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Early neutrophil and persistent eosinophil-associated gene signature in childhood asthma

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Abstract

Rationale: **Early childhood represents a critical window for asthma susceptibility, marked by developmental and molecular changes, yet their longitudinal pattern remains unclear.**

Objectives: **To identify differences in longitudinal whole-blood gene expression during early childhood in future asthmatics compared to healthy children.**

Methods: **We conducted a longitudinal whole-blood transcriptomic analysis at 4 timepoints (1, 4.5, 6, 10.5 years) in a sample of the birth cohort Protection against Allergy Study in Rural Environments (PASTURE) ($n = 378$), comparing children who developed asthma between ages 6 and 10.5 years with nonasthmatic controls (83/295). Analyses included longitudinal differential gene expression, weighted gene co-expression network analysis, and cis-expression quantitative trait loci analysis.**

Measurements and main results: **At age 1 year, 42 genes, mostly upregulated in future asthmatics, were associated with neutrophilic inflammation and NLRP3 inflammasome-markers. By 4.5 years, this shifted to a novel eosinophil-related signature (40 genes), remaining increased in asthmatics until 10.5 years. Co-expression analysis confirmed a neutrophilic module at 1 year and eosinophilic modules at 4.5, 6, and 10.5 years, all associated with asthma. Fractional exhaled nitric oxide was associated with the eosinophilic module at age 6 years ($P = .003$). A total of 86 SNPs were identified modulating the expression of 10 eosinophil-associated genes and GSDMB from this eosinophilic signature. A variant-based genetic risk score was associated with asthma diagnosis (adjusted odds ratio [aOR], 1.47; 95% CI, 1.13-1.93).**

Conclusions: **We identified a shift from a neutrophil-driven gene signature at age 1 year to a persistent eosinophilic signature at 4.5-10.5 years in asthmatic children, highlighting the 1- to 4.5-year period as the most vulnerable period. Genetic variants strongly influenced the persistent eosinophilic gene signature, comprising potential novel therapeutic targets.**

Keywords childhood asthma, transcriptomics, longitudinal study, eosinophilic inflammation

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At a Glance Commentary

Scientific Knowledge on the Subject: Asthma is a heterogenous inflammatory disease that often begins in early childhood. This period constitutes a sensitive time window, during which the immune system is developing. Within this period, environmental exposures and genetic factors lead to molecular changes that modulate disease risk. Neutrophilic inflammation has been associated with early asthma-like symptoms (eg, wheezing) and asthma risk factors (eg, viral infections), whereas eosinophilic inflammation is linked to disease progression. However, the longitudinal pattern of molecular and immunological changes remains unclear.

What This Study Adds to the Field: This study uniquely assessed longitudinal whole-blood RNA-sequencing data from 4 timepoints starting at age 1 year until 10.5 years in children with and without subsequent school-age asthma. We identified an early neutrophil-associated gene signature at age 1 year, associated with future asthma, being absent at age 4.5 years. An eosinophilic gene signature positively associated with asthma, emerged at 4.5 years and persisted until 10.5 years of age. This later signature showed a strong genetic predisposition and included novel potential therapeutic targets for childhood asthma. Our results identify the 1- to 4.5-year period as a critical window of opportunity when key molecular and immunological changes linked to disease development have their onset, highlighting the importance of focusing future research on this specific maturational period.

Introduction

The understanding of childhood asthma has advanced in recent decades, yet critical questions remain, particularly concerning the timing and molecular mechanisms that drive its onset.[1] Although pathophysiology can develop at any age, first symptoms begin in early childhood, making this a vulnerable time window and key for understanding underlying biological and immune pathways.[2]

The specific time frame and order within this period, at which asthma risk factors exert their influence, are still unclear, likely involving a complex interplay of genetic, environmental, and host factors.[3] Epidemiological studies highlight the perinatal period and preschool age as especially sensitive, with protective exposures (eg, farm milk consumption, animal, stable contact) linked to lower asthma and allergy prevalence.[4-7] Conversely, early viral infections, caused by respiratory viruses including respiratory syncytial virus (RSV) and human rhinovirus (hRV) are associated with asthma and atopic sensitization.[8,9] Moreover, microbiome maturation in the first year of life was shown to shape immune development and asthma susceptibility in the Protection against Allergy Study in Rural Environments (PASTURE) study.[10] Notably, inflammatory features in the lung are first visible by age 3 years in children with recurrent wheeze, narrowing this critical window to this period.[11,12]

Clustering analysis and multiomics studies point to early predominance of neutrophilic inflammation that may later transition to T2-high/eosinophilic profiles during established asthma.[13,14] Blood transcriptomics, a minimally invasive method to track systemic immune changes, supports these observations, where dysfunctional neutrophil gene expression has been linked to recurrent wheezing in preschool children,[15] while eosinophil-associated genes predominate in older asthmatic children and adults.[16,17] Whether these patterns reflect a true longitudinal progression or distinct disease pathways remains unresolved due to the cross-sectional nature of these studies.

To understand the molecular mechanisms and the time window driving childhood asthma pathogenesis, we performed a longitudinal transcriptomic analysis within the in-depth characterized international birth cohort PASTURE. We aimed to identify specific gene expression profiles early in life in children with asthma diagnosis between ages 6 and 10.5 years compared to nonasthmatic peers, using whole blood samples collected at 4 timepoints across the first 10.5 years of life. We focused particularly on the first 4 years, a key developmental window, to identify early molecular changes associated with asthma onset and progression.

Methods

Study population

Our sample included 378 children from the birth cohort PASTURE recruited from rural areas in Austria, Finland, France, Germany, and Switzerland.[18] Briefly, 1133 pregnant women were enrolled; 530 of these women lived on family-run farms. Detailed questionnaires and blood samples were collected at birth, 1, 4.5, 6, and 10.5 years. The study included data from these visits and children with complete questionnaire and sensitization data, asthma diagnosis, and available RNA-sequencing data in at least 1 visit. Children from the Austrian cohort were excluded because no blood was collected during the 10.5-year visit (Figure 1). The study was approved by the ethical committees of the participating institutions, and written informed consent was obtained from parents/guardians of the children.

Asthma definition

Asthma was defined as a reported doctor's diagnosis of asthma or more than once a diagnosis of recurrent obstructive or asthmatic bronchitis between ages 6 and 10.5 years and current symptoms. In children with reported doctor's diagnosis before 6 years, additional asthma symptoms (eg, wheeze and/or cough without a cold) and/or asthma medication between 6 and 10.5 years had to be reported.[19]

Specific IgE measurement and differential blood counts

Specific IgE (sIgE) measurements were assessed from peripheral blood as described before.[20] A child was considered sensitized at a specific visit if sIgE for any allergen was higher than 0.7 IU/ml. Differential blood count was performed at 10.5 years in the French and Swiss arms (n = 175). Cell fractions were deconvoluted from

transcriptomic data using CIBERSORTx for all children (online supplement).

