Multi-omics approaches to understand respiratory disease

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CHAPTER 4

4

Nasal DNA methylation profiling of asthma and rhinitis

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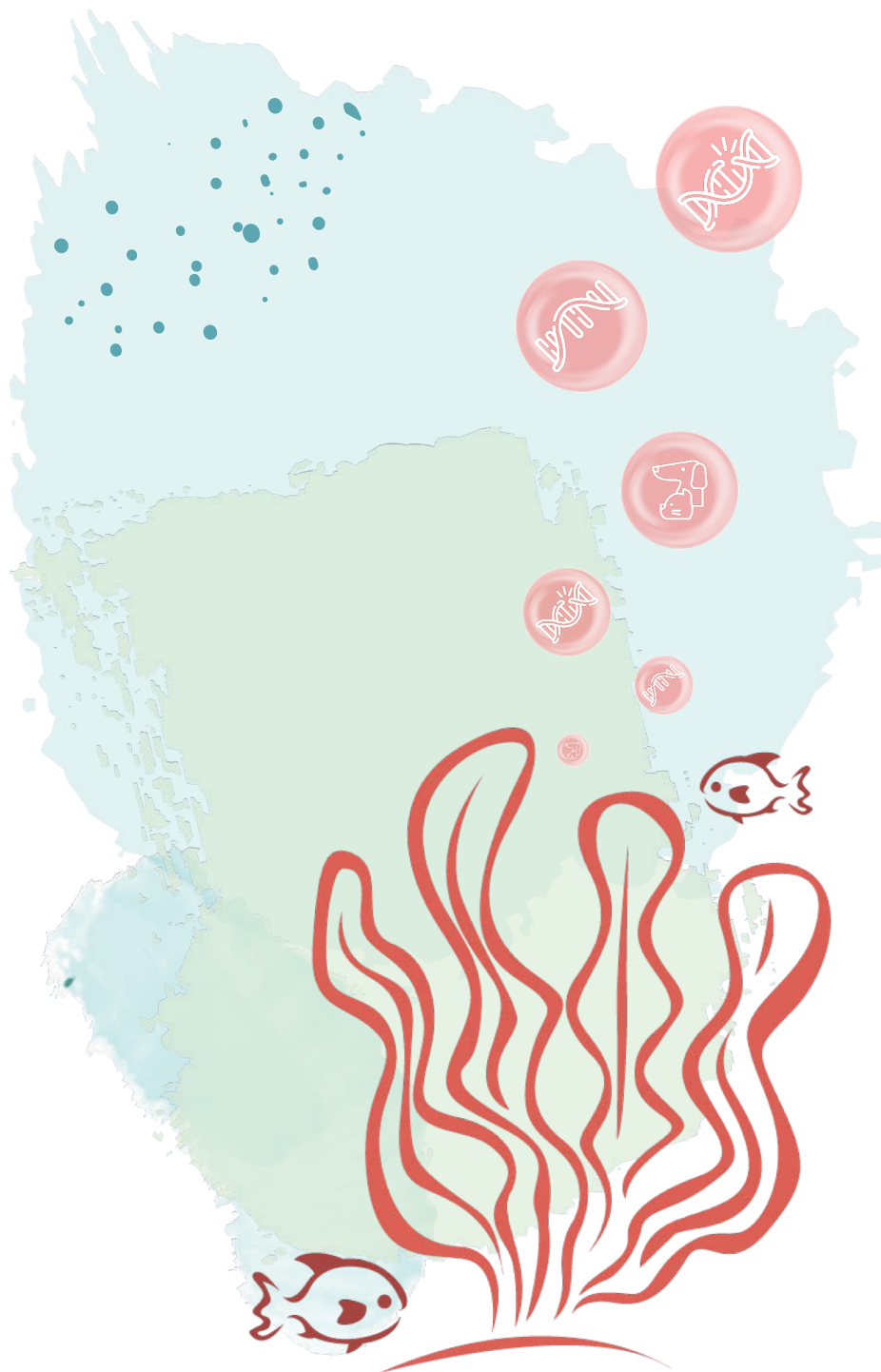
Abstract

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 PIAMA birth cohort study. Epigenome-wide association studies (EWAS) were performed on asthma, rhinitis and asthma and/or rhinitis (AsRh) using logistic regression, and top results were replicated in two independent cohorts of African American and Puerto Rican children. Significant CpG sites (CpGs) were related to environmental exposures (pets, active and passive smoking and molds) during secondary school, and correlated to gene expression by RNA-sequencing (n=244).

