

Fungal Exposure Modulates the Effect of Polymorphisms of Chitinases on Emergency Department Visits and Hospitalizations

Ann Chen Wu¹⁻³, Jessica Lasky-Su⁴, Christine A. Rogers⁵, Barbara J. Klanderman³, and Augusto A. Litonjua^{3,4,6}

¹Department of Population Medicine, Harvard Pilgrim Health Care Institute and Harvard Medical School, Boston, Massachusetts; ²Department of Pediatrics, Children's Hospital, Boston, Massachusetts; ³Harvard Medical School, Boston, Massachusetts; ⁴Channing Laboratory and ⁶Division of Pulmonary and Critical Care Medicine, Department of Medicine, Brigham and Women's Hospital, Boston, Massachusetts; ⁵Environmental Health Sciences, University of Massachusetts, Amherst, Massachusetts

Rationale: Chitinases are enzymes that cleave chitin, which is present in fungal cells. Two types of human chitinases, chitotriosidase and acidic mammalian chitinase, and the chitinase-like protein, YKL-40, seem to play an important role in asthma. We hypothesized that exposure to environmental fungi may modulate the effect of chitinases in individuals with asthma.

Objectives: To explore whether interactions between high fungal exposure and common genetic variants in the two chitinases in humans, *CHIT1* and *CHIA*, and the chitinase 3-like 1 gene, *CHI3L1*, are associated with severe asthma exacerbations and other asthma-related outcomes.

Methods: Forty-eight single nucleotide polymorphisms (SNPs) in *CHIT1*, *CHIA*, and *CHI3L1* and one *CHIT1* duplication were genotyped in 395 subjects and their parents as part of the Childhood Asthma Management Program. Household levels of mold (an index of fungal exposure) were determined on house dust samples. We conducted family-based association tests with gene-environment interactions. Our outcome was severe exacerbation, defined as emergency department visits and hospitalizations from asthma over a 4-year period, and our secondary outcomes included indices of lung function and allergy-related phenotypes.

Measurements and Main Results: Of the 395 subjects who had mold levels at randomization, 24% (95 subjects) had levels that were greater than 25,000 units per gram of house dust (high mold exposure). High mold exposure significantly modified the relation between three SNPs in *CHIT1* (rs2486953, rs4950936, and rs1417149) and severe exacerbations (*P* for interaction 0.0010 for rs2486953, 0.0008 for rs4950936, and 0.0005 for rs1417149). High mold exposure did not significantly modify the relationship between any of the other variants and outcomes.

Conclusions: Environmental exposure to fungi, modifies the effect of *CHIT1* SNPs on severe asthma exacerbations.

Keywords: chitinase; asthma; *CHIA*; *CHIT1*; *CHI3L1*

The pathogenesis of asthma, which affects up to 300 million people of all ages in the world and 15 million people in the United States (1), is mediated by immunologic responses that parallel those induced by parasitic infections. Furthermore,

(Received in original form March 1, 2010; accepted in final form June 10, 2010)

Supported by the Childhood Asthma Management Program through contracts NO1-HR-16044, 16045, 16046, 16047, 16048, 16049, 16050, 16051, and 16,052 with the National Heart, Lung, and Blood Institute and General Clinical Research Center grants M01RR00051, M01RR0099718-24, M01RR02719-14, and RR00036 from the National Center for Research Resources. This work was also supported by U01 HL65899. Dr. Litonjua is supported by R01 AI056230.

Correspondence and requests for reprints should be addressed to Ann Wu, M.D., M.P.H., Department of Population Medicine, 133 Brookline Avenue, 6th Floor, Boston, MA 02215-5301. E-mail: ann.wu@childrens.harvard.edu

Am J Respir Crit Care Med Vol 182, pp 884-889, 2010

Originally Published in Press as DOI: 10.1164/rccm.201003-0322OC on June 10, 2010
Internet address: www.atsjournals.org

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Chitinases are enzymes that cleave chitin, which is present in fungal cells. Two types of human chitinases, chitotriosidase and acidic mammalian chitinase, and the chitinase-like protein, YKL-40, seem to play a role in asthma.

