Shared DNA methylation signatures in childhood allergy: The MeDALL study

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Background: Differential DNA methylation associated with allergy might provide novel insights into the shared or unique etiology of asthma, rhinitis, and eczema.

Objective: We sought to identify DNA methylation profiles associated with childhood allergy.

Methods: Within the European Mechanisms of the Development of Allergy (MeDALL) consortium, we performed an epigenome-wide association study of whole blood DNA methylation by using a cross-sectional design. Allergy was defined as having symptoms from at least 1 allergic disease (asthma, rhinitis, or eczema) and positive serum-specific IgE to common aeroallergens. The discovery study included 219 case patients and 417 controls at age 4 years and 228 case patients and 593 controls at age 8 years from 3 birth cohorts, with replication analyses in 325 case patients and 1111 controls. We performed additional analyses on 21 replicated sites in 785 case patients and 2124 controls by allergic symptoms only from 8 cohorts, 3 of which were not previously included in analyses.

Results: We identified 80 differentially methylated CpG sites that showed a 1% to 3% methylation difference in the discovery phase, of which 21 (including 5 novel CpG sites) passed genome-wide significance after meta-analysis. All 21 CpG sites were also significantly differentially methylated with allergic symptoms and shared between asthma, rhinitis, and eczema. The 21 CpG sites mapped to relevant genes, including *ACOT7*, *LMAN3*, and *CLDN23*. All 21 CpG sties were differently methylated in asthma in isolated eosinophils, and 10 were replicated in respiratory epithelium.

Conclusion: Reduced whole blood DNA methylation at 21 CpG sites was significantly associated with childhood allergy. The findings provide novel insights into the shared molecular mechanisms underlying asthma, rhinitis, and eczema. (J Allergy Clin Immunol 2021;147:1031-40.)

Key words: Epigenetics, DNA methylation, allergy, IgE, children

Allergic diseases, such as asthma, rhinitis, and eczema are of great public health concern and constitute the most prevalent childhood illnesses worldwide.[1] Many allergic diseases have a strong hereditary component, yet large-scale genome-wide association studies (GWASs)[2-4] indicate that genetic polymorphisms explain only a limited proportion of the susceptibility to allergy. Moreover, the dramatic rise in prevalence of IgE-mediated allergic diseases in Western European countries over the last decades can be explained not by genetic factors but likely by environmental factors and lifestyle changes.[5] The analysis of DNA methylation in relation to allergic diseases has therefore attracted much interest because epigenetic modification may mediate environmental effects on the development of allergic diseases.[6,7] So far, many studies have linked the DNA methylome to a single trait, such as asthma,[8,9] rhinitis,[10] eczema,[11] total[12,13] and allergen-specific IgE levels,[14-16] and food allergy.[14,17] However, allergic diseases often coexist in the same individual,[18] an overlap that is partly explained by IgE sensitization. A recent large-scale GWAS[2] indicated that the majority of genetic risk factors for allergy are shared between asthma, eczema, and rhinitis, suggesting many shared causal mechanisms.[18,19] So far, no study has assessed shared epigenetic factors related to these 3 allergic diseases.

To address this question, we implemented a large-scale epigenome-wide association study (EWAS) of allergy and whole blood DNA methylation in 3 birth cohorts participating in the Mechanisms of the Development of Allergy (MeDALL) consortium. This is a follow-up of our previous article on childhood asthma.[8] In the current study, we investigated DNA methylation associated with IgE-mediated allergic diseases, asthma, rhinitis, or eczema in children at the ages of 4 and 8 years, followed by replication and meta-analysis of children up to 16 years of age. We further tested whether our differently methylated CpG sites were associated with allergic symptoms only, without inclusion of specific IgE in our case definition, were shared or different between children with asthma, rhinitis, or eczema only, and we also investigated the DNA methylation of replicated CpG sites in isolated eosinophils and nasal epithelial cells. Finally, we functionally annotated these CpG sites.

METHODS

The Methods section in this article's Online Repository (available at www.jacionline.org) provides details on the methods used and cohorts included in this study. The study design and structure of this article are presented in Fig 1.

Allergy definition

The primary outcome of the study was allergy, defined as agreed on by experts from the MeDALL consortium.[18] A child was considered to have an allergy if he or she had at least 1 of 3 diseases—asthma, rhinitis, or eczema—as well as specific IgE against any of the tested allergens (>0.35 IU/mL).

The term *allergy symptoms only* was defined as the situation in which a child had at least 1 of the 3 diseases asthma, rhinitis, and eczema, without consideration of information on the specific IgE results. This definition was also used for comparisons between single diseases.

Detailed definitions of allergy, asthma, rhinitis, and eczema, as well as specific IgE positivity in each cohort, are provided in the Online Repository.

Study population and design

The present study was conducted within the framework of the MeDALL consortium and included cohorts with DNA methylation data measured at birth and/or during childhood. The study consisted of 2 phases, discovery and replication. In discovery and replication, allergic disease end point/IgE data were collected at the same time as DNA methylation analyses.