Results: The EWAS identified CpGs significantly associated with rhinitis (n=81) and AsRh (n=75), but not with asthma. We significantly replicated 62 /81 CpGs with rhinitis, and 60/75 with AsRh, as well as one CpG with asthma. Methylation of cg03565274 was negatively associated with AsRh, and positively associated with pets exposure 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 CpGs performed well in predicting AsRh.

Conclusions: We identified replicable DNA methylation profiles of asthma and rhinitis in nasal brushes. Pets exposure may affect nasal epithelial methylation in relation to asthma and rhinitis.

Key Words: asthma, rhinitis, united airways, epigenetics, environmental exposure

Introduction

The dramatic increase in the prevalence of allergic disease over the last 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 (EWAS) of white blood cells from participants in a multinational consortium, Xu *et al.* identified 14 CpGs significantly associated with childhood asthma[3]. The airway epithelium is also a highly relevant tissue to study allergic respiratory diseases (e.g. 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 of 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 co-exist, and a recent study, which 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, rhinitis and eczema indicated that the overlap between these studies is partly explained by IgE sensitization, but also 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 tested this by combining asthma and rhinitis into one 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 study in Puerto Ricans Study (EVA-PR). In addition, we developed and validated nasal methylation-based prediction models for rhinitis and AsRh. We subsequently functionally interpreted our findings using matched nasal brush bulk and single cell RNA-sequencing (scRNAseq) data. We finally

investigated four different environmental exposures relevant to AsRh in relation to our significant DNA methylation signals.

Methods

A full description of methods is provided in the online supplement.

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 out of the following 3 criteria: 1) Doctor diagnosed asthma ever; 2) Wheeze in the last 12 months; and 3) Prescription of asthma medication in the last 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 hayfever in the previous 12 months. AsRh was defined as the presence of either asthma or rhinitis. Serum specific IgE to house dust mite, cat, dactylis (grass) and birch was measured and classified as positive if $\geq$ 0.35 IU/ml. Pets exposure was defined as the presence of furry pets (dog/ cat/ rodent) in the home during secondary school.

Nasal DNA methylation measurements and RNA sequencing
DNA and RNA were extracted from nasal brushing samples collected from the lower inferior turbinate. Genome-wide DNA methylation was determined using Illumina Infinium HumanMethylation450 BeadChips. After QC, 455 samples and 436,824 probes remained; M-values were used in downstream analyses. We performed replication analyses in two cohorts: 72 children from the US Inner City Asthma Study (GSE65205)[8]; and 487 children from EVA-PR. RNA-seq was performed on Illumina HiSeq2500 platform. After QC, 326 subjects and 17,156 genes were retained. Raw counts were transformed to log2CPM (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 using comb-p[14] and DMRcate[15]. Top CpGs (FDR < 0.05) were selected for replication. If none of the sites met that significance criterium, we used a looser threshold ($p<1\times10^{-4}$) to select potential relevant CpGs for replication. After replication, we performed inverse variance-weighted fixed-effects meta-analyses with METAL[16]. Successful replication was defined as CpGs that showed significance in the meta-analysis of replication cohorts (Bonferroni correction, P < 0.05/number of tests)

and passed epigenome-wide significance (Bonferroni correction, $P<1.14\times10^{-7}$, 436,824 tests) in the meta-analysis of all studies. We performed stratified analysis of significant CpGs in specific IgE positive or negative patients compared to non-allergic controls. We investigated the association of CpGs associated with AsRh with environmental risk factors (active smoking, second-hand 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 CpGs identified by EWAS, with age and sex were used to train the models which were subsequently tested in the EVA-PR cohort. Replicated CpGs were annotated by GREAT 3.0.0[18]. We performed expression quantitative trait DNA methylation (eQTM) analysis in *cis* region (+/- 250kb). Pathway analysis was performed by ConsensusPathDB[19] using eQTM genes, and nasal brush scRNAseq of four subjects was used to annotate eQTM genes to cell types.

