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Improvement Of Asthma Control And Inflammation In Pediatric Patients Undergoing Adenotonsillectomy

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IMPROVEMENT OF ASTHMA CONTROL AND INFLAMMATION IN PEDIATRIC PATIENTS UNDERGOING ADENOTONSILLECTOMY.

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Observational studies have suggested improvement in asthma control after adenotonsillectomy, but longitudinal studies that correlate the effect of the procedure on markers of airway inflammation with changes in asthma control are limited. We conducted a longitudinal, observational study on 130 pediatric patients undergoing adenotonsillectomy, including 66 with asthma and 64 control subjects. Asthma Control Test (ACT) scores, chitotriosidase *(CHIT1)* activity, and YKL-40 *(CHI3L1)* levels in the circulation were measured at the time of surgery and at a 6-month follow-up visit, and genotypes of chitinase family proteins were measured at baseline. Gene expression data was analyzed from blood, tonsil, and nasal epithelial tissue samples at baseline and in the blood at follow-up by microarray analysis. Mean ACT scores improved by 3 points ($p< 0.001$) after 6 months. 85% of children with poorly-controlled asthma demonstrated an increase in ACT score of at least 3 points or a decrease in Emergency Department/Urgent Care visits, oral corticosteroid courses, or rescue short acting bronchodilator usage. Serum chitinase activity decreased significantly in children with asthma ($p< 0.01$), but not in children without asthma ($p= 0.83$) undergoing tonsillectomy. Higher chitinase activity levels at baseline were associated with improved asthma control following surgery in all children with asthma ($p< 0.01$) and in the subgroup of children with poorly-controlled asthma ($p< 0.05$). Subjects with asthma had a higher allele frequency of the *CHIT1* mutation ($p< 0.02$). Gene expression analysis identified a number of inflammatory genes differentially expressed in children who had improved asthma control that were not changed in children without improved control and control subjects. Of particular interest was *SerpinB2*, a plasmin activation inhibitor previously implicated in asthma, significantly downregulated after surgery compared to baseline in children with improved control. This data suggests that adenotonsillectomy improves asthma control by modulation of airway inflammation. Elevated serum chitinase activity may be a clinically useful determinant to identify patients with poorly-controlled asthma that will benefit from the procedure.

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Introduction

Asthma is an inflammatory disease of the lower airways that remains a significant healthcare concern for the pediatric population despite public health and pharmacologic advances to control the disease. (1, 2) Studies have demonstrated a systemic component to asthmatic disease, and that the upper airway inflammation may be specifically involved in the pathogenesis of asthma. (3-5) Upper airway infections and inflammation are common in the pediatric population; likewise, respiratory infection and allergen exposure are frequently indicated as triggers for asthma exacerbations in this population.

Adenotonsillectomy is one of the most common surgical procedures in children, performed to address upper airway lymphoid hypertrophy, infection, and inflammation. (6) Its clinical effect on asthma control has been debated, with observational studies demonstrating a significant improvement in symptoms, lower healthcare utilization, and reduction in controller or rescue medication usage following adenotonsillectomy. (7-10) Although there are clear effects of adenotonsillectomy on the upper airway, to our knowledge the effect of this procedure has not yet been studied on any markers of airway inflammation.

Pediatric Asthma – Clinical Features and Pathophysiology

Asthma is the most common chronic disease of childhood; data from the 2009 National Health Statistics Reports showed that 9.6% of children 0-17 years of age (a total of 7.1 million) had asthma. 4.0 million children had at least one attack in 2009 and were at risk for adverse outcomes such as Emergency Department visits or hospitalizations. Asthma prevalence increased from 2001 to 2009. (1) Pediatric asthma has a significant burden; in 2003, there were 12.8 million reported days of missed school due to childhood asthma. In 2004, there were 198,000 total hospitalizations (3% of all pediatric admissions)

and 750,000 visits to the emergency room (2.8% of pediatric visits) attributed to childhood asthma; mortality rate was 2.5 per 1 million. (2)

Asthma is characterized by intermittent attacks that include symptoms of cough, wheezing, and difficulty breathing. Attacks can be triggered by respiratory infections, allergen exposure (e.g. dust mites, cockroach allergens, animal dander, and molds), exposure to irritants (e.g. tobacco smoke), changes in weather, or exercise. Inflammation results in hyperresponsive airways and causes bronchoconstriction and obstruction of airflow. By definition, these symptoms are at least partially reversible. Obstructive changes in airway physiology cause reductions in Forced Expiratory Volume (FEV_1) > Forced Vital Capacity (FVC) and a low FEV_1/FVC ratio. In children, low FEV_1 and FVC, along with a low Forced Expiratory Flow (FEF) 25%-75%, are better predictors of disease than FEV_1/FVC ratio. (11)

Asthmatic inflammation is mediated by a variety of cell types including Th2 lymphocytes, eosinohpils, and mast cells. Th2 cytokines including IL-4, IL-5, and IL-13 produce inflammation that causes the pathophysiologic changes seen in asthma including narrowing of the airways, mucus hypersecretion, and airway hyperresponsiveness. IgE plays a pivotal role in this process as well. Airway remodeling occurs due to chronic inflammation and leads to bronchial smooth muscle hypertrophy, mucous gland hyperplasia, thickening of the subepithelial basemsent membrane due to collagen deposition, and fibrotic changes. (12)

A Disease Beyond the Lower Airway

There is evidence that asthmatic disease exists beyond the lower airways. (3) Upper airway respiratory infection and allergen exposure are frequently invoked as triggers for exacerbations. Rhinovirus infection in the nasal airway has been shown to significantly increase airway reactivity to both histamine and ragweed antigen, and predisposed patients

to late asthmatic reactions to the challenge. (4) Another study showed that over 70% of patients with asthma also have rhinitis, and this link has led to the "one airway hypothesis", defining asthma and rhinitis as two manifestations of one inflammatory process. (13) In patients with rhinitis, provocation with a nasal allergen produces eosinophilic inflammation in both the upper and lower airways, and provocation of the lower airway with an allergen likewise produces in eosinophilic inflammation in both the upper and lower airways. (5) Airway and nasal epithelial cells in both disease entities undergo similar structural and inflammatory changes; likewise, corticosteroid therapy targeted at these epithelial cells is effective for treatment of asthma as well as rhinitis. (14)

There are systemic components to this Th2 inflammation as well, reflected by markers in the circulation. Inflammatory activity in the airways triggers eosinophil and basophil recruitment from the bone marrow, producing a significantly higher number of these cell types in the peripheral blood. (15) IL-4 and IL-13 induce B-lymphocytes to produce IgE, which is elevated in the serum of asthmatics. (16) Blocking the effect of IgE systemically with omalizumab (anti-IgE) has been shown to be an effective adjunctive therapy for asthma in uncontrolled atopic patients with elevated IgE levels and allergen hypperactivity proven by testing. (17) A number of other inflammatory markers in the serum have also been shown to be associated with asthma, including IL-5 (18), eosinophil cationic protein (19), IL-8, and TNF-α. (20) Asthma has also been associated with diseases of other organ systems including atopic dermatitis, gastroesophageal reflux disease, inflammatory bowel disease, obesity, obstructive sleep apnea, and cardiovascular disease. (3)

Current Modalities of Treatment

The current cornerstone of recommendations for the management of asthma is the control of symptoms and prevention of adverse outcomes. The Healthy People 2010

initiative focuses on increasing the proportion of asthmatics receiving formal education and appropriate care according to National Asthma Education and Prevention Program (NAEPP) guidelines. However, it fell short of its stated goals, particularly in patients receiving an asthma management plan with specific instructions on how to change the amount or type of medicine taken, when to call a doctor for advice, and when to go to the ED. Report of receiving this asthma education differed among groups, with generally higher rates among children than adults and among non-Hispanic black patients than non-Hispanic white and Mexican patients. (1)

Medical management is divided into two categories – controller medications to reduce inflammation and "rescue" medications to reverse bronchospasm. NAEPP guidelines recommend a stepwise treatment to asthma. Children with intermittent asthma are treated with rescue medications as needed. Albuterol, a short acting bronchodilator (SABA) that relaxes airway smooth muscle and reverses bronchoconstriction, is the cornerstone of such treatment.

Children with persistent asthma, as well as children whose asthma is not well-controlled, require "step ups" of treatment to daily controller medication. Not well-controlled asthma is defined by frequent symptoms and/or frequent use of SABAs (>2 days/week), nighttime awakenings, interference with normal activity, and 2 or more exacerbations in a year requiring oral corticosteroid therapy. (21). The Asthma Control Test (ACT) is a paper and web-based validated tool developed to assess level of disease control over the last 4 weeks; the adult version assesses limitation at work or school, shortness of breath, frequency of symptoms, frequency of rescue inhaler or medication use, and self rating of control. (22) A pediatric version, validated for ages 4 to 11, includes four questions directed at the child with a face and corresponding text (rate asthma today, problem with exercise, cough, wake up at night), and 3 questions directed at the parent

(frequency of daytime symptoms, frequency of wheeze, and frequency of nighttime awakening). In both tests, a score of 19 or less suggests poor asthma control. (23) Data supports that the minimally important difference for the ACT is 3 points. (24)

Daily controller medications include therapies targeting asthmatic inflammation, such as inhaled corticosteroids (ICS), leukotriene modifiers, and the anti-IgE antibody omalizumab. Long acting bronchodilators (LABAs) are also used in conjunction with ICS therapy for patients with persistent asthma that do not achieve good control with inhaled ICSs alone.

Adenotonsillectomy in Children and its Effect on Asthma

Adenotonsillectomy is one of the most common surgical procedures performed in children. The superior part of the pharynx contains a ring of lymphoid tissue consisting of the pharyngeal tonsils on the posterior wall of the nasopharynx (commonly known as the adenoids) and two palatine tonsils (commonly known as "tonsils") on either side of the oropharynx. The tonsils and adenoids are most active between the ages of 4 and 10, and then usually involute starting at puberty. As lymphoid tissue exposed to the environment, the tonsils and adenoids can directly transport environmental antigens encountered on their surfaces to the lymphoid tissue on the interior. (6)

There were 530,000 tonsillectomies (with or without adenoidectomy) performed on children under fifteen in 2006; although this number is less than it once was (1.4 million in 1959), the prevalence of the procedure has increased in recent years (315,000 in 1996). (25) Indications for tonsillectomy have shifted over time. The most common current indication is hypertrophied tonsillar tissue that causes upper airway obstruction "sleep-disordered breathing." Symptoms and signs can include snoring and sleep fragmentation, leading to daytime exhaustion, with possible effects on behavior and/or school

performance. Obstructive sleep apnea (OSA) is diagnosed when there is a complete blockage of the airway during sleep and intermittent hypopnea, leading to hypercarbia and hypoxia; the gold standard for diagnosis of OSA is overnight polysomnography. (6) Recent studies have also shown activation of inflammatory pathways as a result of sleep disordered breathing, including activation of cytokine cascades. (26) Adenotonsillectomy is a well proven intervention for sleep disordered breathing in children. (27)

Recurrent or persistent pharyngitis is the second most common indication. Classic studies have used as criteria for tonsillectomy: 7 infections in one year, 5 in two years, 9 in three years, or chronic tonsillitis defined as sore throat lasting for at least 3 months. (28) Studies have shown there to be a benefit from adenotonsillectomy in reducing the number of infections; however, the improvement in a recent meta-analysis was not dramatic. (29) The exact role of tonsillectomy as therapy for recurrent pharyngitis remains somewhat controversial.