In the discovery phase, whole blood DNA methylation was measured in participants of 3 cohorts, namely, Barn/Children, Allergy, Milieu, Stockholm, Epidemiology (Sweden), Infancia y Medio Ambiente (INMA) (Sabadel, Spain), and Prevention and Incidence of Asthma and Mite Allergy (PIAMA) (The Netherlands) (Table I and see Table E1 in the Online Repository at
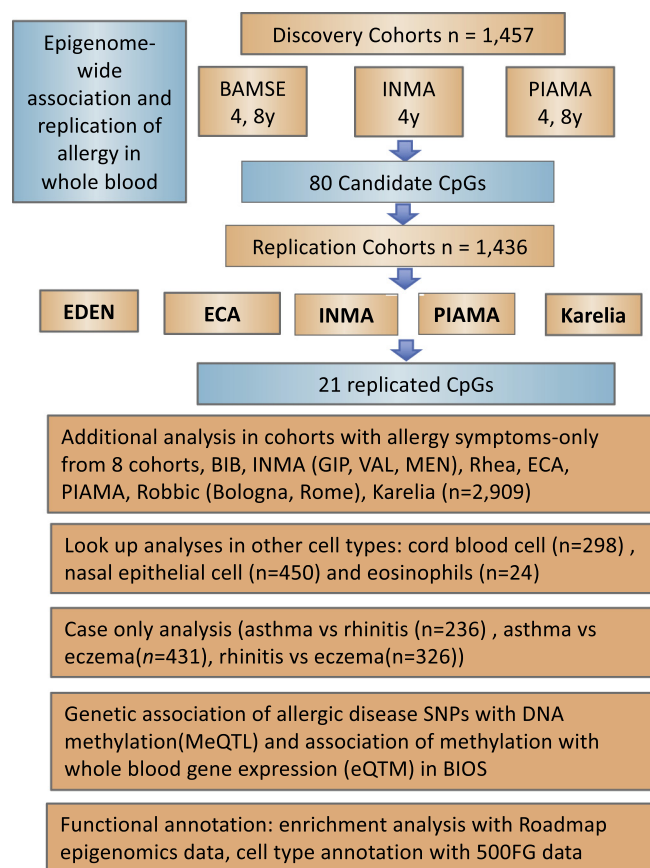
FIG 1. Overview of the study design. The present EWAS study consisted of a discovery and replication phase, followed by a meta-analysis. The top significant CpG sites from the discovery phase (n = 1457) were selected for replication from 5 European cohorts (n = 1436). Subsequently, results from the discovery and replication phases were combined in meta-analysis. The 21 replicated CpG sites were further investigated in an allergy symptoms–only analysis based on 8 cohorts (n = 2909), the lookup analysis in the cord blood and nasal epithelia cell, and case patient–only analysis, as well as functional analyses, including methylation quantitative trait loci (MeQTLs), eQTM, functional enrichment, and cell type annotation. *BIB*, Born in Bradford; *ECA*, Environment and Childhood Asthma Study in Oslo.

www.jacionline.org). As DNA methylation is age specific,[20] we specified beforehand that the discovery analysis would be performed separately in 2 age groups, 4 years and 8 years. The discovery study was enriched with case patients with allergic disease and consisted of 219 case patients with allergy and 417 controls at age 4 years, as well as 228 case patients with allergy, and 593 controls at age 8 years. After identification of differentially methylated CpG sites, we performed replication analysis in 325 case patients with allergy and 1111 controls from 5 cohorts, namely, the cohorts EDEN (France), Environment and Childhood Asthma Study in Oslo (Norway), PIAMA (The Netherlands), INMA (Menorca, Spain), and Karelia (Finland), after which we meta-analyzed the results (Table I and see Table E1). Any individuals whose samples had been used in the discovery studies have been excluded in the replication.

We conducted an additional allergy symptoms–only analysis to test the association of allergy-replicated CpG sites in 785 case patients and 2124 controls from 8 cohorts, including the cohorts Born in Bradford (United Kingdom), EDEN (France), Environment and Childhood Asthma Study in Oslo (Norway), Karelia (Finland), PIAMA, Mother-Child Cohort in Crete (Greece), Robbic (Italy [the subsets Bologna and Rome]), and INMA (Spain [the subsets Gipuzkoa, Menorca, and València]) (Table I and see Table E1).

Furthermore, we investigated potential shared or disease-specific DNA methylation markers by comparing 3 nonoverlapping groups of case patients

who had a single allergic disease, namely, asthma only, rhinitis only, or eczema only. Thus, the following groups were compared: with asthma versus with rhinitis (118 vs 118), with asthma versus with eczema (252 vs 179), and with rhinitis versus with eczema (156 vs 170). Information on IgE sensitization status was not considered in the case-only analyses.

We investigated whether cord blood DNA methylation levels at replicated CpG sites could predict allergy at age 4 to 5 years in 298 children from the EDEN, and INMA-Sabadel cohorts (see Table E2 in the Online Repository at www.jacionline.org). We subsequently studied whether the allergy-replicated CpG sites in whole blood were also differentially methylated in nasal brush samples from 16-year-old children in the PIAMA cohort and isolated eosinophils (16 case patients with asthma and 8 controls aged 2-56 years) from families from the Saguenay–Lac-Saint-Jean region in Canada[21] (see Table E2). Detailed information on these cohorts is provided in the Online Repository materials (at www.jacionly.org).

The medical ethics committees of all the institutions approved this study. Written informed consent was obtained from parents or legal guardians of all participating children.

DNA methylation measurements

In the discovery analysis, DNA methylation was measured by Illumina Infinium HumanMethylation450 BeadChips (Illumina, San Diego, Calif), whereas in the cohorts contributing to the replication, it was measured by either Illumina Infinium HumanMethylation450 BeadChips or iPlex (Agena Biosciences, Hamburg, Germany). We applied the same quality control of DNA methylation data and covariates as published previously.[8]

Functional follow-up of significant DNA methylation findings

We searched for the effects of genetic variants on DNA methylation variation (*cis* and *trans* methylation quantitative trait loci) by using the public Biobank-Based Integrative Omics Studies (BIOS) consortium database (the BIOS QTL browser, http://genenetwork.nl/biosqtlbrowser/). We associated blood CpG methylation with whole genome gene expression by RNA sequencing in the BIOS consortium data set.[22] CpG sites were annotated by using Genomic Regions Enrichment of Annotations Tool.[23] Functional enrichment analysis was done by overlapping our replicated CpG sites with histone marks and chromatin states of 27 blood cell types in the Roadmap Epigenome Project.[24] Gene expression sets associated with replicated CpG sites were interrogated for cell type specificity using the 500 Functional Genomics Project data set.[25,26]

Statistical analyses

Allergy-associated differentially methylated CpG sites were identified by robust linear regression to account for potential outliers and heteroscedasticity in the data,[27] adjusted for sex, cohort, and technical covariables. We used models without adjustment for blood cell type as the main model for discovery, as well as replication and report cell type–corrected models by inclusion of estimated cell type proportions[28] as covariates in sensitivity analyses. For regional analysis, comb-p[29] was used to identify differentially methylated regions by using the summary statistics from models of single CpG site analysis.

