UCSF
UC San Francisco Electronic Theses and Dissertations

Title
The Genetics and Function of Chitinases in Human Asthma

Permalink
https://escholarship.org/uc/item/13m6p09x

Author
Seibold, Max Anthony

Publication Date
2008-08-06

Peer reviewed|Thesis/dissertation

The Genetics and Function of Chitinases in Human Asthma

by

Max Anthony Seibold

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Pharmaceutical Sciences and Pharmacogenomics

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO

in memory of Max R. Seibold,

Whose sacrifices have made my scientific endeavors possible

Acknowledgements

Abstract

The Genetics and Function of Chitinases in Human Asthma

Max A. Seibold

Asthma is a disease characterized by chronic inflammation of the airway, thought to result from inappropriate activation of the Th2 immune response. Recent work has shown that chitinase proteins are strongly upregulated during Th2 inflammation in the lungs of mice. Studies in mouse models have implicated chitinase activity in both promotion of Th2 inflammation and protection from the Th2-priming effects of chitin in the lungs.

Herein we detail investigation of chitinases in human asthma. Namely, we explored the possibility that variants affecting the enzymatic activity of the two active chitinase proteins, acidic mammalian chitinase (AMCase) and chitotriosidase (CHIT1), may alter risk for human asthma. Secondly, we have determined expression and activity of these enzymes in lungs of healthy and asthmatic subjects.

We identified a coding haplotype in the *AMCase* gene strongly associated with protection from asthma among African American asthma case-control groups. This association was also observed in family-based analysis of a group of Latino American asthmatic trios. Through recombinant protein expression and biochemical characterization we have found the protective AMCase isoform to be considerably more active at pH values corresponding to the lung, stomach, and

lysosomes. In contrast, no association was observed with a common null genetic variant in the *CHIT1* gene in either African Americans, or Latinos.

We found CHIT1 to be responsible for the majority of chitinase activity in the human lung based on pH profile of enzymatic activity and genetic analysis of a *CHIT1* null variant. Median chitinase activity in BAL tended to be lower than normal in asthma. AMCase protein was detectable in the lung, but mRNA transcripts expressed from lung tissues was consistent with an isoform lacking enzymatic activity. AMCase protein expression was observed to be lower in the BAL of asthmatics.

This work is the first evidence that functional genetic variants in chitinase genes are associated with risk for asthma, and are consistent with possible Th2-priming effects of environmental chitin. Additionally, we find differential expression of the two active chitinases in the human lung and reveal differences in chitinase protein expression and enzymatic activity in lung disease.

Table of Contents ....**Page**

List of Tables

List of Figures

List of Appendix Tables

Chapter 1. Background

1.1 Asthma Genetics in African Americans and Latinos

1.1.1 Race and Ethnicity

Although there is much debate regarding the precise definition and boundaries of race and ethnicity, both of these classifications capture important information which can be used to tease apart the complex etiologies of asthma. Definitions of race are generally based on ancestral geographical origin. Since genetic differentiation in human populations is based upon mating patterns largely dependent on geographical factors, race is often representative of a shared genetic makeup [1]. Ethnicity is a more general term describing groups with shared characteristics including language, religious beliefs, dietary habits, and culture. Latino is an ethnic term that encompasses multiple racial or national groups that share a common culture and language [2]. As a whole, Latinos account for 14% of the U.S. population and are the fastest growing minority population [2].   In the United States, Latinos include Mexicans, Puerto Ricans, Cubans, Dominicans, Central Americans and South Americans. Despite being all considered Latinos these groups exhibit great diversity with regards to socioeconomic status, education, and genetic ancestry [2].   African American refers to a U.S. racial group defined by people deriving at least a portion of their ancestry from Sub-Saharan African nations, and account for 12.3% of the U.S. population [3]. Most African Americans are descendents from people forcibly

migrated to the United States in the African Diaspora and therefore sharing common historical and cultural contexts [4]. Similar to Latinos, within the African American population there exist differences in social class, economic status, and education level. The diversity in these outcomes, which are related to asthma susceptibility and severity, has great value in epidemiological studies for discerning the role of multiple risk factors in asthma traits.

1.1.2 Racial Disparities in Asthma

Asthma prevalence has increased dramatically in the past several decades in the United States with an overall prevalence of 13.1% [5]. However, there are significant racial and ethnic disparities in the prevalence of and outcomes for asthma in the U.S [6]. Current data indicate that African American children have an asthma prevalence of 15.8%, compared to only 13.1% for Caucasian children [5]. Interestingly, despite higher poverty levels and reduced access to care compared to Caucasians, the overall asthma prevalence in Latinos (12.5%) is slightly lower than that of Caucasians [5]. This phenomenon in the Latino population has been referred to as the "Hispanic Paradox," and is conjectured to be due to protective dietary and psychosocial factors experienced by new immigrants [5]. However, the situation is much more complex when examining the multiple subgroups contained under the Latino designation separately. In fact, it was found that Mexicans and Puerto Ricans, have the lowest (10.1%) and highest (25.8%) prevalence of asthma in the U.S., respectively [5]. Similar trends are observed with respect to asthma mortality with Mexicans (9.2/million) and

Caucasians (14.7/million) experiencing the lowest mortality rates, whereas African Americans (38.1/million) and Puerto Ricans (40.9/million) suffer much higher mortality rates [7]. Results from the Genetics of Asthma in Latino Americans study (GALA), which includes Mexican and Puerto Rican subjects, found that Puerto Ricans had higher rates of emergency department visits for an asthma exacerbation (OR = 2.63), as well as higher odds of previous hospitalization for asthma (OR = 1.94) [8].

Disparities have also been observed with respect to asthma medication response. Results from the GALA study indicated that Puerto Rican subjects have significantly reduced response to the most common asthma bronchodilator medicine, albuterol, in comparison to Mexican subjects [8, 9]. A similar reduction in bronchodilator response has also been observed among moderate-to-severe African American asthmatics [9]. Disparities in asthma traits have not been fully accounted for by socioeconomic and environmental risk factors, but may also include genetic components acting alone or in concert with other risk factors [2].

1.1.3 Benefits of Genetic Studies in Diverse Populations

Despite the high prevalence of asthma in ethnic minority populations in the U.S. their inclusion into genetics studies of asthma has been limited. In particular Latino and African American groups have only participated in 8% and 5% of the genetic association studies completed, to date, respectively [6]. This is of concern for multiple reasons including the fact that many genetic variants associated with disease in one ethnic group do not consistently confer risk across other ethnic

populations. An excellent example of this is one of the few positionally cloned asthma genes, G-protein coupled receptor for asthma susceptibility (GPRA). Haplotypes within this gene have been associated with asthma traits in multiple European populations including Finnish, Swedish, Italian and German, and in Chinese [10-14]. Despite this wealth of replication in populations of European descent, two studies in Latinos and the only study in African Americans failed to find significant association with variants in the GPRA gene [15, 16]. Additionally, pharmacogenetic associations in asthma have been shown to vary greatly between ethnic groups. Namely, the Arg allele of the Arg16Gly polymorphism in the beta-2-adrenergic receptor has been shown to be strongly associated with response to albuterol in Puerto Ricans. However, this SNP was not observed to be a genetic determinant of response in either African Americans or Mexicans [17, 18]. While these results could be due to methodological issues (sample size, type of analysis, etc.) it is also possible that the genetic risk is modified by real factors differing between these populations. This is especially true for complex diseases like asthma where disease susceptibility is believed to depend on interactions among genetic and other risk factors that vary by race including environmental, socioeconomic, and psychosocial [6]. Furthermore, examination of diverse populations with varying exposures can help elucidate the mechanism of genetic association and resolve inconsistent results. A prime example of this is with the CD14 gene, a receptor for bacterial endotoxin. Endotoxin is an environmental exposure that has previously been shown to influence development of asthma. A polymorphism observed in the CD14 gene, -C159T, was determined to modulate

circulating CD14 levels [19]. Although initial studies associated the T allele with protection from asthma traits [19-22] some subsequent studies failed to replicate this result and even observed the opposite: that the T allele was a risk factor for asthma traits [23, 24]. However, when studies were extended from urban to rural populations researchers observed consistent risk for asthma traits being conferred by the TT genotype [25]. This resulted in the hypothesis that differential endotoxin exposure between these populations, which is higher in rural populations, may be modifying –C159T genetic risk. Subsequent studies reporting endotoxin levels have confirmed this hypothesis, revealing a complex interplay between –C159T genotype, where the TT genotype is protective in the setting of high endotoxin levels, but increases risk in the setting of low endotoxin exposure [26, 27]. This result shows the potential benefits of examining diverse populations in genetic research.

Another important reason to include diverse populations in genetic research is the potential for race-specific genetic variation to influence disease risk. The magnitude of this potential is revealed in a study done by Stephens et al. In this work they examined variation in human genes across 4 U.S. racial and ethnic groups, Caucasian, African American, Asian, and Latino [28]. Through resequencing of 313 genes in these 4 groups the authors found 3,899 SNPs. Interestingly, 21% of the SNPs were pan-racial, but 15% of the SNPs were race-specific [28]. As genic SNPs, these race-specific variants have the potential to modulate expression and function of the corresponding protein and thus disease risk. This raises the possibility that race-specific genetic variation may in part

contribute to the increased prevalence and more severe asthma observed in ethnic minority populations of the U.S. In our work we have recently discovered an African-specific, non-synonymous variant in the KCNMB1 gene that significantly alters the corresponding protein's function [29]. This variant was strongly associated with a clinically significant decrement in lung function (a measure of asthma severity) in two populations of African American males [29]. Clearly, these race-specific genetic risk factors cannot be discovered unless we study the populations in which they occur. These examples show the benefits of including diverse populations in genetic research, both to the population studied and among other populations.

1.1.4 Genetic Ancestry in Admixed Populations

Latinos and African Americans are genetically admixed groups deriving their ancestry from several different continental populations including Africans, Europeans, and Native Americans. Prior studies have shown self-report of ancestry in Latino groups is not indicative of genetically determined ancestry [30, 31]. The application of Ancestry Informative Markers (AIMs), which are private to a specific continental group or nearly so, have made possible the precise determination of individual genetic ancestry in admixed groups [30].

Genotype data from AIMs markers can be analyzed by recently developed statistical programs to infer the precise ancestry of admixed individuals. Both Mexicans and Puerto Ricans were formed from mixing between the European colonists, West Africans brought to North America as part of the slave trade, and

the new world Native American inhabitants [31]. As a tri-hybrid population, to determine ancestry, markers must be typed that differentiate between all three of these groups. We have typed 112 AIMs markers in the 686 asthmatic probands from the Genetics of Asthma in Latino Americans study, which includes 300 Mexican and 386 Puerto Rican trios. The genotypes from these markers were analyzed by a model-based clustering program called STRUCTURE to infer individual ancestry estimates (IAE) [32]. Results from this analysis show significant differences in mean ancestry between Mexican and Puerto Rican subjects despite both being classified within the Latino ethnic group (Figure 1.1). Namely, Puerto Ricans have twice the African ancestry, one-third of the Native American ancestry, and over 1.5x the European ancestry of Mexican subjects (Figure 1.1). Despite clear differences in mean ancestry proportions between Puerto Rican and Mexican Latino groups, there is still significant overlap in ancestral proportions at the individual level, due to the wide spectrum of ancestry proportion across individuals (Figure 1.2). This overlap in individual ancestry between Mexicans and Puerto Ricans occurred despite strict self-identification criteria.

Figure 1.1
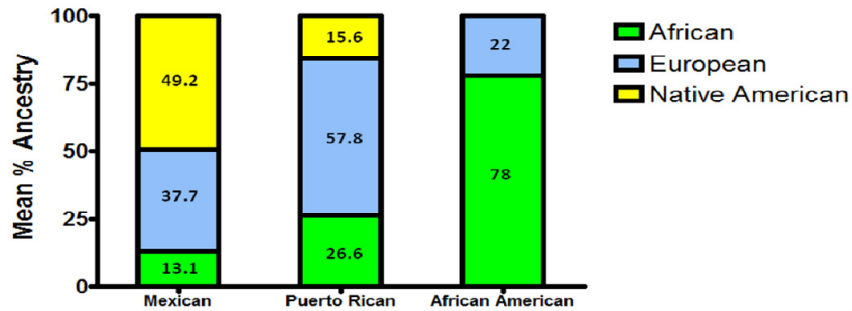
Fig. 1.1 Mean ancestral proportions of Mexicans, Puerto Ricans, and African Americans.

Figure 1.2
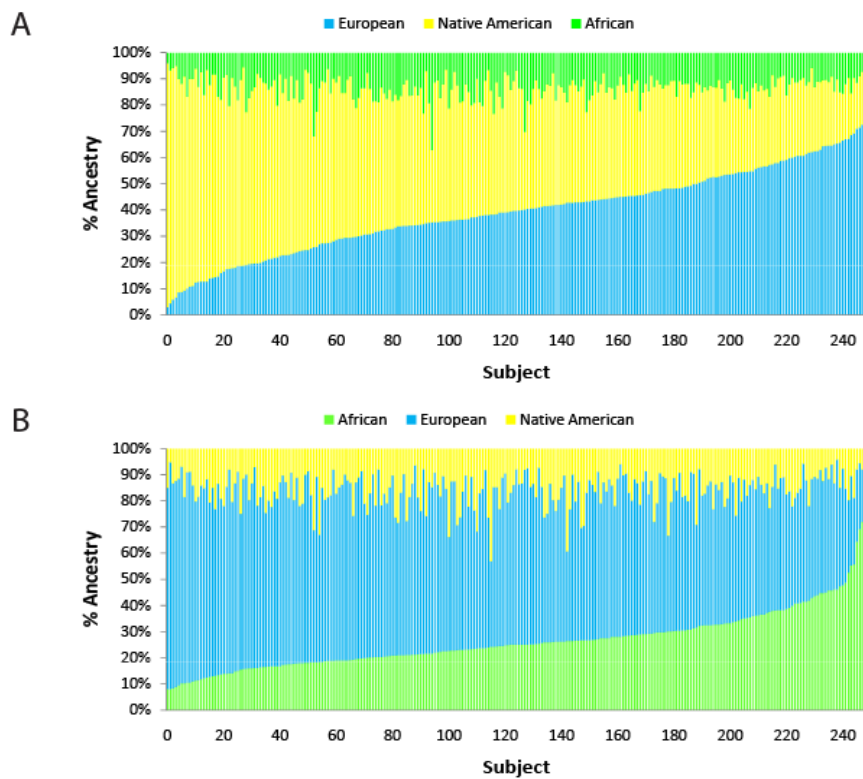
Fig. 1.2 Individual ancestry proportions of Latino asthmatic probands. (A) Estimates are shown for 250 Mexican subjects. (B) Estimates are shown for 250 Puerto Ricans subjects. Each bar represents the ancestry of a single subject.

