DNA methylation and allergic sensitizations, a genome-scale longitudinal study during adolescence

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Keywords

Adolescence, Allergic sensitization. DNA methylation, Longitudinal, Skin prick test.

Abstract (250/250)

Background

The presence of allergic sensitization has a major influence on the development and course of common childhood conditions such as asthma and rhinitis. The etiology of allergic sensitization is poorly understood and its underlying biological mechanisms are not well established. Several studies showed that DNA methylation (DNAm) at some CpGs is associated with allergic sensitization. However, no studies have focused on the critical adolescence period.

Methods

We assessed the association of pre- and post-adolescence genome-wide DNAm with allergic sensitization against indoor, outdoor and food allergens, using linear mixed models. We hypothesized that DNAm is associated with sensitization in general, and with poly-sensitization status, and these associations are age- and gender-specific. We tested these hypotheses in the IoW cohort (n=376) and examined the findings in the BAMSE cohort (n=267).

Results

Via linear mixed models, we identified 35 CpGs in IoW associated with allergic sensitization (at false discovery rate of 0.05), of which 33 were available in BAMSE and replicated with respect to the direction of associations with allergic sensitization. At the 35 CpGs except for cg19210306 on *C13orf27*, a reduction in methylation among atopic subjects was observed, most notably for cg21220721 and cg11699125 (*ACOT7*). DNAm at cg10159529 was strongly correlated with expression of *IL5RA* in peripheral blood (p-value=6.76×10^{-20}). Three CpGs (cg14121142, cg23842695, and cg26496795) were identified in IoW with age-specific association between DNAm and allergic sensitization.

Conclusion

In adolescence, the status of allergic sensitization was associated with DNAm differentiation and at some CpGs the association is likely to be age-specific.

Introduction (3,482/3,500)

The presence of allergic sensitization has a major influence on the development and course of common childhood conditions such as asthma and rhinitis.[1, 2] In clinical practice, allergic sensitization is defined as the presence of sensitization to allergen by skin prick testing (SPT),[3, 4] or by the presence of serum-specific immunoglobulin E (IgE).[5] The etiology of allergic sensitization is poorly understood and its underlying biological mechanisms are not well established, although it was suggested that gene-environment interactions in early-life may play a role in atopy and atopic-disease susceptibility.[6]

A covalent addition of a methyl group to the DNA at a cytosine residue that is followed by a guanine (CpG site), denoted in our study as DNA methylation (DNAm) at CpG sites, is an important epigenetic mechanism that regulates gene transcription.[7] Several studies have shown that epigenetic changes, in particular methylation of CpGs, are associated with allergic sensitization. Most studies in humans have focused on methylation measured in DNA from peripheral blood cells. Studying DNAm of Alu and LINE-1 repetitive elements (as a proxy for global methylation levels) in older adults (mean age 73 years) from the Normative Ageing Study cohort, Sordillo et al. showed that sensitization to aeroallergens was associated with increased Alu methylation.[8] White et al. examined the association between atopic status and epigenetic changes in peripheral blood among children (age 6 years or younger) and adults in the promoter region of *IFNγ* gene.[9] They found that methylation was reduced in naïve CD8+ T cells among atopic children, implying that differential regulation of *IFNγ* promoter methylation in T cells may contribute to development of allergic sensitization. However, this difference was not observed in cord blood CD8+ T cells between infants who subsequently went on to be sensitized to $\geqslant 1$ allergen by age 2 years compared to those remained non-atopic.

The studies described above have focused on candidate genes. In one cross-sectional study, the association of DNAm with allergic sensitization was examined at a genome-scale and 13 CpGs were identified.[10] Participants in this study were young females aged 18 years, while participants in the aforementioned studies were either in early childhood (6 years or younger), adults, or older men. To our

knowledge, no studies have focused on the critical period of adolescence. Adolescence is accompanied by significant changes, e.g., puberty, rapid growth, and often body mass index (BMI) increase, which may be associated with changes in sensitization and DNAm.[11-13] We also detected changes in DNAm in genes in the Th2 pathways between pre- and post-adolescence and their association with risk of asthma and with changes in asthma status between pre- and post-adolescence.[14] These findings led to the present study to examine whether similar association patterns between allergic sensitization and DNAm would be observed in childhood and young adults, and if not, then to what extent they differ. To this end, we assessed the association of pre- and post-adolescence genome-scale DNAm with any sensitizations (yes vs. no) against 11 allergens, including indoor, outdoor and food allergens, using linear mixed models. We hypothesized that DNAm is associated with allergic sensitization and the associations are gender-specific and are different between pre- and post-adolescence. We tested these hypotheses in subsamples from two independent cohorts.

Methods

Participants and Allergic Sensitization Assessments

Data analyzed in this study were from a birth cohort of children born between January 1, 1989 and February 28, 1990 on the Isle of Wight (IoW), United Kingdom.[15] Of the 1,536 children born and recruited in this period, 1,456 were available for further follow-up with data collected at ages 1, 2, 4, 10, and 18 years. Questionnaires that included the questions of the International Study of Asthma and Allergy in Childhood (ISAAC) were administered at each follow-up. Information such as disease status, age of disease onset, and tobacco smoke exposure were collected based on responses to the questionnaires. The local Research Ethics Committee approved this study (06/Q1701/34). The informed consent was written for in person visits.

Skin prick test (SPT) assessment was conducted at ages 4, 10 and 18 years for 11 common allergens (house dust mite, cat dander, dog dander, grass pollen mix, tree pollen mix, *Alternaria alternata*, *Cladosporium herbarium*, cow's milk, hen's egg, peanut and cod). Standard lancets (ALK, Horsholm, Denmark) were used. Participants were asked to avoid taking oral antihistamines for 72 hours. The average of the longest diameter and the diameter perpendicular to it was assessed at 15 minutes. Results were discarded if the wheal size of positive control was smaller than 3mm in diameter. Allergic sensitization to an allergen was defined as a mean wheal size of 3 mm greater than the negative control. Specific IgEs were measured at ages 10 and 18 using the FX5 test for a mixture of 5 common food allergens (milk, egg, cod, peanut, and soy), and the Phadiatop test for mix inhalant allergens (grasses, trees, weeds, cat, dog, mites and mold). Positivity was defined as serum IgE > 0.35 kU/L.

