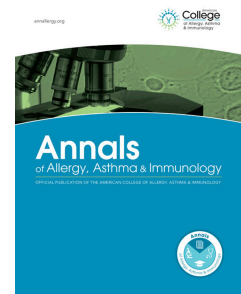


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Type 2 Inflammation in the Sputum of Adolescents with Asthma

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Author contributions: AMC is responsible for the study conception, data acquisition, analysis and interpretation; drafting of the final work. JSB is responsible for data interpretation and critical revision of the manuscript. DL is responsible for data analysis and interpretation and critical revision of the manuscript. LBB is responsible for study design; data acquisition and interpretation and critical revision of the manuscript. MC is responsible for study conception, data acquisition, analysis and interpretation, drafting of the final work. All authors approve of the final version of the manuscript and agree to be accountable for all aspects of the work.

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Abbreviations:

- ANOVA: analysis of variance
- BAL: bronchoalveolar lavage
- FeNO: fraction of exhaled nitric oxide
- FEV₁: forced expiratory volume in one second
- FVC: forced vital capacity
- HC: healthy control
- ICS: inhaled corticosteroids
- IL: interleukin
- NSA: non-severe asthma
- PCR: polymerase chain reaction
- ppb: parts per billion
- RIN: RNA integrity number
- SA: severe asthma
- SARP: Severe Asthma Research Program
- SC: systemic corticosteroids
- T2: type 2

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Type 2 (T2) inflammation, which includes cytokines interleukin (IL)-4, -5 and -13, is a major molecular mechanism in asthma pathogenesis. While extensively studied in adults, pediatric-specific data on these pathways in children with asthma are limited¹⁻⁴. The Severe Asthma Research Program (SARP), a longitudinal cohort study with 7 US clinical sites, enrolled children 6-17 years of age with asthma, who underwent detailed characterization and collection of various biomarkers to understand the molecular mechanisms of the different asthma endotypes. We measured T2 gene expression within sputum of adolescents with asthma to determine if a T2-high endotype was associated with asthma severity, and to examine an evoked response after administration of systemic corticosteroids (SC).

Demographics, clinical characteristics, sputum samples and lung function measures were obtained at baseline and 18±3 days following injection of 1mg/kg (max 40mg) intramuscular triamcinolone. Sputum collection was limited to subjects ≥ 12 years of age with an FEV₁ ≥ 70% predicted. Due to lack of healthy control (HC) children, subjects were compared to a cohort of 29 adult HC who were free from pulmonary and atopic disease and had normal spirometry and airway responses to methacholine.

Sputum induction and processing were performed using previously described methods^{4,5}. RNA extracted from sputum with RNA Integrity Number (RIN) ≥ 5 and less than 80% squamous cell contamination were considered adequate for gene expression profiling⁶. Real-time quantitative PCR (rt-qPCR) was performed on purified RNA using Taqman-based assays³. Gene expression for *IL4*, *IL5* and *IL13* was normalized to the geometric mean of four housekeeping genes (*GAPDH*, *PPIA*, *YWHAZ*, and *PSMB2*) and log2-transformed. The T2 cytokine gene mean expression is the geometric mean of the normalized absolute expression of *IL4*, *IL5*, and *IL13*, log-transformed then centered and scaled³. Statistical Analyses were performed with SAS 9.4

(Cary, NC) using t-tests, Chi-square, ANOVA, Pearson's correlations and multivariate regression when indicated. *P* values of less than 0.05 were considered significant.

Thirty-four (22 severe asthma [SA], 12 non-severe asthma [NSA]) of 66 (52%) sputum inductions were of adequate quality for gene expression analysis. Children with NSA had higher sputum *IL4* gene expression than HC (15.4 ± 1.9 vs 13.1 ± 3.6 , $p=0.03$), while children with any asthma had higher sputum *IL5* gene expression (17.0 ± 4.4 vs 13.7 ± 6.4 , $p=0.02$) and T2 gene mean expression (0.06 ± 0.98 vs -0.54 ± 1.2 , $p=0.03$) than HC. Gene expression for the other cytokines otherwise did not differ between HC and children with any asthma, NSA or SA. Expression of *IL4*, *IL5*, *IL13*, and the T2 gene mean did not differ between children with SA and NSA.

