Nasal DNA methylation profiling of asthma and rhinitis

Qi, Cancan; Jiang, Yale; Yang, Ivana V; Forno, Erick; Wang, Ting; Vonk, Judith M; Gehring, Ulrike; Smit, Henriëtte A; Milanzi, Edith B; Carpaij, Orestes A

Published in:
Journal of Allergy and Clinical Immunology

DOI:
10.1016/j.jaci.2019.12.911

Publication date:
2020

Nasal DNA methylation profiling of asthma and rhinitis

Cancan Qi, MsC[1,2*], Yale Jiang, MsC[3,4,12*], Ivana V. Yang, PhD[5], Erick Forno, MD, MPH[3,4], Ting Wang, PhD[3,4], Judith M. Vonk, PhD[2,6], Ulrike Gehring, PhD[7], Henriëtte A. Smit, PhD[8], Edith B. Milanzi, MsC[7], Orestes A Carpaij, MD[2,9], Marijn Berg,BsC[2,10], Laura Hesse, MsC[2,10], Sharon Brouwer, BsC[2,10], Jonathan Cardwell, MsC[5], Cornelis J. Vermeulen, PhD[2,9], Edna Acosta-Pérez, PhD[11], Glorisa Canino, PhD[11], Nadia Boutaoui, PhD[3,4], Maarten van den Berge, MD, PhD[2,9], Sarah A. Teichmann, PhD[13,14], Martijn C. Nawijn, PhD[2,10], Wei Chen, PhD[3,4], Juan C. Celedón, MD, DrPH[3,4+], Cheng-Jian Xu, PhD[1,2,15,16+] and Gerard H. Koppelman, MD, PhD[1,2+]

1 University of Groningen, University Medical Center Groningen, Dept. of Pediatric Pulmonology and Pediatric Allergy, Beatrix Children's Hospital, Groningen, The Netherlands.

2 University of Groningen, University Medical Center Groningen GRIAC Research Institute, Groningen, The Netherlands.

3 Division of Pulmonary Medicine, UPMC Children's Hospital of Pittsburgh, Pittsburgh, PA.

4 Dept. of Pediatrics, University of Pittsburgh School of Medicine, Pittsburgh, PA.

5 Department of Medicine, University of Colorado, Aurora, CO, USA.

6 University of Groningen, University Medical Center Groningen Dept. of Epidemiology, Groningen, The Netherlands.

7 Institute for Risk Assessment Sciences, Utrecht University, Utrecht, the Netherlands.

8 Julius Center for Health Sciences and Primary Care, University Medical Center Utrecht, Utrecht, The Netherlands.

9 University of Groningen, University Medical Center Groningen, Department of Pulmonary Diseases, Groningen, Netherlands.

10 University of Groningen, University Medical Center Groningen, Department of Pathology & Medical Biology, Groningen, The Netherlands.

11 Behavioral Sciences Research Institute, University of Puerto Rico, San Juan, Puerto Rico.

12 School of Medicine, Tsinghua University, Beijing, China.

13 Wellcome Sanger Institute, Wellcome Genome Campus, Hinxton, Cambridge, CB10 1SA, UK.

14 Theory of Condensed Matter Group, Cavendish Laboratory/Dept Physics, University of Cambridge, JJ Thomson Avenue, Cambridge CB3 0EH, UK.

15 Department of Gastroenterology, Hepatology and Endocrinology, Centre for Individualised Infection Medicine, CiiM, a joint venture between the Hannover Medical School and the Helmholtz Centre for Infection Research, Hannover, Germany.

16 TWINCORE, Centre for Experimental and Clinical Infection Research, a joint venture between the Hannover Medical School and the Helmholtz Centre for Infection Research, Hannover, Germany

* authors share the first authorship; + authors share the senior authorship.

Address of correspondence:

Gerard H. Koppelman, MD PhD

Department of Pediatric Pulmonology and Pediatric Allergology

Beatrix Children's Hospital,

University Medical Center Groningen

PO Box 30.001

9700 RB Groningen, the Netherlands

e-mail: g.h.koppelman@umcg.nl

Phone: + 31 50 3611036

Fax: + 31 50 3614235

Author contributions: CQ, YJ, WC, JCC, CJX and GHK contributed to the design of the study, and JCC and GHK obtained funding for the study. CQ, YJ, CJX, EF,

TW, EBM, MB, JC and CJV contributed to data analysis. IVY, JMV, UG, HAS, OAC, SB, LH, EA-P, GC, NB, M van den B,ST and MCN contributed to study procedures, participant recruitment or laboratory procedures. All authors contributed to results interpretation. CQ, YJ, WC, JCC, CJX and GHK contributed to the initial manuscript draft. All authors revised the manuscript draft for important intellectual content, and approved the final version for submission.

