

DR. ANNE KATHLEEN ELLIS (Orcid ID : 0000-0002-0725-2353)

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Blood and Nasal Epigenetics Correlate to Allergic Rhinitis Symptom Development in the Environmental Exposure Unit

Michelle L. North PhD DABT^{1,3}, Meaghan J. Jones PhD^{2,4}, Julia L. MacIsaac PhD²,
Alexander M. Morin BSc², Lisa M. Steacy BSc CCRP³, Alexander Gregor MD¹, Michael S.
Kobor PhD^{2,4}, and Anne K. Ellis MD MSc FRCPC^{1,3*}

1) Department of Biomedical & Molecular Sciences and Division of Allergy & Immunology,
Department of Medicine, Queen's University, Kingston, ON, Canada.

2) BC Children's Hospital Research Institute and Centre for Molecular Medicine &
Therapeutics, University of British Columbia, Vancouver, BC, Canada.

3) Allergy Research Unit, Kingston General Hospital, Kingston, ON, Canada.

4) Department of Medical Genetics, University of British Columbia, Vancouver, BC, Canada.

* Anne K. Ellis MD MSc FRCPC FAAAAI

Associate Professor and Chair,

Division of Allergy & Immunology

Department of Medicine, Queen's University

Watkins 1D, Kingston General Hospital

76 Stuart Street, Kingston, ON K7L 2V7

Telephone: (613) 548-2336

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Fax: (613) 546-3079

ellisa@queensu.ca

Short Title: Epigenetics in allergic rhinitis symptom development

Abstract

Background

Epigenetic alterations may represent new therapeutic targets and/or biomarkers of allergic rhinitis (AR). Our aim was to examine genome-wide epigenetic changes induced by controlled pollen exposure in the Environmental Exposure Unit (EEU).

Methods

38 AR-sufferers and 8 non-allergic controls were exposed to grass pollen for 3h on two consecutive days. We interrogated DNA methylation at baseline and 3h in peripheral blood mononuclear cells (PBMCs) using the Infinium Methylation 450K array. We corrected for demographics, cell composition, and multiple testing (Benjamini-Hochberg), and verified hits using bisulfite PCR-pyrosequencing and qPCR. To extend these findings to a clinically relevant tissue, we investigated DNA methylation and gene expression of mucin 4 (*MUC4*), in nasal brushings from a separate validation cohort exposed to birch pollen.

Results

In PBMCs of allergic rhinitis participants, 42 sites showed significant DNA methylation changes of 2% or greater. DNA methylation changes in tryptase gamma 1 (*TPSG1*), schlafen 12 (*SLFN12*) and *MUC4* in response to exposure were validated by pyrosequencing. *SLFN12* DNA methylation significantly correlated with symptoms ($p < 0.05$), and baseline DNA

methylation pattern was found to be predictive of symptom severity upon grass allergen exposure ($p < 0.05$). Changes in *MUC4* DNA methylation in nasal brushings in the validation cohort correlated with drop in peak nasal inspiratory flow (Spearman $r = 0.314$, $p = 0.034$), and *MUC4* gene expression was significantly increased ($p < 0.0001$).

Conclusion

This study revealed novel and rapid epigenetic changes upon exposure in a controlled allergen challenge facility, identified baseline epigenetic status as a predictor of symptom severity.

Key Words

Allergen challenge, allergic rhinitis, Environmental Exposure Unit (EEU), epigenetics, pollen

Abbreviations

AR; allergic rhinitis, CT; cycle threshold, CYP2E1; Cytochrome P450 IIE, EEU; Environmental Exposure Unit, FC; fold change, MUC4; Mucin 4, PBMC; peripheral blood mononuclear cell, PCR; polymerase chain reaction, PPIA; peptidylprolyl isomerase A, SLFN12; schlafen 12, SEM; standard error of the mean, TPSG1; tryptase gamma 1, TSS; total symptom score, Ubiquitin C (UBC).

Declaration of Sources of Funding

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Author Contributions

MLN contributed to the conception/design of the work, processed clinical samples, isolated cells and DNA, analyzed pyrosequencing and qPCR data and drafted the manuscript. MJJ analyzed the 450k data, and contributed to the design of follow-up studies on genes of interest. JLM performed the 450k array experiment and pyrosequencing. AMM performed qPCR and pyrosequencing. LMS contributed to the clinical study design, ethical approvals, and design of clinical study procedures. AG performed differential cell counts. MSK contributed to the conception/design of the work and data analysis bioinformatics strategies. AKE contributed to the conception/design of the work, design and performance of the clinical procedures, and data analysis strategies for clinical data. All authors contributed to critical revision of the manuscript and gave approval of the final submission.

Conflict of Interest Statement

AKE declares the following potential conflicts of interest: Participated in advisory boards for Circassia Ltd, GSK, Merck and Novartis, been a speaker for Novartis, Merck, Meda, Pfizer, Takeda and AstraZeneca, and institution received research grants from GSK, Circassia Ltd, SunPharma, Merck, Pfizer and Novartis. All other authors declare they have no potential conflicts of interest.

Introduction

Allergic rhinitis (AR) is an IgE-mediated disorder involving symptoms of rhinorrhea, sneezing, nasal congestion, and aggravation of comorbid asthma (1, 2). Approximately 25% of the world population is affected (2). AR has significant socioeconomic and quality of life impacts (3, 4). Although some genetic associations have been made, genetics alone cannot predict allergic disease or variability in phenotype (5). Epigenetic modifications, such as DNA methylation, may play a role in mediating environmental effects, genome accessibility, transcription factor binding, and gene expression (6-8).

Thus, DNA methylation may play a role in allergic responses. In this investigation we employed the Environmental Exposure Unit, a controlled allergen challenge facility that offers the unique ability to study the induction of symptoms, while removing many of the confounding variables in the participant's natural environment (9, 10). Allergen exposure studies also offer the ability to collect blood, nasal and other samples at well-defined time points, and to perform studies out of season in order to isolate the early DNA methylation changes associated with the induction of allergy symptoms.

We interrogated lymphocyte-enriched peripheral blood mononuclear cells (PBMCs) before and after grass pollen exposure with the Infinium Methylation 450K BeadArray. While controlling for inter-individual variability in blood cell types, we demonstrated that AR sufferers underwent rapid DNA methylation changes in response to allergen, while concurrently-exposed non-allergic controls did not. Pyrosequencing and qPCR, revealed correlations between DNA methylation in *Schlafen 12* (*SLFN12*), gene expression and allergic symptoms, and differences in baseline that predicted symptom severity. Finally, we

found that mucin 4 (*MUC4*) in the blood corresponded to nasal DNA methylation and gene expression in a separate cohort.

Materials and Methods

Participant Screening and Enrolment

The Queen's University Health Sciences and Affiliated Teaching Hospitals Research Ethics Board approved the study, and all participants provided written informed consent before undergoing any study-specific procedures. Persons 18-65 years old were eligible to participate. We conducted skin testing to rye grass, timothy grass, blue grass, grass-mix, ragweed, *Alternaria*, birch, tree-mix, dog, cat, *D. pteronyssius*, and *D. farinae*) and read the early-phase response at 15 minutes post-application, as it has been demonstrated to correlate to nasal symptoms in AR (11). Allergic participants had a positive test to rye grass (defined as a wheal diameter of 3mm or greater in excess of the negative control) (12), and reported symptoms during the two preceding grass seasons. Non-allergic controls were negative to all allergens and reported no allergy symptoms. The study was conducted out of season, and participants with symptoms due to other allergens were excluded. Exclusion criteria and medication washouts were detailed previously (13).