Fractional exhaled nitric oxide measurements

Fractional exhaled nitric oxide (FeNO) measurements were performed at 6 years as previously described[21] (online supplement).

DNA and RNA extraction, sequencing, and preprocessing

DNA extraction and genotype imputation were performed as described before.[19] Standard protocols were used for RNA extraction from whole blood, quality assessment, and sequencing. Preprocessing of raw RNA sequences followed GENCODE recommendations (online supplement).

Statistical analysis

A longitudinal differential gene expression analysis (DGEA) was performed by normalizing count data using the variance-stabilizing transformation from DESeq2 R package and fitting linear mixed models.[22] Models included asthma diagnosis, follow-up visit, and their interaction, adjusted for sex, study center, farming environment, sensitization status, RNA integrity number, and unique mapping rate included as fixed effects. A random intercept for child identity was included to account for repeated measurements. Weighted gene co-expression networks analysis (WGCNA) was performed using the WGCNA R package at each timepoint, and the association of co-expression modules with asthma was assessed using logistic regression. An overrepresentation analysis was conducted on differentially expressed genes and asthma-associated modules using gene sets from REACTOME. To assess genetic effects on the expression of asthma-associated genes, cis-expression quantitative trait loci (eQTL) analyses were performed using MatrixEQTL R package at all visits. The association of the cumulative effect of the eQTLs and asthma was assessed by computing a genetic risk score (Figure E3) (online supplement).

Results

Study sample characteristics

The sample comprised 378 children with 892 available timepoints with RNA-sequencing and sensitization data (Figure E1) of whom 83 children developed asthma between ages 6 and 10.5 years (Table 1; Figure E2). Asthmatic children were more likely to be male, come from nonfarming environments, and have parental history of atopy ($P \leq .05$; Table 1). Sensitization was significantly higher among cases at 4.5, 6, and 10.5 years compared to controls, whereas no significant differences were observed at the 1-year visit. In a subsample of 175 children (French and Swiss arm) with available blood cell counts at 10.5 years, asthmatic children had significantly higher relative blood eosinophils and basophils, but lower neutrophils (Table 1).

Age-dependent differential gene expression associated with asthma development

To assess if age had an influence on gene expression, a principal component analysis was performed, where a clear separation by age was observed (Figure E4A). Gene expression data from visits at age 4.5 and 6 years showed the highest overlap. This pattern was not explained by RNA-sequencing quality parameters (Figure E4B).

To identify age-dependent differences in gene expression associated with asthma, a DGEA was conducted using linear mixed models. In total, expression of 98 of 12481 genes significantly differed in at least 1 visit between children with and without subsequent asthma (false discovery rate [FDR] ≤ 0.05) (Figure 1A; Table E1). A post hoc test was conducted to identify differences at specific timepoints ($P \leq .05$) (Figure 1A; Table E1). Two distinct gene expression patterns associated with school-age asthma were identified. At 1 year, 42 genes were differentially expressed (Figure 1A in orange). Most of these genes ($n = 38/42$) showed higher expression in subsequent asthmatics (Figure 1A, B; Table E1). Noteworthy, between ages 4.5 and 6 years, part of these genes switched to an inverse association with asthma as shown for MEFV (Figure 1A, B). The genes included key components of the innate immune system (eg, TLR8, ICAM1, VCAN) being enriched for lymphoid and nonlymphoid cell interaction, NLPR3-inflammasome, neutrophil degranulation, and extracellular matrix organization (FDR ≤ 0.05) (Figure 1C; Table E2). Given the association of these genes with neutrophilic degranulation, we assessed their correlation with imputed cell fractions at 1 year and measured relative blood cells at 10.5 years. All upregulated genes demonstrated a strong positive correlation with neutrophils and intermediate correlation with monocytes (Figure E5A, Figure E6A). This pattern was consistent with single-cell gene expression data from the Human Protein Atlas (HPA) (Figure E6B).

In parallel to the switch in the neutrophil-associated gene signature, we identified a second signature starting around age 4.5 years. In total, 54 genes were expressed significantly higher in asthmatics at 4.5, 6, and/or 10.5 years. We focused on 40 genes with significant differences at all 3 timepoints (Figure 1A, in purple). Many of these genes are known to be associated with eosinophilic inflammation (eg, SIGLEC8, IL5RA, CLC, ALOX15) (Figure 1A, B; Table E1). The GSDMB gene, previously linked to increased childhood asthma risk, followed a similar pattern. These 40 genes were enriched for G-protein coupled receptors (GPCR) signaling pathways, eicosanoids ligand-binding receptors, arachidonic acid, and leukotriene and eoxin synthesis (Figure 1C; Table E2). All genes in this signature showed a strong positive correlation with relative blood eosinophils at 10.5 years, except for GSDMB (Figure E5B).

Gene co-expression modules associated with subsequent asthma

Weighted gene co-expression networks analyses were performed at each timepoint, and 21, 18, 22, and 19 co-expression modules were identified at 1, 4.5, 6, and 10.5 years, respectively (Figure 2; Table E3). The association of these modules with asthma was assessed using logistic regression models against the scaled

Table 1 Characteristics of the study sample.

	Nonasthmatics	Asthmatics	*P*-value
No. of participants	295[a]	83[b]	
Farmers, No. (%)	150 (50.8)	29 (34.9)	**.013**
Female, No. (%)	149 (50.5)	27 (32.5)	**.004**
Study center, No. (%)			.12
Finland	90 (30.5)	22 (26.5)	
France	86 (29.2)	19 (22.9)	
Germany	53 (18.0)	25 (30.1)	
Switzerland	66 (22.4)	17 (20.5)	
Parental atopy,[c] No. (%)	133 (45.1)	60 (72.3)	**1.2×10^{-5}**
Sensitized, No. (%), 1 y	57 (21.0)	17 (22.1)	.98
Sensitized, No. (%), 4.5 y	125 (45.5)	47 (66.2)	**.002**
Sensitized, No. (%), 6 y	128 (43.4)	48 (67.6)	**5.0×10^{-4}**
Sensitized, No. (%), 10.5 y	139 (47.3)	46 (71.9)	**5.0×10^{-4}**
Blood eosinophils in %, median (IQR), 10.5 y	3.0 (2.1-5.0)	7.3 (4.0-10.8)	**6.6×10^{-7}**
Blood basophils in %, median (IQR), 10.5 yr	0.6 (0.5-0.8)	0.8 (0.6-1.0)	**.006**
Blood neutrophils in %, median (IQR), 10.5 y	49.0 (43.4-54.9)	45.6 (40.6-51.2)	**.031**
Blood lymphocytes in %, median (IQR), 10.5 y	38.6 (33.0-43.3)	37.0 (32.2-44.8)	.58
Blood monocytes in %, median (IQR), 10.5 y	7.1 (6.0-8.4)	7.5 (6.3-8.1)	.55