DNA Methylation

Peripheral blood samples were collected in IoW at ages 10 and 18 years. DNA was extracted using a standard salting out procedure [16]. The methylation level for each CpG was assessed using one of two platforms, the Illumina Infinium HumanMethylation450 BeadChip or the MethylationEPIC BeadChip (Illumina, Inc., San Diego, CA, USA). DNA concentration was estimated by fluorometric quantitation. For each sample, one microgram DNA was bisulfite-treated for cytosine to thymine conversion using the EZ 96-DNA methylation kit (Zymo Research, Irvine, CA, USA), following the manufacturer's protocol. HumanMethylation450 BeadChip interrogates > 484,000 CpGs and MethylationEPIC BeadChip > 850,000 CpGs associated with over 24,000 genes. Arrays were processed using a standard protocol [6] with multiple identical control samples assigned to each bisulfite conversion batch to assess assay variability. DNAm was measured by a ratio of intensities, denoted as β values for each CpG site. It is a ratio of methylated (M) over the sum of methylated and unmethylated (U) probes ($\beta=M/[c+M+U]$), where c is a constant to prevent zero in the denominator. In total, 376 subjects with DNAm at both ages and allergic sensitization status on 11 allergens available at one or both ages were included in the study.

Pre-processing

Probes not reaching a detection p-value of 10^{-16} in at least 95% of samples were excluded. CpGs on sex chromsomes were also excluded to avoid bias. DNAm were then pre-processed using the CPACOR pipeline for data from both platforms (HumanMethylation450 and MethylationEPIC).[17] Specifically, the data were quantile normalized using the R package, *minfi*.[18] Next, principal components (PCs) inferred based on control probes were used to represent latent chip-to-chip and technical variations. Since DNAm data were from two different platforms, we determined the PCs based on DNAm at shared control probes. In total, 195 control probes were shared between the two arrays, and used to calculate the control probe PCs with the top 15 to represent latent batch factors.[17]

After pre-processing, a total of 439,635 CpGs overlapped between the two platforms were included in subsequent analyses. Probes that contained single nucleotide polymorphisms (SNPs) within 10 base pairs of a targeted CpG site with minor allele frequency in at least 0.7% subjects (corresponding to at least 10 subjects in IoW with n=1,456) were excluded due to their influence to DNAm.

Statistical Methods

Comparing with the complete cohort: To compare the data used in our study and in the complete cohort at ages 10 and 18 years, respectively, we tested proportions of subjects being sensitized at each age and different demographic variables such as gender and socio-economic status.

Screening CpGs: We excluded CpGs potentially not associated with allergic sensitization and CpGs showing no difference between mono- and poly-sensitization in DNAm. An R package *ttScreening* [19, 20] was implemented for this purpose. The method in *ttScreening* utilizes training and testing data in robust linear regressions with surrogate variables included to adjust for unknown effects (Supplemental material). We applied the package to assess the association of genome-scale DNAm with the status of sensitization (yes/no) and with the status of poly-sensitization status (poly- vs. mono-sensitization). The

screening was performed for each gender at ages 10 and 18 years, separately. Subjects with DNAm and allergic sensitization data at one or both ages were included in the screening. CpGs that passed the screening at either age for one or both genders were included in subsequent analyses.

Detecting CpGs associated with status of sensitization or status of poly-sensitisation: CpGs that passed the screening were further examined. We implemented two linear mixed models to assess the associations with DNAm, one model focusing on the effect of sensitization and the other on the effect of poly-sensitization. The dependent variable was DNAm of a CpG at each age (10 and 18 years). To remove effects of batches and technical variations on DNAm measurements, we first regressed logit-transformed DNAm at each CpG on the aforementioned 15 PCs and an indicator for the two platforms (450 and EPIC). The residuals were treated as batch-effect-adjusted DNAm, and included in the models as dependent variables. The main independent variable was the atopic status at each age or the status of mono-/poly-sensitization. Gender and age were included in the model as covariates.[21-24] DNAm is influenced by heterogeneity of cell compositions in peripheral blood. Thus, cell type proportions at each age were inferred using the *minfi* R package[25] and included in the model.[26] Finally, to test the moderating effects of gender and age on the association of DNAm with sensitizations, we considered additional linear mixed models with two-way interactions, one for moderating effect of gender and the other for age. Multiple testing was corrected by controlling for a false discovery rate (FDR) of 0.05.

Replication Cohort - The BAMSE Cohort

CpGs shown to be associated with allergic sensitization or poly-sensitization in IoW were further tested in an independent cohort, the BAMSE study (sensitization defined as specific IgE > 0.35 kU/L to cat, dog, grass, house dust mite, egg or peanut).[27] As in IoW, DNA was extracted from peripheral whole blood. DNAm were analyzed for 267 subjects at 16 years of age using the Illumina HumanMethylation450 array and quality control details have been presented elsewhere.[28] Robust linear regressions were implemented with similar covariates, gender and cell type proportions, included in the analyses.

Genome-scale gene expressions in peripheral white blood cells of the 267 participants aged 16 years were used to assess biological relevance for the identified CpGs.[29]

Bioinformatics Assessment

Genes annotated to the CpGs replicated in BAMSE were identified based on Illumina's manifestation file. Bioinformatic assessment of the genes was conducted using ToppFun, available in the ToppGene Suite.[30] ToppFun is for functional enrichment analyses based on a list of genes in Gene Ontology (GO) for molecular functions and biological processes. Multiple testing was adjusted by controlling FDR of 0.05.

Results

The subsamples included in the study represented the whole cohort with respect to demographics related to allergy, except for maternal smoking during pregnancy (p-value=0.04) (Table 1a). Proportions of allergic sensitization to any allergen between the subsample and the whole cohort was also compared. No statistically significant difference was observed at the age of 10 years, but at age 18 years, the proportion of being sensitized was higher in the subsamples (p-value=0.01, Table 1b).