Results

Characteristics of the study population
The characteristics are shown in Table 1 and E1. 455 PIAMA participants were included in the analyses, which corresponds to 56.7% of the total 16 years follow-up, and 11.5% of the total PIAMA population (Table E2). The prevalence of asthma, rhinitis and AsRh at age 16 years was 8.1%, 45.1% and 46.4% respectively. The combined AsRh phenotype was dominated by rhinitis (97.2% cases had rhinitis, 17.5 % had asthma, 14.7 % had both), and 64.9% of children with AsRh showed positive IgE sensitization (Figure E1). The mean age of the discovery and replication cohorts were 16 years (PIAMA), 15.5 years (EVA-PR) and 11 years old (Inner City Asthma study). The distribution of ethnicity of study participants differed: in PIAMA, ~97% children had European white ancestry, whereas the US Inner City study included ~92% African American and the EVA-PR study included Puerto Rican children who were 100% Hispanic or Latino.

Table 1 Characteristics of study populations from discovery and replication cohorts

	Discovery cohort	Replication cohorts	
	PIAMA	Yang et al.	EVR-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%)
Asthma and/or rhinitis (%)	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%[a]	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

Numbers represent number of participants (%) for categorical variables and mean ± SD for continuous variables. Allergic respiratory disease is defined as the presence of asthma and/or rhinitis. *a*Does not add up to 100% because participants could report more than one ethnicity. *Data shown as number of "Yes" / number of all available samples (%); in PIAMA cohort, the number represented participants exposed to listed exposures during secondary school.

EWAS discovery and replication in nasal epithelium

In total, 81 CpGs were significantly associated with rhinitis and 75 were associated with AsRh (FDR<0.05), and were thus selected for replication. In addition, 95 CpGs associated with asthma were selected for replication using a less stringent threshold ($P < 1.0×10^{-4}$), since no CpG passed the threshold of FDR <0.05 (Figure 1 and E2). Although no DNA methylation signal at single CpG level was significantly associated with asthma, we identified 16 significant DMRs associated with asthma (Table E3). Moreover, significant DMRs associated with rhinitis (n=20) and AsRh (n=20) were identified (Table E3).

After applying cohort specific QC, 74 out of the 95 CpGs associated with asthma, 72 out of the 81 CpGs associated with rhinitis and 66 out of the 75 CpGs associated with AsRh were available in EVA-PR. The US Inner City Asthma Study could assess all 95

