



Published in final edited form as:

*Pediatr Pulmonol.* 2014 February ; 49(2): 132–139. doi:10.1002/ppul.22784.

## Atopy, Cytokine Production, and Airway Reactivity as Predictors of Pre-School Asthma and Airway Responsiveness

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### Summary

**Background**—Childhood asthma is often characterized by recurrent wheezing, airway hyper-reactivity, atopy, and altered immune characteristics; however, our understanding of the development of these relationships from early in life remains unclear. The aim of our study was to evaluate whether atopy, cytokine production by peripheral blood mononuclear cells (PBMCs), and airway responsiveness, assessed in infants and toddlers, are associated with asthma and airway responsiveness at 4-years of age.

**Methods**—Infants with eczema (N = 116), enrolled prior to wheezing, were assessed at entry (mean age of 10.7 months), at 1-year follow-up (N = 112), and at 4-years of age (N = 94). Total serum IgE, specific IgE to allergens, and cytokines produced by stimulated PBMCs, were assessed at entry and 1-year follow-up. Spirometry was obtained at all 3-visits, while airway reactivity to methacholine was assessed at entry and 1-year follow-up, and bronchodilator (BD) responsiveness, as well as current asthma was assessed at 4-years of age.

**Results**—We found that pre-school children with asthma had lower spirometry and a greater BD-response. Serum IgE, particularly to egg and/or milk, and altered cytokine production by PBMCs at entry to the study were associated with asthma, lower spirometry, and greater airway responsiveness at 4-years of age. In addition, we found that airway responsiveness, as well as spirometry, tracked from infancy to 4-years of age.

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Conflict of interest: None.

Additional supporting information may be found in the online version of this article.

**Conclusions**—While spirometry and airway responsiveness track longitudinally from early in life, atopy and cytokine production by PBMCs are associated not only with an increased risk of pre-school asthma, but also lower spirometry and increased airway responsiveness.

### Keywords

airway reactivity; bronchodilator response; infants; pre-school children; spirometry

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## INTRODUCTION

Childhood asthma is often characterized by recurrent wheezing, airway hyper-reactivity, atopy, and alterations in immune characteristics.<sup>1-4</sup> However, our understanding of the development of these relationships from early in life remains unclear, as there have been few cohort studies assessing all of these components. While atopy is a risk factor for childhood asthma,<sup>5,6</sup> a study that evaluated airway reactivity during infancy in a non-selected cohort did not find an association with atopic status or a consistent relationship between airway reactivity in infants and wheezing or childhood asthma.<sup>7</sup> Cohort studies have reported that cytokine production by stimulated peripheral blood mononuclear cells (PBMCs) obtained during infancy is associated with the risk of wheezing and childhood asthma<sup>8-13</sup>; however, whether these cytokines are also risk factors for decreased airway function or heightened airway responsiveness during childhood has not been evaluated.

We have previously described that among infants enrolled with eczema prior to wheezing, titers of IgE to egg and/or cow's milk was associated with heightened airway reactivity upon entry to the study, as well as at 1-year follow-up.<sup>13,14</sup> Cytokine production by PBMCs was also associated with the subsequent risk of wheezing.<sup>13</sup> While recurrent wheezing early in life is a risk factor for childhood asthma, not all infants with recurrent wheeze develop childhood asthma.<sup>15</sup> Therefore, biomarkers to predict childhood asthma might differ when applied to infants prior to wheezing compared to infants who have developed wheezing. Our cohort at 4-years of age performed spirometry to assess airway function and bronchodilator (BD) responsiveness. We hypothesized that atopy, particularly to egg and/or milk, as well as cytokine production by PBMCs, assessed in infants and toddlers, would be associated with lower airway function, greater BD-response, and current asthma at 4-years of age. We also hypothesized that airway responsiveness would track longitudinally from infancy to 4-years of age and be a risk factor for asthma at 4-years of age. Our analysis included wheezing during the first year of the study as a covariate to determine whether the biomarkers we assessed at entry to the study, prior to wheezing, differ from those at 1-year follow-up, after onset of wheezing.