Grass Pollen Exposures in the Environmental Exposure Unit

38 allergic participants and 8 non-allergic controls chose to enroll. All participants who took part were exposed to 2000-3500 grains/m³ of rye grass pollen in the unit for 3h on two consecutive days. All participants in the nasal brushing cohort were exposed to 3500 grains/m³ of birch pollen for one 4h session (± 500 grains/m³). Air quality, pollen characteristics and distribution have been described previously (9, 13, 14).

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Participants recorded their symptoms every 30m using a four-point (0–3) scale for; rhinorrhea, nasal congestion, sneezing, itchy nose, itchy/gritty eyes, watery eyes, red/burning eyes, and ear/palate/throat itching. Total symptom score (TSS) was the sum of symptom scores (maximum 24). Additionally, participants measured their Peak Nasal Inspiratory Flow (PNIF), a measure of nasal congestion, using the In-Check meter (Clement Clark International) (13, 15). Physician observers circulated inside the unit during all exposure sessions. They were present for safety, to help correct participant PNIF technique, and to periodically record objective signs of allergy for each participant. Physician observations were not used in analyses but were cross-referenced with self-reported symptoms to check for gross inaccuracies.

Lymphocyte-Enriched Cell Preparation

We collected heparinized blood at baseline, 3h (after the first exposure) and 27h (after the second exposure). The Kingston General Hospital core laboratory performed complete blood counts. Within 1h of collection we layered blood, diluted 1:1 with McCoy's 5A medium (Gibco, Burlington, Canada), onto Lymphoprep (Axis, Norway) for lymphocyte-enriched PBMC isolation. Post-enrichment, we performed differential counts on cytospin slides stained with Diff-Quik (Siemens, Newark, DE). Samples were homogenized using QiaShredder Columns (Qiagen, Germantown, MD), and DNA was isolated using the DNeasy kit (Qiagen).

Genome-Wide DNA methylation Analysis

The EZ-DNA methylation kit (Zymo Research, Irvine, CA) was used for bisulfite conversion and DNA from the 15 allergic participants with the highest TSS and 8 non-allergic controls were interrogated using the Illumina Infinium HumanMethylation450 BeadChip (Illumina,

San Diego, CA). We used Illumina GenomeStudio software to extract raw signal intensities, check two technical replicates, and sex of each participant. Overall intensity and probe-type were normalized by quantile and peak-based correction (16, 17). Probes with poor design features, SNPs, X/Y chromosomes, very-low-variability (standard deviation <0.05), or not-above-background were excluded (28,092 probes analyzed) (18). Cell type was controlled for using post-enrichment differentials and multiple linear regression (16, 19). To compare DNA methylation between allergics and controls at baseline, and within participants before/after exposure, we used the `lm` function in RStudio with the Benjamini-Hochberg correction (20). We considered a mean difference in beta ($\Delta\beta$) $\geq \pm 0.02$ and an adjusted p-value ≤ 0.1 to be significant. For all other comparisons $p \leq 0.05$ was considered significant. Data has been deposited in the Gene Expression Omnibus (GSE100386).

Bisulfite PCR-Pyrosequencing

We validated DNA methylation changes including *MUC4*, *TPSG1* and *SLFN12* as they demonstrated the most significant changes and biological plausibility. We performed bisulfite PCR-pyrosequencing in triplicate using a Pyromark™ Q96 MD and Pyro Q-CpG software (Biotage, Sweden). Validated pyrosequencing assays for human *SLFN12*, *TPSG1*, and *MUC4* were purchased from EpigenDx (Hopkinton, MA). To compare pyrosequencing to the array we used Bland-Altman plots and Spearman correlations. Pyrosequencing and qPCR analyses were carried out in Prism statistical software (Graphpad Software Inc.).

qPCR

To assess gene expression, we reverse-transcribed RNA with the QuantiTect Kit (Qiagen) according to the manufacturer's protocol. We used a Rotor-Gene 6000 (Corbett) and PerfeCTa SYBR Green FastMix (Quanta Biosciences) and the primers detailed in

Supplemental Table 1. Average Ct values of triplicates were normalized to peptidylprolyl isomerase A (*PPIA*) for blood, and polyubiquitin-C (*UBC*) for nasal brushings.

Results

Participants and AR symptom development

Our goal was to identify epigenetic changes underlying allergic symptom development. To isolate early epigenetic changes, we performed allergen challenges out-of-season, focused on the 3h time-point, and compared non-allergic controls to the 15 allergic participants who developed the most severe symptoms. Factors such as age, sex, and number of positive allergy tests did not differ (**Table 1**).

Table 1: Participant Characteristics

Group	n	F	Age	+SPT Results	Grass +SPT	Mean TSS at 3h
Allergic participants included in 450k analysis	15	47%	22 - 58	9.1 ± 0.3	yes	18.2 ± 0.7
Allergic participants not included in 450k analysis	23	56%	21 - 63	8.3 ± 0.4	yes	11.3 ± 1.1
Controls (all controls were included)	8	50%	21 – 61	0	no	0.27 ± 0.1

Table 1: Characteristics of participants. We averaged symptom scores between 30 min and 3h (inclusive) to obtain the mean total symptom scores (TSS) (13). We interrogated lymphocyte-enriched PBMCs from the 15 allergic participants with the highest average TSS for genome-wide DNA methylation using the Infinium 450K BeadArray. F; percent female, +SPT; number of positive skin prick test results. SEM; standard error of the mean.

Lymphocyte enrichment and cellular composition

Differential cell counts before and after lymphocyte enrichment revealed a 220% increase of lymphocytes to 66.3% of the total sample. To control for cell composition we used multiple linear regression including neutrophils, lymphocytes, monocytes, eosinophils and basophils (19). We did not find differences between allergic participants and controls.

DNA methylation changes after allergen exposure

There were no significant differences in DNA methylation between AR and controls at baseline (**Figure 1A**). However, allergic participants experienced changes following the 3h exposure, while non-allergic controls did not (**Figure 1B and C**). When we restricted our findings to $\Delta\beta$ values of greater than ± 0.02 (at least 2%), 42 CpGs showed significant differences (adjusted $p \leq 0.1$, **Supplemental Table 2**).

Pyrosequencing Genes of Interest

After identifying DNA methylation changes using the array, we validated three biologically-relevant genes; *TPSG1*, *SLFN12*, and *MUC4*. Correlation analysis and Bland-Altman plots established good concordance between array and pyrosequencing (**Supplemental Figure 1**).

We then took advantage of the simplicity of pyrosequencing to examine more participants (including those who were not included in the array), add the 27h time point (blood drawn after the second consecutive daily 3h pollen exposure session), and examine broader DNA methylation neighbourhoods.

