12* CpG sites in children having persistent wheezing at age 4 years compared with children who never wheezed could be replicated (Table 2).

DNA Methylation of ALOX12 and Risk of Persistent Wheezing

In the Menorca study results from the array showed that lower levels of DNA methylation at *ALOX12* CpG2 (E85) at age 4 years were significantly associated with a higher risk of persistent wheezing at age 6 years (OR per a decrease in 1% of methylation, 1.07; 95% confidence interval [CI], 1.02–1.12) (Table 3). Although the association with the four pyrosequenced CpGs in *ALOX12* was not statistically significant, the direction and magnitude of the association was essentially the same (Table 3). Accordingly, in the Sabadell study, DNA hypomethylation at *ALOX12* CpG2 (E85) at birth was associated with higher risk of having persistent wheezing at age 4 years (OR per a decrease in 1% of methylation, 1.19; 95% CI, 1.03–1.37; $P = 0.017$) (Table 3).

Prenatal Exposures and DNA Methylation of *ALOX12*

In the Menorca study, higher levels of DDE in cord blood were associated with a decrease in DNA methylation at *ALOX12* CpG2 (E85) (upper vs. lower tertile of DDE; $P = 0.033$) (Table 4). However, in the Sabadell study prenatal exposure to DDE, measured as DDE levels in maternal serum in first trimester of pregnancy, was not significantly associated with DNA methylation at *ALOX12* CpG2 (E85) site in cord blood (Table 4).

We found no evidence of an association between maternal smoking or folate supplement use during pregnancy and DNA methylation at *ALOX12* CpG sites (Table 4).

Genetic Variants in *ALOX12*, DNA Methylation of *ALOX12*, and Risk of Persistent Wheezing in Childhood

We first tested if SNPs in *ALOX12* gene and surrounding regions (50 kb upstream and downstream) were associated with DNA methylation at CpG sites of *ALOX12* (*see* Tables E5 and E6). We found that genotypes of the rs312466 (A/G), a haplotype-tagging SNP that lies within the CpG island (*see* Figure E1), were significant associated with DNA methylation at *ALOX12*. Children carrying the minor allele (A) showed lower DNA methylation levels at *ALOX12* CpG2 (E85) site compared with those carrying the major allele (G) (Figure 2). Other SNPs in the region, especially those tagged by rs312466, were also associated with DNA methylation levels of *ALOX12*. Moreover, in the Sabadell study, children carrying the minor allele of the rs312466 tended to have higher risk of persistent wheezing in childhood than those homozygote for the major allele (GA/AA vs. GG genotype; OR, 2.62; 95% CI, 1.02–6.75; $P = 0.036$). Although statistically nonsignificant, similar results were found in the Menorca cohort (GA/AA vs. GG genotype; OR for persistent wheezing, 3.12; 95% CI, 0.37–26.41; $P = 0.274$). The results of the pooled analysis of the Menorca and Sabadell studies

TABLE 3. ASSOCIATION BETWEEN DNA METHYLATION LEVELS IN *ALOX12* CPG SITES AND RISK OF PERSISTENT WHEEZING IN CHILDHOOD

		Menorca Cohort*				Sabadell Cohort†			
Method	CpG	N (*Never/Persistent*)	Odds Ratio	95% Confidence Interval	*P* Value	N (*Never/Persistent*)	Odds Ratio	95% Confidence Interval	*P* Value
Array	CpG2 (E85)	61/17	1.07	1.02–1.12	0.006	—	—	—	
Pyrosequencing	CpG1	60/17	1.13	0.99–1.29	0.077	109/37	1.16	0.97–1.38	0.111
Pyrosequencing	CpG2 (E85)	60/17	1.07	0.97–1.17	0.165	109/37	1.19	1.03–1.37	0.017
Pyrosequencing	CpG3	59/17	1.07	0.98–1.16	0.134	107/36	1.10	0.99–1.22	0.062
Pyrosequencing	CpG4	59/14	1.08	0.95–1.22	0.254	109/34	1.25	1.04–1.50	0.015

Definition of abbreviation: CpG = cytosine-guanine dinucleotide site.