Prior studies have found little evidence of Native American admixture in contemporary African Americans with ancestry proportions estimated at 1-2% [4, 33]. Therefore, we only typed 60 AIMs markers, all discriminatory for European-African ancestry in African American subjects from the Study of African Americans, Asthma, Genes, and Environments (SAGE). The markers were analyzed by the STRUCTURE program using a 2 population model. Mean European and African ancestry was 22% and 78%, respectively, among the 264 African American asthmatics (Figure 1.1). However, similar to Latino subjects there was a large distribution of European ancestry among individual subjects ranging from less than 5% to greater than 50%, revealing the heterogeneity in this population (Figure 1.3).

Figure 1.3
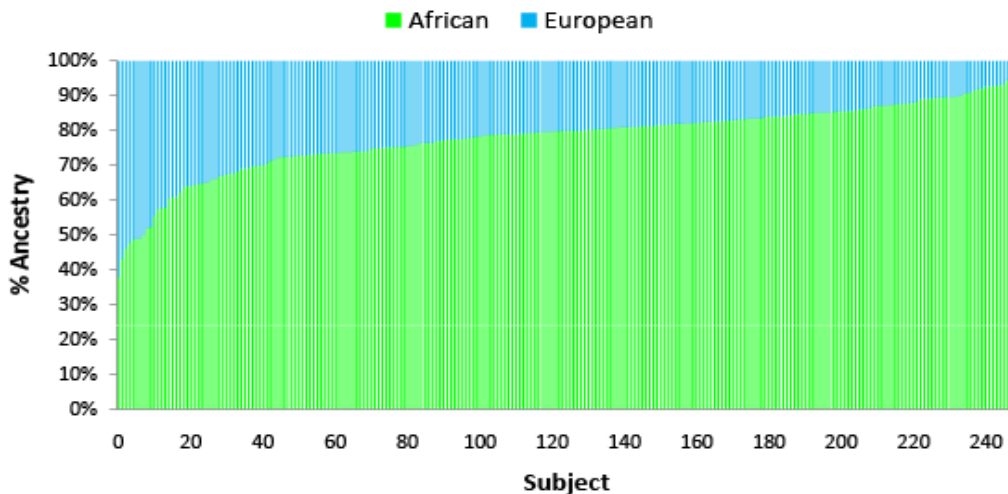
Fig. 1.3 Individual ancestry proportions of African American asthmatics. Each bar represents the ancestry of a single subject.

This large variation in percent ancestry was observed despite requirement that the subject, both of their parents, and all four grandparents must self-identify as African American. Furthermore, all subjects were recruited from the same geographic area of the U.S., the San Francisco Bay Area. These results show the scope of variation in genetic ancestry among African Americans, Mexicans, and Puerto Ricans, despite the careful design of recruitment to ensure homogeneous groups. Although this variation can confound genetic studies (Section 1.1.7) it can also be used in deciphering the inheritance of complex diseases like asthma (Section 1.1.5 & 1.1.6)

1.1.5 Asthma Traits and Ancestry

As noted in Section 1.1.2 there exist dramatic differences in both the prevalence of and outcomes for asthma among U.S. racial and ethnic groups. Although socioeconomic status, environmental risk factors, and social risk factors partially explain these disparities, it is also possible that genetic ancestry or interactions between genetic ancestry and these factors can contribute to these differences. Admixed populations provide a unique opportunity to explore the relationship between genetic ancestry and asthma traits, due to the large variation in individual ancestry estimates observed in admixed populations such as African Americans and Latinos. This has been demonstrated in Mexican subjects from the GALA study, in which the relationship between ancestry proportions and airway function was evaluated [34]. The measure of airway function tested in this study was forced expiratory volume in one second (FEV_1), a reproducible,

quantitative measure of asthma severity. In this study there was a strong inverse relationship observed between the degree of European admixture and FEV_1 % of predicted. Since the degree of African ancestry observed in these subjects was minimal (3%), a strong direct correlation between Native American ancestry and FEV_1 was also observed. In fact, it was determined that every 10% increase in European ancestry resulted in a 1.8% decrease in FEV_1 [34]. These results withstood correction for multiple confounding variables including asthma duration, medication usage, age, and sex. In the same study similar results were observed for asthma severity defined as a qualitative trait (mild or severe) using medication usage and symptoms as the criteria for classification. Reflecting the FEV_1 analysis, a 10% increase in European admixture was associated with a 37% increase in risk for severe asthma [34]. Although studies comparing admixture in African Americans and asthma traits have not been conducted yet, similar studies have associated admixture among African Americans with other traits including the inflammatory marker IL-6, LDL-cholesterol, triglycerides, and insulin resistance [35, 36]. These links between genetic ancestry and traits have led to development of admixture mapping as a technique to identify disease alleles in admixed groups (Section 1.1.6)

1.1.6 Admixture Mapping

A logical extension to studies that identify a particular genetic ancestry is associated with a biological trait is the process of admixture mapping. In theory, the association between genetic ancestry and a trait could be due to a functional

genetic marker that influences that trait and is private or much more common to that ancestral population. Admixture mapping takes advantage of the fact that in populations where the admixture is recent, there exists large linkage disequilibrium (LD) blocks that are associated with a particular ancestry. Therefore, a limited number of AIMs markers can be typed that cover the admixture LD blocks across the entire genome. Using AIMs to screen these LD blocks investigators can identify discrete genome regions where cases share more ancestry of the high risk ancestral group than do control subjects. These regions should contain the risk variants responsible for the association with ancestry, and can be fine-mapped down to the causative variant. The efficiency of admixture mapping depends on the time since admixture began [37]. This time determines the size of the admixture LD blocks, with the length of time inversely related with the size of the blocks, as they are diced into smaller sizes by recombination events. Ideally, blocks would be large enough to allow fewer markers to be screened, but small enough to allow manageable size blocks to be identified. Work has shown the optimal time period since admixture to be between 5-20 generations [37]. Admixture in both African Americans and Latinos is estimated to have occurred 20 generations ago, making both these populations amenable to admixture mapping [38]. No admixture mapping analyses have been conducted for asthma to date. However, admixture mapping studies in African Americans have identified loci that are associated with atherosclerosis[39], prostate cancer[40], IL-6 levels[35], and white blood cell count[41]. The recent work showing associations between genetic ancestry and asthma severity in Mexicans, detailed

in Section 1.1.5, coupled with the success of this technique in other diseases, suggest admixture mapping may be an appropriate approach for identification of genetic risk factors among African Americans and Latinos.

1.1.7 Population Stratification and Ancestry

Population stratification in mixed ethnicity genetic association studies is defined as the under- or over-representation of a particular ethnic group among case versus control subjects [42]. Population stratification can result in genetic confounding, if markers typed are linked with the ethnicity that is differentially represented between cases and controls [42]. The genetic confounding that occurs due to population stratification can be the cause of both false-positive and false-negative results [42]. In theory this can be avoided in non-admixed groups by careful matching of case and control subjects with regards to ethnicity. However, the situation is more complex in admixed populations, even when restricting analysis to only one admixed group. As formerly shown in section 1.1.4 there is a large amount of variation in ancestral proportions within contemporary Mexican, Puerto Rican, and African American populations. This variation sets up the potential for differential distribution of ancestral proportions between cases and controls. Although this can occur by chance, it can also happen systematically if the prevalence of traits under study are strongly related to a particular ancestral group, as described in Section 1.1.5. Since self-report of ancestry is unreliable, especially for Mexicans and Puerto Ricans, genetically determined Individual Ancestry Estimates (IAE) can instead be used to control for this confounding.

These ancestry estimates can be determined for all subjects, as detailed in Section 1.1.4, and incorporated as a covariate into genetic disease models tested, to correct for any differences in ancestry between cases and controls. The potential problem of population stratification in admixed groups, and the utility of the IAE method to resolve this dilemma was demonstrated in recent work [30]. In this study 44 AIMs markers were tested in a large Latino case-control group containing both Mexican and Puerto Rican subjects [30]. They found AIMs on different chromosomes in both groups were more strongly associated than expected by chance, suggesting admixture LD in these groups and the potential for stratification. The AIMs markers were tested for association with asthma finding among Puerto Ricans significantly more associations, 8 markers (18%), than expected by chance alone (5%) [30]. Repeat of these analyses incorporating IAE as a covariate resulted in only 2 markers remaining significant for disease status. These results strongly support the notion that population stratification can significantly alter results in genetic studies of Latinos and other admixed groups, and show the power of genetically determined ancestry to account and control for this phenomenon.

1.2 Role of Chitin and Chitinases in Asthma

1.2.1 Chitinases in Mouse Models of Asthma

The pathology of allergic asthma is characterized by persistent airway inflammation, which is driven in large part by a dysregulated Th2 immune response. Inappropriate activation of Th2 cells leads to the production of a panel of pro-allergic cytokines such as IL-4, IL-5, and IL-13. IL-13 appears to be foremost in this group of inflammatory cytokines, being both necessary and sufficient for the reproduction of allergic asthma in mouse models of asthma [43, 44]. The pulmonary manifestations of IL-13 over-expression in mouse models include eosinophilic infiltration, excessive mucus secretion, and airway hyperresponsiveness to cholinergic stimuli [43, 44]. Many of these inflammatory effects are mediated through signaling resulting in the activation of the inflammatory transcription factor STAT6 [45]. In addition, both IL-13 and STAT6 have been observed to be over-expressed in the lungs of subjects with asthma [46, 47]. Therefore, genes regulated by (IL-13/STAT6)-dependent signaling have been explored as effectors of Th2 inflammation in asthma [48, 49].

The acidic mammalian chitinase (AMCase) was investigated as a potential mediator of Th2 airway inflammation and IL-13 signaling in the seminal work by Zhu et al. They demonstrated in an ovalbumin mouse model of asthma that sensitized and challenged mice elicited a dramatic increase in the expression of AMCase mRNA and protein in the lung and a corresponding increase in the level of chitinase activity in the bronchoalveolar lavage (BAL) [50]. However, no effect

on chitotriosidase mRNA expression was observed. Using immunohistochemistry they revealed that the source of this expression was airway epithelial cells and macrophages. In experiments comparing mice reconstituted with either Th1 or Th2 polarized cells they found AMCase upregulation to be a result of Th2 mediated inflammation. Moreover, it was determined that AMCase upregulation was dependent on IL-13 rather than IL-4 signaling. Inhibition of AMCase enzymatic activity with either allosamidin (specific chitinase inhibitor) or anti-AMCase antibody resulted in a significant decrease in airway hyperresponsiveness, tissue eosinophilia, and inflammation. Importantly, this inhibition did not affect either IL-4 or IL-13 signaling nor did it inhibit activation of STAT6. Rather inhibition of AMCase diminished the induction of multiple IL-13 effector molecules including the chemokines eotaxin, eotaxin-2, monocyte chemotactic protein (MCP-1), MCP-2, macrophage inflammatory protein (MIP-1β), C10 and ENA-78 [50]. These experiments suggest that within the Th2 inflammatory cascade that AMCase is downstream of IL-13 cytokine production. Additionally, AMCase expression and activity is required for airway hyperresponsiveness and inflammation, possibly through mediating expression of chemokines (See Figure 1.4).

Figure 1.4
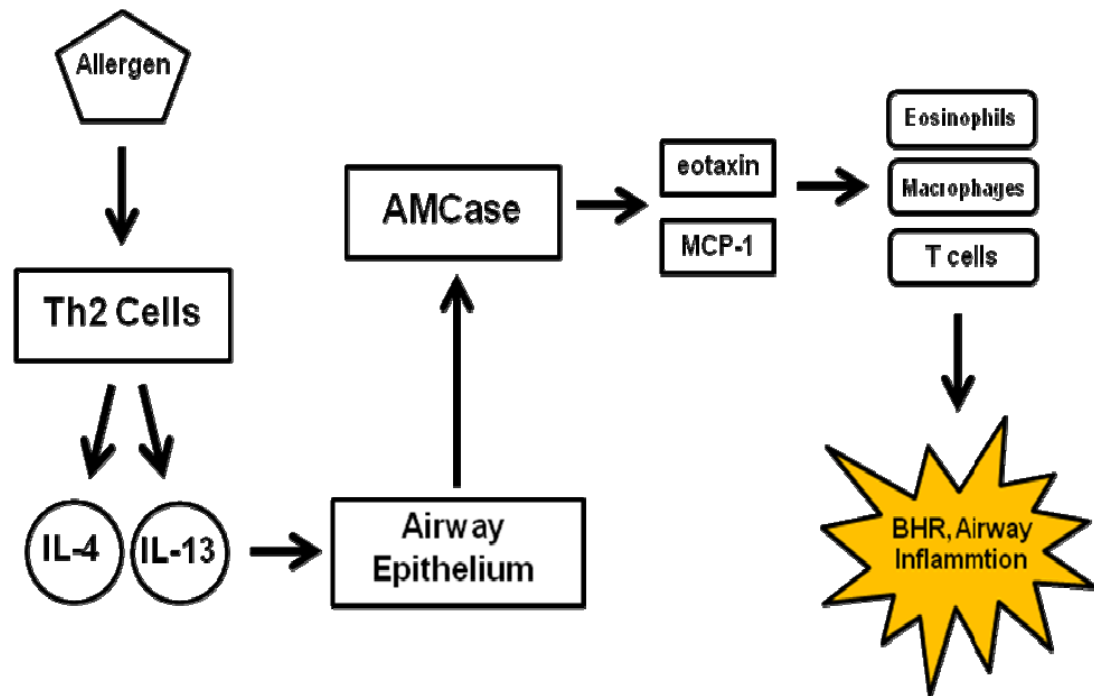
Fig. 1.4 AMCase is a central mediator of the allergen induced Th2 inflammation in the airway of mice. (BHR – bronchial hyperresponsiveness).