CpGs associated with asthma, but did not include a rhinitis phenotype and therefore this study only participated in the asthma replication.
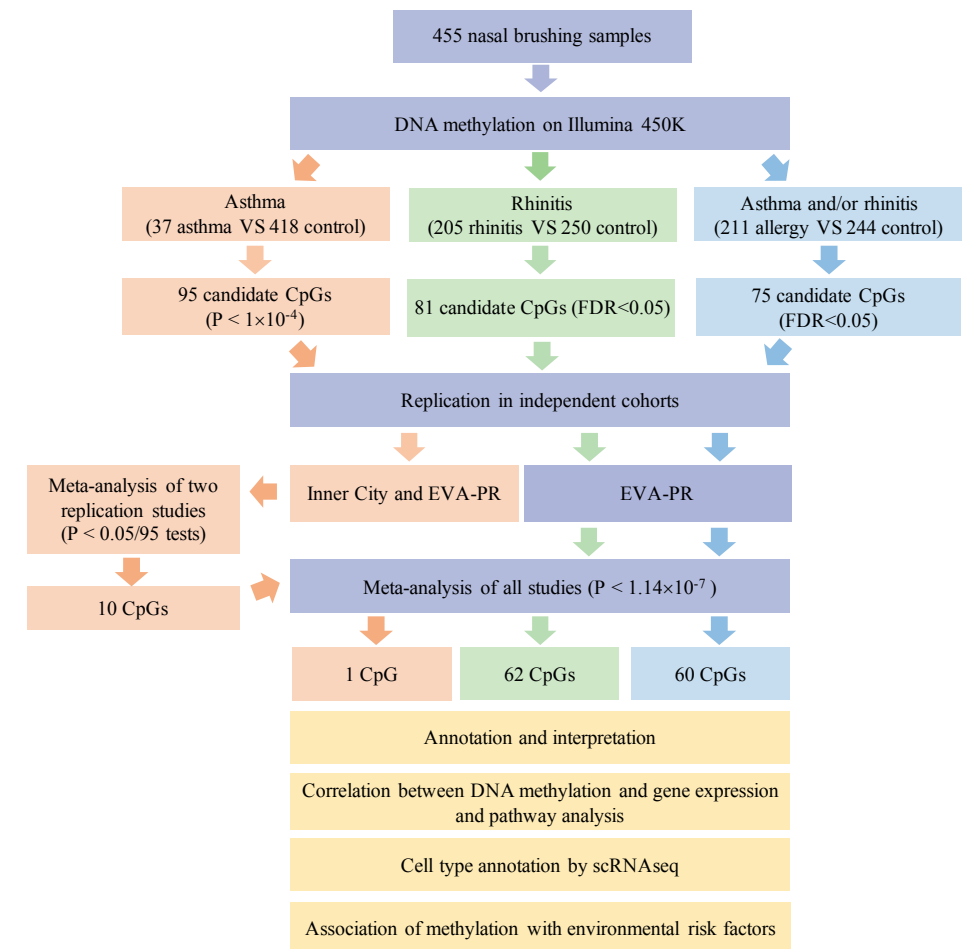

Figure 1: Study design. EWAS on three phenotypes (asthma, rhinitis and asthma and/or rhinitis) was conducted on 455 samples obtained by nasal brushing. Significant CpGs with FDR<0.05 were selected for replication. EWAS on asthma did not identify CpGs that passed the threshold of FDR<0.05, so therefore a looser threshold of P value<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 was analyzed to link the observed methylation to gene expression, while 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 association of CpGs associated with asthma and/or rhinitis with four environmental risk factors (active smoking, second-hand smoking, pets, dampness and molds).
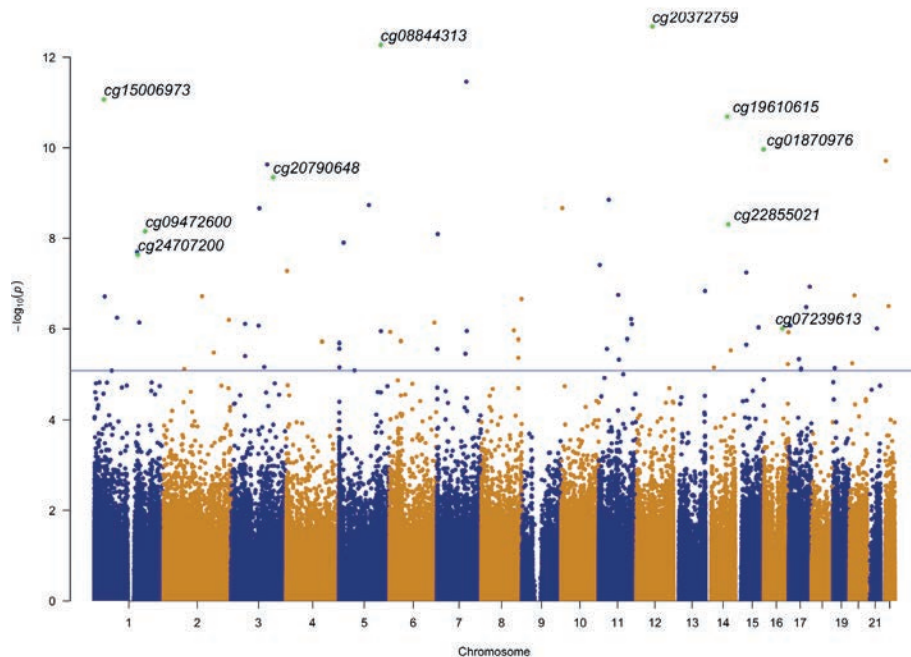
Figure 2: A manhattan plot of association between asthma and/or rhinitis and DNA methylation at 16 years using nasal epithelial samples in PIAMA cohort (discovery). In total, 436,824 CpGs were tested. The blue line represents the FDR corrected threshold (FDR<0.05) of significance. Highlighted sites represent the top 10 replicated CpGs associated with asthma and/or rhinitis.

