Ten-eleven translocation 1 (*TET1*) methylation is associated with childhood asthma and traffic-related air pollution

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Background: Asthma is a complex disorder influenced by genetics and the environment. Recent findings have linked abnormal DNA methylation in T cells with asthma; however, the potential dysregulation of methylation in airway epithelial cells is unknown. Studies of mouse models of asthma have observed greater levels of 5-hydroxymethylcytosine (5-hmC) and ten-eleven translocation 1 *(TET1)* expression in lungs. TET proteins are known to catalyze methylation through modification of 5-methylcytosine to 5-hmC.

Objective: We sought to examine the association of *TET1* methylation with asthma and traffic-related air pollution (TRAP).

Methods: *TET1* methylation levels from DNA derived from nasal airway epithelial cells collected from 12 African American children with physician-diagnosed asthma and their nonasthmatic siblings were measured by using Illumina 450K arrays. Regions of interest were verified by means of locus-specific pyrosequencing in 35 sibling pairs and replicated in an independent population (n = 186). Exposure to TRAP in participants' early life and at current home addresses was estimated by using a land-use regression model. Methylation studies in saliva, PBMCs, and human bronchial epithelial cells were done to support our findings.

Results: Loss of methylation at a single CpG site in the *TET1* promoter (cg23602092) and increased global 5-hmC levels were significantly associated with asthma. In contrast, TRAP exposure at participants' current homes significantly increased methylation at the same site. Patterns were consistent across tissue sample types. 5-Aza-2′-deoxycytidine and diesel exhaust particle exposure in human bronchial epithelial cells was associated with altered *TET1* methylation and expression and global 5-hmC levels.

Conclusions: Our findings suggest a possible role of *TET1* methylation in asthmatic patients and response to TRAP. (J Allergy Clin Immunol 2015;■■■:■■■-■■■.)

Key words: *DNA methylation, TET1, 5-hmC, nasal epithelial cells, cross-tissue marker, traffic-related air pollution, asthma*

Asthma is a complex, heterogeneous,[1] and inheritable disorder. The prevalence of asthma has increased dramatically worldwide, making it a primary clinical and economic burden. Asthma is known to be influenced by gene-environment interactions.[2] In children asthma exacerbations are commonly triggered by environmental exposures, including traffic-related air pollution (TRAP). A ubiquitous environmental exposure in urban areas, TRAP has been shown to induce inflammatory and immunologic responses, leading to asthma exacerbation.[3-8] Both early childhood and longitudinal TRAP exposure have been associated with asthma incidence in epidemiologic studies.[9-13] Despite detailed phenotypic characterization of asthma,[14] the molecular mechanisms of the initiation and progression of asthma and the effect of environmental exposure on these processes are not fully understood.

Epigenetic regulation in asthmatic patients has recently become an intensely studied area because of its effect on transgenerational disease susceptibility, phenotypic variability, and association with environmental exposures.[15,16] DNA methylation is a heritable epigenetic modification that can be altered by environmental exposures. Its essential role in the regulation of gene expression makes it a potential mechanism in the etiology of complex diseases, such as asthma. Previous studies have indicated that pathways involved in asthma, such as T-cell differentiation and cytokine production, can be regulated by DNA methylation.[17-22] Pharmacologic demethylation has been reported to alleviate asthma symptoms in an experimental mouse model of asthma, possibly by altering the methylation on cytokine genes.[23] Moreover, exposure to TRAP has been associated with DNA methylation variation in asthma-related genes,[22,24-29] and DNA methylation levels at specific loci have also been suggested to be used as a biomarker for asthma severity and exposure-related asthma exacerbations.[30] Therefore the epigenome has been suggested to be a mechanistic bridge between exposures in the environment and asthma development, possibly through mediating gene-environment interactions. A better understanding of the epigenome will offer new pathophysiologic insights into the relationship between genetics and the environment and might provide therapeutic targets to prevent the onset and delay the progression of asthma.

The ten-eleven translocation 1 *(TET1)* enzyme is a candidate that might play an essential role in asthma development. A known epigenetic modulator, TET1 promotes DNA

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Abbreviations used
DEP: Diesel exhaust particle
ECAT: Elemental carbon attributable to traffic
ESS: Exposure Sibling Study
HBEC: Human bronchial epithelial cell
5-hmC: 5-Hydroxymethylcytosine
PEES: Pediatric Environmental Exposure Study
TET1: Ten-eleven translocation 1
TRAP: Traffic-related air pollution

demethylation by catalyzing the conversion of 5-methylcytosine to 5-hydroxymethylcytosine (5-hmC), 5-formylmethylcytosine, and ultimately 5-carboxylcytosine.[31] It is well characterized in cellular differentiation, cancer development, and response to hypoxia.[32-41] Recently, by using a mouse model of house dust mite–induced asthma, a study reported an altered methylome, increased levels of 5-hmC, and increased *TET1* expression in the lungs,[42] implicating a possible role of *TET1* in asthma. In this article we evaluated the associations of *TET1* methylation and expression levels with childhood asthma and determined whether these associations were modified by TRAP exposure.

METHODS

Study population

The Exposure Sibling Study (ESS) and the Pediatric Environmental Exposure Study (PEES) were used as the discovery and replication cohorts, respectively. Both studies were approved by the Institutional Review Board at Cincinnati Children's Hospital Medical Center. Written informed consent was obtained from each participant or the participant's parent or legal guardian before study participation.

ESS is a cross-sectional study consisting of African American siblings between the ages of 5 and 18 years. Participants were recruited from the Greater Cincinnati Pediatric Clinical Repository[43] and from the general public in response to flyers posted around the medical center and online. Eligible siblings (nontwins) discordant for asthma were born and raised in the same household in the Cincinnati Metropolitan area. Asthma diagnosis was obtained from the parental report and confirmed through electronic medical records. Children receiving medication for any heart or lung condition besides asthma were excluded. Nasal, saliva, and blood samples were collected as described below, and height and weight were recorded. The parent or guardian was given an electronic survey (Research electronic data capture [REDCap] software)[44] that characterized asthma onset, diagnosis, symptoms, severity, quality of life, medication, environmental exposures, social histories, and residential address for the first year of life and for the past 5 years. For the discovery phase of the analysis, nasal epithelial cells from 12 of the 35 sibling pairs participating in the ESS were assayed for DNA methylation by using the Infinium HumanMethylation450 BeadChip (Illumina, San Diego, Calif; detailed study design shown in Fig 1).

