Nasal DNA methylation profiling of asthma and rhinitis

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Background: Epigenetic signatures in the nasal epithelium, which is a primary interface with the environment and an accessible proxy for the bronchial epithelium, might provide insights into mechanisms of allergic disease.

Objective: We aimed to identify and interpret methylation signatures in nasal epithelial brushes associated with rhinitis and asthma.

Methods: Nasal epithelial brushes were obtained from 455 children at the 16-year follow-up of the Dutch Prevention and Incidence of Asthma and Mite Allergy birth cohort study. Epigenome-wide association studies were performed on children with asthma, rhinitis, and asthma and/or rhinitis (AsRh) by using logistic regression, and the top results were replicated in 2 independent cohorts of African American and Puerto Rican children. Significant CpG sites were related to environmental exposures (pets, active and passive smoking, and molds) during secondary school and were correlated with gene expression by RNA-sequencing (n = 244).

Results: The epigenome-wide association studies identified CpG sites significantly associated with rhinitis (n = 81) and AsRh (n = 75), but not with asthma. We significantly replicated 62 of 81 CpG sites with rhinitis and 60 of 75 with AsRh, as well as 1 CpG site with asthma. Methylation of cg03565274 was negatively associated with AsRh and positively associated with exposure to pets during secondary school. DNA methylation signals associated with AsRh were mainly driven by specific IgE–positive subjects. DNA methylation related to gene transcripts that were enriched for immune pathways and expressed in immune and epithelial cells. Nasal CpG sites performed well in predicting AsRh.

Conclusions: We identified replicable DNA methylation profiles of asthma and rhinitis in nasal brushes. Exposure to pets may affect nasal epithelial methylation in relation to asthma and rhinitis. (J Allergy Clin Immunol 2020;145:1655-63.)

Key words: *Asthma, rhinitis, united airways, epigenetics, environmental exposure*

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Abbreviations used
ANO1: Anoctamin 1 gene
AsRh: Asthma and/or rhinitis
AUC: Area under the curve
CISH: Cytokine inducible SH2 containing protein gene
CYP27B1: Cytochrome p450 family 27 subfamily b member 1 gene
DMR: Differentially methylated regions
eQTM: Expression quantitative trait DNA methylation
EVA-PR: Epigenetic Variation and Childhood Asthma in Puerto Ricans
EWAS: Epigenome-wide association study
FDR: False discovery rate
FBXL7: F-box and leucine rich repeat protein 7 gene
GJA4: Gap junction protein alpha4 or connexin 37 gene
NCF2: Neutrophil cytosolic factor 2 gene
NTRK1: Neurotrophic tyrosine kinase receptor 1 gene
PCSK6: Proprotein convertase subtilisin/kexin type 6 gene
PIAMA: Prevention and Incidence of Asthma and Mite Allergy
QC: Quality control
scRNAseq: Single-cell RNA-sequencing
TREM1: Triggering receptor expressed on myeloid cells 1 gene
ZMYND10: Zinc finger MYND-type containing 10 gene

The dramatic increase in the prevalence of allergic disease over the past 50 years in westernized countries indicates that environmental exposures may play an important role in the development of allergic disease.[1] Epigenetic variation such as DNA methylation changes might mediate these environmental effects.[2] DNA methylation refers to the addition of a methyl group to DNA, which may regulate gene expression.

In recent epigenome-wide association studies (EWASs) of white blood cells from participants in a multinational consortium, Xu et al identified 14 CpG sites significantly associated with childhood asthma.[3] The airway epithelium is also a highly relevant tissue to study allergic respiratory diseases (eg, asthma and rhinitis), as it is the first barrier to inhaled environmental agents.[4,5] Moreover, current evidence suggests that nasal epithelial cells can be used as a proxy for bronchial epithelial cells in the lower airways,[6,7] which are not easily accessible in children.

A study of 72 predominantly African American children identified associations between nasal epithelial DNA methylation markers and allergic asthma, providing a basis for methylation studies in larger populations.[8] Our previous study showed highly replicable associations between nasal epithelial DNA methylation and atopy and atopic asthma.[9] However, the role of rhinitis in relation to nasal DNA methylation is less clear. Rhinitis and asthma often coexist, and a recent study that combined asthma, rhinitis, and eczema as a shared phenotype suggested strong genetic overlap among these diseases, supporting the concept of a united airway disease.[10] Moreover, investigations of the comorbidity of asthma, rhinithis, and eczema indicated that the overlap between these studies is partly explained by IgE sensitization, but is also partly explained by non–IgE-dependent mechanisms.[11]

In the present study, we hypothesized that DNA methylation profiles of the nasal epithelium are associated with rhinitis and asthma. We considered the possibility of shared epigenetic associations of asthma and rhinitis, and we tested this possibility by combining asthma and rhinitis into 1 shared asthma and/or rhinitis (AsRh) phenotype. To test this hypothesis, we conducted EWAS in 16-year-old participants of the Dutch PIAMA (Prevention and Incidence of Asthma and Mite Allergy) birth cohort,[12] and replicated our top findings in the Inner City Asthma Study and the Epigenetic Variation and Childhood Asthma in Puerto Ricans (EVA-PR) study. In addition, we developed and validated nasal methylation–based prediction models for rhinitis and AsRh. We subsequently functionally interpreted our findings by using matched nasal brush bulk and single-cell RNA sequencing (scRNAseq) data. We finally investigated 4 different environmental exposures relevant to AsRh in relation to our significant DNA methylation signals.

METHODS

A full description of the methods used in this study is provided in the Online Data Supplement, which is available in this article's Online Repository at www.jacionline.org.

Study population and phenotypes

The discovery analysis was performed in the PIAMA birth cohort at age 16 years.[12] Asthma was defined as the presence of at least 2 of the following 3 criteria: (1) doctor-diagnosed asthma ever, (2) wheeze in the past 12 months, and (3) prescription of asthma medication in the past 12 months. Rhinitis was defined as the presence of sneezing or a runny or stuffed nose without having a cold in the previous 12 months or the presence of hay fever in the previous 12 months. AsRh was defined as the presence of either asthma or rhinitis. Levels of serum-specific IgE to house dust mite, cat, dactylis (grass), and birch were measured and classified as positive if at least 0.35 IU/mL. Pet exposure was defined as the presence of furry pets (dog, cat, or rodent) in the home during secondary school.