Ten out of the 95 asthma-associated CpGs were significant in the meta-analysis of the two replication cohorts after Bonferroni correction (95 tests, $P < 5.26 \times 10^{-4}$), which were used in downstream analysis. One CpG, 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 (Table E4). Sixty-two of the 72 tested CpGs associated with rhinitis and 60 of the 66 tested CpGs associated with AsRh passed the genome-wide significance threshold using Bonferroni correction ($P < 1.14 \times 10^{-7}$, Figure 2) in the meta-analysis of all cohorts (Table 2, E5-6). The results were robust when using different rhinitis definitions (online supplements, Table E7-8).

In total, 68 unique CpGs were identified to be associated with one or more phenotypes. Additional adjustment for sampling season did not change the results indicating that sampling season was not a confounder (Table E9). None of the replicated probes showed significance in Hartigan's dip test[20], indicating no significant SNP effect under the probe sequence; eight of these were additionally

validated by pyrosequencing (online supplements)[9]. The Q-Q plots and inflation factors are shown in Figure E3. P values of discovery CpGs after BACON[21] correction are shown in Table E10. Asthma-associated CpGs also showed strong associations with rhinitis and AsRh (Table E11); and rhinitis-associated CpGs showed strong associations with AsRh, but less strongly with asthma (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 to the same controls who were specific IgE-negative and without AsRh (Table 3). The same tendency was also found for asthma and rhinitis (Table E12-14).

Prediction of asthma and rhinitis with methylation levels

We used CpGs selected for replication to train the models. CpGs that did not pass QC in EVA-PR were excluded, so that models could be tested independently. After training, the final sets consisted of 70 CpGs in asthma prediction, 48 CpGs in rhinitis prediction, and 26 CpGs in AsRh prediction. Coefficients of CpGs in each model are shown in Table E15. In the PIAMA cohort, the areas under the curve (AUC) for asthma, rhinitis and AsRh were 0.98, 0.74 and 0.70 respectively. In EVA-PR, 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 the replication cohort are shown in Figure E4.

Table 2 Description of top 10 replicated CpGs associated with asthma and/or rhinitis (AsRh)

CpG ID	CHR[a]	Basepair position[b]	Discovery_PIAMA		Replication1_EVAPR		Meta_analysis_all[c]		Great gene annotation[d]
			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)

OR (95% CI): Odds Ratio and 95% Confidence Interval; [a] CHR: Chromosome; [b] Basepair postion: Basepair position according to Genome build 37; [c] Meta_analysis_all: meta-analysis of discovery and replication; [d] Great gene annotation: the CpGs were annotated by GREAT version 3.0.0 (Genomic Regions of Annotations Tool, http://bejerano.stanford.edu/great/); Information on all replicated CpGs for asthma, rhinitis and AsRh is presented in the Online Supplement table E4-6

Table 3 IgE stratified analysis of top 10 replicated CpGs associated with asthma and/or rhinitis (AsRh)

CpG ID	Specific IgE positive (137 cases VS 155 controls)				Specific IgE negative (70 cases 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)	0.68
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)	0.17
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)	0.39
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)	0.27
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)	0.30
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)	0.64
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)	0.74
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)	0.19
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)	0.49
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)	0.09

OR*(95% CI): Odds Ratio and 95% Confidence Interval of 10% absolute change in methylation level of M value. OR (95% CI): Odds Ratio and 95% Confidence Interval of 1 absolute change in methylation level of M value; Specific IgE positive: Specific IgE positive AsRh cases versus (vs) non AsRh and IgE negative controls; Specific IgE negative: Specific IgE negative AsRh cases vs non AsRh and IgE negative controls. 23 subjects that did not have IgE sensitization data and 70 subjects that were IgE positive and had no AsRh were not included in this analysis.