To replicate the finding from the discovery population (ESS), we assayed saliva DNA from 158 asthmatic and 28 nonasthmatic African American PEES participants. PEES is a case-control study of nearly 400 asthmatic and nonasthmatic children aged 5 to 18 years living in the Cincinnati Metropolitan area.[45] Children with asthma in PEES were diagnosed according to American Thoracic Society criteria.[46]

Elemental carbon, or soot, is produced by incomplete combustion, and the dominant contributors of outdoor elemental carbon are traffic (diesel combustion) and wood burning.[47] However, in the Cincinnati metropolitan area outdoor wood burning is not a major source of particulate matter. In both studies elemental carbon attributable to traffic (ECAT; a proxy of diesel exhaust particles [DEPs], herein referred to as TRAP) was estimated by using a land-use regression model from the latitude and longitude coordinates from participants' home addresses by using previously established methods.[48] Two estimates were generated: birth or early exposure to TRAP was defined as exposure during the first year of life, and current exposure was derived by using the current address. Children with 85th or greater body mass index percentiles (considering age and sex based on the Centers for Disease Control and Prevention curves) were considered overweight.[49]

Sample collection and DNA/RNA extraction

Nasal mucosal sampling was performed for each participant with a CytoSoft Brush (Medical Packaging Corp, Camarillo, Calif), and the samples were immediately taken to the laboratory for processing. Information regarding DNA/RNA extraction, cell-culture studies, 450k array processing, bisulfite pyrosequencing (see Table E1 for primers), and quantitative PCR (see Table E2 for primers) can be found in the Methods section in this article's Online Repository at www.jacionline.org.

Statistical analysis

Demographics and characteristics were compared between the ESS and PEES cohorts by using Wilcoxon rank sum or χ^2 tests according to data distribution.

In the discovery phase, by using the 450K methylation array, 24 ESS nasal epithelial samples were assayed. The β values of each CpG site were first modeled with age and sex in all 24 samples by using linear regression to remove the effects of age and sex on the methylation level; residuals were calculated and used in subsequent association tests with asthma status. Because the siblings were paired by family, we compared the residuals between asthmatic patients and control subjects by using paired *t* tests for each CpG site. Sites with *P* values (paired *t* test) of .05 or less, and absolute β differences between asthmatic patients and control subjects of 0.1 or greater were selected as differentially methylated sites. Because the sample size is small, the nonparametric Wilcoxon signed-rank test was also performed for the significant findings to ensure the robustness of the conclusion. All analyses on the microarray data were conducted in R software.

To examine whether the methylation levels of the *TET1* site from the 450K array agreed with those from pyrosequencing, we correlated the data from the 24 ESS samples used in the discovery phase using Pearson and Spearman correlation. Agreement was also visually judged by using a Bland-Altman plot. Correlation was also examined among PBMCs, saliva, and nasal epithelial cells from the ESS cohort to evaluate tissue specificity of the methylation of *TET1*.

To verify the *TET1* association with asthma identified by using the 450K methylation array, we analyzed the methylation level measured by means of pyrosequencing in all 70 ESS samples. Because ESS samples were paired by family, to account for the interfamily variability, we used a mixed-model approach to test the association of methylation percentages with asthma status and ECAT values. Age and sex were tested in the same model as potential covariables. One hundred eighty-six saliva samples from the PEES cohort were examined to replicate the *TET1* association with asthma and ECAT. The association of *TET1* methylation with asthma and ECAT values was tested by using linear regression with age and sex adjustment.

To elucidate the role of methylation on *TET1* expression, we first correlated methylation levels with expression levels by using Pearson correlation. Methylation levels were also dichotomized into 2 groups (high- and low-methylation groups) by using hierarchical and k-means clustering. A 2-sided *t* test was then performed to test whether the expression level was different in the low-methylation group compared with the high-methylation group. *TET1* expression, methylation, and global 5-hmC levels between control subjects and the 5-aza-2′-deoxycytidine– or DEP-treated groups in human bronchial epithelial cells (HBECs) were compared by using the 2-sided *t* test. Analyses were performed with SAS, version 9.3 (SAS Institute, Cary, NC), unless otherwise specified. A *P* value cutoff of .05 was used to indicate statistical significance.

RESULTS

Population characteristics

The demographics and characteristics of the ESS and PEES subjects included in the analyses are shown in Table I. No

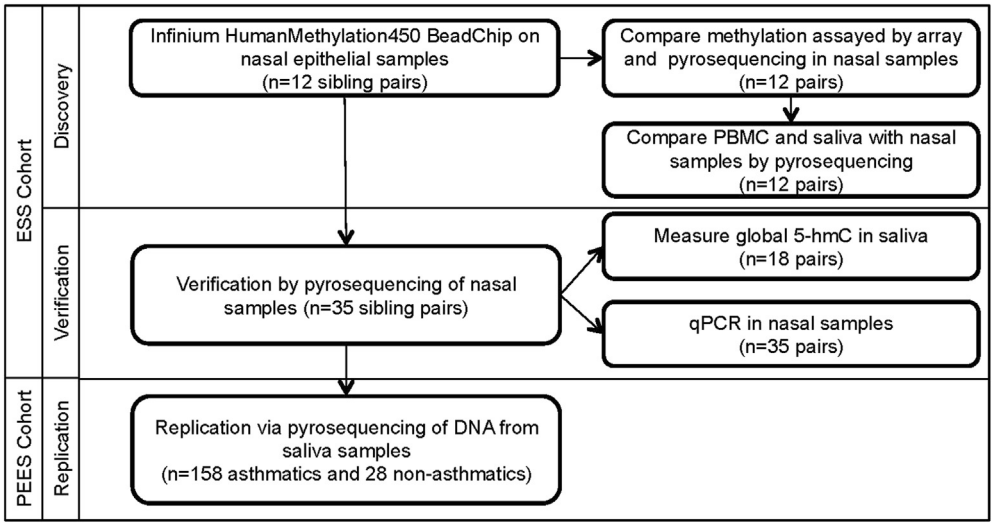

FIG 1. Study schematic. There were 35 sibling pairs in the discovery ESS population. Of these, nasal samples from 12 pairs were selected to run on the Infinium HumanMethylation450 BeadChips. We then verified the results using pyrosequencing on PBMCs and saliva in these same 12 pairs and in nasal epithelial samples from all 35 sibling pairs. We also measured 5-hmC levels in saliva from 18 pairs. To replicate our findings, we performed pyrosequencing on 186 saliva samples from children participating in the PEES cohort. *qPCR*, Quantitative PCR.

statistically significant differences were detected between the ESS and PEES populations in age, sex, exposure to secondhand smoke, body mass index percentile, obesity, current ECAT value, or ECAT value during infancy. Because the ESS by design used siblings, it had 50% asthmatic participants, whereas PEES targeted and therefore had more asthmatic participants (85%). We also compared the demographics of the participants used in the discovery phase with those of the rest of the ESS cohort; no significant differences were detected (see Table E3 in this article's Online Repository at www.jacionline.org).

