Predominant DNMT and TET mediate effects of allergen on the human bronchial epithelium in a controlled air pollution exposure study

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Abstract

Background: Epidemiological data show that traffic-related air pollution contributes to the increasing prevalence and severity of asthma. DNA methylation (DNAm) changes may elucidate adverse health effects of environmental exposures.

Objectives: To assess the effects of allergen and diesel exhaust (DE) exposures on global DNAm and its regulation enzymes in human airway epithelium.

Methods: 11 participants, including 7 with and 4 without airway hyperresponsiveness (AHR), were recruited for a randomized, double-blinded crossover study. Each participant had 3 exposures: filtered air + saline (FA-S), filtered air + allergen (FA-A), and DE + allergen (DE-A). 48 hours post-exposure, endobronchial biopsies and bronchoalveolar lavages (BAL) were collected. Levels of DNA methyltransferases (DNMTs) and ten-eleven translocation enzymes (TETs), 5-methylcytosine (5mC), and 5-hydroxymethylcytosine (5hmC) were determined by immunohistochemistry. Cytokines and chemokines in BAL were measured by electrochemiluminescence multiplex assays.

Results: *Predominant* DNMT (pDNMT, the most abundant among DNMT1, DNMT3A, and DNMT3B), and *predominant* TET (pTET, the most abundant among TET1, TET2, and TET3) were participant-dependent. 5mC and its regulation enzymes differed between participants with and without AHR at baseline (FA-S) and in response to allergen challenge (regardless of DE exposure). pDNMT and pTET correlated with lung function. Allergen challenge effect on interleukin-8 in BAL was modified by TET2 baseline levels in the epithelium.

Conclusions: Response to allergen challenge is associated with key DNAm regulation enzymes. This relationship is generally unaltered by DE co-exposure but is rather dependent on AHR status. These enzymes therefore warranted further inquiry regarding their potential in diagnosis, prognosis, and treatment of asthma.

Clinical implications:

In those with hyperresponsive airways, lung function changes induced by allergen inhalation may be due to changes in enzymes that regulate DNA methylation.

Capsule Summary

This randomized, double-blinded, controlled human exposure, crossover study reveals that response to allergen challenge is associated with key DNA methylation regulation enzymes, especially in participants with hyperresponsive airways.

Keywords: Crossover human study, ten-eleven translocation (TET), DNA methylation

(DNAm), asthma, allergen, diesel exhaust (DE)

Abbreviations:

5hmC:	5-Hydroxymethylcytosine
5mC:	5-Methylcytosine
AHR:	Airway Hyperresponsiveness
AR:	Allergic Rhinitis
BAL:	Bronchoalveolar Lavages
DE:	Diesel Exhaust
DEP:	Diesel Exhaust Particle
DNAm:	DNA Methylation
DNMT:	DNA Methyltransferase
FA:	Filtered Air
FEF 25-75%:	Forced Expiratory Flow at 25–75% of FVC
FEV_1:	Forced Expiratory Volume in 1 Second
FVC:	Forced Vital Capacity
GM-CSF:	Granulocyte-macrophage Colony-Stimulating Factor
HBECs:	Human Bronchial Epithelial Cells
HDM:	House Dust Mite
Iavg	Average Intensity of all Pixels
IHC:	Immunohistochemistry
IL:	Interleukin
IP-10:	IFN-γ-induced Protein 10
LLOD:	Lower Limit of Detection
LME:	Liner Mixed Effects
MCP-1:	Monocyte Chemoattractant Protein-1
PBMCs:	Peripheral Blood Mononuclear Cells
pDNMT:	Predominant DNMT
$PM_{2.5}$:	Particulate Matter with a Diameter of 2.5 Micromole or Less
pTET:	Predominant TET
RT	Room Temperature
TARC:	Thymus and Activation Regulated Chemokine
TET:	Ten-Eleven Translocation
TRAP:	Traffic-related Air Pollution

Introduction

Traffic-related air pollution (TRAP) contributes to increased morbidity and mortality of respiratory diseases, especially asthma (1). Epidemiological studies show that chronic exposure to TRAP, of which diesel exhaust (DE) is a major component, is related to an increased incidence of asthma, and short-term spikes in TRAP can induce airway hyperresponsiveness (AHR) in asthmatic patients (2, 3). We have previously demonstrated using controlled human exposure studies that short-term DE exposure alters DNA methylation (DNAm) in the human lung epithelium and peripheral blood mononuclear cells (PBMCs) (4, 5).

DNA methylation (5mC) is the addition of methyl groups to primarily DNA cytosines(6). DNA methyltransferases (DNMTs) are the enzymes responsible for 5mC deposition and include DNMT3A and DNMT3B which perform de-novo 5mC and DNMT1 which is involved in the maintenance of 5mC following DNA replications (7, 8). DNA demethylation can occur through passive or active mechanisms. Passive DNA demethylation occurs in the absence of maintenance methylation during DNA replication (9). Active DNA demethylation is mediated by the ten-eleven translocation (TET) methylcytosine dioxygenases (10) which include TET1, TET2, and TET3. TET enzymes catalyze the oxidation of 5-methylcytosine to 5-hydroxymethylcytosine (5hmC), 5-formylcytosine, and 5-carboxylcytosine (11, 12). Of these, 5hmC is the most abundant, playing poorly understood roles in genome function (13). Dynamic regulation of DNA methylation and demethylation modulates a wide range of processes, including cell differentiation, cellular ageing and oncogenic transformation (14-17).

Recently, the role of TET enzymes in asthma has gained significant interest. Somineni *et al.* showed decreased methylation of cg23602092 in the TET1 promoter and increased global 5hmC in nasal mucosa samples of asthmatic individuals compared to healthy participants (18). This study reported 5mC at cg23602092 positively correlated with TRAP concentrations by utilizing a land-use regression model as a proxy estimate of DE particles (DEP), and showed that *in vitro* DEP exposures induced cg23602092 methylation in human bronchial epithelial cells (HBECs) at 24 hours (h). Additionally, in TET1-knockout mice, loss of the TET1 enzyme aggravated allergen-induced airway inflammation (19). The evidence above supports the notion of TET enzymes contributing to the effects of environmental exposures (e.g. allergen and TRAP) on asthma pathophysiology (20), but the overall outcomes are currently unclear. Given the data from epidemiological and animal studies, evaluating the role of TET family enzymes, including the understudied TET2 and TET3, in human airways is warranted, and may elucidate additional pathways linking environmental exposures to asthma.

In this study, we performed a controlled human exposure crossover study to investigate changes in DNA methylation, DNA hydroxymethylation and their regulation enzyme expression, including DNMTs and TETs, in response to acute allergen challenge and DE exposures *in vivo* in the human bronchial epithelium. We also analyzed the correlation between these biomarkers and the allergic airway inflammation, which was evaluated by the cytokines and chemokines in the bronchoalveolar lavages (BAL) and the lung function.