After 3h of pollen exposure, there was a significant decrease in DNA methylation by pyrosequencing at the site of interest in *TPSG1* across all allergic individuals in the study ($p = 0.025$, **Figure 2A**). Despite this analysis including those with relatively milder allergy

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symptoms who were not selected for the array, the magnitude of the change was comparable to that detected on the array, as evidenced by overlapping 95% confidence intervals (pyrosequencing mean $\Delta\beta$ = -1.19; -0.16 to -2.22, array mean $\Delta\beta$ = -2.29; -1.59 to 2.99). However, this change appeared transient, as it was not significantly different at the 27h time point, and no other site in the region changed significantly ($p>0.05$, **Figure 2A**).

In contrast to the single-site transient DNA methylation effect observed in *TPSG1*, we found changes at surrounding sites, and at multiple time points, upon examination of *SLFN12*. Significant increases in mean $\Delta\beta$ were detected at 3h ($p = 0.003$, **Figure 2B**), and became progressively larger at 27h ($p = 0.004$, **Figure 2B**). The magnitude of the increase, when all allergic participants were considered by pyrosequencing, was smaller than the increase detected on the array in the high-responders (3h pyrosequencing mean $\Delta\beta$ = 0.24; 0.13 to 0.35, 27h pyrosequencing mean $\Delta\beta$ = 0.97; 0.47 to 1.47, array mean $\Delta\beta$ = 3.67; 2.25 to 5.10). At 27h, DNA methylation at two CpGs were also significantly elevated at the individual level (CpG₁ $p = 0.015$, CpG₆ $p = 0.0085$, **Figure 2B**).

MUC4 DNA methylation also changed, with a significant increase in mean $\Delta\beta$ in *MUC4* ($p = 0.026$, **Figure 2C**) evident at 3h. Pyrosequencing on the full cohort demonstrated changes with a magnitude similar to what the array revealed in high-responders, as evidenced by the overlapping 95% confidence intervals (3h pyrosequencing mean $\Delta\beta$ = 2.6; 0.34 to 4.83, array mean $\Delta\beta$ = 2.2; 1.2 to 3.2). This was encouraging, as it indicated that the changes in *MUC4* were not only experienced by participants with high TSS, but were detectable across a spectrum of symptom severity. After 27h, methylation in *MUC4* remained significantly elevated relative to baseline ($p = 0.0005$, **Figure 2C**).

DNA methylation at SLFN12 correlates with symptoms

Next we examined whether the changes in DNA methylation at the loci of interest correlated with symptoms. *TPSG1* and *MUC4* did not ($p > 0.05$), however, at *SLFN12*, changes in DNA methylation at the site of interest correlated with increases in TSS (**Figure 3A**, Spearman $r = 0.43$, $p = 0.029$).

Lower baseline SLFN12 is associated with increased allergic symptoms

Having established increased DNA methylation at *SLFN12* upon allergen exposure, we sought to determine if this was associated with gene expression. A significant correlation emerged between methylation and gene expression when we performed qPCR on PBMC RNA (**Figure 3B**, Spearman $r = -0.308$, $p = 0.013$). This prompted us to examine baseline *SLFN12* expression relative to subsequent symptom development. We found that those participants with the lowest expression of *SLFN12* at baseline exhibited the most extreme allergic symptoms (**Figure 3C**, $p = 0.0199$), suggesting that the pre-existing level of *SLFN12* expression before allergen exposure can affect the response. For DNA methylation, we observed a different pattern. While the direction of DNA methylation change in response to allergen exposure was related to symptoms, as shown above, we did not find a relationship between DNA methylation at baseline and the subsequent development of symptoms (data not shown).

MUC4 in nasal brushings

MUC4 is a mucin, and thus is a member of a family of proteins that are critical constituents of mucus, which plays a prominent role in rhinorrhea and congestion (1, 2). To determine if the changes we observed in *MUC4* DNA methylation were associated with gene expression, we performed pilot qPCR studies in PBMC RNA. However, we were not able to detect

MUC4 transcripts in the blood, consistent with previous reports of somatic expression patterns (21). Some blood epigenetic changes have been shown to correlate with other tissues (22). We thus sought to discover if in this case the blood could be a biomarker of modifications in a relevant tissue, such as the nasal epithelia (23). As nasal samples were not available in the grass-exposed group, we examined *MUC4* DNA methylation and gene expression in nasal brushings from an independent cohort exposed to birch pollen for 4h. Nasal brushings have been shown to capture primarily nasal epithelial cells (23-25).

The *MUC4* DNA methylation pattern we observed in nasal brushings mirrored the pattern in blood. Similar to blood, mean $\Delta\beta$ was significantly elevated post-exposure (**Figure 4A**, $p = 0.017$). At 4h post-exposure, DNA methylation at CpG2 was significantly elevated at the individual level ($p = 0.004$, **Figure 4A**). In addition, although blood and nasal samples were not matched between individuals, the magnitude of the increase in the nose across all participants was similar to the blood, as evidenced by overlapping 95% confidence intervals (4h nasal pyrosequencing mean $\Delta\beta = 1.643$; 0.2967 to 2.989, PBMC array mean $\Delta\beta = 2.2$; 1.2 to 3.2). This indicated similar changes in *MUC4* methylation across tissues in response to allergen-induced AR symptoms.

Upon deeper analysis, we found a trend towards association between nasal DNA methylation and lower *MUC4* expression (**Supplemental Figure 2**, $p = 0.12$). We observed a significant correlation between PNIF, an indicator of congestion, and the mean $\Delta\beta$ in the nose (**Figure 4B**, Spearman $r = 0.314$, $p = 0.034$). Birch pollen exposure was significantly associated with an up-regulation of gene expression at *MUC4*, relative to the ubiquitin C (UBC) reference gene (**Figure 4C**, $p < 0.0001$).

Discussion

Evidence that epigenetic mechanisms are involved in allergic airways diseases is accumulating (7, 26). We studied DNA methylation and AR symptoms in a controlled setting, using the Environmental Exposure Unit, and found 42 significant ($p < 0.1$) changes of greater than 2%. Baseline DNA methylation at one of these genes, *SLFN12*, was associated with symptom severity. Additionally, changes in blood prompted us to investigate nasal *MUC4* DNA methylation and expression, both of which were altered by birch pollen exposure in an independent cohort.

Early epigenetic changes triggered by allergen exposure in symptom-free individuals are important to isolate, as they represent potential therapeutic targets. One recent allergen-challenge study demonstrated DNA methylation in blood to be a useful biomarker of early vs dual response (27). That study harnessed epigenetics as a signal of underlying changes in lymphocyte subsets, while we focused on changes that were independent of cell composition (27). Both studies highlight the responsiveness of blood DNA methylation to allergens and the potential utility of epigenetic biomarkers in allergy (27). We also examined changes in *SLFN12* by pyrosequencing. These changes may suggest monocyte differentiation, or T cell activation (28). However, as our blood samples were lymphocyte-enriched, the signal was likely predominated by T cells. In T cells, *schlafens* (*SLFN*) promote quiescence and repress activation (29, 30).