TABLE I. Population characteristics

	ESS cohort (n = 70)	PEES cohort (n = 186)	*P* value
Age (y)	11.0 (9.0-14.0)	12.0 (8.0-15.0)	.62
Male sex (%)	38 (54)	97 (52)	.76
Asthmatic (%)	35 (50)	158 (85)	<.001
Secondhand smoke exposure (%)	33 (47)	97 (52)	.48
BMI percentile	72.9 (46.3-96.8)	76.5 (49.1-96.9)	.49
Overweight/obese (%)	29 (41)	83 (45)	.65
ECAT during infancy	0.42 (0.34-0.51)	0.41 (0.34-0.53)	.61
Current ECAT	0.34 (0.31-0.42)	0.37 (0.30-0.49)	.18

Note: Age, BMI percentile, ECAT value during infancy, and current ECAT value are shown as medians (interquartile ranges) and compared by using the Wilcoxon rank sum test. Other variables are shown as numbers (percentages) and compared by using χ^2 tests.
BMI, Body mass index.

cg23602092 methylation at the *TET1* promoter is associated with asthma

Of the 312,516 CpG sites that passed the filtering procedure described in the Methods section, 237 had *P* values of .05 or less and an absolute difference in β values between asthmatic patients and control subjects of 0.1 or greater, including a CpG in the promoter region of the *TET1* gene (cg23602092; $P = .014$, paired *t* test; Fig 2, *A*). Because *TET1* is a candidate gene that might be involved in asthma, we assayed this CpG site in all 70 ESS samples using pyrosequencing. We observed a good agreement in methylation levels between pyrosequencing and the 450K array (Pearson $r = 0.96$, Spearman $r = 0.86$; Fig 2, *B* and *C*). Using the 70 ESS samples, we then verified the association of *TET1* methylation percentages with asthma in a mixed model. Age and sex were tested as potential covariables. Because no significant effects were detected, age and sex were excluded from the final model. Although age and sex did not show effects on the methylation at cg23602092, a statistically significant association was detected between asthma status and cg23602092 methylation ($P = .040$), with the mean methylation level being 22% ± 17% and 15% ± 15% in control subjects and asthmatic patients, respectively (Fig 2, *D*).

Effect of TRAP exposure on cg23602092 methylation differs in asthmatic patients versus control subjects

To assess the influence of TRAP exposure on the methylation level of cg23602092 at the *TET1* promoter and the *TET1* association with asthma, we added TRAP exposure (current or birth estimates) to the mixed model. As shown in Fig 3, the correlation between current TRAP exposure (estimated as current ECAT) and cg23602092 methylation significantly differed in asthmatic patients and control subjects ($P = .033$). In control subjects higher cg23602092 methylation was associated with higher current ECAT values ($P < .001$); in asthmatic patients a similar trend was observed, but the association was not statistically significant ($P = .23$). Interestingly, in addition to an overall significant cg23602092-asthma association when current ECAT values were not considered, our results suggested that the difference in cg23602092 methylation between asthmatic patients and control subjects was larger in children who were