## METHODS

### Subjects

Full-term infants with eczema (N = 116) were recruited from general pediatric clinics and advertisements, and excluded for prior wheezing or treatment with asthma medications.<sup>13,14</sup> Subjects were evaluated at entry to the study (median age of 10.7 months, range 2.6–19.1

months), 1-year follow-up (N = 112), and 4-years of age (N = 94) (Fig. 1). The study was approved by the Institutional Review Board and written parental consent was obtained.

### Immunologic Characteristics

At entry and 1-year follow-up, total serum IgE (IU/ml) and 10 allergen-specific IgE (egg white, cow's milk, wheat, cat, house dust mite, timothy grass, Bermuda grass, ragweed, *Alternaria* species, and cedar; Immune Tech, Foster City, CA) were assessed. Cytokine production (IL-4, IL-5, IL-13, IL-17, IL-9, IL-10, and IFN $\gamma$ ) by stimulating isolated and cultured peripheral mononuclear cells (PBMC) were measured with PMA/ionomycin.<sup>9,10,13</sup> The ratios of individual cytokines to IFN $\gamma$  were employed to assess the balance between the Th2 and Th1.<sup>13,16,17</sup>

### Eczema

Eczema was doctor-diagnosed and severity quantified using SCORAD (Scoring Atopic Dermatitis) scale at each visit.<sup>18</sup>

### Airway Function

**Spirometry**—At entry to the study and 1-year follow-up, forced expiratory flows were obtained in sedated infants using the raised volume technique.<sup>19</sup> Forced vital capacity (FVC) and forced expiratory flow at 25–75% of expired volume (FEF<sub>25–75</sub>) were expressed as z-scores.<sup>19,20</sup> At 4-years of age, spirometry was performed; FVC and FEF<sub>25–75</sub> were expressed as z-scores.<sup>21,22</sup> Forced expiratory flows at 50% and 75% of expired volume (FEF<sub>50</sub> and FEF<sub>75</sub>) were also measured at entry, 1-year follow-up and 4-years of age, and are expressed as absolute values adjusted by gender and height.

**Airway Responsiveness**—At entry and 1-year follow-up, airway reactivity to inhaled methacholine (MCh) was expressed as PC<sub>30</sub>, the concentration to decrease by 30% the forced expiratory flows at 75% expired volume (FEF<sub>75</sub>).<sup>23</sup> At 4-years of age, BD-response to inhaled Albuterol (0.5 mg) was assessed as the change in z-FEF<sub>25–75</sub>.

### Respiratory History and Asthma Diagnosis

Histories for maternal cigarette smoking during pregnancy, cigarette smoking by household members or caregivers, as well as family (parent/sibling) asthma and/or allergy were obtained at study visits. Episodes of wheezing and medications usage were updated by monthly telephone contact. At 4-years of age, current asthma was defined as: (a) physician diagnosis of asthma at any time and a history of wheezing in previous 12-months, or (b) use of asthma medication (BDs or inhaled corticosteroids) in previous 12-months.<sup>21</sup>

### Statistical Analysis

Demographics were summarized and compared between asthma and non-asthma subjects using two-sample *t*-test or Pearson's chi-square test. Logistic models were used to evaluate the relationship between infant atopy, cytokines, SCORAD and spirometry, with asthma at 4-years of age, adjusted for gender race, and history of cigarette smoking exposure. Linear mixed-models were used to evaluate the effect of atopy, cytokines, SCORAD and airway

function at entry to the study and 1-year follow-up on spirometry and BD-response at 4-years of age, adjusted for gender and race. For the 1-year follow-up we also included the number of wheezing episodes. An interaction with pre and post-BD spirometry was included as an effect modification; a significant interaction indicated the predictor had a significant effect on BD-response. Cytokine values were transformed as  $\log(1 + \text{cytokine})$ , due to skewness and to avoid extreme value in log scale. The transformed value was divided by its standard deviation so that the coefficient corresponded to the change of cytokine response with respect to one standard deviation increase of  $\log(1 + \text{cytokine})$ . All the analyses were performed using SAS 9.2.