Paradoxically, AMCase is also reported as a negative regulator of chitin induced recruitment of innate immune cells associated with allergy (discussed in Section 1.2.5) [51]. Although the case may be made that AMCase is only protective in the presence of chitin-induced inflammation, most puzzling is the fact that transgenic mice over-expressing lung AMCase were not predisposed to any exaggerated inflammation [51]. Despite these inconsistencies it is clear that AMCase is strongly induced by Th2 inflammatory signaling and may well play multiple contextual roles in allergic inflammation. Further experiments elucidating the precise biology of chitinases and chi-lectins will be necessary to clarify the roles of AMCase in Th2 inflammation of the airway.

1.2.2 Active Chitinases in Human Asthma

The role of AMCase in mediating the Th2 inflammation and airway
hyperresponsiveness in mouse models of asthma has led to examination of
AMCase expression in human asthma. *In-situ* hybridization probing for AMCase
mRNA in bronchial biopsies from both fatal asthmatics and subjects who have
died of natural causes, revealed AMCase expression only among asthmatics [50].
AMCase expression was observed in both the epithelial and sub-epithelial cells
of the fatal asthmatics [50]. These results are congruent with the overexpression of
AMCase observed during Th2 inflammation in mice. However, a larger more
recent study has examined airway epithelial cells from both asthmatics (N=30)
and controls (N=28) and failed to observe any AMCase expression in either
group [49]. This discrepancy may reflect the heterogeneous nature of asthma and
in particular severe asthma, as the latter study only included mild to moderate
asthmatics rather than severe asthmatics. Additionally, biopsies from the first
study were taken from lung tissue isolated after a fatal asthma attack versus the
second study which collected cells from stable asthmatics.

It is also important to note that AMCase mRNA expression cannot be equated
with enzymatically active AMCase in the lung. The enzymatic activity of AMCase
in humans is particularly relevant since the results of Zhu et al. revealed specific
inhibition of AMCase enzymatic activity alleviated much of the bronchial
hyperresponsiveness and inflammation in a mouse model of asthma. In fact, an
AMCase transcript (TSA-1902S) with the active site spliced out has been

reported to be expressed in the human lung [52]. Moreover, to date there has been

no report of AMCase protein expression or specifically AMCase enzymatic

activity in the lungs of human subjects. Any exploration into the role of AMCase

activity in the human lung should take into account the presence of the other

active chitinase in the human genome, chitotriosidase (CHIT1). Indeed, the

presence of CHIT1 protein and chitinase activity has been observed in the lungs

of healthy human controls [53, 54]. Moreover, these studies found CHIT1

expression and chitinase activity were both dramatically upregulated in the lungs

of patients with interstitial lung diseases (ILD), such as sarcoidosis and

pulmonary fibrosis. Due to the involvement of macrophages in ILDs the authors

attributed this chitinase activity to CHIT1. However, the authors did not account

for AMCase expression. Additional studies will be needed to determine the

protein expression and activity of both active chitinases in human asthma.

1.2.3 Chi-lectins in Human Asthma

Chi-lectins, such as YM1 and YM2 are some of most highly expressed proteins

during Th2 inflammation of the lung in mouse models of asthma [55, 56]. This

observation raises the potential that chi-lectins may also modulate human

asthma phenotypes [55, 56]. This hypothesis has been tested in examination of the

human chi-lectin YKL-40 (also called CHI3L1) in asthma. Specifically, serum

levels of YKL-40 were examined in two cohorts of asthmatic cases and healthy

controls [57]. In this study YKL-40 levels were determined specifically by ELISA

assay. In the Yale University cohort median serum levels of YKL-40 were found

to be slightly higher in asthmatics (Median= 69.7 ng/ml, N=97) versus controls (Median= 58.3 ng/ml, N=38). These results were subsequently confirmed in the smaller replication cohort from Paris University, with asthmatics (Median=97.7 ng/ml, N=40) displaying over twice the median YKL-40 levels of control subjects (41.5 ng/ml, N=12). Using cross-sectional analysis the authors tested whether serum YKL-40 levels were correlated with asthma severity, as defined by the amount of medication required to achieve asthma control. They observed a significant positive correlation between YKL-40 levels and asthma severity in both the Paris and Yale cohorts, and an additional replication cohort of asthmatics (Wisconsin Cohort). Furthermore, the authors found YKL-40 serum levels were also negatively correlated with lung function, as measured by forced expiratory volume in one second (FEV_1) in all three groups of asthmatics [57]. Although these results suggest serum YKL-40 levels to be a marker of asthma and disease severity in general, it is important to note the considerable overlap of YKL-40 distributions observed between asthmatic cases and controls. Moreover, the correlations with asthma severity appear to be driven in large part by a few severe asthmatic cases with extremely high YKL-40 serum levels.

Extending this study to bronchial biopsy samples in the Paris cohort, immunohistochemistry revealed significantly larger numbers of YKL-40 expressing sub-epithelial cells and airway macrophages in asthmatics as compared to controls [57]. Positive correlation was also observed in the second cohort between asthma severity and the number of YKL-40 expressing cells in bronchial biopsies. Moreover, serum YKL-40 levels were associated with

subepithelial basement membrane thickness, an important measure of airway remodeling [57, 58].

These results are supported by a recent report of significant increases in YKL-40 protein levels in the bronchoalveolar lavage (BAL) of subjects with allergic asthma, 24 hours after segmental allergen challenge [59]. Moreover, BAL eosinophil counts at 24 hours correlated positively with YKL-40 levels. These results extend earlier findings by revealing upregulation of YKL-40 levels during active inflammation in the human lung. However, the authors do not mention whether significant YKL-40 levels were observed in the asthmatics prior to allergen challenge. As this was a short letter to the editor, further comparison with the earlier results is difficult as clinical characteristics and severity of these patients was not reported. Nonetheless, these are important observations and should drive the examination of other chi-lectin proteins in asthma such as OVGP1 and CHI3L2 [60].

1.2.4 Chitinase Genetics in Human Asthma

The functional data in mouse models of asthma and differential expression of chitinases in human asthma has generated interest in the possible effects of chitinase genetic variation in susceptibility to asthma. Genetic variants within the AMCase gene have recently been tested for association with asthma in a German case-control cohort [61]. In this study, all exonic portions of the AMCase gene were resequenced in 30 German subjects to determine all coding gene variation. The AMCase gene exhibited considerable variation in this population

with 12 polymorphisms being identified including 5 non-synonymous (amino acid changing) variants. All 6 exonic variants (rs12033184, rs2275253, rs2275254, rs2256721, F269S, and K17R) and one promoter polymorphism (rs3818822) were genotyped in the case-control group. Interestingly, the authors found significant associations, using both adult and pediatric controls, between asthma and the promoter SNP rs3818822 (P=0.0003, P=0.017). Additionally, one of the novel coding SNPs, K17R, was also associated with asthma using both adult and pediatric controls (P=0.019, P=0.0003). Haplotype analysis of the seven SNPs was then performed, identifying 16 haplotype combinations with an allele frequency of at least 1%. Analysis of the distribution of haplotypes in cases versus adult controls resulted in a χ^2 P-value of 0.000039. Similar haplotype analysis between cases and adult controls resulted in a χ^2 P-value of less than 10^{-10}. Closer examination of the results finds four of the six common haplotypes (those with > 5% frequency) differed in haplotype frequency by greater than 4% between asthmatic cases and pediatric controls. However, haplotype tests were not performed on individual haplotypes, precluding the identification of a particular risk haplotype. These results strongly suggest a role for AMCase polymorphisms in the predisposition toward asthma. The associated polymorphisms included both a non-synonymous (amino acid changing) and a promoter SNP. Therefore, to aide in the elucidation of AMCase's role in human asthma, it will be important to determine how these variants may affect enzyme function and expression, respectively.

Based on the functional and expression overlap of AMCase and the chitotriosidase (CHIT1) enzymes, genetic variants in the CHIT1 gene were also tested for association with asthma disease status in the same German case-control cohort [62]. Three coding variants of the CHIT1 gene were tested including SNPs Gly102Ser and Ala442Gly, and a 24 base pair duplication in exon 10. This duplication polymorphism has been fully characterized and results in a misspliced mRNA that is quickly degraded [63]. Therefore, if loss of CHIT1 activity plays a role in predisposition to asthma this null variant should be strongly predictive. However, no association was observed between the 322 asthmatic cases and 270 adult controls for either SNPs or haplotypes. Despite the negative results it must be noted that resequencing analysis was not completed for the CHIT1 gene and judging by the importance of haplotypic structure across the AMCase gene in asthma risk, further analysis of sequence variation in the CHIT1 gene will be needed to confirm this result.

In addition to genetic studies on active chitinases, the chi-lectin YKL-40 has also been screened for association with asthma. As discussed in Section 1.2.3, YKL-40 expression levels in the serum were associated with asthma severity and tissue remodeling, making it an excellent biological candidate gene. Using a genome-wide screen several promoter SNPs in tight linkage disequilibrium were determined to be strongly associated with YKL-40 serum protein levels (p = 1.1×10^{-13}), among a Hutterite founder population. A SNP (rs4950928) tagging this block was tested for association with several asthma traits. Associations were observed between FEV1/FVC (p=0.002), bronchial hyperresponsiveness

(p=0.002), and asthma disease status (p=0.047) among the Hutterites. The investigators also replicated the asthma association in 2 out of 3 outbred populations tested. The SNP allele associated with asthma in all three of these studies was the one linked with higher serum YKL-40 levels. These results are supportive of chi-lectins contributing in a deleterious manner to the pathology of asthma. However, results will need to be replicated in larger populations, and it will be interesting to see if lung levels of YKL-40 are also correlated with disease and genotype influenced.

These studies have just begun to scratch the surface of the potential involvement of chitinase genetics in asthma traits. Future studies should explore the possibility of interactions between AMCase and CHIT1 genetic variants, since both genes are expressed in the human lung and overlap functionally. Furthermore, there is a possibility for effect modification based on exposure to environmental chitin, which has been shown to have inflammatory properties in the lung (discussed in Section 1.2.5). Therefore, it will be important to determine whether chitinase genetic results are consistent in different environmental settings (rural versus urban) which may act as a surrogate of environmental chitin exposure.

1.2.5 Immunomodulatory Properties of Chitin

Although the formerly discussed results reveal a role for AMCase in a mouse model of asthma, which is likely unrelated to chitin, recent work has implicated environmental chitin in polarizing the T-helper cell response in the lungs [51, 64]. The interface of environmental factors (*i.e.* chitin) and effectors of asthma (*i.e.* AMCase) are thought to underlie the complex etiology of asthma. Exposure to chitin by inhalation is very common and it is an important component of aeroallergens. Furthermore, researchers have noted the similarities between the immune responses to chitin-containing parasites and those observed in allergic asthma, including Th2 inflammation driven by IL-4 and IL-13 expressing cells (eosinophils and basophils) [65].

The most comprehensive study in this area was recently completed by Reese et al. In this study they used the chitin-containing parasitic helminth, *Nippostrongylus brasiliensis,* to investigate chitinase expression in Th2 immune responses [51]. They found in mice infected with *Nippostrongylus brasiliensis* that STAT6-dependent expression of AMCase, YM-1, and YM-2 was induced in the lung and that the immune response was dependent on IL-4 and IL-13 expression. Since these worms molt in the lungs the authors hypothesized that the structural chitin remodeled during molting acts as a pattern recognition particle, triggering accumulation of IL-4 and IL-13 expressing innate immunity cells. This was tested by administering chitin to the lungs of genetically engineered 4get mice. These mice contain an IL-4 green fluorescent protein

(GFP) knock-in, allowing detection of IL-4 expressing cells. Chitin challenge led to the accumulation of eosinophils and basophils in the lungs. To ensure that the immune cell recruitment was not due to contaminating lipopolysaccharide (LPS), these experiments were repeated in TLR4 and MyD88 deficient mice (mice unresponsive to LPS). Furthermore, pretreatment of chitin with enzymatically active mouse AMCase blocked this response. These results suggest that the observed effect was dependent on intact chitin. The chitin challenge experiments were repeated in AMCase over-expressing transgenic mice and immune cell recruitment was strongly reduced compared to wild type mice. Moreover, these effects were determined to be mediated by innate immunity cells, as chitin-induced eosinophil recruitment was functional in recombination activating gene (RAG) knockout mice (no adaptive immune system). Based on the presence of alternatively activated macrophages in allergic and parasitic immunity, the authors explored the role of these cells in mediating eosinophil and basophil recruitment. To test this hypothesis they used reporter mice (termed YARG) which co-express enhanced yellow fluorescent protein (eYFP) and arginase I, allowing for the detection of alternatively activated macrophages. Both *Nippostrongylus brasiliensis* infection and intranasal chitin challenge resulted in the accumulation of eYFP-positive macrophages in the lung. Through *in vitro* experiments they implicated the macrophage produced Leukotriene B_4 in eosinophil recruitment. Chitin challenge experiments in mice lacking the high-affinity receptor for leukotriene B4 (BLT1) and additional experiments in mice depleted of macrophages supported this hypothesis. When the chitin challenge

was administered in YARG + AMCase over-expressing transgenic mice, the tissue accumulation of alternatively activated macrophages was greatly diminished. These experiments strongly implicate chitin as a pattern recognition molecule stimulating the tissue recruitment of innate immunity cells associated with allergic asthma, including eosinophils, basophils, and alternatively activated macrophages. In these experiments, rather than being pro-inflammatory, (see Section 1.2.1) AMCase fulfills the role of a strong negative regulator of chitin-induced allergic inflammation. These results are especially interesting considering the widespread presence of chitin in many aeroallergens.