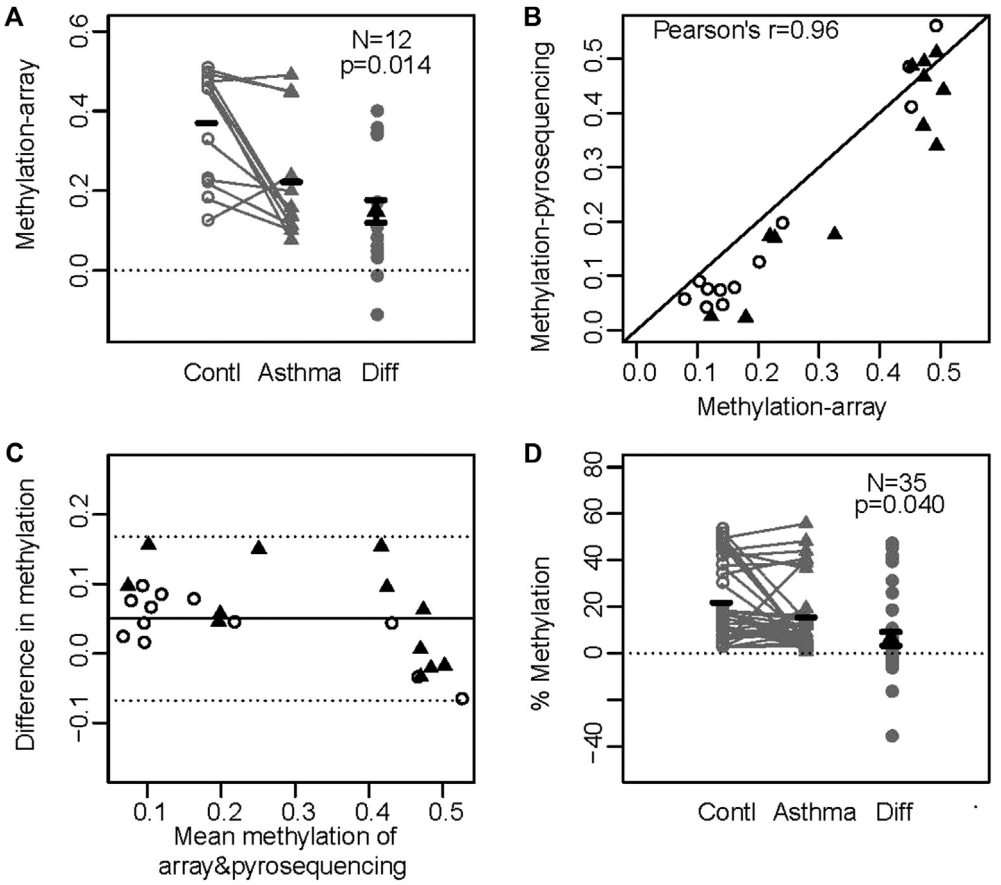

FIG 2. Hypomethylation of cg23602092 located at the *TET1* promoter is associated with childhood asthma in the ESS cohort. **A,** Differentially methylated cg23602092 on the Infinium HumanMethylation450 BeadChip. β Values and differences of each of the asthma-control pairs are shown as *gray dots*. The mean β value for control subjects or asthmatic patients is shown as the *black bar*. The mean difference is shown as a *black triangle* together with the SEM. **B,** Correlation between bisulfite pyrosequencing and bead array measurements. Control subjects and asthmatic patients are represented by *solid triangles* and *open dots*, respectively. **C,** Bland-Altman plot showing limits of agreement. *Solid line*, Mean difference; *dotted lines*, 95% confidence limits of the mean. **D,** DNA methylation in all ESS participants.

exposed to high TRAP levels. M values were modeled similarly to ensure statistical robustness. The results were consistent with those when methylation percentages were used. The effect of birth TRAP was tested similarly, but no significant association was detected with *TET1* methylation.

Correlation of DNA methylation between tissue types

Epigenetic modifications have been reported to be tissue specific, but both saliva and PBMCs have previously been used to profile DNA methylation changes associated with environmental exposures and respiratory diseases caused by their accessibility, especially for younger kids. Using bisulfite pyrosequencing, we measured methylation levels of *TET1* cg23602092 in PBMCs and saliva and compared these values with those measured in nasal cells from the ESS cohort. As shown in Fig 4, methylation levels of cg23602092 were highly correlated in all 3 tissues (Pearson $r \geq 0.75$). These consistent results suggest that cg23602092 methylation changes are robust and that methylation at cg2360292 might be a useful cross-tissue biomarker for asthma.

Hypomethylation of the *TET1* promoter was associated with asthma in the PEES cohort

To substantiate our findings and replicate our results, we assessed the same CG site in saliva DNA from an independent cohort (PEES) containing asthmatic (n = 158) and nonallergic nonasthmatic (n = 28) children. Using linear regression, we detected a statistically significant association between *TET1* cg23602092 and asthma after adjusting for age ($P < .001$, Fig 5). Potential effects of sex and current or birth ECAT values were also tested; no significant associations were detected. M values were similarly evaluated, and our results support the findings when we modeled methylation percentages.

TET1 expression was negatively associated with the promoter CG site methylation

DNA methylation is an important epigenetic mechanism involved in gene expression regulation. It was recently shown that the *TET1* promoter is hypermethylated and transcriptionally silenced in human non-Hodgkin B-cell lymphoma[50] and murine cell lines.[51] To investigate the role of methylation modification

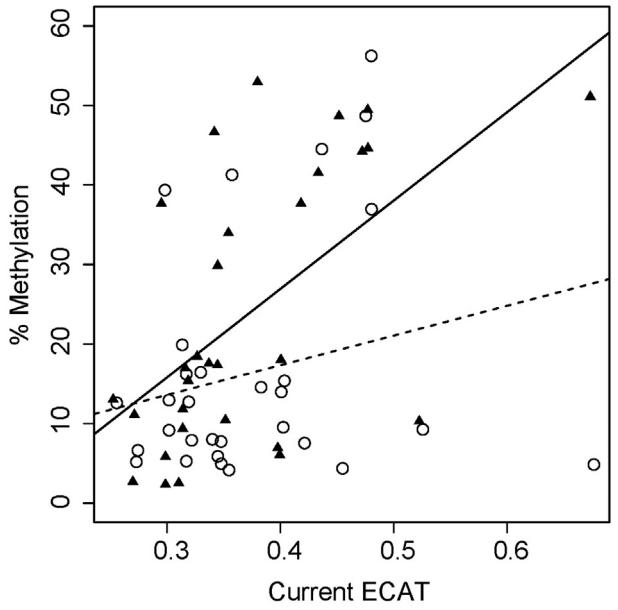

FIG 3. Influences of TRAP on cg23602092 methylation and the association between cg23602092 methylation and asthma. Percentage methylation was shown for control subjects *(solid triangles)* and asthmatic patients *(open circles)*. Model-predicted relationship between TRAP exposure (current ECAT value) and methylation was shown in control subjects *(solid line)* and asthmatic patients *(dashed line)*. Distance between the *dashed* and *solid lines* represents the differences in methylation between control subjects and asthmatic patients at different levels of current ECAT exposure.

at cg23602092 on transcription in nasal cells, we evaluated expression profiles of *TET1* by using quantitative PCR analysis. Interestingly, although expression levels were not significantly correlated with pyrosequencing methylation values, we observed a bimodal distribution of the DNA methylation data (Fig 6, *A*). Hierarchical and k-means clustering further support that high- and low-methylation groups might exist. Therefore we dichotomized the methylation data and found that the expression level of *TET1* was marginally lower in samples with higher cg23602092 methylation ($P = .107$, 2-sided t test; Fig 6, *B*). On treating HBEC cells with 5-aza-2′-deoxycytidine, a DNA methylation inhibitor, we found that the *TET1* expression is significantly upregulated after 24 hours and that cg23602092 is significantly demethylated ($P < .0001$; Fig 6, *C* and *D*), which supports the negative correlation between cg23602092 methylation and *TET1* expression.

Upregulation of global 5-hmC levels in asthmatic patients

TET enzymes regulate DNA methylation by catalyzing the conversion of 5-methylcytosine to 5-hmC in an Fe(II)- and α-ketoglutarate–dependent manner.[52] *TET1* in particular has been shown to play a pivotal role in DNA demethylation.[53] Knockdown of *TET1* resulted in reduced global 5-hmC levels,[53,54] whereas increased *TET1* expression was associated with increased 5-hmC levels in murine lung tissues.[42] Consistently, we observed a significant global increase in 5-hmC levels when *TET1* expression was induced by 5-aza-2′-deoxycytidine ($P < .0001$; Fig 6, *E*). We then measured global 5-hmC levels in the saliva of the ESS sibling pairs and again observed higher levels of 5-hmC in asthmatic patients compared with their nonasthmatic siblings (Fig 6, *F*), suggesting *TET1* expression in asthmatic children is increased.