Odds ratios are presented for a decrease in 1% of DNA methylation levels at 4 years of age in the Menorca cohort and at birth in the Sabadell cohort. For all comparisons reference group is children having had never wheeze from birth to the age of 6 years in the Menorca cohort and from birth to the age of 4 years in the Sabadell cohort.

*All models adjusted for child sex, gestational age, maternal prepregnancy body mass index, eosinophil count at age 4 years, and child body mass index z-score at age 6 years.

†All models adjusted for child sex, gestational age, and maternal prepregnancy body mass index.

TABLE 4. ASSOCIATION BETWEEN PRENATAL EXPOSURES AND DNA METHYLATION LEVELS (%) AT CPG2 (E85) OF *ALOX12*

	Menorca Cohort*				Sabadell Cohort†			
	N (%)	Mean	SE	*P* Value	N (%)	Mean	SE	*P* Value
DDE tertile‡								
Low	40 (34.8)	19.3	1.9		80 (33.3)	6.4	0.2	
Medium	37 (32.2)	17.3	1.6	0.244	80 (33.3)	6.6	0.2	0.750
High	38 (33)	15.8	1.9	0.033	80 (33.3)	5.9	0.2	0.377
Maternal smoking								
No	85 (69.7)	17.6	1.1		205 (86.5)	6.2	0.1	
Yes	37 (30.3)	18.2	2	0.688	32 (13.5)	6.6	0.4	
Folate use								
No	48 (39.3)	18.3	1.7		14 (5.8)	6.6	0.8	
Yes	74 (60.7)	17.5	1.2	0.571	227 (94.2)	6.2	0.1	0.687

Definition of abbreviation: DDE = dichlorodiphenyldichloroethylene.

*DNA methylation levels measured at blood collected at age 4 years. All models adjusted for child sex, gestational age, maternal prepregnancy body mass index, and eosinophil count at age 4 years.

†DNA methylation levels measured in cord blood. All models adjusted for child sex, gestational age, and maternal prepregnancy body mass index.

‡Cord blood levels (nanograms per milliliter) in the Menorca cohort and maternal serum levels during pregnancy in the Sabadell cohort. Menorca cohort: lower tertile = 0.13–0.63; medium tertile = 0.64–1.68; higher tertile greater than 1.68. Sabadell cohort: lower tertile = 0.11–0.53; medium tertile = 0.54–0.90; higher tertile greater than 0.90.

showed a statistically significant association between rs312466 genotypes and persistent wheezing at age 4 years (GA/AA vs. GG genotype; OR for persistent wheezing, 2.49; 95% CI, 1.19–5.23; $P = 0.011$).

DISCUSSION

The discovery study in the Menorca cohort identified lower DNA methylation levels at a CpG site in *ALOX12* at age 4 years between children having persistent wheezing and those who never wheezed at age 6 years. This finding was validated by pyrosenquencing and replicated in an independent pregnancy cohort (the Sabadell cohort) in which DNA methylation was assessed at birth and wheezing phenotypes at age 4 years. In the Menorca study, higher DDE levels in cord blood were associated with DNA hypomethylation of *ALOX12* gene at age 4 years; however, we failed to find an association between prenatal DDE levels (measured in maternal blood serum during pregnancy) and DNA methylation of *ALOX12* at birth in the Sabadell cohort. We did not find evidence of an association between maternal smoking or folate use during pregnancy with DNA methylation of *ALOX12*. In contrast, genetic polymorphisms within or near *ALOX12* were strongly associated with DNA methylation and with risk of persistent wheezing in childhood.