## RESULTS

### Subjects

Eighteen subjects were lost to follow-up; they were more likely to be female (69% vs. 50%,  $P = 0.067$ ), non-Caucasian (76% vs. 50%,  $P = 0.021$ ), and less likely to have history of family asthma (54% vs. 95%,  $P = 0.003$ ) or maternal smoking (8% vs. 25%,  $P = 0.018$ ). At 4-year-old follow-up, 74 subjects (79%) performed acceptable spirometry and 20 subjects were not able to perform technically acceptable baseline spirometry. There were no differences between those who could and could not perform spirometry, as to race ( $P = 0.313$ ), gender ( $P = 0.472$ ), maternal smoking ( $P = 0.639$ ), and birth weight ( $P = 0.080$ ), although those without spirometry data were less likely to have asthma ( $P = 0.016$ ). For airway function analysis, we tested FEF<sub>25-75</sub>, FEF<sub>50</sub>, and FEF<sub>75</sub>. Results for FEF<sub>25-75</sub> are presented in the text, as it was consistently more informative than the results for FEF<sub>50</sub> and FEF<sub>75</sub>, which are available as Supplementary Tables.

### Asthma and Airway Function at 4-Years of Age

Fifty-five subjects at 4-year of age (59%) had current asthma with no significant differences for gender, race, maternal smoking during pregnancy, and family asthma/allergy when compared to those without current asthma. Among asthmatic subjects at 4-years of age, 33.3% had a positive history of current cigarette smoking in the household, while 42.3% of non-asthmatic subjects at 4-years of age had a positive history of cigarette smoking in the household. Among subjects with spirometry data, those with asthma had lower z-FEF<sub>25-75</sub> (Estimate =  $-0.473$ ,  $P = 0.015$ ), but no difference in z-FVC ( $P = 0.597$ ) compared to those without asthma. Those with asthma also demonstrated a greater BD-response in z-FEF<sub>25-75</sub> (Estimate =  $0.320$ ,  $P = 0.018$ ), but no significant difference in post-BD z-FEF<sub>25-75</sub> following an inhaled BD (Estimate:  $-0.153$ ;  $P = 0.432$ ).

### Predictors of Asthma at 4-Years of Age

At entry to the study, higher total IgE and higher specific IgE to egg and/or milk were associated with an increased risk for asthma (Table 1). Increased ratios of IL4/IFN $\gamma$  and IL10/IFN $\gamma$  by stimulated PBMCs were also associated with an increased risk of asthma.

At 1-year follow-up, only specific IgE to egg and/or milk were associated with an increased risk for asthma, after adjusting for wheezing, which was a significant covariate. The

association of total IgE to risk of asthma approached statistical significance, while none of the cytokines at 1-year follow-up were significantly associated with asthma (Table 1).

### Predictors of Airway Function at 4-Years of Age

Upon entry to the study, higher total IgE, specific IgE for egg and/or milk, cat and/or mite, or any aero-allergens were associated with lower z-FEF<sub>25-75</sub> at 4-years of age (Table 2). Cytokine production was not associated with z-FEF<sub>25-75</sub>, while higher SCORAD was associated with lower z-FEF<sub>25-75</sub>. Increased total IgE and specific IgE to egg and/or milk were also associated with a greater BD-response, while there was a tendency for an increased IL-9/IFN $\gamma$  ratio to be associated with greater BD-response ( $P = 0.062$ ).