Funding/ Support: The PIAMA study was supported by The Netherlands Organization for Health Research and Development; The Netherlands Organization for Scientific Research; Lung Foundation of the Netherlands (with methylation studies supported by AF 4.1.14.001); The Netherlands Ministry of Spatial Planning, Housing, and the Environment; and The Netherlands Ministry of Health, Welfare, and Sport. Cancan Qi was supported by a grant from the China Scholarship Council. The EVA-PR Study was supported by grants HL079966 and HL117191 from the U.S. National Institutes of Health (NIH). The contributions of JCC and WC were additionally supported by grant MD011764 from the U.S. NIH, and RNA Sequencing was funded by the Department of Pediatrics of UPMC Children's Hospital of Pittsburgh. Research infrastructure for EVA-PR was additionally supported by grant U54MD007587 from the U.S. NIH. Inner City Asthma Consortium was supported by contract N01-AI90052 from the U.S. National Institutes of Health (NIH).

Disclosure: GHK reports grants from Lung Foundation of the Netherlands, during the conduct of the study; grants from Lung Foundation of the Netherlands, TEVA the Netherlands, Vertex, GSK, Ubbo Emmius Foundation, TETRI foundation, outside the submitted work. M van den B reports grants paid to the University from

Astra Zeneca, TEVA, GSK, Chiesi, outside the submitted work. JCC received research materials from GSK and Merck (inhaled steroids) and Pharmavite (vitamin D and placebo capsules) to provide medications free of cost to participants in NIH-funded studies, outside the submitted work. CJV reports grants from GSK, outside the submitted work. MCN reports grants from GSK, Lung Foundation of the Netherlands, outside the submitted work. ST reports consultant for Genentech, Roche and Biogen. The rest of the authors declare that they have no relevant conflict of interests.

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.

Word count: 250

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

Capsule summary: We identify replicable DNA methylation biomarker associated with asthma and rhinitis in nasal brushes, and provide the indication that pet exposure may have an impact on the DNA methylation of cells obtained by nasal brushing.

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

Abbreviations:

EWAS: epigenome-wide association study; PIAMA: Prevention and Incidence of Asthma and Mite Allergy; EVA-PR: Epigenetic Variation and Childhood Asthma study in Puerto Ricans Study; scRNAseq: single cell RNA-sequencing; CPM: counts per million; DMR: Differentially methylated regions; eQTM: expression quantitative trait DNA methylation; FDR: False discovery rate; QC: quality control.

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 methylgroup 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, rhinithis 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 ≥ 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, 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 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.

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 ($P < 1.0 \times 10^{-4}$), since no CpG passed the threshold of FDR <0.05 (Figure 1, 2 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.

Ten out of the 95 asthma-associated CpGs were significant in the meta-analysis of the two replication cohorts after Bonferroni correction (95 tests, $P < 5.26\times10^{-4}$), which were used in downstream analysis. One CpG, annotated to the *PDE6A* gene (cg08844313, $P = 6.72\times10^{-8}$), 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 ($P < 1.14\times10^{-7}$) 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.

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 ($P=3.72\times10^{-11}$) 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, secondhand 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 ($P = 7.57\times10^{-4}$) which passed

335 Bonferroni correction (Table 4, E22), and had a negative correlation with AsRh. We
336 next investigated the association of nasal DNA methylation level of this CpG at 16
337 years with pets exposure in different time windows from birth onwards, and observed
338 consistent patterns from infancy to secondary school: children exposed to pets from
339 birth onwards had higher DNA methylation levels at this CpG (Table E23; Figure E7).
340 This CpG cg03565274 showed positive correlation with expression levels of
341 *ZMYND10* (Zinc Finger MYND-Type Containing 10). The *ZMYND10* gene was found
342 to be highly expressed in ciliated cells using scRNAseq (Figure 3). We also checked
343 the direction of all 60 CpGs associated with AsRh, and found that 56 out of the 60
344 CpGs had a positive association with pets ($P < 0.001$, Monte Carlo resampling
345 method). Active smoking, secondhand smoking and dampness and molds were not
346 significantly associated with the 60 CpGs.

347

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.