Other less common indications include peritonsillar abscess unresponsive to medical therapy and, rarely, suspected malignancy. (6, 28)

The "one-airway hypothesis" suggests that abnormally inflamed tonsillar tissue, whether due to infection or due to hypertrophy and causing sleep disordered breathing, may contribute to upper airway inflammation that is reflected in the lower airways and thus may contribute to asthma. Studies report conflicting results on the relationship between obstructive sleep apnea and asthma; though studies outside of the U.S. report asthma as a risk factor for a diagnosis of OSA (30), one U.S. study found that asthma was not a risk factor and in fact parental report of asthma decreased the risk of an OSA diagnosis. (31) Nonetheless, it is possible that the hypertrophied or inflamed tonsillar lymphoid tissue

chronically or intermittently triggers airway inflammation and asthma; removing the tonsils would eliminate this trigger.

A handful of observational studies support this concept. (7) One early study demonstrated that up to 88% of patients with asthma experience a significant improvement in symptoms and reduce or eliminate asthma controller or rescue medication usage following adenotonsillectomy.(8) A recent retrospective chart review of 93 children with asthma who underwent tonsillectomy for standard indications showed significant improvements in multiple asthma measures including mean hospital visits, systemic steroid administration, asthma medication use, and childhood asthma control test scores.(9) In addition, a recent cohort study identified children with poorly-controlled asthma and referred them for polysomnography; if diagnosed with OSA, the children were offered adenotonsillectomy. Post-tonsillectomy data for 35 children showed a significant improvement from adenotonsillectomy in asthma control, defined by asthma exacerbations, weekly rescue medication usage, asthma symptom score, and FEV_1.(10) Limitations of the study include lack of control groups (non-surgical, well-controlled asthma, non-asthmatics), a study design that included identification of OSA as "part of clinical routine", loss to follow-up, and lack of time frame indicated. (32)

Whether or not this reported improvement is a purely clinical effect on the airway, or is a biological effect on airway inflammation and asthmatic pathogensis, has not yet been studied.

Chitinases in Asthma

Chitinases are a family of hydrolases detectable in the circulation that correlate with inflammation and disease activity in a number of chronic diseases including asthma and adenotonsillar disease. (33) These proteins bind to or cleave chitin, the second most

abundant polysaccharide in nature and the major structural polymer in cell walls of bacteria and fungi, the shells of crustaceans, and the exoskeletons of arthropods such as cockroaches and dust mites, common allergic triggers in asthma. Animal models have shown that chitin can have either pro- or anti- inflammatory effects – when delivered orally or intransally, chitin inhibits Th2 inflammation; when delivered to the lung in isolation, chitin enhances Th2 inflammation. These results suggest a complex interplay between environmental chitin exposure and the pathogenesis of asthma. (33, 34)

Though mammals do not have chitin, they have conserved chitinase proteins that break down chitin from the environment. Like chitin itself, there is evidence in animal models as well as humans that chitinase proteins can act as up- or down-regulators of the innate immune response by interacting with and degrading chitin and by modulating the host's inflammatory response. Chitinases may act as the link between environmental chitin exposure and the Th2 inflammatory response. (33, 35) There are two major chitinases in humans; chitotriosidase (*CHIT1*) is a true enzyme with the ability to hydrolyze chitin, while YKL-40 (*CHI3L1*) is a chitinase-like protein, which has the ability to bind to chitin but not to degrade it. Both chitinase activity and YKL-40 levels are demonstrated to be elevated in the bronchoalveolar lavage fluid of children with asthma. (36)

Chitotriosidase has been shown to be the primary active chitinase enzyme in the human lung. (37) It also is the only active chitinase in the peripheral blood, meaning that measurement of serum chitinase activity is a surrogate for measurement of chitotriosidase levels. Serum chitinase activity has been shown to be elevated in a number of disease states, including Gaucher's disease, thalassemias, arteriosclerosis, and coronary artery disease. It is elevated in the bronchoalveolar lavage fluid in certain lung diseases including sarcoidosis and interstitial pulmonary fibrosis. (33)

Chitotriosidase is overexpressed in adenoid tissue of children undergoing adenotonsillectomy with concurrent chronic rhinosinusitis, otitis media with effusion, and allergic rhinitis compared to subjects without concomitant upper airway disease. (38)

Proteins in the chitinase family have also been demonstrated as markers of the systemic component of asthmatic disease. A cross-sectional study of children with allergic and non-allergic asthma showed that chitotriosidase levels were elevated in the serum of individuals with asthma children versus control subjects. (39) YKL-40, the chitinase-like protein, has been reported to be elevated in the serum of individuals with asthma and levels correlate positively with disease severity. Specifically, Chupp et al. showed that levels correlated with poor pulmonary function tests, subepitithelial basement membrane thickening, and airway remodeling in the Paris cohort. (40) Members of the chitinase family may reflect a biological link between upper airway inflammation alleviated by adenotonsillectomy and the effect of environmental triggers in the pathogenesis of asthma.

Chitinase Genetics

Studies have shown mixed results on the role of genotypes for chitinase proteins on asthma. *CHIT1*, the gene encoding chitotriosidase, has a polymorphism containing a 24-base pair duplication in exon 10 (rs3831317) that produces a non-functional protein. The allele is highest frequency in East Asian, Middle Eastern, and Indian populations. (33) Lee et al. reported associations between the 24-bp duplication and positive TB tests in Europeans but not in Asians, and an association with three or more atopic diseases (asthma, allergic rhinitis, atopic dermatitis, allergies to drugs, cosmetics or food, and frequent sneezing, watering, or nasal congestion) in Asians but not in Europeans. (41) Though significant, the p-values were modest, the definition of atopy broad, and there were differences among ethnic groups, raising the possibility of a false positive result. (33) Vicencio et al. reported in an observational study 6 asthmatic pediatric patients with fungal

sensitization, all of whom had the *CHIT1* 24-bp duplication. (42) However, other larger studies have reported no association between the allele and presence of asthmatic disease, emergency room visits for asthma exacerbations, or hospitalizations due to asthma. (43, 44)

A stronger association between asthma and the genotype of *CHI3L1*, the gene encoding YKL-40, has been reported. *CHI3L1* contains a promoter SNP (rs4950928, 131C→G); the C allele (major allele) is associated with elevated YKL-40 levels. A study looking at frequency of the SNP in a founder population of European descent (the Hutterites) as well as multiple other case-control populations showed an association of the C allele with asthma prevalence, poor pulmonary function, and bronchial hyperresponsiveness. (45) Cunningham et al., in a large cross-sectional study of pediatric patients with asthma, showed that the G allele (minor allele) was protective for asthma-related hospitalizations. (46) A study of Korean children demonstrated an association between C haplotype and atopy, though there was no association with asthma. (47)

Gene Expression in Asthma

Gene expression profiling techniques allow for the high-throughput identification of novel genes and pathways involved in disease pathogenesis. This technique has been increasingly used in asthma to study its complex etiology. There exist a number of studies looking at human tissue in asthmatics. (48)

A number of studies have examined bronchial biopsy tissue, as the principal tissue affected in asthma. Liprase et al. used microarray technology to compare gene expression from 4 subjects with mild asthma (who were not on ICSs) and control subjects. They found that 20 genes were up-regulated and 54 genes were down regulated, including immune signaling molecules, extracellular proteins, immune response proteins, and intracellular signaling molecules. Some genes were identified that were known to be involved in asthma

pathogenesis, including nitric oxide synthase 2A (*NOS2A*), glutathione peroxidase 3 (*GPX3*), and T-cell receptor α (*TCRα*); others were newly implicated including arachidonate 15-lipoxygenase (*ALOX15*), which may be involved in airway remodeling, and fractalkine receptor (*CX3CR1*), previously found to be elevated in peripheral CD4+ lymphocytes of asthmatics, serpin proteinase family inhibitors, some of which have been found to be elevated in the serum of asthmatics. (49) Another study using RT-PCR of bronchial samples confirmed a number of genes identified by Laprise et al. and also identified others, including the Na^+ K^+ Cl^- co-transporter (*NKCC1*) which showed eightfold increased expression in asthmatics. (50) Woodruff et al. studied airway epithelial cells in adults with asthma in a randomized trial of inhaled corticosteroids. A calcium-activated chloride channel (*CLCA1*), periostin, and serine peptidase inhibitor B2 were up-regulated in asthmatics. Corticosteroid therapy down-regulated expression of the three genes, and high baseline expression of the three predicted improvement. (51) The limitation of bronchial studies is that bronchial biopsy samples are often heterogeneous due to differing biopsy techniques and patterns of disease. (48)

Other studies have looked at upper airway epithelial cells. Lilly et al. examined individuals with mild asthma and subjected them to an allergen challenge. A number of genes previously implicated in asthma were found to be differentially expressed genes between pre- and post-challenge airway epithelium, including included IL-1β, IL-8, TNF-α induced protein 6, lipocortin-1, and plasminogen activator inhibitor 2. (52) Guadajero et al. recruited 10 children with stable asthma, 10 children experiencing an acute asthma exacerbation, and 10 nonatopic children without asthma. They used a pooled microarray analysis to compare expression profiles of nasal respiratory epithelial cells from each group. 37 immune-related genes were among the up-related genes in the acute-exacerbation group, with 9 cilia-related genes consistently down-regulated. (53) Limitations for

epithelial studies include sample heterogeneity, questionable representation of the lower airway in the nasal epithelia, and confounders including rhinitis and atopy. (48)