At 1-year follow-up, total IgE, as well as all allergen specific IgE groups, were associated with lower z-FEF<sub>25-75</sub> at 4-years of age, after adjusting for the significant association between increased wheezing and z-FEF<sub>25-75</sub> (Table 2). Increased IL-9/IFN $\gamma$  and IL-10/IFN $\gamma$  ratios were also associated with lower z-FEF<sub>25-75</sub> (Table 2). All of the immunologic characteristics assessed at 1-year follow-up, as well as SCORAD, were also associated with an increased BD-response, after adjusting for wheezing.

### Airway Function Early in Life and at 4-Years of Age

Lower z-FEF<sub>25-75</sub> at entry to the study, prior to wheezing, was associated with lower z-FEF<sub>25-75</sub> at 4-years of age, and a greater BD-response at 4-years of age (Table 3); it also tended to be associated with an increased risk of asthma at 4-years of age ( $P = 0.052$ ). Infants with lower PC<sub>30</sub> (greater airway responsiveness) at entry to the study had lower z-FEF<sub>25-75</sub>, as well as a greater BD-response, but it was not associated with an increased risk of asthma (Table 3).

## DISCUSSION

In our selected cohort enrolled as infants with eczema, we found that, as pre-school children, those with current asthma had lower spirometry and greater BD-response. Atopy in infants and toddlers was not only associated with asthma at 4-years of age, but also with lower spirometry and greater BD-response. Similarly, altered ratios of cytokines produced by stimulated PBMCs were associated with an increased risk of asthma, lower spirometry and greater BD-response. Lastly, heightened airway responsiveness in infants and toddlers was associated with heightened airway responsiveness at 4-years of age, but not asthma. Our findings suggest that, after adjusting for wheezing early in life, which is a well-known risk factor for asthma, atopy and increased cytokine production by PBMCs are not only associated with risk for pre-school asthma, but also with decreased airway function and heightened airway responsiveness.

We found that spirometry during infancy correlated with similar measurements at 4-years of age, which is consistent with the Tucson longitudinal study demonstrating that spirometry tracks from infancy into adulthood.<sup>24</sup> However, our subjects with current asthma at 4-years of age tended to have lower spirometry as infants ( $P = 0.052$ ), prior to episodes of wheezing, which differs from the Tucson cohort, where those with asthma as 6-year olds did not have lower spirometry during infancy.<sup>25</sup> The apparent loss of airway function in the Tucson

subjects may reflect the insensitivity of the lung function testing used during infancy and/or the absence of post-BD spirometry as 6-year olds. A major strength of our study was that spirometry was not only assessed longitudinally in infants and toddlers, but the raised volume rapid thoracic compression (RVRTC) technique was used during infancy, which is more sensitive than partial flow-volume maneuvers used 30-years ago.<sup>25-27</sup> Alternatively, our results may differ from the Tucson cohort since we evaluated a selected population as older infants compared to the Tucson cohort of non-selected neonates. Our findings are consistent with the recent report evaluating a selected high risk cohort at 7-years of age; those with asthma had lower forced expiratory flows during infancy when assessed by the RVRCT technique.<sup>28</sup>

Our pre-school children with asthma had lower  $z$ -FEF<sub>25-75</sub>, but no difference in FVC, which is consistent with airway obstruction. However, the decreased flows were secondary to increased airway tone, as post-BD flows were no longer significantly different between those with and without asthma. We also found that greater airway responsiveness in infants and toddlers assessed by methacholine challenge was associated with greater BD-response as 4-year old, even after adjusting for wheezing. These findings suggest that airway responsiveness tracks longitudinally from infancy to pre-school years; however, airway responsiveness early in life was not associated with the risk of asthma at 4-years of age. We previously found that at 1-year follow-up there was a significant interaction between airway reactivity upon entry to the study, wheezing, and airway reactivity at 1-year follow-up. When our analysis was performed without wheezing as covariate, heightened airway reactivity at 1-year follow-up was associated with an increased risk of asthma at 4-year of age ( $P < 0.017$ ). These findings are consistent with the Perth Cohort study, which found a relationship between diagnosis or symptoms of asthma in childhood and airway responsiveness assessed late in infancy, after the onset of wheezing, but not when assessed early in infancy.<sup>7,29,30</sup> As our cohort becomes older and more removed from the transient-wheeze phenotype, we may find that airway reactivity in infants and toddlers becomes more strongly associated with childhood asthma, as recently reported in a cohort of 7-year olds enrolled as high-risk infants of asthmatic mothers.<sup>28</sup>