466

References

1. Stein MM, Hrusch CL, Gozdz J, Igartua C, Pivniouk V, Murray SE, et al. Innate Immunity and Asthma Risk in Amish and Hutterite Farm Children. N Engl J Med. 2016;375:411–21.

2. Tost J. Epigenetic plasticity of eosinophils and other immune cell subsets in childhood asthma. The Lancet Respiratory Medicine. 2018;6:322–4.

3. Xu C-J, Söderhäll C, Bustamante M, Baïz N, Gruzieva O, Gehring U, et al. DNA methylation in childhood asthma: an epigenome-wide meta-analysis. Lancet Respir Med. 2018;6:379–88.

4. Lambrecht BN, Hammad H. The airway epithelium in asthma. Nat Med. 2012;18:684–92.

5. Lopez-Guisa JM, Powers C, File D, Cochrane E, Jimenez N, Debley JS. Airway epithelial cells from asthmatic children differentially express proremodeling factors. Journal of Allergy and Clinical Immunology. 2012;129:990-997.e6.

6. Bergougnoux A, Claustres M, De Sario A. Nasal epithelial cells: a tool to study DNA methylation in airway diseases. Epigenomics. 2015;7:119–26.

7. Imkamp K, Berg M, Vermeulen CJ, Heijink IH, Guryev V, Kerstjens HAM, et al. Nasal epithelium as a proxy for bronchial epithelium for smoking-induced gene expression and expression Quantitative Trait Loci. J Allergy Clin Immunol. 2018;142:314-317.e15.

8. Yang IV, Pedersen BS, Liu AH, O'Connor GT, Pillai D, Kattan M, et al. The nasal methylome and childhood atopic asthma. Journal of Allergy and Clinical Immunology. 2017;139:1478–88.

9. Forno E, Wang T, Qi C, Yan Q, Xu C-J, Boutaoui N, et al. DNA methylation in nasal epithelium, atopy, and atopic asthma in children: a genome-wide study. The Lancet Respiratory Medicine [Internet]. 2018 [cited 2019 Jan 9]; Available from: https://linkinghub.elsevier.com/retrieve/pii/S2213260018304661

10. Ferreira MA, Vonk JM, Baurecht H, Marenholz I, Tian C, Hoffman JD, et al. Shared genetic origin of asthma, hay fever and eczema elucidates allergic disease biology. Nat Genet. 2017;49:1752–7.

11. Pinart M, Benet M, Annesi-Maesano I, von Berg A, Berdel D, Carlsen KCL, et al. Comorbidity of eczema, rhinitis, and asthma in IgE-sensitised and non-IgE-sensitised children in MeDALL: a population-based cohort study. The Lancet Respiratory Medicine. 2014;2:131–40.

12. Wijga AH, Kerkhof M, Gehring U, de Jongste JC, Postma DS, Aalberse RC, et al. Cohort profile: the prevention and incidence of asthma and mite allergy (PIAMA) birth cohort. Int J Epidemiol. 2014;43:527–35.

13. Leek JT, Storey JD. Capturing Heterogeneity in Gene Expression Studies by Surrogate Variable Analysis. PLoS Genetics. 2007;3:e161.

14. Pedersen BS, Schwartz DA, Yang IV, Kechris KJ. Comb-p: software for combining, analyzing, grouping and correcting spatially correlated P-values. Bioinformatics. 2012;28:2986–8.

15. Peters TJ, Buckley MJ, Statham AL, Pidsley R, Samaras K, V Lord R, et al. De novo identification of differentially methylated regions in the human genome. Epigenetics Chromatin. 2015;8:6.

16. Willer CJ, Li Y, Abecasis GR. METAL: fast and efficient meta-analysis of genomewide association scans. Bioinformatics. 2010;26:2190–1.

17. Friedman J, Hastie T, Tibshirani R. Regularization Paths for Generalized Linear Models via Coordinate Descent. J Stat Softw. 2010;33:1–22.

18. McLean CY, Bristor D, Hiller M, Clarke SL, Schaar BT, Lowe CB, et al. GREAT improves functional interpretation of cis-regulatory regions. Nature Biotechnology. 2010;28:495–501.

19. Herwig R, Hardt C, Lienhard M, Kamburov A. Analyzing and interpreting genome data at the network level with ConsensusPathDB. Nature Protocols. 2016;11:1889–907.