Despite the evidence implicating chitin in Th2 inflammation, prior studies have shown the opposite effects of chitin on immune responses. Namely, Shibata et al. found chitin micro-particles (1-10 μM) are phagocytosed through mannose receptors and stimulate production of INF-gamma (Th1 cytokine) [66]. Additional experiments have demonstrated that the oral administration of chitin microparticles in a ragweed allergen mouse model of asthma reduces serum IgE levels and the recruitment of eosinophils to the lungs [64]. Chitin microparticles have even been proposed as a therapy for allergic diseases and for use as a Th1 adjuvant [64, 67]. Although these results appear to be contradictory, they likely are related to differences in the chitin used including the chitin particle size (phagocytosable micro-particles versus larger fragments) and the route of administration (intranasal and lung *vs.* oral). Additionally, commercial chitin may be contaminated with LPS, which despite purification procedures could account for some of the Th1 stimulatory effects seen in these studies. Reese et al.

controlled for LPS contamination by using TLR4 (receptor for LPS) knockout

mice, increasing confidence in their results. Nonetheless, it is clear that chitin

can modulate immune responses and that more carefully controlled experiments

are needed to further elucidate the role of chitin in mediating and/or inhibiting the

progression of Th2 inflammation in allergic asthma.

1.3 Research Aims

To determine the role of chitinases in human asthma we have used a multi-faceted approach including population genetics, molecular functional analysis, and characterization of clinical samples. Both human chitinase genes, AMCase and Chitotriosidase were selected as candidate genes for asthma based on work implicating both chitin and chitinase activity in Th2 inflammation in the lungs of mice [50, 68]. Both genes were comprehensively resequenced in multiple ethnic groups to identify all amino-acid changing variants in the populations studied. Single SNPs and gene-wide variant haplotypes in the AMCase gene were tested for association with asthma in both African American case-controls groups and in a Latino family-based study. A common null duplication polymorphism in the CHIT1 gene was also tested in these same cohorts. Additionally, we analyzed the effects on chitinase activity of both protective and non-protective gene-wide AMCase haplotypes by biochemical characterization of recombinant proteins. Finally, we determined the level and possible differential expression and activity of both chitinases in asthmatics, smokers, and healthy subjects. This analysis included determination of the effects of pH and the CHIT1 duplication polymorphism on chitinase activity in the lung.

Outline of Aims:

(1) Determination of all the amino acid coding variants and linkage disequilibrium patterns in the AMCase gene among African Americans, Mexicans and Puerto Ricans.

(2) Case-control and family-based tests of association between AMCase genetic variants and asthma among African Americans and Latinos

(3) Haplotype analysis of AMCase polymorphisms with recombinant protein expression and enzymatic characterization of associated AMCase isoforms.

(4) Determination of all coding variants in the CHIT1 gene and test for association of the null CHIT1 duplication variant with risk for asthma traits in African Americans and Latinos.

(5) Characterize the contribution of both AMCase and CHIT1 to human lung chitinase activity, and determine differences in chitinase activity and expression in lung disease.

Chapter 2. The Genetics and Function of the Acidic Mammalian Chitinase in Asthma among Admixed Populations.

2.1 Introduction

Asthma is a complex respiratory disease resulting from the interaction of genetic predisposition and exposure to environmental factors [69]. Interest in the impact of environmental contributions to asthma has been spurred by the marked increase in asthma prevalence in developed Western countries over the past three decades [70]. Epidemiologic studies have raised the possibility that microbial products encountered in early life may influence the likelihood of developing asthma [69, 71, 72]. Chitin, a linear polymer of the sugar *N*-acetyl-glucosamine, is ubiquitous in nature and comprises a structural component in the cell wall of fungi, the exoskeleton of arthropods such as insects and crustaceans, and in the eggs and pharyngeal organs of parasitic nematodes [73-77]. Chitinases are evolutionarily ancient enzymes which hydrolytically cleave the chitin polymer into di- and tri-saccharides. Chitinases serve functionally diverse roles across species, including nutrient scavenging, structural remodeling of chitin constituents, parasitism and innate immunity [78, 79].

Despite the absence of endogenous chitin, humans express two active chitinases, designated Acidic Mammalian Chitinase (AMCase) and chitotriosidase [78]. Chitotriosidase is expressed by phagocytic cells in humans and extremely high levels are seen in lysosomal storage diseases, such as Gaucher's disease [80]. AMCase is expressed in the stomach and lungs, the two

anatomical sites potentially exposed to chitin-containing particles or microorganisms by ingestion or inhalation, respectively [81]. A recent study revealed that AMCase expression is strongly upregulated in response to sensitization and challenge in an ovalbumin mouse model of asthma. The expression of AMCase was found to be induced by IL-13, a cytokine implicated in many of the effects of human asthma [45, 50]. Moreover, they suggested chitinase activity plays a causative role in allergic inflammation, reporting that inhibition of AMCase activity greatly reduced Th2 dependent airway inflammation and bronchial hyperresponsiveness in these mice [50]. Intriguingly, AMCase is induced strongly in both lung epithelial cells and macrophages among patients with asthma [50]. However, a more recent report implicated chitin itself as a pattern recognition molecule stimulating the tissue accumulation of innate immune cells associated with asthma, such as eosinophils and basophils [51]. Furthermore, pretreatment of the polymeric chitin with recombinant AMCase alleviated the inflammatory effects of chitin [51]. These results were confirmed *in vivo*, showing a strong reduction in the inflammatory effects of chitin in AMCase overexpressing transgenic mice compared to wildtype mice [51]. These results suggest AMCase can also act as a strong negative regulator of chitin-induced Th2 inflammation in the lungs.

Thus far little is known regarding the function and involvement of chitinases in human asthma. A German childhood asthma case-control study did find strong associations between genetic variation in the *AMCase* gene and asthma [61]. However, the functional effects of these associated variants were not determined

making interpretation of these results difficult. Herein, we determined all coding

variation in the *AMCase* gene and the association of these variants with asthma

in both African Americans and Latinos, populations with a high asthma

prevalence and mortality. Additionally, we have determined the functional effect

of these associated variants on the ability of the AMCase protein to enzymatically

cleave polymeric chitin, to gain insight into the role of AMCase in human asthma.

2.2 Materials and Methods

Study Participants

Our SNP Discovery Panel includes 24 each of African American, Puerto Rican, and Mexican asthmatic subjects. Recruitment for the Study of African Americans, Asthma, Genes, and Environments (SAGE) study is ongoing and includes 264 asthmatic cases and 176 matched controls, recruited in the San Francisco Bay Area. Eligible asthmatic subjects met the following criteria: (1) between the ages of 8 and 40 years (2) physician-diagnosed asthma (3) symptoms including coughing, wheezing, or shortness of breath for at least 2 years. Matched healthy African American controls were enrolled only if they met the following criteria: (1) no history of asthma or allergy (2) no asthma symptoms including cough, wheeze, or shortness of breath, in the past two years. Additionally, both asthmatic cases and matched controls were enrolled only if both biological parents and all biological grandparents self-identified as African American. Exclusion criteria included >10 pack-year smoking history, smoking in the previous 12 months, and/or history of other lung diseases. The Genetics of Asthma in Latino Americans (GALA) study recruited 301 Mexican and 399 Puerto Rican asthmatics (probands) and their biologic parents from 4 different clinical sites: Mexico City/San Francisco (Mexican), and Puerto Rico/New York (Puerto Ricans). All the parents and grandparents of Puerto Rican and Mexican probands had to self-identify as Puerto Rican or Mexican, respectively. The exclusion and inclusion criterion for the GALA study was identical to that of the SAGE study. A modified version of the 1978 American Thoracic Society-Division

of Lung Diseases Epidemiology Questionnaire was administered to all asthmatics at the time of enrollment to assess individual asthma characteristics and confounders [82]. African American subjects from the Chicago Initiative to Raise Asthma Equity (CHIRAH) and the Asthma Clinical Research Network (ACRN) trials were used to replicate the frequency of the G339T SNP observed in SAGE cases. The CHIRAH cohort is a longitudinal population based cohort of asthmatics, including 321 African Americans, collected from the urban Chicago school system. The ACRN cohort is comprised of 12 separate clinical trials with hypotheses, trial design, and results for completed trials described at http://www.acrn.org/. A general overview of the ACRN purpose is described elsewhere [83]. Local Institutional Review Boards (IRBs) approved all studies, and all subjects provided written, age-appropriate informed consent or assent. Characteristics of the subjects genotyped and analyzed are included in Table 2.1.

Table 2.1 Characteristics of the populations genotyped for AMCase SNPs

Trait	SAGE Controls (n=176)	SAGE Cases (n=264)	Chirah Cases (n=321)	ACRN Cases (n=334)	Mexican Probands (n=281)	Puerto Rican Probands (n=351)
Age (yr)	29.9 (7.8)	19.4 (9.3)	18.8 (10.8)	32.4 (10.2)	16.6 (8.4)	14.1 (6.8)
Sex (% male)	38.1	40.5	38.3	40.4	53.4	56
Serum IgE (IU/ml)	-	286.9 (440.6)	-	494.1 (654.7)	467.8 (624.4)	486.9 (639)
FEV_1 (% of pred.)	-	92.1 (16.7)	92.2 (19.5)	81.8 (15.4)	88.6 (18.6)	83.4 (16.9)

Note: Values in parenthesis are standard deviations, (% of pred.) -% of the predicted value

AMCase Sequencing

All *AMCase* exons (12) and exon-intron boundaries (20 bp from either end of the

exon) were sequenced in the SNP Discovery panel. Resequencing of 48

chromosomes provided power of > 90% to find all genetic variants of 5% or

greater frequency in the general population. Sequencing reactions were

performed using the BigDye Terminator Cycle Sequencing Kit (Applied

Biosystems, Foster City, California) and the reactions were processed on the ABI

Prism 3700 sequencher. PCR primers used to produce AMCase exon PCR

products are listed in Appendix Table 2.1.

Genotyping

AMCase SNPs G339T, G461A, A531G were genotyped in the SAGE and GALA

cohort using the AcycloPrime-FP-TDI (PerkinElmer) method [84].   The PCR

cocktail included: 3.0-5.0 ng genomic DNA, 0.1-0.2 µM primers, 2.5 mM MgCl2,

50 µM dNTPs, 6 µl volume with Platinum Taq PCR buffer and 0.1-0.2 units

Platinum Taq (Invitrogen) plus 1 µl extra water to counteract evaporation. PCR

cycling conditions were as follows: 95°C for 2 minutes, 40 cycles of 92°C for 10

seconds, 58°C for 20 seconds, 68°C for 30 seconds, and final extension at 68°C

for 10 minutes. We used AcycloPrime-FP kits for enzymatic cleanup and single

base extension genotyping reactions. Plates were read on an EnVision

fluorescence polarization plate reader (PerkinElmer). Genotyping of the G339T

SNP in the CHIRAH cohort was accomplished by the same FP-TDI assay used

to genotype this SNP in the SAGE cohort. PCR primers, FP allele-specific

primers, and Fluorescent tagged terminator nucleotides are listed in Appendix

Table 2.2.

AMCase SNPs G1172A, T1218G, G1452T were genotyped in the SAGE and

GALA cohort using fluorogenic allele-specific amplification (Amplifluor,

Chemicon). PCR reactions contained a pair of 5'-tailed allele-specific forward

primers (ASPs, 25 nM each), a single allele-nonspecific reverse primer (375 nM),

and a pair of universal fluorogenic hairpin primers (375 nM). Each universal

primer, one of which is labeled with FAM and the other with JOE, drives

amplification of one of the two allele-specific amplicons through base-pairing to

the complement of the ASP's 5' tail. Incorporation of universal primers into

amplicons generated a fluorescent signal. Reactions were carried out according to the manufacturer's instructions (Chemicon) on an MJ Tetrad thermocycler, and fluorescence read on an Applied Biosystems 7900HT Sequence Detection System. Cluster analysis for calling genotypes was carried out utilizing Applied Biosystems software (SDS version 2.1.1). The G339T SNP was genotyped in the ACRN subjects by the Sequenom iPLEX technology. All plates genotyped contained no template controls to ensure genotyping accuracy and lack of contamination.

Statistical Analysis

The χ^2 goodness-of-fit test was used to determine whether the individual AMCase SNPs were under Hardy Weinberg Equilibrium (HWE). All SNPs genotyped conformed to HWE (p>0.01) in all four cohorts tested. Linkage disequilibrium patterns were determined by using the r^2 statistic. Standard logistic regression was used to test association between AMCase SNP alleles and asthma disease status in the SAGE, CHIRAH, and ACRN cohorts. Age and gender were both included as covariates in the logistic regression models. Additionally, individual ancestry estimates (IAE) were determined on all SAGE and CHIRAH subjects, and IAE was then included as a covariate in these analyses. The IAE variable was generated by genotyping 60 Ancestry Informative Markers (AIMs), with a δ = or > 0.4 between African and European ancestral groups, in all SAGE and CHIRAH subjects. Genotype data from the admixed subjects, African ancestral, and European ancestral subjects were then

analyzed in the program STRUCTURE using a two-population model, as previously described [85].

 In the combined analysis we compared the SAGE, ACRN, and CHIRAH asthmatic cases as one group against the SAGE controls using the same logistic regression model. The Family-based association tests (FBAT) program was used to assess the association between individual SNPs with asthma in the GALA families [86]. All other statistical analyses in these populations were performed using STATA 8.0 S/E statistical software (College Station, TX). Haplotypes and their frequencies were determined with the program HAPSTAT [87].

AMCase expression plasmid cloning

The full-length *AMCase* cDNA was fused to an Fc purification tag and cloned into the pMIB-V5-His-(A) vector (Invitrogen). The Fc hinge region was removed to prevent dimerization of the proteins. Successive rounds of in-vitro mutagenesis were conducted on the AMCase expression construct using the Stratagene Quik-Change Kit, to generate both the protective and non-protective AMCase haplotypes. Detailed methods are listed in Appendix Note 2.1. PCR cloning and mutagenesis primers for all of these cloning and mutagenesis steps are contained in Appendix Tables 2.3 and 2.4.