We defined genome-wide significance by using Bonferroni correction ($P < 1.14 \times 10^{-7}$ [439,306 tests]). In each age group, CpG sites with genome-wide significance were taken further for replication. If fewer than 10 CpG sites met the genome-wide significance level, the top 10 CpG sites were selected for replication. We performed inverse variance-weighted fixed-effects meta-analyses with METAL,[30] or random effects meta-analysis,[31] the latter in the case of a heterogeneity χ^2 test P value less than .05. Our replicated CpG sites comprised those that were significantly associated with allergy in the meta-analysis of the replication samples (Bonferroni correction; $P < .0006$ [80 tests]) and passed the epigenome-wide significance threshold by using Bonferroni correction (P

TABLE I. Study characteristics of the discovery and replication cohorts in the whole blood DNA methylation analyses

Cohort	Country	Age group	n	Age, (y), mean (SD)	Male sex, no. (%)	Individuals with allergy (according to questionnaire + aeroallergen sIgE level)	Design
BAMSE	Sweden	Preschool age	247	4.26 (0.20)	134 (54.3%)	117 (47.4%)	Discovery
INMA-SAB	Spain		166	4.35 (0.23)	84 (50.6%)	38 (22.9%)	
PIAMA	The Netherlands		223	4.08 (0.21)	119 (53.4%)	64 (28.7%)	
BAMSE	Sweden	School age	235	8.32 (0.41)	125 (53.2%)	96 (40.9%)	
BAMSE-epi	Sweden		383	8.30 (0.52)	207 (54.0%)	75 (19.6%)	
PIAMA	The Netherlands		203	8.06 (0.28)	101 (49.8%)	57 (28.1%)	
EDEN	France	Preschool age	143	5.65 (0.10)	87 (60.8%)	22 (15.4%)	Replication
BIB	United Kingdom		514	4.53 (0.36)	278 (54.1%)	NA	
INMA-GIP	Spain		74	4	36 (48.6%)	NA	
Rhea	Greece		490	4.26 (0.27)	254 (51.8%)	NA	
ECA	Norway	School age	213	10.89 (0.78)	112 (52.6%)	35 (16.4%)	
INMA-VAL	Spain		58	7.50 (0.20)	31 (53.4%)	NA	
PIAMA	The Netherlands		361	8.30 (0.46)	193 (53.5%)	46 (12.7%)	
Robbic-Bologna	Italy		123	7.82 (0.51)	63 (51.2%)	NA	
Robbic-Rome	Italy		214	8.73 (0.27)	108 (50.5%)	NA	
Karelia	Finland	Adolescent age	98	15.74 (1.59)	38 (38.8%)	35 (35.7%)	
INMA-MEN	Spain		75	14	35 (46.7%)	14 (18.7%)	
PIAMA	The Netherlands		546	16.35 (0.22)	271 (49.6%)	115 (21.1%)	

BAMSE, Barn/Children, Allergy, Milieu, Stockholm, Epidemiology; *BAMSE-epi,* BAMSE-epigene; *BIB,* Born in Bradford; *ECA,* Environment and Childhood Asthma Study in Oslo; *GIP,* Gipuzkoa; *INMA,* Infancia y Medio Ambiente; *MEN,* Menorca; *NA,* not available; *SAB,* Sabadel; *sIgE,* specific IgE; *VAL,* València.

$<1.14 \times 10^{-7}$) in the meta-analysis of all studies (discovery and replication). To avoid unstable effect estimates owing to small numbers, we set a minimum of 10 case patients per participating cohort to be included in the analyses. Detailed description of the functional follow-up is provided in the Methods section of the Online Repository.

RESULTS
Discovery analyses

The baseline characteristics of the study populations are presented in Tables I and E1. In the discovery population, allergy prevalence ranged from 22.9% to 47.4% at age 4 years and from 19.6% to 40.9% at age 8 years. In the replication population with IgE data available, 12.7% to 16.4% of children fulfilled the definition of allergy at preschool age and school age, respectively, and 18.7% to 35.7% fulfilled the definition during adolescence.

At age 4 years, the discovery EWAS analysis identified 1 genome-wide significant CpG site, namely, cg15344640, annotated to Max dimerization protein 3 (*MXD3*) and lectin, mannose binding 2 (*LMAN2*) gene ($P = 2.97 \times 10^{-8}$ [Fig 2, *A* and see Fig E1, *A* and Table E3 in the Online Repository at www.jacionline. org]; genomic inflation factor $\lambda = 1.15$). According to our prespecified plan, we selected the top 10 CpG sites for replication in participants at age 4 years (see Table E3). In participants at age 8 years, 74 CpG sites passed genome-wide significance threshold, with the strongest association ($P = 1.76 \times 10^{-10}$) found for cg03695871 annotated to the adaptor related protein complex 5 subunit beta 1 (*AP5B1*) and ribonuclease H2 subunit C (*RNASEH2C*) genes (Fig 2, *B* and see and Fig E1, *B* and Table E4 in the Online Repository at www.jacionline.org [$\lambda = 1.09$]). White blood cell composition was estimated in all discovery cohorts; we did not observe significant, consistent cell composition differences between the allergy and control groups with regard to 7 cell types in our discovery cohorts at age 4 and 8 years (see Fig E2 and Table E5 in the Online Repository at www.jacionline.org in the Online Repository at www.jacionline. org). However, the association of CpG methylation with allergy became less significant after eosinophil and neutrophil correction:

the *P* values after correction ranged from 1.71×10^{-2} to 1.12×10^{-5} versus from 3.58×10^{-7} to 8.27×10^{-10} before correction (see Tables E3 and E4), which did suggest that at least part of the results were driven by cell type. Between the top 10 CpG sites at age 4 years and the 74 genome-wide significant CpG sites at age 8 years, 4 CpG sites (cg13628444, cg20885063, cg11456013 and cg03131767) were overlapping; thus, 80 unique CpG sites were selected for replication. Differentially methylated regions analyses identified regions near genes (such as *RUNX3, IL1B, IL13* and *AP5B1*) that were previously implicated in the development of allergy (see Tables E6 and E7 in the Online Repository at www.jacionline.org).