Reduced *TET1* mRNA and global 5-hmC levels in HBECs challenged with DEPs

DEPs constitute a significant portion of particulate matter in TRAP and are recognized as a key component not only in TRAP-related asthma exacerbations[55,56] but also have been implicated in asthma pathogenesis[57,58] and asthma severity.[45] To determine the effect of controlled DEP exposure on *TET1* expression levels, we exposed HBECs to DEPs (5 $\mu g/cm^2$) and measured *TET1* expression and methylation over time. At this dose of DEP, expression of *CYP1A1*, *HMOX1*, *IL1B*, *IL6*, and *TSLP* was induced (see Fig E1 in this article's Online Repository at www.jacionline.org). *TET1* expression was slightly upregulated in HBECs exposed to DEPs for 1 hour, significantly downregulated 4 hours after exposure, and restored to a normal level 24 hours after exposure ($P = .003$; Fig 7, *A*). Associated with this downregulation, *TET1* cg23602092 showed an increase in methylation at 24 hours after exposure ($P < .0001$), supporting a negative association between methylation and expression (Fig 7, *B*). Also associated with the lower *TET1* expression, we observed significantly lower levels of 5-hmC in HBECs exposed to DEPs for 24 hours (Fig 7, *C*).

DISCUSSION

Our study consistently found that loss of nasal cell–derived DNA methylation at a particular CpG (cg23602092) site in the promoter region of *TET1* was associated with childhood asthma. The methylation level at this CpG site was also significantly associated with TRAP exposure, and this was most evident in nonasthmatic subjects. *TET1* expression levels and levels of 5-hmC, a product of TET1, were altered in asthmatic children, as well as in HBECs treated with DEPs, a major component of TRAP. In addition, inhibition of methylation promoted *TET1* expression in HBECs. Collectively, our results demonstrate the function of methylation in *TET1* regulation, which might play a role in asthma etiology and response to traffic pollution. Moreover, the methylation level at this CpG site was highly correlated across nasal cells, PBMCs, and saliva, making it a potential cross-tissue biomarker for childhood asthma.

TET1 encodes a dioxygenase that consecutively converts 5-methylcytosine into 5-hmC, 5-formylcytosine, and 5-carboxylcytosine, thus playing a key role in active DNA demethylation. Redox status, such as oxidative stress, has been shown to increase TET-mediated demethylation.[40,41,59] Oxidative stress has been demonstrated in all forms of asthma by affecting airway epithelial cell damage, airway hyperresponsiveness, airway obstruction, and immune responses.[60] Exposure to TRAP alone or in combination with allergens induces oxidative stress, which contributes to increased asthma risk.[60] Because of the aforementioned associations among *TET1*, oxidative stress, asthma, and TRAP exposure, associations of *TET1* with asthma status and TRAP described in this article are not unexpected. Although the exact role of *TET1* in such a cascade is yet to be elucidated, it is evident that DEP exposure upregulates proinflammatory cytokines in airway epithelial cells with well-established roles in asthmatic patients.[61-67] Therefore it is

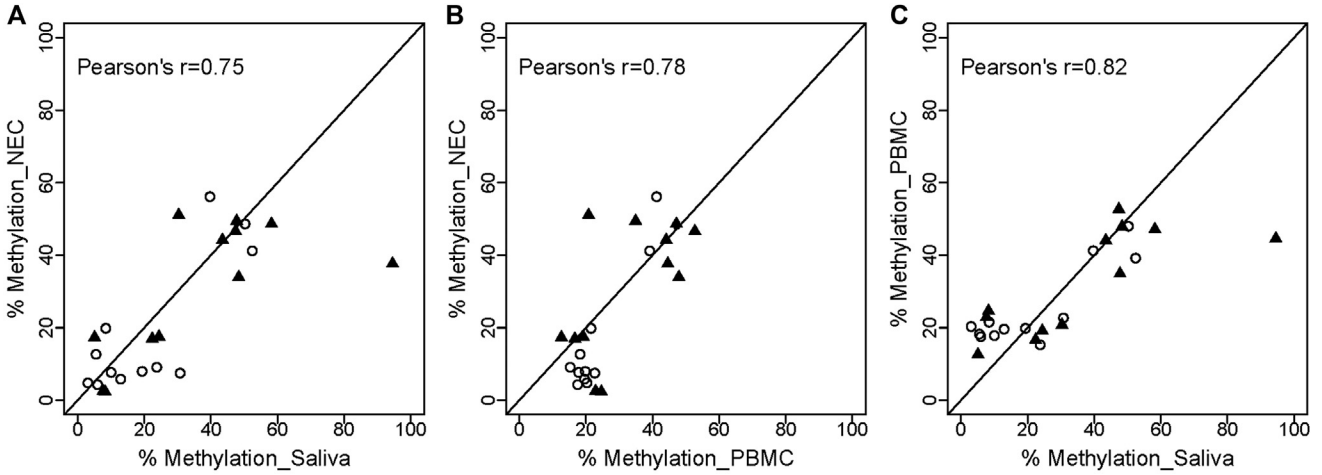

FIG 4. Methylation of cg23602092 is correlated among 3 tissues. Shown are comparisons of cg23602092 methylation between nasal cells and saliva **(A)**, nasal cells and PBMCs **(B)**, and saliva and PBMCs **(C)** in the ESS cohort. Control subjects and asthmatic patients are represented by *solid triangles* and *open dots*, respectively.