What This Study Adds to the Field

Fungal levels may modify the effect of variants in the chitinase gene, *CHIT1*, and chitinases may play an important role in asthma.

fungal exposure seems to be associated with severe asthma and admission to the intensive care unit for asthma (2, 3). Mammalian chitinases and a chitinase homolog that lacks chitinase activity may contribute to the pathogenesis of T-helper 2 (Th2) immune responses and thereby play an important role in asthma (4-6). Chitin is a polysaccharide that is present in fungal cells, crustaceans, insects, and parasitic nematodes, and chitinases cleave chitin (7-9). Chitin is present in the cell walls of fungi and provides architectural reinforcement to the cell walls; thus, exposure to fungi correlates with exposure to chitin (10). Chitinases seem to play an important role in Th2-mediated inflammation and allergic diseases, such as asthma. A study in rats found that intratracheal infection with a fungus, *Cryptococcus neoformans*, induces generalized chitinase activity in bronchoalveolar lavage fluid and lung, suggesting a link between respiratory fungal infection and asthma through the induction of chitinase (11).

Although chitin does not exist in humans, two chitinases, acidic mammalian chitinase (*CHIA*) and chitotriosidase (*CHIT1*), have been described in humans (7). A third protein, chitinase-like protein YKL-40 (also known as "human cartilage glycoprotein 39" and "chitinase 3-like 1") also seems to play an important role in asthma (6). These two chitinases perform important defensive functions against chitin-containing pathogenic organisms in both the gastrointestinal tract and lungs. *CHIT1* is primarily expressed in the lung, whereas *CHIA* is highly expressed in the lungs of patients with asthma, but not in normal subjects. In studies of mice, *CHIA* seems to play an influence the development of bronchial asthma (4). Inhibition of *CHIA* in mice leads to an abrogated Th2 inflammation, reduced bronchial hyperreactivity, and lower eosinophil counts (4). Studies have also found that chitin may protect mice from asthma (12). Levels of YKL-40 are higher in patients with asthma than healthy persons, and patients with more severe asthma have higher levels of YKL-40 (6).

We have previously reported that variants in *CHITI*, *CHIA*, and *CHI3LI* are not associated with asthma or asthma-related phenotypes (13). However, we hypothesized that the effects of these genes may only be increased in the appropriate environmental context, in this case exposure to high levels of house dust fungi.

The objectives of this study were to assess whether fungal exposure modulates the effect of variants in *CHITI*, *CHIA*, and *CHI3LI* and one *CHITI* duplication on emergency department visits and hospitalizations from asthma. We hypothesized that exposure to fungi, as a source of environmental chitin exposure, would influence the association of single nucleotide polymorphisms (SNPs) in the genes of chitinases and chitinase-like protein because these genes have been found to be associated with asthma severity, fungal exposure is known to be associated with asthma symptoms, and higher fungal levels suggest higher chitin levels. Our secondary objectives were to assess whether fungal exposure modulates the association of these SNPs and changes in lung physiology that are associated with asthma or allergy-related phenotypes.

METHODS

Study Population

We used data from the Childhood Asthma Management Program (CAMP), a multicenter trial that enrolled children between the ages of 5 and 12 years with mild to moderate persistent asthma between 1993 and 1995 (14). We included data from 395 white subjects and their parents. Subjects were randomly assigned to receive budesonide, nedocromil, or placebo and were followed every 2 to 4 months for 4 years to study the long-term use of the medications. Details of this study have been previously published (14). The institutional review board at each of the eight participating institutions approved the study and parents or guardians of the subjects gave informed consent (14).