Replication and meta-analysis

Iplex assays for 70 of these 80 CpG sites were available with use of the Iplex design and passed quality control. Two of 10 the CpG sites selected at age 4, as well as 19 of 74 significant CpG sites at age 8 years, were significantly associated with allergy across childhood in the replication setting (Table II). With regard to all 21 replicated CpG sites, subjects with allergy had lower methylation levels than the controls did. Genes annotated to these 21 CpG sites were investigated via literature review (see Table E8 in the Online Repository at www.jacionline.org). In the meta-analysis of the discovery and replication results (Table II), the most significant association ($P = 9.84 \times 10^{-21}$) was observed for cg06483820 annotated to A-kinase anchoring protein 1 (*AKAP1*) and Musashi RNA binding protein 2 (*MSI2*). Forest plots showing cohort-specific and combined estimates and 95% CIs of all replicated and nonreplicated CpG sites are shown in Fig E3 to E6 (in this article's Online Repository at www.jacionline.org).

Additional replication and functional analyses

In an additional analysis, all 21 aforementioned CpG sites were significantly associated with allergy defined by symptoms only
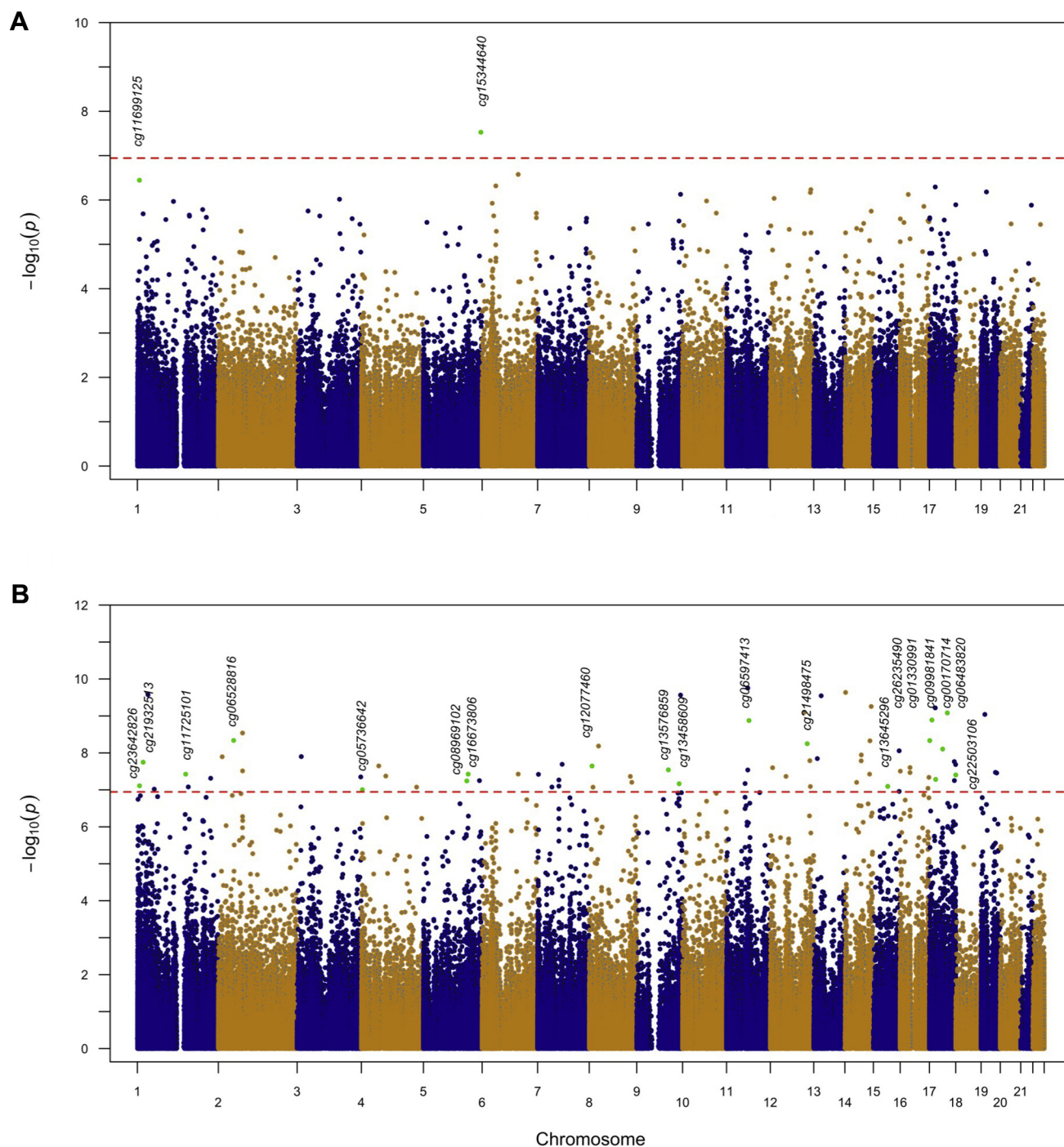
FIG 2. Manhattan plots from the EWASs performed in 3 European birth cohorts of childhood allergic disease (discovery). A total of 439,306 CpG sites were tested for association. The red dotted horizontal line represents the Bonferroni-corrected threshold ($P < 1.14 \times 10^{-7}$) of genome-wide significance. All 21 replicated CpG sites are marked as green circles and annotated with CpG sites name. **A**, Results for the EWAS analysis in children aged 4 years (n = 636) from the 3 European cohorts Barn/Children, Allergy, Milieu, Stockholm, Epidemiology (BAMSE), Infancia y Medio Ambiente (INMA), and PIAMA. **B**, Results for the EWAS meta-analysis in children aged 8 years (n = 821) from the BAMSE and PIAMA cohorts.