In total, 1,437 CpGs passed screening at either age for one or both genders for their potential association with allergic sensitization against one or more allergens, and these CpGs were analysed further using linear mixed models. Of the 1,437 CpGs, DNAm at 35 CpGs were shown to be associated with the status of allergic sensitization (FDR=0.05), controlling for the effects of age, gender, cell type compositions, and random subject effects and technical variation (Table 2). Consistent findings were observed when sensitization were defined using specific IgE (Supplemental material, Table S1). Out of the 35 CpGs, four (14%) were on CpG islands, and 17 CpGs (48.6%) were not close to any islands. Except for cg19210306 (5' UTR of gene *C13orf27*, alias for *TEX30*, coefficient=0.22, p-value=4.63×10^{-4}), being atopic was associated with lower DNAm at other 34 CpGs (Table 2, Figure 1). Three CpGs are

on one gene, *ACOT7* (cg09249800, cg11699125, and cg21220721), and two of these 3 CpGs, cg21220721 and cg11699125, exhibited the largest DNAm reduction (coefficients =-0.20 with p-values=1.12×10^{-4} and $4.34\times10^{-5,}$ respectively).

We also assessed whether the association of DNAm at each of the 1,437 CpGs with allergic sensitization was gender-specific. No statistically significant interaction effects were identified at FDR=0.05. In addition, stability of DNAm at all these 35 identified CpGs was suggested by results from linear mixed models.[24] Stability in DNAm was defined as statistically insignificant changes in DNAm between ages 10 and 18 years.

Replication analyses were carried out in BAMSE. Of the 35 CpGs, DNAm on 33 CpGs was available in BAMSE (cg05072552 and cg09249800 were not available). Altogether, 27 of the 33 CpGs had a nominal p-value <0.05 in BAMSE. Notably, at all the 33 CpGs, the pattern of DNAm differentiation due to atopy was replicated (Table 2). We also assessed the association of methylation with expression of the genes in Table 2 (Supplemental material, Tables S2a,b). The most significant association was observed at cg10159529 with expression of *IL5RA* (coefficient=-12.58, p-value=6.76×10^{-20}). CpG cg10159529 was located in the TSS1500 of *IL5RA.*

We further conducted bioinformatic functional analyses on the 33 CpGs using ToppFun, based on 29 genes that these 33 CpGs mapped to. We identified seven enriched GO molecular functions (Table 3). Five of the 29 genes are involved in these seven enrichment terms, *B4GALT7, SEC14L1, GPR44, PCYT1A,* and *IL5RA.* A further 15 pathways enriched in GO biological processes are also identified (Table 3). The findings were not statistically significant at FDR=0.05.

For the 33 CpGs tested in BAMSE, 13 were on a list of 190,672 potentially spurious CpGs.[31] These sites may be more likely to contain outliers that influence results. To examine the possibility of this concern, we tested for multimodality using the R package diptests[32] and visually inspected the density plots of DNAm beta values. None of the multimodality tests were statistically significant at the 0.05 significance level, and the sample density plots of all the 13 CpGs did not support multimodality either (Supplemental material, Table S3 and Figure S1).

Of the 1,437 CpGs, we identified 3 CpGs (cg14121142 on *DDN*, cg23842695 on *PRKD2*, and cg26496795 on *ANKRD20A8P*) showing an interaction effect between any allergic sensitization and age (FDR=0.05). At these 3 CpGs, the effect of sensitization on DNAm was strengthened at age 18 years compared to 10 years (Table 4a). Associations at these CpGs were consistent with those when specific IgEs were applied to determine allergic sensitization status (Supplemental material, Table S4). In addition, DNAm at these 3 CpGs was shown to be stable between ages 10 and 18 years.[24] A ToppFun analysis indicated that *PRKD2* is possibly involved in one enriched GO pathway of molecular functions (protein kinase C activity, p-value=0.077) and in five GO biological processes (Table 4b, p-value=0.022).

Turning to mono-sensitization v.s poly-sensitizations, 48 CpGs passed screening based on comparison in DNAm between the two groups at each age for each gender (Table S5). Via linear mixed models, DNAm at 3 of the 48 CpGs was shown to be significantly different between mono- and poly-sensitized subjects, adjusting for the same confounders and covariates as in the analyses for status of sensitization to any allergens. At cg11371879 (in the body of *DENND3* gene) and cg08762603 (intergenic), DNAm was lower among subjects with poly-sensitization (cg11371879, coefficient=-0.31, p-value 5.03×10^{-4}; and cg08762603, -0.32 and 8.46×10^{-4}, respectively). At cg01381613 (TSS200 of *PRM3* gene), DNAm was higher for poly-sensitized subjects (coefficient=0.18, p-value 2.25×10^{-4}). However, when testing these 3 CpGs in BAMSE, none were statistically significant at 0.05 level.

For the testing of moderating effects of gender or age in IoW on the association of mono- vs poly-sensitization with DNAm, no statistically significant interactions were identified at FDR=0.05.

Discussion

We examined the association of allergic sensitization with DNAm at the genome-scale. In the IoW, 35 CpGs showed an association with the status of sensitization, of which 33 were replicated in BAMSE. The consistent findings between the two independent cohorts suggested informativeness of the detected CpGs.

In IoW, DNAm at 3 CpGs were shown to be different between subjects allergic to one allergen and subjects allergic to more than one allergens, but the findings were not replicated in BAMSE.

Based on the 33 CpGs, genes were identified with biological functions relevant to allergic sensitization. The top enriched pathways in GO biological processes were involved in the regulation of *IL-5* production and these processes included *IL5RA* and *EPX*. Furthermore, when assessing the association of DNAm with gene expression, the strongest association was found between cg10159529 (in the promoter region of *IL5RA*) and expression of *IL5RA*. The alpha sub-unit of the *IL-5* receptor complex is expressed on eosinophils and B-cells and plays an important role in allergic inflammation through the regulation of expression of genes involved in proliferation, cell survival and maturation and effector functions of B cells and eosinophils. Genetic variation in *IL5RA* has been found to be associated with allergic sensitization,[33] asthma,[34-36] and atopic dermatitis.[37, 38] Likewise *EPX* encodes eosinophil peroxidase, a component of the matrix of the cytoplasmic granules of eosinophils. It is likely that both these signals arise from the higher numbers of blood eosinophils present in atopic individuals.[39]

The *ACOT7* gene was found enriched in four biological processes. Effect sizes of 3 CpGs (cg09249800, cg11699125, and cg21220721) in this gene were among the largest. These 3 CpGs were also detected in a genome-scale epigenetic meta-analysis of serum total IgE in Hispanic children by Chen et al,[40] and in an epigenome-scale association study of asthma and wheeze in a European cohort. [34] In a recent association study of allergen-specific IgE,[41] *ACOT7* was identified as well, but it was not associated with self-reported allergies. The previous findings and results in the present study indicate a potentially critical role of CpGs on the *ACOT7* gene in allergic sensitizations and related conditions. It was observed that in the assessment of association of DNAm with expression of *ACOT7*, DNAm at two CpGs available in BAMSE (cg11699125, and cg21220721 located in gene body) did not show any statistically significant association.