We found that atopic status, particularly increased specific IgE to egg and/or milk, was associated with asthma at 4-years of age, even after adjusting for wheezing, which was also associated with asthma. In addition, egg and/or milk sensitization upon entry to the study, as well as at 1-year follow-up, were associated with lower spirometry and greater BD-response, independent of wheezing early in life. These findings are consistent with our previous report that sensitization to egg and/or milk at entry to the study, was associated with heightened airway responsiveness, which persisted at 1-year follow-up.<sup>13</sup> Other antigen specific IgE indicators of atopy were less consistently associated with lower spirometry, greater BD-response, and asthma, which may reflect that sensitization to food most often precedes sensitization to other antigens.<sup>31,32</sup> Although atopy as infants and toddlers was associated with asthma at 4-years of age, we previously reported in this cohort that atopy upon entry to the study, prior to any wheezing, was not associated with an increased risk for wheezing during the 1-year follow-up. These findings are consistent with viral respiratory illness, rather than to atopy being a more important determinant of wheezing as infants.

Our current findings indicate that altered cytokine production by PBMCs upon entry to the study was associated with asthma at 4-years of age, which is consistent with our previous report that cytokine production at entry to the study was associated with wheezing at 1-year follow-up.<sup>13</sup> However, in the current study, cytokine production at 1-year follow-up was not associated with asthma at 4-years of age, after adjusting for wheezing. Our finding is consistent with the Tucson Cohort, where Th2 cytokine production at 3-months of age was associated with asthma through 5-years of age; however, this association was not present for PBMCs obtained at 1-year follow-up.<sup>11</sup> In older children and adults increased cytokine production by stimulated PBMCs has been associated with lower spirometry, greater airway reactivity, and greater exhaled nitric oxide, when measured at the same time point as the PBMCs were obtained.<sup>33–35</sup> Our findings are novel as we found that altered cytokine production at 1-year follow-up was associated with lower spirometry and greater BD-response at 4-years of age, which suggests that cytokine production in infants and toddlers may be a biomarker not only for asthma, but also the phenotypic characteristic of airway hyper-reactivity.

There are several limitations to our study. We maintained most of our cohort to 4-years of age; however, female and non-Caucasian subjects were more often lost to follow-up; therefore, our analysis was adjusted for these factors. Twenty subjects did not perform technically acceptable spirometry, which is consistent with the difficulties of assessing pre-school subjects.<sup>21,36</sup> Our subjects are also an enriched population with increased atopy and family asthma; therefore, our results cannot be extrapolated to cohorts of non-selected subjects. Lastly, multiple cytokines were assessed, which are not all independent. However, correlations among the different cytokines ranged from a high of  $r = 0.94$  between IL-4 and IL-5 to a low of  $r = 0.10$  between IL-4 and IL-9, which probably reflects co-expression of Th2 cytokines and discordant expression of cytokines from distinct Th subsets.

In summary, the relationships of atopy, immune characteristics, and airway function from infancy into childhood, and the associations of these factors with childhood asthma are complex. Our pre-school children with asthma had lower spirometry and a greater BD-response. More importantly, we found that atopy and increased cytokine production by stimulated PBMCs from infants and toddlers were associated with an increased risk for asthma, lower spirometry, and greater BD-response at 4-years of age. In addition, airway responsiveness, as well as spirometry, tracked from infancy to 4-years of age.