Methods

Study design and participants

11 allergen-sensitized participants, including 7 with AHR and 4 without AHR, were recruited. These participants were recruited based on sensitization to common allergens, and were secondarily grouped by AHR status as an objective way to stratify based on objective physiological measures. While participants' responding differently by AHR status was not the primary concern in the initial design of this study, it allowed for some important questions to be asked in the current analysis in spite of modest sample size. AHR was evaluated by measurement of forced expiratory volume in 1 second (FEV_1) during methacholine challenge, using the 2-minute tidal breathing technique, and participant-specific allergen (either house dust mite (HDM), pacific grass or birch pollen) concentration determined with skin prick testing during screening visits (21). The provocative concentration of methacholine eliciting a 20% drop in FEV_1 (PC_{20}) during screening classified participants as either being AHR ($PC_{20} \leq$ 8 mg/mL) or not AHR ($PC_{20} > 8$ mg/mL). During screening, each participant underwent an allergen inhalation challenge to determine a dose of allergen that induce 20% decrease in FEV_1. We used the consistent dose and allergen throughout the study for each participant. This randomized, double-blinded, controlled human exposure crossover study took place between April 2013 and April 2017 (Clinical Trials ID: NCT02017431). Specimens and clinical data were collected with informed consent using protocols approved by the University of British Columbia Clinical Research Ethics Board (H11-01831) in Vancouver, BC, Canada. The study design and exposure procedures have been described previously (22). Briefly, each participant underwent 3 distinct exposures, in a random order, with at least a 4-week washout period between exposures: filtered air (FA) + 0.9% saline (FA-S), FA + participant-specific allergen (FA-A), and DE (diluted to 300 $\mu g/m^3$ of particulate matter sized 2.5 microns in diameter or less ($PM_{2.5}$)) + allergen (DE-A). FA and DE exposures were 2 h in duration. 1 h post-exposure, a 2-minute inhaled allergen challenge was conducted (22, 23). An allergen PC_{20} dose, which was determined at screening based on PC_{20} and skin prick wheal size for each participant (24), was applied for allergen challenge. The characteristics of 11 participants are listed in Table 1.

Lung function

Spirometry measurements were performed based on the American Thoracic Society's guidelines (25), and related results were previously reported (22).

Bronchoscopy procedures and processing of endobronchial biopsy

The bronchoscopy procedure (26) and endobronchial biopsy processing techniques (27, 28) were adapted from work described previously. In brief, a bronchoscopy was performed post-exposure (48h after the start of each exposure), for each condition. The biopsies collected were fixed in ice-cold acetone with protease inhibitors iodoacetamide (20 mM, Sigma, Oakville, ON) and phenylmethylsulfonylfluoride (2 mM, Sigma, Oakville, ON) at 4 °C overnight (16–24h).

On the second day, the protease inhibitors were washed out of biopsies by replacing the acetone solution with fresh acetone at room temperature (RT) and then with 100% methyl benzoate (Sigma, Oakville, ON). Biopsies were infiltrated with glycol methacrylate acrylic (GMA) solution A containing 5% methyl benzoate (replaced 3 times for 2h each at 4 °C). Biopsies were embedded in polymerized glycol methacrylate acrylic (GMA) (29). The JB-4 Embedding Kit (Polysciences, Warrington, PA) was used following manufacturer's instructions. The blocks were stored with desiccant at -20°C until sectioned. 2 μm thick sections from biopsies were cut using an ultra-microtome (Leica EM UC6) and placed on poly-l-lysin-coated slides for immunohistochemistry (IHC) staining.

IHC staining and quantification

IHC was performed to evaluate the levels of DNMT1, DNMT3A, DNMT3B, TET1, TET2, TET3, 5mC, and 5hmC,based on previous work (27, 28), with modifications optimized antibody concentration and antigen retrieval steps. Heat-induced epitope retrieval was applied. Sodium citrate buffer was used for TETs staining, and Tris-EDTA buffer was applied for DNMTs, 5mC, and 5hmC staining. After antigen retrieval, the slides were washed twice with ddH_2O. The sections were permeabilized twice with 0.4% Triton-100 in Phosphate Buffered Saline with 0.1% Tween 20 (PBST) for 10 min and washed with Tris-buffered saline (TBS, 5 mins × 3). 0.3% H_2O_2 in 0.1% aqueous sodium azide was applied to inhibit endogenous peroxidase activity following washes with TBS (5 mins × 3). 20% fetal calf serum and 1% bovine serum albumin in Dulbecco's modified Eagle's minimal essential medium (DMEM) was applied at RT for 30 mins to block the sections from non-specific antibody binding. Primary antibodies (see Table S1) were incubated with each specimen at 4°C overnight. On the next day, the sections were washed with TBS (5 mins × 3) and incubated with biotinylated secondary antibodies at RT for 1h. After washes with TBS (5 mins × 3), the sections were incubated with VECTASTAIN® Elite ABC (avidin-biotin complex) HRP Kit (Vector, PK6100) at RT for 2h and washed with TBS (5 mins × 3). AEC (3-amino-9-ethylcarbazole) peroxidase substrate kit (Vector, SK-4200) was used for color development with 20 mins incubation. The sections were counterstained with Mayer's Hematoxylin (Sigma-Aldrich, MHS16) and covered with CC/mount (Sigma-Aldrich, C9368) and Permount (Fisher Scientific, SP15100) successively for long-term storage.

The slides were scanned with Aperio digital pathology slide scanners (ScanScope AT2) (Leica Biosystem, Buffalo Grove, IL, USA) and quantified with Aperio Positive Pixel Count Algorithm (v9) using Aperio ImageScope software (v12.4.0.5043). The epithelium was gated along the basement membrane (Figure S1). Only the epithelial layer was included in the analysis of this study. The average intensity of all pixels (Iavg) was utilized to quantify the level of the target protein. Higher values of Iavg indicate lower levels of the target.

Electrochemiluminescent multi-plex assay

The cytokines and chemokines secreted into the BAL were measured using the V-PLEX Human Cytokine 30-Plex Kit (Meso Scale Diagnostics, Rockville, Maryland, USA). The list of the cytokines and chemokines is shown in the supplementary materials. The assay was performed following the manufacturer's instructions with 2-fold (cytokine panel 1 and proinflammatory panel 1) and 4-fold (chemokine panel 1) dilution of BAL in assay diluent. The lower limit of detection (LLOD) was set at the signal intensity that was 2.5 standard deviations above the background noise in the blank. For statistical analysis, values below the LLOD were replaced with ½ of the respective LLOD value. Eotaxin, IFN-γ, IL-4, IL-8(HA), IL-10, IL-12p70, IL-13, MCP-4, MDC, MIP-1α, and TNF-β were not reliably detectable above the LLOD and were excluded from statistical analysis.

Statistical analysis

Exposure effects were assessed using linear mixed-effects (LME) models (nlme package version 3.1-142) in R (version 3.6.1). Conditions (FA-S, FA-A, and DE-A) were used as fixed effects and participant identification as a random effect. This model was conducted in 11 participants (Figure 2-4), no AHR group (Figure 5), and AHR group (Figure 5), respectively. A second model was employed to test the baseline level (FA-S) of the biomarkers of interest, between the no AHR group and AHR group. Therefore, AHR status was used as a fixed effect and participant identification as a random effect (Figure 5). A third model was applied to test potential role of DNAm biomarkers levels (5mC, 5hmC, 5mC/5hmC ratio, DNMTs, TETs, pDNMT, and pTET) at baseline in modulating the exposure responses, and condition-by-DNAm biomarker baseline level (e.g. low TET2 vs. high TET2) interaction was the fixed effect in this model (Figure 7G). Spearman's correlation was applied to test the correlation between DNA methylation biomarkers, including 5mC, 5hmC, DNMTs, TETs, pDNMT, and pTET, and clinical lung function (Figure 6) or cytokine and chemokine secretion in the BAL (Figure 7A-E). Spearman's correlation coefficient R and *p*-value were computed. A $p < 0.05$ was considered to indicate a statistically significant difference. The details of missing data are shown in Table S2.