Among all the detected CpGs, decreased DNAm was observed in subjects allergic to any allergen, except for cg19210306 located on the 5' UTR region of the *TEX30* gene. DNAm reduction on most CpGs has also been observed in a recent epigenome-scale study on asthma.[28] The *TEX30* gene has

been shown to be mutated in patients with allergic eczema and their fathers.[42] A further investigation is warranted to examine the role of cg19210306 in the development of allergic sensitization leading to atopic eczema.

At 3 CpGs (cg14121142, cg23842695, and cg26496795) in IoW, the period of adolescence transition seemed to strengthen the association of overall sensitization with DNAm. However, insignificant moderating effects of age was suggested on the association of mono- vs poly-sensitizations with DNAm. It is likely that the moderating effects of age only occurred to atopic status and are irrelevant to the number of allergic sensitizations.

It is not clear if there is a fundamental difference in IgE responses in mono- and poly sensitized subjects or poly-sensitized subject are merely "more reactive" than mono-sensitized subjects; a quantitative rather than qualitative difference.[43] In this study, a statistically significant difference in DNAm between mono- and poly-sensitized subjects was observed on 3 CpGs in IoW, but the findings were not replicated in BAMSE. Immune responses in poly-sensitized subjects are likely not to be "qualitatively" different. Pepys' detailed atopic status in quantity terms based on the number of positive SPT results, and classified patients to 0, 1, 2 or 3 or more groups.[44] Although disagreement between IgE and SPT has been discussed,[43] there is evidence that compared to mono-sensitized patients, poly-sensitized patients tend to have a higher serum total and specific IgE levels.[45-47] Our findings with specific screening IgE also demonstrated the general agreement between IgE and SPT with respect to their association with DNAm. It is possible that the sample sizes in both cohorts were insufficient to identify CpGs showing differentiation in DNAm between mono- and poly-sensitized patients and further studies in larger cohorts are warranted.

The findings of this study were based on data such that allergic sensitization status was assessed at the same age when blood samples collected, and that only pre- and post-adolescence in IoW were considered. We thus were not able to assess the trend of DNAm changes and differentiate whether DNAm changes were casual or a consequence of allergic sensitization. It would be desirable to have allergic sensitization assessment at denser time points (starting from non-sensitization) during adolescence along

with DNAm at those time points or at lagged time points. In addition, BAMSE did not have paired pre-adolescence DNAm available in sufficient numbers and we thus did not replicate age-specific associations with allergic sensitization. Finally, it would be necessary to include information on genetic polymorphisms that may modify susceptibility of allergic sensitization with similar allergen exposure.

Author contributions

HZ, WK, SHA, and JWH conceived and designed the study. HZ, AK, FIR, and SKM analysed and interpreted the data. LH assisted with the analyses and provided results on DNAm stability, EM, SE, and GP provided guidance on the study design and data analyses. HZ, WK, JWH, and EM obtained funding. HZ wrote the manuscript. All authors commented on the manuscript and approved the final version.

Acknowledgement

The authors are thankful to Ramya Velampati for the help in the analyses, and the nurses and staff at the David Hide Asthma & Allergy Research Centre, Isle of Wight, UK, for their help in recruitment and sample collections. Our special thanks also go to the High Performance Computing facility provided by the University of Memphis. The BAMSE team would like to thank Professors Jean Bousquet and Josep Maria Antó for coordinating the MeDALL project and Professor Charles Auffray and his team for generating the transcriptomics data in BAMSE.

Funding

This study was supported by the National Institutes of Health research funds R21AI099367 (PI: H Zhang), R01AI121226 (MPI: H Zhang and JW Holloway), and R01AI091905 (PI, W Karmaus). BAMSE was supported by The Swedish Heart-Lung Foundation, The Swedish Research Council, Stockholm

County Council (ALF), The Swedish Research Council Formas and the Mechanisms of the Development of ALLergy (MeDALL) project, European framework programme 7, Project No: 261357. Erik Melen was supported by grants from the Strategic Research Programme (SFO) in Epidemiology at Karolinska Institutet and the European Research Council (ERC) under the European Union's Horizon 2020 research and innovation programme (grant agreement n° 757919, TRIBAL).

Conflicts of interest

The authors declare that they have no conflicts of interest.

Table 1a. Comparing the subsample included in the current study with the complete Isle of Wight cohort on demographics via proportion tests.

Variables		Complete cohort	Subsample	p-value
Gender (Female)		786; 51.2% (N*=1,536)	193; 51.3% (N=376)	0.99
Season of birth	Winter	499; 32.5%	116; 30.9%	0.78
	Spring	364; 23.7%	86; 22.9%	
	Summer	353; 23.0% (N=1,536)	87; 23.1% (N=376)	
Low birth weight (Yes)		66; 4.4% (N=1,497)	17; 4.7% (N=360)	0.80
Socio-economic Status	Low	209; 15.4%	51; 13.8%	0.73
	Medium	1,037; 76.4% (N=1,357)	287; 77.6% (N=370)	
Maternal smoking during pregnancy (Yes)		384; 25.3% (N=1,521)	75; 20.1% (N=374)	0.04
Parental allergy (Yes)		756; 50.0% (N=1,514)	206; 55.4% (N=372)	0.06

Note: *: All Ns denote the number of subjects with no missing values for each variable. In total, N=376 subjects were included in the analyses.

Table 1b: Comparing the subsample included in the current study with the complete Isle of Wight cohort on allergic sensitizations via proportion tests.