Given that asthma is a systemic disease, a number of studies have looked at expression in circulating cells. Brutsche et al. examined peripheral blood mononuclear cells. They developed gene expression based score (composite atopy gene expression, or CAGE) to predict atopy with 96% sensitivity and 92% specificity; the score is based on the expression of 10 genes including IL-1 receptor, IL-6, and the IFN-αβ receptor. Additionally, a number of B-cell isotype, cell survival, and IgE production genes were up-regulated in severe asthma. (54) Aoki et al. performed expression profiling of genes related to asthma exacerbations in the peripheral blood mononuclear cells of children. They found during an exacerbation 137 up-regulated and 16 down-regulated genes; 62 were also differentially expressed during an upper respiratory infection. Many of these genes were related to immune responses to external stimuli, supporting the notion that asthma exacerbations and respiratory infections share a common mechanism. (55) In peripheral blood lymphocytes, Hansel et al. found up-regulated genes in asthma to include TGF-β and genes involved in T cell activation. (56) The involvement of TGF-β was confirmed by another microarray study of CD4+ lymphocytes in asthmatics versus controls. (57)

Hypothesis and Aims

Asthma is a heterogeneous disease of the lower airways that remains a significant healthcare concern for the pediatric population despite numerous public health and pharmacologic advances to control the disease. Recent studies have shown a link between upper airway inflammation and asthma, and respiratory infection and allergen exposure are frequently indicated as triggers for asthma exacerbations in pediatric patients. Adenotonsillectomy is common surgical procedure in children performed to address upper

airway hypertrophy, infection, and inflammation. A number of observational studies have demonstrated a positive clinical effect of this surgery on asthma control; however these studies have limitations due to study design, and no previous studies have examined the effect of adenotonsillectomy on biomarkers on inflammation. Chitinases have been implicated in the pathogenesis of inflamed adenotonsillar tissue, and in the presence and severity of asthma, and may reflect a biological link between upper airway inflammation alleviated by adenotonsillectomy and the effect of environmental triggers in the pathogenesis of asthma. Gene expression profiling in the blood and upper airway tissue of patients undergoing adenotonsillectomy may be able to characterize biological changes due to the procedure and identify genes uniquely affected in children with asthma undergoing the procedure.

We hypothesize that *asthmatic control will improve in children undergoing adenotonsillectomy.* Using chitinase levels and gene expression profile data in the serum and upper airway, we postulate *it is possible to characterize unique biological changes among children with improvement in asthma control undergoing the surgery compared to children without improvement and children without asthma.*

Specific Aims

1. Conduct a prospective observational study of pediatric patients, with and without asthma, undergoing tonsillectomy for standard indications. Asthma will be characterized by clinical symptoms and pulmonary function tests. Serum biomarkers including chitotriosidase activity, YKL-40 levels, and IgE will be measured, and expression profile data of tonsillar tissue, nasal epithelium, and peripheral blood will be collected. Questions, PFTs, serum biomarkers, and peripheral blood expression data will be repeated 6 months post-operatively.

2. Compare asthma control pre- and post-operatively, and retrospectively identify any clinical characteristics pre-operatively that predict a positive outcome.
3. Determine the changes in chitotriosidase activity, YKL-40 levels, and genetic expression profiles pre- and post-operatively of asthmatics and non-asthmatics undergoing tonsillectomy. *CHIT1* and *CHI3L1* genotypes will be determined at baseline.
4. Correlate clinical findings (in Aim 2) with biomarker and genetic findings (in Aim 3) to determine if there is (a) a characteristic biological response in patients who see symptom improvement after tonsillectomy and (b) a set of biomarkers that could be measured pre-operatively that would predict clinical improvement after operative intervention.

Methods

Enrollment and Follow-up

Pediatric patients ages 2-18, with and without asthma, who were undergoing adenotonsillectomy for standard indications at Yale New Haven Children's Hospital and North Haven Surgery Center were recruited over a 13-month period. Informed parental consent and child assent for patients age ≥ 7 were obtained. All procedures were approved by the Yale University and the Yale New Haven Hospital Human Investigation Committee.

On the day of surgery, a study member administered a questionnaire to the subject's parent. Definition of asthma was based on a previous physician diagnosis prior to surgery. The questionnaire consists of pulmonary history, including history of asthmatic disease; a list of common triggers with examples including infection, allergy, household (dust, smoke, carpet), environment (pollution, cold weather), and exercise; Emergency Department (ED)

or urgent care visits related to asthma; steroid courses; comorbidities including history of sinus disease, gastroesophageal reflux disease (GERD), allergic rhinitis, and eczema; medications, including compliance, perception, and frequency of usage in the last 4 weeks; and days of school missed and parental days of work missed due to the child's asthma and other illnesses. Subjects with asthma also completed an age-appropriate version of the Asthma Control Test (ACT), with the assistance of parents or guardians as needed. (22, 23) Adult ACT scores were rescaled from maximum 25 to 27 in order to compare to childhood ACT scores, which also have a maximum score of 27. Children were defined as having poorly-controlled asthma at baseline by one of the following criteria, based on asthma and ACT guidelines: pediatric or adult ACT < 20, two or more ED/urgent care visits in the previous year, two or more oral corticosteroid courses in the previous year, or using SABA medication more than twice per week in the last month. (21) Children age six and above completed Pulmonary Function Testing (PFTs) if they were able to comply with the instructions given. Intraoperatively, the anesthesiology team obtained venous blood in serum.

Six months after surgery, parents were contacted by telephone or email. Primary study outcome for children with asthma was measured by retesting an ACT score. The follow-up questionnaire was also administered to parents, with rates calculated for variables measuring number of incidences over a given time (Emergency/Urgent Care visits, steroid courses, school and work days missed). Changes were used to calculate a composite variable of improvement in asthma control, with improvement defined as one of the following: increase in ACT score of 3 or greater, decreased rate of Emergency/Urgent Care visits, a decreased rate of oral corticosteroid courses, or a decrease in rescue short acting bronchodilator (SABA) usage in the previous month. PFTs were also repeated for

those study subjects with initial testing. Repeat blood samples were drawn at this time as well. Study participants were compensated for completing follow-up.

Chitotriosidase Activity, YKL-40 levels, and IgE levels

Serum chitinase activity was determined using a fluorimetric assay as previously described, and reported as nmol/mL/hr. (37, 58) Briefly, 44 µmol/L substrate was prepared by mixing 1 mg 4-methylumbelliferyl-β-D-N,N',N''-triacetylchiotrioside in 2mL H_2O and 26.9 mL of McIlvain buffer, then sonicated and incubated at 37°C until dissolved. Serum was immediately spun down at 1500rpm for 10 min after collection; the supernatant was extracted and stored at -20°C. To measure the enzyme activity, 180µL substrate was mixed with 20µL serum. The mixture was incubated at 37°C for 30 minutes. 2mL Stop Solution (0.3mol/L glycine-NaOH, pH 10.6) was added to each sample, and then each sample was measured using fluorometry (Sequioa Turner). Enzyme activity was calculated in nmol/mL*h using a standard curve.

YKL-40 levels were determined using commercially available enzyme-linked immunosorbent assay (ELISA) kits (MicroVue), as previously described. (40). Briefly, serum from each subject was measured in duplicate. 100 µL capture solution was added to 20 µL of each sample in plates included with the kit. After 1 hour incubation at room temperature, sample wells were emptied and then washed three times with 300 µL wash buffer. 100 µL enzyme conjugate was then added to each well. Samples were incubated for another hour at room temperature, followed by three more washes. 100 µL substrate buffer was then added to each well. After incubation for 1 hour at room temperature, 100 µL stop solution was added to each sample. Levels were measured by plate reader (BioTek) based on a standard curve. Median values between sample duplicates are presented. If measured

levels between the duplicates showed a more than 30% discordance, the sample was repeated.

IgE levels were measured by commercially available services (Healthpoint Diagnostix, Inc).

Genotyping

We extracted DNA from blood using the QuickGene DNA whole blood extraction kit (Fujifilm). Briefly, blood was stored in Solution A tubes and immediately refrigerated at 4°C. 300 µL protease and 2.5 mL lysis buffer were added to each 2 mL blood, and the samples were incubated 30-40 minutes at 56°C. 2.5 mL 100% EtOH was added to neutralize the lysis buffer, and then the DNA was extracted using the QuickGene system. The concentration of DNA was confirmed using the NanoDrop spectrophotometry system.

We examined the prevalence of a promoter SNP in the *CHI3L1* gene encoding YKL-40, rs4950928 (131 C→G). The C allele has been shown to be associated with elevated levels of YKL-40. The SNP was genotyped with differentially labeled primers containing the C and G allele using TaqMan q-PCR Assay-on-Demand (ABI). Briefly, 1 µL of sample DNA was added to an Assay Mix that included the labeled rs4950928 primers, dNTPs, and enzyme. After the PCR reaction was complete, each sample was scanned (Applied Biosystems 7500 Fast Real Time-PCR); based on the incorporated label, each sample was identified as having the C or G SNP. (45)

We also examined the prevalence of the 24-bp exon 10 duplication in the *CHIT1* gene encoding chitotriosidase. The duplication results in a nonfunctional protein. We performed PCR on the DNA using primers for exon 10; the duplication genotype was differentiated from the wild-type using gel electrophoresis. Briefly, 1 µL of sample DNA and exon 10 primers were added to premixed solutions with dNTPs and polymerase. After the

PCR reaction was complete, gel electrophoresis was performed; based on segment length, each sample was determined to either be wild-type or include the duplication.

Statistical Analysis

All clinical and experimental data was uploaded onto the Yale Center for Asthma and Airway Diseases online database. Statistical analyses were performed using IBM SPSS Statistics version 19. Nominal data was compared using Fisher's Exact test and Pearson's chi-square; all scaled data was confirmed to be of a non-normal distribution with the Shapiro-Wilk test for normality; thus, non-parametric methods including Mann-Whitney U and Wilcoxon Signed Rank Tests were employed. p-values <0.05 were considered significant.

RNA Extraction

Tonsillar and nasal epithelial tissue were immediately stored in 500 µL RNALater™ (Invitrogen) solution and refrigerated at 4°C overnight to allow for the solution to permeate the tissue, then stored long term at -80°C.

To extract DNA from tonsillar tissue, 50-100 mg sample was cut and transferred to a fresh 14mL Falcon tube. 1 mL Trizol solution (Invitrogen) was added. The sample was homogenized using sonication for 30 seconds, with 15 second PBS washes of the sonication probe between samples. The homogenized mixture was then transferred to a 1.5 mL centrifugation tube. The solution was spun down at 12000g x 10 min at 4°C, and then incubated at room temperature for 5 minutes. 750 µL supernatant (aqueous phase) was extracted; 150 µL chloroform was added. After vortexing to mix, the sample was set to incubate at room temperature for 3 minutes. Afterwards, it was spun down again at 12000g x 15 min at 4°C. At this point, 300 µL supernatant was transferred to a DNA column from the RNA Easy Mini Plus Kit (Qiagen).