Nasal DNA methylation measurements and RNA sequencin

DNA and RNA were extracted from nasal brushing samples collected from the lower inferior turbinate. Genome-wide DNA methylation was determined with use of Infinium HumanMethylation450 BeadChips (Illumina, San Diego, Calif). After quality control (QC), 455 samples and 436,824 probes remained; M values were used in downstream analyses. We performed replication analyses in 2 cohorts: 72 children from the US Inner City Asthma Study (GSE65205)[8] and 487 children from the EVA-PR study.

RNA-seq was performed on the Illumina HiSeq2500 platform. After QC, 326 subjects and 17,156 genes were retained. Raw counts were transformed to $\log_2$ counts per million.

Statistical analyses

Multivariable logistic regression was used for the analysis of DNA methylation and asthma, rhinitis, and AsRh, which was adjusted for age, sex, batch, study center, and surrogate variables.[13] Differentially methylated regions (DMRs) were identified by using comb-p[14] and DMRcate.[15] The top CpG sites (false discovery rate [FDR] <0.05) were selected for replication. If none of the sites met that significance criterion, we used a looser threshold ($P < 1 \times 10^{-4}$) to select potentially relevant CpG sites for replication. After replication, we performed inverse variance–weighted fixed-effects meta-analyses with METAL.[16] Successful replication was defined as CpG sites that showed significance in meta-analysis of the replication cohorts (Bonferroni correction; $P < .05$ divided by the number of tests) and passed epigenome-wide significance (Bonferroni correction; $P < 1.14 \times 10^{-7}$; 436,824 tests) in the meta-analysis of all studies. We performed stratified analysis of significant CpG sites in specific IgE–positive or specific IgE–negative patients compared with in allergy-free controls. We investigated the association of CpG sites associated with AsRh with environmental risk factors (active smoking, secondhand smoking, pets, and dampness and molds) during secondary school.

A logistic regression model with elastic net regularization[17] was used to predict current disease. The top CpG sites identified by EWAS, along with age and sex, were used to train the models that were subsequently tested in the EVA-PR cohort.

Replicated CpG sites were annotated by using the GREAT 3.0.0 software package.[18] We performed expression quantitative trait DNA methylation (eQTM) analysis in the *cis* region (±250 kb). Pathway analysis was performed by using ConsensusPathDB[19] with eQTM genes, and nasal brush scRNAseq of 4 subjects was used to annotate eQTM genes to cell types.

RESULTS

Characteristics of the study population

The characteristics are shown in Table I and Table E1 (for Table E1, see this article's Online Repository at www.jacionline.org). A total of 455 PIAMA participants were included in the analyses, which corresponds to 56.7% of the total 16-year follow-up population and 11.5% of the total PIAMA population (see Table E2 in this article's Online Repository at www.jacionline.org). The prevalences of asthma, rhinitis, and AsRh at age 16 years were 8.1%, 45.1%, and 46.4%, respectively. The combined AsRh phenotype was dominated by rhinitis (97.2 % of AsRh case patients had rhinitis, 17.5 % had asthma, and 14.7 % had both); 64.9% of children with AsRh showed positive IgE sensitization (see Fig E1 in this article's Online Repository at www.jacionline.org). The mean ages of the discovery and replication cohorts were 16 years (PIAMA), 15.5 years (EVA-PR study), and 11 years (Inner City Asthma study). The distribution of ethnicities of the study participants differed: in PIAMA, approximately 97% of children had European white ancestry; in the US Inner City study, approximately 92% were of African American ancestry; and in the EVA-PR study, 100% of the children included were Puerto Rican (Hispanic or Latino).

TABLE I. Characteristics of study populations from the discovery and replication cohorts

	Discovery cohort	Replication cohorts	
	PIAMA	Yang et al[8]	EVA-PR
Total	455	72	483
Age	16.3 ± 0.2	11.0 ± 0.9	15.5 ± 3.0
Male sex (%)	217 (47.7%)	36 (50.0%)	252 (51.8%)
Asthma (%)	37 (8.1%)	36 (50.0%)	237 (48.7%)
Rhinitis (%)	205 (45.1%)	NA	299 (61.4%)
AsRh (%)	211 (46.4%)	NA	352 (72.3%)
Allergen-specific IgE (%)	207 (45.5%)	36 (50.0%)	311 (63.9%)
Ethnicity			
Hispanic/Latino	0%	12.9%*	100%
African American	0%	91.7%*	0%
Non-Hispanic white	97.1%	6.9%*	0%
Other/missing	2.9%	4.2%*	0%
Environmental exposures†			
Pets	227/380 (59.7%)	NA	NA
Dampness and molds	55/430 (12.8%)	NA	NA
Active smoking	44/384 (11.5%)	0	5/483 (1.0%)
Secondhand smoking	47/384 (12.2%)	29 (40.3%)	NA

NA, Not available.
Numbers represent the numbers of participants (%) for categoric variables and means ± SDs for continuous variables.
Allergic respiratory disease is defined as the presence of AsRh.
*Does not add up to 100% because participants could report more than 1 ethnicity.
†Data shown as number of yes responses among all available samples (%); in the PIAMA cohort, the number represents participants exposed to the listed factors during secondary school.

EWAS discovery and replication in nasal epithelium

In total, 81 CpG sites were significantly associated with rhinitis and 75 were associated with AsRh (FDR <0.05) and were thus selected for replication. In addition, 95 CpG sites associated with asthma were selected for replication using a less stringent threshold ($P < 1.0 \times 10^{-4}$), because no CpG site passed the threshold of an FDR less than 0.05 (Figs 1 and 2 and see Fig E2 in this article's Online Repository at www.jacionline.org). Although no DNA methylation signal at the single–CpG site level was significantly associated with asthma, we identified 16 significant DMRs associated with asthma (see Table E3 in this article's Online Repository at www.jacionline.org). Moreover, significant DMRs associated with rhinitis (n = 20) and AsRh (n = 20) were identified (see Table E3).