Measures

Mold measures (an index of fungal exposure) in the home environment were taken by research assistants. Details have been described previously (14, 15). Briefly, during a study visit, a CAMP-certified technician used a vacuum cleaner (Douglas Redivac model 6735; Scott-Fetzer, Walnut Ridge, AZ) fitted with a disposable filter to collect a dust sample 2 m² from the upper part of the patient's mattress cover, 1 m² of bedroom floor or carpet, 1 m² of living room–family room floor or carpet, 1 m² of the kitchen floor, and a major item of upholstered furniture to which the child was exposed. Each area was vacuumed for 2 minutes. The specimens were shipped frozen to the Dermatology Allergy Clinical Immunology Laboratory at Johns Hopkins University, Baltimore, MD, where they were analyzed. An agar plate was streaked for enumeration of mold colonies, and the colonies were not further identified. The mold colonies were reported in units per gram of house dust. We chose to stratify the mold levels by greater than or less than or equal to 25,000 units per gram as used in a previous CAMP study (15).

At each study visit, which occurred every 4 months, subjects reported on the number of hospitalizations and emergency department visits they had experienced since the last study visit (16). Research assistants obtained spirometry measurements on the subjects both before and after a bronchodilator at each study visit. Bronchodilator response was calculated at each visit as FEV₁ ([postbronchodilator FEV₁ – prebronchodilator FEV₁]/prebronchodilator FEV₁). Each year, the subjects' airway responsiveness to methacholine was measured by calculating the concentration of methacholine that caused a 20% decrease in the FEV₁. The concentration that provoked a 20% decrease from postdiluent FEV₁ was obtained by linear interpolation of logarithmic dose–response curve expressed as PC₂₀. Additional demographic information was obtained at an initial prerandomization study visit.

SNP Genotyping

SNPs in *CHITI*, *CHIA*, and *CHI3LI* were genotyped, in addition to a *CHITI* duplication, which has been previously studied (17). SNPs in CAMP were genotyped using the Infinium HumanHap550 genotyping at Illumina (San Diego, CA). Genotyping quality was evaluated using the program PLINK (V1.01). SNPs with low Illumina gencall scores, poor completion rates, or four or more parent–offspring genotyped inconsistencies were dropped. Using the Basic Local Alignment Search Tool, SNPs were further limited to those whose flanking sequences were reliably mapped to unique autosomal locations in the hg17 reference genome sequence. Mitochondrial and sex-linked markers were not included. The *CHITI* fragment analysis was performed using the Applied Biosystems 3100 Genetic Analyzer platform. Primers CHIT1_A1FGTCTGGATGAGGGGATCG-FAM and CHIT1_A1RGTTCCTCCCTGCACAGGTCAGCTATC were used to polymerase chain reaction amplify the region containing the 24-bp duplication, and peaks were analyzed with Applied Biosystems GeneMapper software (Carlsbad, CA). Genotyping completion rate was 94%.

Family-Based Association Test Generalized Estimating Equations

Family-based association test generalized estimating equations (FBAT-GEE) is a method that has the ability to use genetic data from family members to assess potential associations between a disease phenotype and a gene allele (18, 19). This methodology has been robust in identifying the associations between SNPs with complex diseases, particularly in genome-wide association studies. We performed association analyses for each SNP and each phenotype using the FBAT-GEE approach, which has been described previously (20). We used Vansteelandt and coworkers' method (21), which uses causal inference to derive estimating equations that generate an estimate of the main genetic effect, β_1 , and the gene-by-environment interaction, β_2 , after accounting for the main genetic effect. The general principle behind FBAT interaction is that after removing the overall main genetic effect, the phenotype should not depend on the genotypes conditional on the environmental exposure under the null hypothesis (22).

In the analysis for this study, the additive genetic model was used and a minimum of 20 informative families were required. We used an FBAT approach with GEE (23) for our outcomes. The main outcome was severe exacerbations defined as one or more hospitalizations or emergency department visits experienced during the 4 years of the study.