20. Hartigan JA, Hartigan PM. The Dip Test of Unimodality. The Annals of Statistics. 1985;13:70–84.

21. the BIOS Consortium, van Iterson M, van Zwet EW, Heijmans BT. Controlling bias and inflation in epigenome- and transcriptome-wide association studies using the empirical null distribution. Genome Biol. 2017;18:19.

22. Raedler D, Ballenberger N, Klucker E, Böck A, Otto R, Prazeres da Costa O, et al. Identification of novel immune phenotypes for allergic and nonallergic childhood asthma. Journal of Allergy and Clinical Immunology. 2015;135:81–91.

23. Vieira Braga FA, Kar G, Berg M, Carpaij OA, Polanski K, Simon LM, et al. A cellular census of human lungs identifies novel cell states in health and in asthma. Nat Med [Internet]. 2019 [cited 2019 Jun 24]; Available from: http://www.nature.com/articles/s41591-019-0468-5

24. Ordovas-Montanes J, Dwyer DF, Nyquist SK, Buchheit KM, Vukovic M, Deb C, et al. Allergic inflammatory memory in human respiratory epithelial progenitor cells. Nature. 2018;560:649–54.

25. Zhang X, Biagini Myers JM, Burleson JD, Ulm A, Bryan KS, Chen X, et al. Nasal DNA methylation is associated with childhood asthma. Epigenomics. 2018;10:629–41.

26. Zhu Z, Lee PH, Chaffin MD, Chung W, Loh P-R, Lu Q, et al. A genome-wide cross-trait analysis from UK Biobank highlights the shared genetic architecture of asthma and allergic diseases. Nat Genet. 2018;50:857–64.

27. Liang L, Willis-Owen SAG, Laprise C, Wong KCC, Davies GA, Hudson TJ, et al. An epigenome-wide association study of total serum immunoglobulin E concentration. Nature. 2015;520:670–4.

28. Chen W, Wang T, Pino-Yanes M, Forno E, Liang L, Yan Q, et al. An epigenome-wide association study of total serum IgE in Hispanic children. J Allergy Clin Immunol. 2017;140:571–7.

29. Cardenas A, Sordillo JE, Rifas-Shiman SL, Chung W, Liang L, Coull BA, et al. The nasal methylome as a biomarker of asthma and airway inflammation in children. Nat Commun. 2019;10:3095.

30. Nicodemus-Johnson J, Naughton KA, Sudi J, Hogarth K, Naurekas ET, Nicolae DL, et al. Genome-Wide Methylation Study Identifies an IL-13-induced Epigenetic Signature in Asthmatic Airways. Am J Respir Crit Care Med. 2016;193:376–85.

31. Rochman M, Kartashov AV, Caldwell JM, Collins MH, Stucke EM, Kc K, et al. Neurotrophic tyrosine kinase receptor 1 is a direct transcriptional and epigenetic target of IL-13 involved in allergic inflammation. Mucosal Immunol. 2015;8:785–98.

32. Park SJ, Lee KS, Kim SR, Min KH, Lee KY, Choe YH, et al. Change of connexin 37 in allergen-induced airway inflammation. Exp Mol Med. 2007;39:629–40.

33. Yip K-H, Kolesnikoff N, Yu C, Hauschild N, Taing H, Biggs L, et al. Mechanisms of vitamin D_3 metabolite repression of IgE-dependent mast cell activation. J Allergy Clin Immunol. 2014;133:1356–64, 1364.e1-14.

34. Jang Y, Oh U. Anoctamin 1 in secretory epithelia. Cell Calcium. 2014;55:355–61.

35. Wu D, Mueller SK, Nocera AL, Finn K, Libermann TA, Bleier BS. TREM-1 Neutrophil Activation Pathway Is Suppressed in Eosinophilic Nasal Polyps. Am J Rhinol Allergy. 2018;1945892418782233.

36. Jiang H, Wang L, Wang F, Pan J. Proprotein convertase subtilisin/kexin type 6 promotes in vitro proliferation, migration and inflammatory cytokine secretion of synovial fibroblast-like cells from rheumatoid arthritis via nuclear-κB, signal transducer and activator of transcription 3 and extracellular signal regulated 1/2 pathways. Mol Med Rep. 2017;16:8477–84.

37. Park H-W, Dahlin A, Tse S, Duan QL, Schuemann B, Martinez FD, et al. Genetic predictors associated with improvement of asthma symptoms in response to inhaled corticosteroids. J Allergy Clin Immunol. 2014;133:664-669.e5.