	10 years (n[$]; %)					**18 years (n; %)**				
	Complete cohort		**Subsample**		**p-value**	**Complete cohort**		**Subsample**		**p-value**
Allergic sensitization	279; 26.9% (N*=1,036)		108; 29.8% (N=363)		0.26	353; 41.4% (N=853)		176; 48.2% (N=365)		0.01
Mono-sensitization[#]	**Female** (N=520)	**Male** (N=516)	**Female** (N=168)	**Male** (N=195)		**Female** (N=446)	**Male** (N=407)	**Female** (N=172)	**Male** (N=193)	
Alternaria	1; 0.19%	3; 0.58%	1; 0.60%	3; 1.54%		6; 1.35%	3; 0.74%	4; 2.33%	2; 1.04%	
Cladosporium	1; 0.19%	1; 0.19%	---	1; 0.51%		1; 0.22%	---	---	---	
HDM	38; 7.31%	55; 10.66%	15; 8.93%	18; 9.23%		38; 8.52%	48; 11.79%	14; 8.14%	22; 11.40%	
Cat dander	4; 0.77%	5; 0.97%	---	4; 2.05%		2; 0.45%	1; 0.25%	---	---	
Dog dander	2; 0.38%	---	---	---		1; 0.22%	1; 0.25%	1; 0.58%	1; 0.52%	
Grass	13; 2.50%	17; 3.29%	6; 3.57%	7; 3.59%		25; 5.61%	7; 1.72%	15; 8.82%	4; 2.07%	
Cod	---	1; 0.19%	---	1; 0.51%		---	---	---	---	
Peanut	---	1; 0.19%	---	1; 0.51%		1; 0.22%	1; 0.25%	1; 0.58%	1; 0.52%	
Total[#]	59; 11.35%	83; 16.09%	22; 13.10%	34; 17.44%		74; 16.59%	61; 14.99%	35; 20.34%	30; 15.54%	
Poly-sensitization[#]	**Female** (N=520)	**Male** (N=516)	**Female** (N=168)	**Male** (N=195)		**Female** (N=446)	**Male** (N=407)	**Female** (N=172)	**Male** (N=193)	
	59; 11.35%	77; 14.92%	20; 11.90%	32; 16.41%		83; 18.61%	129; 31.70%	42; 24.42%	68; 35.23%	

Note: *: All Ns denote the number of subjects with no missing values for each variable. In total, N=376 subjects were included in the analyses

#: Number and % of subjects mono-sensitized against one allergen for each gender at each age (10 and 18 years), and "Total" refers to the number and % of subjects mono-sensitized against one of the 11 allergens, stratified by gender and age. For mono-sensitizations, only allergens with at least one subject with positive SPT at ages 10 or 18 are included in the table.

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Table 2. Effects of any allergic sensitization (based on SPT) on DNAm via linear mixed models in the IoW cohort and in the replication cohort BAMSE on each of the 35 identified CpGs.

					IoW Cohort			BAMSE	
Name	**Relation to CpG islands**	**Gene Name**	**Location**	**Chr.**	**Est.**	**Raw p-value**	**FDR p-value**	**Est.**	**Raw p-value**
cg01888561	Island	SEC14L1	TSS1500	17	-0.070	1.36×10^{-4}	0.050	-0.036	0.13
cg02245534	N_Shore	METRNL	Body	17	-0.061	4.41×10^{-5}	0.016	-0.060	0.14
cg02475695	S_Shore	NHLRC4	TSS1500	16	-0.077	1.22×10^{-4}	0.045	-0.111	**0.0026**
cg02803925	—	PCYT1A	Body	3	-0.178	1.14×10^{-4}	0.042	-0.136	**0.016**
cg03493123	N_Shore	B4GALT7	Body	5	-0.062	6.17×10^{-5}	0.023	-0.012	0.69
cg05072552	N_Shore	CFL1	Body	11	-0.053	1.26×10^{-4}	0.046	NA	NA
cg05390183	N_Shelf	Intergenic		1	-0.058	5.57×10^{-5}	0.020	-0.053	**0.010**
cg06070625	—	MITF	Body; TSS200	3	-0.075	8.71×10^{-5}	0.032	-0.086	**0.0065**
cg06099697	Island	ZFPM1	Body	16	-0.067	8.79×10^{-5}	0.032	-0.024	0.44
cg07124719	—	GDEP	TSS200	4	-0.075	7.85×10^{-5}	0.029	-0.064	**0.031**
cg07721901	—	FAM81B	TSS1500	5	-0.073	3.32×10^{-5}	0.012	-0.042	0.11
cg09249800	Island	ACOT7	Body	1	-0.160	1.17×10^{-4}	0.043	NA	NA
cg09705784	—	DNAH17	Body	17	-0.105	8.74×10^{-5}	0.032	-0.145	**0.00066**
cg10159529	—	IL5RA	TSS1500	3	-0.085	5.49×10^{-5}	0.020	-0.113	**0.00026**
cg11699125	Island	ACOT7	Body	1	-0.198	4.34×10^{-5}	0.016	-0.264	**0.00011**
cg11988722	S_Shelf	Intergenic		20	-0.067	9.04×10^{-5}	0.033	-0.086	**0.0029**
cg12077460	—	MFHAS1	Body	8	-0.077	2.98×10^{-5}	0.011	-0.133	$\mathbf{7.03\times10^{-8}}$
cg12105691	—	C3orf50	Body	3	-0.100	2.25×10^{-5}	0.008	-0.133	$\mathbf{8.74\times10^{-5}}$
cg14011077	—	Intergenic		9	-0.183	2.83×10^{-5}	0.010	-0.235	$\mathbf{1.16\times10^{-4}}$
cg14436861	—	WEE1	3'UTR	11	-0.089	6.74×10^{-5}	0.025	-0.108	$\mathbf{4.26\times10^{-4}}$
cg15482717	S_Shelf	FADD	3'UTR	11	-0.063	1.32×10^{-4}	0.048	-0.054	**0.020**
cg15710961	—	DST	Body	6	-0.113	1.15×10^{-5}	0.004	-0.097	**0.0018**
cg17203290	—	C8orf47	3'UTR	8	-0.086	4.38×10^{-5}	0.016	-0.101	**0.0085**
cg17429587	S_Shelf	NCOR2	Body	12	-0.086	2.69×10^{-5}	0.010	-0.117	$\mathbf{1.83\times10^{-4}}$
cg17933300	S_Shelf	SCOC	TSS200	4	-0.079	1.03×10^{-5}	0.037	-0.061	**0.028**
cg17971251	—	SEC16B	Body	1	-0.089	1.46×10^{-5}	0.005	-0.159	$\mathbf{3.38\times10^{-7}}$
cg18666454	N_Shore	KCNH2	Body	7	-0.135	1.11×10^{-4}	0.040	-0.089	**0.018**
cg19210306	Island	C13orf27	5'UTR	13	0.217	4.63×10^{-5}	0.017	0.014	0.72
cg20315954	—	PMP22	Body	17	-0.087	7.91×10^{-5}	0.029	-0.097	**0.0091**
cg21220721	Island	ACOT7	Body	1	-0.202	1.12×10^{-4}	0.041	-0.294	$\mathbf{7.07\times10^{-4}}$
cg25087851	S_Shelf	GPR44	TSS1500	11	-0.069	1.37×10^{-4}	0.050	-0.082	**0.011**
cg25479097	S_Shelf	C13orf35	5'UTR	13	-0.092	4.36×10^{-5}	0.016	-0.093	**0.0066**
cg25854298	—	ASCC1	Body	10	-0.102	6.28×10^{-5}	0.023	-0.131	$\mathbf{6.38\times10^{-4}}$
cg26508444	—	FAM53B	Body	10	-0.054	1.37×10^{-4}	0.050	-0.047	**0.0087**
cg27469152	—	EPX	3'UTR	17	-0.070	1.62×10^{-5}	0.006	-0.071	**0.016**