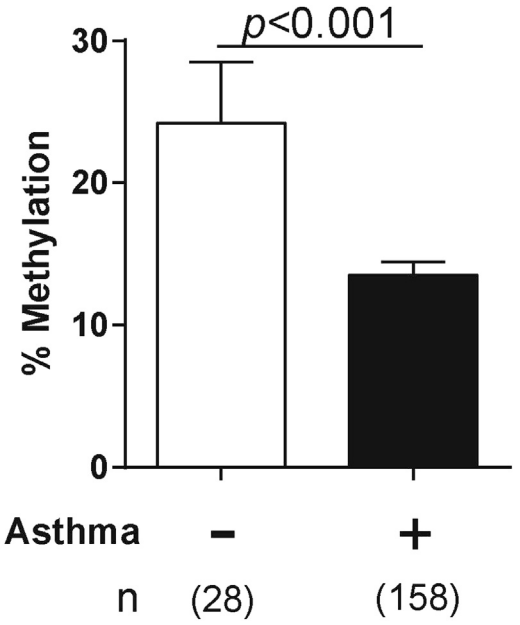

FIG 5. Hypomethylation of cg23602092 is associated with childhood asthma in the PEES cohort. Methylation levels were measured by means of locus-specific bisulfite pyrosequencing in the saliva of asthmatic (n = 158) and nonasthmatic (n = 28) children enrolled in the PEES cohort. A statistically significant association between TET1 hypomethylation and asthma was detected, thus replicating our finding from the ESS cohort. *Bars* represent means ± SEMs.

plausible that DEP-associated oxidative stress and inflammation in airways regulate *TET1*, which in turn modulates 5-hmC levels, resulting in transcriptional activation of downstream genes, such as *VEGFA*, known to be associated with lung function,[68] particulate matter exposure, and asthma.[69-71] Interestingly, our study showed methylation of the *TET1* promoter might be a mechanism for regulation of *TET1*.

Methylation signatures are tissue specific, and therefore selection of relevant tissues and cell types to identify disease-specific modifications and disease etiology is crucial. To date, a majority of the studies have used either blood or saliva to profile DNA methylation patterns associated with complex diseases and environmental exposures. Although there are well-founded reasons why these surrogate tissues are used, the validity of the associations identified by using surrogate tissues is questionable. In asthmatic patients the airway epithelium is a major driver of allergic responses through its interaction with immune cells, which play an essential role in the origin and persistence of the disease.[72,73] Therefore it would be optimal to use lung epithelial cells for methylation profiling, but obtaining such samples in a clinical setting is impractical, particularly in children. Therefore we sampled nasal epithelial cells as a proxy of bronchial epithelial cells to study biologically relevant changes occurring in the lung.[74] Strikingly, when we quantified *TET1* methylation levels at the site of interest in PBMCs, saliva, and nasal cell DNA from our discovery cohort, a strong correlation was observed across all 3 disease-relevant tissues and cell types, indicating that *TET1* dysregulation could be used as a systemic biomarker for childhood asthma.

We observed a bimodal distribution of *TET1* methylation in our cohort. To further investigate this phenomenon, we dichotomized the ESS cohort into high and low *TET1* methylation levels (cutoff = 0.2) and TRAP exposure (cutoff median = 0.3445). In the high-methylation group 83% had been exposed to high TRAP levels compared with 36% of the low-methylation group ($P = .001$, χ^2 test). Therefore TRAP exposure might contribute to the bimodal distribution, and it is possible that *TET1* methylation changes markedly once TRAP exposure reaches a certain level. Another contributing factor might be a family effect. Of 35 families, siblings in 28 had concordant methylation grouping. In our final mixed models both TRAP exposure and a random family effect were included. Nevertheless, after adjusting for both TRAP exposure and family effect, we still observed a significant difference in *TET1* methylation levels between asthmatic patients and control subjects.

The incidence of asthma is more prevalent among low-income African American children, who are more likely to reside near high TRAP and industrial areas.[75-77] Accumulating evidence suggests that TRAP mediates asthma susceptibility and aggravate

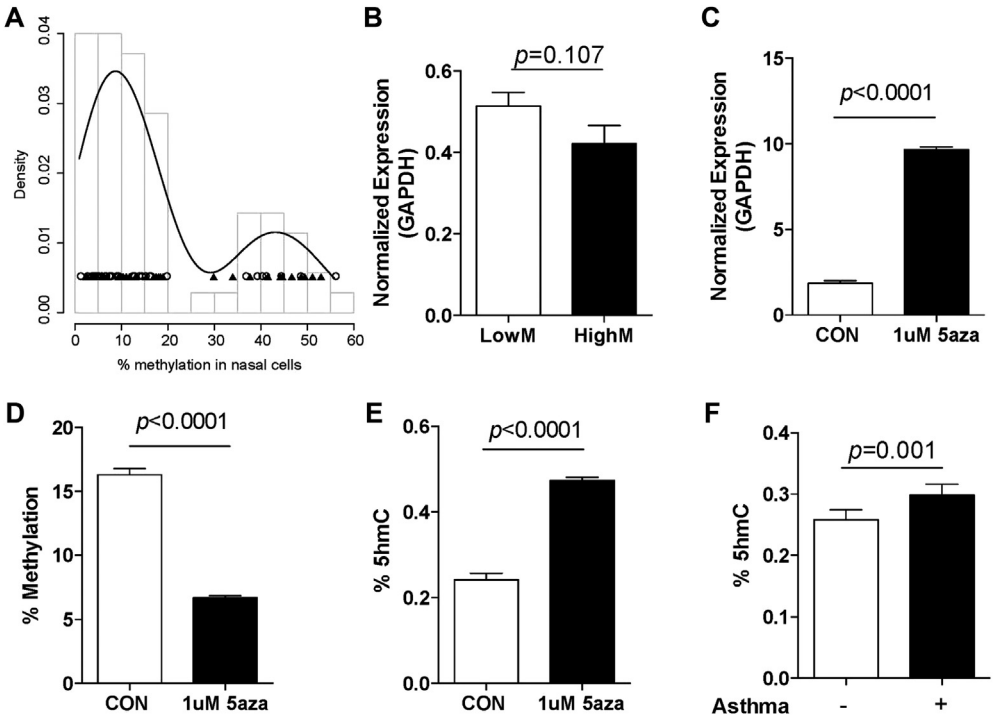

FIG 6. cg23602092 methylation is negatively correlated with *TET1* expression in nasal mucosa samples. **A,** Bimodal distribution of cg23602092 methylation shown as a histogram *(gray bars)* and as density *(solid curve)*. *Solid triangles*, Control subjects; *open circles*, asthmatic patients. **B,** *TET1* expression levels in nasal epithelial cells. *HighM*, Methylation greater than 20%; *LowM*, methylation less than 20%. Values are presented as means ± SEMs. **C-E,** *TET1* expression (Fig 6, *C*), cg23602092 methylation (Fig 6, *D*), and global 5-hmC levels (Fig 6, *E*) in HBECs treated with 1 μmol/L 5-aza-2′-deoxycytidine. Data represent 2 technical duplicates of 3 biological replicates and are shown as means ± SDs. **F,** 5-hmC levels in saliva DNA from 18 pairs of siblings from the ESS cohort. Values are presented as means ± SEMs.