Expression and purification

In parallel, High Five insect cells (Invitrogen) were transfected with the pMIB-V5-His-(A)-AMCase-Fc vectors containing both the protective (GATGAGCG), non-protective (AGGGAATT) haplotypes, and mock transfection. Blasticidin was used to select for stable cell lines. These cell lines were grown in culture dishes for 5 days and AMCase was purified from the supernatants using Immobilized Protein G resin (Pierce Biotechnology). A detailed purification protocol is described in Appendix Note 2.2. Eluted fractions were run on SDS-PAGE gels to ensure the purity of the AMCase protein. AMCase protein concentration was determined with the Micro BCA assay (Pierce Biotechnology) according to the manufacturer's instructions.

AMCase Enzymatic Assays

AMCase activity was determined using the synthetic chitin substrate 4-MU-(4-deoxy)chitobiose [88]. All enzymatic reactions for both determination of optimium pH and kinetic parameters were conducted in a volume of 100 μl, containing 100 ng of AMCase protein in McIlvaine's Buffer. All reactions were conducted in triplicate in 96 well fluorescent spectroscopy plates (Sigma) with 4-Methylumbelliferone also loaded as a standard. Reactions for both pH and kinetic assays were incubated in a SpectraMax Gemini XS fluorescence plate reader for 20 minutes at 37 degrees Celsius. Reactions were stopped by the addition of 120 μl of 1M glycine/NaOH pH 10.5 to reaction wells. Plates were

then immediately read on the SpectraMax fluorometer at an excitation wavelenght of 365nm and an emission wavelength of 460nm.

AMCase pH Assays

We tested activity of both AMCase isoforms in McIlvaine's buffer at pH's 2.2, 2.6, 3.0, 3.4, 3.8, 4.2, 4.6, 5.0, 5.4, 6.0, 7.0, 8.0 to determine the AMCase pH profiles. Activity at all pH values were tested at a substrate concentration of 75 μM.

AMCase Kinetic Reactions

Michaelis-Menton kinetic curves were generated by performing AMCase enzymatic reactions with the substrate 4-MU-(4-deoxy)chitobiose at concentrations of 10, 25, 50, 75, 100, 150, and 200 μM. Assays for kinetic measurements were generated for both AMCase isoforms at pH's of 2.2, 4.6, and 7.0. Reactions were conducted as described in General.

AMCase Enzymatic Data Analysis

Activity in pH profiles was displayed as a percent of activity at the pH where the isoform was most active (pH 4.6 for both isoforms). The pH profiles diplayed in Figure 2.3A are the result of a single experiment representative of at least two independent experiments.

For determination of enzyme velocities the mean fluorescence units detected from the triplicate assays were converted to picomoles of product produced by extrapolation using the 4-MU standard curves generated on each plate, and

divided by reaction time and enzyme amount. Enzyme velocities divided by the

amount of enzyme in the reaction were plotted versus the substrate

concentration and fitted to the Michaelis-Menton equation by non-linear

regression to determine the kinetic parameters, Km and Kcat of each experiment,

using the program Graphpad Prism 4.0. The kinetic parameters listed in the

paper are the mean of 4 independent experiments for the analysis at pH 4.6 and

2 independent experiments for the analysis at pH 2.2 and 7.0. Statistical

comparison of the kinetic parameters was by two-sample two-tailed T-Test

assuming equal variances.

2.3 Results

AMCase Resequencing and Linkage Disequilibrium

We resequenced all 12 *AMCase* exons and exon-intron boundaries in our SNP

Discovery panel, which includes African Americans, Mexicans, and Puerto

Ricans. We identified 21 variants including 2- 5' untranslated region (UTR)

insertions, 9 non-synonymous single nucleotide polymorphisms (SNPs), 4

synonymous SNPs, 3 UTR SNPs, and 3 intronic SNPs, of which 9 were

previously undescribed (Appendix Table 2.5).

In an effort to identify functionally significant variants, we restricted our analysis

to the 8 common (>5% allele frequency) non-synonymous SNPs: A290G

Asn45Asp, G296A Asp47Asn, G339T Arg61Met, G461A Gly102Arg, A531G

Lys125Arg, G1172A Val339Ile, T1218C Phe354Ser, and G1452T Gly432Val

(Figure 2.1). Linkage disequilibrium (LD) analysis (Table 2.2) revealed complete

LD between the three novel SNPs: A290G, G296A, and G339T, in all three

populations.

Figure 2.1
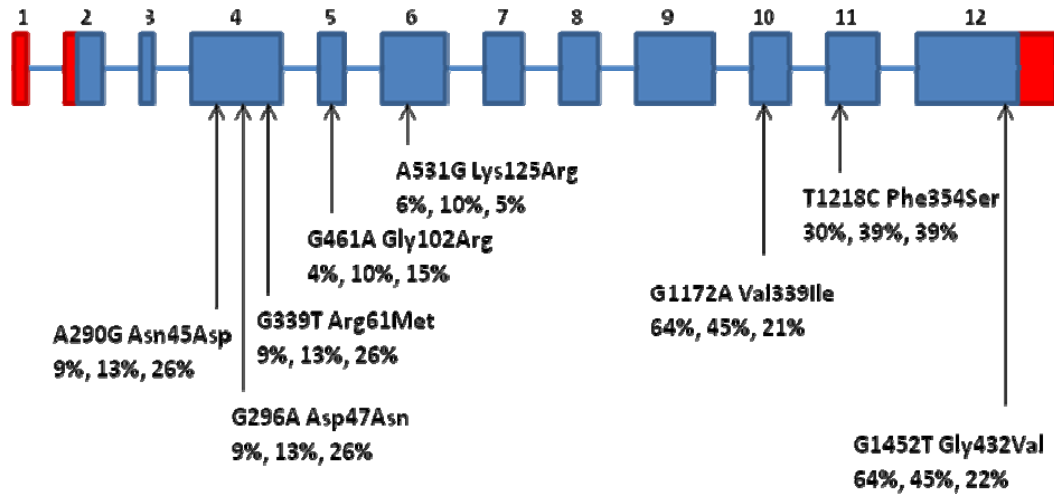
Fig. 2.1 The structure of the human AMCase gene, with accompanying common non-synonymous SNPs present among Mexicans, Puerto Ricans and African Americans. Exons are identified by blue and red block structures and numbered accordingly. The red portion of exons represent untranslated regions and the blue represent the coding portion of exons. All variant mRNA and amino acid positions are reported with regard to mRNA sequence uc001eas.1 (UCSC Genome Browser). Percentages listed refer to minor allele frequencies in Mexicans, Puerto Ricans, and African Americans, respectively. The minor allele and amino acid is listed second.

Table 2.2 Linkage Disequilibrium values for African Americans and Latinos

SNPs	G339T			G461A			A531G			G1172A			T1218C		
G339T		*													
G461A	6	2	0		*										
A531G	2	2	1	1	1	0		*							
G1172A	9	11	16	2	9	7	1	7	10		*				
T1218C	38	19	21	5	7	2	8	12	11	18	53	75		*	
G1452T	10	12	15	2	9	7	1	8	10	84	97	99	19	61	74

Note: Linkage disequilibrium values listed are pairwise r2. LD values in black, red, and blue refer to those determined among African Americans, Puerto Ricans, and Mexicans, respectively. LD values were determined from the SAGE controls, and GALA parents. SNPs A290G and G296A were in complete linkage disequilibrium in all three populations.

Asthma association analysis among African Americans

Based on the linkage disequilibrium patterns, we genotyped and analyzed 6 of

the 8 common non-synonymous SNPs (G339T, G461A, A531G, G1172A,

T1218G, G1452T) in the SAGE cohort. The SAGE study includes 264 asthmatic

cases and 176 healthy controls all self-identified as African Americans. SNP

G339T served as a proxy for both SNPs A290G and G296A, due to the complete

linkage disequilibrium exhibited between these three SNPs. Allele frequencies in

SAGE cases and controls are displayed in Table 2.3. SNPs were tested for

association with asthma disease status by standard logistic regression correcting

for age, gender, and percent ancestry. The only SNP to achieve statistical

significance between asthma cases and controls was SNP G339T (proxy for

SNPs A290G and G296A) located in exon 4 of *AMCase* (Table 2.3).

Table 2.3 Association analysis of AMCase SNPs with asthma in SAGE

SNP	Allele Frequency (%)		O.R. (95% C.I.)	P Value
	Cases	Controls		
G339T	16.8	25.9	0.57 (0.38-0.83)	0.004
G461A	17.1	14.5	1.15 (0.74-1.78)	0.525
A531G	4	4.6	0.90 (0.42-1.90)	0.774
G1172A	22.4	20.7	1.25 (0.85-1.83)	0.255
T1218C	32	39.2	0.81 (0.58-1.13)	0.216
G1452T	23.2	21.7	1.26 (0.86-1.84)	0.23

Notes: O.R. = Odds Ratio, C.I. = Confidence Interval, Odds Ratio and P value are generated from a logistic
regression model.

The minor T allele of SNP G339T was present at a frequency of 25.9% in the

controls and 16.8% in asthmatic cases, revealing a protective effect (Odds Ratio

(OR) = 0.57, 95% Confidence Interval (CI)=0.38-0.83, P=0.004). To confirm the

lower allele frequency of SNP G339T among asthmatics we genotyped two

additonal independent groups of African American asthmatic cases. In African

Americans with asthma from the CHIRAH cohort (n=321) we observed an allele

frequency of 16.7%, nearly identical to that of SAGE asthmatic cases. We

analyzed a third group of African American asthmatics from the ACRN trials

(n=334) to further confirm the allele frequency of SNP G339T. The frequency of

the 339 T allele in these subjects was 18.2%. Due to the absence of a control

population for the CHIRAH and ACRN case groups, we tested association of the

G339T SNP with asthma disease status using the common set of SAGE controls.

Comparing allele frequency of the G339T SNP in CHIRAH cases to SAGE

controls by logistic regression resulted in an Odds Ratio of 0.62, CI=0.43-0.89,

P=0.01 Table 2.4. IAE was also included in this model to correct for the potential

confounding effect of ancestry differences between CHIRAH cases and SAGE

controls. Comparison of the 339 T allele frequency in the ACRN cases to SAGE

controls resulted in an OR=0.64, CI=0.47-0.88, P=0.005 Table 2.4. Combining

the cases from the SAGE, CHIRAH, and ACRN (N=919) studies and comparing

339 T allele frequency to SAGE controls (N=176) resulted in an OR=0.62,

CI=0.47-0.81, P=0.001 (Table 2.4). Although these additional analysis with the

CHIRAH and SAGE cases have the limitation of using the same control set, the

allele frequency in cases is supportive of the original result in the SAGE case-

control set.

Table 2.4 Frequency and Asthma Association Analysis of SNP G339T in additional African American case groups

Subject Group	G339T Allele Freq (%)	O.R. (95% C.I.)	P Value
SAGE Controls (n=176)	25.9		
CHIRAH Cases (n=321)	16.7	0.62 (0.43-0.89)	0.01
ACRN Cases (n=334)	18.2	0.64 (0.47-0.88)	0.005
Combined Cases (n=919)	17.3	0.62 (0.47-0.81)	0.001

The CHIRAH and ACRN analysis used the SAGE controls. Analysis of the CHIRAH cohort included percent ancestry in the model. The combined analysis included all cases from the SAGE, ACRN, and CHIRAH compared to SAGE controls.

Asthma association analysis among Latinos

The same 6 SNPs tested among African Americans were also tested for association with asthma in the GALA trios by transmission disequilibrium test (TDT). Subjects in this study are part of family-based trios, which include both parents and an asthma affected child (proband). With respect to specific ethnicity the GALA cohort contains both Mexican (301) and Puerto Rican trios (389). When the entire GALA cohort was analyzed as a whole, the G allele of SNP A531G was undertransmitted to asthmatic subjects (p=0.05). In a stratified analysis this association was only observed among Puerto Ricans (P=0.03) and not Mexicans (p=0.8) (See Table 2.5).

Table 2.5 Family-based analysis of AMCase SNPs in GALA subjects

Variant	Mexicans		Puerto Ricans	
	OT Allele	p-value	OT Allele	p-value
G339T	G	0.17	G	0.6
G461A	A	0.34	A	0.49
A531G	A	0.8	A	*0.03*
G1172A	G	0.63	G	0.16
T1218C	C	0.89	T	0.91
G1452T	G	0.55	G	0.33

Note: OT-over transmitted allele

We did not observe a significant association between SNP G339T and asthma among Mexicans or Puerto Ricans separately. To determine if genetic risk differed by U.S. versus foreign residency, we performed a stratified analysis comparing Latino American trios (n = 274) to foreign Latino trios (n = 416). Analyzing all Latino Americans we observed significant undertransmission of the 339 T allele (p=0.04). This significant undertransmission of the 339 T allele among Latino Americans was due to undertranmission among both Mexican Americans (P=0.10) and Puerto Rican Americans (P=.22). These results replicate in Latino Americans both the presence and direction of the SNP G339T association observed in African Americans, as the 339 T allele is associated with protection from asthma in both populations. We did not find transmission distortion of SNP G339T among Foreign Latinos (p=0.94). The protective effect of the 339 T allele observed in African Americans and Latino Americans but not

foreign Latinos indicate the effects of this SNP could be modified by U.S. residency or associated environmental and/or socio-economic variables.

Haplotype Analysis and Enzymatic Characterization

The observed association with SNP G339T, which is also linked to SNPs A290G, and G296A, could be due to any of these SNPs or due to haplotypic combinations of these SNPs with the other 5 SNPs in the *AMCase* gene. Therefore, we determined 8 SNP haplotypes in both SAGE and GALA subjects using the program HAPSTAT to determine the haplotype context in which the protective alleles occured [87]. We observed 6 different common haplotypes in African Americans, and Latinos (Figure 2.2). The T allele of SNP G339T only occurred on one haplotypic background in both the African American, and Latino populations. Therefore, we defined the haplotype containing the protective alleles, GATGAGCG, as the protective haplotype, and the other five haplotypes as non-protective. The haplotypes and SNPs contained therein are overlayed on a domain model of the AMCase protein shown in Figure 2.2.