(Table III and see Fig E7 in the Online Repository at www.jacionline.org). In a comparison of case patients with patients with asthma only, rhinitis only, or eczema only while not taking specific IgE into account, none of the 21 CpG sites were differentially methylated (ie, disease specific) in relation to any of these 3 individual diseases (see Table E9 in the Online Repository at www.jacionline.org).

Methylation of the 21 allergy-associated CpG sites in 298 cord blood DNA samples at birth was not associated with allergy development during early childhood (see Table E10 in the Online

TABLE II. Description of 21 unique replicated allergic disease–related CpG sites across childhood

CpG sites	Age group	Chr	bp Position*	Gene name†	Discovery coef‡	Discovery P value§	Houseman-corrected P value‖	Eosinophil-corrected P value¶	Replication Coef#	Replication P value**	Meta-analysis Coef††	Meta-analysis P value‡‡
cg11699125	4 y	1	6341327	GPR153, ACOT7	−0.0190	3.58×10^{-7}	1.02×10^{-5}	1.66×10^{-4}	−0.0306	2.87×10^{-14}	−0.0271	5.84×10^{-19}
cg15344640		5	176774729	MXD3, LMAN2	−0.0100	2.97×10^{-8}	9.46×10^{-7}	1.12×10^{-5}	−0.0104	1.62×10^{-7}	−0.0100	2.53×10^{-14}
cg23642826	8 y	1	6645463	ZBTB48, KLHL21	−0.0178	7.77×10^{-8}	7.87×10^{-5}	1.71×10^{-2}	−0.0127	2.88×10^{-8}	−0.0140	2.60×10^{-14}
cg21932513		1	17751974	RCC2, PADI4	−0.0161	1.79×10^{-8}	2.74×10^{-5}	2.81×10^{-3}	−0.0140	3.31×10^{-7}	−0.0150	5.34×10^{-14}
cg11725101		1	148739457	NBPF16	−0.0125	3.77×10^{-8}	9.03×10^{-7}	1.50×10^{-3}	−0.0077	5.22×10^{-4}	−0.0100	3.58×10^{-10}
cg06528816		2	47242277	TTC7A, CALM2	−0.0209	4.61×10^{-9}	1.81×10^{-6}	3.50×10^{-4}	−0.0169	1.57×10^{-10}	−0.0183	8.47×10^{-18}
cg05736642		4	3123132	RGS12, HTT	−0.0128	9.90×10^{-8}	4.67×10^{-6}	2.46×10^{-4}	−0.0129	2.90×10^{-6}	−0.0124	1.37×10^{-12}
cg16673806		5	137771872	REEP2	−0.0160	3.76×10^{-8}	4.94×10^{-6}	1.51×10^{-3}	−0.0098	6.56×10^{-5}	−0.0116	5.74×10^{-11}
cg08969102		5	133563532	PPP2CA	−0.0168	5.73×10^{-8}	1.09×10^{-5}	1.25×10^{-3}	−0.0103	3.74×10^{-5}	−0.0129	3.81×10^{-11}
cg12077460		8	8702053	ERI1, CLDN23	−0.0156	2.27×10^{-8}	1.03×10^{-6}	6.39×10^{-5}	−0.0144	2.98×10^{-6}	−0.0150	3.73×10^{-13}
cg13576859		9	97403129	FBP1	−0.0196	2.88×10^{-8}	5.63×10^{-7}	1.64×10^{-4}	−0.0128	1.05×10^{-7}	−0.0147	4.91×10^{-14}
cg13458609		9	130608923	ENG, FPGS	−0.0188	6.84×10^{-8}	1.52×10^{-6}	8.92×10^{-4}	−0.0164	1.53×10^{-8}	−0.0174	7.08×10^{-15}
cg06597413		11	69251883	CCND1, TPCN2	−0.0172	1.33×10^{-9}	3.91×10^{-6}	3.98×10^{-4}	−0.0123	2.25×10^{-7}	−0.0137	4.20×10^{-15}
cg21498475		12	113737469	SLC24A6, TPCN1	−0.0155	5.64×10^{-9}	2.20×10^{-6}	1.69×10^{-3}	−0.0121	2.20×10^{-9}	−0.0134	1.78×10^{-16}
cg13645296		15	64275810	HERC1, DAPK2	−0.0208	8.06×10^{-8}	1.41×10^{-7}	4.61×10^{-5}	−0.0220	3.17×10^{-11}	−0.0215	1.70×10^{-17}
cg26235490		17	1047567	ABR, TIMM22	−0.0132	4.64×10^{-9}	9.61×10^{-7}	2.20×10^{-3}	−0.0153	1.92×10^{-10}	−0.0142	1.07×10^{-17}
cg01330991		17	7648108	KDM6B, DNAH2	−0.0166	1.29×10^{-9}	1.58×10^{-6}	4.23×10^{-4}	−0.0098	9.32×10^{-6}	−0.0122	2.42×10^{-13}
cg09981841		17	19177387	EPN2, B9D1	−0.0171	5.21×10^{-8}	1.89×10^{-4}	2.50×10^{-2}	−0.0090	4.25×10^{-4}	−0.0122	4.63×10^{-10}
cg00170714		17	40724562	PSMC3IP, MLX	−0.0147	7.87×10^{-9}	1.23×10^{-5}	1.57×10^{-3}	−0.0113	6.92×10^{-7}	−0.0129	2.13×10^{-14}
cg06483820		17	55167149	MSI2, AKAP1	−0.0159	8.27×10^{-10}	7.96×10^{-7}	4.21×10^{-4}	−0.0140	1.41×10^{-12}	−0.0146	9.84×10^{-21}
cg22503106		17	81040906	METRNL	−0.0198	3.95×10^{-8}	3.07×10^{-6}	3.32×10^{-3}	−0.0143	2.28×10^{-5}	−0.0174	5.68×10^{-12}

Chr, Chromosome; *Coef*, coefficient.