38. Burnham ME, Koziol-White CJ, Esnault S, Bates ME, Evans MD, Bertics PJ, et al. Human airway eosinophils exhibit preferential reduction in STAT signaling capacity and increased CISH expression. J Immunol. 2013;191:2900–6.

39. Gur M, Hakim F, Bentur L. Better understanding of childhood asthma, towards primary prevention – are we there yet? Consideration of pertinent literature. F1000Research. 2017;6:2152.

40. Pelucchi C, Galeone C, Bach J-F, La Vecchia C, Chatenoud L. Pet exposure and risk of atopic dermatitis at the pediatric age: A meta-analysis of birth cohort studies. Journal of Allergy and Clinical Immunology. 2013;132:616-622.e7.

41. O'Connor GT, Lynch SV, Bloomberg GR, Kattan M, Wood RA, Gergen PJ, et al. Early-life home environment and risk of asthma among inner-city children. Journal of Allergy and Clinical Immunology. 2018;141:1468–75.

42. Wijga A, Smit HA, Brunekreef B, Gerritsen J, Kerkhof M, Koopman LP, et al. Are children at high familial risk of developing allergy born into a low risk environment? The PIAMA Birth Cohort Study. Prevention and Incidence of Asthma and Mite Allergy. Clin Exp Allergy. 2001;31:576–81.

43. van Strien RT, Koopman LP, Kerkhof M, Spithoven J, de Jongste JC, Gerritsen J, et al. Mite and pet allergen levels in homes of children born to allergic and nonallergic parents: the PIAMA study. Environmental Health Perspectives [Internet]. 2002 [cited 2019 Jan 15];110. Available from: https://ehp.niehs.nih.gov/doi/10.1289/ehp.021100693

44. Zariwala MA, Gee HY, Kurkowiak M, Al-Mutairi DA, Leigh MW, Hurd TW, et al. ZMYND10 Is Mutated in Primary Ciliary Dyskinesia and Interacts with LRRC6. The American Journal of Human Genetics. 2013;93:336–45.

45. Saulyte J, Regueira C, Montes-Martínez A, Khudyakov P, Takkouche B. Active or Passive Exposure to Tobacco Smoking and Allergic Rhinitis, Allergic Dermatitis, and Food Allergy in Adults and Children: A Systematic Review and Meta-Analysis. Novotny TE, editor. PLoS Medicine. 2014;11:e1001611.

46. Thacher JD, Gruzieva O, Pershagen G, Melén E, Lorentzen JC, Kull I, et al. Mold and dampness exposure and allergic outcomes from birth to adolescence: data from the BAMSE cohort. Allergy. 2017;72:967–74.

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.

611

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.

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)			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	0.11	cg08844313	0.14	3.25×10^{-3}	cg04206484	-0.21	5.37×10^{-2}
cg24224501	0.06	7.83×10^{-2}	cg08175352	-0.10	0.13	cg10054641	0.12	5.72×10^{-3}	cg08175352	-0.12	6.09×10^{-2}
cg00664723	0.13	8.14×10^{-2}	cg27058763	-0.05	0.16	cg20372759	0.11	9.48×10^{-3}	cg00664723	-0.12	6.58×10^{-2}
cg10549071	0.12	8.56×10^{-2}	cg04206484	0.13	0.19	cg19610615	0.07	1.35×10^{-2}	cg12716639	-0.08	8.67×10^{-2}
cg00049323	-0.09	9.44×10^{-2}	cg01870976	-0.08	0.20	cg22855021	0.09	2.16×10^{-2}	cg07239613	-0.05	9.20×10^{-2}
cg23005227	0.08	0.10	cg21291385	0.07	0.23	cg10549071	0.10	2.51×10^{-2}	cg09562938	-0.05	0.11
cg12875548	0.10	0.11	cg04891688	0.06	0.25	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 Legends:

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, secondhand smoking, pets, dampness and molds).