Results

The *predominant* DNMT and TET enzymes in human bronchial epithelium were participant-dependent

Figure 1A-1B show the IHC staining levels of DNMT family (DNMT1, DNMT3A, and DNMT3B) and TET family (TET1, TET2, and TET3) in the bronchial biopsies from the same participant (Participant ID: 5, condition: FA-S). It's worth noting that, the levels of DNMT family and TET family in human bronchial epithelium under FA-S condition varied from person to person. To better understand the role of the most abundant enzyme, we defined, for each study participant, the most abundant DNMT among DNMT1, DNMT3A, and DNMT3B in the airway epithelium under FA-S condition as the *predominant DNMT* (pDNMT), and the most abundant TET among TET1, TET2, and TET3 as the *predominant TET* (pTET). For the example in Figure 1A-1B, the pDNMT was DNMT3B and the pTET was TET3 in this participant. At baseline (FA-S), 6 out of 11 participants were DNMT3B predominant and 8 out of 11 participants were TET3 predominant (See Figure 1 and Table 1). Similar results were found in an *in vitro* analysis of human primary bronchial epithelial cells from another independent study (Table S3). Among those 8 participants, 4 were DNMT3B predominant and 6 were TET3 predominant.

pDNMT and pTET decreased following allergen challenge, irrespective of DE exposure

To investigate the effects of allergen mono-exposure (FA-A) and DE + allergen co-exposure (DE-A) on the DNMT and TET family expression, IHC was applied with the bronchial biopsies which were collected at 48h post-exposure. Representative staining levels of DNMT1, DNMT3A, and DNMT3B levels are shown in Figure 2A. Examining the levels of DNMT1, DNMT3A and DNMT3B individually following FA-A or DE-A exposure (Figure 2B-2D) revealed no significant changes in DNMT1, DNMT3A or DNMT3B, respectively. However, a focus on the pDNMT level showed a significant decrease following FA-A and DE-A exposures (FA-A vs. FA-S, $p = 0.02$; DE-A vs. FA-S, $p = 0.006$, Figure 2E). The levels of TET family members were also evaluated following three exposures in the 11 participants (Figure 3A-3D). Following allergen mono-exposure, TET1 and TET3 were not significantly changed while TET2 was decreased ($p = 0.02$, Figure 3C). With co-exposure to DE and allergen, both TET2 and TET3 were decreased (both $p = 0.02$, Figure 3C-D). Corresponding to the changes of pDNMT, pTET was also decreased following both FA-A and DE-A exposures (FA-A vs. FA-S, $p = 0.04$; DE-A vs. FA-S, $p = 0.009$, Figure 3E).

The ratio of 5mC to 5hmC decreased following allergen challenge, irrespective of DE exposure

Global DNA methylation and hydroxymethylation in the human bronchial epithelium were measured by IHC (Figure 4A). 5mC showed non-significant decreasing trend following both

FA-A and DE-A exposures, relative to FA-S ($p = 0.06$ and $p = 0.16$, respectively, Figure 4B). 5hmC is an emerging epigenetic modification that plays poorly understood roles in genome function. It is also an intermediate in the DNA demethylation pathway allowing us to examine the ratio of 5mC to 5hmC as a rough measure of the balance between DNA methylation and DNA demethylation (30). The ratio of 5mC to 5hmC was decreased following both FA-A and DE-A exposures ($p = 0.006$ and $p = 0.03$, Figure 4D).

5mC levels and associated enzymes differed between participants with and without AHR under baseline conditions and in response to allergen challenge

We explored whether the levels of these DNAm biomarkers (5mC, 5hmC, 5mC/5hmC ratio, DNMTs, TETs, pDNMT, and pTET) in the bronchial epithelium are different at the baseline between AHR group and non-AHR group. The baseline (FA-S) levels of TET1, 5mC, and the 5mC/5hmC ratio in the bronchial epithelium were higher in participants with AHR than those without AHR ($p = 0.03$, $p = 0.04$, and $p = 0.04$, respectively, Figure 5E, 5I, 5K). We further investigated whether these DNAm biomarkers between AHR group and non-AHR group had different responses following exposures by using LME models. pDNMT, pTET, 5mC, and the 5mC/5hmC ratio in the bronchial epithelium decreased following both FA-A and DE-A in participants with AHR, but not in non-AHR (Figure 5D, 5H, 5I, 5K). *P*-values are listed in Table S4. Therefore, global DNA methylation and its regulation enzymes are more susceptible to allergen challenge in the context of AHR.

DNMT and TET were correlated with lung function

To investigate the association between DNAm biomarkers and lung function, including FEV_1, FVC, FEV_1/FVC, and FEF 25-75%, at 48h post-exposure, Spearman's correlation was computed (Selected results are shown in Figure 6, and more related results are shown in Figure S2. The correlation analyses grouped by condition and AHR status are shown in Figure S3 and Figure S4). FEV_1/FVC was positively correlated with DNMT1 ($R = 0.4$, $p = 0.027$, Figure 6A), DNMT3B ($R = 0.4$, $p = 0.028$, Figure 6C), and pDNMT ($R = 0.43$, $p = 0.018$, Figure 6D) levels, respectively, in the bronchial epithelium. FEV_1 was positively correlated with the levels of TET2 ($R = 0.46$, $p = 0.0099$, Figure 6F) and pTET ($R = 0.42$, $p = 0.019$, Figure 6H), respectively, in the bronchial epithelium. These data suggest that DNMT and TET family involve with the regulation of lung function.

TET levels were correlated with cytokines and chemokines in the BAL

To understand the association between DNAm biomarkers and lung inflammation, we correlated DNAm biomarkers with cytokine and chemokine levels in BAL by Spearman's correlation (including 3 conditions). Figure 7A-E only show those plots when the Spearman's correlation coefficient R is above 0.4 and the *p*-value is below 0.05. TET1 levels negatively

correlated with monocyte chemoattractant protein-1 (MCP1), a chemokine that functions in the recruitment of monocytes, memory T-helper cells and dendritic cells (31, 32), in the BAL (R = -0.43, p = 0.016, Figure 7A), and TET2 levels negatively correlated with granulocyte-macrophage colony-stimulating factor (GM-CSF, R = -0.5, p = 0.0037, Figure 7B), a cytokine known to stimulate the differentiation of macrophages and eosinophils (33, 34), and thymus and activation regulated chemokine (TARC, R = -0.44, p = 0.011, Figure 7C), which is also called CCL17 and is known as a chemotactic factor for T cells (35). pTET levels in the bronchial epithelium were negatively correlated with the secretion of Eotaxin-3 (R = -0.52, p = 0.0025, Figure 7D), a chemoattractant of eosinophils and basophils (36), and IL-5 (R = -0.5, p = 0.0033, Figure 7E), a colony-stimulating factor for eosinophils that induces the differentiation of B cells to immunoglobulin secreting cells (37, 38). The correlation analyses grouped by condition and AHR status are shown in Figure S5 and Figure S6. These results suggest that TET levels involve with the development of allergic airway inflammation.

To investigate whether these DNAm biomarkers' baseline levels can be applied in the prognosis of the allergen and DE exposure responses, we did the subgroup analysis based on the baseline (FA-S) levels of these biomarkers one by one. For example, based on the median of TET2 level at baseline, the 11 participants were split into two groups, a low TET2 group (n = 6) and a high TET2 group (n = 5). Focusing on these two groups (Figure 7E) we next asked if there were any differences in the baseline levels of cytokines and chemokines and different responses following exposures (FA-A and DE-A). Intriguingly, IL-8 levels at baseline were higher in the high TET2 group than in those in the low TET2 group (means ± SEM = 38.8 ± 14.1 pg/mL vs. 9.1 ± 1.2 pg/mL, p = 0.01). The exposure effects of FA-A and DE-A on IL-8 secretion in the BAL were modified by TET2 levels (p = 0.03 and p = 0.01, respectively, Figure 7F). The same analysis was also accomplished in the rest DNAm biomarkers, and the interaction between allergen (with or without DE) and TET2 level on IL-8 secretion in the BAL is the most impressive.

Finally, since our *in vivo* study samples did not include a DE mono-exposure so we added an *in vitro* component to the study and found that SRM2975, standardized diesel exhaust particles, decreased the mRNA levels of TET1 and TET2 in the BEAS-2B cell line (Figure S7).