Allergy sufferers with the lowest *SLFN12* expression at baseline developed the most acute symptoms. While DNA methylation at baseline was not predictive of subsequent symptoms, higher *SLFN12* DNA methylation post-exposure and its association with allergic symptoms was consistent with *SLFN12* acting to contain immune responses. The location of the CpG

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sites also suggest plausible connections to gene regulation, as they are in the first exon of *SLFN12*, in a DNase I hypersensitive site, overlapping with histone H3K27 enrichment and predicted transcription factor binding sites.

It is known that AR patients exhibit different levels of reactivity to allergen, but data on the underlying mechanisms are scarce. We previously demonstrated this is unrelated to skin test wheal size, but potentially to allergen exposure (14). Recently, another study demonstrated that exposures to allergen and diesel exhaust in sequence 4 weeks apart induced more significant alterations than either exposure individually (31). Thus, previous exposures such as allergens or pollutants may affect future responses (31). Aside from excluding participants with recent viral infections, we did not collect information about prior exposures. Future studies should evaluate environmental exposures in the weeks prior to allergen challenge, and how they relate to the baseline epigenetic and gene expression status of the individual. The technique of grouping participants based on molecular biomarkers has previously been used in cancer research, and we demonstrate here that it may also have value in allergy (32).

In addition to *SLFN12*, we also investigated *MUC4*. Mucin proteins are major constituents of mucus, which plays a role in AR-related congestion (33, 34). We found that *MUC4* underwent similar DNA methylation changes in the blood and nasal epithelia, concurrent with the development of symptoms. The suitability of blood DNA methylation as an indicator of target tissues is an area of great interest, but has also been debated (22, 35). We did not examine *SLFN12* DNA methylation in the nose, as expression in the nasopharynx and bronchi have not been reported (21). Additionally, *SLFN12* functions are immunomodulatory, and expression has been shown to be cytoplasmic in blood immune cells (29, 30). Thus, while our study does not indicate that all genes experience

harmonious changes in the blood and target tissues, our data suggests that perhaps blood methylation can be a suitable indicator in a subset of genes that are biologically relevant to the exposure of interest. Future studies could probe farther through a genome-wide approach in both blood and nasal tissue.

MUC4 has been shown to be susceptible to epigenetic control, upregulated by proinflammatory stimuli, and to be involved in nasal polyps and cystic fibrosis (36-39). Our data extends these observations by demonstrating the involvement of *MUC4* DNA methylation in the onset of allergic symptoms. It is known that mucus hypersecretion plays a large role in AR (40). However, we also observed a trend between increased DNA methylation and lower gene expression, which appears paradoxical. We hypothesize that although overall *MUC4* gene expression is higher after allergen exposure, as mucus is known to be induced, the increased DNA methylation response has a modifying effect in the direction of preventing excessive *MUC4* expression. This idea is supported by our finding that participants who demonstrated higher DNA methylation at 4 hours experienced less severe symptoms. The four sites of interest in *MUC4* are in a middle intron/gene body, in a DNase I hypersensitive site region, partially overlapping with a CpG island. Thus, this region has regulatory potential, but the changes observed could also be secondary to gene expression. This study was not designed to capture the time points necessary to fully understand this phenomenon or to imply causation. *MUC4* may represent a future therapeutic target, as there is a need for non-sympathomimetic treatments for congestion, the AR symptom that is consistently cited by patients as being the most bothersome (15, 41).

This study has various limitations. Although we did not find any cases of gross inaccuracy when comparing to the observations of physician observers, self-reported symptoms remain a subjective measure. Despite this limitation, we chose to use self-reported symptoms rather than the physician observations in our analyses, in accordance with the FDA guidance on clinical outcomes for allergic rhinitis studies (42). An additional limitation was the fairly small sample size and effect sizes for our genes of interest. However, we validated several of the changes using pyrosequencing, and observed that the effects were generally larger in the population that was examined using the array. This is likely due to the inclusion of the allergic individuals with the most severe symptoms. We also investigated gene expression and confirmed changes in *SLFN12* and *MUC4*. Another potential approach would be a method such as RNAseq, which could extend the inquiry into transcriptome-wide changes in future studies. Additionally, this study did not include measures of nasal inflammation, such as lavage or nasal nitric oxide. In the future, correlating inflammation with epigenetics may bring additional insights.

A strength of this study was the inclusion of non-allergic individuals, which ensured that the DNA methylation changes we identified were allergy-specific, by controlling for the ‘white-coat effect’, and diurnal variation (43). However, the number of non-allergic participants was smaller than the allergic individuals. Future studies should aim to have matched controls to further explore allergen-induced changes in DNA methylation.

Conclusions

In conclusion, this study demonstrated that participants with grass pollen-induced AR undergo epigenetic changes within 3h upon exposure to grass pollen. We validated epigenetic changes in *TPSG1*, *SLFN12*, and *MUC4*, and follow-up studies indicated that *SLFN12* and

MUC4, two genes with plausible connections to allergic airways diseases, may represent future therapeutic targets. Stratification of AR participants based on *SLFN12* expression at baseline predicted the severity symptoms, suggesting potential effect modification of the epigenetic code in that region. This study also highlights the possibility that epigenetic changes in the blood may be indicative of similar modifications in target tissues, and that pre-existing epigenetic marks, potentially due to environmental exposures, may have effects on subsequent responses to allergen.

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Figure Legends

Figure 1: DNA methylation dynamics in participants with allergic rhinitis and controls at baseline and upon exposure to pollen. Volcano plots of **A)** allergic vs non-allergic control at baseline, **B)** Non-allergic control 0h vs 3h, and **C)** Allergic 0h vs 3h, show statistical significance (-log of nominal P value, y axis) and biological significance (mean difference

between groups, x axis), with vertical thresholds indicating a minimum absolute difference between groups of 0.02, and horizontal threshold indicating an false discovery rate of 0.1. Note that baseline (A) (top) and control 0 versus 3h (B) did not have any sites that met false discovery rate of 0.1, and so thresholds are not indicated.

Figure 2: Pyrosequencing at baseline and following 3h of grass pollen exposure

These panels present pyrosequencing results from lymphocyte-enriched blood samples from participants included on the array as well as all additional participants in the study for whom sufficient DNA was available. A) 7 sites in tryptase gamma 1 (*TPSG1*), B) 6 sites in schlafen 12 (*SLFN12*), and C) 3 sites in mucin 4 (*MUC4*). ϕ site of interest identified by the array. # single CpG site ($p < 0.05$). * mean $\Delta\beta$ $p < 0.05$. Error bars indicate standard error of the mean.

Figure 3: Schlafen 12 (*SLFN12*) DNA methylation relationships to gene expression and allergic symptoms

These panels examine DNA methylation and gene expression in lymphocyte-enriched blood samples. A) Change in total symptom score during the 3 hour grass pollen exposure significantly correlated with change in % DNA methylation at *SLFN12* ($p = 0.029$, Spearman $r = 0.427$, $n = 26$) B) Fold change in *SLFN12* RNA expression, relative to the reference gene peptidylprolyl isomerase A (PPIA)), significantly correlated with % DNA methylation at the site of interest ($p = 0.013$, Spearman $r = -0.308$) across all time points ($n = 65$). C) Change in total symptom score at the 3h time point experienced by allergic participants, by quartile of baseline *SLFN12* expression. The mean increase in symptoms experienced by the participants in the quartile with the lowest expression at baseline (Quartile 1), was significantly higher than the participants in Quartile 4, who had the highest *SLFN12*

expression at baseline (n = 38, 9-10 per quartile, p < 0.05). Error bars indicate standard error of the mean.