Abbreviations: eQTL, expression quantitative trait loci; IQR, interquartile range.
The *P*-value is derived from comparisons of the 2 groups using chi-square test for categorical variables and Wilcoxon rank sum test for cell counts. *P*-values lower or equal 0.05 are highlighted in bold.
[a]Subsets of samples were available for the following analyses: transcriptomic analyses: 116, 137, 130, and 289 children from the 1, 4.5, 6, and 10.5 years visit, respectively; cis-eQTL analyses: 109, 128, 122, and 263 children from the 1, 4.5, 6, and 10.5 years visit, respectively; sensitization data: 272, 275, 288, and 294 children from the 1, 4.5, 6, and 10.5 years visit, respectively; fractional exhaled nitric oxide analysis: 121 children from the 6 years visit; cell counts: 148 children from Switzerland and France from the 10.5 years visit.
[b]Subsets of samples were available for the following analyses: transcriptomic analyses: 36, 70, 52, and 62 children from the 1, 4.5, 6, and 10.5 years visit, respectively; cis-eQTL analyses: 34, 69, 52, and 62 children from the 1, 4.5, 6, and 10.5 years visit respectively; sensitization data: 77, 71, 71, and 64 children from the 1, 4.5, 6, and 10.5 years visit, respectively; fractional exhaled nitric oxide analysis: 50 children from the 6 years visit; cell counts: 27 children from Switzerland and France from the 10.5 years visit.
[c]Parental atopy was defined as any parent reporting a doctor diagnosis of asthma, hay fever, or atopic dermatitis.

eigengene values of each module adjusting for sex, study center, farming status, and sensitization (Figure 2). At 1 year, we identified 4 modules significantly associated with asthma. Module 8 remained significant after adjusting for multiple testing (bold, FDR ≤ 0.05). This module was positively associated with asthma (odds ratio [OR] = 2.17; 95% CI, 1.4-3.4]), comprised 405 genes, and the gene phosphatidylinositol-binding clathrin assembly protein (PICALM) was identified as hub gene (Figure 2A; Tables E3 and E4). This module was enriched for neutrophil degranulation, toll-like receptor-associated processes, and caspase-activation signaling pathways (Figure E7). Similar to the DGEA results, this module was strongly correlated with imputed neutrophils at 1 year ($r_s = 0.80$, $P < 2.2 \times 10^{-16}$) (Figure E8A). The first principal component of genes in the PICALM module using data from the 10.5-years visit confirmed this correlation ($r_s = 0.71$, $P = 1.5 \times 10^{-21}$) (Figure E9A), and single-cell data from HPA confirmed a strong expression of these genes in neutrophils (Figure E9B). A PICALM-homologue module was not identified at later timepoints, suggesting that this signature does not persist in later timepoints in our cohort (Figure 3A).

Among the identified modules in later timepoints, modules 11, 14, and 15 from 4.5, 6, and 10.5 years, respectively, were significantly associated with asthma (Figure 2B-D). These modules shared an overlap of 60 genes, including the solute-carrier family 29 member 1 (SLC29A1) gene as hub gene (Table E3, E5-E7). However, module 14 at 6 years was no longer significant after adjustment for multiple testing. These 60 genes showed a high overlap ($n = 34$) with the 40 differentially expressed genes at these timepoints (Figure 1A, purple). After stratifying for sensitization at each timepoint, we identified a significant positive association of the modules with asthma at 6 and 10.5 years only among sensitized children (Figure 3B). However, at 4.5 years, a strong positive association with asthma was observed independent of sensitization. Similar to DGEA results, these 60 genes were enriched for GPCR signaling pathways and eicosanoids binding receptors. At 10.5 years, these genes also showed a strong positive correlation with eosinophils ($r_s = 0.90$, $P < 2.2$ x 10-16) (Figures E7 and E8B). Moreover, a strong association between FeNO and the eosinophilic module at 6 years was identified independent of sensitization using multiple linear regression (beta = 0.05, $P = .003$) (Figure E10). Noteworthy, 3 other modules (3, 15, and 17) were also associated with asthma, but only at nominal level (Figure 2C). As these modules were not present in other timepoints, we did not further explore them. Of note, a similar eosinophilic module was not identified at 1 year, confirming the results from the DGEA (Figure 3A). A potential explanation for the absence of the eosinophilic module, despite eosinophils being present at 1 year in blood, is that these genes may be involved in eosinophil activation and/or maturation.

Additionally, we modeled longitudinal trajectories of imputed neutrophils and our eosinophilic signature using linear mixed models. Besides significant differences at age 1 year, the trajectories of predicted neutrophils did not differ significantly between asthmatic and nonasthmatic children (Figure 4A; Table E8). In contrast, the eosinophilic signature showed an increasing pattern in children who developed asthma, while remaining stable in controls (Figure 4B; Table E8).