Discussion

This study documents, for the first time, changes in the levels of DNAm regulation enzymes, including pDNMT and pTET, in human airway epithelium following allergen mono-exposure and DE + allergen co-exposure. These results provide solid evidence for the notion that environmental exposure can affect human airway through epigenetic regulation, even the acute allergen inhalation effect can last 48h. No significant difference was found in the comparison between DE-A and FA-A, which infers that the effect of allergen challenge on global DNAm biomarkers were not significantly affected by DE. Given the lack of a DE mono-exposure in our intact human model, which is a recognized limitation, we used an *in vitro* correlate to show decreased the mRNA levels of TET1 and TET2 in the BEAS-2B cell line (Figure S7). Whether more effects will be found on DNA methylation of specific genes or not needs further investigation. Correspondingly, DEP exposures induced the methylation of cg23602092 in TET1 promoter in HBECs (19).

Our data shows higher TET1 levels in AHR participants' bronchial epithelial cells, in comparison to normally responsive participants. Meanwhile, Somineni *et al*. showed that methylation of the TET1 promoter was lower in asthmatic children than non-asthmatic controls (27). These results suggest that higher TET1 levels in the asthmatic airway epithelium might be due to the hypomethylation of site(s) within the TET1 promoter. On the other hand, higher levels of TET1 seems paradoxical to global DNA hypermethylation in participants with AHR. Coincidentally, higher TET levels and global DNA hypermethylation was also found in the PBMCs of allergic rhinitis (AR) patients (39). TET-mediated 5hmC increases may also trigger passive replication-dependent DNA demethylation (40). The activity of DNMT1 can be dramatically suppressed (by a factor of >60) on a 5hmC-abundant DNA substrate (40). This suggests, therefore, that the increased TET level seen in atopic participants may not represent causation, but instead feedback regulation to counter the accumulation of aberrant DNA methylation. In interpreting these results, one should note that while we avoided experimental exposure to grass and tree allergen during their peak season, we cannot rule out some exposure to these allergens outside of the study setting. Any such exposure would bias results away from significant effects in our analysis, leading to potential type 2 error (falsely supporting null hypothesis).

In our study, global DNA methylation and its regulation enzymes were more vulnerable to allergen challenge in participants with AHR, compared to those without AHR. These responses might be related to the higher TET1 and 5mC levels at baseline (FA-S condition) in the bronchial epithelium of AHR group than that of no AHR group. The results above suggest the underlying mechanism of AHR might be due to the differences in epigenetic stabilities between those with and without AHR. Several asthma-related genes, including forkhead box P3 (Fox P3), IFN-γ, IL-4, IL-13, and IL-17, have been found to be more susceptible to the modulation of DNA methylation in asthmatic participants (41-44). The evidence suggests that those with AHR may experience greater inflammatory responses following allergen challenge

due to changes in the epigenome relative to those without AHR, and that those with AHR may have higher susceptibility to greater subsequent responses. Note however that not all our participants with AHR had clinical asthma, and those who were diagnosed with asthma were mild, so our results may not be generalizable to asthmatics of greater severity.

The mechanism by which DNAm regulates the development of AHR is still not fully understood. The known mechanisms of DNAm include not only directly limiting the access to transcription factors and suppress the expression of the target genes (45), but also coordinating with histone variants, histone modifications, and non-coding RNAs in regulating gene expression (46, 47). The DNAm-regulated genes which are associated with asthma phenotypes are involved in the immune response, NO synthesis, lipid pathway, and pharmacologic receptor (48). DE and allergen *in vitro* exposure modified DNAm levels of genes in oxidative stress response, epithelial adherence junction signaling and immune cell responses, including Th1, Th2, macrophage and dendritic cell maturation pathway in HBECs (49). Our previous *in vivo* human study reveals that the order of exposure to DE and allergen determines the epigenetic signals (5). The effect of subsequent exposure to allergen and DE on DNAm of genes involved in cell adhesion and migration, protein localization/transport, angiogenesis, while DNAm of genes involved in protein metabolism and hormone/steroid stimulation are associated with subsequent exposure to DE and allergen (5). The potential role of DNA methylation in mediating the effect of allergen and DE exposures and the development of AHR still needs further investigation.

Zhang *et al*. demonstrated that the mRNA levels of TET1 in HBECs were increased at 1h following diesel exhaust particles (DEP) and house dust mite (HDM) exposures (*in vitro*), respectively, and returned to baseline levels at 4h (49). Earlier, the same team reported that TET1 mRNA was decreased at 4h by the same dose of DEP in HBECs (18). Our *in vitro* study found that SRM2975 decreased the mRNA levels of TET1 and TET2 in BEAS-2B at 2h, 6h, and 18h (Figure S7). While contrary to Zhang *et al.*, perhaps due to the specific DEP used, its dose, the timing of sampling, or cell type examined, this is consistent with our *in vivo* finding that DE (in the context of allergen) did not increase TETs levels in human bronchial epithelium.

TET family members have been demonstrated to have a catalytic domain, comprising a Cys-rich and a double-stranded β helix domain, with all three TET members having 5mC oxidation properties (50). However, there are known differences among the TET members. Both TET1 and TET3 have CXXC domains, which have a high affinity for unmethylated CpG dinucleotides, whereas TET2 does not possess this domain(11). It has been widely reported that TET family enzymes are expressed and regulated in a dynamic and tissue-specific manner (11-13). TET1 and TET2 are highly expressed in mouse embryonic stem cells, while TET3 has a high abundance in oocytes and one-cell zygotes (51, 52). We found that TET1 is consistently predominant in human circulating dendritic cells among different participants (39). Unexpectedly, pTET was participant-dependent in human bronchial epithelium, as was pDNMT. This observation is important, especially when researchers are investigating the role

of these enzymes in primary human samples or in *in vivo* human studies.

As lung epithelial cells serve as barriers to environmental exposures, DNA methylation of these cells has been demonstrated to be affected by various inhaled insults, including particulate matter, allergens, and tobacco smoke (5, 20, 53). We show global hypermethylation in AHR participants' bronchial epithelium, compared to those without AHR, but demonstrate no significant difference between the global 5hmC levels in the epithelium of AHR and non-AHR participants. In an independent study, global 5hmC levels in saliva were higher in asthmatics than non-asthmatics (n = 18 pairs) (18). Higher levels of global 5hmC was also found in the PBMCs from AR patients than those from healthy volunteers (39). This discrepancy might be due to the different sample types across these studies or the relatively small sample size of this study. Intriguingly, we observed that changes in 5mC and 5hmC following allergen challenge tended to be in the opposite directions. As a result, we showed that the 5mC/5hmC ratio significantly decreased following allergen challenge. Moreover, the ratio of 5mC to 5hmC declines with aging, caloric restriction (30), and mucosal hypertrophy in oral ulcers (54), but increases with prenatal mercury exposure (55) and colorectal cancer (56). The ratio of 5mC to 5hmC may be a sensitive marker to monitor environmental exposures and the development of inflammation and we recommend considering this when studying DNA methylation patterns following such exposures.