## Acknowledgments

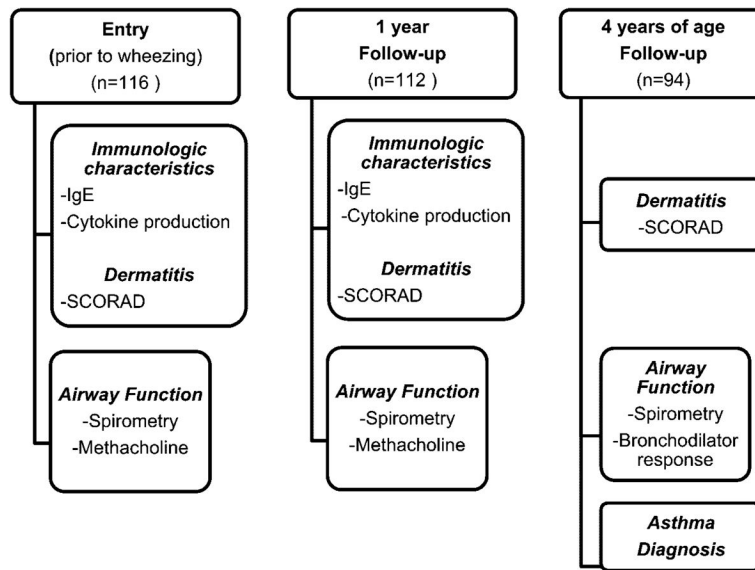
Funding source: NIH, Numbers: HL080071, AI070448, AI057459.

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**Fig. 1.**

Flow-chart representing the assessment and number of subjects in the cohort at three time points of the study.

Serum IgE, Cytokine Production, and SCORAD at Entry to Study and at 1-Year Follow-Up and Predictors of Current Asthma at 4-Years of Age

TABLE 1

Parameters	At entry			At 1-year follow-up		
	OR	95% CI	P-value	OR	95% CI	P-value
Total IgE	1.33	1.02–1.74	0.039	1.29	0.99–1.69	0.058
IgE egg/cow's milk	2.66	1.10–6.45	0.030	2.89	1.11–7.51	0.029
IgE cat/mite	1.86	0.86–4.00	0.048	1.19	0.71–1.99	0.511
IgE aero-allergen	3.62	0.72–18.29	0.120	1.46	0.69–3.10	0.327
IL-4/IFN- $\gamma^a$	3.42	1.15–10.17	0.027	0.68	0.42–1.11	0.123
IL-5/IFN- $\gamma^a$	1.85	0.902–3.79	0.093	0.89	0.54–1.48	0.660
IL-9/IFN- $\gamma^a$	1.51	0.793–2.87	0.211	2.46	0.96–6.30	0.619
IL-10/IFN- $\gamma^a$	6.46	1.28–32.51	0.024	0.75	0.45–1.26	0.278
IL-13/IFN- $\gamma^a$	1.23	0.76–1.99	0.391	0.84	0.51–1.40	0.512
IL-17/IFN- $\gamma^a$	1.29	0.79–2.09	0.307	0.76	0.44–1.31	0.320
SCORAD	1.01	0.96–1.07	0.666	1.01	0.94–1.08	0.835

OR = odds ratio; CI = confidence interval; BD (bronchodilator) response: (z-*FEF*<sub>25-75</sub> post-BD minus z-*FEF*<sub>25-75</sub> pre-BD); SCORAD = Scoring Atopic Dermatitis. Allergen-specific IgE is defined as serum IgE to timothy grass, bermuda grass, ragweed, alternaria species, and/or cedar.

<sup>a</sup>Standardized as log(cytokine + 1)/STD (cytokine + 1). The model at entry was adjusted for race and gender. The model at 1-year follow-up was adjusted for race, gender, and number of wheezing episodes during first year of study.