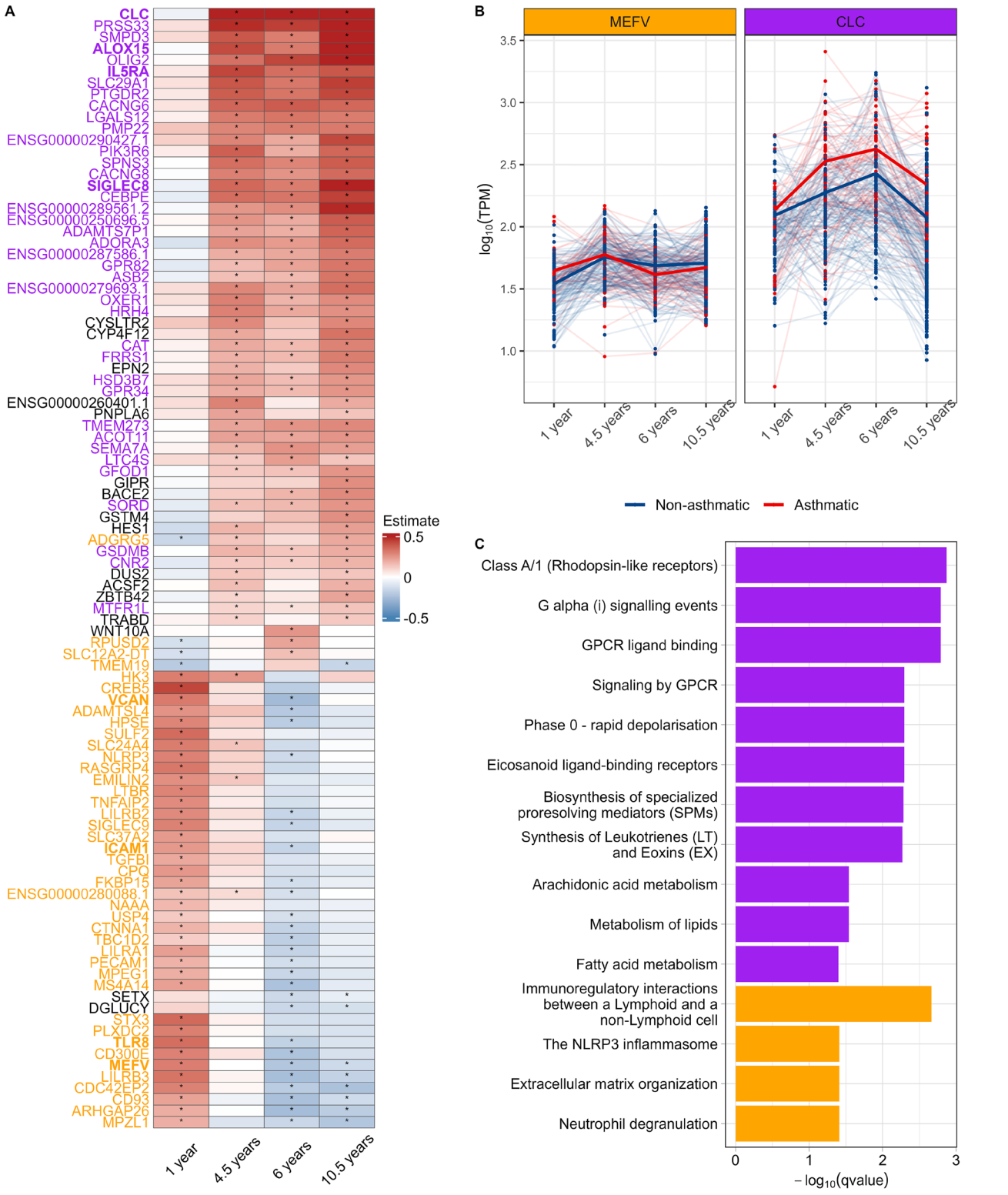


Figure 1 Longitudinally differentially expressed genes and associated gene sets between asthmatic and nonasthmatic children. (A) Heatmap summarizing the results from the longitudinal differential gene expression analysis adjusted for sex, study center, farming status, cross-sectional sensitization status, RNA integrity number (RIN) values, and unique mapping rate. Rows correspond to the 98 significant genes from the *F* test (false discovery rate [FDR] ≤ 0.05). Each cell shows the mean difference of the normalized data between cases and controls at specific timepoints; *indicates significant differences ($P \leq .05$) at that specific timepoint. In purple are 40 genes all significantly differentially expressed at the 3 later timepoints, and in orange are 42 genes differentially expressed at the age of 1 year visit. (B) Longitudinal trend of the genes showing the biggest size effect estimate in the purple and orange group, genes within each category follow similar trends. Gene expression is expressed in transcript per million (TPM). In red, the mean longitudinal trend of asthmatic children and in blue of nonasthmatic children. (C) Barplot summarizing the enriched gene sets from REACTOME among the purple and orange marked genes. *Q*-values correspond to Benjamini–Hochberg adjusted *P*-values, and only significant gene sets are shown (FDR ≤ 0.05).