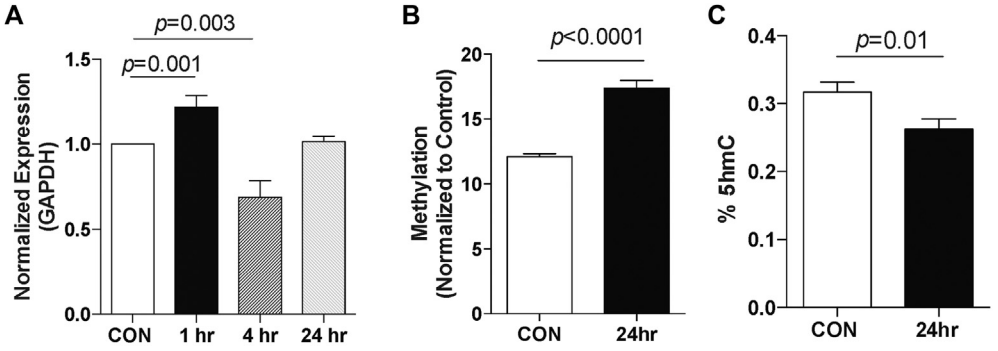

FIG 7. DEP exposure induced changes in cg23602092 methylation, *TET1* expression, and 5-hmC. **A,** *TET1* expression in HBECs cultured with DEPs (5 μg/cm^2) was measured and normalized to that in untreated control subjects at indicated time points. **B,** Percentage methylation values were measured and normalized to those of control subjects. **C,** 5-hmC ELISA. Data represent 3 technical replicates of biological duplicates and are shown as means ± SDs.

asthmatic symptoms.[9,10,13] Recently, it was suggested that the association between TRAP and asthma is mediated by epigenetic mechanisms.[28] Given the shared mechanisms and co-occurrence of asthma and TRAP exposure, it is expected that the correlation of *TET1* with asthma and TRAP will be in the same direction. However, in our study, nonasthmatic children with current exposure to higher TRAP levels demonstrated increased methylation at the *TET1* promoter, whereas children with asthma exhibited lower *TET1* methylation levels. Because the current study used a cross-sectional design, subjects were only examined at a particular time point. However, the biological processes are often dynamic and interrelated, and thus data from one time point cannot capture all dynamic changes. It is likely that the response of *TET1* to TRAP is time specific, as suggested by its response to acute DEP exposure in HBECs (Fig 7, *A*). Nevertheless, our findings clearly showed a role of *TET1* in asthmatic patients, and response to TRAP and future longitudinal studies are needed to further dissect the relationship between TRAP, DNA methylation, and asthma in conjunction with a cell-culture model of real-life TRAP exposure.

One of the strengths of our research design is the recruitment of African American sibling pairs discordant for asthma who were born and have been living in the same household. Asthma is a complex disease with many influencing risk factors, such as race, family history, socioeconomic status, and various environmental exposures, most of which have been shown to modify epigenetic landscapes.[15,78] The paired siblings allowed us to better control for genetic factors, as well as other unknown environmental factors, thus increasing the power to identify asthma-specific variation.

In conclusion, for the first time, our study demonstrated that DNA hypomethylation at the *TET1* promoter was associated with childhood asthma in African Americans. We also showed that methylation at the same CpG site was significantly associated with current exposure to TRAP in an opposite direction of the asthma association. Further studies are warranted to understand the underlying mechanism or mechanisms of the opposite directionality in these associations and to determine whether they are present in children of various races at crucial developmental windows. Our *in vitro* human cell line experiments support the involvement of *TET1* in response to DEP exposure. Further experiments will be needed to address the role of *TET1* in asthma development and response to TRAP exposure.

We thank the participating families and staff from the ESS and PEES, as well as the Genomics, Epigenomics, and Sequencing Core at the University of Cincinnati for array processing and the Cincinnati Children's Pyrosequencing Core Laboratory for Genomic and Epigenomic Research for assistance in pyrosequencing. We also thank the Cincinnati Children's Clinical and Translational Research Center for assistance in patient recruitment and sample collection.

Key messages

- **Increased *TET1* promoter methylation and global 5-hmC levels are associated with childhood asthma.**
- **Increased TRAP levels are associated with decreased *TET1* promoter methylation.**
- ***TET1* promoter methylation is conserved between different tissue types.**
- **There is a negative correlation between *TET1* methylation and its expression.**

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METHODS

DNA and RNA extraction

DNA and RNA were extracted from nasal epithelial cells with the AllPrep DNA/RNA Micro Kit (Qiagen, Hilden, Germany), according to the manufacturer's protocols. Saliva was collected with Oragene Saliva DNA and Oragene Saliva RNA kits (DNA Genotek, Ottawa, Canada). Blood was collected in one Serum Separator Tube (4 mL) and 2 Cell Preparation Tubes (8 mL; BD Vacutainer, BD Biosciences, Franklin Lakes, NJ) and taken to the laboratory for immediate processing. Serum from the Serum Separator Tube was separated, placed in aliquots, and then frozen. From the Cell Preparation Tubes, plasma was separated, placed in aliquots, and frozen. PBMCs (from the buffy coat layer) were put into new tubes, washed twice with cold 1× PBS, and then frozen for later use or pelleted by means of centrifugation for DNA and RNA extraction.

Cell-culture studies

HBECs[E1] were grown to confluence in keratinocyte serum-free medium (Life Technologies, Grand Island, NY) and supplemented with human recombinant epidermal growth factor and bovine pituitary extract. Cells were exposed to DEPs (5 μg/cm^2) or 5-aza-2′-deoxycytidine (1 μmol/L) for indicated times before harvest. DNA/RNA extraction from HBECs was performed with the AllPrep DNA/RNA Mini Kit (Qiagen), according to the manufacturer's protocols.