Figure 2.2

Risk Conferred	A290G	G296A	G339T	G461A	A531G	G1172A	T1218C	G1452T	AA	PR	MX
Protective	G / Asp	A / Asn	T / Met	G / Gly	A / Lys	G / Val	C / Ser	G / Gly	24.1	12.5	8.4
Non-protective	A / Asn	G / Asp	G / Arg	G / Gly	A / Lys	A / Ile	T / Phe	T / Val	18.4	44.1	63.9
Non-protective	A / Asn	G / Asp	G / Arg	G / Gly	A / Lys	G / Val	T / Phe	G / Gly	24.9	4.9	2.4
Non-protective	A / Asn	G / Asp	G / Arg	A / Arg	A / Lys	G / Val	T / Phe	G / Gly	10.2	9.6	3.4
Non-protective	A / Asn	G / Asp	G / Arg	G / Gly	A / Lys	G / Val	C / Ser	G / Gly	10.2	17.9	15.4
Non-protective	A / Asn	G / Asp	G / Arg	G / Gly	G / Arg	G / Val	C / Ser	G / Gly	4.5	8.6	5.3

Fig. 2.2 AMCase protein domain structure and common haplotypes with frequencies in African Americans (AA), Puerto Ricans (PR), and Mexicans (MX). Protective SNPs are shown in red. The haplotype alleles for SNPs A290G and G296A are inferred based on the linkage disequilibrium patterns defined in resequencing. Haplotypes frequencies reported are for SAGE controls, and GALA parents.

As observed from Figure 2.2, the AMCase protein is composed of an N-terminal signal peptide, followed by a glycosyl hydrolase 18 domain, containing a highly conserved active site, linked to the chitin binding domain by a hinge region. The amino acid changes associated with asthma protection, Met61 and the linked SNPs Asp45 and Asn47, are clustered together in the glycosyl hydrolase domain and are in close proximity to the active site of the enzyme. Therefore, we

hypothesized that the protective isoform of the AMCase protein might modulate

the kinetics of chitin cleavage compared to a non-protective isoform. To

investigate this hypothesis, we expressed the protective isoform and a non-

protective recombinant isoform of AMCase fused to an Fc purification tag, in

insect cells. The protective isoform (Asp-Asn-Met-Gly-Lys-Val-Ser-Gly) contains

the variant amino acids designated by the protective haplotype GATGAGCG and

the non-protective isoform (Asn-Asp-Arg-Gly-Lys-Ile-Phe-Val) was encoded for

by the haplotype AGGGAATT. The non-protective haplotype AGGGAATT was

chosen because it is common in African Americans, Puerto Ricans, and

Mexicans. After purification of the proteins by affinity chromatography, chitinase

activity was assessed by enzymatic digestion of synthetic substrates. Although

chitinase activity is commonly determined using fluorogenic substrates, such as

4-methylumbelliferyl-β-N,N'-diacetylchitobioside(4-MU-chitobioside: two N-

acetylglucoamine sugars linked to the fluorescent 4-MU molecule), AMCase

enzymatic activity is inhibited at high substrate concentrations, thus precluding

accurate determination of kinetic parameters by this method [88]. Therefore, we

used a chemically altered version of 4-MU-chitobioside, 4-MU-(4-deoxy)-

chitobiose, which has been modified to prevent substrate inhibition, thus allowing

accurate determination of enzyme kinetics [88]. Previous work has shown the

optimal pH of AMCase enzymatic activity is 4.6, with sharply declining activity at

more acidic and basic pH values [79]. We determined the pH profile of enzymatic

activity for both isoforms of the protein over a range of pHs from 2.2-8.0, and

corroborated that both isoforms display optimal enzymatic activity at pH 4.6 [79].

Enzymatic activity of the AMCase isoforms is expressed as a percentage of their respective activity at the optimal pH 4.6. Interestingly, we found the protective isoform retained near optimal activity at acidic pH values (Figure 2.3A). In contrast, enzymatic activity of the non-protective isoform declined sharply with decreasing pH, as previously reported for AMCase (Figure 2.3A) [79]. Furthermore, the protective isoform maintains 57% and 28% of optimal enzymatic activity at pH's of 6.0 and 7.0, respectively, whereas the non-protective isoform has only 31% of optimal enzymatic activity at pH 6.0 and is essentially devoid of activity at pH 7.0.

We next kinetically characterized the AMCase isoforms to identify any differences in absolute enzyme activity between the protective and non-protective AMCase isoforms. To conduct the kinetic analysis we selected three pH values, which reflect the three pH environments likely to contain AMCase in the human body: (1) pH 2.2, reflecting the stomach acidity where AMCase is highly expressed; (2) pH 4.6, reflecting the pH of lysosomes where some AMCase likely resides; and (3) pH 7.0, reflecting the pH of the airway lumen. AMCase isoforms were tested at a range of substrate concentrations from 10-200 μM, and data tightly fit the Michaelis-Menton (MM) equation at all pH values for both isoforms ($R^2 > 0.95$). The kinetic parameters determined for the two AMCase isoforms at pH 2.2 revealed a significant increase in enzymatic activity of the protective isoform vs. the non-protective isoform at both sub-saturating (protective Km=48 μM, non-protective Km=171.6 μM, P=1.8 X 10^{-3}) and

saturating substrate concentrations (protective Kcat=1.43 s^{-1}, non-protective

Kcat=0.34 s^{-1}, P=1.9 X 10^{-3}) (Figure 2.3B). The protective isoform was similarly

more active than the non-protective isoform at pH 4.6 (protective Kcat=1.83 s^{-1},

Km=30.18 μM; non-protective Kcat=0.71 s^{-1}, Km=70.3 μM, P=6.2 X 10^{-8} and 4.6

X 10^{-4}, respectively) (Figure 2.3C). While enzymatic activity for the protective

isoform was considerablely lower at pH 7.0 (Kcat=0.56 s^{-1}, Km=71.75 μM),

activity was barely detectable for the non-protective isoform (Kcat=0.05 s^{-1}

Km=134 μM, Kcat P=8.5 X 10^{-3}, Km P=5.4 X 10^{-3}) (Figure 2.3D).

Figure 2.3
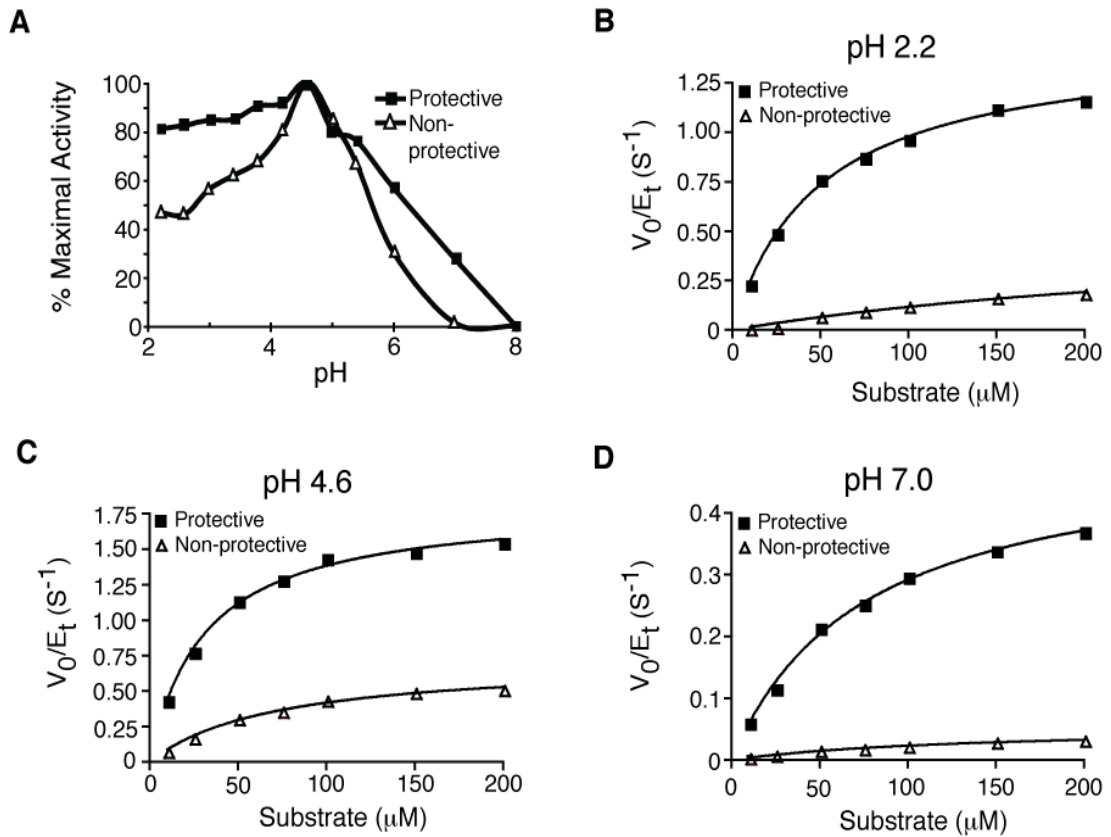
Fig. 2.3 pH and kinetic characterization of AMCase isoforms. AMCase activity was determined using the synthetic chitin substrate 4-MU-(4-deoxy)-chitobiose. (A) pH activity profile of protective and non-protective AMCase isoforms. Activity at each pH is expressed as a percentage of activity at optimum pH. Michaelis-Menton kinetic profiles of AMCase isoforms. (B) Assays conducted at a pH of 2.2. (C) Assays conducted at a pH of 4.6. (D) Assays conducted at a pH of 7.0. Substrate concentrations tested were 10, 25, 50, 75, 100, 150, and 200 μM. Curves were fit to data by nonlinear regression using the Michaelis-Menton equation. All graph points are the mean of triplicate measurements and representative of multiple experiments.

2.4 Discussion

Our results indicate that subjects haboring the novel 339 T allele, tagging an AMCase haplotype, will produce an enzymatically enhanced isoform of the AMCase protein. This gain-of-function AMCase haplotype was associated with protection from asthma in an African American case-control study, with the lower frequency of the 339 T allele among cases confirmed in two independent groups of asthmatic cases. Furthermore, we validated the G339T association with asthma protection in another ethnic group, Latino Americans. These results provide the first evidence that differential AMCase enzymatic activity may modulate risk for human asthma.

Although a former study has associated both SNPs and haplotypes in the *AMCase* gene with asthma, in a German case-control cohort, functional effects of these SNPs and haplotypes were not determined, making their functional interpretation difficult. Furthermore, our study is not directly comparable to theirs as our analysis was based on the full length AMCase gene (AF290004) and their analysis on a shorter version of the AMCase transcript, lacking the fourth exon, which contains the novel SNPs associated with asthma and enzymatic activity in this study. However, it is interesting to note that the same SNP they found to be associated with asthma protection in their German cohort, (K17R) named A531G in our study, was also associated with asthma protection among Puerto Ricans, but not Mexicans and African Americans. These discrepancies could result from differences in linkage disequilibrium patterns between this SNP and

another causative SNP, or may be due to effect modification by different environmental factors. Further analysis will be needed to confirm this result.

Our examination of human AMCase genetics was based upon studies implicating chitinases in both the promotion and inhibition of Th2 inflammation in the airway. Specifically, Zhu *et al.* has reported overexpression of AMCase in ovalbumin sensitized mice after antigen challenge. Furthermore, blockade of AMCase activity significantly reduced bronchial hyperresponiveness and Th2 inflammation in this model [50]. Human AMCase mRNA has also been observed to be overexpressed in the lungs of fatal asthmatics. However, a recent mouse study revealed chitin can trigger the accumulation of immune cells associated with allergy, such as eosinophils and basophils, with AMCase negatively regulating this process [51]. These seemingly disparate results could result from multiple roles for the AMCase protein, particularly in the presence or absence of chitin.

Interestingly, we find the protective AMCase isoform was significantly more active than the non-protective isoform at all three pH's tested. Although we only tested one of the five non-protective AMCase isoforms, the haplotype encoding this isoform is found at a high frequency in Puerto Ricans (44.1%), Mexicans (63.9%), and among African Americans (18%). Furthermore, the kinetic parameters we determined for the protective isoform ($Kcat=1.83$ s^{-1}, $Km=31\mu M$) are two-fold greater than those reported by Chou *et al.* for AMCase ($Kcat=0.81$s^{-1}, $Km=61$ μM), whereas parameters determined for the non-protective isoform are quite similar ($Kcat=0.71$ s^{-1}, $Km=70.3$ μM), giving further credence to the

anomalous nature of the protective haplotype and the representative nature of the non-protective haplotype [79]. Additionally, pH profile results of Chou *et al.* mirrored those determined for the non-protective haplotype tested and differed dramatically from the protective haplotype at acidic and neutral pH values. These dramatic increases in chitinase activity of the protective AMCase isoform at pH 2.2 (4-fold higher) and 7.0 (10-fold higher), corresponding to the pH of the lungs and stomach respectively, is especially relevant since the lumen of these organs are exposed to exogenous chitin through inhalation and ingestion. Indeed, some of the most common indoor allergens in Western societies, such as the U.S., include dust mites, molds, and cockroachs, which are all chitin-containing organisms. In fact, dust mite and cockroach feces, components of house dust and a major source of allergy, are surrounded by a chitinous peritrophic membrane [89, 90]. Lack of childhood exposure to bacterial products such as endotoxin has been associated with increased risk of asthma in some studies, supporting the hygiene hypothesis of allergic diseases. In this context, it is noteworthy that chitinases produced by bacteria comprise an important mechanism for degradation and turnover of the massive amounts of chitin produced in marine environments [91]. The widespread use of anti-microbial cleaning products and antibiotics in modern Western society may have inadvertantly contributed to the accumulation of environmental chitin through suppression of bacterial populations producing chitinases critical to environmental chitin degradation (Figure 2.4).