The DNA column was spun at 10000 rpm x 30 sec at room temperature. Flow through was collected and the column discarded. 300 µL (1:1 volume) 70% EtOH was added to the flow through and mixed well by pipetting. This solution was transferred to the RNA Column from the kit. It was spun at 10000 rpm x 30 sec at room temperature. With the RNA in the column, the flow through was discarded. 700 µL RW1 Wash Buffer was added and spun down at 10000 rpm x 30 sec; another two washes were completed with 500 µL RPE Wash Buffer and spun down at 10000 rpm x 30 sec; this was followed by a dry spin of 10000 rpm x 2 min. Lastly, 50 µL water was added to the column and spun down at 10000 rpm x 1 minute to elute the RNA. The presence of RNA and concentration was measured using the NanoDrop spectrophotometer; if more concentration was desired, the 50 µL elution was re-spun through the column for a higher RNA yield.

This method was a modification over previously attempted methods to isolate the RNA using Trizol solution (Invitrogen). At first, samples were stored in Trizol. RNA was extracted after homogenization, separation of the aqueous phase once chloroform was added, and then purified by spinning down to create an RNA pellet. However, it was found that despite good concentration, RINs (RNA Integrity Numbers) were poor, indicating RNA degradation. This method was modified to use the Qiagen RNA Easy Mini Plus Kit for column purification of the RNA, rather than using an RNA pellet, but RINs were still poor. Finally, RNALater™ was used instead of Trizol as the storage solution for the tissue samples. This drastically improved RINs, though there was found to be some protein contamination (low A260/A230, as measured by NanoDrop). The final modification to create the protocol as listed above, was adding an extra spin down step after homogenization but before adding chloroform to remove insoluble protein precipitate from the Trizol mixture before separation of layers. This procedure allowed us to consistently produce high concentrations of high quality RNA from tonsillar samples. However, the first 60 tonsillar

samples collected were stored in Trizol and therefore have low quality RNA that was not used in expression analysis.

A similar process was employed to extract RNA from nasal epithelial tissue. Again, tissue was stored in 500 µL of RNALater™, and refrigerated at 4°C for one night before long term storage in -80°C. Preparation for extraction included mixing 3.5 µL β-mercaptoethanol and 350 µL RLT buffer (Qiagen RNA Easy Mini Plus Kit). The nasal swab brush was then transferred from the RNALater™ to the RLT buffer with β-mercaptoethanol and allowed to sit on ice for 30 minutes. After incubation, the mixture, with the swab, was vortexed for at least 15 seconds. The solution was then transferred to a QIAShredder column. Finally, the original tube with the swab was spun at low speed for 1 min, and any remaining solution was added to the QIAShredder column. The QIAShredder was spun at 10000rpm x 2min; flow through was transferred to the DNA column and the procedure was carried out with the Qiagen RNA Easy Mini plus Kit, exactly as stated above for the tonsillar tissue RNA extraction. Again, this method was a modification after samples that were stored in Trizol produced low RIN numbers. The first 60 samples collected were stored in Trizol and therefore have low quality epithelial RNA.

To extract RNA from whole blood, peripheral blood was collected in a Tempus™ tube (Applied Biosystems) and stored at -20°C. It was found to be important that the blood was mixed well with the solution in the Tempus™ tube immediately after collection to ensure RNA preservation. To extract the blood RNA, 3 mL PBS and the peripheral blood were mixed and vortexed, then spun at 3000g x 30 minutes at 4°C. The supernatant was discarded after the spin (though kept in case RNA extraction failed and needed to be repeated). Tubes were air dried, facing upside down on a paper towel for 5 minutes, then dried with a cotton swab (being careful not to swab the bottom of the tube, the location of the RNA pellet). Using the Total RNA Purification Kit (Norgen), 400 µL Lyse Buffer was

added to each sample and vortexed. 200 µL 100% EtOH was added and mixed by pipetting. The solution was then transferred to the Norgen RNA extraction columns. Samples were centrifuged at 13000 rpm x 1min at room temperature. The flow through was discarded. Each sample was washed three times with 400 µL Wash Buffer, 13000 rpm x 1 min for the first two washes and 13000 rpm x 3 min for the last wash. After a dry spin of 13000 rpm x 2 min, 50 µL Elution buffer was added. The sample was let to sit for 1-2 minutes, and then spun at 13000 rpm x 2 min.

Extracted RNA from all tissue types was sent to the Keck Laboratory on the Yale West Campus, where RNA quality was measured by looking at the integrity of 18S and 28S ribosomal RNA. Their integrity indicated good sample quality; their absence reflected RNA degradation in the sample. The computer generated an RNA Integrity Number (RIN) for each sample based on the 18S and 28S rRNA integrity; RINs ≥ 6 were generally considered acceptable; however if spectrophotometric peaks for the 18S and 28S rRNA looked acceptable to the eye but the computer calculated a low RIN or could not calculate a RIN, the sample was also included.

Microarray Analysis

Affymetrix™ Gene Chip Human Exon 1.0 ST microarrays were used for analysis. Gene Chip Human Exon 1.0 ST Arrays contain 5,362,207 features with 1.4 million probesets that cover over one million exon clusters, 289,961 known genes by mRNA transcript, and 665,175 genes by Expressed Sequence Tags. The Keck Laboratory performed all microarray readings according to Affymetrix protocols. Blood samples were cleaned of hemoglobin prior to reading. All samples underwent cDNA amplification using PCR prior to read.

Scanned output files were analyzed using Affymetrix™ software, and cell image data files were loaded into GeneSpring GX software version 11 (Agilent). Data were log-transformed, normalized, and baseline-converted was performed to median values by RMS for each individual analysis group (tonsil samples, nasal epithelial samples, baseline blood samples, and matched baseline & follow-up blood samples) Quality control on each set was performed using PCA plot. Interpretations were performed for baseline blood, nasal epithelium, and tonsil samples according to improvement in asthma control. Differential gene expression was determined by t-test; genes for analysis were limited to differential expression $p<.05$ and fold change > 1.5. No *post hoc* testing for multiple comparisons, including Benjamini-Hochberg method, was used due to insufficient results after corrections. Interpretations for improved control, not improved control, and control subjects were also performed on matched baseline and follow-up blood samples; differential gene expression was determined by paired t-test without *post hoc* testing for multiple comparisons; again, genes for analysis were limited to differential expression $p<.05$ and fold change >1.5. Gene Ontology categories and pathways that were well represented among the gene lists were determined, with $p < 0.1$. (53, 59) Individual genes were searched for relevance in the NCBI Gene databases.

Results

Cohort Characteristics

130 patients total undergoing adenotonsillectomy were enrolled, ages 2-18, over a 13 month period. 66 subjects had diagnoses of asthma; 64 subjects were controls (Figure 1). 75% of study participants had a primary indication of tonsillar hypertrophy, including symptoms of sleep-disordered breathing, for surgery. 23% of participants had a primary

indication of recurrent tonsillitis/infection for surgery; there was no significant difference between asthma and control groups. Compared to controls, children with asthma had higher incidence of expected comorbidities including atopy (seasonal allergies and/or eczema) and a history of GERD, and were more common of Hispanic origin (p=0.01, 0.01, 0.03, respectively). (60) Children with asthma had a lower median FEV_1 (p=0.03), consistent with a physician diagnosis of asthma (Table A). Baseline ACT scores ranged from 6-27, with a median of 22 (interquartile range 16-22). 46 children completed the pediatric ACT and another 5 children completed the adult ACT at baseline; 15 children never completed a baseline ACT. 29 subjects were administered ACT questionnaires following surgery during the follow-up period, rather than prior to surgery. These families completed the ACT prior to follow-up by phone or at the follow-up appointment. There was no relation between indication for surgery and baseline ACT score. 38 out of 66 (58%) had poorly-controlled asthma (ACT < 20, ≥ 2 ED/urgent care visits in the previous year, ≥ 2 oral corticosteroid courses in the previous year, or using SABA medication more than twice per week in the last month).

Follow-up was achieved in 81% of the enrolled subjects (N=105), 58 subjects with asthma and 47 control subjects. Bloodwork was obtained for 122 participants (94%) at baseline; 67 parents gave consent (64% of those achieving follow-up) to have bloodwork performed post-operatively. Patients were primarily lost to follow-up due to failure to respond or unable to contact (moved or changed phone number). Mean time to follow up was 7.0 months (range 5-12, SD 1.5), and was not statistically different between study groups. Follow-up rate was also not statistically different for patient age, gender, race, ethnicity, or indication for tonsillectomy.

Effect of Adenotonsillectomy on Asthma Control

Asthma control and asthma-related healthcare utilization were significantly improved after adenotonsillectomy. There was a clinically significant improvement in ACT scores after 6 months (median increase from 22 to 25, $p< 0.001$, Figure 2a) and subjects with asthma reported significant decreases in the rate of ED/Urgent Care visits, oral corticosteroid courses, missed school days due to asthma, and total missed parental work days due to illness (Table B). An improvement in score of 3 or more was considered clinically significant, previously shown to be the minimally important difference in score. (24) Of 35 individuals with rescaled ACT scores < 25, 24 subjects (69%) had increases in score of 3 or more ($p< 0.001$, Figure 2b). 12 of 27 children (44%) on controller medications for asthma at baseline were no longer on controller medication at follow-up. Two children "developed" asthma during the follow-up period; these children were characterized as controls since they were classified as without asthma at the time of surgery. PFT scores did not significantly change for either study group between baseline and follow-up. Subgroup analysis of children with poorly-controlled asthma at baseline demonstrated a more pronounced increase in ACT score, from a median of 18 to 24. (21)

The composite variable of improvement in asthma control was defined by fulfilling one of the following categories: increase in ACT score of 3 or greater, decreased rate of Emergency/Urgent Care visits, a decreased rate of oral corticosteroid courses, or a decrease in rescue short acting bronchodilator (SABA) usage in the previous month. Of the 48 children with asthma who were not already maximally controlled at baseline, and therefore had room to improve (pediatric ACT <25, adult ACT <23, one or more ED/urgent care visit in the previous year, one or more oral corticosteroid course in the last year, or using SABA medication in the last month), 36 (75%) had an improvement in symptoms in at least one

category. When limited to the 34 subjects with poorly-controlled asthma at baseline, 29 (85%) had an improvement in symptoms in at least one category.