Figure 4: Mucin 4 (*MUC4*) DNA methylation and expression in nasal brushings

A) This panel presents pyrosequencing results from nasal brushings as changes from baseline after 4h of birch pollen exposure. The mean $\Delta\beta$ changed significantly following exposure (p = 0.017). **B)** Average change in DNA methylation correlated with drop in peak nasal inspiratory flow, a measure of congestion (Spearman r = 0.314, p = 0.034). **C)** *MUC4* gene expression was significantly higher in the nasal epithelia of birch-allergic participants after 4h of exposure to pollen in the environmental exposure unit (n = 39 for allergic 0h, n = 38 for allergic 3h, ***p < 0.0001 2-way ANOVA). *MUC4* expression did not change in non-allergic participants exposed concurrently (n = 10 for 0h and 4h).

Figure 1

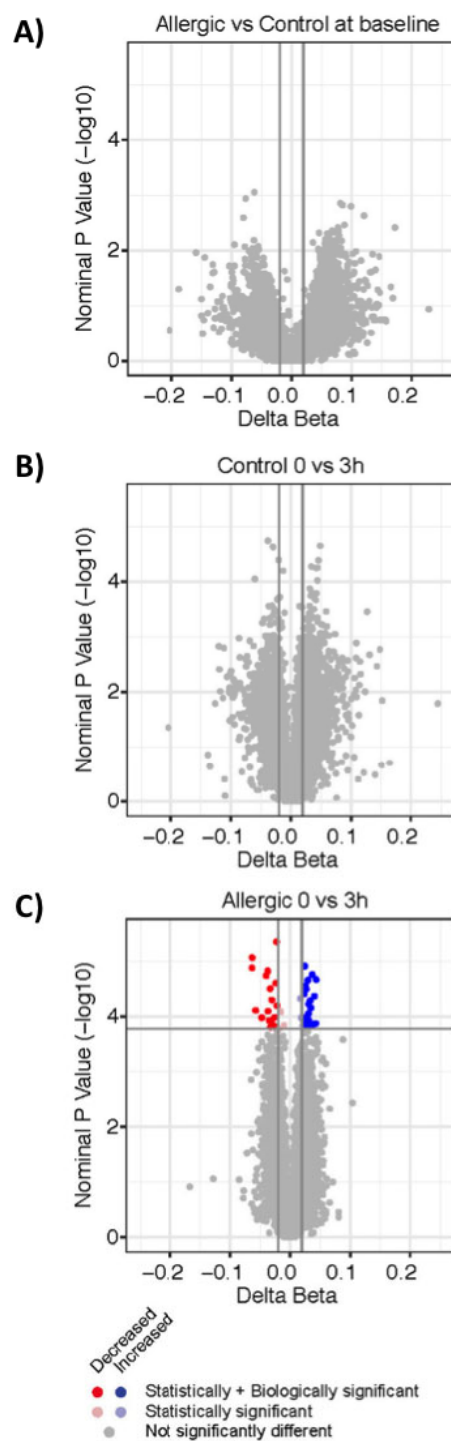
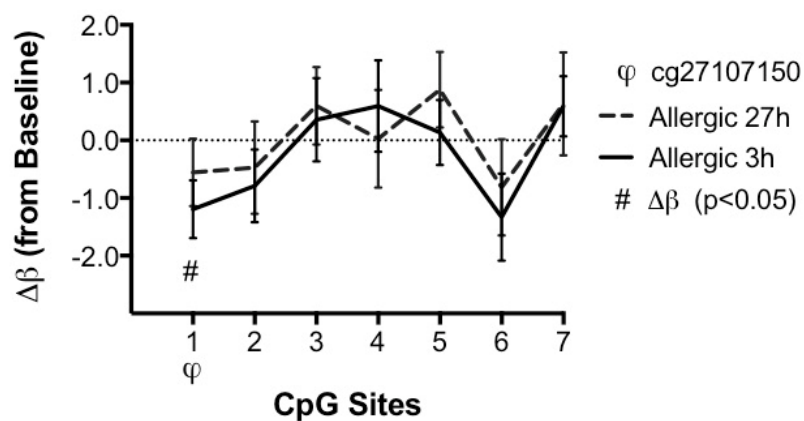
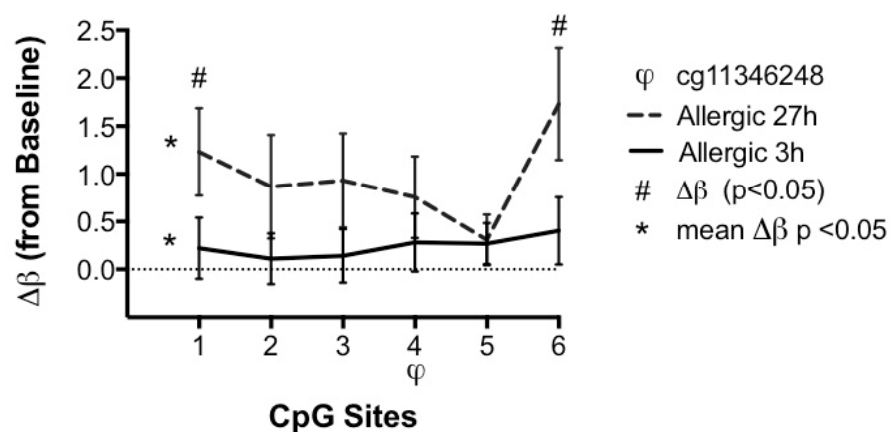


