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ORIGINAL RESEARCH

Elevated fractional exhaled nitric oxide and blood eosinophil counts are associated with a 17q21 asthma risk allele in adult subjects

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Background and objectives: Genome-wide association studies identified single-nucleotide polymorphisms (SNPs) at the 17q21 locus conferring increased risk for childhood-onset asthma. Little is known about how these SNPs impact adult asthma patients. We sought to examine an adult population for associations between rs7216389 (17q21-associated SNP) and features of asthma including fractional exhaled nitric oxide (FeNO), eosinophil counts, and age of asthma onset.

Methods: Subjects were genotyped at SNP rs7216389. The geometric mean of FeNO measurements and peripheral blood eosinophil counts from 2008 to 2015 were collected. Demographics and medical history were collected including self-reported allergy diagnoses and age of asthma onset. Eosinophils, monocytes, and peripheral blood mononuclear cells (PBMCs) were isolated for the examination of ORMDL3 expression.

Results: FeNO levels from 157 genotyped subjects (31CC, 72CT, and 54TT) and peripheral eosinophil counts from 252 genotyped subjects (46CC, 122CT, and 84TT) were analyzed. In a sub-group analysis of asthma subjects, the number of attributable T alleles was associated with significantly lower age of asthma onset (P=0.03) and greater FeNO levels (geometric mean 30.0 ppb TT, 20.0 ppb CT, 20.0 ppb CC, P=0.02). In the total cohort of subjects, the T allele was associated with a higher percentage of individual eosinophil counts >200/mm^3 (45% TT, 26% CT, 24% CC, P=0.005). Eosinophils expressed ORMDL3 mRNA and protein.

Conclusion: In adult subjects, the number of T alleles at SNP rs7216389 corresponds to significantly greater FeNO levels and peripheral eosinophil counts. The expression of ORMDL3 in eosinophils suggests that they may participate in mediating the asthma risk associated with the 17q21 locus.

Keywords: asthma, eosinophils, fractional exhaled nitric oxide, 17q21

Introduction

Asthma is a chronic inflammatory disease of the airways that affects 7.8% of the US population.[1] A family history of allergic disease is a significant risk factor for the diagnosis of asthma. Thus, there is a great interest in identifying the genetic determinants of asthma, which in turn would lead to targeted therapies. Among these efforts to identify asthma-related genes, there have been several genome-wide association studies.[2–6] One locus identified by genome-wide association studies, replicated in several different cohorts, is 17q21.[6–10] Additionally, multiple single-nucleotide polymorphisms (SNPs) at the 17q21 locus are expression quantitative trait loci (eQTLs) for genes in this region, [11,12] and these SNPs are specifically associated with childhood-onset asthma.[2]

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At one of the SNPs at the 17q21 locus, rs7216389, the TT genotype carries a 1.5 times increased risk of developing asthma.[13] Other studies have shown that a combination of the risk genotype and either smoke exposure or respiratory infections in childhood further amplifies asthma development risk.[8,14–17] In addition, the risk genotype at SNP rs7216389 is associated with disease severity in childhood-onset asthma.[7]

Long-term implications of the 17q21 genotype as children with asthma progress into adulthood are unknown. One study of adult asthmatics demonstrated decreased forced expiratory volume in 1 second (FEV1) levels with the risk allele.[18] However, little else is known about the relevance of this locus in adult asthmatics. We sought to determine whether the genotype at rs7216389 is associated with differences in FeNO and peripheral blood eosinophil counts, which are two well-characterized biomarkers that are often used to distinguish asthma phenotypes and to identify responders to inhaled corticosteroid therapy.

Methods

A cohort of 255 subjects provided written informed consent to participate in the University of Wisconsin–Madison's institutional review board-approved blood donation protocol. Individuals had physician-diagnosed asthma and/or allergic rhinitis. Individuals were between the ages of 18 and 55 years with no history of tobacco use within the past year. From 2008 to 2015, participants underwent single or repeated blood draws and fractional exhaled nitric oxide (FeNO) measurements and provided a medical history, which included self-reported ethnicity and age of asthma onset.

Genotyping

Whole blood was processed for DNA using the Gentra Puregene Blood Kit (Qiagen NV, Venlo, the Netherlands) according to the manufacturer's protocols. SNP rs7216389 genotypes were assessed using the TaqMan SNP genotyping assay (Thermo Fisher Scientific, Waltham, MA, USA).