After application of cohort-specific QC, 74 of the 95 CpG sites associated with asthma, 72 of the 81 CpG sites associated with rhinitis, and 66 of the 75 CpG sites associated with AsRh were available in the EVA-PR study. The US Inner City Asthma Study was able to assess all 95 CpG sites associated with asthma, but it did not include a rhinitis phenotype; therefore, this study participated only in the asthma replication.

Of the 95 asthma-associated CpG sites, 10 were significant in the meta-analysis of the 2 replication cohorts after Bonferroni correction (95 tests; $P < 5.26 \times 10^{-4}$) and were used in the downstream analysis. One CpG site, annotated to the *PDE6A* gene (cg08844313; $P = 6.72 \times 10^{-8}$), was statistically significantly associated with asthma after Bonferroni correction in the meta-analysis of all cohorts (see Table E4 in this article's Online Repository at www.jacionline.org). Of the 72 tested CpG sites, 62 associated with rhinitis and 60 of the 66 tested CpG sites associated with AsRh passed the genome-wide significance threshold with use of Bonferroni correction ($P < 1.14 \times 10^{-7}$) in the meta-analysis of all cohorts (Table II and see also Tables E5 and E6 this article's Online Repository at www.jacionline.org). The results were robust when different rhinitis definitions were used (see the Online Data Supplement and Tables E7 and E8, which are available in this article's Online Repository at www.jacionline.org).

In total, 68 unique CpG sites were identified as being associated with 1 or more phenotypes. Additional adjustment for sampling season did not change the results, indicating that sampling season was not a confounder (see Table E9 in this article's Online Repository at www.jacionline.org). None of the replicated probes showed significance in the Hartigan dip test,[20] indicating no significant SNP effect under the probe sequence; 8 of these were additionally validated by pyrosequencing (see the Online Data Supplement).[9] The Q-Q plots and inflation factors are shown in Fig E3 (available in this article's Online Repository at www.jacionline.org). The *P* values of the discovery CpG sites after BACON[21] correction are shown in Table E10 (which is available in this article's Online Repository at www.jacionline.org). Asthma-associated CpG sites also showed strong associations with rhinitis and AsRh (see Table E11 in this article's Online Repository at www.jacionline.org), and rhinitis-associated CpG sites showed strong associations with AsRh but were less strongly associated with asthma (see Table E11).

In stratified analysis, strong associations were observed in specific IgE–positive children and virtually no association in the IgE-negative children with AsRh when compared with the same