DNA hypomethylation at *ALOX12* CpG sites in relation to persistent wheezing was found as early as birth, which suggests that methylation status of *ALOX12* gene could be a potential early epigenetic biomarker of susceptibility to asthma-related phenotypes in childhood. Key elements in asthma pathophysiology include the chronic airway inflammation and remodeling associated with exaggerated Th2 over Th1 responses to allergic and nonallergic stimuli. *ALOX12* gene (also known as *12S-LOX* or platelet-type lipoxygenase 12) encodes for the 12-LOX enzyme that is involved in the metabolism of arachidonic acid leading to the generation of the inflammatory eicosanoids 12(S)-hydroxyeicosatetraenoic acid (12[S]-HETE) (34). Mice 12-LOX and 15-LOX are referred as 12/15-LOX because of their high homology and the fact that they can form 12(S)-HETE and 15(S)-HETE from arachidonic acid (35). The 12/15-LOX is constitutively expressed at high levels in immature red blood cells, eosinophils, and airway epithelial cells (30). Interestingly, experimental studies have shown that 12/15-LOX knockout mice were almost completely protected from the induction of specific IgE antibodies after airway exposure to allergens; were at least fourfold more protected from the induction of specific IgG1 antibodies and from allergen-induced increases in expression of Th2 cytokines (IL-4 and IL-13); and had a lower number of eosinophils in bronchoalveolar lavage fluid (31). Moreover, increased levels of 15(S)-HETE are found in bronchoalveolar lavage fluid from persistently wheezing children (32) and adults with asthma (36). Finally, genetic polymorphisms in *ALOX12* gene and other family members have been investigated in relation to asthma with inconclusive results (37, 38).

Environmental insults during development could leave permanent epigenomic marks that might lead to increased risk of developing asthma phenotypes later in life. Current evidence of effects of early life environmental exposures on asthma risk in childhood mediated by epigenetic changes has so far been limited to air pollutants, such as tobacco smoke (17, 18) and polycyclic aromatic hydrocarbons (24). We previously reported that DDE levels at birth but not DDE measured at 4 years of age increased the risk of physician-diagnosed asthma and of persistent wheezing at age 6 years (16), which supports the hypothesis of developmental programming of asthma by DDE. Here, results from the Menorca study suggest a link between higher prenatal DDE levels to DNA hypomethylation of *ALOX12* gene; however, this was not replicated in an independent cohort. There may be several possible explanations for this. First, assessment of DDE exposure differed between both cohorts in

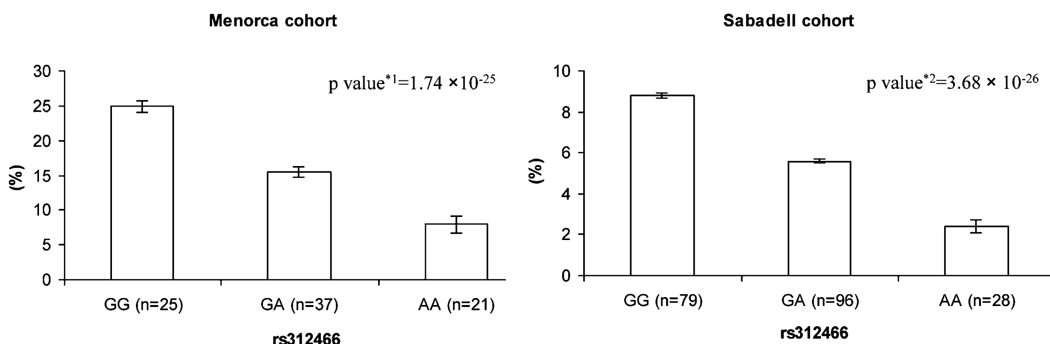

Figure 2. DNA methylation levels in cytosine-guanine (CpG) dinucleotide E85 site at *ALOX12* gene by genotype for haplotype-tagging single-nucleotide polymorphism rs312466. Bars represent means and standard errors. **P* value under an additive model. [1]Adjusted for child sex, gestational age, maternal prepregnancy body mass index, eosinophil count at age 4 years, and child body mass index *z*-score at age 6 years. [2]Adjusted for child sex, gestational age, and maternal prepregnancy body mass index.

regards to the cohort set up, developmental timing at assessment, and biologic matrices of measurement. There is a 7-year gap between the two cohort recruitments, which may explain why levels of DDE tended to be lower in the Sabadell than in the Menorca cohort. In addition, temporal and local differences in agriculture and industrial activities could determine differences in exposure to a diverse mixture of POPs between cohorts. Second, different timing and biologic matrix of exposure assessment could also explain lack of replication. Also, differences between cohorts in relation to maternal age and maternal obesity (*see* Table E1) may account for different levels of exposure in utero. Third, DNA methylation levels of *ALOX12* were measured at different ages and using different tissues between both cohorts that can limit direct comparisons. In the Sabadell cohort we found lower methylation levels in *ALOX12* CpGs in cord blood compared with levels in blood at age 4 years, although a strong correlation was found. Because cell count information was not available, we cannot rule out whether the change in absolute values between these two time points is caused by an increase in *ALOX12* methylation over time or a change in cell count between cord blood and whole blood at age 4 years. Further research is needed to confirm these findings and elucidate potential biologic mechanisms involved in DDE impact on DNA methylation.