FeNO measurements

The NIOX MINO (Aerocrine, Stockholm, Sweden) was used for FeNO measurements which were performed according to the American Thoracic Society guidelines.[19] For subjects with multiple measurements, the geometric mean was used for analysis.

Eosinophil counts

Whole blood was stained with eosin dye, and eosinophils were counted via hemacytometer. For subjects with multiple measurements, the geometric mean was used for analysis.

Cell isolation

Whole blood was centrifuged over Percoll (1.090 g/mL) to obtain the peripheral blood mononuclear cell (PBMC) fraction and the granulocyte fraction. Eosinophils were purified from the granulocyte fraction by negative selection using anti-CD16 immunomagnetic beads (MACS System; Miltenyi Biotec, San Jose, CA, USA). Blood eosinophils were >98% viable and pure. PBMCs were washed, and half the cells were collected as PBMCs and the remaining cells were used for monocyte isolation. Monocytes were isolated by negative selection using the Monocyte Isolation Kit II (Miltenyi Biotec), as per the manufacturer's instructions.

RNA isolation

Total RNA was isolated using an RNeasy Mini Kit (Qiagen NV) according to the manufacturer's instructions, including DNase I digestion to remove contaminating DNA. cDNA was generated using GoScript Reverse Transcriptase with random primers, nucleotides, and RNasin (Promega Corporation, Fitchburg, WI, USA). TaqMan primer and probe sets for the target gene (ORMDL3) and housekeeping/reference gene (β-actin) were obtained commercially (Thermo Fisher Scientific). Samples were run in duplicate with each reaction containing Universal Master Mix (Thermo Fisher Scientific), the primer and probe sets, and cDNA for a total reaction volume of 25 μL. Fluorescence was measured for each cycle using the StepOnePlus Real-Time PCR System (Thermo Fisher Scientific). Results were calculated using the $2^{-\Delta Ct}$ method relative to the endogenous control and β-actin and normalized to a donor monocytes' expression.

Western blot analysis

Whole-cell protein lysates were prepared from eosinophils or PBMCs in radioimmunoprecipitation assay buffer that included phosphatase inhibitors and complete protease inhibitors (Hoffman-La Roche Ltd., Basel, Switzerland/Thermo Fisher Scientific). Protein lysates were heated for 5 minutes at 75°C in 1× Laemmli sample buffer prior to loading, and the samples were resolved on 12% SDS-PAGE under reducing conditions. Proteins were transferred to polyvinylidene fluoride-Fl (EMD Millipore, Billerica, MA, USA), and the membranes were blocked with TBS blocking buffer (LI-COR Biosciences, Lincoln, NE, USA) +0.1% Tween for 1 hour at room temperature. Western blots were probed with rabbit anti-ORMDL3 (1:1,000; EMD Millipore) or mouse-anti-beta-actin (1:10,000; Sigma-Aldrich, St Louis, MO, USA). Blots were washed with TBS +0.05% Tween and then probed with secondary antibodies (680RD anti-mouse and 800CW

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anti-rabbit, 1:10,000; LI-COR Biosciences) diluted with TBS blocking buffer for 1 hour at room temperature. Western blots were washed with TBS +0.05% Tween, dried, and scanned using the Odyssey System (LI-COR Biosciences). Density measurements were captured using the Odyssey software.

Statistics

The number of measurements provided by each subject ranged from 1 to 27 for eosinophil counts and from 1 to 21 for FeNO levels. Eosinophil counts and FeNO levels for subjects providing multiple measurements were analyzed using the geometric mean of their measurements. FeNO levels and eosinophil counts were each log-transformed for analysis. Eosinophil counts were categorized as >200 or <200/mm^3, which is the historical cutoff used in our laboratory to distinguish "low" and "high" eosinophil blood donors. Age of onset was square root transformed for analysis. Genotype at rs7216389 was analyzed using the additive genetic model of risk according to the number of T alleles (CC =0, CT =1, and TT =2). Eosinophil counts, FeNO levels, and age of asthma onset were each examined for associations with genotype using the linear regression. The associations between eosinophil count category and genotype were examined using the logistic regression. A two-sided *P*-value of <0.05 was regarded as statistically significant. Analyses were conducted using R Version 2.15 (R Foundation for Statistical Computing, Vienna, Austria).