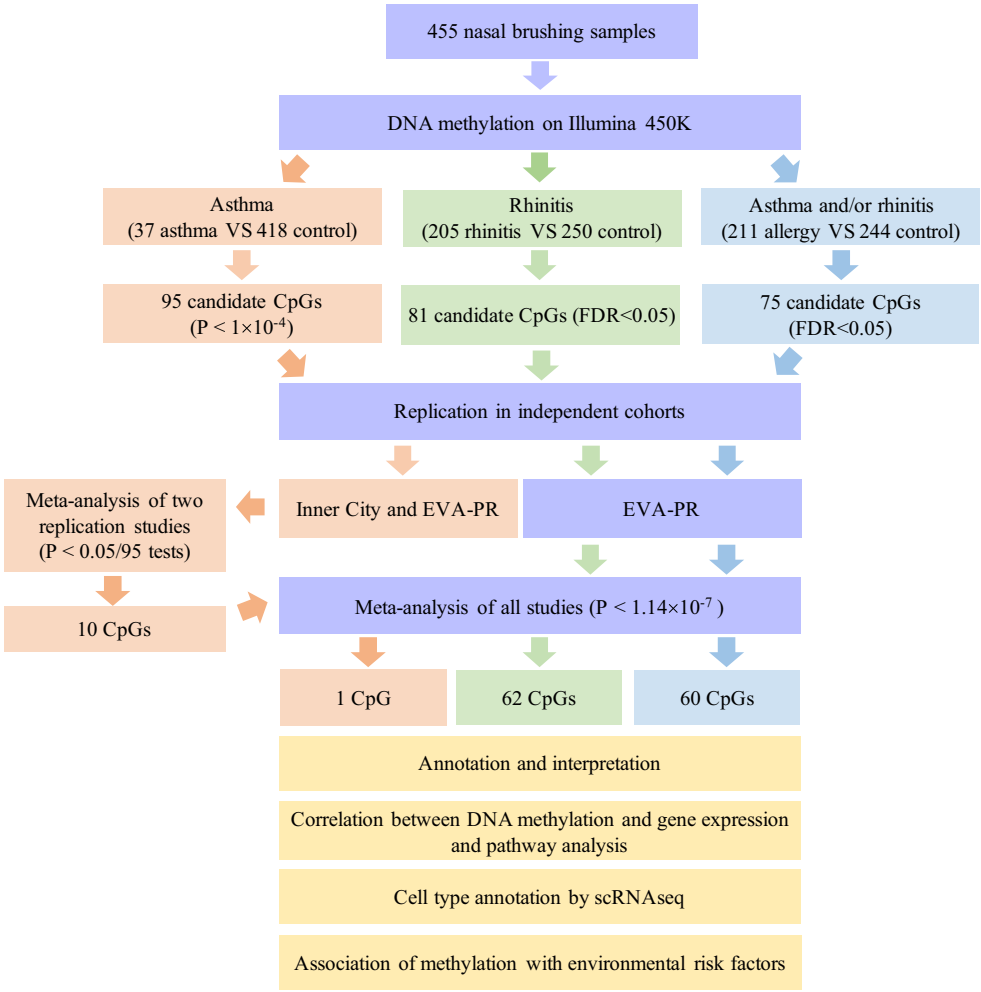

FIG 1. Study design. EWAS on 3 phenotypes (asthma, rhinitis, and AsRh) was conducted on 455 samples obtained by nasal brushing. Significant CpG sites (CpGs) with an FDR less than 0.05 were selected for replication. EWAS on asthma did not identify CpGs that passed the threshold of an FDR less than 0.05; therefore, a looser threshold *P* value of less than 10^{-4} was used to select CpGs for replication. After replication and meta-analysis, 123 CpGs (68 unique CpGs) were replicated. Matched nasal epithelial transcriptome data were analyzed to link the observed methylation to gene expression, whereas the functional enrichment analysis gave insight into potentially involved pathways. Nasal epithelium scRNAseq data were used to annotate eQTM genes to cell types. We investigated the relationship between CpGs associated with AsRh and 4 environmental risk factors (active smoking, secondhand smoking, pets, and dampness and molds).

controls who were specific IgE–negative and without AsRh (Table III). The same tendency was also found for asthma and rhinitis (see Tables E12 to E14 in this article's Online Repository at www.jacionline.org).

Prediction of asthma and rhinitis with methylation levels

We used CpG sites selected for replication to train the models. CpG sites that did not pass QC in the EVA-PR study were excluded so that the models could be tested independently. After training, the final sets consisted of 70 CpG sites in asthma prediction, 48 CpG sites in rhinitis prediction, and 26 CpG sites in AsRh prediction. The coefficients of the CpG sites in each model are shown in Table E15 (which is available in this article's Online Repository at www.jacionline.org). In the PIAMA cohort, the areas under the curve (AUCs) for asthma, rhinitis, and AsRh were 0.98, 0.74, and 0.70, respectively. In the EVA-PR study, we obtained AUCs of 0.55 for asthma, 0.67 for rhinitis, and 0.73 for AsRh. The ROC curve, sensitivity, specificity, PPV, and NPV from the discovery and replication cohorts are shown in Fig E4 (available in this article's Online Repository at www.jacionline.org).

Association between methylation and gene expression

Of 68 unique CpG sites, 24 were significantly associated with gene expression levels *in cis,* resulting in 66 unique CpG site–gene expression pairs, of which 29 pairs showed negative correlation (see Table E16 in this article's Online Repository at www.jacionline.org). The 66 CpG site–gene pairs include 59 unique genes, which were called eQTM genes. The most significant association ($P = 3.72 \times 10^{-11}$) was between the methylation level of cg18297196 and gene expression of *TREM1* (triggering receptor expressed on myeloid cells 1), a gene previously associated with asthma.[22]

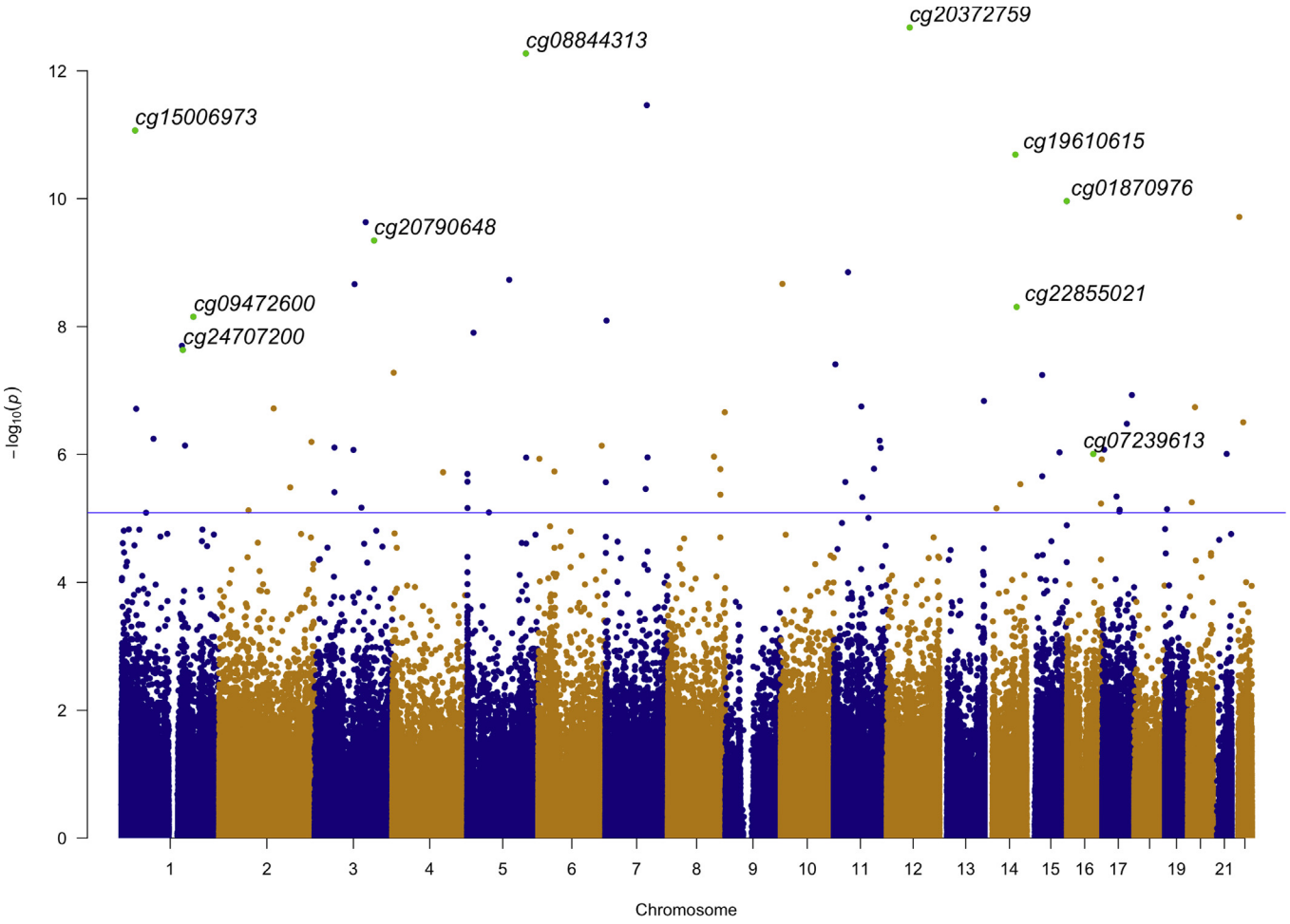

FIG 2. A Manhattan plot of association between AsRh and DNA methylation at 16 years with use of nasal epithelial samples in the PIAMA (discovery) cohort. In total, 436,824 CpG sites were tested. The blue line represents the FDR-corrected threshold (FDR <0.05) of significance. Highlighted sites represent the top 10 replicated CpG sites associated with asthma and/or rhinitis.