Association between methylation and gene expression

Of 68 unique CpGs, 24 CpGs were significantly associated with gene expression levels in cis, resulting in 66 unique CpG-gene expression pairs, of which 29 pairs showed negative correlation (Table E16). The 66 CpG-gene pairs include 59 unique genes which were called eQTM genes. The most significant association (P=3.72× 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].

Pathway analysis

Four eQTM genes related to asthma were significantly enriched (P < 0.01) in 11 pathways (Table E17). Fifty-seven eQTM genes related to rhinitis were significantly enriched in 23 pathways, of which 6 were related to immune function including Microglia Pathogen Phagocytosis Pathway, DAP12 interactions, adaptive Immune System, IL-2 Signaling Pathway, T cell receptor signaling pathway and Immune System (Table E17). One pathway (Bacterial invasion of epithelial cells) related to epithelial function. Twenty-five pathways were enriched for 51 eQTM genes related to AsRh, and the immune related pathways mentioned above were also found for AsRh (Table E17).

Cell type annotation

We performed scRNAseq in independent nasal brush samples from 2 asthma patients and 2 healthy controls[23] (Table E18). After stringent QC and doublet removal we aligned the samples using Canonical Correlation Analysis (CCA) on 2356 shared variable genes. Clustering these aligned samples produced 5 clusters. We annotated the clusters based on gene expression[23,24] (Table E19) to represent 4 epithelial cell types (club, goblet, ciliated and basal cells) and one cluster of mixed immune cells (Figure E5). This suggested that epithelial brushes yielded mostly epithelial cells in combination with some immune cells, with seven eQTM genes (*DNALI1, ZMYND10, CCDC153, MEAF6, C11orf70, DUSP14, APOBEC4*) that were also marker gene of ciliated cells and one (*RHOG*) that represents a marker gene of the immune cell cluster. Other eQTM genes did not show significant differential expression among cell clusters (Figure E6). To investigate if the association of CpG methylation with AsRh was due to methylation differences within epithelial cells, we replicated our top CpGs in nasal epithelial cells sorted by CD326 EpCAM microbeads in a small subset of the EVA-PR cohort (n=31), and 13 out of 60 CpGs associated with AsRh remained nominally significant (P < 0.05) with the same direction (Table E20). In the sorted epithelial cells, 11 out of 66 CpG-gene pairs (eQTM) were also found nominally significant with the same direction as the bulk analysis (P < 0.05) (Table E21).

Association between environmental risk factors and nasal methylation levels

We investigated the association between four environmental factors relevant for allergic disease (active smoking, second-hand smoking, exposure to pets, and dampness and molds in the house) during secondary school and the 60 replicated AsRh-associated CpGs , and identified one CpG (cg03565274) that showed significant positive association with pets exposure (P = 7.57×10^{-4}) which passed Bonferroni correction (Table 4, E22), and had a negative correlation with AsRh. We next investigated the association of nasal DNA methylation level of this CpG at 16 years with pets exposure in different time windows from birth onwards, and observed consistent patterns from infancy to secondary school: children exposed to pets from birth onwards had higher DNA methylation levels at this CpG (Table E23; Figure E7). This CpG cg03565274 showed positive correlation with expression levels of *ZMYND10* (Zinc Finger MYND-Type Containing 10). The *ZMYND10* gene was found to be highly expressed in ciliated cells using scRNAseq (Figure 3). We also checked the direction of all 60 CpGs associated with AsRh, and found that 56 out of the 60 CpGs had a positive association with pets (P < 0.001, Monte Carlo resampling method). Active smoking, second-hand smoking and dampness and molds were not significantly associated with the 60 CpGs.

Table 4 Association between methylation level of CpGs associated with asthma and/or rhinitis (AsRh) in nasal epithelium and four environmental factors during secondary school (top 10 CpGs for each environmental factor).