Figure 2

A) *TPSG1* Changes from Baseline



B) *SLFN12* Changes from Baseline



C) *MUC4* Changes from Baseline

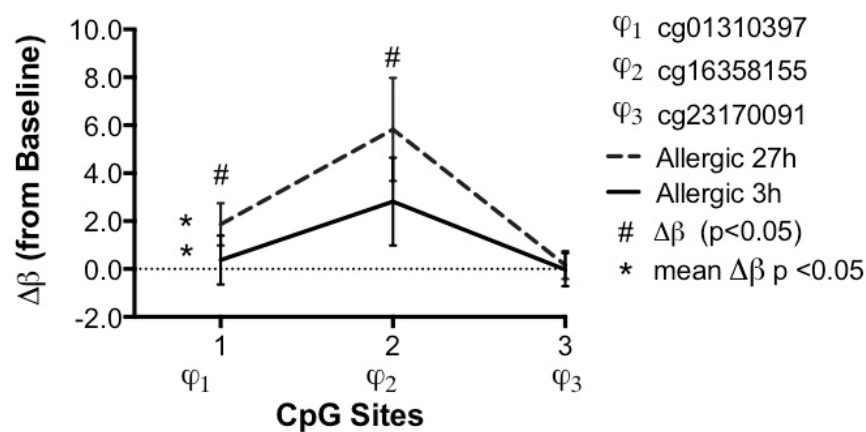
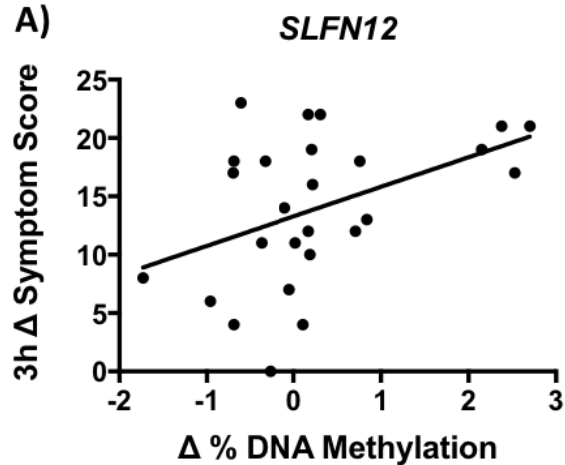
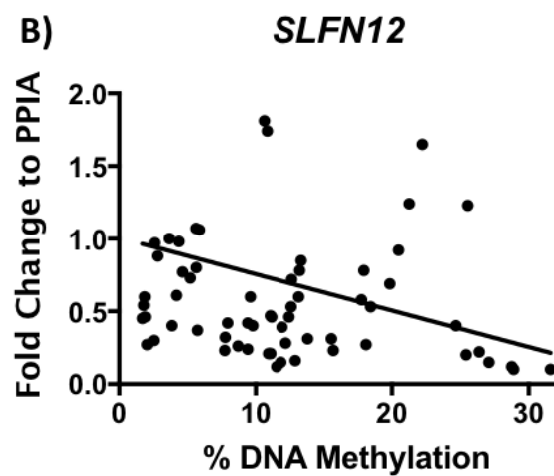


Figure 3 A)



B)



C)

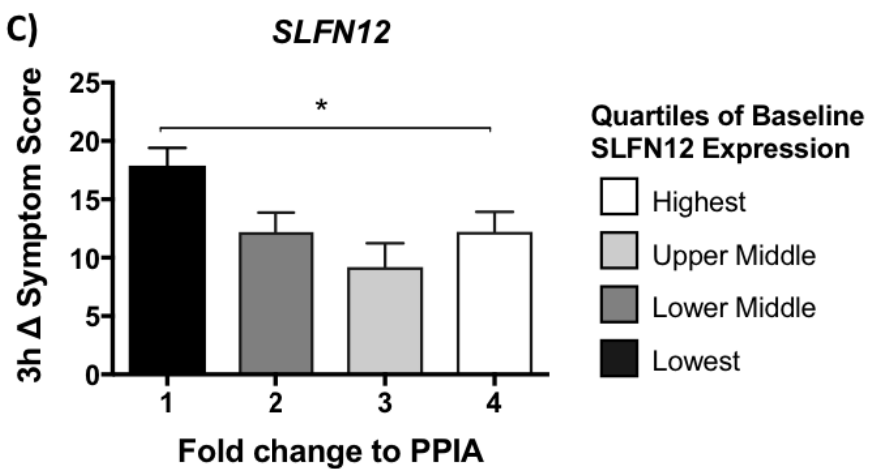
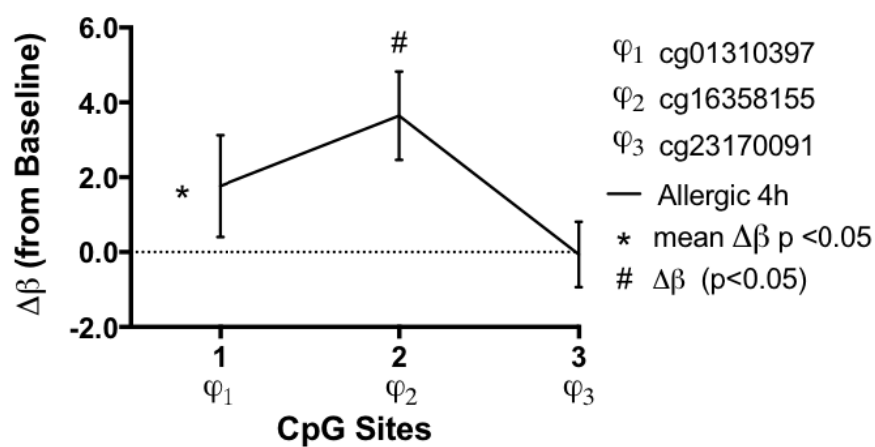
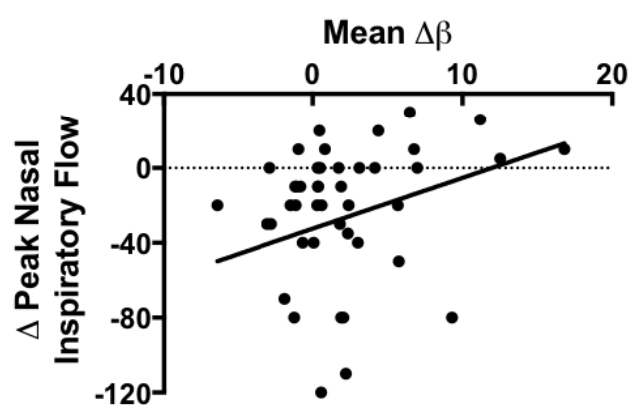


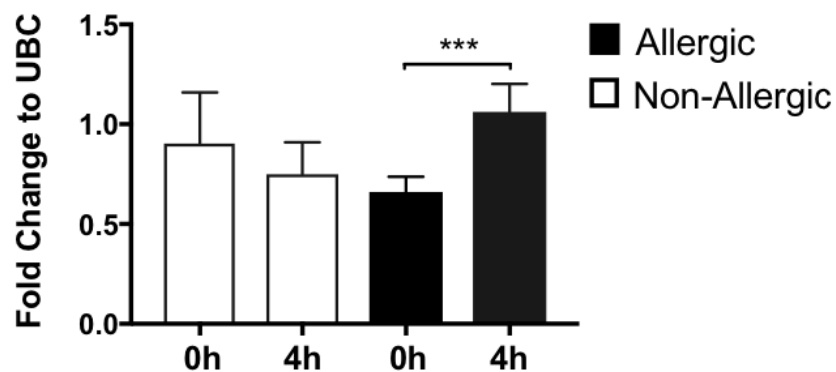
Figure 4 A) *MUC4* Nasal Brushing DNAm



B) *MUC4* Nasal DNA Methylation



C) *MUC4* Nasal Expression



Supplemental Materials

Methods

Supplemental Table 1: qPCR Primers

Gene	Forward Primer	Reverse Primer
SLFN12	TGTGTTTGTCCAGTTCTTTGTTT	AGCTATGCAGTGTCCAAGC
MUC4	CCAACCCACTCCCAACAG	G TTCACCATAGAACGTCTCGT
PPIA	TGGTATAAAAGGGGCGGGAG	CTGCAAACAGCTCAAAGGAGAC
UBC	GGATTTGGGTCTGCAGTTCTT	GCCCATCTTCCAGCTGTTT

Forward and reverse primers used for qPCR gene expression analyses of blood and nasal RNA.