Pathway analysis

A total of 4 eQTM genes related to asthma were significantly enriched ($P < .01$) in 11 pathways (see Table E17 in this article's Online Repository at www.jacionline.org). In all, 57 eQTM genes related to rhinitis were significantly enriched in 23 pathways, of which 6 were related to immune function, including the microglia pathogen phagocytosis pathway, *DAP12* interactions, adaptive immune system, *IL-2* signaling pathway, T-cell receptor signaling pathway, and immune system (see Table E17). One pathway (bacterial invasion of epithelial cells) was related to epithelial function. A total of 25 pathways were enriched for 51 eQTM genes related to AsRh, and the aforementioned immune-related pathways were also found for AsRh (see Table E17).

Cell type annotation

We performed scRNAseq in independent nasal brush samples from 2 patients with asthma and 2 healthy controls[23] (see Table E18 in this article's Online Repository at www.jacionline.org). After stringent QC and doublet removal, we aligned the samples by using canonical correlation analysis on 2356 shared variable genes. Clustering these aligned samples produced 5 clusters. We annotated the clusters based on gene expression[23,24] (see Table E19 this article's Online Repository at www.jacionline.org) to represent 4 epithelial cell types (club, goblet, ciliated, and basal cells) and 1 cluster of mixed immune cells (see Fig E5 in this article's Online Repository at www.jacionline.org). This suggested that epithelial brushes yielded mostly epithelial cells in combination with some immune cells, with 7 eQTM genes (*DNALI1, ZMYND10, CCDC153, MEAF6, C11orf70, DUSP14,* and *APOBEC4*) that were also marker genes of ciliated cells and 1 gene (*RHOG*) that represents a marker gene of the immune cell cluster. Other eQTM genes did not show significant differential expression among cell clusters (see Fig E6 in this article's Online Repository at www.jacionline.org). To investigate whether the association of CpG methylation with AsRh was due to methylation differences within epithelial cells, we replicated our top CpG sites in nasal epithelial cells sorted by CD326 EpCAM microbeads in a small subset of the EVA-PR cohort (n = 31), and 13 of 60 CpG sites associated with AsRh remained nominally significant ($P < .05$) with the same direction (see Table E20 in this article's Online Repository at www.jacionline.org). In the sorted epithelial cells, 11 of 66 CpG site–gene pairs (eQTM) were also found to be nominally significant, with the same direction as in the bulk analysis ($P < .05$) (see Table E21 in this article's Online Repository at www.jacionline.org).

TABLE II. Description of the top 10 replicated CpG sites associated with AsRh

CpG site ID	CHR	bp position*	Discovery PIAMA		Replication1 EVA-PR		Meta-analysis, all†		GREAT gene annotation‡
			OR (95% CI)	*P* value	OR (95% CI)	*P* value	OR (95% CI)	*P* value	
cg20372759	12	58162287	0.15 (0.09-0.25)	2.10×10^{-13}	0.47 (0.38-0.57)	2.13×10^{-14}	0.41 (0.34-0.49)	1.60×10^{-22}	*METTL21B* (–4095), *CYP27B1* (–1254)
cg08844313	5	149240529	0.17 (0.10-0.27)	5.36×10^{-13}	0.43 (0.34-0.55)	3.80×10^{-13}	0.36 (0.30-0.44)	6.39×10^{-22}	*PDE6A* (+83826), *PPARGC1B* (+130656)
cg20790648	3	151619923	0.27 (0.18-0.41)	4.52×10^{-10}	0.48 (0.40-0.59)	1.56×10^{-13}	0.44 (0.37-0.52)	9.73×10^{-21}	*MBNL1* (–365905), *SUCNR1* (+28493)
cg15006973	1	35258933	0.08 (0.04-0.16)	8.58×10^{-12}	0.30 (0.22-0.42)	1.12×10^{-12}	0.24 (0.18-0.32)	1.86×10^{-20}	*GJA4* (+335)
cg24707200	1	156833163	0.08 (0.03-0.19)	2.32×10^{-8}	0.19 (0.12-0.30)	1.05×10^{-12}	0.16 (0.11-0.24)	6.26×10^{-19}	*INSRR* (–4354), *NTRK1* (+2478)
cg07239613	16	67051005	0.07 (0.02-0.20)	9.86×10^{-7}	0.11 (0.06-0.20)	1.12×10^{-12}	0.10 (0.06-0.16)	7.48×10^{-18}	*CBFB* (–12142), *CES4A* (+28514)
cg01870976	15	101887154	0.18 (0.11-0.30)	1.09×10^{-10}	0.52 (0.43-0.64)	2.71×10^{-11}	0.46 (0.38-0.55)	1.99×10^{-17}	*SNRPA1* (–51699), *PCSK6* (+142718)
cg09472600	1	183537770	0.20 (0.11-0.34)	7.03×10^{-9}	0.41 (0.32-0.53)	4.86×10^{-11}	0.36 (0.28-0.45)	3.37×10^{-17}	*NCF2* (+21945), *SMG7* (+96133)
cg22855021	14	81610812	0.19 (0.11-0.33)	4.93×10^{-9}	0.46 (0.37-0.59)	2.00×10^{-11}	0.41 (0.33-0.50)	4.14×10^{-17}	*GTF2A1* (+76453), *TSHR* (+189426)
cg19610615	14	78446340	0.07 (0.03-0.15)	2.05×10^{-11}	0.34 (0.25-0.48)	3.73×10^{-10}	0.27 (0.20-0.37)	4.69×10^{-17}	*NRXN3* (–423752), *ADCK1* (+179915)