Improvement in asthma control, using the composite variable, did not significantly correlate with baseline patient characteristics including age, years since asthma diagnosis, comorbidities, smoke exposure, indication for adenotonsillectomy, or PFTs. In the subgroup analysis of children with poorly-controlled asthma at baseline, asthma control in all 19 subjects who listed "Environment" as a trigger improved, while control in only 10 of 15 (67%) of those who did not improve ($p=0.01$). Improvement in control was also significantly associated with a lower baseline ACT score among those with poor control at baseline (median score of 22 for subjects with improved asthma control versus score of 16 for those without improved control, $p< 0.05$).

Analysis of Chitinase Genotypes and Levels

Genotypic analysis of the *CHIT1* 24-bp exon 10-duplication showed a significantly higher allele frequency of the mutation in the asthmatic population undergoing adenotonsillectomy, 26% vs. 14%, (t-test $p< 0.02$, Figure 3). Analysis of the *CHI3L1* rs4950928 (131 C→G) promoter SNP showed a lower allele frequency of the minor G allele in the asthma populations (14% vs. 18%, respectively), a result consistent with previous findings though not significant in this study ($p=0.39$). Of note, *CHIT1* and *CHI3L1* genotypes did not significantly associate with gender, race, or ethnicity. *CHIT1* and *CHI3L1* genotypes also did not significantly associate with improvement in asthma control following surgery.

Chitinase activity was evaluated at baseline and follow-up. Corresponding to the increased prevalence of the exon 10-duplication in children with asthma, chitinase activity was significantly lower in the asthmatic population, median 3.1 nM/ml*hr (interquartile range 1.9-5.3) vs. 3.9 nM/ml*hr for controls (interquartile range 3.0-6.6), ($p< 0.01$, Figure

4a). While there was a significant decrease in circulating chitinase activity in children with asthma (median decrease 0.4 nM/ml*hr, $p< 0.01$), there was no significant change in chitinase activity in the control subjects after surgery (median no change, $p=0.83$). Within the asthma group, chitinase activity significantly decreased in subjects with improved control ($p=0.001$), while it was unchanged in those who did not improve ($p=0.73$, Figure 4b). Baseline chitinase activity was also significantly higher in subjects whose asthma control improved versus those whose control did not, median 3.5 nM/ml*hr (interquartile range 2.6-6.6) versus 2.2 nM/ml*hr (interquartile range 1.4-3.5) nM/ml*hr, $p< 0.01$). YKL-40 levels were higher among children with asthma (median 41.5 ng/mL versus 35.5 ng/mL , interquartile ranges 27.5-62.4 versus 25.8-50.1), but this was result was not statistically significant (Figure 4a). This trend corresponded to the lower prevalence in the asthmatic population of the promoter SNP 131 G→C, which increases YKL-40 expression. YKL-40 levels not change significantly in either study group over the study period (Figure 4c). Improvement in asthma control did not correlate with baseline YKL-40 level, change in YKL-40 level over the study period, or baseline IgE levels.

In the subgroup analysis of children with poorly-controlled asthmatic subjects at baseline, the decrease in chitinase activity remained statistically significant when disease activity asthma control improved (median decrease 0.9 nM/ml*hr, $p< 0.01$), but not when disease activity control remained unchanged (median no change, $p=1.00$), as defined by the composite variable (Figure 4d). In this subgroup, baseline chitinase activity was again significantly higher in asthmatic subjects whose asthma control improved versus those whose asthma control did not (median 3.4 versus 1.9 nM/ml*hr, interquartile ranges 2.4-5.4 versus 0.8-3.0, $p< 0.05$). A baseline chitinase activity ≥ 2.35 predicted improvement with 82% sensitivity and 80% specificity among children with poorly-controlled asthma.

Gene Expression Analysis

In nasal epithelial samples, 46 exon clusters including 32 known genes were differentially expressed among children with asthma between those with improved control and those without(unadjusted p<0.05, |Fold change (FC)|>1.5, Table C). Using NCBI databases, transcripts related to asthma, the upper airway, or the inflammatory response were identified. Serpin Peptidase inhibitor B2 (*SerpinB2*), previously associated with asthma, was down-regulated in the nasal epithelium of children with asthma with improved control versus those without improved control (p<0.01, FC -1.96). It was also down-regulated in children with improved asthma versus control subjects (p=0.03, FC -1.61). Other inflammatory-related genes with differential expression in the nasal epithelium of children with improved control versus those without improved control included two glutathione S-transferases (*GSTM1*, p=0.03, FC -2.45; *and GSTM4*, p<0.01, FC -1.51) and IGF binding protein 2 (*IGFBP2*, p=0.01, FC -1.50), which were down-regulated at baseline among children with improved control. A leukocyte immunoglobulin-like receptor (*LILRB4*, p=0.04, FC 1.5), cytolysis protein perforin 1 *(PRF1*, p=0.02, FC 1.52*)*, and a GTPase involving T-cell survival (*GIMAP5*, p=0.02, FC 1.66) were up-regulated in baseline nasal epithelium among children with improved asthma control. None of the above, aside from *SerpinB2*, was differentially expressed in children with improved asthma versus control subjects.

In baseline blood samples, 7 exon clusters including 5 known genes were differentially expressed (Table C); these included an interferon induced protein (interferon-induced protein with tetratricopeptide repeats 1-like protein - *IFIT1L*, p=0.02, FC -1.51) and a carboxypeptidase produced by mast cells (*CPA3*, p<0.001, FC -1.51) which were both down-regulated at baseline among children with improved control. Interestingly, *IFIT1* (not *IFIT1L*) was *up-regulated* in the blood of children with improved asthma control versus control subjects. In tonsil tissue, 9 differentially expressed clusters and 7 known genes

were identified, interestingly all of which are on the Y chromosome; none were differentially expressed compared to control subjects (Table C).

In matched blood samples using pairwise comparisons (Table D), expression of *SerpinB2* decreased from baseline to follow-up in children with asthma that improved ($p<0.01$, FC -1.54), but not in children with asthma that did not improve or in control patients. This was the only differentially expressed gene among all children with improved asthma; when limited to asthma that was poorly controlled at baseline, 25 exon clusters with 15 known genes were differentially expressed. These included *SerpinB2*, which decreased in expression ($p=0.01$, FC -1.6), as well as interferon induced protein *(IFIT1*, $p=0.02$, FC 2.27), *HERC5* ($p=0.04$, FC 2.07), *CD274* ($p=0.04$, FC 1.69), *LAMP3* (associated with Influenza A, $p<0.05$, FC 1.6), and an olfactory receptor (*OR2W3*, $p=0.04$, FC 1.52) which all increased in expression. None of these significantly changed in expression in either children with asthma whose control did not improve or control subjects. In control subjects, 29 known genes showed differential expression pre- and post-operatively, including interleukin 1B (*IL1B*, $p=0.03$, FC 1.53), cyclooxygenase-2 (*PTGS2,* the inducible form, $p=0.03$, FC 1.5), adrenomedullin (*ADM*, $p=0.04$, FC 1.66), and free fatty acid receptor 2 (*FFAR2* or *GPR43*, $p=0.03$, FC 1.64), all of which increased in expression. Granzyme to eliminate transformed and virus-infected cells (*GZMH*, $p=0.04$, FC -1.65) and NK cell lectin receptor (*KLRF1*, $p=0.03$, FC -1.65*)* both decreased in expression over the study period.

Gene Ontology analysis of differentially expressed genes between children with improved asthma control versus those without improved control demonstrated significance ($p<0.1$) in the baseline nasal epithelium for the classes of pathogenesis ($p<0.01$), MHC Class II protein complex ($p<0.01$), and membrane parts ($p=0.08$). In the baseline blood samples, serine-type endopeptidase inhibitor activity ($p<0.05$) was the only significant class.

Discussion

Though a number of observational studies have demonstrated a positive clinical effect of adenotonsillectomy on asthma control, these studies have contained limitations in size and study design. (32) No study to our knowledge has examined the effect of adenostonsillectomy on markers of airway inflammation; we chose to explore chitinases given previous, separate findings liking them to both asthma and adenotonsillectomy. (38, 40) In this study, we have demonstrated that asthma control and healthcare utilization significantly improve after adenotonsillectomy and that this improvement is associated with a decrease in chitinase activity in the circulation that is not evident in children without asthma. Therefore, while this procedure has effects on upper airway inflammation and mechanics that contributes to improved asthma control, these results suggest that adenotonsillectomy also modulates airway inflammation in asthma.

This longitudinal, observational study followed children with asthma undergoing adenotonsillectomy for standard clinical indications, using both clinical and biological characterizations. We used a past physician diagnosis of asthma as our identification criteria, which was supported by the statistically lower $\%FEV_1$ in the asthmatic group. The asthmatic group also had statistically more children of Hispanic or Latino heritage; however, this did not significantly correlate with chitinase activity or YKL-40 levels. A six month follow-up was chosen to minimize any natural changes of asthma control with age, but to provide enough time to see clinical and biological changes in airway inflammation.

The study demonstrated that a vast majority of subjects with asthma undergoing adenotonsillectomy reported improvement in symptoms after 6 months. 75% of all children with asthma not already maximally controlled at baseline, and therefore with room to improve, reported symptom improvement. 85% of children with poorly-controlled

asthma at baseline reported improvement. Response was significantly associated with a lower baseline ACT score, indicating that those with poorly-controlled asthma were most likely to see a benefit from the surgery, and listing environmental exposures as a trigger (including exposures outside the home, weather changes, and pollution).

ACT score, a validated tool to monitor asthma symptoms and severity, increased on average over 3 points from baseline to post-operative follow-up. An initial group of 29 subjects with asthma enrolled in the study had baseline scores collected after the procedure; median baseline ACT score in this group was identical to baseline ACT scores collected pre-operatively (score of 22), and thus, all scores were included in analysis.

A strength of this study is that we have identified a serum biological marker, chitinase activity, which decreases with improvement in asthma after adenotonsillectomy but does not change in subjects without asthma undergoing the procedure. Given the difficulties of performing a randomized controlled trial of surgery for pediatric asthma patients, identifying positive clinical and biological predictors for symptom improvement would help to categorize patients most likely to benefit from adenotonsillectomy and further justify a biological effect of the surgery on asthmatic inflammation. Higher serum chitinase activity was also significantly associated with improvement in disease activity in both the entire cohort of individuals with asthma as well as the subgroup with poorly-controlled asthma at baseline. This data suggest that the surgery improves not only clinical asthma symptoms, but also has a biological effect on inflammation associated with asthma.