There are important implications of our findings. Firstly, our data suggest that allergen exposure may have a great effect on triggering epigenetic regulation leading to the development of inflammation. However, it is important to consider the acute DE mono-exposure and DE + allergen co-exposure effects, to further investigate the complex interplay between different environmental exposures and asthma pathophysiology. Secondly, we show that TET levels in HBECs were correlated with not only allergen-induced FEV_1 decline, but also with increased cytokines and chemokines in the BAL; this suggests that the TET family may play a role in instigating airway inflammation in the development of asthma. In support of this, TET2 SNP (rs10010325) was shown to correlate with lung function (57). To some extent, these findings indicate that compared to DNMT, TET changes may be a more sensitive biomarker for airway inflammation and, therefore, a more relevant focus for diagnosis and determining the prognosis of asthma. Interestingly, we demonstrated a correlation, between levels of these fundamental enzymes of DNA methylation and lung function, that suggests a potential physiologic relationship therein even after the primary effect of acute exposure (allergen leading to drop in FEV_1) has resolved. However, further investigation is needed to uncover the mechanism by which TETs mediate the development of airway inflammation. Burleson *et al*. showed that IFN signaling and the aryl hydrocarbon receptor pathway are modulated by TET1 in an allergic airway inflammation mouse model (19). NF-κB activation leads to the repression of TET1 and higher immune infiltration in breast cancer, melanoma, lung cancer, and thyroid cancer (58). Increasing our knowledge of the upstream and downstream pathways of TET regulation could provide further mechanistic insight that may enhance asthma treatment or prevention.

Conclusions

Our data suggest that AHR participants whose bronchial epithelium is globally hypermethylated with higher TET enzyme expression may be more susceptible to environmental exposures. This controlled human exposure study provides new evidence of the acute effects of allergen challenge on epigenetic marks, which could be potentially used as biomarkers for the diagnosis, prognosis, and treatment of asthma.

Author contributions

H.L.—formulated and designed this study, performed experiments, analyzed the data, produced most of the tables and figures, and drafted the manuscript; M.H.R.—performed experiments, provided conceptual and logistical support for the study, and reviewed and edited the manuscript; C.R.—performed experiments, provided conceptual and logistical support for the study, and reviewed and edited the manuscript; W.T.—performed experiments, and reviewed and edited the manuscript; R.L.C.—conceptual and logistical support of the study, and reviewed and edited the manuscript; M.J.A.—conceptual and logistical support of the study, and reviewed and edited the manuscript; W.P.W.—helped with study design, reviewed and edited the manuscript; C.C.—obtained funding, designed the study, provided supervision, and reviewed and edited the manuscript.

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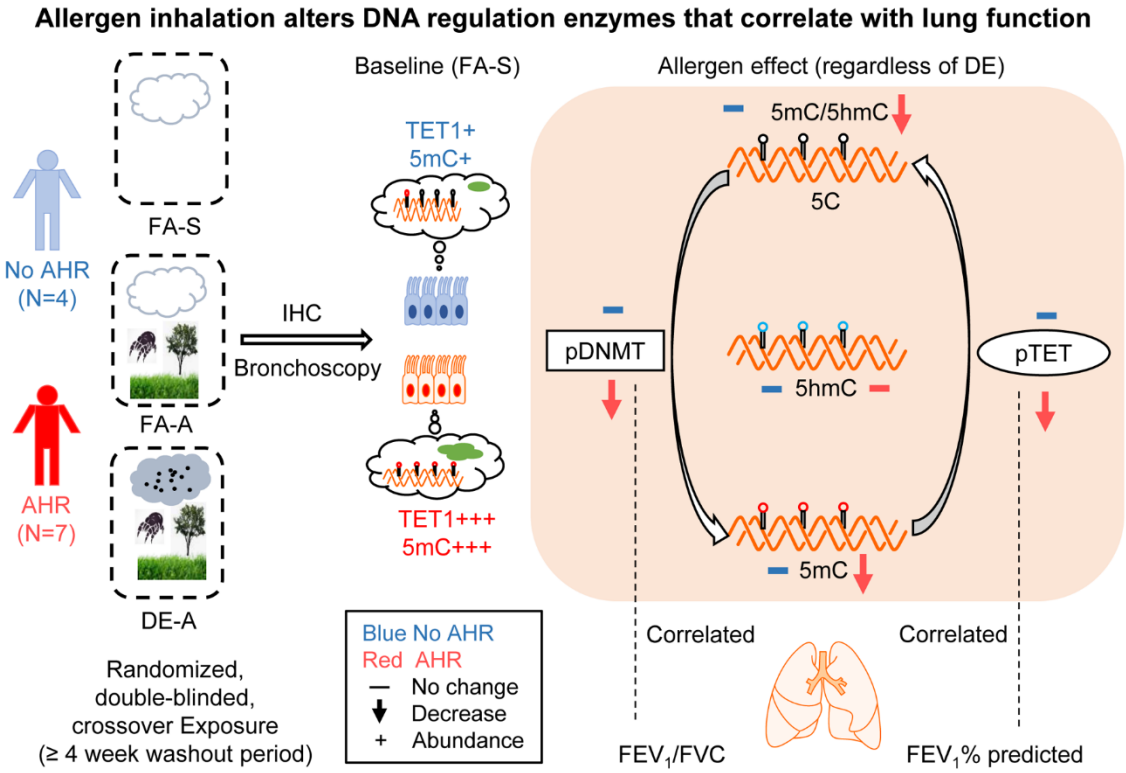
Graphical abstract



AHR, airway hyperresponsiveness; **FA-S**, filtered air (FA) + saline; **FA-A**, FA + allergen; **DE-A**, diesel exhaust (DE) + allergen; **IHC**, immunohistochemistry; **pDNMT**, predominant DNA methyltransferase (the most abundant DNMT among DNMT1, DNMT3A, and DNMT3B); **pTET**, predominant ten-eleven translocation enzyme (the most abundant TET among TET1, TET2, and TET3); **5mC**, 5-methylcytosine; **5hmC**, 5-hydroxymethylcytosine; **FEV_1**, forced expiratory volume in 1 second; **FEV_1% predicted**, FEV1% of the participant divided by the average FEV1% in the population for any person of similar race, sex, age, and height; **FVC**, forced vital capacity.

Figure and figure legends

Fig 1.

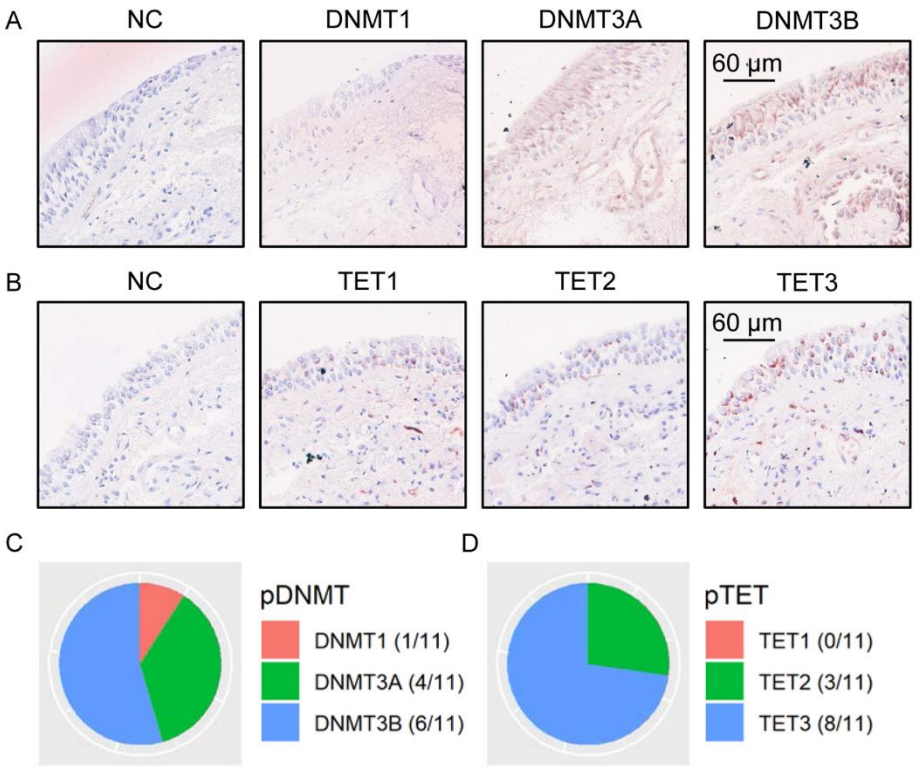


Figure 1. Predominant DNMT and TET enzymes in the human bronchial epithelium were participant-dependent.