Note: 1) Chr.=Chromosome, Est.=Estimation. 2) P-values that are statistically significant at the 0.05 level in the BAMSE are in bold. 3) The "—" in the second column represents CpGs not identified as being related to CpG islands. Following Illumina's definition, a CpG island is defined as regions > 500 bp with more than 55% GC and expected/observed CpG ratio of more than 0.65. Around 40% of gene promoters contain islands. CpG shelves are ~4Kbp from islands, and CpG shores are ~2Kbp from islands.[48]

Table 3: Enriched pathways in GO molecular function and biological processes identified using genes from the longitudinal assessment of SPT on DNAm. For molecular functions, all detected pathways are with p-value of 0.091, and for biological processes, the p-values are 0.099.

Pathway ID	Name	Raw p-value	FDR p-value	Genes from our study involved in each term
	GO molecular function			
GO:0046525	Xylosylprotein 4-beta-galactosyltransferase activity	0.0014		*B4GALT7*
GO:0039552	RIG-I binding	0.0014		*SEC14L1*
GO:0004956	Prostaglandin D receptor activity	0.0028		*GPR44*
GO:0001785	Prostaglandin J receptor activity	0.0028	0.091	*GPR44*
GO:0004105	Choline-phosphate cytidylyltransferase activity	0.0028		*PCYT1A*
GO:0004958	Prostaglandin F receptor activity	0.0028		*GPR44*
GO:0004914	Interleukin-5 receptor activity	0.0028		*IL5RA*
	GO biological process			
GO:0032674	Regulation of interleukin-5 production	0.0004		*EPX,IL5RA*
GO:0032634	Interleukin-5 production	0.0004		*EPX,IL5RA*
GO:0045598	Regulation of fat cell differentiation	0.0006		*NCOR2, ZFPM1, METRNL*
GO:0002761	Regulation of myeloid leukocyte differentiation	0.0007		*FADD, ZFPM1, MITF*
GO:0032673	Regulation of interleukin-4 production	0.001	0.099	*ZFPM1,EPX*
GO:0045403	Negative regulation of interleukin-4 biosynthetic process	0.0014		*ZFPM1*
GO:0036116	Long-chain fatty-acyl-CoA catabolic process	0.0014		*ACOT7*
GO:2000454	Positive regulation of CD8-positive, alpha-beta cytotoxic T cell extravasation	0.0014		*FADD*
GO:0036114	Medium-chain fatty-acyl-CoA catabolic process	0.0014		*ACOT7*

GO:0072365	Regulation of cellular ketone metabolic process by negative regulation of transcription from RNA polymerase II promoter	0.0014	*NCOR2*
GO:0060377	Negative regulation of mast cell differentiation	0.0014	*ZFPM1*
GO:1902303	Negative regulation of potassium ion export	0.0014	*KCNH2*
GO:1900533	Palmitic acid metabolic process	0.0014	*ACOT7*
GO:1900535	Palmitic acid biosynthetic process	0.0014	*ACOT7*
GO:0032633	Interleukin-4 production	0.0015	*ZFPM1,EPX*

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Table 4a. Identified 3 CpGs such that age moderates the association of DNA-m with SPT (genome-scale study).

				Main effects (SPT)			Interaction effects (SPT×Age)		
CpG Name	**Gene Name**	**Location**	**Chr.**	**Est.**	**Raw p-value**	**FDR p-value**	**Est.**	**Raw p-value**	**FDR p-value**
cg14121142	*DDN*	Body	12	-0.073	0.0003	0.090	0.95	4.7×10^{-6}	0.0058
cg23842695	*PRKD2*	Body	19	0.012	0.0005	0.11	-0.24	1.2×10^{-5}	0.015
cg26496795	*ANKRD20B*	Body	2	0.050	0.94	0.99	-0.10	1.9×10^{-5}	0.023

Note: Chr.=Chromosome, Est.=Estimation.

Table 4b: Enriched pathways identified based on the 3 CpGs in GO biological process with gene PRKD2 included. All the detected pathways are with FDR adjusted p-value of 0.022 .

Pathway ID	**Name**	**Raw p-value**	**FDR p-value**	**Genes from our study included in the pathways**
GO:0089700	Protein kinase D signaling	0.00032		
GO:1901727	Positive regulation of histone deacetylase activity	0.00054		
GO:0038033	Positive regulation of endothelial cell chemotaxis by VEGF-activated vascular endothelial growth factor receptor signaling pathway	0.00064	0.022	*PRKD2*
GO:0038089	Positive regulation of cell migration by vascular endothelial growth factor signaling pathway	0.00075		
GO:1901725	Regulation of histone deacetylase activity	0.00075		