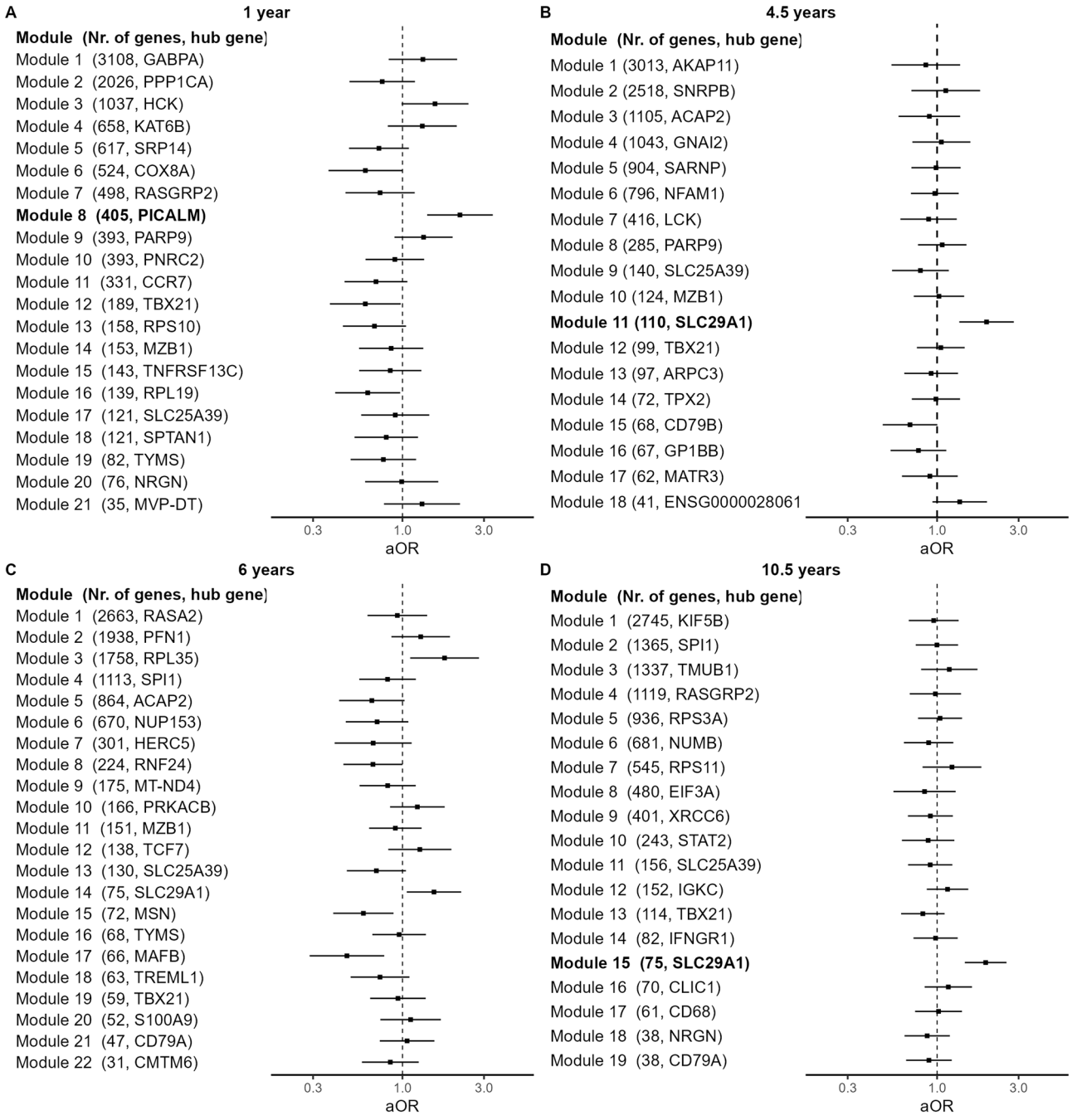
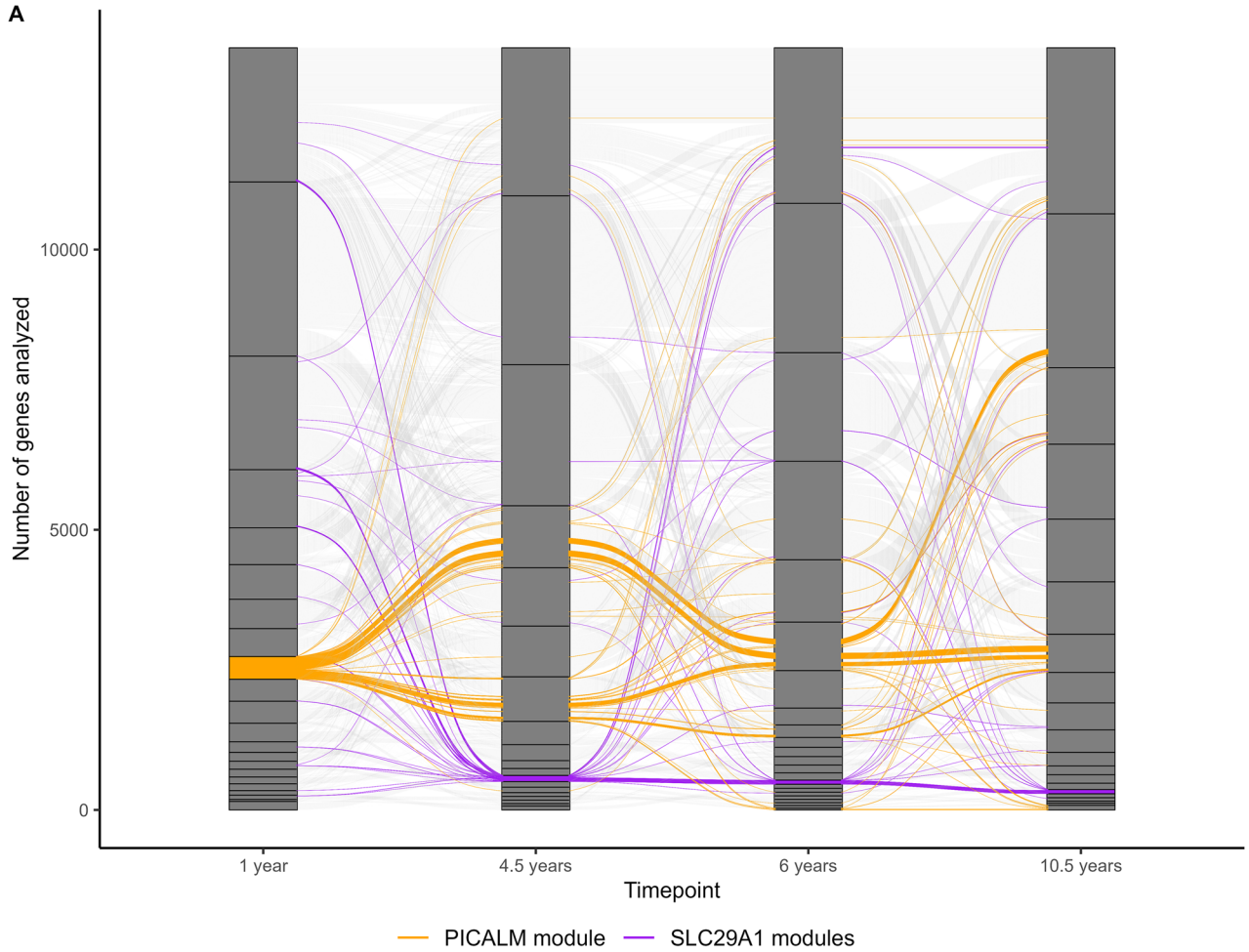


Figure 2 Summary of the co-expression modules and their association to asthma diagnosis at each visit. Module numbers are designated according to the number of genes within the module; total number of genes and hub genes of each module are also indicated. Estimates in forest plots correspond to adjusted odds ratio (aOR) and their 95% confidence intervals between asthma and eigengene values adjusted for sex, study center, farming status, and sensitization status. The odds ratios are expressed as an increase in 1 standard deviation of the eigengene value. Modules in bold are significantly associated to asthma after correcting for multiple testing (false discovery rate [FDR] ≤ 0.05). (A) Summarizes results from 1 year follow-up visit. (B) Summarizes the results from the 4.5 year follow-up visit. (C) Summarizes the results from the 6 year follow-up visit. (D) Summarizes the results from the 10.5 year follow-up visit.