Representative images showing the immunohistochemistry (IHC) staining of DNA methyltransferases (DNMTs) (A), ten-eleven translocation enzymes (TETs) (B) and the negative control (NC) in human bronchial biopsies (Participant 5, condition: FA-S). DNMT3B was the predominant DNMT (pDNMT) and TET3 was the predominant TET (pTET) in the bronchial epithelium from this participant. Red staining shows specific target signals (DNMT or TET enzyme) from HRP/AEC/ABC detection kit, and blue staining shows hematoxylin counterstaining. Pie chart showing the number of individuals with predominant DNMT1, DNMT3A or DNMT3B (pDNMT) (C) and predominant TET1, TET2 or TET3 (pTET) (D) in human bronchial epithelial samples (taken from FA-S exposure) from 11 participants.

Fig 2.

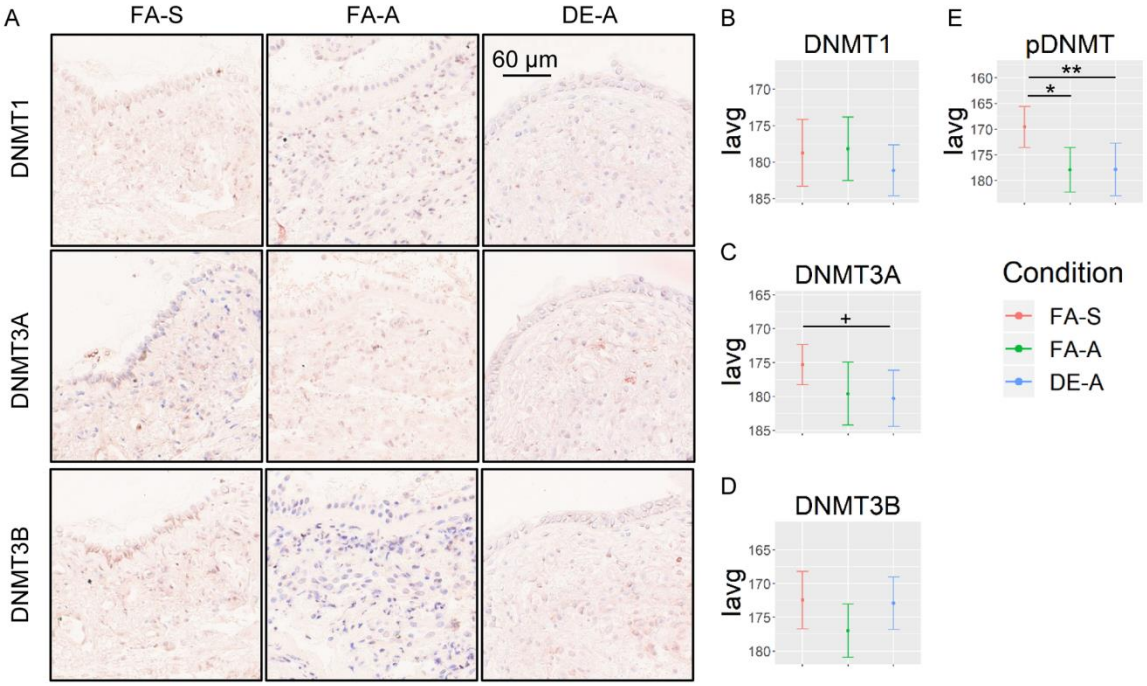


Figure 2. pDNMT decreased following allergen challenge, irrespective of DE exposure. (A) Representative IHC staining images show DNMT1, DNMT3A, and DNMT3B levels in human bronchial epithelial samples (Participant ID: 3) at 48h after exposure to FA-S (negative control), FA + allergen (FA-A), or diesel exhaust (DE) (diluted to 300 µg/m^3 of particulate matter sized 2.5 microns in diameter or less ($PM_{2.5}$)) + allergen (DE-A), respectively. Means ± SEM of DNMT1, DNMT3A, DNMT3B, and pDNMT levels are shown in B, C, D, and E, respectively. The Aperio Positive Pixel Count Algorithm (v9) was applied in the quantification of the epithelial layer. Higher average intensity of all pixels (Iavg) indicates a lower level of the target. Linear mixed-effects (LME) model analysis was conducted. +: $p < 0.1$, *: $p < 0.05$, **: $p < 0.01$.

Fig 3.

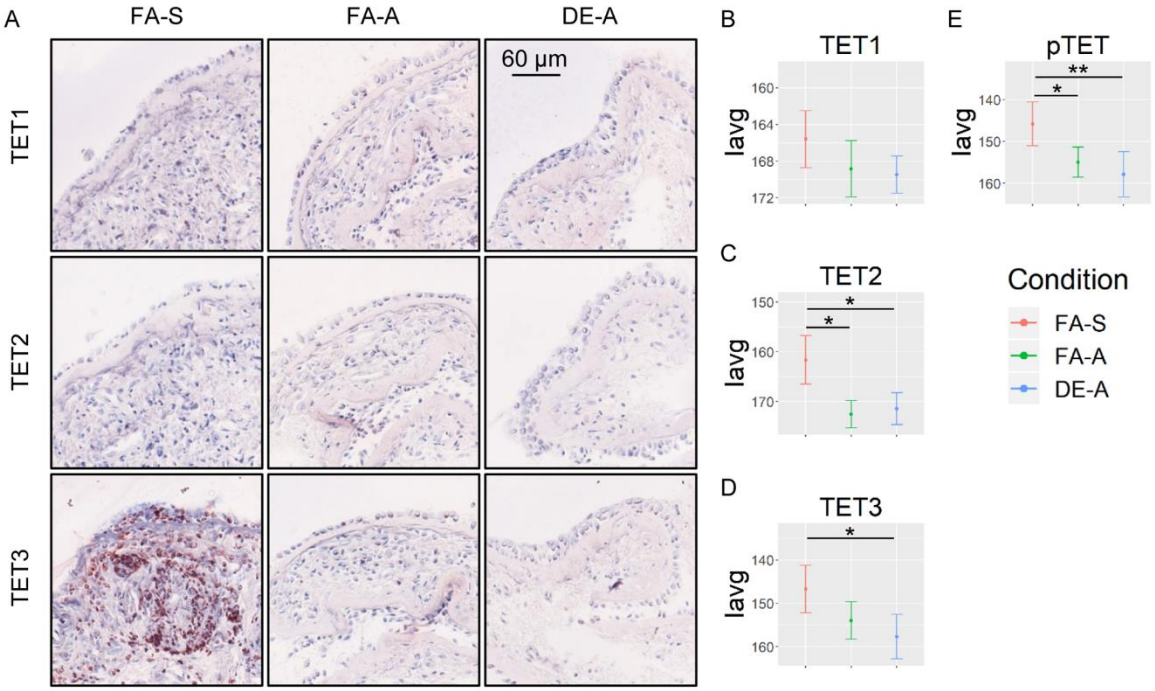


Figure 3. pTET decreased following allergen challenge, irrespective of DE exposure.
(A) Representative IHC staining images show TET1, TET2, and TET3 levels in the human bronchial epithelium (Participant ID: 6) at 48h following exposure to FA-S, FA-A, and DE-A, respectively. Means ± SEM of TET1, TET2, TET3, and pTET levels are shown in B, C, D, and E, respectively. LME model analysis was conducted. Data are presented as means ± SEM. *: $p < 0.05$, **: $p < 0.01$.