Genotype analysis of our baseline population showed a higher prevalence of the *CHIT1* 24-bp exon 10 duplication in children with asthma who were undergoing adenotonsillectomy. Correspondingly, baseline chitinase activity was significantly lower in this population. These results are somewhat surprising since chitinase activity also

decreased in children whose asthma control improved following the procedure, and genotype did not associate with improvement. One possibility is differences in genetic predisposition versus a response to an environmental effect (Figure 5). Specifically, our data suggest that among children with tonsillar disease requiring adenotonsillectomy, children with the mutation are more likely to have asthma, due to an inherently decreased ability to modulate the effect of environmental substances on the airway (including chitin), potentially related to the tonsils. This may result in more inflammation in the airway and the development of asthma. Because we only explored this in a population of children with tonsillar pathology, these conclusions cannot be applied to children with asthma as a whole.

The decline in chitinase activity following the surgery may represent an environmental response rather than an inherent trait. The results suggest that in patients who improve after adenotonsillectomy, tonsillar inflammation and hypertrophy may contribute to inflammation of the lower airways. In accordance with the "one-airway hypothesis," removing the tonsils may reduce inflammation in the lower airway, decrease chitinase activity, and improve asthma control. Those who do not improve control may have different triggers or a distinct form of asthma, and thus chitinase levels are likely to remain unchanged following the procedure in this group. Chitotriosidase has been shown to have both pro-and anti-inflammatory properties. Whether the enzyme is responding to inflammation in the airways induced in the environment as an attempt at negative feedback or mediating the inflammation occurring in the airways cannot be discerned by this study.

While prior studies have demonstrated a strong association between serum YKL-40 and asthma severity (40), it was another chitinase family protein, chitotriosidase, whose activity was found in this study to correlate with asthma and improvement in symptoms after surgery. This distinction may be due to differences in the mechanism of chitin response and Th2 inflammation in children and adults. Additionally, as *CHIT1* expression is

elevated in hypertrophied adenoid tissue of children with chronic upper airway and sinus disease (38), chitotriosidase may play a unique role in airway inflammation triggered by hypertrophied adenotonsillar tissue. A future direction will be to measure chitotriosidase activity and YKL-40 levels in adenotonsillar tissue of children with and without asthma.

To our knowledge, global gene expression has never been studied in patients undergoing adenotonsillectomy. This analysis demonstrated a number of relevant genes that may act as biomarkers for changes in asthma control following to adenotonsillectomy. The most promising is Serpin Peptidase inhibitor B2 (*SerpinB2*) since it has been implicated in asthma previously, and it was differentially expressed in multiple compartments - blood and nasal epithelial tissue. Interestingly, *SerpinB2* was downregulated in the nasal epithelium among children whose asthma control improved versus children in which it did not, and also decreased in expression in the blood from baseline to follow-up in children that improved (but not in controls or children that did not improve). Serpin peptidase inhibitor 2 inhibits tPA and uPA, thus preventing the activation of plasmin and promoting fibrin formation. Its expression is provoked by IL-13. (51) Fibrinogen and thrombin accumulation in the airways has been shown to be involved in airway hyperresponsiveness. (61) *SerpinB2* and carboxypeptidase A3, a mast cell product, were previously shown to be up-regulated in genome wide profiling of asthmatic bronchial epithelium. (51) Response to corticosteroids was associated with decreased expression of *SerpinB2*; likewise, in our study serum expression of *SerpinB2* decreased in children with improved asthma control after adenotonsillectomy. Both higher *SerpinB2* levels and carboxypeptidase A3 levels have been shown, in separate studies, to predict response to corticosteroids. (62) However, we identified that *lower* expression of *SerpinB2* and carboxypeptidase A3 in the nasal epithelium at baseline predicted improvement after surgery. This pattern may identify a subgroup of children with asthma, possibly of a non-allergic type (given low levels of

carboxypeptidase A3), unlikely to be controlled by conventional therapies and likely to improve by adenotonsillectomy. Additionally, *SerpinB2* knockout mice poorly regulate Th1 responses. (63) Thus, children with low baseline *SerpinB2* levels may have an underlying susceptibility to adenotonsillar-induced airway inflammation.

Glutathione S-transferases have also been implicated in asthma, and two (*GSTM1* and *GSTM4*) were found to be down-regulated in the nasal epithelium of children whose control improved. Children null for the *GSTM1* allele were more susceptible to asthma associated with environmental tobacco smoke; in older children, the *GSTM1* null status interacted with smoke exposure to reduce peak expiratory flow rate. (64) Likewise, children with asthma undergoing tonsillectomy with low glutathione s-transferase expression in the airway may be more susceptible to environmental stress on the airway from tonsillar inflammation, and more likely to improve from adenotonsillectomy.

An interferon induced protein, *IFIT1,* also increased in expression in matched blood samples of children with uncontrolled asthma at baseline that improved control after surgery, but not in control subjects or children with uncontrolled asthma that did not improve. This may be relevant since *IFIT1L* was found to be down-regulated at baseline in the blood of children with improved control as well. *IFIT1* is an antiviral protein expressed highly after viral infection. (65)

A number of other inflammatory proteins also changed in expression in the blood in control subjects. This is the first time that changes in gene expression related to adenotonsillectomy are reported. These inflammatory proteins include interleukin-1 β, cyclooxygenase-2 (PTGS2, the inducible form), adrenomedullin (ADM), and free fatty acid receptor 2 (FFAR2, or Gpr43), all of which increased in expression. Granzyme-H, which eliminates transformed and virus-infected cells, and NK cell lectin receptor (KLRF1) both

decreased in expression over the study period. These changes support the notion that adenotonsillectomy affects inflammatory pathways, regardless of asthma status. Of these, IL-1β has previously been shown to exist in high concentrations in chronically inflamed adenotonsillar tissue in children. (66) Others have been implicated in airway inflammation. COX-2 is implicated in asthma, particular in nasal polyposis and aspirin-associated asthma. (67) Adrenomedullin has been shown to suppress Th-2 related inflammation in mice. (68) Mice deficient for Gpr43, a G protein coupled receptor in the gut for short chain fatty acids, have hyperractive neutrophilic responses. (69) Granzyme-H targets adenovirus-infected cells by neutralizing an inhibitor of Granzyme B. (70) KLRF1 has been shown to stimulate CD8 T cells with an inflammatory NK-like phenotype. (71)

Limitations to the observational study include regression to the mean; however longitudinal measurement of ACT scores and other measures of asthma control support our findings. Though 29 'baseline' ACT scores were collected during the post-operative follow-up period due to a change in study design, the median of these scores did not differ from the median of scores truly collected at baseline. While this minimizes the possibility of a recall bias due to the timing of the questionnaire, it does not eliminate such a possibility. Our asthmatic group also had a high prevalence of poorly-controlled asthma and was thus more severe than population data; however, this may be explained given our starting point of children who were candidates for adenotonsillectomy, and thus, may have been more connected to the medical system or more likely to have concurrent illnesses. Another limitation is the effect of seasonal variability with a 6 month follow-up period; however, this was controlled by enrolling participants year round. Though follow-up was targeted at six months, actual mean follow-up time was 7 months, and ranged from 5 to 12 months. This variability may have likewise affected post-operative results. Additionally, limited PFT data in a young population made objective measures of asthma improvement difficult to obtain.

Though this study was designed to specifically examine patients undergoing adenotonsillectomy, the results suggest further studies comparing changes in asthma control and chitinase activity to a non-surgical pediatric asthma population.

Finally, limitations to the gene expression analysis include heterogeneous cell types in all three tissues, inability to perform *post hoc* correction for multiple comparisons due to lack of results, and batch effect due to differences in sample processing including RIN number and processing date. It is also curious that in the baseline tonsil samples, all differentially expressed genes were on the Y chromosome, indicating an amplification or detection anomaly.

This study demonstrates that a vast majority of children with asthma, and particularly those with poorly-controlled asthma, undergoing adenotonsillectomy for standard indications are likely to see an improvement in disease control. Clinical markers associated with response include lower ACT scores at baseline, environmental triggers of asthma, and higher serum chitinase activity at baseline. Additionally, a number of asthma-related or inflammatory-related genes were found to be differentially expressed between children with improved asthma control and children without improved control. *SerpinB2*, a plasmin activation inhibitor previously shown to be related with asthma, may be of particular interest as a marker of change in airway inflammation. This data suggest that the surgery affects asthma control by modulating inflammation, rather than just altering airway mechanics. The clinical, serum chitinase, and expression patterns could identify a subgroup of children with poorly-controlled asthma likely to benefit from the surgery, and support the rationale behind adenotonsillectomy as an intervention for asthma. Prospective studies of children with asthma will be required to determine the clinical and biologic phenotype of those most likely to have clinical improvement of asthma control from surgery.

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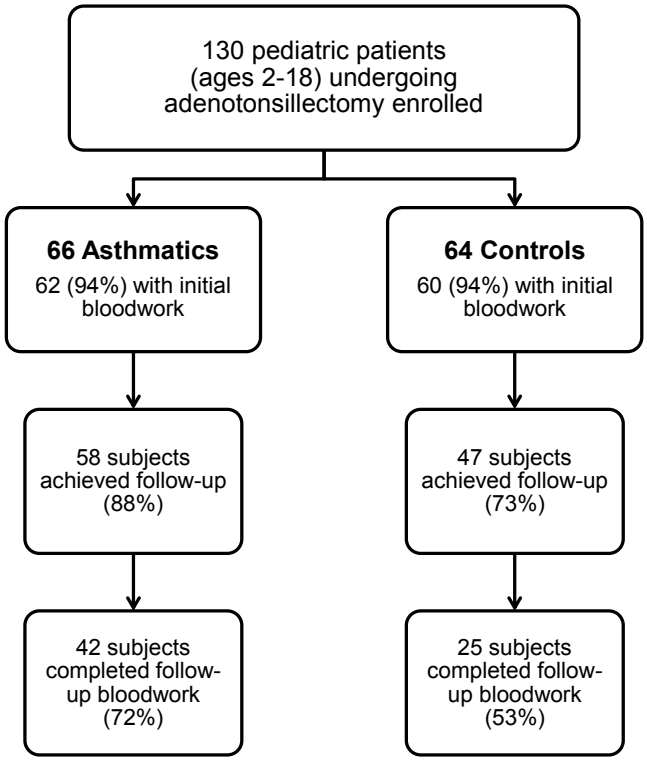
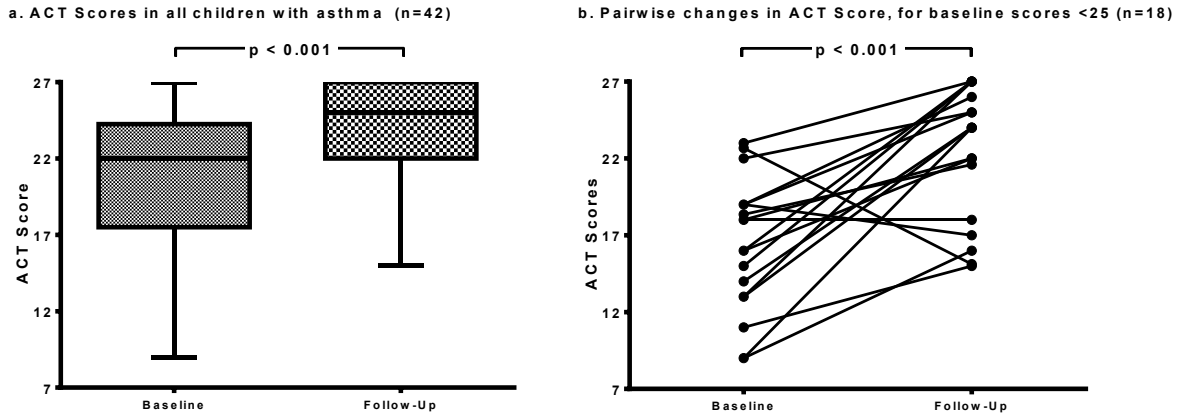
Figures and Tables