RESULTS

Descriptive Statistics

Our study population included 395 white subjects who had available genotype information and mold levels. The mean age was 8.7 years (SD 2.1). Table 1 provides the baseline demographic characteristics measured in our study population. The mean age of the subjects with mold levels of greater than 25,000 units per gram was 8.7 years, and 8.8 years for subjects with mold level of less than or equal to 25,000 units per gram ($P = 0.68$). Of the subjects with mold level greater than 25,000 units per gram, 33% were in the budesonide group, whereas 26% of subjects with mold level less than or equal to 25,000 units per gram were in the budesonide group; this difference was not statistically significant. A slightly higher percentage of subjects in the group with mold level greater than 25,000 units per gram were male (65% vs. 61%; $P = 0.49$). There were no differences between the groups of subjects with mold level greater than 25,000 units per gram and less than or equal to 25,000 units per gram with respect to the total number of hospitalization and emergency department visits over the 4-year period of the trial, pre-FEV₁, bronchodilator response, FEV₁ percent predicted, ln PC₂₀, log₁₀ IgE, log₁₀ eosinophil, parental history of asthma or atopy.

TABLE 1. DEMOGRAPHICS

N = 395 Mean [SD] or Percent (n)	Mold Level >25,000 Units Per Gram (n = 95)	Mold Level ≤25,000 Units Per Gram (n = 300)	P Value
Age in years [SD] (range)	8.70 [1.95]	8.80 [2.16]	0.68
Treatment group			0.23
Budesonide	31 (33%)	78 (26%)	
Nedocromil	22 (33%)	95 (32%)	
Placebo	42 (44%)	127 (42%)	
Sex			0.49
Male	62 (65%)	184 (61%)	
Female	33 (35%)	116 (39%)	
Weight at baseline, kg	30.76 [9.36]	32.30 [11.07]	0.22
Height at baseline, cm	131.08 [12.75]	133.09 [14.04]	0.22
Total number of hospitalization and emergency department visits over 4-yr period			0.64
0	67	209	
1	14	41	
2	7	19	
≥3	7	31	
Baseline pre-FEV ₁	1.59 [0.43]	1.64 [0.48]	0.33
Baseline bronchodilator response	0.105 [0.093]	0.11 [-0.11]	0.79
Baseline FEV ₁ percent predicted	93.60 [13.79]	93.3 [13.94]	0.88
Baseline ln PC ₂₀	-0.095 [1.24]	0.069 [1.11]	0.23
Baseline log ₁₀ IgE	2.61 [0.65]	2.61 [0.66]	0.98
Baseline log ₁₀ eosinophil count	2.56 [0.48]	2.57 [0.48]	0.91
Paternal history of asthma			0.27
Present	24 (26%)	61 (21%)	
Absent	67 (74%)	231 (79%)	
Paternal history of atopy			0.29
Present	42 (45%)	112 (38%)	
Absent	52 (55%)	179 (62%)	
Maternal history of asthma			0.40
Present	22 (23%)	82 (28%)	
Absent	72 (77%)	213 (72%)	
Maternal history of atopy			0.86
Present	43 (46%)	50 (47%)	
Absent	50 (54%)	156 (53%)	

FBAT Analysis

We studied 395 subjects and their parents, and there was one affected offspring within each family. Table 2 shows the findings for the SNP-by-mold level interaction on hospitalizations and emergency department visits. We present the number of informative families for each SNP and the uncorrected FBAT–interaction *P* values. Ten SNPs had significant FBAT–GEE *P* values (*P* < 0.05) for the interaction. After adjusting for multiple comparisons, the significant SNPs are bolded and the interaction estimate, β_2 , is given. Mold exposure significantly modified the relation between three SNPs in *CHIT1* (rs2486953, rs4950936, and rs1417149) and one or more emergency department visit or hospitalization from asthma. We found that a mold level of greater than 25,000 units per gram modified the relationship of rs2486953 with one or more emergency department visits or hospitalizations (FBAT–interaction *P* value 0.0010); a mold level of greater than 25,000 units per gram modified the relationship of rs4950936 (FBAT–interaction *P* value 0.0008) and mold level modified rs1417149 (FBAT–interaction *P* value 0.0005). These three SNPs in *CHIT1* are in linkage disequilibrium (Figure 1), and are located within an intron of *CHIT1*. Figure 2 depicts the increased number of emergency department visits and hospitalizations when exposed to a mold level of greater than 25,000 units per gram when possessing two copies of the genotype compared with one copy for rs2486953.