ID, Identification number; *CHR*, chromosome; *OR*, odds ratio.
*bp postion refers to bp position according to Genome build 37.
†Meta analysis, all refers to meta-analysis of the discovery and replication cohorts.
‡GREAT gene annotation: CpG sites were annotated by GREAT, version 3.0.0 (Genomic Regions of Annotations Tool; http://bejerano.stanford.edu/great/); information on all replicated CpG sites for asthma, rhinitis, and AsRh is presented in Tables E4 to E6.

TABLE III. IgE-stratified analysis of the top 10 replicated CpG sites associated with AsRh

CpG site ID	Specific IgE–positive (137 case patients vs 155 controls)				Specific IgE–negative (70 case patients vs 155 controls)			
	Coef	SE	OR* (95% CI)	*P* value	Coef	SE	OR (95% CI)	*P* value
cg20372759	–7.37	0.91	0.48 (0.40-0.57)	5.15×10^{-16}	0.28	0.67	1.32 (0.36-4.92)	.68
cg08844313	–2.74	0.35	0.76 (0.71-0.81)	3.62×10^{-15}	–0.45	0.33	0.64 (0.33-1.22)	.17
cg20790648	–4.01	0.50	0.67 (0.61-0.74)	8.69×10^{-16}	0.45	0.53	1.57 (0.55-4.43)	.39
cg15006973	–7.09	0.90	0.49 (0.41-0.59)	2.57×10^{-15}	–0.72	0.65	0.49 (0.14-1.74)	.27
cg24707200	–5.39	0.76	0.58 (0.50-0.68)	1.20×10^{-12}	0.83	0.81	2.29 (0.47-11.22)	.30
cg07239613	–4.97	0.77	0.61 (0.52-0.71)	1.39×10^{-10}	0.37	0.79	1.45 (0.31-6.81)	.64
cg01870976	–5.80	0.73	0.56 (0.49-0.65)	1.87×10^{-15}	–0.22	0.66	0.80 (0.22-2.93)	.74
cg09472600	–3.78	0.51	0.69 (0.62-0.76)	1.19×10^{-13}	–0.67	0.51	0.51 (0.19-1.39)	.19
cg22855021	–3.41	0.50	0.71 (0.64-0.78)	6.26×10^{-12}	–0.35	0.51	0.70 (0.26-1.91)	.49
cg19610615	–5.21	0.71	0.59 (0.52-0.68)	2.05×10^{-13}	–1.22	0.72	0.30 (0.07-1.21)	.09

ID, Identification number; *Coef*, coefficient; *OR*, odds ratio.
OR*(95% CI) refers to the OR and 95% CI of 10% of the absolute change in methylation level of the M value; OR (95% CI) refers to the OR and 95% CI of 1 absolute change in methylation level of the M value. Specific IgE–positive refers to case patients with specific IgE–positive AsRh versus AsRh-free, IgE-negative controls; specific IgE–negative refers to case patients with specific IgE–negative AsRh versus AsRh-free, IgE-negative controls. A total of 23 subjects for whom there were no IgE sensitization data and 70 subjects who were IgE-positive and had no AsRh were not included in this analysis.

Association between environmental risk factors and nasal methylation levels

We investigated the association between 4 environmental factors relevant for allergic disease (active smoking, secondhand smoking, exposure to pets, and dampness and molds in the house) during secondary school and the 60 replicated AsRh-associated CpG sites and identified 1 CpG site (cg03565274) that showed significant positive association with pet exposure ($P = 7.57 \times 10^{-4}$), which passed Bonferroni correction (Table IV and see also Table E22 in this article's Online Repository at www.jacionline.org) and had a negative correlation with AsRh. We next investigated the association of nasal DNA methylation level of this CpG site at 16 years with pet exposure in different time windows from birth onward and observed consistent patterns from infancy to secondary school: children exposed to pets from birth onward had higher DNA methylation levels at this CpG site (see Tables E23 and Fig E7, both of which are available in this article's Online Repository at www.jacionline.org). This CpG site, cg03565274, showed positive correlation with expression levels of *ZMYND10* (zinc finger MYND-type containing 10). The *ZMYND10* gene was found by scRNAseq to be highly expressed in ciliated cells (Fig 3). We also checked the direction of all 60 CpG sites associated with AsRh and found that 56 of the 60 CpG sites had a positive association with pets ($P < .001$; Monte Carlo resampling method). Active smoking, secondhand smoking and dampness and molds were not significantly associated with the 60 CpG sites.

TABLE IV. Association between methylation level of CpG sites associated with AsRh in nasal epithelium and 4 environmental factors during secondary school (top 10 CpG sites for each environmental factor)