Results

Characteristics of the 255 subjects based on rs7216389 genotype classifications (47 CC, 123 CT, and 85 TT) are listed in Table 1. There were no associations between rs7216389 genotype and FEV1, self-reported allergies, age, or gender distribution. The TT genotype exhibited a higher rate of asthma diagnosis (CC 63.0%, CT 52.5%, and TT 76.2%; *P*=0.09), but this was not statistically significant. Subset subject characteristics with available FeNO data are listed in Table S1.

The at-risk T allele at SNP rs7216389 has been linked to early childhood-onset asthma; therefore, we examined the age of asthma onset by genotypes (Figure 1). Based on the self-reported age of asthma onset, a T allele was associated with lower age of asthma onset (CC median 12.0 years [25th–75th percentiles, 8.0–18.2], CT median 7.0 years [25th–75th percentiles, 6.0–17.0], and TT median 8.0 years [25th–75th percentiles, 4.0–15.5]; *P*=0.03).

FeNO levels from asthma subjects across the three genotypes are shown in Figure 2. The T allele was associated with increased FeNO levels in the subset of asthma subjects (CC 21 ppb [25th–75th percentiles, 16–33], CT 22 ppb [25th–75th percentiles, 15–35], TT 36 ppb [25th–75th percentiles, 25–48]; *P*=0.02). The use of inhaled corticosteroid can affect FeNO levels; therefore, we also analyzed a model of FeNO levels with both inhaled corticosteroid use and genotype

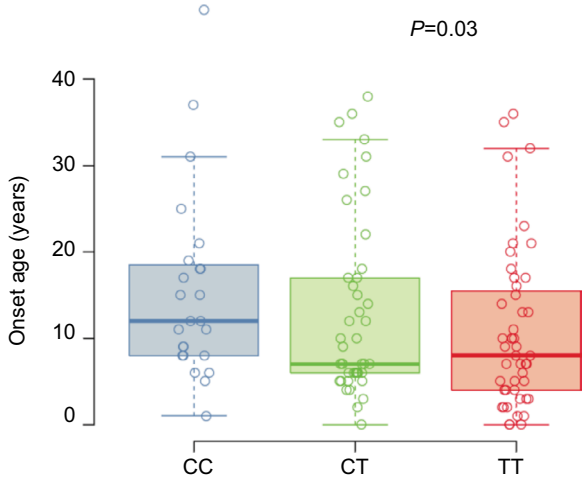

Figure 1 Self-reported age of asthma onset.
Notes: Box plots for the self-reported age of asthma onset in years for each subject (n=252) stratified by their genotype at SNP rs7216389, CC, CT, or TT. Boxes denote the median and 25th to 75th percentiles with whiskers extending to the farthest points within 1.5 times the box height. The *P*-value indicates the significance of the association between the number of T alleles and age of onset.
Abbreviation: SNP, single-nucleotide polymorphism.

Table 1 Study population

Genotype	CC	CT	TT
Population (n)	47	123	85
Age (mean)	28.7	27.6	28.0
Gender (male %)	45.7	40.2	47.6
Asthma (%)	63.0	52.5	76.2
Self-reported allergies (%)	91.3	85.2	84.5
Ethnicity (% Hispanic)	15.2	23.8	26.2
Race	2% American Indian	2% American Indian	1% American Indian
	2% Pacific Islander	3% Asian	4% Asian
	96% Caucasian	4% African American	9% African American
		91% Caucasian	86% Caucasian

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terms included. The estimated genotype effect was similar in the unadjusted and adjusted analyses indicating that the relationship between FeNO and rs7216389 genotype does not differ with the use of inhaled corticosteroid.

Subjects' average (geometric mean) eosinophil counts by genotype are shown in Figure 3A. The T allele at rs7216389 was associated with a higher percentage of subjects having an average eosinophil count of >200 cells/mm^3 (CC 23.9%, CT 26.2%, and TT 45.2%; *P*=0.005; Figure 3B). In the subset of asthma subjects, average eosinophil counts by genotype followed a similar pattern (CC 32.3%, CT 35.9%, and TT 46.9%; *P*=0.13) but was not statistically significant.