Mold exposure did not modify the relationship between polymorphisms in *CHIA* or *CH13LI* with hospitalizations or emergency department visits. Furthermore, SNP-by-mold level

interaction was not associated with the secondary asthma or allergy phenotypes. More specifically, mold exposure did not modify the relationship between polymorphisms of *CHIT1*, *CHIA*, or *CH13LI* and pre-FEV₁, bronchodilator response, FEV₁ percent predicted, ln PC₂₀, log₁₀ IgE, and log₁₀ eosinophil.

DISCUSSION

Mold levels may modify the effect of variants in the chitinase gene, *CHIT1*, on emergency department visits and hospitalizations from asthma. We also found that mold levels do not modify the association between other variants in *CHIT1*, and variants in both *CHIA* and *CH13LI* and childhood asthma or asthma-related phenotypes. To our knowledge, this was the first study to examine the effect of mold levels on the association of SNPs in the genes of both chitinases and chitinase-like proteins with asthma and allergy-related phenotypes. Strengths of our study include the availability of mold levels in a well-defined clinical trial, the availability of outcomes over a 4-year time period, and a family-based design that avoids issues with population stratification.

Our results support increasing evidence that *CHIT1*, which is primarily expressed in the lung, plays an important role in the pathophysiology of asthma in the proper environmental context of exposure to chitin, which was approximated by mold levels (24). Our results are supported by a study of workers in the snow crab–processing industry who are exposed to high levels of chitin (found in the exoskeletons of crustaceans), which found that cumulative exposure to snow crab allergens is associated

TABLE 2. ASSOCIATION OF THE SINGLE NUCLEOTIDE POLYMORPHISMS IN CHIT1, CHIA, AND CHI3L1 WITH ONE OR MORE HOSPITALIZATIONS OR EMERGENCY DEPARTMENT VISITS WITH GENE-ENVIRONMENT INTERACTION