T2 gene mean expression positively correlated with FeNO, blood and sputum eosinophil percentage, total IgE, and the number of positive serum-specific IgE tests (Figure 1a-e). In addition, sputum T2 expression positively correlated with maximum bronchodilator reversibility, while there was a negative correlation with baseline and maximum FEV₁/FVC% predicted (Figure 1f-h). The positive association of T2 gene expression and bronchodilator response persisted after correcting for baseline lung function ($R^2=0.04$, $p=0.02$). T2 gene mean expression was significantly higher for those with a 12% or greater bronchodilator response following albuterol administration (-0.5 ± 1 vs 0.49 ± 0.72 , $p=0.002$). We found no correlation between sputum T2 gene mean expression and age, asthma duration, baseline FEV₁% predicted, or asthma control.

Twenty-four subjects (17 SA, 7 NSA) had paired sputum samples of adequate quality obtained both before and after triamcinolone injection. There were no differences in T2 cytokine gene expression between subjects with SA and NSA following SC administration nor was there a

significant change in expression following SC administration. Higher baseline maximum FEV₁% predicted ($R^2=0.03$, $p=0.01$), maximum FEV₁/FVC % predicted ($R^2=0.04$, $p=0.05$), and BMI ($R^2=0.02$, $p=0.02$) were weakly associated with an increase in T2 expression after SC, after controlling for baseline T2 expression.

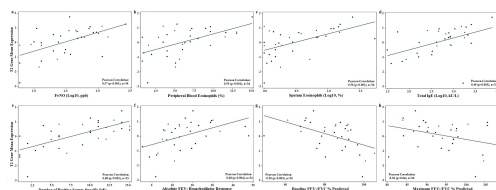
We anticipated that children with SA might have higher expression of T2 cytokine genes in their sputum than children with NSA. Bossley, et al. previously failed to show a difference in T2 cytokine detection in BAL fluid in children with SA compared to controls, though detection rate was overall low². Interestingly, children with any detectable T2 cytokines in the BAL had lower lung function and more bronchodilator responsiveness than those without detectable T2 cytokines. Similarly, we found a significant association of sputum T2 cytokine gene expression with airflow limitation and bronchodilator response; however, we found no association with baseline FEV₁ or markers of asthma control. We have recently shown that the maximal bronchodilator response in children may serve as an indicator of a more severe asthma phenotype and those who might respond to corticosteroid treatment⁷. Adults with high sputum T2 gene expression despite treatment with inhaled corticosteroids (ICS) were more likely to have SA with lower lung function and increased numbers of asthma exacerbations⁴.

The inability to distinguish SA and NSA in adolescents by T2 gene expression may be due to the generally higher T2 inflammatory profile of children with asthma. Additionally, the influence of age-related changes to the immune system may also explain these differences⁸. Within SARP, children with asthma had higher peripheral blood eosinophils, FeNO, and total serum IgE, and a greater number of positive serum-specific IgE tests than adults with asthma⁹. Other research has indicated that these non-invasive markers of T2 inflammation identify children with SA¹⁰. FeNO was a reasonable predictor of corticosteroid-resistant T2-high asthma

in adults, with both blood and sputum eosinophils performing as the best predictors^{3,4}. While blood and sputum eosinophils correlated well with sputum T2 gene expression, we found similarly strong correlations with FeNO, IgE and allergy tests in our study⁴.

We should recognize several limitations, including the relatively small sample size, lack of a pediatric healthy control group for comparison, and limitation of sputum induction to those 12 and older with preserved lung function, as this may not be reflective of all children with SA. Additionally, subjects were allowed to continue ICS, which may have influenced the expression of these cytokines and response to SC. It is also possible that the timing of repeated measures was not appropriate for measurement of a corticosteroid response for airway inflammation.

In summary, while we did not find a difference in sputum T2 gene expression between severe and non-severe adolescents with asthma, we found differences between adolescent asthmatics and adult healthy controls as well as correlations of T2 gene expression and biomarkers of T2 inflammation. These are important findings as there are limited data regarding the molecular phenotypes of the pediatric asthma population, particularly in those with severe asthma. It is especially important in pediatrics to identify surrogate and less-invasive biomarkers of T2-high asthma in order to appropriately tailor therapy and identify those patients that would best benefit from addition of a biologic agent targeting T2 inflammation. Further studies in a larger cohort that includes younger children and those with more limited lung function are warranted.



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Figure Legend

Figure 1. Pearson's correlation of sputum T2 gene mean expression with a) FeNO, b) peripheral blood eosinophil %, c) sputum eosinophil % (log transformed), d) total IgE (log transformed), e) number of positive serum-specific IgE tests , f) absolute change in FEV₁ after maximal bronchodilation, g) baseline FEV₁/FVC % predicted and h) maximum FEV₁/FVC % predicted after bronchodilation.

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