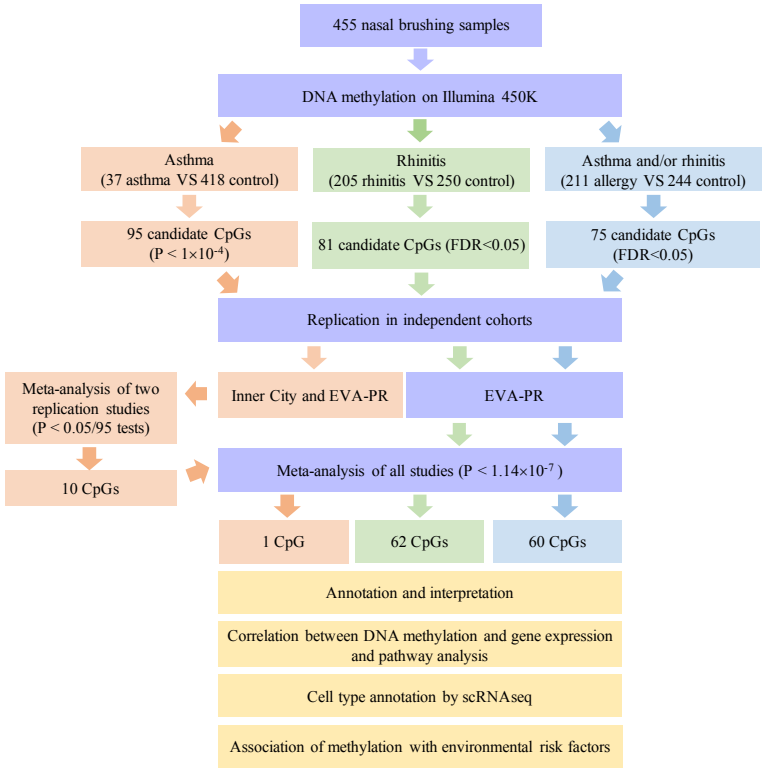


Figure 2: A manhattan plot of association between asthma and/or rhinitis and DNA methylation at 16 years using nasal epithelial samples in PIAMA cohort (discovery). In total, 436,824 CpGs were tested. The blue line represents the FDR corrected threshold (FDR<0.05) of significance. Highlighted sites represent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

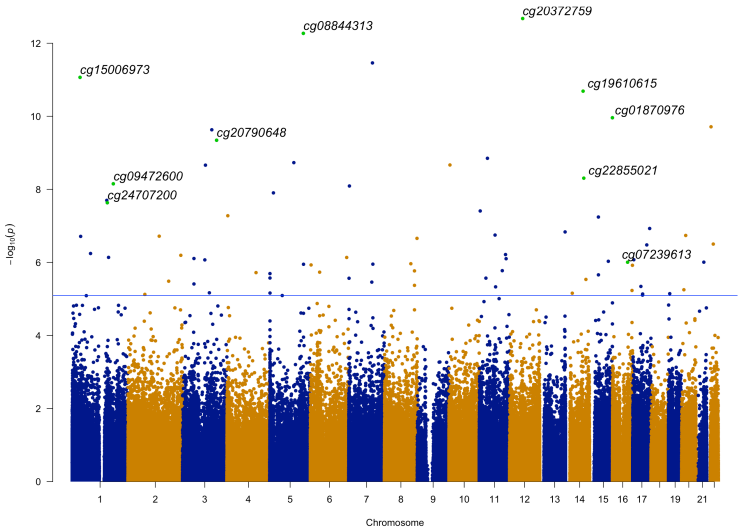
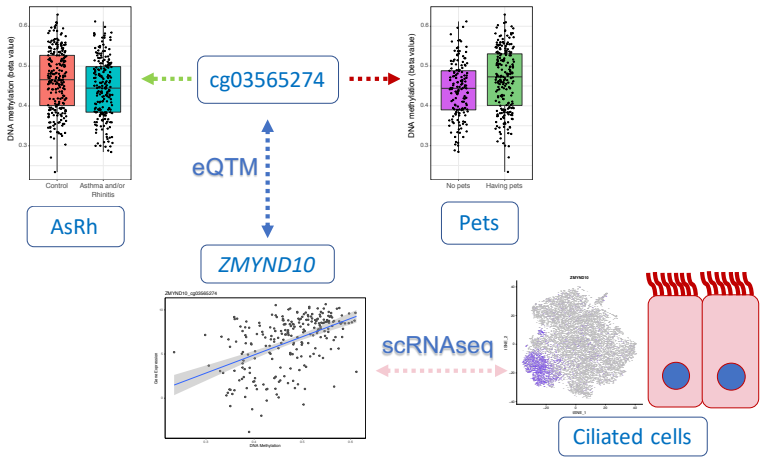


Figure 3: The relationship among asthma and/or rhinitis (As/Rh), DNA methylation, environmental factors (pets), gene expression and nasal epithelial cell type. Methylation level of cg03565274 was negatively correlated to AsRh status, and positively correlated to pets. Methylation levels of cg03565274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


cg03565274
eQTM
ZMYND10
AsRh
Pets
scRNAseq
Ciliated cells