Figure 2.4
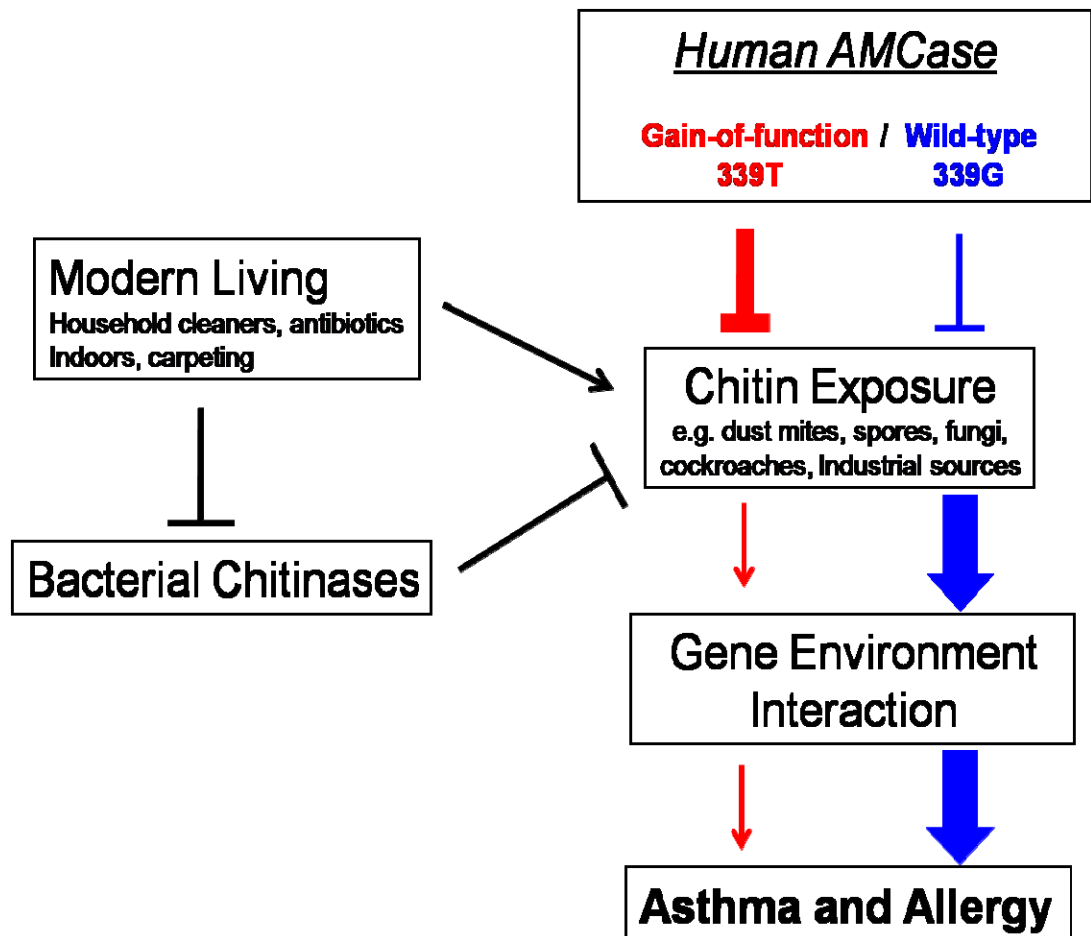
Fig. 2.4 Potential causal model describing the interaction of pro-inflammatory environmental chitin exposures with chitinase genetics. In line with the hygiene hypothesis we predict decreased bacterial populations (and accompanying bacterial chitinases) due to the prevalence of anti-microbial products in developed societies, which may increase environmental chitin exposure. In this environment of increased chitin exposures a gain-of-function AMCase variant would be protective against development of allergy and asthma.

Interestingly, individuals employed in industries where exposure to chitin would be anticipated to be high, such as shellfish processors, have a high prevalence of new onset asthma [92]. The development of asthma in these industries has been

linked with processing steps involving high pressure shell meat removal, which produce vapors and particulate aerosols [92]. In settings of high environmental chitin burden it would prove beneficial for subjects to produce a hyperfunctional AMCase enzyme capable of degrading this inflammatory stimulus (Figure 2.4). Within this paradigm the lack of association between SNP G339T and asthma among foreign Latinos could be explained by social and environmental differences giving rise to differences in environmental chitin burden or exposure. Indeed, home levels of chitinous dust mite aeroallergens, measured in Boston area homes, were inversely correlated with poverty level [93].

Intriguingly, the enhanced acidic pH activity of the protective isoform of AMCase may reflect improved activity in the stomach, where the degradation of ingested polymeric environmental chitin or chitin-containing micro-organisms could lead to modifications of the bowel commensal flora or to alterations in immune responses to ingested allergens. Ingested polymeric chitin has been observed to disrupt interactions with host proteins involved in regulating bacterial adherance to the gastrointestinal epithelium, such as RegIIIγ, and to be used as the preferred energy source by certain gut bacteria [94, 95]. Alterations in the intestinal microflora modified the subsequent immune response to allergens in the lung in experimental models [96].

 In summary, our findings indicate that increased AMCase enzymatic activity is associated with protection against the development of human asthma. Further studies are warranted in larger populations to confirm our results. Moreover,

these studies should take into account variables which may act as surrogates of environmental chitin exposure, a possible effect modifier of AMCase genetic variation.

Chapter 3 Genetics of Chitotriosidase in Asthma among admixed populations.

3.1 Introduction

As discussed in Chapter 1 chitin has been observed to function as a sort of pattern recognition molecule triggering Th2 type immune responses in the lungs of mice [51]. Furthermore, the inflammatory effect of chitin depended on it being present in a polymeric form, as digestion of the chitin polymer by AMCase to oligosaccharides alleviated the inflammatory effects [51].

These results fit well with the association between asthma protection and the gain-of-function AMCase haplotype we observed in both African Americans and Latino Americans, detailed in Chapter 2. However, there are two active chitinases in the human genome, the other being chitotriosidase (CHIT1). Both AMCase and CHIT1 belong to the Family 18 of glycosyl hydrolases and cleave chitin polymers by the same substrate-assisted hydrolytic mechanism [97]. In humans, CHIT1 is recognized to be the primary chitinase in the blood, while AMCase is the dominant chitinase in the digestive tract [97]. Both AMCase and CHIT1 expression have been observed in the human lung, although enzymatic activity resulting from each specific chitinase has not been determined [50, 53]. Based upon the expression and possible activity of the CHIT1 protein in the human lung, we hypothesized that genetic variants affecting function of the CHIT1 protein may also be associated with asthma traits.

Boot et al. determined that 5% of the Dutch population is totally deficient in CHIT1 activity [63]. Subsequent studies have determined the source of this deficiency to be a 24 bp duplication in the 10th exon of the CHIT1 gene, resulting in an unstable mRNA that is quickly degraded [63]. Subsequent studies have established this duplication variant is present in several other ethnic groups [98, 99]. In this chapter we explore the potential role of the null 24 bp duplication variant in the CHIT1 gene on risk for asthma and related traits among admixed populations.

3.2 Materials and Methods

Study Populations

Subjects genotyped and analyzed in this chapter include trios from the Genetics of Asthma in Latino Americans (GALA) study, and the asthmatic cases and matched controls from the Study of African Americans, Asthma, Genes and Environments (SAGE). Resequencing analysis was completed in the SNP discovery series subjects, which include 24 African Americans, 24 Puerto Ricans, and 24 Mexican subjects. All of these study subjects and their recruitment is described in further detail in Section 2.2. We also obtained DNA samples from 24 healthy Caucasian subjects for use in this SNP discovery analysis.

Genotyping

The CHIT1 24bp duplication (rs3831317) was genotyped using the AcycloPrime-FP-TDI (PerkinElmer) method [84]. The PCR cocktail included: 3.0-5.0 ng genomic DNA, 0.1-0.2 µM primers, 2.5 mM MgCl2, 50 µM dNTPs, 6 µl volume with Platinum Taq PCR buffer and 0.1-0.2 units Platinum Taq (Invitrogen) plus 1 µl extra water to counteract evaporation. PCR cycling conditions were as follows: 95°C for 2 minutes, 40 cycles of 92°C for 10 seconds, 57°C for 20 seconds, 68°C for 30 seconds, and final extension at 68°C for 10 minutes. We used AcycloPrime-FP kits for enzymatic cleanup and single base extension genotyping reactions. Plates were read on an EnVision fluorescence polarization plate reader (PerkinElmer). Forward and Reverse PCR primers used in the FP assay

are, CCAAGGCTGTCTCCAGAATC and AGGTGGGGTACAAACACAGC. The

FP primer used in the allele-specific amplification was

GTCATCTAAGTCCAGTGCCCAGACCATGGCCCCGCCCAGTCCCT. An A/T

dye combination was used in the FP assay, with the A allele representing the

duplication and T allele representing absence of the duplication.

Statistical Analysis

Deviation from Hardy-Weinberg Equilibrium (HWE) was tested by χ^2 goodness-

of-fit test, using 0.01 as the cut-off for significance. Test for association with

asthma disease status in the African American case-control cohort was

conducted by logistic regression including the covariates age, gender, and

individual ancestry estimate (IAE). Generation of the IAE variable is described in

Section 2.2. Duplication genotypes were coded 0 for subjects homozygous for no

duplication and coded 1 if they were either heterozygous or homozygous for the

duplication. Both quantitative traits, IgE levels and forced expiratory volume in 1

second (FEV$_1$ % of predicted) were tested by linear regression including the

same covariates as in the asthma model. IgE levels were log transformed to

achieve normal distribution of the variable.

Tests for association with asthma, FEV$_1$ % of predicted, and IgE levels among

Latino trios was conducted by transmission disequilibrium test (TDT) using the

Family Based Association Tests (FBAT) [86].

CHIT1 gene resequencing

All *CHIT1* exons (12) and exon-intron boundaries (20 bp from either end of the exon) were sequenced in the SNP Discovery panel. Resequencing of 48 chromosomes provided power of > 90% to find all genetic variants of 5% or greater frequency in the general population. Sequencing reactions were performed using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, California) and the reactions were processed on the ABI Prism 3700 sequencher. PCR primers used to produce CHIT1 exon PCR products are listed in Appendix Table 3.1.

3.3 Results

Genetic Association Analysis in African Americans

We genotyped the 24bp duplication in the CHIT1 gene in 264 African American asthmatic cases and 176 matched African American healthy control subjects from the SAGE study. Genotyping results among both cases and controls were consistent with Hardy-Weinberg Equilibrium (HWE) (p>0.9). The duplication was present at frequency of 6.3% in cases and 8.0% in healthy controls, frequencies much lower than that observed in populations of European (17%) and Asian (56%) descent [98]. Therefore, all genotype models tested were dominant due to the dearth of subjects homozygous for the duplication. No significant association between the duplication polymorphism and asthma disease status was detected among the SAGE study subjects (Table 3.1). Additional quantitative tests for association with FEV1 % of predicted, a measure of asthma severity, and IgE levels did not yield significant results (p>0.3).

Table 3.1 African American case-control association analysis of 24bp duplication

Group	Genotype	Counts	Genotype Freq.	Allele Freq.	Odds Ratio / P Value
Cases	*No Duplication*	229	87.7		
	Heterozygous	31	11.9	6.3%	
	Homozygous Duplication	1	0.38		0.76 / 0.67
Controls	*No Duplication*	149	84.7		
	Heterozygous	26	14.8	8%	
	Homozygous Duplication	1	0.6		

Note: Odds ratio and p value are for genotypic logistic regression

Genetic Association analysis in Latinos

We genotyped the CHIT1 24bp duplication in Latino asthmatics trios, including both Puerto Ricans and Mexicans. The duplication frequency was much greater among Latino parents compared to African American controls, displaying allele frequencies of 24.4% in Mexicans and 22% in Puerto Ricans. Transmission disequilibrium test (TDT) analysis was conducted to discern over or under transmission of the duplication polymorphism, and thus association with asthma, in both Mexican and Puerto Rican trios. We did not observe significant transmission distortion in Mexicans or Puerto Ricans by either allelic or recessive risk models (Table 3.2). Quantitative TDT analysis was also conducted for both FEV_1 % of predicted and IgE levels, again with insignificant transmission distortion.

Based on our prior AMCase association in Latino Americans we also examined the Latinos stratified by U.S. or foreign residency. Tests for all three asthma traits failed to reach significance in residence stratified analysis. However, in the analysis for IgE levels we did notice a trend for both over and under transmission of the homozygous duplication genotype (recessive model) in foreign Latinos (p=0.1) and Latino Americans (p=0.08), respectively (See Table 3.2).

Table 3.2 Transmission disequilibrium analysis of GALA trios for asthma traits

Population	Asthma		FEV$_1$ % of predicted		LogIgE	
	Allelic	Rec.	Allelic	Rec.	Allelic	Rec.
Mexicans	**0.85**	**0.69**	**0.2**	**0.26**	0.68	0.46
Puerto Ricans	**0.72**	1	**0.72**	**0.18**	0.61	**0.23**
Foreign Latinos	**0.16**	**0.21**	**0.43**	**0.16**	**0.95**	**0.1**
Latino Americans	0.29	0.38	**0.35**	**0.32**	0.2	0.08

Note: Results listed under Rec. refer to recessive TDT analysis. P values obtained from FBAT are listed.

SNP Discovery

Due to the lack of association observed for the 24 bp duplication and the low

allele frequency observed among African Americans; we screened the entire

coding region of the *CHIT1* gene for other potential risk variants. Resequencing

analysis of all 12 *CHIT1* exons was conducted in 4 U.S. ethnic groups including

African Americans, Mexicans, Puerto Ricans, and Caucasians for comparison.

We identified 5 non-synonymous SNPs and 3 insertion/deletion polymorphisms

in the *CHIT1* gene. Allele frequencies varied greatly between ethnic groups, with

the C776G SNP being African-specific (Figure 3.1). Interestingly, we identified

two novel deletion polymorphisms including, a 29 bp deletion in exon 9 at

position 1083, and a 4 bp deletion in exon 11 at position 1166. The 29 bp

deletion was only observed among African Americans (2.1%) and Puerto Ricans

(6.3%). The 4 bp deletion was only observed among African Americans (8.3%) and Mexicans (2.1%). Both of these SNPs are predicted to alter the reading frame and truncate the CHIT1 protein. One of the SNPs identified, C1337G, was bi-allelic in all populations except African Americans, where a third (T) allele was also observed.