Fig 4.

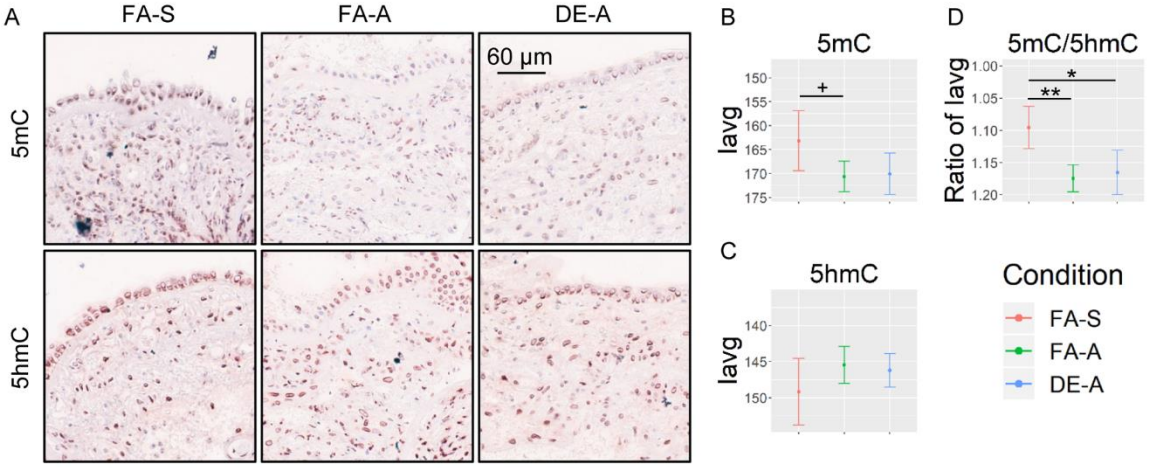


Figure 4. The ratio of 5mC to 5hmC decreased following allergen challenge, irrespective of DE exposure.
(A) Representative IHC staining images show global 5mC and 5hmC levels in the human bronchial epithelium (Participant ID: 3) at 48h following three exposures. Means ± SEM of global 5mC, 5hmC, and the ratio of 5mC to 5hmC are shown in B, C, and D, respectively. LME model analysis was conducted. Data are presented as means ± SEM. +: $p < 0.1$, *: $p < 0.05$, **: $p < 0.01$.

Fig 5.

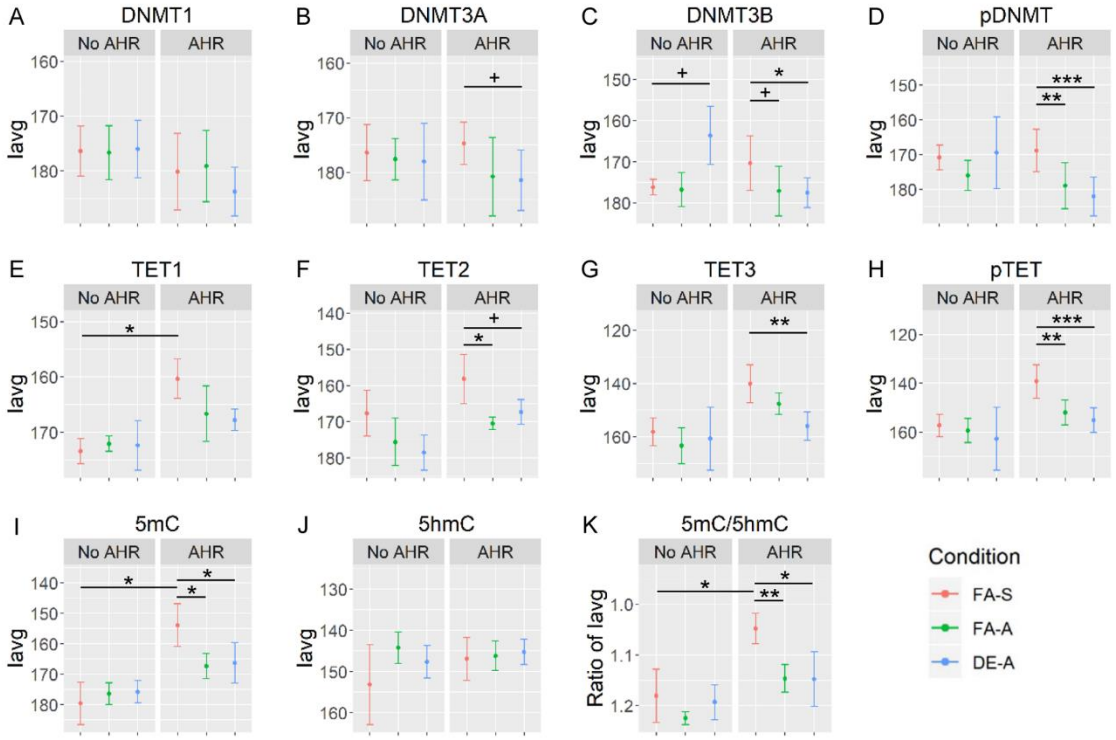


Figure 5. Global 5mC levels and associated enzymes differed between participants with and without AHR under baseline conditions and in response to allergen challenge.
The level of DNMTs (A-D) and TETs (E-H), global levels of 5mC (I) and 5hmC (J), and the 5mC/5hmC ratio were grouped by participants' airway hyperresponsiveness (AHR) status (7 with AHR and 4 without AHR). The levels of TET1, 5mC, and the 5mC/5hmC ratio in the negative control (FA-S) were higher in the bronchial epithelium of AHR group than those of the non-AHR group (E, I, K). Global 5mC levels (I), 5mC/5hmC ratio (K) and their regulation enzymes, including pDNMT (D) and pTET (H), are more susceptible to allergen challenge (irrespective of DE exposure) in AHR group compared to non-AHR group. LME model analysis was conducted. Data are presented as means ± SEM. +: $p < 0.1$, *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$.

Fig 6.

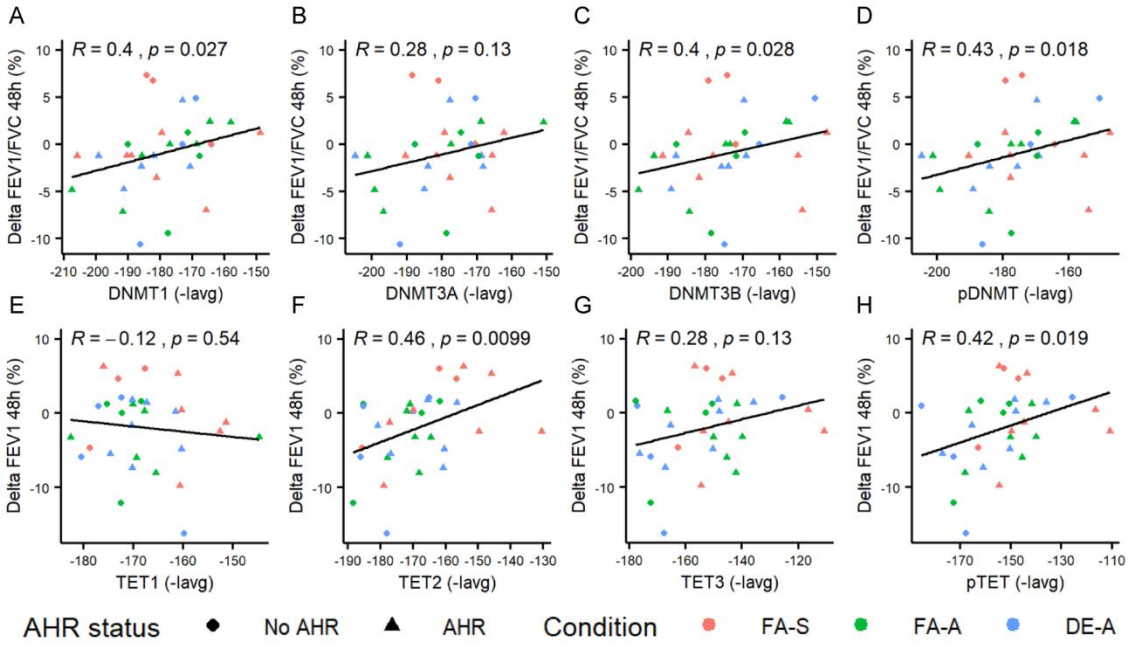


Figure 6. DNMT and TET were correlated with lung function.