Figure 1



Study design and follow-up rates for Asthma and Control cohorts undergoing adenotonsillectomy

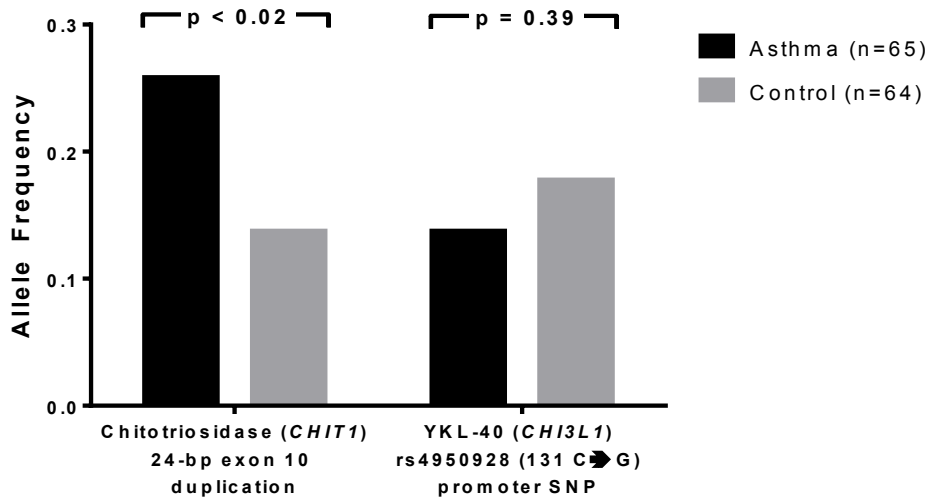
Figure 2



Asthma Control Test (ACT) scores at baseline and follow-up

Median score displayed with interquartile ranges, error bars represent range of minimum and maximum scores. p-values calculated by Wilcoxon Signed Rank Test.

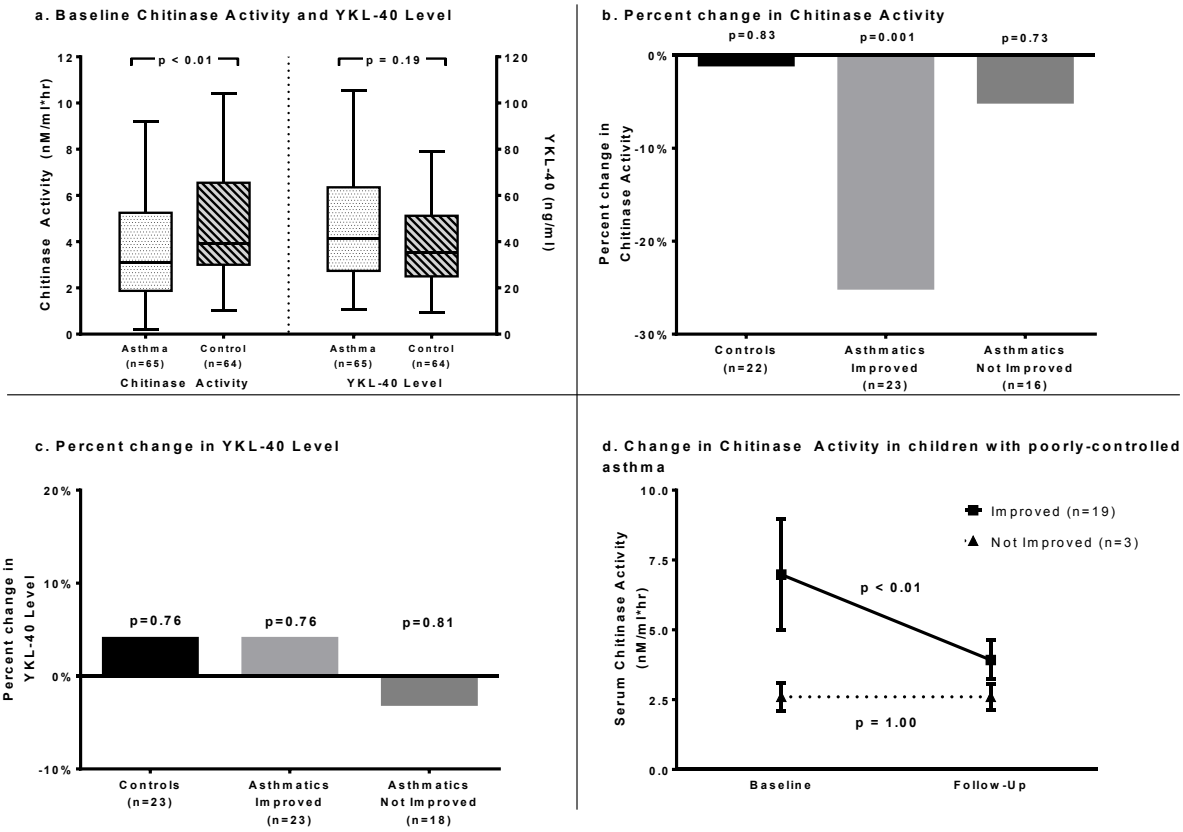
Figure 3



Chitotriosidase (*CHIT1*) and YKL-40 (*CHI3L1*) Genotypes in Asthma and Control cohorts

p-values calculated by t-test.

Figure 4

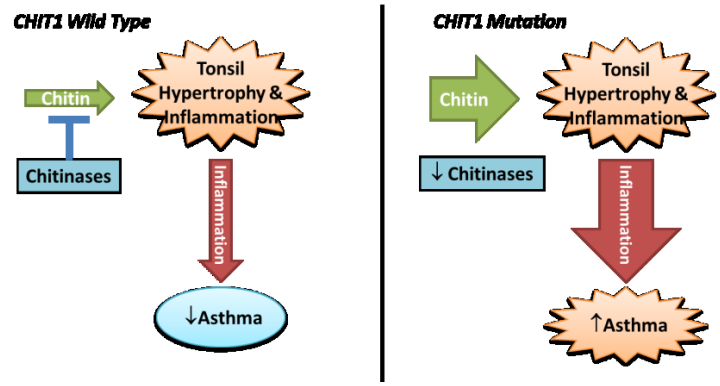


Chitinase Activity and YKL-40 Level in Asthma and Control cohorts

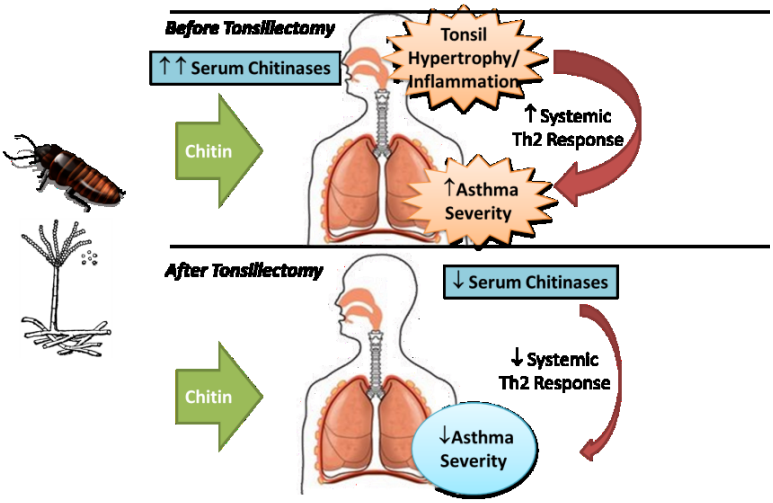
a. p-values calculated by Mann Whitney U Test. b-c. p-values calculated by Wilcoxon Signed Rank Test. d. Poorly-controlled defined as baseline Asthma Control Test (ACT) Score < 20, rescue SABA usage > 2 times/week, Emergency Room / Urgent Care Visits ≥ 2/year, or Oral Corticosteroid (OCS) courses ≥ 2/year. p-values calculated by Wilcoxon Signed Rank Test. Baseline chitinase activity of the improved group was also significantly higher than the not-improved group, $p<0.05$ by Mann Whitney U Test.

Figure 5

a.



b.



Concept: Adenotonsillectomy, Chitinases, and Asthma Control

a. CHIT1 polymorphism affects asthma prevalence in patients undergoing adenotonsillectomy

The genotypic data suggests that the CHIT1 polymorphism, which results in lower chitotriosidase activity, correlates with asthma prevalence among patients undergoing adenotonsillectomy. Chitinases may modulate the effect of environmental stressors (including chitin) on airway inflammation, thus lowering asthma prevalence. Those with the mutant *CHIT1* allele have lower chitinase activity, and may be unable to modulate this inflammation, possibly relating to upper airway disease including adenotonsillar hypertrophy and inflammation, raising asthma prevalence.

b. Tonsillectomy induces changes in systemic inflammation associated with asthma

Meanwhile, longitudinal results suggest that patients with higher chitinase levels at baseline with tonsillar hypertrophy/inflammation & asthma are more likely to see improvement after tonsillectomy. This may be a reflection of both the airway-specific and systemic inflammatory response to tonsillar hypertrophy and inflammation, and thus chitinase levels decline after adenotonsillectomy correlating with a decrease in asthma severity.