Results

Concordance between 450K arrays and pyrosequencing

In pyrosequencing experiments to validate changes in *TPSG1*, *SLFN12*, and *MUC4*, we found a significant correlation between levels of DNA methylation determined by pyrosequencing and those determined by the 450k array (**Supplemental Figure 1B, D, F-H**). Using Bland-Altman plots, we determined the mean difference between measurements made using the array vs. pyrosequencing to be -5.53 for *TPSG1* and -1.4 for *MUC4* (**Supplemental Figure 1A and E**). *SLFN12* demonstrated a slightly higher mean difference, at 13.7 (**Supplemental Figure 1C**), potentially related to the wider distribution of observed methylation levels between individuals (**Supplemental Figure**

1D). However, for *TPSG1*, *SLFN12* and *MUC4*, respectively, only 5.7%, 2.9% and 2.0% of data points fell outside the 95% limits of agreement defined by the Bland-Altman analysis (**Supplemental Figure 1A, C, E**). Therefore the agreement was deemed acceptable overall.

Supplemental Figure Legends

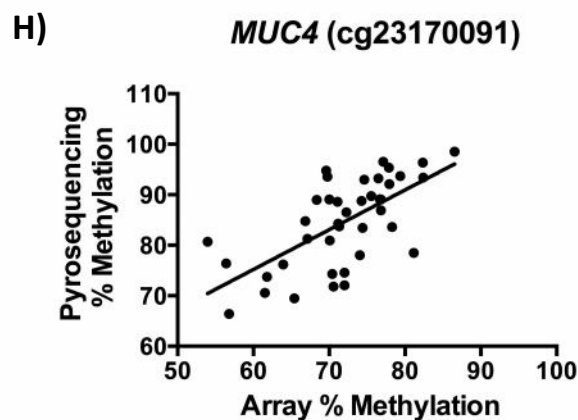
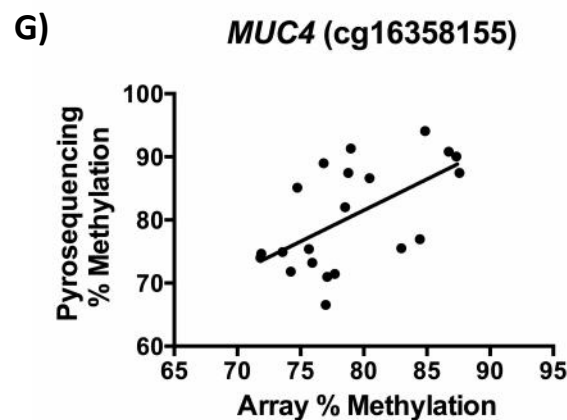
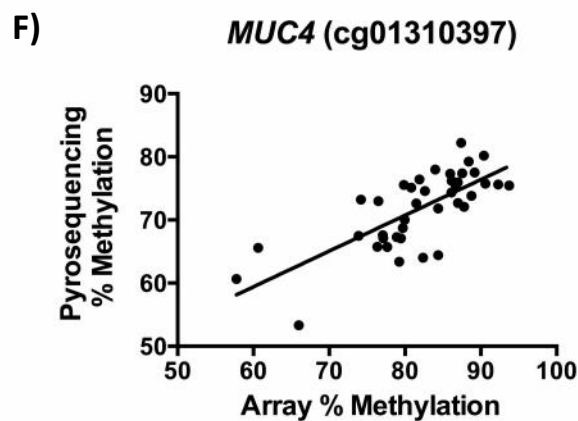
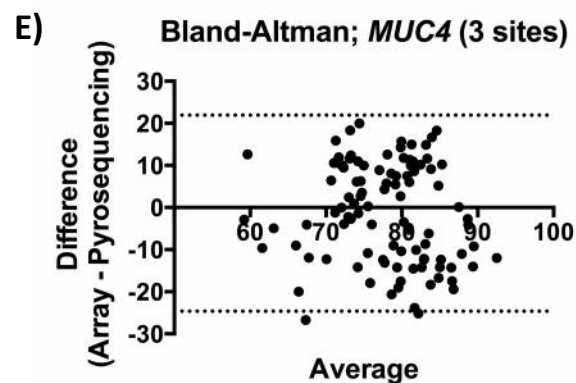
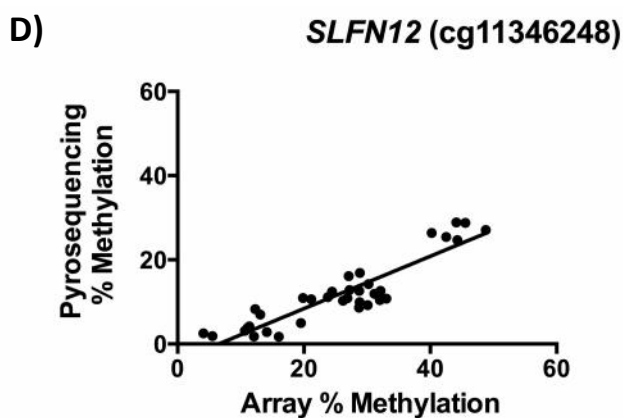
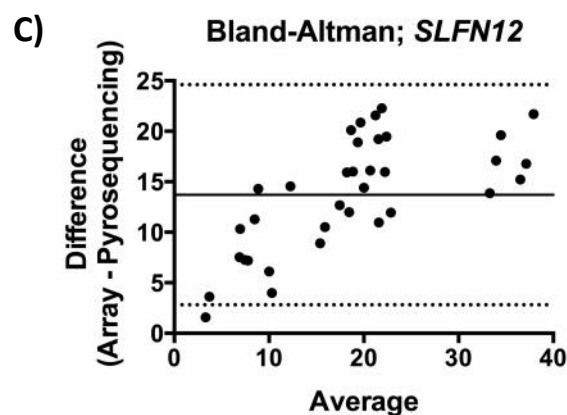
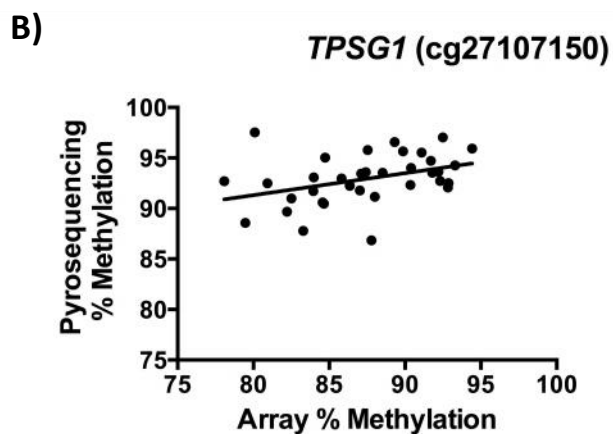
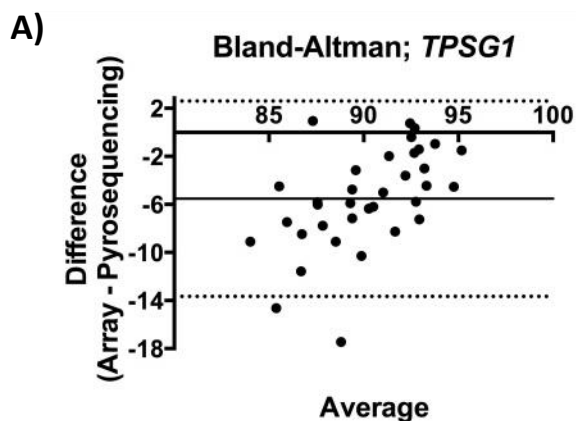
Figure 1: Validation of 450k array data by pyrosequencing; Bland-Altman analysis and correlations