Genetic influence on asthma-associated eosinophilic gene signature

Given the consistent gene expression pattern starting around the fourth year of life associated with asthma, we assessed if genetic variants had an influence on the expression of these genes. We selected 66 genes comprising the results from the DGEA or genes within SLC29A1 modules and conducted a cross-sectional cis-eQTL analysis at each visit (Table E9). We identified 1948, 2757, 3400, and 4831 SNPs at 10.5-, 6- , 4.5-, and 1-year visits, respectively, associated to different genes (Table E9-E13). As we were mainly interested in genetic variants showing similar effects at all timepoints, we focused on cis-eQTL variants that were significantly associated to the same genes at all timepoints (FDR ≤ 0.05). In total, we identified



B

	Odds Ratio [95% CI]
Follow-up: 10.5 years (Cases/Total)	
Full model (61/350)	1.92 [1.45,2.55]
Sensitized (45/182)	2.34 [1.6,3.41]
Not sensitized (16/166)	1.29 [0.76,2.19]
Follow-up: 6 years (Cases/Total)	
Full model (52/182)	1.53 [1.06,2.21]
Sensitized (37/86)	1.82 [1.11,2.98]
Not sensitized (15/96)	1 [0.5,1.98]
Follow-up: 4.5 years (Cases/Total)	
Full model (69/204)	1.95 [1.35,2.81]
Sensitized (46/101)	2.12 [1.27,3.53]
Not sensitized (23/103)	2.11 [1.14,3.9]

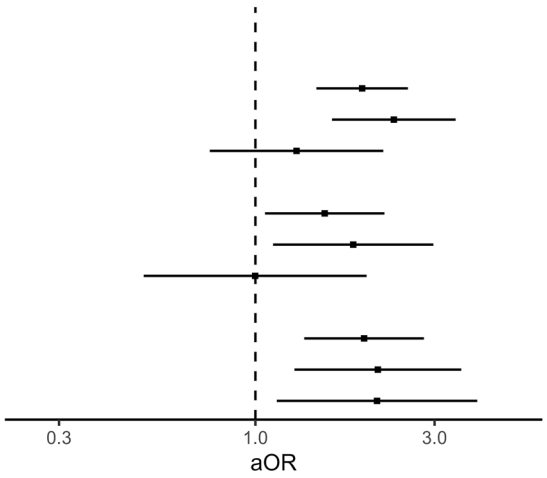


Figure 3 Dynamic changes of co-expression modules across time. (A) Alluvial plot summarizing the identified modules from all cross-sectional co-expression networks. Each of the vertical bars correspond to 1 timepoint, and the rectangles within the bars to the different modules. Rectangle height is proportional to the number of genes within a module. In purple are all the genes that are part of module 11, module 14, and module 15 at 4.5, 6, and 10.5 years of follow-up, respectively (SLC29A1 modules). In orange are the genes that are part of module 8 at follow-up 1 (phosphatidylinositol-binding clathrin assembly protein [PICALM] module). (B) Association stratified by sensitization between solute-carrier family 29 member 1 (SLC29A1) modules and asthma. Estimates in forest plots correspond to adjusted odds ratios (aOR) and their 95% confidence intervals between asthma and eigengene values from SLC29A1 modules. Full models: Adjusted for sex, study center, farming status, and sensitization status. Sensitized: Same model only including sensitized children. Not sensitized: Same model only including not sensitized children.

1403 SNPs associated with 11 genes independent of age (Table 2). After clumping, we reported 86 independent lead cis-eQTL variants showing consistent effects on asthma-associated genes across the first 10.5 years of life (Table 2; Table E14; Figure 5). Most of these genetic variants constitute novel findings in association with eosinophilic inflammation and asthma (Table E15). Noteworthy, genetic variants from the 17q21 locus were consistently associated with GSDMB expression. To explore their cumulative contribution, we constructed a genetic risk score based on these 86 variants, which was significantly associated with asthma diagnosis (adjusted odds ratio [aOR] = 1.47; 95% CI, 1.13-1.93) (Table E16).

Discussion

In the international PASTURE birth cohort, we compared the longitudinal gene expression profiles of children with and without subsequent asthma at school-age using 2 methodological approaches: DGEA and WGCNA. Our results demonstrated a neutrophil-driven gene signature at 1 year in children with subsequent asthma, which shifted to an eosinophilic signature starting at 4.5 years and persisting until 10.5 years. Both signatures were supported by WGCNA confirming a neutrophil-associated module at 1 year and a novel eosinophil-associated module starting at 4.5 years and persisting until 6 and 10.5 years. The eosinophilic module was also associated with T2 inflammation showing a strong correlation with FeNO independent of sensitization at 6 years. Ten genes within the eosinophilic gene signature and GSDMB were strongly influenced by genetics at all timepoints, and their cumulative effect was associated with asthma. This highlights the period between 1 and 4.5 years as critical for disease prevention. Moreover, the subsequent eosinophilic signature points to possible therapeutic targets for early prevention strategies.

Table 2 Summary of identified cis–expression quantitative trait loci (eQTL) variants for each gene at all visits.

Ensembl ID	Gene name	Chromosome	Start position	End position	eQTL variants	eQTL lead variants
ENSG00000174837.15	ADGRE1	19	6887566	6940459	1	1
ENSG00000161905.13	ALOX15	17	4630919	4642294	162	14
ENSG00000142408.7	CACNG8	19	53962937	53990215	2	2
ENSG00000121691.7	CAT	11	34438934	34472060	102	12
ENSG00000183625.16	CCR3	3	46130890	46266706	4	3
ENSG00000073605.19	GSDMB	17	39904595	39919854	495	27
ENSG00000135116.9	HRK	12	116856144	116881441	101	5
ENSG00000117461.15	PIK3R3	1	46040140	46133036	168	4
ENSG00000255587.9	RAB44	6	36697826	36733184	31	4
ENSG00000189068.11	VSTM1	19	54040825	54063953	12	5
ENSG00000250696.5		4	69182100	69216766	262	15
Total					1340	86