Table A

Baseline demographics and clinical characteristics of Asthma and Control cohorts

		Asthmatics (n=66)	Controls (n=64)	p-value
Age - Mean (Range)		6.4 (2-17)	5.8 (2-18)	0.17*
Females – n (%)		25 (38)	33 (52)	0.16‡
Males – n (%)		41 (62)	31 (48)	
Race – n (%)	White/Caucasian	54 (82)	43 (67)	0.13‡‡
	Black/African American	11 (17)	20 (31)	
	Other	1 (1)	1 (2)	
Hispanic and/or Latino Ethnicity – n (%)		32 (49)	19 (30)	0.03‡
Comorbidities	Atopy – n (%)	49 (77)	32 (51)	<0.01‡
	History of GERD – n (%)	25 (38)	9 (14)	<0.01‡
	Sinusitis – n (%)	22 (33)	12 (19)	0.11‡
Second Hand Smoke Exposure – n (%)		18 (29)	17 (27)	1.00‡
Weight (Described) – n (%)				0.31‡
	Normal or Underweight	47 (71)	51 (80)	
	Overweight/Obese	19 (29)	13 (20)	
Residence	Urban – n (%)	24 (37)	20 (31)	0.72‡‡
	Suburban – n (%)	34 (52)	38 (60)	
	Rural – n (%)	7 (11)	6 (9)	
Tonsillectomy Indication – n (%)				0.30‡
	Hypertrophy, including OSA	46 (70)	52 (81)	
	Chronic/Recurrent Infection	18 (27)	12 (19)	
FEV1 % Predicted – Median		93	112%	0.03*
IgE Levels (IU/mL) – Median		45.7	25.4	0.23*

* Mann-Whitney U Test; ‡Fisher's Exact Test ; ‡‡Chi-Squared Test

Table B

Change of clinical characteristics from baseline to follow-up in Asthma cohort (n=58)

Event frequency per 12 months	Baseline (mean)	Follow-up (mean)	Wilcoxon Signed Rank Test
Emergency Room / Urgent Care Visits for Asthma	1.88	0.40	0.01
Oral corticosteroid courses	1.11	0.21	<0.01
Hospitalizations for Asthma	0.09	0.00	0.06
Missed School Days due to Asthma	3.86	2.00	0.01
Total Missed Parental Work Days	2.79	1.13	0.03

Table C

Differentially expressed transcripts at baseline between children with asthma with improved control versus those without improved control

Nasal Epithelium

p-value	Fold Change	Gene	Description
0.014	2.57	HLA-DQB1| HLA-DRB1| HLA-DQB2| HLA-DRB2| HLA-DRB3| HLA-DRB4| HLA-DRB5| ZNF749| RNASE2| hCG_1998957	major histocompatibility complex, class II, DQ beta 1 | major histocompatibility complex, class II, DR beta 1 | major histocompatibility complex, class II, DQ beta 2 | major histocompatibility complex, class II, DR beta 2 | major histocompatibility complex, class II, DR beta 3 | major histocompatibility complex, class II, DR beta 4 | major histocompatibility complex, class II, DR beta 5 | zinc finger protein 749 | ribonuclease, RNase A family, 2 (liver, eosinophil-derived neurotoxin) | similar to hCG199264
0.030	-2.45	GSTM1	glutathione S-transferase M1
0.003	2.44	HLA-DRB5| HLA-DQB1| HLA-DQB2| HLA-DRB1| HLA-DRB2| HLA-DRB3| HLA-DRB4| ZNF749| RNASE2| hCG_1998957	major histocompatibility complex, class II, DR beta 5 | major histocompatibility complex, class II, DQ beta 1 | major histocompatibility complex, class II, DQ beta 2 | major histocompatibility complex, class II, DR beta 1 | major histocompatibility complex, class II, DR beta 2 | major histocompatibility complex, class II, DR beta 3 | major histocompatibility complex, class II, DR beta 4 | zinc finger protein 749 | ribonuclease, RNase A family, 2 (liver, eosinophil-derived neurotoxin) | similar to hCG199264
0.012	-2.10	FLJ21511	
0.009	-1.96	SERPINB2	serpin peptidase inhibitor, clade B (ovalbumin), member 2
0.047	-1.96	CD177	
0.002	1.93	ND6	NADH dehydrogenase, subunit 6 (complex I)
0.014	-1.91	SLC22A16	solute carrier family 22 (organic cation/carnitine transporter), member 16
0.043	-1.82	NELL2	NEL-like 2 (chicken)
0.034	1.80	HLA-DQA1	major histocompatibility complex, class II, DQ alpha 1
0.003	-1.78	ST6GALNAC2	ST6 (alpha-N-acetyl-neuraminyl-2,3-beta-galactosyl-1,3)-N-acetylgalactosaminide alpha-2,6-sialyltransferase 2
0.012	1.77	ADAMDEC1	ADAM-like, decysin 1
0.018	1.76	KIR2DL3| KIR2DS2| KIR2DS4| KIR2DL2| KIR2DS1| KIR2DL1| KIR3DL2	killer cell immunoglobulin-like receptor
0.016	-1.74	CLDN3	claudin 3
0.010	-1.73	CYP2S1	cytochrome P450, family 2, subfamily S, polypeptide 1
0.005	-1.72	AHNAK2	AHNAK nucleoprotein 2
0.037	-1.68	VSNL1	visinin-like 1
0.044	1.67	SORD	sorbitol dehydrogenase

0.021	1.66	GIMAP5	GTPase, IMAP family member 5
0.015	-1.62	ATP12A	ATPase, H+/K+ transporting, nongastric, alpha polypeptide
0.014	-1.60	PTPRT	protein tyrosine phosphatase, receptor type, T
0.040	1.60	SIGLEC10	sialic acid binding Ig-like lectin 10
0.009	-1.58	CLDN16	claudin 16
0.009	-1.56	LAMC2	laminin, gamma 2
0.029	-1.55	CKB	creatine kinase, brain
0.003	-1.54	KCND3	potassium voltage-gated channel, Shal-related subfamily, member 3
0.026	1.52	PRF1	perforin 1 (pore forming protein)
0.017	-1.52	CDH13	cadherin 13, H-cadherin (heart)
0.003	-1.51	GSTM4| GSTM2	glutathione S-transferase M4 | glutathione S-transferase M2 (muscle)
0.010	-1.50	IGFBP2	insulin-like growth factor binding protein 2, 36kDa
0.031	-1.50	DENND2C	DENN/MADD domain containing 2C
0.043	1.50	LILRB4	leukocyte immunoglobulin-like receptor, subfamily B (with TM and ITIM domains), member 4

Blood

p-value	Fold Change	Gene	Description
0.001	-1.62	XRRA1	X-ray radiation resistance associated 1
0.001	-1.61	OVOS	ovostatin
0.001	-1.60	OVOS2	ovostatin 2
0.023	-1.51	IFIT1L	interferon-induced protein with tetratricopeptide repeats 1-like
0.0004	-1.51	CPA3	carboxypeptidase A3 (mast cell)

Tonsil

p-value	Fold Change	Gene	Description
0.031	-3.39	UTY	ubiquitously transcribed tetratricopeptide repeat gene, Y-linked
0.035	-3.05	RPS4Y1	ribosomal protein S4, Y-linked 1
0.019	-2.80	JARID1D	jumonji, AT rich interactive domain 1D
0.030	-2.79	EIF1AY	eukaryotic translation initiation factor 1A, Y-linked
0.029	-2.79	DDX3Y	DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, Y-linked
0.027	-2.66	USP9Y	ubiquitin specific peptidase 9, Y-linked (fat facets-like, Drosophila)
0.025	-1.85	ZFY	zinc finger protein, Y-linked

Differentially expressed genes determined by non-corrected p-value <0.05, |Fold change|>1.5.

Table D

Differentially expressed transcripts at follow-up versus baseline in matched blood samples

Improved Asthma Control

p-value	Fold Change	Gene	Description
0.009	-1.54	SERPINB2	serpin peptidase inhibitor, clade B (ovalbumin), member 2

Non-Improved Asthma Control

p-value	Fold Change	Gene	Description
0.029	1.52	SNHG10| SCARNA13	small nucleolar RNA host gene (non-protein coding) 10 | small Cajal body-specific RNA 13

Improved Asthma Control Among Children with poorly controlled asthma

p-value	Fold Change	Gene	Description
0.012	2.59	RSAD2	radical S-adenosyl methionine domain containing 2
0.022	2.27	IFIT1	interferon-induced protein with tetratricopeptide repeats 1
0.039	2.07	HERC5	hect domain and RLD 5
0.024	1.73	EPSTI1	epithelial stromal interaction 1 (breast)
0.040	1.69	CD274	
0.004	1.65	XK	X-linked Kx blood group (McLeod syndrome)
0.024	1.62	CMPK2	cytidine monophosphate (UMP-CMP) kinase 2, mitochondrial
0.011	-1.60	SERPINB2	serpin peptidase inhibitor, clade B (ovalbumin), member 2
0.048	1.60	LAMP3	lysosomal-associated membrane protein 3
0.006	1.58	DDX60L	DEAD (Asp-Glu-Ala-Asp) box polypeptide 60-like
0.040	1.55	ELOVL7	ELOVL family member 7, elongation of long chain fatty acids (yeast)
0.037	1.52	OR2W3	olfactory receptor, family 2, subfamily W, member 3
0.040	-1.51	SH2D2A	SH2 domain protein 2A
0.038	1.51	FECH	ferrochelatase (protoporphyria)
0.013	1.51	TSPAN7	tetraspanin 7

Control Subjects

p-value	Fold Change	Gene	Description
0.042	1.77	KCNJ15	potassium inwardly-rectifying channel, subfamily J, member 15
0.011	1.75	GPR109A	G protein-coupled receptor 109A
0.014	1.74	GPR109B	G protein-coupled receptor 109B
0.023	1.70	ACSL1	acyl-CoA synthetase long-chain family member 1
0.009	1.69	PROK2	prokineticin 2
0.041	1.68	GK|GK3P	glycerol kinase | glycerol kinase 3 pseudogene
0.013	1.67	MGAM	maltase-glucoamylase (alpha-glucosidase)
0.040	1.66	ADM	adrenomedullin
0.042	-1.65	GZMH	granzyme H (cathepsin G-like 2, protein h-CCPX)

0.027	-1.65	KLRF1	killer cell lectin-like receptor subfamily F, member 1
0.029	1.64	FFAR2	free fatty acid receptor 2
0.005	1.63	ROPN1L	ropporin 1-like
0.007	1.63	VNN3	vanin 3
0.032	1.58	GPR97	G protein-coupled receptor 97
0.046	1.58	UNQ9368	RTFV9368
0.030	1.53	IL1B	interleukin 1, beta
0.013	1.52	STEAP4	STEAP family member 4
0.036	1.51	KREMEN1	kringle containing transmembrane protein 1
0.031	1.50	PTGS2	prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)

Differentially expressed genes determined by non-corrected p-value <0.05, |Fold change|>1.5.