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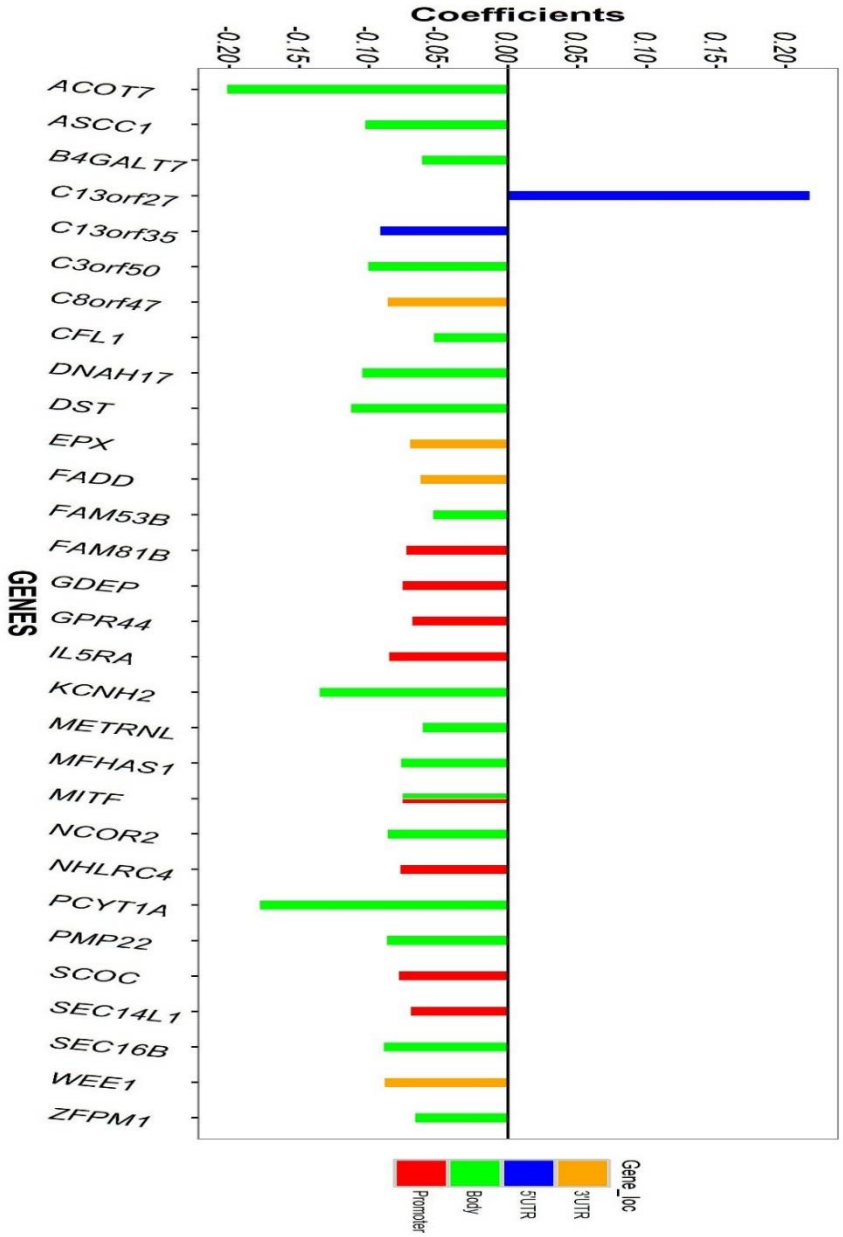
Figure 1. Bar plots of the regression coefficients and the location of CpGs on their corresponding genes. The coefficients represent changes in DNA methylation associated with allergic sensitizations. Negative coefficients reflect decrease in DNA methylation in sensitized patients.



References:

1. Sly, P.D., et al., *Early identification of atopy in the prediction of persistent asthma in children.* The Lancet, 2008. 372(9643): p. 1100-1106.
2. Arshad, S.H., et al., *Early life risk factors for current wheeze, asthma, and bronchial hyperresponsiveness at 10 years of age.* CHEST Journal, 2005. 127(2): p. 502-508.
3. Heinzerling, L., et al., *Standard skin prick testing and sensitization to inhalant allergens across Europe–a survey from the GA2LEN network.* Allergy, 2005. 60(10): p. 1287-1300.
4. Education, F.A.R.a., *Skin Prick Tests*, 2016, Food Allergy Research and Education: VA. p. 1.
5. Roberts, G., et al., *Trends in cutaneous sensitization in the first 18 years of life: results from the 1989 Isle of Wight birth cohort study.* Clinical & Experimental Allergy, 2012. 42(10): p. 1501-1509.
6. Lockett, G.A., J. Huoman, and J.W. Holloway, *Does allergy begin in utero?* Pediatric Allergy and Immunology, 2015. 26(5): p. 394-402.
7. Jones, P.A., *Functions of DNA methylation: islands, start sites, gene bodies and beyond.* Nature Reviews Genetics, 2012. 13(7): p. 484.
8. Sordillo, J.E., et al., *Allergen sensitization is associated with increased DNA methylation in older men.* International archives of allergy and immunology, 2012. 161(1): p. 37-43.
9. White, G.P., et al., *CpG methylation patterns in the IFNgamma promoter in naive T cells: variations during Th1 and Th2 differentiation and between atopics and non-atopics.* Pediatr Allergy Immunol, 2006. 17(8): p. 557-64.
10. Everson, T.M., et al., *DNA methylation loci associated with atopy and high serum IgE: a genome-wide application of recursive Random Forest feature selection.* Genome Med, 2015. 7: p. 89.
11. Guthikonda, K., et al., *Oral contraceptives modify the effect of GATA3 polymorphisms on the risk of asthma at the age of 18 years via DNA methylation.* Clin Epigenetics, 2014. 6(1): p. 17.
12. Yousefi, M., et al., *The methylation of the LEPR/LEPROT genotype at the promoter and body regions influence concentrations of leptin in girls and BMI at age 18 years if their mother smoked during pregnancy.* International Journal of Molecular Epidemiology and Genetics, 2013. 4(2): p. 86.
13. Roberts, G., et al., *Trends in cutaneous sensitization in the first 18 years of life: results from the 1989 Isle of Wight birth cohort study.* Clin Exp Allergy, 2012. 42(10): p. 1501-9.
14. Zhang, H., et al., *The interplay of DNA methylation over time with Th2 pathway genetic variants on asthma risk and temporal asthma transition.* Clin Epigenetics, 2014. 6(1): p. 8.
15. Arshad, S.H., et al., *Cohort Profile: The Isle Of Wight Whole Population Birth Cohort (IOWBC).* Int J Epidemiol, 2018.
16. Miller, S.A., D.D. Dykes, and H.F. Polesky, *A simple salting out procedure for extracting DNA from human nucleated cells.* Nucleic Acids Res, 1988. 16(3): p. 1215.
17. Lehne, B., et al., *A coherent approach for analysis of the Illumina HumanMethylation450 BeadChip improves data quality and performance in epigenome-wide association studies.* Genome Biol, 2015. 16(1): p. 37.
18. Aryee, M.J., et al., *Minfi: a flexible and comprehensive Bioconductor package for the analysis of Infinium DNA methylation microarrays.* Bioinformatics, 2014. 30(10): p. 1363-9.
19. Ray, M., X. Tong, and H. Zhang, *ttScreening: Genome-wide DNA methylation sites screening by use of training and testing samples (R Package)*, 2014.
20. Ray, M.A., et al., *An Efficient Approach to Screening Epigenome-Wide Data.* Biomed Res Int, 2016. 2016: p. 16.