Gene	Marker	Allele	Minor Allele Frequency	Number of Informative Families	Beta Estimate for Interaction	FBAT-GEE P Value for Interaction	Beta Estimate for Main Effect	FBAT-GEE P Value for Main Effect	
<i>CHIT1</i>	rs4950934	1	0.11	160	0.0652	0.572	0.442	-0.0462	
	rs2486953	2	0.47	340	0.278	0.0010	0.173	-0.0562	
	rs2486954	3	0.20	250	0.0871	0.283	0.794	-0.0115	
	rs12141375	1	0.20	251	0.0871	0.283	0.794	-0.0115	
	rs4950936	3	0.47	340	0.284	0.0008	0.138	-0.0609	
	rs4950937	1	0.28	275	0.297	0.006	0.283	-0.0561	
	rs872583	2	0.19	252	0.0688	0.399	0.810	-0.0108	
	rs1417149	2	0.47	339	0.309	0.0005	0.148	-0.0607	
	rs3831317*	2	0.17	272	0.0050	0.961	0.525	-0.0334	
	rs2486958	2	0.49	339	0.223	0.0100	0.364	-0.0399	
	rs1556854	2	0.49	340	0.247	0.007	0.371	-0.0397	
	rs2486959	3	0.17	234	0.0397	0.649	0.935	0.0038	
	rs946257	3	0.31	289	0.270	0.0082	0.311	-0.0567	
	rs2486068	3	0.17	233	0.0449	0.634	0.871	-0.0080	
	rs2297950	4	0.31	288	0.259	0.0121	0.345	-0.0539	
	rs2486070	1	0.17	232	0.0522	0.578	0.782	-0.0136	
	rs3766537	4	0.19	241	0.154	0.204	0.347	0.0538	
	rs1417150	2	0.47	330	0.1200	0.0122	0.133	-0.0646	
	rs2486072	3	0.35	322	0.0533	0.558	0.365	0.0408	
	rs12747110	4	0.01	28	0.901	0.0141	0.681	0.0597	
	rs2494287	4	0.13	177	0.202	0.156	0.9208	-0.0060	
	<i>CHIA</i>	rs4240529	1	0.28	222	0.0783	0.456	0.1138	-0.0693
		rs4272622	2	0.19	167	-0.158	0.194	0.4753	-0.0447
		rs11102233	4	0.26	208	-0.249	0.061	0.6353	-0.0274
		rs12401737	4	0.46	264	-0.0252	0.775	0.5242	-0.0299
		rs10857871	2	0.21	188	0.191	0.089	0.0112	-0.1237
rs3806448		1	0.48	261	-0.0786	0.471	0.8169	-0.0118	
rs10494132		2	0.22	208	0.0817	0.597	0.0036	0.2003	
rs3806446		2	0.45	270	-0.0072	0.943	0.3928	-0.0435	
rs7411387		2	0.40	239	-0.0299	0.763	0.0591	-0.0904	
rs11584291		4	0.31	243	0.0633	0.581	0.6792	-0.0233	
rs4240530		2	0.29	245	-0.0876	0.414	0.3206	0.0506	
rs12127313		1	0.14	149	0.118	0.425	0.3740	0.0545	
rs10494133		2	0.14	159	-0.0685	0.601	0.5605	0.0444	
rs3818822		1	0.10	124	-0.0623	0.672	0.5302	0.0387	
rs12034576		3	0.33	242	-0.0233	0.846	0.9829	0.0011	
rs10494134		4	0.46	269	0.0301	0.789	0.6086	0.0236	
rs2275253		1	0.29	236	0.0180	0.893	0.1084	-0.0948	
rs2275254		4	0.40	261	-0.0062	0.964	0.3007	-0.0573	
rs2256721		4	0.29	225	0.0061	0.968	0.1387	-0.0955	
rs2820093		4	0.10	126	-0.0605	0.685	0.5542	0.0378	
rs2282290		3	0.47	267	-0.0563	0.609	0.6820	0.0208	
rs12034177		2	0.33	241	-0.0204	0.864	0.9840	-0.0010	
rs10776724		2	0.45	262	-0.0202	0.886	0.3560	-0.0539	
rs12137697		4	0.13	141	0.146	0.329	0.6063	0.0330	
<i>CHI3L1</i>		rs7542294	1	0.15	168	-0.212	0.399	0.8317	-0.0186
		rs880633	2	0.49	254	0.047	0.585	0.8918	0.0068
	rs10399805	1	0.13	149	-0.178	0.505	0.3133	-0.0958	
	rs946261	2	0.40	252	-0.105	0.342	0.8310	-0.0115	

Definition of abbreviation: FBAT-GEE = family-based association test generalized estimating equations.

* rs3831317 is a 24-bp duplication in *CHIT1*.

Significant SNPs are bolded.

with prevalence of occupational asthma and allergy in a dose-response manner, even after adjusting for age, sex, and smoking (25). Furthermore, intranasal administration of chitin to mice induces the accumulation of IL-4 expressing innate immune cells, inducing eosinophils and basophils (26), further lending support to the notion that exposure to chitin induces an allergic phenotype. Chitinases seem to be able negatively to regulate the tissue infiltration of eosinophils and basophils (26). Thus, the level of enzymatic activity of chitinases may be protective against development of allergies or asthma by breaking down chitin. Additional evidence supporting our hypothesis is a pilot study that found that subjects with the *CHIT1* genotype that correlates with decreased levels of chitotriosidase had increased

susceptibility to filarial infection (27). These findings in the pathophysiology of asthma support our finding that *CHIT1* may be associated with hospitalizations and emergency department visits from asthma in the setting of varying mold exposures.