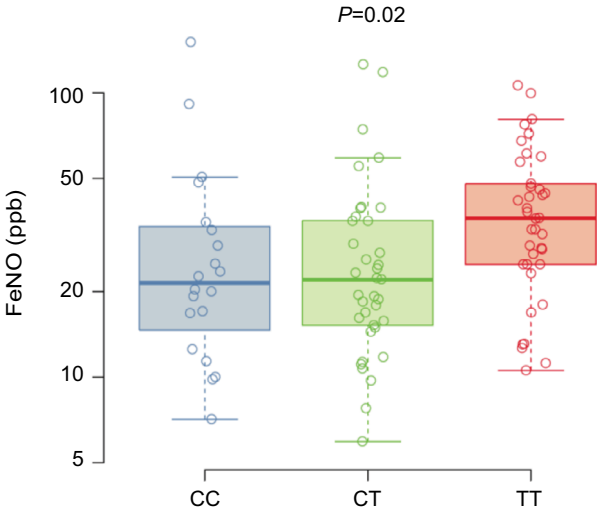

Figure 2 FeNO.
Notes: Box plots for the FeNO measured in ppb for the asthma subgroup (n=95) stratified by genotype at SNP rs7216389, CC, CT, and TT. Boxes denote the median and 25th to 75th percentiles with whiskers extending to the farthest points within 1.5 times the box height. The *P*-value indicates the significance of the association between the number of T alleles and FeNO levels.
Abbreviations: FeNO, fractional exhaled nitric oxide; SNP, single-nucleotide polymorphism.

In order to address a potential confounding influence of ethnicity/race on the genetic analyses, an additional analysis of the subset of Caucasian non-Hispanic individuals was completed. FeNO levels (Figure S1) and eosinophil counts (Figure S2) were analyzed. In this subset, the T allele remains associated with both FeNO levels (CC 20 ppb [25th–75th percentiles, 11–35], CT 19 ppb [25th–75th percentiles, 14–35], and TT 33 ppb [25th–75th percentiles, 22–46]; *P*=0.03; Figure S1) and eosinophil counts >200 cells/mm^3 (CC 24.3%, CT 33.3%, and TT 48.1%; *P*=0.02; Figure S2). Therefore, our observations persisted with the exclusion of a potential ethnic/racial confounder.

In order to address the possibility that the genotype association with FeNO and blood eosinophil count is confounded by the age of asthma onset, we adjusted for the age of onset. Among subjects with asthma, there was no significant difference in the FeNO fold increase per T allele unadjusted versus adjusted (1.22 versus 1.25, *P*=0.73). There was also no significant difference in the eosinophil count >200 cells/mm^3 odds ratio per T allele unadjusted versus adjusted (1.39 versus 1.32, *P*=0.29).

To determine whether eosinophils may be one of the cell types through which the 17q21 genes may be influencing asthma, we determined the expression of *ORMDL3*, one of the leading candidate genes at the 17q21 locus.[2,20] We examined the mRNA and protein expressions of *ORMDL3* in PBMCs and eosinophils. As shown in Figure 4, eosinophils expressed *ORMDL3* mRNA (Figure 4A) at levels compa-