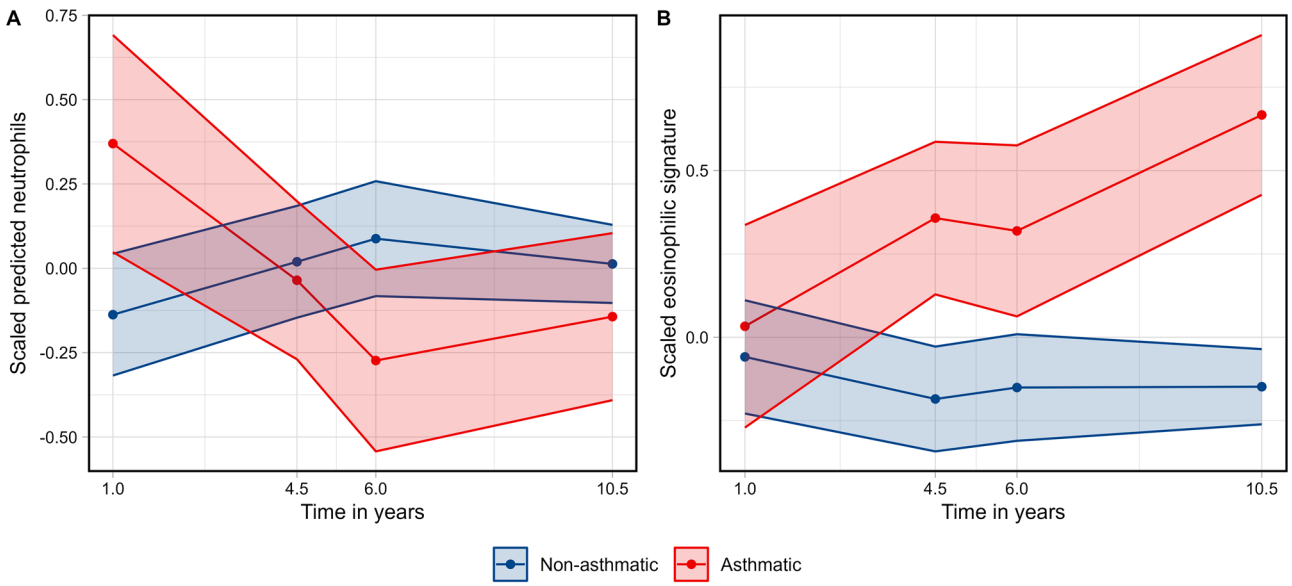


Figure 4 Longitudinal immune trajectories by asthma outcome. Marginal mean trajectories estimated from linear mixed models are shown, with points representing mean values and shaded areas indicating 95% confidence intervals, stratified by asthma outcome. (A) Scaled predicted neutrophils. (B) Scaled eigengene values of eosinophilic modules.

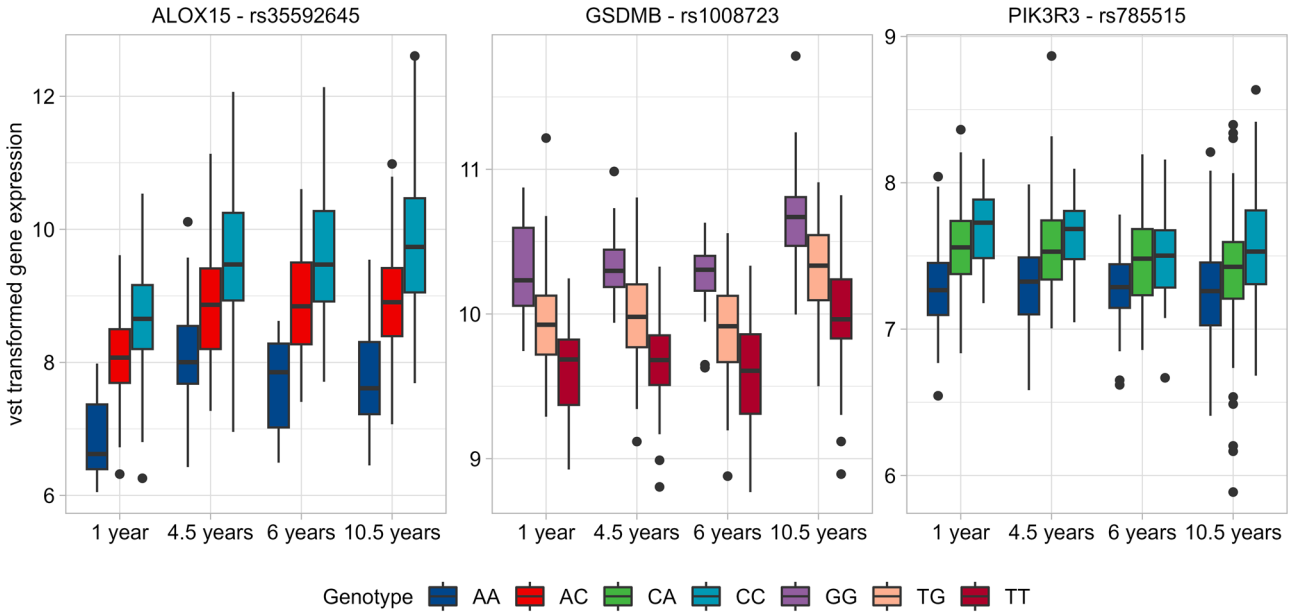


Figure 5 Top 3 most significant expression quantitative trait loci (eQTL) lead variant–gene pairs across the different follow-ups. Box plots summarize the relationship between top 3 eQTL lead variants and gene expression stratified by follow-up and genotype. Gene expression data are transformed using the variance stabilizing transformation (vst) function. Similar patterns are observed in the other eQTL lead variant–gene pairs.

Neutrophil-associated signature: critical window of opportunity for asthma development

At 1 year, we report 42 differentially expressed genes, with 38 being significantly higher expressed in later asthmatics. This early gene signature, being positively associated with relative blood neutrophils and monocytes, indicates strong neutrophilic inflammation before asthma diagnosis is confirmed at school age. It included ICAM1, LILRB2, LILRB3, CREB5, MEFV, and TLR8, which have been associated with neutrophilic inflammation.[23-27] Using WGCNA, we confirmed this finding by identifying a novel co-expression module associated with a higher risk of subsequent asthma and blood neutrophils. PICALM was identified as hub gene, encoding a protein involved in vesicle formation during endocytosis. A strong expression of PICALM in neutrophils points to its potential function during inflammation.[28]

Consistent with our findings, a recent multiomics study from lower airways identified a neutrophil-associated latent factor differentiating preschool children with severe recurrent wheezing from preschool controls and school-aged severe asthmatic children.[14] Our study is also in line with Guiddir et al.[13] observing a "neutrophilic steroid-refractory recurrent wheeze" cluster in preschool wheezers, with elevated blood neutrophils,[13] and extending this further by demonstrating that neutrophilic inflammation not only marks early wheeze but also precedes and potentially contributes to later asthma development. Genes associated with neutrophilic inflammation in our study can point to early viral infections (eg, RSV or hRV), being 1 major risk factor for asthma and wheezing.[29] Supporting this, ICAM1, a gene present in our signature, was upregulated in peripheral blood neutrophils from children with RSV bronchiolitis.[30] Also, a longitudinal study comparing children with severe RSV-bronchiolitis at 1 year with healthy counterparts identified a higher risk of asthma and allergic sensitization at 18 years.[8] Further strengthening the early role of neutrophils for later asthma, a recent study showed that children with high blood neutrophils during bronchiolitis have stronger subsequent lung impairment.[31] Our study design did not allow to determine a causal role of infections in the early neutrophilic signature. Yet, our findings suggest that immune dysregulation, potentially triggered by viral infections (eg, RSV or hRV) may begin as early as the first year of life in children predisposed to asthma, pointing to this period as critical for disease development. Although strongly associated with neutrophils, this early signature may also reflect a broader type-1 inflammatory response, as strictly neutrophil-specific patterns are unlikely in whole blood and require single-cell approaches to resolve.