Active smoking (n = 381)			Secondhand smoking (n = 384)			Pets (n = 380)			Dampness and molds (n = 430)		
CpG ID	Coef	*P* value	CpG ID	Coef	*P* value	CpG ID	Coef	*P* value	CpG ID	Coef	*P* value
cg11058904	0.11	5.64×10^{-2}	cg23005227	0.09	2.93×10^{-2}	cg03565274*	0.07	7.57×10^{-4}	cg12875548	–0.17	1.55×10^{-3}
cg25020944	–0.06	6.13×10^{-2}	cg06675531	–0.07	4.21×10^{-2}	cg23387401	0.13	1.23×10^{-3}	cg27058763	–0.08	1.06×10^{-2}
cg04206484	0.23	6.31×10^{-2}	cg01062020	0.19	5.82×10^{-2}	cg24707200	0.07	1.38×10^{-3}	cg03668556	–0.14	4.07×10^{-2}
cg07686035	0.08	6.52×10^{-2}	cg03668556	–0.10	.11	cg08844313	0.14	3.25×10^{-3}	cg04206484	–0.21	5.37×10^{-2}
cg24224501	0.06	7.83×10^{-2}	cg08175352	–0.10	.13	cg10054641	0.12	5.72×10^{-3}	cg08175352	–0.12	6.09×10^{-2}
cg00664723	0.13	8.14×10^{-2}	cg27058763	–0.05	.16	cg20372759	0.11	9.48×10^{-3}	cg00664723	–0.12	6.58×10^{-2}
cg10549071	0.12	8.56×10^{-2}	cg04206484	0.13	.19	cg19610615	0.07	1.35×10^{-2}	cg12716639	–0.08	8.67×10^{-2}
cg00049323	–0.09	9.44×10^{-2}	cg01870976	–0.08	.20	cg22855021	0.09	2.16×10^{-2}	cg07239613	–0.05	9.20×10^{-2}
cg23005227	0.08	.10	cg21291385	0.07	.23	cg10549071	0.10	2.51×10^{-2}	cg09562938	–0.05	.11
cg12875548	0.10	.11	cg04891688	0.06	.25	cg01062020	0.18	2.53×10^{-2}	cg21291385	–0.10	.12

ID, Identification number; *Coef*, coefficient.
*CpG site passed Bonferroni correction. Information on association between all 60 CpG sites associated with AsRh and 4 environmental factors is presented in the Table E22.

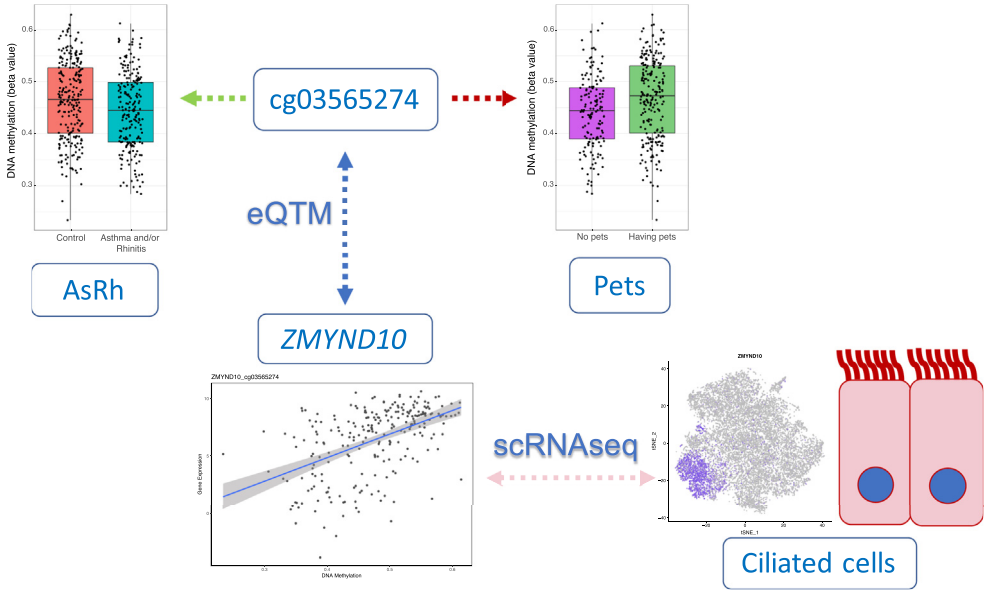

FIG 3. The relationship among As/Rh, DNA methylation, environmental factors (pets), gene expression, and nasal epithelial cell type. The methylation level of cg03565274 was negatively correlated with AsRh status and positively correlated with pets. Methylation levels of cg03565274 was also positively correlated with expression level of the gene *ZMYND10*, which is highly expressed in ciliated cells.

DISCUSSION

This EWAS of cells obtained by nasal brushings identified replicable DNA methylation profiles associated with asthma and rhinitis. We observed a strong overlap between nasal methylation profiles associated with asthma and rhinitis and showed that these epigenetic profiles were mainly driven by children with IgE sensitization to aeroallergens. Our results also implicate an epigenetic association of pet exposure on nasal DNA methylation in relation to the development of asthma and rhinitis. Finally, our results show that nasal methylation patterns can be used across different populations to predict the presence of asthma and rhinitis in children.

The nasal epithelium is considered a noninvasive proxy for bronchial epithelium in children,[6,7] and it has been used as target tissue to study asthma.[8,25] However, rhinitis is highly prevalent and shows comorbidity and shared genetic origins with asthma.[10,11,26] Taking the shared mechanisms of asthma and rhinitis into consideration, we used a combined phenotype of asthma and rhinitis (AsRh). In our study, 83.8% of patients with asthma also had rhinitis, which may explain why a significant proportion of nasal DNA methylation signals related to rhinitis also showed association with asthma. Thus, it is important to consider the presence of rhinitis when assessing the association of DNA methylation with asthma in nasal epithelium.

IgE is a key mediator of allergic disease, and epigenetic markers associated with total serum IgE have been identified in blood.[27,28] However, part of the overlap between asthma and rhinitis is due to non–IgE-mediated mechanisms.[11] Considering this, we defined the main phenotypes by symptoms of asthma and rhinitis but did not include IgE. In addition, we did an IgE-stratified analysis of replicated CpG sites; the results showed that DNA methylation signals in nasal epithelium were mainly driven by IgE-positive subjects with AsRh and not by IgE-negative subjects with AsRh. This indicates that the signals we identified were mainly associated with IgE sensitization and not driven by the presence of AsRh symptoms. These results are consistent

with the findings of Forno et al, who identified a strong correlation between IgE sensitization and DNA methylation profiles in nasal epithelium.[9] In fact, when the results of our clinical AsRh definition were compaed with their IgE sensitization results, 21 of 60 CpG sites associated with AsRh were also on their list of the top 30 CpG sites. Both results indicate that nasal DNA methylation might be a biomarker for IgE sensitization.