Previous studies have found conflicting results on whether genes of chitinases and chitinase-like proteins are not associated with asthma-related phenotypes. Some studies have suggested that variants in *CHIT1*, *CHIA*, and *CHI3L1* are not associated with asthma or other asthma phenotypes (13, 28, 29). On the other hand, previous studies found that polymorphisms in *CHIA* are associated with asthma and IgE levels (30, 31). Ober and co-workers (32) concluded that SNPs in *CHI3L1* are associated with bronchial hyperresponsiveness in the Hutterite population. Be-

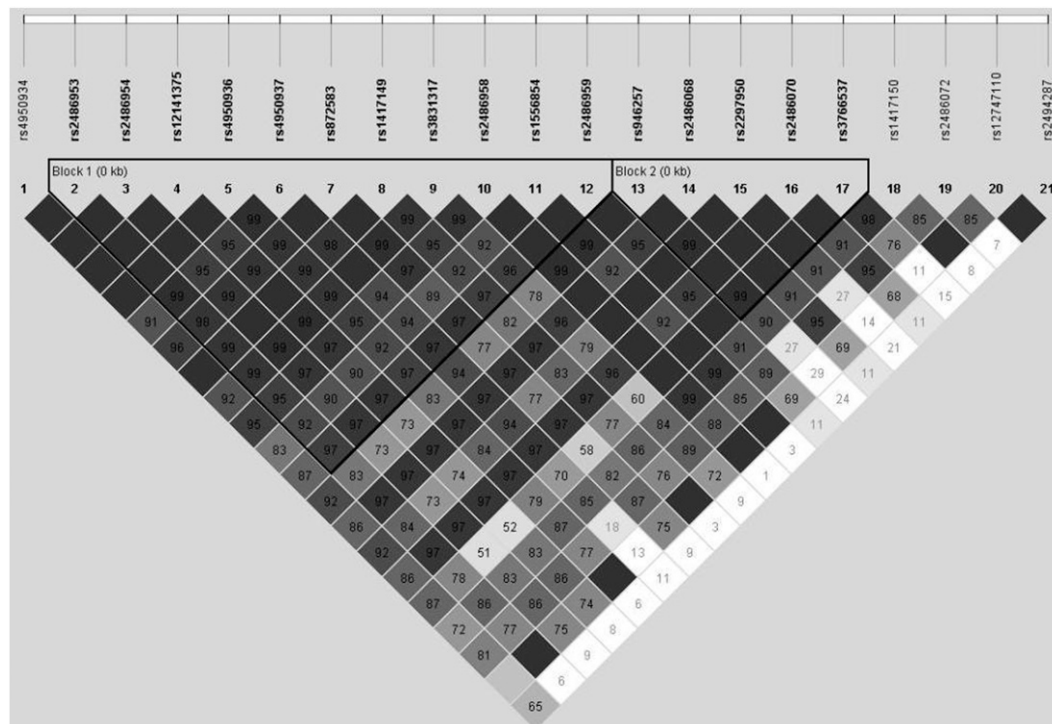


Figure 1. Linkage disequilibrium plot for *CHIT1* demonstrating that rs2486953, rs4950936, and rs1417149 are in linkage disequilibrium with each other. Linkage disequilibrium is measured as D' , with darker gray colors indicating higher values. The number in each box represents the r^2 between the two corresponding SNPs.

cause this is a relatively isolated population, with similar environmental exposure, this finding may avoid some confounding effects of genetic and environmental heterogeneity (32). One potential reason for the conflicting findings in the literature is environmental heterogeneity in exposure to sources of chitin between populations, and we show that accounting for environmental exposure to mold levels may help to clarify these genetic associations.