Illumina 450K array processing

Genomic DNA from the nasal cells of the 12 sibling pairs was bisulfite treated and assayed with the Illumina Infinium HumanMethylation450 BeadChip (Illumina). Quality of the array was assessed by using sample-independent and dependent internal control probes included on the array for staining, extension, hybridization, specificity, and bisulfite conversion. All the samples passed quality control and were included in the discovery analysis. The signal intensities were then background adjusted and normalized by using the methylation module and used to calculate β values as follows:

$$\beta = \frac{Signal_{methylation}}{Signal_{methylation} + Signal_{unmethylation} + 100}.$$

In some analyses M values were also generated as follows:

$$M = log_2 \frac{Methylation\ \%}{1 - Methylation\ \%}$$

and examined. The following CpG sites were excluded from analysis: (1) CpG sites that were not detected in all samples at a *P* value of .01; (2) CpG sites on X and Y chromosomes; (3) CpG sites that have <5 bead numbers in 1 or more samples; and (4) CpG sites with single nucleotide polymorphisms present nearby (>10 or ≤10 bp from query site). These procedures resulted in 24 samples and 312,516 CpG sites for analyses.

Bisulfite pyrosequencing

A total of 200 ng of genomic DNA was subjected to sodium bisulfite treatment and purified by using the EZ DNA Methylation-Gold Kit (Zymo Research, Irvine, Calif), according to the manufacturer's specifications. A standard bisulfite PCR amplification reaction was performed to amplify the *TET1* gene fragment at an annealing temperature of 50°C. Pyrosequencing was carried out with Pyro Gold reagents with a PyroMark vacuum prep workstation and a PyroMark Q96 MD instrument (Qiagen, Valencia, Calif), according to the manufacturer's instructions. The generated pyrograms were automatically analyzed with PyroMark analysis software (Qiagen). The pyrosequencing assay was validated by using SssI-treated human genomic DNA as a 100% methylation control, and human genomic DNA was amplified by using the GenomePlex Complete WGA kit (Sigma, St Louis, Mo) as a 0% methylation control. *TET1* pyrosequencing assay design and genomic coordinates are as documented in Table E1.

Quantitative PCR

Total RNA isolated as described above was reverse transcribed to cDNA with the Superscript III Kit (Life Technologies) by using random hexamers, according to the manufacturer's instructions. Real-time quantitative PCR was performed with the SYBR Green Master Kit and LightCycler 480 instrument (Roche Diagnostics, Indianapolis, Ind). PCR was carried out in triplicate from each fraction, and the mean cycle threshold (Ct) value of the triplicate reaction was normalized against the mean Ct value of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Primer sequences for *TET1* and *GAPDH* are described in Table E2.

5-hmC ELISA

Quantitative estimation of saliva 5-hmC levels was performed by using the Quest 5-hmC DNA ELISA kit (Zymo Research). A 96-well plate was coated overnight at 4°C with anti–5-hydroxymethylcytosine polyclonal antibody (200 ng/well) and then blocked for 2 hours at 37°C. Denatured control and sample DNA (200 ng) were added in duplicate and incubated for 1 hour at 37°C. The plate was washed, and then 100 μL of diluted anti-DNA HRP antibody (1:100 in 1× ELISA buffer) was added to each well and incubated for an hour at 37°C. The plate was washed again. Color developer was added and incubated for 15 minutes at room temperature, and the absorbance was measured with a Hybrid Multi-Mode Microplate Reader at 450 nm. Unknown saliva 5-hmC concentrations were determined from a standard curve plotted by using assay standards in the range of 0% to 0.55%.

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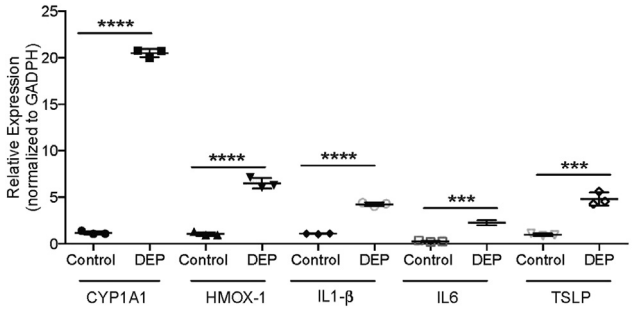

FIG E1. DEP exposure in HBECs induced expression of genes encoding cytoprotective enzymes and proinflammatory cytokines. HBECs were treated with 5 $\mu g/cm^2$ DEPs, and cells were harvested at 4 hours for RNA extraction and quantitative PCR. Mean values from technical duplicates of 3 biological replicates are presented as means ± SDs. ***$P < .001$ and ****$P < .0001$.

TABLE E1. Primers used for pyrosequencing of cg23602092

Primer	Sequence (5′→3′)	Chromosomal coordinates†	
		Chromosome	Location
Forward	TTTTGGATTTTTTTGTTTGTGTTAT	10	70319645
Reverse	ACAAAATTCATCATAATATTTCTATTAATT		
N_Forward	AATGTTATTGGTTATTTTTTTTAATT		
N_Reverse*	TTAACCCTAAACCTCAATAATAATC		
Sequencing	TTGTAGTGGGGGTTAGGAGGGATTG		

*5′ Biotinylated.
†Chromosomal coordinates are based on the UCSC Genome Browser Human February 2009 Assembly (hg19).

TABLE E2. Primers used for real-time quantitative PCR

Gene	Forward	Reverse
TET1	5′-CCCGGGCTCAAAGTTGTG-3′	5′-GCAGGAAACAGAGTCATTGGTCCT-3′
GAPDH	5′-GGGGAAGGTGAAGGTCGGAGTCA-3′	5′-AGCCTTGACGGTGCCATGGAAT-3′

GAPDH, Glyceraldehyde-3-phosphate dehydrogenase.

TABLE E3. Compare 12 and 23 pairs of the ESS cohort

	ESS		
	n = 46	n = 24	*P* value
Age	11.0 (10.0-13.0)	11.0 (9.0-14.5)	.87
Sex			.13
Male	28 (61%)	10 (42%)	
Asthma			1.00
Yes	23 (50%)	12 (50%)	
Secondhand smoke			.18
Yes	19 (41%)	14 (58%)	
BMI percentile	69.4 (30.4-97.4)	77.2 (56.4-95.2)	.33
Overweight/obese			.59
Yes	18 (39%)	11 (46%)	
Allergy			.73
Yes	18 (39%)	8 (35%)	
ECAT during infancy	0.40 (0.34-0.50)	0.45 (0.34-0.52)	.51
Current ECAT	0.34 (0.30-0.40)	0.35 (0.33-0.46)	.10

Note: Age, BMI percentile, ECAT value during infancy, and current ECAT value are shown as medians (interquartile ranges) and compared by using the Wilcoxon rank sum test. Other variables are shown as numbers (percentages) and compared by using χ^2 tests.
BMI, Body mass index.