*Base pair position according to Genome Build 37.

†The CpG sites were annotated by the Genomic Regions of Annotations Tool (GREAT), version 3.0.0 (available at http://bejerano.stanford.edu/great/).

‡Regression coefficient from the discovery study.

§P value from discovery study.

‖P value from the discovery study after correction for estimated cell counts with the default Houseman setting (T cells [CD8T], T cells [CD4T], NK cells, B cells, monocytes, and granulocytes.

¶P value from the discovery study after correction for estimated cell counts (T cells [CD8T], T cells [CD4T], NK cells, B cells, monocytes, eosinophils, and neutrophils).

#Regression coefficient from replication study.

**P value from meta-analysis of replication study.

††Regression coefficient from meta-analysis of discovery and replication.

‡‡P value from meta-analysis of discovery and replication.

Repository at www.jacionline.org), whereas 10 of 21 tested CpG sites were associated with allergic disease with nominal significance ($P < .05$) in nasal respiratory epithelial cells of 16-year-old children of the PIAMA cohort, with matching direction of effect (see Table E11 in the Online Repository at www.jacionline.org).

In the Saguenay–Lac-Saint-Jean cohort, we found all 21 CpG sites to be significantly associated with asthma within eosinophils: on average, participants with asthma had 19% lower DNA methylation of these 21 CpG sites than did participants without asthma. (see Table E12 in the Online Repository at www.jacionline.org).

Functional analyses using Roadmap epigenomics data showed that 17 of 21 allergy-associated CpG sites were significantly enriched for enhancer markers in the whole blood Roadmap and Encode data (see Tables E13 and E14 in the Online Repository at www.jacionline.org).

We next investigated whether DNA methylation of the 21 allergy-associated CpG sites is driven by allergy-associated genetic variants. Looking up methylation quantitative trait

TABLE III. Independent meta-analysis of the association of DNA methylation at 21 replicated CpG sites with allergic disease defined on the basis of symptoms without consideration for IgE levels in 8 replication cohorts

CpG site	Gene name*	Meta-analysis coefficient	Meta-analysis *P* value	Direction
cg11699125	*GPR153, ACOT7*	−0.0189	1.78E−09	——+——+−+
cg15344640	*MXD3, LMAN2*	-0.0064	2.95E−04	—+−++——+—
cg23642826	*ZBTB48, KLHL21*	−0.0079	8.06E−07	——++———
cg21932513	*RCC2, PADI4*	−0.0093	8.26E−07	——+++—+—
cg11725101	*NBPF16*	−0.0074	5.28E−05	——?+−+−+−+
cg06528816	*TTC7A, CALM2*	−0.0111	3.51E−08	——+——?—
cg05736642	*RGS12, HTT*	−0.0069	4.73E−05	——++———
cg16673806	*REEP2*	−0.0064	3.43E−05	——++—+—
cg08969102	*PPP2CA*	−0.0089	2.73E−06	——?+———
cg12077460	*ERI1,CLDN23*	−0.0117	1.90E−06	——++—+—
cg13576859	*FBP1*	−0.0083	1.88E−08	——++—+—
cg13458609	*ENG, FPGS*	−0.0122	5.63E−08	—+−?+———
cg06597413	*CCND1, TPCN2*	−0.0084	1.71E−09	——+——+—
cg21498475	*SLC24A6, TPCN1*	−0.0088	1.48E−07	—+———?−+
cg13645296	*HERC1, DAPK2*	−0.0172	2.78E−12	———————?—
cg26235490	*ABR, TIMM22*	−0.0097	3.17E−09	——+—+—
cg01330991	*KDM6B, DNAH2*	−0.008	3.28E−08	——++—+—
cg09981841	*EPN2, B9D1*	−0.0075	7.01E−05	—+−++———
cg00170714	*PSMC3IP, MLX*	−0.007	1.74E−04	——?+——+
cg06483820	*MSI2, AKAP1*	−0.0086	1.01E−09	——+———
cg22503106	*METRNL*	−0.0108	8.34E−07	——++—+—

Coefficient is the regression coefficient from the meta-analysis. *P* value is the *P* value from meta-analysis of the replication cohorts only, without consideration for the discovery population. For each cohort participating in the analysis, the symbol + indicates a positive direction of effect, whereas the symbol – indicates a negative direction of effect and the symbol ? indicates missing information for that CpG site in a given cohort. The cohort order is as follows: Born in Bradford (BIB), Rhea, INMA (Gipuzkoa), EDEN, INMA (València), PIAMA (age 8 years), Robbic (Rome), Robbic (Bologna), Environment and Childhood Asthma Study in Oslo (ECA), INMA (Menorca), PIAMA (age 16 years), and Karelia.
*The CpG sites were annotated by using the Genomic Regions of Annotations Tool, version 3.0.0 (available at http://bejerano.stanford.edu/great/).