Despite the strengths of our study, a few caveats deserve mention. First, our sample size of 395 subjects was relatively

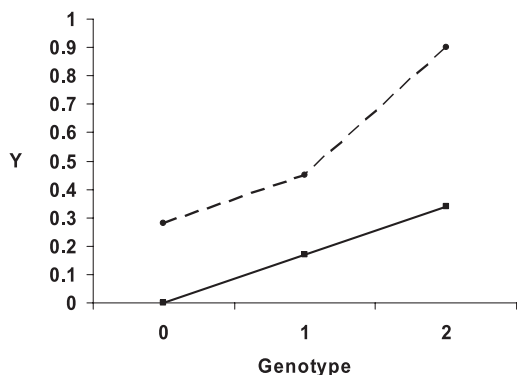


Figure 2. Depiction of the effect of mold level >25,000 units per gram and genotype on the outcome of hospitalizations and emergency department visits for rs2486953. E = 1 refers to exposure to a mold level of >25,000 units per gram and E = 0 refers to a mold level of ≤25,000 units per gram. G stands for genotype and there are three possibilities for the number of copies of rs2486953: 0 copies, 1 copy, or 2 copies. Y refers to the probability of having one or more hospitalizations and emergency department visits. This figure demonstrates that with exposure to a mold level of >25,000 units per gram, having two copies of the minor allele produces a much higher probability of experiencing hospitalizations and emergency department visits than with no exposure to this mold level.

small. Nevertheless, we did find that mold exposure significantly modified the relation between three SNPs in *CHIT1* and one or more emergency department visits or hospitalizations from asthma. Second, our analysis was limited to one population and we did not have a replication population for study; thus, our results may not be generalizable to other populations. To our knowledge, no other longitudinal clinical trials of asthma have measured mold levels as an exposure; thus, we do not have other populations to replicate our findings. A recent review article on gene-by-environment interaction in asthma mentioned that the study of gene-environment interaction in relation to asthma is in its infancy (33). Thus, our findings provide support for future studies examining gene-environment interactions in asthma, and should encourage evaluation of environmental exposures in these studies. In addition, we only included mold measurement from the randomization visit. Although a second mold measurement was attempted at the 3-year visit, 23% of subjects did not have a mold measurement, and FBAT does not allow repeated measurements for the environment variable. Finally, it is likely that mold exposure is only one source of chitin exposure. A comprehensive evaluation of all sources of environmental chitin exposure was beyond the scope of this study.

Both *in vitro* and *in vivo* studies have demonstrated that chitin and chitin derivatives have important immunologic effects and play an important role in pulmonary inflammation (34). The literature suggesting the importance of chitinases in the pathophysiology of asthma is strong (4–9, 12), and chitinases may play a role in future targets for asthma therapy (24). In future genetic studies of asthma, measurements of fungal levels could contribute important knowledge on the pathophysiology of asthma.

In conclusion, fungal levels may modulate the effect of variants in the chitinase gene, *CHIT1*, on emergency department visits and hospitalizations. This finding supports the important role that chitinases have in asthma.

Author Disclosure: A.C.W. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. J.L.S. received up to \$1,000 from Golden Helix for providing statistical support. C.A.R. does not

have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. B.J.K. holds a patent from the Massachusetts Institute of Technology (US Patent 6,703,228) for methods and products related to genotyping and DNA analysis, receiving \$0 in the past 3 years. A.A.L. received \$1,001–\$5,000 from Up To Date in author royalties and more than \$100,001 from the National Institutes of Health in sponsored grants.

Acknowledgment: The authors thank Brooke Schuemann, M.P.H., for her assistance with preparation of the phenotype data. The authors also thank all subjects for their ongoing participation in this study. We acknowledge the CAMP investigators and research team, supported by the National Heart, Lung, and Blood Institute, for collection of CAMP Genetic Ancillary Study data. All work on data collected from the CAMP Genetic Ancillary Study was conducted at Channing Laboratory of the Brigham and Women's Hospital under appropriate CAMP policies and human subject's protections. CAMP is supported by U01 HL076419, U01 HL65899, P01 HL083069, and T32 HL07427 from the National Heart, Lung, and Blood Institute, National Institutes of Health.

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