These panels present Bland-Altman plots of the difference between 450k data and pyrosequencing at sites of interest in **A)** tryptase gamma 1 (*TPSG1*), **C)** schlafen 12 (*SLFN12*), and **E)** mucin 4 (*MUC4*). The dashed lines represent the 95% limits of agreement defined by Bland-Altman analysis. The remaining panels present the results of correlations between pyrosequencing data and 450k data; **B)** cg27107150 in *TPSG1* ($p = 0.006$, $r_s = 0.452$, $n = 35$), **D)** cg11346248 in *SLFN12* ($p < 0.0001$, $r_s = 0.962$, $n = 35$), **F)** cg01310397 in *MUC4* ($p < 0.0001$, $r_s = 0.710$, $n = 40$), **G)** cg16358155 in *MUC4* ($p = 0.0024$, $r_s = 0.626$, $n = 21$), and **H)** cg23170091 in *MUC4* ($p < 0.0001$, $r_s = 0.625$, $n = 40$).

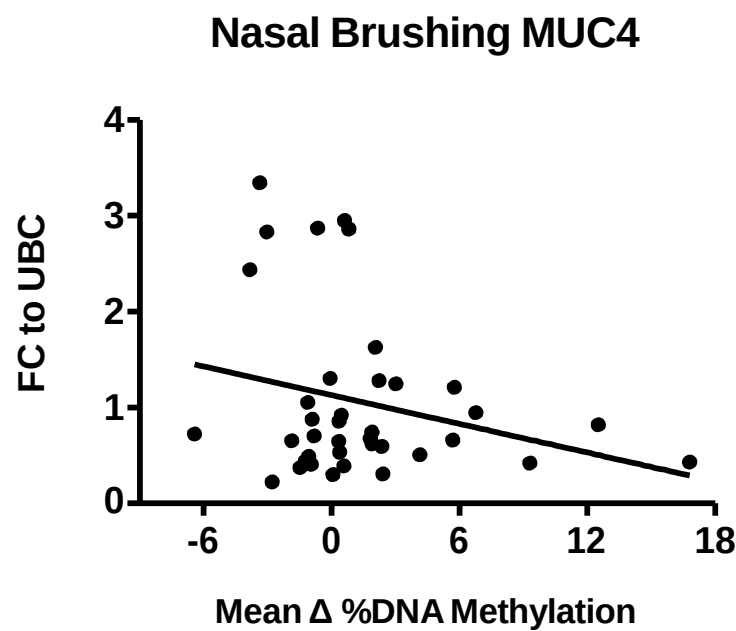
Figure 2: Correlation between *MUC4* gene expression and change in DNA methylation

We observed a trend towards association between higher levels of nasal DNA methylation and lower MUC4 expression ($p = 0.12$). MUC4 gene expression was normalized to the ubiquitin C (UBC) reference gene.

Supplemental Figure 1



Supplemental Figure 2



Supplemental Table 2: Significant changes in DNA methylation in allergic participants following allergen exposure

	p	adj. p	Chr	Co-ordinates	Gene	Δ Beta	
Significantly higher post-exposure methylation	cg06680906	1.48E-04	0.097	15	67112614	MIR548H4; NOX5	0.022
	cg06841052	3.77E-05	0.068	10	34448536	PARD3	0.023
	cg04707706	3.88E-05	0.068	2	50428830	NRXN1	0.023
	cg23170091	1.13E-04	0.092	3	196975765	MUC4	0.024
	cg19240319	1.22E-05	0.065	1	159693725		0.024
	cg27200869	1.12E-04	0.092	7	157738741	PTPRN2	0.025
	cg12709207	1.59E-04	0.098	17	75223669		0.025
	cg11530693	1.04E-04	0.092	1	119966880	ZNF697	0.025
	cg16358155	2.78E-05	0.065	3	196975840	MUC4	0.025
	cg08186935	3.24E-05	0.065	14	78914321	NRXN3	0.026
	cg10473311	3.18E-05	0.065	7	157739119	PTPRN2	0.027
	cg01310397	1.35E-04	0.097	3	196975704	MUC4	0.028
	cg05104581	2.19E-05	0.065	16	87692491	ACSF3	0.030
	cg10157098	1.60E-04	0.098	7	128142203	FAM71F1	0.030
	cg19056772	8.86E-05	0.092	17	7282340	FGF11	0.031
	cg07676709	1.18E-04	0.092	17	44028441	HOXB6	0.031
	cg09068086	5.92E-05	0.079	2	110327493	NCRNA00116	0.031
	cg03102494	5.18E-05	0.073	1	146268345		0.033
	cg18918831	6.96E-05	0.085	3	196975453	MUC4	0.035
	cg11346248	1.73E-05	0.065	17	30784042	SLFN12	0.037
	cg23099839	1.38E-04	0.097	8	2573383		0.038
	cg02770061	4.28E-05	0.071	7	157738927	PTPRN2	0.040
	cg06905511	1.34E-04	0.097	5	139042677	CXXC5	0.043
	cg02564175	2.13E-05	0.065	16	55891403		0.043
Significantly lower post-exposure methylation	cg25134647	8.59E-06	0.065	12	25346257		-0.063
	cg19636627	7.72E-05	0.088	6	29757063		-0.057
	cg02072495	1.05E-04	0.092	15	58476577	ANXA2	-0.047
	cg00021855	1.82E-05	0.065	8	144762099	PYCRL	-0.040
	cg14737484	1.49E-05	0.065	6	32162539	TNXB	-0.037
	cg19645298	7.98E-05	0.088	8	99146993	C8orf47	-0.037
	cg07178994	1.18E-04	0.092	8	806998		-0.035
	cg00495303	3.12E-05	0.065	18	3761110	DLGAP1	-0.033
	cg02505117	1.55E-04	0.098	5	43191431	ZNF131	-0.033
	cg18310658	1.18E-04	0.092	2	229846448		-0.032
	cg17188169	5.03E-05	0.073	6	74161109	DDX43	-0.031
	cg26012482	1.45E-04	0.097	2	8340606		-0.029
	cg03228974	1.49E-04	0.097	6	30175834		-0.027
	cg19188572	1.03E-04	0.092	15	57737304	GTF2A2	-0.026
	cg15255086	2.47E-05	0.065	17	5614605		-0.024
	cg27107150	4.39E-06	0.065	16	1212276	TPSG1	-0.023
	cg10237252	1.66E-04	0.098	17	50332212	TOM1L1	-0.023
	cg08294772	6.35E-05	0.081	8	138204574		-0.022

Supplemental Table 2: Significant changes (adjusted $p \leq 0.1$, $\Delta \text{Beta} \geq \pm 0.02$) in DNA methylation following a 3 hour allergen exposure. Adjusted p-value (adj. p) is after Benjamini-Hochberg correction. Chr; chromosome, Gene: gene that the CpG of interest is contained within, if applicable. ΔBeta ; the median difference between 3h post-exposure and pre-exposure methylation.