21. **Zhang, F.F., et al., *Significant differences in global genomic DNA methylation by gender and race/ethnicity in peripheral blood.* Epigenetics, 2011. 6(5): p. 623-9.**
22. **El-Maarri, O., et al., *Gender specific differences in levels of DNA methylation at selected loci from human total blood: a tendency toward higher methylation levels in males.* Hum Genet, 2007. 122(5): p. 505-14.**
23. **Xu, C.J., et al., *The emerging landscape of dynamic DNA methylation in early childhood.* BMC Genomics, 2017. 18(1): p. 25.**
24. **Han, L., et al., *Stability of DNA methylation pre- and post-adolescence and related risk factors*, 2018, Division of Epidemiology, Biostatistics, and Environmental Health, School of Public Health, University of Memphis, Memphis, TN.**
25. **Hansen, K.D., et al., *minfi: Analyze Illumina Infinium DNA methylation arrays*, in *R package*2018.**
26. **Houseman, E.A., et al., *DNA methylation arrays as surrogate measures of cell mixture distribution.* BMC Bioinformatics, 2012. 13: p. 86.**
27. **Wickman, M., et al., *Childhood-to-adolescence evolution of IgE antibodies to pollens and plant foods in the BAMSE cohort.* J Allergy Clin Immunol, 2014. 133(2): p. 580-2.**
28. **Xu, C.J., et al., *DNA methylation in childhood asthma: an epigenome-wide meta-analysis.* Lancet Respir Med, 2018. 6(5): p. 379-388.**
29. **Gref, A., et al., *Genome-Wide Interaction Analysis of Air Pollution Exposure and Childhood Asthma with Functional Follow-up.* Am J Respir Crit Care Med, 2017. 195(10): p. 1373-1383.**
30. **Chen, J., et al., *Improved human disease candidate gene prioritization using mouse phenotype.* BMC bioinformatics, 2007. 8(1): p. 392.**
31. **Naeem, H., et al., *Reducing the risk of false discovery enabling identification of biologically significant genome-wide methylation status using the HumanMethylation450 array.* BMC Genomics, 2014. 15: p. 51.**
32. **Maechler, M., *Diptest: Hartigan's dip test statistic for unimodality—corrected.* R package version 0.75-7. See https://CRAN. R-project. org/package= diptest, 2015.**
33. **Tripathi, P., et al., *Genetic determinants in the development of sensitization to environmental allergens in early childhood.* Immun Inflamm Dis, 2014. 2(3): p. 193-204.**
34. **Arathimos, R., et al., *Epigenome-wide association study of asthma and wheeze in childhood and adolescence.* Clin Epigenetics, 2017. 9: p. 112.**
35. **Forno, E., et al., *A Multiomics Approach to Identify Genes Associated with Childhood Asthma Risk and Morbidity.* Am J Respir Cell Mol Biol, 2017. 57(4): p. 439-447.**
36. **Cheong, H.S., et al., *Association analysis of interleukin 5 receptor alpha subunit (IL5RA) polymorphisms and asthma.* J Hum Genet, 2005. 50(12): p. 628-34.**
37. **Namkung, J.H., et al., *IL-5 and IL-5 receptor alpha polymorphisms are associated with atopic dermatitis in Koreans.* Allergy, 2007. 62(8): p. 934-42.**
38. **Kotsimbos, A.T. and Q. Hamid, *IL-5 and IL-5 receptor in asthma.* Mem Inst Oswaldo Cruz, 1997. 92 Suppl 2: p. 75-91.**
39. **Lewis, S.A., et al., *The relation between peripheral blood leukocyte counts and respiratory symptoms, atopy, lung function, and airway responsiveness in adults.* Chest, 2001. 119(1): p. 105-114.**
40. **Chen, W., et al., *An epigenome-wide association study of total serum IgE in Hispanic children.* J Allergy Clin Immunol, 2017. 140(2): p. 571-577.**
41. **Ek, W.E., et al., *Epigenome-wide DNA methylation study of IgE concentration in relation to self-reported allergies.* Epigenomics, 2017. 9(4): p. 407-418.**
42. **Luo, W., et al., *Family-based whole exome sequencing of atopic dermatitis complicated with cataracts.* Oncotarget, 2017. 8(35): p. 59446-59454.**

43. **Bousquet, J., et al.,** ***Are allergic multimorbidities and IgE polysensitization associated with the persistence or re-occurrence of foetal type 2 signalling? The MeDALL hypothesis.*** **Allergy, 2015. 70(9): p. 1062-1078.**

44. **Pepys, J.,** ***"Atopy": a study in definition.*** **Allergy, 1994. 49(6): p. 397-9.**

45. **Lee, J.H. and G. Noh,** ***Clinical significance and evaluation of polysensitisation using skin sensitisation profiling in atopic dermatitis.*** **Inflammation & Allergy-Drug Targets (Formerly Current Drug Targets-Inflammation & Allergy), 2013. 12(3): p. 212-220.**

46. **Rudin, A., et al.,** ***"Bystander" amplification of PBMC cytokine responses to seasonal allergen in polysensitized atopic children.*** **Allergy, 2001. 56(11): p. 1042-1048.**

47. **Bousquet, J., et al.,** ***Heterogeneity of atopy. I. Clinical and immunologic characteristics of patients allergic to cypress pollen.*** **Allergy, 1993. 48(3): p. 183-8.**

48. **Illumina,** ***Field Guide to Methylation Methods*** **Illumina Sequencing Methods, 2016.**