Active smoking (N=381)			Secondhand smoking (N=384)		
CpG ID	**Coef**	**P-value**	**CpG ID**	**Coef**	**P-value**
cg11058904	0.11	5.64×10^{-2}	cg23005227	0.09	2.93×10^{-2}
cg25020944	-0.06	6.13×10^{-2}	cg06675531	-0.07	4.21×10^{-2}
cg04206484	0.23	6.31×10^{-2}	cg01062020	0.19	5.82×10^{-2}
cg07686035	0.08	6.52×10^{-2}	cg03668556	-0.10	0.11
cg24224501	0.06	7.83×10^{-2}	cg08175352	-0.10	0.13
cg00664723	0.13	8.14×10^{-2}	cg27058763	-0.05	0.16
cg10549071	0.12	8.56×10^{-2}	cg04206484	0.13	0.19
cg00049323	-0.09	9.44×10^{-2}	cg01870976	-0.08	0.20
cg23005227	0.08	0.10	cg21291385	0.07	0.23
cg12875548	0.10	0.11	cg04891688	0.06	0.25
Pets (N=380)			**Dampness and molds (N=430)**		
CpG ID	Coef	P-value	CpG ID	Coef	P-value
cg03565274*	0.07	7.57×10^{-4}	cg12875548	-0.17	1.55×10^{-3}
cg23387401	0.13	1.23×10^{-3}	cg27058763	-0.08	1.06×10^{-2}
cg24707200	0.07	1.38×10^{-3}	cg03668556	-0.14	4.07×10^{-2}
cg08844313	0.14	3.25×10^{-3}	cg04206484	-0.21	5.37×10^{-2}
cg10054641	0.12	5.72×10^{-3}	cg08175352	-0.12	6.09×10^{-2}
cg20372759	0.11	9.48×10^{-3}	cg00664723	-0.12	6.58×10^{-2}
cg19610615	0.07	1.35×10^{-2}	cg12716639	-0.08	8.67×10^{-2}
cg22855021	0.09	2.16×10^{-2}	cg07239613	-0.05	9.20×10^{-2}
cg10549071	0.10	2.51×10^{-2}	cg09562938	-0.05	0.11
cg01062020	0.18	2.53×10^{-2}	cg21291385	-0.10	0.12

* CpG site passed Bonferroni correction. Information of association between all 60 CpGs associated with *AsRh* and four environmental factors is presented in the Online Supplement table E22.
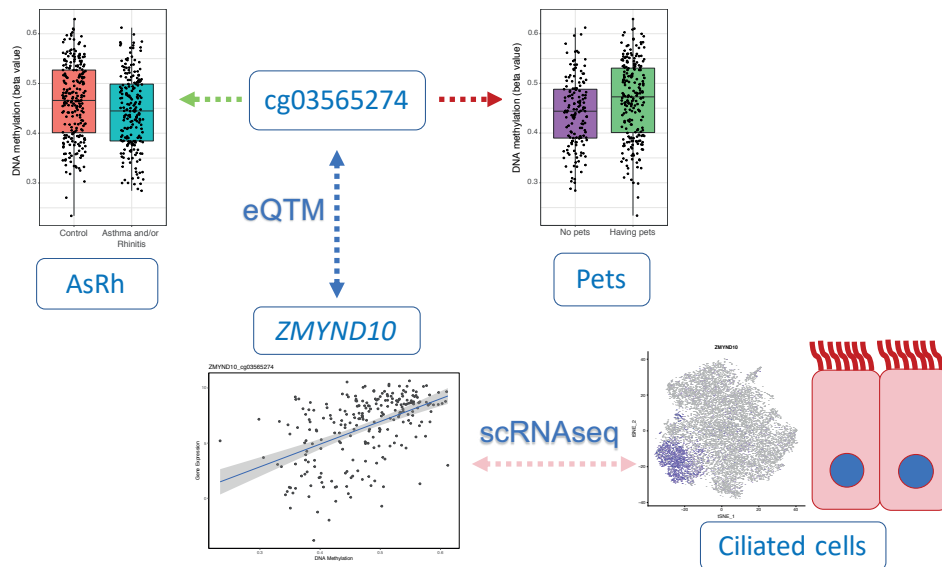
Figure 3: The relationship among asthma and/or rhinitis (As/Rh), DNA methylation, environmental factors (pets), gene expression and nasal epithelial cell type. Methylation level of cg03565274 was negatively correlated to AsRh status, and positively correlated to pets. Methylation levels of cg03565274 was also positively correlated to the expression level of 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 pets 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 non-invasive proxy for bronchial epithelium in children[6,7], and has been used as target tissue to study asthma[8,25]. However, rhinitis is highly prevalent, and shows co-morbidity 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. In our study, 83.8% of asthma patients also had rhinitis, which may explain that 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. Besides, we did an IgE stratified analysis of replicated CpGs, and the results showed that DNA methylation signals in nasal epithelium were mainly driven by IgE positive subjects with AsRh and not by IgE negative AsRh subjects. 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 comparing the results of our clinical AsRh definition to their IgE sensitization results, 21 out of 60 CpGs associated with AsRh were also in their top 30 CpGs list. Both results indicate that nasal DNA methylation might be a biomarker for IgE sensitization.