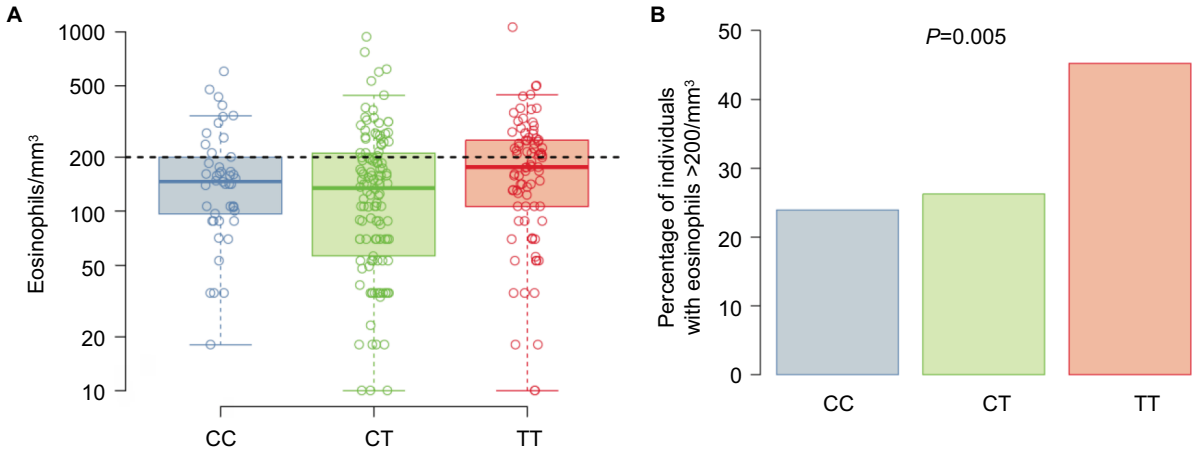

Figure 3 Eosinophil counts.
Notes: (**A**) Box plots for the geometric mean eosinophil counts for the entire cohort (n=252) stratified by genotype at SNP rs7216389, CC, CT, and TT. Boxes denote the median and 25th to 75th percentiles with whiskers extending to the farthest points within 1.5 times the box height. The eosinophil count of 200/mm^3 is shown as dashed line. (**B**) Percentage of subjects in entire cohort with geometric mean eosinophil count >200/mm^3 stratified by genotype at SNP rs7216389, CC, CT, and TT. The *P*-value indicates the significance of the association between the number of T alleles and the percentage of individuals with eosinophil counts >200/mm^3.
Abbreviation: SNP, single-nucleotide polymorphism.

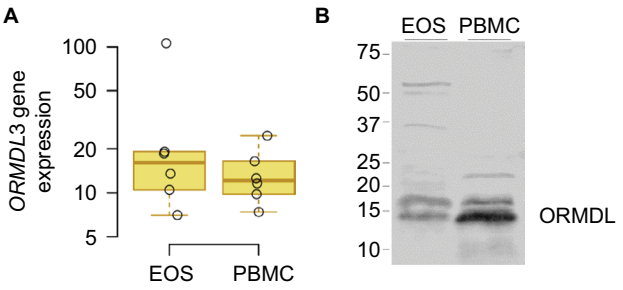

Figure 4 *ORMDL3* expression.
Notes: (**A**) EOS and PBMCs were isolated from six blood donors, mRNA was purified, and gene expression was measured by Q-PCR. Box plots of *ORMDL3* gene expression normalized to b-actin and shown as fold inductions relative to the control (monocyte population) from the same donor. Boxes denote the median and 25th to 75th percentiles with whiskers extending to the farthest points within 1.5 times the box height. (**B**) EOS and PBMC cell lysates were prepared from a representative donor and protein expression of *ORMDL* measured by Western blot.
Abbreviations: EOS, eosinophils; PBMCs, peripheral blood mononuclear cells.

rable to PBMCs, while ORMDL proteins (Figure 4B) were expressed, but fourfold less in total on a per cell basis. Of note, the antibody specificity in Western blots likely includes other isoforms of ORMDL and not just ORMDL3.

Discussion

Very little is known about the impact of genotypic differences at the 17q21 locus on the biology of asthma in adult subjects. In our cohort, regardless of allergy diagnosis, adults with the T allele at rs7216389 were more likely to have higher FeNO levels and eosinophil counts. In addition, a greater number of T alleles in the asthma population were associated with an earlier age of asthma onset compared to those individuals with more C alleles. In order to address whether eosinophils could mediate these genotypic effects at the 17q21 locus, we examined the mRNA and protein expressions of one of the genes encoded in the locus, ORMDL3. We found that eosinophils expressed appreciable levels of ORMDL3 mRNA and ORMDL protein indicating that eosinophils may be one of the cells mediating the risk associated with the 17q21 locus. These data are consistent with several reports in the literature; however, additional analyses of large cohorts will be necessary to confirm these findings.

There are several limitations of this study. The cohort examined was not specifically recruited for this study, and thus, this study has variability in the number of blood eosinophil and FeNO observations for each subject. We chose to use a single data point for each subject based on the geometric mean of their data. There is a recall bias associated with the determination of age of asthma onset; however, we expect that the error should be similarly present in each genotype group. The asthma population was generally mild asthma patients with only 19% of subjects using controller inhaled corticosteroid therapy; thus, it is unknown whether the genotype associations with blood eosinophil counts and FeNO would be stronger in more severe disease.