It is also known that underlying genotype influences epigenetic variation. Thus, we evaluated the possible *cis* genetic effects on *ALOX12* DNA methylation. We found that methylation differences at *ALOX12* CpG sites were highly attributable to genetic variation acting in *cis*, which is consistent with previous studies (39). In addition, we found some evidence that genetic variants in *ALOX12* were associated with the risk of persistent wheezing. These results strengthen the idea of using epigenomic studies to investigate genetic predispositions that could exert their action through epigenetic mechanisms. In addition, assessment of associations between genetic variants (which are not related to confounding factors, and not altered by disease processes and thus subject to reverse causation) and epigenetic patterns may elucidate causal pathways in epidemiologic studies (40).

The strengths of this study are the prospective nature and the population-based cohort design. In addition, assessment of DNA methylation changes was performed before the occurrence of asthma-related phenotypes that precludes reverse causation. The study has some limitations. First, characterization of asthma-related phenotypes was based on questionnaires used to ascertain whether subjects have had symptoms of asthma (i.e., wheezing) or have ever received a diagnosis of asthma from a physician. However, children with persistent wheezing at age 6 years had significantly reduced lung function, which is consistent with the substantial deficits in lung function reported in older children with asthma (28). Further research is warranted to assess if DNA hypomethylation of *ALOX12* is also observed among children with physician-diagnosed asthma or reduced lung function. Second, dramatic differences in methylation levels are reported in cancerous versus noncancerous tissue but not in other complex diseases and phenotypes, where methylation at any given CpG island or specific CpG sites in affected versus unaffected individuals may vary by less than 10% (3). Smaller effect sizes may be a general phenomenon because they have been reported in other human epigenetic studies involving famine exposure, neuropsychiatric disorders, and assisted reproductive technologies (3, 41, 42). Moreover, for some genes, evidence exists that a small change in the level of DNA methylation, especially in the lower range, can dramatically alter gene expression levels (43, 44). Third, we did not assess if changes in DNA methylation levels at *ALOX12* CpG sites affected gene transcription; thus, evidence from human- or animal-based designs is necessary to demonstrate that demethylation of the *ALOX12* enhances gene expression. Fourth, we used whole blood cells as a surrogate to predict the pathophysiologic changes in the target tissues (airway and the immune cells). However, previous studies suggest that whole blood is a good surrogate because of its high levels of T cells, which are important producers of cytokines and other asthma mediators (45). Finally, we used the HumanMethylation Cancer Panel I (Illumina) for screening of DNA regions differently methylated. The CpG sites included in this array are based on their different methylation in tumor tissues and cancer processes, which in some aspects can be related to fetal development. However, this array was not designed for epigenome-wide analyses, and methylation changes at other loci and genes related with asthma may have been overlooked. Future larger prospective cohort studies and genome-wide epigenetic screening are essential to unravel the role of epigenetic mechanisms in programming susceptibility to asthma and asthma-related phenotypes and in mediating effects of environmental early life exposures.

In conclusion, DNA hypomethylation at *ALOX12* CpG sites, which was highly determined by underlying genetic variation, was associated with higher risk of persistent wheezing in childhood. DNA methylation status of *ALOX12* gene could be an epigenetic biomarker of susceptibility to asthma-related phenotypes in childhood. Whether effects of prenatal exposure to DDE on risk of asthma-related symptoms in childhood are mediated by epigenetic changes deserves further investigation.

Author disclosures are available with the text of this article at www.atsjournals.org.

Acknowledgment: The authors acknowledge all teachers and parents of the children from Menorca Island for patiently answering the questionnaires; the nurses and administrative personal from the Primary Health Care Center of Maó for administrative, technical, and material support; and the CeGen-ISCIII Barcelona-CRG Node for technical assistance. The authors are also grateful to Silvia Fochs, Anna Sànchez, Maribel López, and Nuria Pey for their assistance in contacting the families and administering the questionnaires in the Sabadell study and all the participants for their generous collaboration.

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