Eosinophil-associated signature: persistent inflammation during childhood asthma

At age 4.5 years persisting until at least 10.5 years, we identified a novel eosinophilic gene signature strongly associated with school-aged asthma and atopy. In total, 40 genes were persistently higher expressed in asthmatic children at ages 4.5, 6, and 10.5 years. This eosinophil-related signature included key genes for eosinophil function (eg, CLC, SIGLEC8, CEBPE, CNR2)[32-34] and key targets of T2 inflammation and eosinophilic therapies (eg, IL5RA, PTGDR2, LTC4S).[35-38] Noteworthy, GSDMB gene, previously associated with childhood asthma, was among the 40 genes, but its expression was independent of eosinophil counts.

We also confirmed these findings with WGCNA by identifying at each of these timepoints co-expression modules associated to eosinophils, atopy, and asthma. These modules shared 60 genes and SLC29A1 as hub gene, potentially indicating a central role of this gene in our signature. Being implicated in IgE-dependent degranulation of mast cells,[39] it is possible that SLC29A1 has a similar function in eosinophils and therefore a potential therapeutic target. This signature also reflects T2-high inflammation, as evidenced by a strong association with FeNO levels at age 6 years, an established biomarker of asthma severity and T2 inflammation, highlighting its clinical relevance.[40] In adults and older children, several genes from our eosinophilic signature were similarly associated with allergic asthma or sensitization.[16,17,41,42] Also, genes present in this signature were associated with response to mepolizumab and anti–IL-13.[43,44] Our data extend these findings to preschool-aged children, suggesting that this eosinophilic gene signature emerges early in life, has high temporal stability, and may help identify candidates for targeted biologics (eg, anti–IL-13) in this younger age group.

In contrast to the early neutrophilic pattern, the eosinophilic signature was not detectable at age 1 year by differential expression or co-expression analysis. This clearly indicates that this gene signature is not yet established at this early stage, being supported by a recent study showing that higher blood eosinophils at age 18 months were not associated with an increased asthma risk.[45] Similarly, histological evidence indicates that eosinophilic airway inflammation and reticular basement membrane thickening are first visible at 3 years in children with wheeze.[11,46] Taken together, our gene signature reflects eosinophil activation and/or inflammatory programming associated with immune maturation, rather than merely serving as proxy for eosinophil abundance. In line with this hypothesis, the cannabinoid receptor 2 (CNR2) gene, found in our gene signature, was upregulated in eosinophils from asthmatic patients independent of blood eosinophil counts and plays a role in eosinophil inflammation.[47,48] The emergence of this signature by age 4 years, and its persistence into later childhood, further supports the idea that targeted interventions may need to occur before this inflammatory profile is established.

Finally, we showed that our eosinophilic gene signature is genetically predetermined by identifying 10 genes whose expression is consistently associated with genetic variants over all assessed timepoints. Variants in the gene ALOX15 have been previously associated with eosinophil counts; however, other variants represent novel findings in relation to eosinophilic inflammation (Table E15). The gene expression of GSDMB was also strongly determined by genetic variants from the 17q21 locus as previously reported in whole blood. A previous report in our PASTURE cohort has shown how these genetic variants modulate the effect of environmental exposures on wheeze and asthma.[20] The joint effect of our 86 lead variants was associated with asthma, warranting further investigation of these loci to assess their significance for additional clinically relevant endpoints.

Strengths and limitations

One limitation was the unbalanced study sample, as controls had more available timepoints than cases. This could lead to biased estimates in the linear mixed models, potentially affecting group comparisons. However, the cross-sectional network analyses yielded similar results, and being unaffected by biases from repeated measurements, this consistency strengthens the validity of our findings. Of note, the 1-year follow-up visit did not include measured differential blood count data to assess the association of gene signatures with neutrophils. Thus, we used deconvoluted cell fractions, which have been shown to accurately predict cell fractions.[49] Moreover, absent signals of adaptive immunity-associated genes may be due to the nature of unstimulated blood cells including a high frequency of other cells like neutrophils, highlighting the importance of conducting similar studies using PBCMs. For analyses of this early neutrophil signature with asthma endotypes on an individual level, a large number of future asthmatic children with available transcriptomic data at 1 year and longitudinal follow-up over several years until phenotype manifestation are required. In addition, gene expression trajectories showed individual-level heterogeneity that was not further explored, as methods to identify such trajectories (eg, longitudinal clustering) require much larger and more balanced datasets. Both these in-depth multicohort analyses require large, international cooperative efforts.

One major strength of our study is its longitudinal design, leveraging data from an in-depth characterized birth cohort. This is important, as major changes in the immune system relevant for the development of asthma take place during this early sensitive time window. Our well-characterized longitudinal birth cohort offers a unique opportunity to better understand the molecular mechanisms driving asthma onset and disease manifestation. We also chose a longitudinal analytical approach that was particularly strong in a previous benchmark analysis.[22]

In conclusion, we identified distinct, age-specific gene expression signatures associated with onset and progression of childhood asthma. Our findings suggest a shift from early neutrophilic to later eosinophilic immune activity, underscoring the dynamic nature of asthma-related inflammation during early life. Likewise, our study emphasizes that major changes take place in the first years and may persist at least into early adolescence. It will be crucial to identify children at risk before this inflammatory signature settles. Future mechanistic studies require focusing on this vulnerable time window with higher precision.

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Author contributions

F.F. performed sample preparation, study design, data analysis and interpretation and wrote the manuscript. A.B. performed genotype data imputation and data analysis. C.Be. participated in study design, sample preparation, and study coordination. K.U. participated in study coordination and sample preparation. M.E. provided input to study design and data analysis. C.S. performed sIgE measurements. U.F. performed lung function measurements. E.v.M., J.R., J.P., and R.L. obtained funds, set up the PASTURE birth cohort, and together with A.D.C., M.R., E.S.H., C.Ba., A.M.K., C.R., R.F., and B.S., was responsible for data collection and management of the study. B.S. was responsible for financing, study design, data analysis, interpretation, and the final manuscript version. All authors contributed to and approved the final manuscript.

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Supplementary material

Supplementary material is available at *American Journal of Respiratory and Critical Care Medicine* online.

Conflicts of interest

Please see the ICMJE disclosure forms, which have been provided as supplementary material.

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Data availability

The PASTURE study is a longitudinal birth cohort with ongoing analyses. Data currently cannot be anonymized due to local requirements. According to European law, health-related data can only be shared on an aggregate level to avoid identification of individuals. Data will be made available from the corresponding author upon request with study proposal and completion of a satisfactory data transfer agreement. This article has an online data supplement, which is accessible from this issue's table of content online at www.atsjournals.org.

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ChatGPT was used only for final editing purposes.

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Original Research | Asthma and Allergy