In previous ethnically diverse cohort studies, several SNPs at the 17q21 locus were linked to a diagnosis of childhood asthma.[2–5,9,21,22] The risk alleles at 17q21 were associated with an interaction between early infection and early onset asthma.[15] More recently, 17q21 SNPs were also linked to the increased risk of wheezing upon rhinovirus exposure and the development of asthma in a pediatric population.[17] However, whether 17q21 genotype has any relevance for asthma diagnosis, pathophysiology, or disease severity beyond childhood is not clear. In a European large cohort study, a 17q21 SNP exhibited strong association with childhood-onset asthma but did not show any association with adult onset asthma.[20] In three cohorts separately examining association of the rs7216389 T allele with asthma severity in early-age onset asthma patients, statistically significant associations were not present; however, the combined analysis of all three cohorts exhibited a significant association between the T allele and greater odds for severe asthma. In a study of adult severe asthma patients, the odds for an rs7216389 T allele versus C allele were significantly elevated;[7] however, this signal was entirely due to the severe asthma patients who had childhood onset of asthma.[23] In another cohort, there was no association between the rs7216389 SNP and a diagnosis of asthma in adults;[18] however, T allele was associated with both a lower FEV1 and the presence of allergic sensitization. Notably, the authors did not stratify the adult subjects based on childhood onset versus adult onset of asthma. It is intriguing to consider the possibility that the rs7216389 SNP may also be a risk factor for specific phenotypes of asthma; note that the asthma phenotype clusters from the NIH Severe Asthma Research Program identified one cluster that was notable for early age of onset, though it was also a milder form of asthma compared to the other phenotypes.[24] Our results confirm that the risk allele at rs7216389 SNP has relevance in adult patients. We demonstrated that the risk allele at rs7216389 SNP is associated with greater FeNO levels and higher peripheral blood eosinophil counts. Interestingly, these observations were not restricted to the early-onset asthma patients. We did not observe an association between the rs7216389 SNP and FEV1; however, our cohort consisted of primarily mild asthma patients with essentially normal FEV1.

The elevated FeNO levels and eosinophil counts in our cohort associated with the risk alleles at SNP rs7216839 represent two biomarkers for asthma. In a study designed

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to identify SNPs associated with elevated FeNO levels in children, one of the SNPs (rs8069176) was within the 17q21 locus.[25] Recent clinical trials with anti-IL-5 therapies have shown that clinical benefit is greatest in subjects with higher eosinophil counts.[26,27] In these studies, the peripheral eosinophil count was used as an inclusion criterion to enrich the study with potential responders. Based on our report, the SNP rs7216839 represents a genotypic biomarker in asthma. We speculate that the SNP rs7216839, possibly in conjunction with other SNPs, may serve as a genotypic biomarker and predictor of response to asthma therapies such as those currently available and in development for asthma. Traditional biomarkers are subject to variability due to medication use and comorbidities. Therefore, identification of combinations of genotypic biomarkers may provide a more accurate stratification of asthma phenotypes for achieving personalized care of asthma. Further studies of large cohorts will be essential to confirm and validate these results.

Efforts to understand the mechanism of the 17q21 association with asthma have largely centered on the function of two specific genes expressed at the loci, *ORMDL3* and *GSDMB*. Our study and others have shown that the expressions of these two genes are tightly correlated, making it difficult to identify which of these genes is directly involved in asthma pathophysiology.[17,28] In asthma, whether this genotype affects the functional activity of one or multiple cell types is also unknown. Previous studies demonstrated that the risk allele at the 17q21 locus is associated with greater ex vivo rhinovirus-induced expression of *ORMDL3* in PBMCs[17] and increased ex vivo IL-4 and IL-13 secretions from PHA-stimulated PBMCs.[29] Using an in vitro rhinovirus infection model, we recently demonstrated that increased *ORMDL3* in PBMC corresponded to increased *IFN-β*, *CXCL10*, and genes associated with endoplasmic reticulum stress.[30]

A mouse study has shown that Ormdl3 overexpression in eosinophils induced features consistent with asthma, including spontaneous airway remodeling, increased fibrosis, mucus production, inflammation, and methacholine-induced hyper-responsiveness.[31] Murine eosinophils expressed high levels of *Ormdl3* mRNA and protein, and Ormdl3 was shown to have a role in eosinophil activation.[32]