When we compared our results with those of another recent nasal EWAS,[29] only 2 of our AsRh-associated CpG sites were also on their list of significant CpG sites for IgE sensitization, and none of the rhinitis-associated CpG sites was present in their results for rhinitis. Reasons for this may be that the prevalence of rhinitis was lower in their cohort (~17%) and that they used nasal swab samples from the anterior nares, whereas we used nasal brushes from the inferior turbinate, which may be different in cell type composition.

A total of 8 CpG sites in nasal epithelium showed association with all 3 phenotypes, 5 of which are near known biologically plausible genes related to allergic disease, including *NCF2* (neutrophil cytosolic factor 2), which is involved in the oxidative stress pathway and related to asthma[30]; *NTRK1* (neurotrophic tyrosine kinase receptor 1), an epigenetic target of IL-13 involved in allergic inflammation[31]; *GJA4* (gap junction protein alpha4 or connexin 37), the expression of which has been associated with airway inflammation and bronchial hyperresponsiveness[32]; *CYP27B1* (cytochrome p450 family 27 subfamily b member 1), an enzyme, the activity of which has been associated with IgE-dependent mast cell activation[33]; and *ANO1* (anoctamin 1), which is related to chloride conductance in airway epithelial cells and is upregulated in epithelial cells of patients with asthma.[34]

DNA methylation may be related to gene expression. We therefore examined whether DNA methylation was associated with local gene expression by *cis*-eQTM analyses. Association was found for 24 of the 68 investigated CpG sites. The most significant negative association was cg18297196-*TREM1*. *TREM1*-associated neutrophilic signaling pathway proteins have been reported to be significantly suppressed in eosinophilic nasal polyps of patients with chronic rhinosinusitis.[35] A total of 20 CpG site–gene pairs showed significant association between CpG sites and genes where the CpG sites were located, including *PCSK6* (proprotein convertase subtilisin/kexin type 6), *FBXL7* (F-box and leucine rich repeat protein 7), and *CISH* (cytokine inducible SH2 containing protein). The following genes have previously been associated with allergic diseases or inflammation: *PCSK6*, which can activate the NF-κB signaling pathway and is involved in the inflammatory response[36]; *FBXL7*, expression of which is involved in the inhaled corticosteroid response in asthma[37]; and *CISH*, which showed increased expression levels in human airway eosinophils after allergen challenge.[38] Genes identified by eQTM were enriched in pathways related to immune functions and inflammatory responses.

DNA methylation can be cell type–specific. We determined that the majority of cells in the nasal epithelial brushes were epithelial cells, with some contribution of immune cells by scRNAseq. Indeed, we were able to show that 13 CpG sites were associated with AsRh in isolated nasal epithelial cells, confirming DNA methylation changes within the airway epithelium in rhinitis and asthma.

The DNA methylation profiles identified in nasal epithelium performed well in predicting rhinitis and AsRh, and they showed similar performance in the replication cohort. The prediction model for asthma did not perform well in the replication cohorts, which possibly can be explained by overfitting in the discovery cohort because PIAMA is an unselected birth cohort with low prevalence of asthma. However, our model was still able to classify subjects with rhinitis (AsRh) with an AUC larger than 0.6 (0.7) across different populations with different ethnicities, which indicates that nasal methylation signals can help to predict rhinitis and AsRh in children, especially for IgE-positive AsRh.

We found that residential pet exposure at secondary school age was positively associated with current nasal methylation levels of cg03565274, whereas its methylation level was negatively associated with AsRh. Thus, subjects having pets and subjects without AsRh have higher methylation levels at this site. This pattern was consistent from infancy to the secondary school period, which may suggest that environmental exposures could affect DNA methylation in the nasal epithelium, which may have protective effects on AsRh. Several studies found that pet exposure in early life was associated with a lower risk of developing asthma and allergic diseases in children of both school age and preschool age.[39-41] However, studies also showed that parents with allergy may tend to avoid pets, especially cats, in their family,[42,43] which may be an alternative explanation for our finding. Further studies are needed to disentangle the causal effects of pet exposure on DNA methylation and the development of asthma and rhinitis. Methylation levels of cg03565274 were positively correlated with expression level of the gene *ZMYND10*, which is highly expressed in ciliated cells. *ZMYND10* is related to primary ciliary dyskinesia, which causes respiratory distress and impaired mucociliary clearance[44] but has not been previously reported to be associated with asthma or rhinitis. Our findings could indicate that methylation-related expression of *ZMYND10* in AsRh is lower in nasal epithelial cells, or alternatively, it may be explained by a lower subset of differentiated ciliated cells in AsRh compared with in healthy controls, as was recently discovered in patients with chronic rhinosinusitis through use of scRNA-seq.[24] We also investigated active smoking, secondhand smoking, and molds and dampness, which were also reported to be potential risk factors for allergic disease[45,46]; however, we did not identify significant associations between these exposures and CpG sites associated with AsRh in this study.

Despite the overall robustness of our study findings, there are some limitations to consider. First, we had relatively low power in our asthma analysis on account of the low prevalence of asthma. Consequently, the results forAsRh were largely overlapping with the results for rhinitis. Second, our single-cell analyses were performed on a small data set (4 individuals); therefore, we did not have enough power to disentangle the immune cell types and instead present results for 1 mixed immune cell cluster. Third, our prediction models were trained in a limited age range (around 16 years of age), and then were replicated in a wider age range (9 to 20 years of age), which may underestimate the performance of the prediction model. Finally, using the current data, we were not able to investigate whether DNA methylation mediates the effect of pet exposure on the development of asthma and rhinitis.

In conclusion, our study shows replicable DNA methylation sites in nasal brushes that may serve as biomarkers of asthma and rhinitis, and it provides the first indication that early pet exposure may have an impact on development of asthma and rhinitis later in life.

Clinical implications: Nasal DNA methylation profiles may serve as biomarkers of asthma and rhinitis and can be used across different populations to predict the presence of asthma and/or rhinitis in children.

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