loci from the BIOS QTL browser (https://genenetwork.nl/biosqtlbrowser/), we found 1 of 21 CpG sites annotated with tetratricopeptide repeat domain 7A (*TTC7A*) and calmodulin 2 (*CALM2*) (ie, cg06528816) to be significantly associated *in trans* with the asthma-associated polymorphism rs2381416 in the *IL33* gene (false discovery rate *P* value = .03) (see Table E15 in the Online Repository at www.jacionline.org).[32] Interestingly, rs2381416-A has been associated with higher methylation and lower eosinophil levels and higher neutrophil percentage levels (see Tables E15 and E16 in the Online Repository at www.jacionline.org).[33] Expression quantitative trait methylation (eQTM) of the 21 CpG sites with gene expression *in cis* in 2353 adults identified 15 CpG sites to be associated with 38 blood gene transcripts (see Table E17 in the Online Repository at www.jacionline.org). All CpG sites that showed significant *trans*-eQTM with whole blood gene expression are shown in Table E18 (in the Online Repository at www.jacionline.org). In the *trans*-eQTM analysis, we identified 4 gene expression patterns in whole blood by hierarchic clustering (see Fig E8 in the Online Repository at www.jacionline.org), with clusters 1 and 3 showing distinct patterns. In cluster 1, CpG methylation was correlated with gene expression of C-C chemokine receptor type 7 (*CCR7*), charged multivesicular body protein 7 (*CHMP7*), and lymphoid enhancer binding factor 1 (*LEF1*). In cluster 3, CpG methylation was correlated with expression of the genes sialic acid binding Ig like lectin 8 (*SIGLEC8*), oligodendrocyte transcription factor 2 (*OLIG2*), adenosine A3 receptor (*ADORA3*), and Il-5 receptor subunit alpha (*IL5RA*), all of which are genes related to blood eosinophils. An RNA sequencing data set containing data on 89 individuals with 75 measured immune cell (sub)populations[25] from the 500 Functional Genomics

Project showed clustered RNA-transcripts associated with allergy CpG sites to be strongly associated with effector and memory CD8 and natural killer (NK) cells and naive CD8+ and CD4+ cells (see Fig E9 in the Online Repository at www.jacionline.org).

DISCUSSION

This large consortium-based meta-analysis identified 21 CpG sites in whole blood to be associated with childhood allergic disease. Consistently lower methylation levels at 2 CpG sites were observed in children with allergy at the age of 4 years and 19 CpG sites in children with allergy at the age of 8 years, all of which were replicated in independent cohorts up to adolescence. These CpG sites were associated not only with allergy defined by asthma, rhinitis, or eczema in combination with specific IgE but also when allergy was defined by disease symptoms only. We found no evidence for single disease–specific CpG methylation signatures, suggesting shared DNA methylation signatures of allergy in blood. DNA methylation at these 21 CpG sites in cord blood was not predictive of allergy during early childhood, indicating that postnatal changes in DNA methylation may play an important role in the development of allergy.

Five of 21 differentially methylated CpG sites represent novel findings in the context of EWAS on allergy. One of them is cg21932513, which is annotated to regulator of chromosome condensation 2 (*RCC2*) and peptidyl arginine deiminase 4 (*PADI4*). Being a well-established genetic risk factor for rheumatoid arthritis,[34] *PADI4* has recently been implicated in acute allergic reactions to peanut.[35] Other novel findings include cg11725101, mapping to neuroblastoma breakpoint family member 16 (*NBPF16*); cg16673806 - receptor accessory protein

2 (*REEP2*); cg26235490 - ABR activator of RhoGEF and GTPase (*ABR*) and translocase of inner mitochondrial membrane 22 (*TIMM22*), as well as cg01330991 - lysine demethylase 6B (*KDM6B*) and dynein axonemal heavy chain 2 (*DNAH2*). These genes have not been previously implicated in allergic disease.

Some of the differentially methylated CpG sites found in this study were previously reported. We identified 2 overlapping CpG sites, namely, cg15344640 (*MXD3, LMAN2*) and cg06483820 (*MSI2, AKAP1*), which were found in our previous MeDALL EWAS on asthma,[18] as well as 6 overlapping CpG sites (cg11699125, cg21498475, cg00170714, cg13576859, cg08969102, and cg13458609) in the Pregnancy and Childhood Epigenetics consortium study on asthma.[8] Further, 2 of 21 CpG sites were previously reported in relation to total or specific IgE levels, namely, cg11699125 (belonging to G protein–coupled receptor 153 [*GPR153*]) and acyl-CoA thioesterase 7 (*ACOT7*), was found in the Project Viva birth cohort in the United States,[36] as well as in 2 studies of Hispanic children.[11] The CpG sites cg11699125 (*GPR153, ACOT7*) and cg12077460, mapping to exoribonuclease 1 (*ERI1*), and claudin 23 (*CLDN23*), were previously reported in the Isle of Wight cohort.[16] Taken together, these 9 CpG sites represent replicated DNA methylation markers of allergic disease across multiple populations.

Our top significant CpG, cg06483820, mapped to *AKAP1* and *MSI2*. A-kinase anchoring proteins have been identified in T cells; they contribute to the maintenance of T-cell homeostasis.[37] The second most significant finding was cg11699125 in *ACOT7*, which is known to play a role in inflammation responses through the production of arachidonic acid and to be involved in increased prostaglandin production.[38] *ACOT7* is particularly interesting because differential methylation in *ACOT7* was previously identified in an EWAS of blood in relation to both total and allergen-specific IgE level[13,39] and asthma,[8,40,41] as well as in an EWAS of nasal epithelium to asthma.[42] In this study, we also observed differential methylation of cg11699125 in nasal respiratory epithelial cells associated with allergy. Thus, *ACOT7* methylation may be related to asthma and IgE sensitization in both blood and epithelial cells, and it may provide a cross-tissue allergy-associated methylation site. In fact, we observed replication of 10 of the 21 CpG sites in respiratory nasal epithelial cells that further support our findings in whole blood. This suggests that many allergy-associated DNA methylation markers may be replicable across different tissues. To further investigate this possibility, we did a lookup of published nasal methylation sites that were significantly associated with allergic asthma[43] or IgE sensitization[15] in our whole blood DNA methylation data on children at ages 4 and 8 years; we found many CpG sites to be significantly associated in blood (see Tables E19 and E20 in the Online Repository at www.jacionline.org).