The function of ORMDL3 appears to involve the regulation of endoplasmic reticulum-based calcium homeostasis facilitating the upregulation of the cellular unfolded protein response.[33] There are also suggestions of ORMDL3 involvement in the inhibition of sphingolipid signaling, specifically sphingosine-1-phosphate. Further studies will be required to determine how these signaling events promote eosinophil production and activation.[33] We speculate that the increased signaling events in eosinophils in conjunction with other cell types such as monocytes may play a significant role in mediating the 17q21 genotype-related risk and severity of asthma in adult asthmatics.

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Disclosure

The authors report no conflicts of interest in this work.

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Supplementary materials

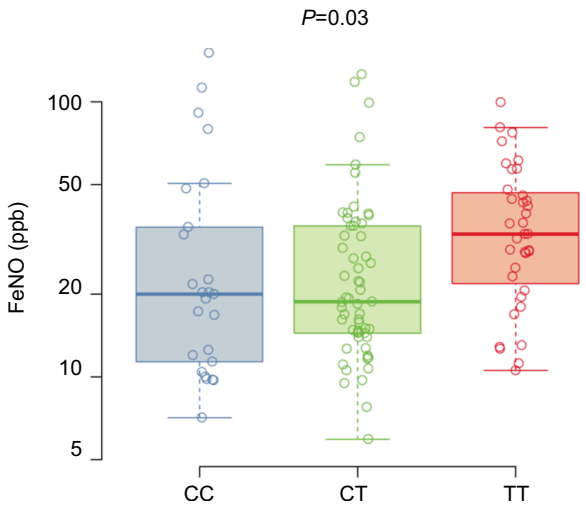

Figure S1 FeNO.
Notes: Box plots for the FeNO measured in ppb for the Caucasian non-Hispanic subgroup (n=120) stratified by genotype at SNP rs7216389, CC, CT, and TT. Boxes denote the median and 25th to 75th percentiles with whiskers extending to the farthest points within 1.5 times the box height. The *P*-value indicates the significance of the association between the number of T alleles and FeNO levels.
Abbreviations: FeNO, fractional exhaled nitric oxide; SNP, single-nucleotide polymorphism.

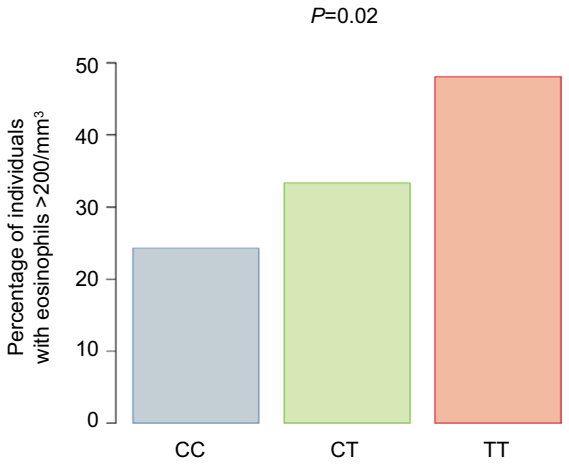

Figure S2 Percentage of donors in Caucasian non-Hispanic cohort (n=176) with geometric mean eosinophil counts >200/mm^3 stratified genotype at SNP rs7216389, CC, CT, and TT. The *P*-value indicates the significance of the association between the number of T alleles and the percentage of individuals with eosinophil counts >200/mm^3.
Abbreviation: SNP, single-nucleotide polymorphism.

Table S1 FeNO study population

Genotype	CC	CT	TT
Population (n)	20	37	38
Age (mean)	31.0	29.0	30.0
Gender (male %)	70.0	40.5	55.3
Self-reported allergies (%)	95.0	100	100
Ethnicity (% Hispanic)	15.0	5.4	10.5
Race	5% Pacific Islander 95% Caucasian	3% Asian 97% Caucasian	5% Asian 5% African American 90% Caucasian
FEV1% pred. (25th–75th percentile)	88.6% (80.1–98.5)	95.2% (85.5–109.0)	92.7% (84.5–103.5)

Abbreviations: FeNO, fractional exhaled nitric oxide; FEV1, forced expiratory volume in 1 second.

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