Spearman's correlation was computed to analyze the association between DNAm biomarkers and lung function at 48h post-exposure. The correlation analyses between FEV_1/FVC and DNMT1 (A), DNMT3A (B), DNMT3B (C), and pDNMT (D) are shown. The correlation analyses between delta FEV_1 and TET1 (E), TET2 (F), TET3 (G), and pTET (H) are shown. Spearman's correlation coefficient R and *p*-values are shown. Y-axis values are expressed as changes in FEV_1/FVC% or FEV_1% predicted from baseline (pre-exposure) measurements. The X-axis shows the negative $\log_2$(Iavg). The points are shape-coded by AHR status and color-coded by condition.

Fig 7.

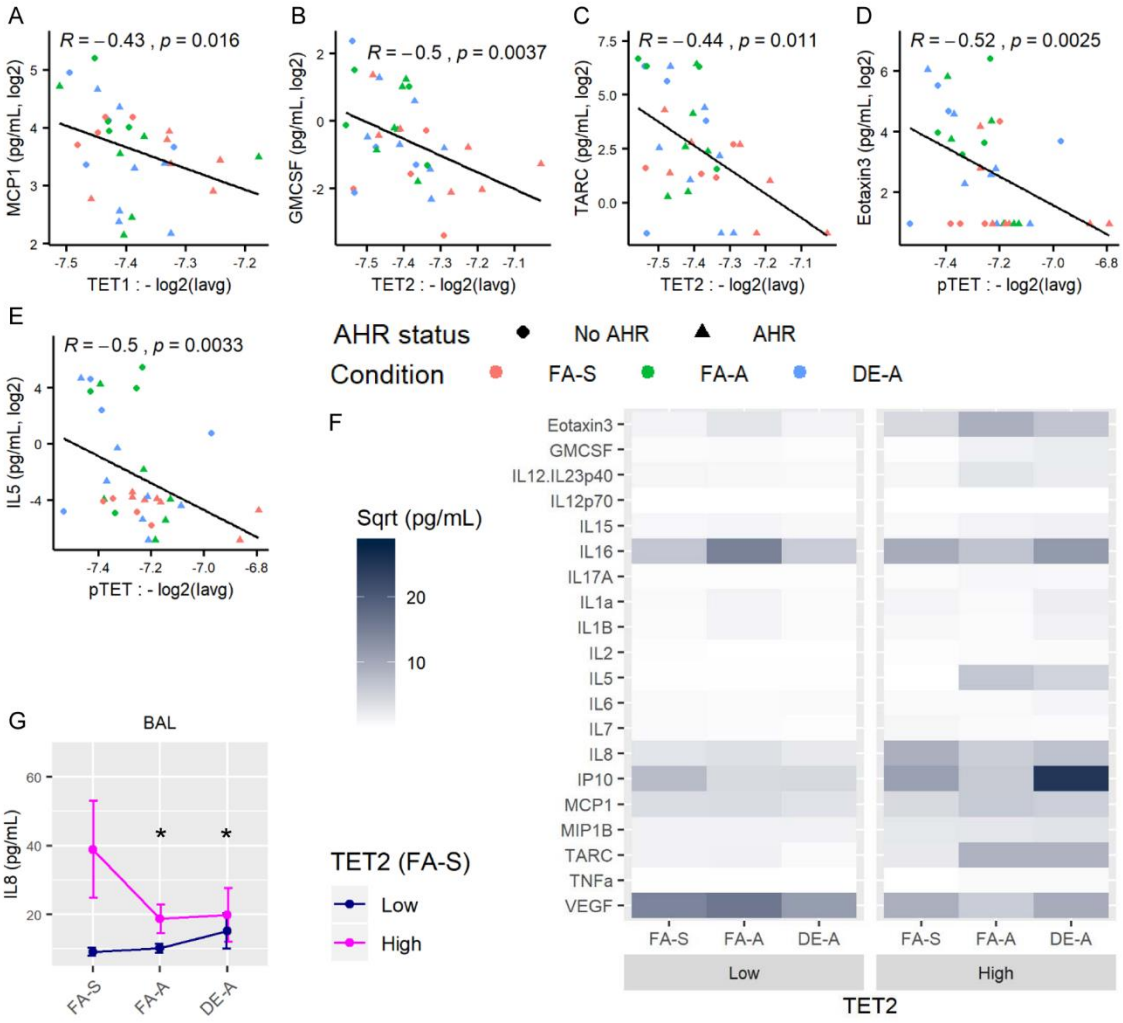


Figure 7. TET level was correlated with cytokines and chemokines in the bronchoalveolar lavage.

Spearman's correlation was computed to analyze the association between TET level and cytokine and chemokine secretions in bronchoalveolar lavages (BAL). Correlation analyses between MCP1 and TET1 (A), GM-CSF and TET2 (B), TARC and TET2 (C), Eotaxin-3 and pTET (D), and IL-5 and pTET (E) are shown. Spearman's correlation coefficient R and *p*-values are listed. The x-axis shows the negative $\log_2$(Iavg). (F) Using the median of TET2 level in the negative control (FA-S), 11 participants were split into two groups, including a low TET2 group (n = 6) and a high TET2 group (n= 5). The heatmap shows the cytokine and chemokine secretions following exposures grouped by TET2 levels. Concentrations are shown as the square root (Sqrt) of the raw data. (G) The exposure effects of FA-A and DE-A on IL-8 secretion in the BAL were modified by TET2 levels. LME model analysis where exposure condition-by-TET2 level (low TET2 vs. high TET2) interaction as the fixed effect was applied. *p<0.05. The points are shape-coded by AHR status and color-coded by condition.

Table 1. Participants' characteristics and their pDNMT and pTET for *in vivo* study

ID	AHR	Sex	Age (yr.)	FEV_1% predicted	Allergen	pDNMT	pTET
1	Yes	F	23	84	Grass	3A	3
2	Yes	F	24	100	HDM	3B	3
3	Yes	F	32	111	HDM	3B	2
4	Yes	F	44	114	HDM	3A	2
5	Yes	M	25	107	HDM	3B	3
6	Yes	M	28	123	Birch	3A	3
7	Yes	M	33	86	Grass	3B	3
8	No	F	28	105	HDM	3B	3
9	No	F	46	97	Grass	3A	2
10	No	M	23	105	Grass	3B	3
11	No	M	30	108	HDM	1	3
Summary	7 Yes	6 F	30.6 ± 7.9*	103.66 ± 11.48*	6 HDM	6 DNMT3B	8 TET3
	4 No	5 M			4 Grass	4 DNMT3A	3 TET2
					1 Birch	1 DNMT1	0 TET1

*Mean ± SD.

AHR, airway hyperresponsiveness; yr., year; FEV_1% predicted, FEV_1% of the patient divided by the average FEV_1% in the population for any person of similar race, sex, age, and height; pDNMT, predominant DNA methyltransferase; pTET, predominant ten-eleven translocation; F, female; M, male; HDM, house dust mite; Grass, pacific grass, Birch, birch pollen.