A recent large GWAS on allergic disease defined by having either asthma, rhinitis, or eczema identified 136 single-nucleotide polymorphisms (SNPs) as being significantly associated with allergic disease,[2] with case patient–only analyses strongly suggesting that 130 of these 136 SNPs are associated with multimorbidity rather than with single disease phenotypes. Our epigenetic study is the first to confirm this finding for DNA methylation in blood: all 21 CpG sites were similarly associated with either allergic disease, and no disease specificity could be shown. This could be due to shared epigenetic signatures of IgE sensitization in blood cells, as disease specificity may be more visible in the end organ. We acknowledge that a limitation of this analysis is the lack of data on IgE sensitization, so we suggest that further studies comparing epigenetic patterns in lung, nose, and skin in combination with IgE sensitization are needed to address this important question.

Integration of genetic and DNA methylation data revealed 2 genes that were implicated by both genetic and epigenetic studies. First, *LMAN2* encodes a type I transmembrane lectin that shuttles between the endoplasmic reticulum, the Golgi apparatus, and the plasma membrane. This gene has previously been reported in an allergy GWAS[2] and an EWAS of childhood asthma.[8] Thus, both genetic and epigenetic markers in *LMAN2* are associated with allergy, which prompted us to investigate whether allergy-associated SNPs could be related to our top CpG sites. However, no significant association between SNPs and CpG sites in *LMAN2* was found, suggesting that genetic and epigenetic factors may independently relate to or cointeract in allergy. In contrast, we did identify a strong association between the allergy-associated SNP rs2381416 in *IL33* at chromosome 9 and CpG methylation in cg06528816 at chromosome 2. The latter finding may be driven by a direct effect of rs238141 on cg06528816 methylation in this gene or by an indirect effect of this SNP regulating blood eosinophils, which is then reflected by the eosinophil-associated CpG site cg06528816.

Correlation of our 21 CpG sites with genome-wide gene expression revealed clusters of associated genes. One cluster included genes such as *CCR7*, which plays a role in allergic airway inflammation,[44] and *TCF7*, which is involved in the pathogenesis of lung diseases.[45] Another cluster was inversely associated with cg11699125 and contained genes such as *IDO1* (which was implicated earlier in the development of immune responses[46]), *HRASLS5, SPNS3, CCL23, ASB2, OLIG2,* and *SEMA7A* (which is known to be correlated with blood eosinophil counts), *CEBPE,* (which is involved in activation of granulocyte production),[47] and *PTGDR2*, which is linked to asthma.[48]

Through clustering of the 21 allergy-associated CpG sites with immune cell–specific gene expression signatures, we were also able to annotate the allergy-associated CpG sites to activated immune cell subsets. We observed 1 cluster consisting of genes related to eosinophil function, such as *SIGLEC8, OLIG2, ADORA3,* and *IL5RA*. The direction of effect suggests that the lower DNA methylation in allergy that was found in this study is related to a higher eosinophil level. The second cluster is in line with previous findings from the MeDALL EWAS asthma study that whole blood DNA from children with allergic diseases carries CpG methylation markers associated with lower activity of naive T cells and higher activity of effector and memory CD8 T-cells and NK cells.[18] This allergy-associated shift in cell state might be due to past exposure, such as childhood infections or microbial stimulation. Thus, we interpret the DNA methylation signatures as markers of activated cell subsets in peripheral blood, reflecting current or past cell activation or suppression events in allergy development.

Our study has some strengths and limitations. This is the largest DNA methylation study in childhood allergy to use independent laboratory methods and cohorts for replication. The study uses detailed definition of allergy phenotype, as well as availability of samples from patients at multiple ages and with multiple nationalities. We further replicated our findings in 5 cohorts that defined allergy by symptoms and IgE sensitization, but we also found that all 21 sites were replicated in 8 cohorts in which allergy

was defined by symptoms only. This suggests that our allergy-associated CpG sites represent robust findings in the context of childhood allergy from the age of 4 years onward.

Previous studies have shown that differences in methylation patterns can be attributed to variability in cell type composition when whole blood is used in DNA methylation studies.[26] Thus, DNA methylation may reflect allergy-associated changes in cell type composition in whole blood, cell-specific changes within the DNA methylome, or a combination of thereof, as was shown for asthma in the MeDALL study.[18] Because no blood cell–specific DNA methylation data in allergic disease are available, we could replicate our findings only in purified eosinophils for asthma. Here, we found that all CpG sites were significantly associated with asthma, indicating that our results are at least partly driven by DNA methylation changes within eosinophils. We also replicated 10 of the 21 CpG sites in nasal epithelial brush cells, further underlining the robustness of our findings. Another limitation is that the Illumina Infinium HumanMethylation450 BeadChip platform covered only about 1.6% of all methylation sites of the genome. One would expect to find more allergy-associated CpG sites by using whole methylome sequencing in the future. Because of the small numbers of case patients with single diseases in the case patient–only analysis, we had limited power to detect unique signatures of single diseases. In our analysis of allergy prediction at birth, we investigated cord blood samples and not peripheral blood, as was done in subjects later in life. Recent analyses showed that 70 % of DNA methylation sites are in good agreement between cord and peripheral blood,[49] which suggests that analyzing cord blood may have introduced an additional source of variation into our analysis.

Our analyses were based on white populations, and whether the findings can be extrapolated to other ethnic groups remains to be investigated.

In conclusion, our study identified consistently lower DNA methylation levels in 21 CpG sites to be associated with allergy in childhood from ages 4 to 16 years, showing that these methylation sites are shared between asthma, rhinitis, and eczema and are implicated eosinophils. Our allergy-associated DNA methylation profiles are indicative of lower presence or activity of naive T cells and higher activity or presence of eosinophils and effector and memory CD8 T cells and NK cells in childhood allergy.

Key messages

- **A consistently lower level of DNA methylation in whole blood DNA of 21 CpG sites was significantly associated with childhood allergy.**
- **The allergy-associated DNA methylation profiles are shared between asthma, rhinitis, and eczema and implicate the presence of and changes in DNA methylation within eosinophils.**

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