**TABLE 2**  
 Immunologic Characteristics and SCORAD at Entry to Study and Airway Function at 4-Years of Age

Airway function at 4-years of age	Parameters	At entry		At 1 year follow-up		
		Estimate	P-value	Estimate	P-value	
z-FFEF25-75	Total IgE	-0.213	0.001	-0.201	0.001	
	IgE egg/cow's milk	-0.232	0.010	-0.235	0.017	
	IgE cat/mite	-0.288	0.008	-0.204	0.024	
	IgE aero-allergen	-0.018	0.017	-0.284	0.019	
	IL-4/IFN- $\gamma^a$	-0.147	0.138	-0.158	0.084	
	IL-5/IFN- $\gamma^a$	-0.143	0.159	-0.161	0.090	
	IL-9/IFN- $\gamma^a$	-0.136	0.259	-0.231	0.020	
	IL-10/IFN- $\gamma^a$	-0.135	0.158	-0.225	0.016	
	IL-13/IFN- $\gamma^a$	-0.070	0.496	-0.163	0.093	
	IL-17/IFN- $\gamma^a$	-0.124	0.216	-0.181	0.087	
	SCORAD	-0.029	0.017	-0.004	0.771	
	BD-response	Total IgE	0.133	<0.001	0.139	0.001
		IgE egg/cow's milk	0.143	0.017	0.266	0.001
IgE cat/mite		0.116	0.099	0.162	0.004	
IgE aero-allergen		0.006	0.244	0.251	0.001	
IL-4/IFN- $\gamma$		-0.016	0.780	0.127	0.034	
IL-5/IFN- $\gamma$		-0.030	0.623	0.151	0.014	
IL-9/IFN- $\gamma$		0.149	0.062	0.216	0.0004	
IL-10/IFN- $\gamma$		-0.006	0.918	0.166	0.0053	
IL-13/IFN- $\gamma$		0.035	0.587	0.177	0.004	
IL-17/IFN- $\gamma$		0.042	0.506	0.123	0.050	
SCORAD		0.003	0.759	-0.026	0.011	

BD (bronchodilator) response: (z-FFEF25-75 post-BD minus z-FFEF25-75 pre BD); SCORAD, Scoring Atopic Dermatitis. Allergen-specific IgE is defined as serum IgE to timothy grass, bermuda grass, ragweed, alternaria species, and/or cedar.

The model at entry was adjusted for race and gender. The model at 1-year follow-up was adjusted for race, gender, and number of wheezing episodes during first year; exposure to smoking and age at enrollment were also adjusted for.

$\alpha$  Standardized as  $\log(\text{cytokine} + 1)/\text{STD}(\text{cytokine} + 1)$ .

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Relationships Between Airway Function at Entry to Study and Airway Function or Current Asthma at 4-Years of Age

TABLE 3

Parameter at 4-years of age	Parameter at entry to study and 1-year FU	At entry to study		At 1-year follow-up	
		Estimate	P-value	Estimate	P-value
z-FEF <sub>25-75</sub>	z-FEF <sub>25-75</sub>	0.281	0.003	0.110	0.257
	PC <sub>30</sub> Ln	0.224	0.031	0.178	0.093
BD-response	z-FEF <sub>25-75</sub>	-0.224	<0.001	-0.004	0.954
	PC <sub>30</sub> Lnb	-0.182	0.004	-0.262	<0.001
<b>Current asthma</b>					
z-FEF <sub>25-75</sub>	OR	0.64	0.40-1.00	0.052	1.00
	95% CI	0.40-1.00	0.052	1.00	0.59-1.70
PC <sub>30</sub> Lna	OR	0.88	0.57-1.37	0.571	0.68
	95% CI	0.57-1.37	0.571	0.68	0.39-1.19
	P-value				0.177

BD (bronchodilator) response: (z-FEF<sub>25-75</sub> post-BD minus z-FEF<sub>25-75</sub> pre BD); PC<sub>30</sub> Ln = natural logarithm of PC<sub>30</sub>; OR = odds ratio; CI = confidence interval. The model at entry was adjusted for race and gender. The model at 1-year FU was adjusted for race, gender, and number of wheezing episodes in first year.