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

Eight CpGs in nasal epithelium showed association with all three 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), whose expression has been associated with airway inflammation and bronchial hyperresponsiveness[32]; *CYP27B1* (Cytochrome P450 Family 27 Subfamily B Member 1), an enzyme, whose activity 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 was upregulated in epithelial cells of asthma patients[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, which was found for 24 of the 68 investigated CpGs. 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 chronic rhinosinusitis patients[35]. Twenty CpG-gene pairs showed significant association between CpGs and genes where the CpGs were located, including *PCSK6*, *FBXL7* and *CISH*. These genes were previously associated with allergic diseases or inflammation: *PCSK6* (Proprotein Convertase Subtilisin/Kexin Type 6) can activate the NF-κB signaling pathway and is involved in the inflammatory response[36]; *FBXL7* (F-Box And Leucine Rich Repeat Protein 7) expression is involved in the inhaled corticosteroid response in asthma[37]; and *CISH* (Cytokine Inducible SH2 Containing Protein) 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 identified that the majority of cells in the nasal epithelial brushes were epithelial cells, with some contribution of immune cells by scRNAseq. Indeed, we could show that 13 CpGs 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 showed similar performance in the replication cohort. The prediction model for asthma did not perform well in the replication cohort, which possibly can be explained by overfitting in the discovery cohort, since PIAMA is an unselected birth cohort with low prevalence of asthma. However, our model could still classify subjects with rhinitis/ AsRh with an AUC larger than 0.6/ 0.7 across different populations with different ethnics, which indicates that nasal methylation signals can help to predict rhinitis and AsRh in children, especially for IgE positive AsRh.

We found that residential pets 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 level at this site. This pattern was consistent from infancy to 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 pets exposure in early life was associated with a lower risk of developing asthma and allergic diseases in children of both school and preschool age[39,40,41]. However, studies also showed that allergic parents 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 pets exposure on DNA methylation and the development of asthma and rhinitis. Methylation levels of cg03565274 were positively correlated to the expression level of 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 may be explained by a lower subset of differentiated ciliated cells in AsRh compared to healthy controls, as recently reported in patients with chronic rhinosinusitis using scRNAseq[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], but did not identify significant associations between these exposures and CpGs associated with AsRh in this study.

Despite the overall robustness of our study findings, there are some limitations to consider. Firstly, we had relatively low power in our asthma analysis, due to the low prevalence of asthma. Consequently, the results of AsRh were largely overlapping with the results of rhinitis. Secondly, our single cell analyses were performed on a small dataset (4 individuals), therefore, we did not have enough power to disentangle the immune cell types, but present results for one mixed immune cell cluster. Thirdly, our prediction models were trained in a limited age range (around 16 years old), and then were replicated in a wider age range (9 to 20 years old), 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 pets exposure on the development of asthma and rhinitis.

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

Supplementary Materials

Online Repository:

https://www.sciencedirect.com/science/article/pii/S0091674920300348?via%3Dihub

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