Figure 3.1
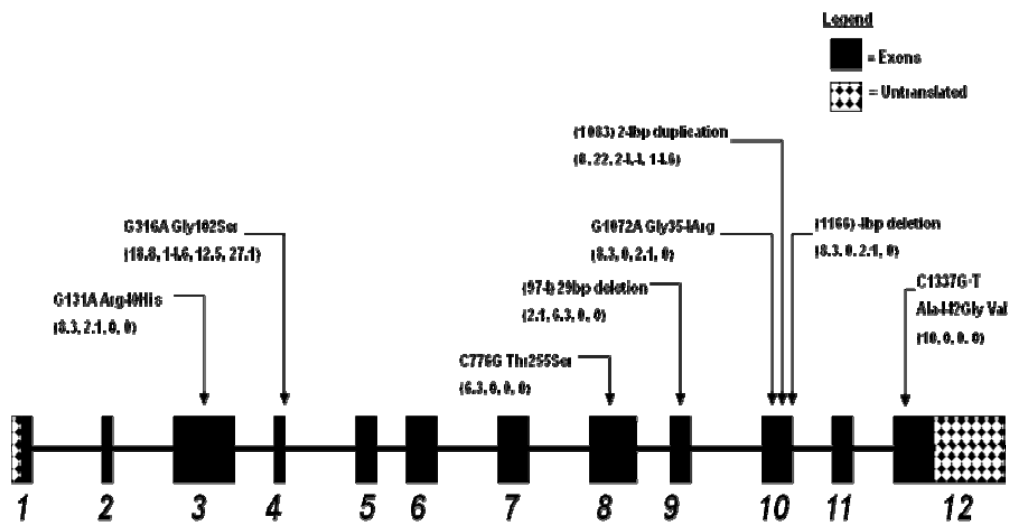
Fig. 3.1 CHIT1 gene structure with genetic variants in African Americans and Latinos

3.4 Discussion

Prior data examining the effects of the AMCase enzyme on the development of asthma and Th2 inflammation in mouse models have strongly implicated it's enzymatic activity in either the promotion or alleviation of Th2 inflammation in the lung. Furthermore, our work in humans has also found a gain-of-function haplotype in the AMCase gene to be associated with protection from the development of asthma. Acknowledging the possible critical role of enzymatic activity on contribution of AMCase to asthma pathology; we explored the role of a common null polymorphism in the *CHIT1* gene on predisposition to asthma traits. We did not observe significant associations between asthma traits and the null 24 bp duplication in African Americans, Mexicans, or Puerto Ricans. Interestingly, we found the duplication to be present at a much lower frequency among African Americans compared to Caucasians and Latinos. Therefore, our results will need to be replicated in a much larger subject group to ensure our results were not due to Type II error. Additionally, one could hypothesize that effects of the duplication variant may only be relevant to disease status in the homozygous form, in which subjects would be totally deficient in chitinase activity. Testing this hypothesis would require a much larger subject group in African Americans. However, the allele frequency was much greater among both Puerto Ricans and Mexicans allowing a more powerful analysis. Despite this, results were similarly negative for all asthma traits, including TDT using the recessive model. In Chapter 2 we described association between an AMCase haplotype in Latino Americans but not foreign Latinos. Hypothesizing similar

effects for the CHIT1 duplication we performed analysis of Latinos stratified by U.S. or foreign residence. Although results for both asthma and IgE levels trended in opposite directions between foreign and U.S. Latinos, all of these results failed to reach statistical significance. This stratified analysis may have suffered from inadequate power, most especially in the recessive model, and further studies will be needed to conclusively determine risk. We note however, that our results are supportive of the only other genetic study of the CHIT1 duplication, in which the duplication was not observed to confer risk for asthma in a German case-control cohort.

There are several other limitations to this analysis including the possibility that significant allelic heterogeneity contributed to the negative results for the *CHIT1* duplication. In exploring this possibility we resequenced the entire *CHIT1* gene coding region in multiple ethnic groups. Interestingly, we found two deletion polymorphisms (974) and (1083), likely to result in inactive CHIT1 protein being produced. Both of these deletions are present among African Americans at a frequency of 2.1%, and 8.3%, displaying a combined allele frequency 1.5x that of the 24 bp duplication in African Americans. Tests of clinical samples in patients carrying these indels will be needed to confirm the likely null enzymatic phenotype of these deletions. If proved to be null polymorphisms a combined analysis of these and the 24 bp duplication could be conducted. This analysis would allow better determination of the effects of CHIT1 deficiency on risk for asthma among African Americans. The deletion at position 974 is also present at a frequency of 6.3% in Puerto Ricans and could similarly be included in a

combined analysis examining the effect of CHIT1 deficiency on asthma risk. Furthermore, additional genotyping of the multiple other amino-acid changing variants, followed by complete haplotype analysis is needed to accurately determine the risk of CHIT1 genetics on asthma. This approach will be especially relevant to populations of African descent due to the high frequency of these SNPs.

In summary, we find no significant results implicating the CHIT1 24 bp duplication, and thus CHIT1 deficiency, in predisposition to asthma traits among multiple U.S. ethnic groups. Comprehensive resequencing of the CHIT1 gene did however reveal other common polymorphisms in the CHIT1 gene exist which may affect enzymatic activity. Further analysis is needed to confirm the role of CHIT1 genetics in asthma among these populations.

Chapter 4. Expression and Activity of Chitinases in the Human Lung

4.1 Introduction

Chitin, a linear polymer of β(1-4)-linked N-acetyl-D-glucosamine, is a component of the exoskeletons of mites and other arthropods, the lining of the insect gut, and the microfilarial sheath of parasitic nematodes [73, 74, 76, 100]. Chitin is also an important structural polymer in fungal cell walls, where it is functionally analogous to peptidoglycan in bacteria.

Chitinases are the enzymes that digest the chitin polymer, and humans have two chitinases encoded in their genome: chitotriosidase (CHIT1) and acidic mammalian chitinase (AMCase) [97]. The substrate for these chitinases is presumably environmental chitin, because all mammals lack a chitin synthase. CHIT1 is prominently expressed in macrophages, but the biological consequences of its over-expression in diseases associated with macrophage activation (lysosomal lipid storage disorders, thalassemia, sarcoidosis, and visceral Leishmaniasis) are unknown [101, 102]. A relatively common 24 base pair duplication in exon-10 of CHIT1 activates a cryptic 3' splice site and results in an enzymatically inactive protein deficient in 29 amino acids [63]. The relatively high prevalence of CHIT1 deficiency from this duplication prompted a search for other chitinases that may compensate for the lack of functional CHIT1. This led to the discovery of AMCase, named for its pronounced pH optimum at pH 2.3 [103]. Like CHIT1, AMCase is capable of cleaving artificial chitin-like substrates as well as natural substrates. The full-length cDNA for human AMCase is almost identical to

TSA1902-L and TSA1902-S - two isoforms previously described as splice variants of a gene encoding a chitinase-like protein in man [52]. These TSA transcripts are actually splice variants of the AMCase gene, but the proteins they encode do not have chitinolytic activity [52]. CHIT1 and AMCase belong to the family 18 of glycosyl hydrolases, which also includes other proteins structurally related to chitinases but lacking in chitinolytic activity [97]. These "chi-lectins" include YKL-40 (also called CHI3L1), a protein of uncertain function, which is elevated in serum in a variety of inflammatory diseases, including severe asthma [57]. Genetic variation in the YKL-40 gene has also been associated with asthma [104].

Insights into the role of chitinases and chitin in allergy and asthma have been greatly advanced by two recent studies. Zhu *et al* [50] showed that AMCase expression and activity is upregulated in an ovalbumin mouse model of asthma and that it's expression is dependent on IL-13. Furthermore, they found that inhibition of AMCase enzymatic activity prevents much of the airway hyperresponsiveness and inflammation present in these mice after challenge. In stark contrast, Reese *et al* [51] showed that polymeric chitin administrated to the lungs of mice could induce the recruitment of immune cells associated with allergy and asthma, such as eosinophils and basophils. Moreover, AMCase mediated degradation of chitin acted as a negative regulator of this process. Despite these important but contrasting roles for chitinase activity in the development of allergic inflammation in the lungs of mice, only sparse clinical data is available to guide opinion on which of these conflicting roles operates in

human asthma. Although Zhu *et al* [50] used *in situ* hybridization to show that AMCase gene expression is increased in airway mucosal biopsies and in small airway tissues from asthmatics, these authors did not report on CHIT1 expression or on levels of chitinase activity in lung secretions. Therefore, in this study, we set out to determine the relative contributions of AMCase and CHIT1 to lung chitinase activity and to determine if chitinase activity differs from normal in asthma or among habitual smokers.

4.2 Materials and Methods

Subjects and clinical samples

We studied biological samples stored in the Airway Tissue Bank at the University of California, San Francisco (UCSF) that had been collected during research bronchoscopy from 40 non-smoking subjects with asthma, 25 habitual smokers without asthma, and 26 healthy non-smoking controls (Table 4.1). Asthmatic subjects had a prior physician diagnosis of asthma, PC_{20} methacholine <8 mg/mL, and were using only inhaled beta-agonist medications for therapy (additional data on the asthma subgroup is provided in supplementary Appendix Table 4.1). Smokers had been smoking at least 10 cigarettes per day and had at least a 10 pack-year total consumption. Healthy controls were non-smokers with no history of lung disease and PC_{20} methacholine >16 mg/mL. The samples withdrawn from the tissue bank for this study were aliquots of bronchoalveolar lavage (BAL) fluid supernatant, RNA from BAL macrophages, RNA from epithelial brushings, and DNA (extracted from either venous blood cells [n=40] or from frozen unfixed bronchial mucosal biopsies [n=18]). Epithelial brushings and macrophages from some of the subjects in all subject subgroups have been used in previously reported studies [49, 105-108].

Table 4.1 Characteristics of patients screened for chitinase activity

Characteristic	Controls	Asthmatics	Smokers
N	26	40	25
Gender	13F/13M	22F/28M	6F/19M*
Age (years)	40 ± 9	36 ± 12	51 ± 10*
FEV1 (% predicted)	103 ± 12	85 ± 11*	83±16*
FEV1/FVC	0.80 ± 0.05	0.85 ± 0.11*	0.66 ± 0.12*
PC20 (mg/dl methacholine)	64.0 (20.4, 64.0)	0.3 (0.1, 1.0)*	26.8 (6.9, 64.0)*
Pack Years Smoking	0 (0, 0)	0 (0, 0)	38 (27, 44)
GOLD Classification			
Smokers without airflow obstruction	-	-	16
Stage 1	-	-	4
Stage 2	-	-	5
DLCO (% predicted)†	-	-	86 ± 19%
No. with DLCO < 80% predicted	-	-	8
No. with chronic bronchitis (%)	-	-	8 (32%)

Note: Data are presented as mean +/- SD or median (interquartile range).

Abbreviations: FEV1, volume of air exhaled in the first second of a forced exhalation; FVC, forced vital capacity (volume of air exhaled in an entire forced exhalation); PC20, the concentration of methacholine that caused a 20% decline in FEV1; DLCO, diffusing capacity to carbon monoxide (†data for n = 24). GOLD classification denotes presence and severity of COPD. No subjects with asthma were using corticosteroids or long-acting β-agonists prior to enrollment.*, $P < 0.05$ compared with nonsmoking healthy control subjects based on Mann-Whitney two-sample ranksum test for quantitative traits and $\chi 2$ analysis for gender.

Clinical procedures

Spirometry, methacholine challenge, measurement of diffusing capacity and bronchoscopy had been performed using methods described previously [105]. At

bronchoscopy, bronchoalveolar lavage was performed in either the right middle lobe or lingula before brushings were obtained from lower lobe bronchi in the ipsilateral lung; the bronchoscope was then moved to the contralateral lung where bronchial biopsies were taken from 2nd through 4th order carinae of lower lobe, middle lobe, and upper segments.

Chitinase Activity Assays

Chitinase activity was determined using the synthetic chitin substrate 4-MU-(4-deoxy)chitobiose [88]. All chitinase assays were performed in a total volume of 100 microliters containing 15 microliters of unconcentrated BAL and 75 micromolar 4-MU-(4-deoxy)chitobiose in McIlvaine's buffer at three different pH values (pH 2.2, 4.6, or 7.0). Reactions were incubated in the dark for 2 hours at 37 degrees Celsius. All reactions were conducted in duplicate in 96 well fluorescent spectroscopy plates (Sigma) with 4-Methylumbelliferone also loaded as a standard. Reactions were stopped by the addition of 120 μl of 1M glycine/NaOH pH 10.5 to reaction wells. Plates were then immediately read on the SpectraMax Gemini XS fluorescence plate reader at an excitation wavelenght of 365nm and an emission wavelength of 460nm. For determination of BAL sample velocities, the mean fluorescence units detected from the duplicate assays were converted to nanomoles of product produced by extrapolation using the 4-MU standard curves generated on each plate, and divided by reaction time and volume of BAL loaded in the assay (15 μl).

Gene Profiling

Gene expression for CHIT1 and AMCase in epithelial cells and macrophages was measured using methods of real time RT-PCR described previously [105-107, 109]. For CHIT1, we designed one primer set against a region common to all known variants (Appendix Table 4.2). For AMCase, we designed two primer sets (Appendix Table 4.2 and Figure 4.3A). The first primer set (primer set 1) was designed to include a region across exon 6 that is known to result in an inactive variant when it is spliced out [52]. The second primer set (primer set 2) was designed against a portion of the AMCase mRNA contained in all known transcripts.

Genotyping

The CHIT1 24bp duplication (rs3831317) was genotyped using the AcycloPrime-FP-TDI (PerkinElmer) method [84]. The PCR cocktail included: 3.0-5.0 ng genomic DNA, 0.1-0.2 µM primers, 2.5 mM MgCl2, 50 µM dNTPs, 6 µl volume with Platinum Taq PCR buffer and 0.1-0.2 units Platinum Taq (Invitrogen) plus 1 µl extra water to counteract evaporation. PCR cycling conditions were as follows: 95°C for 2 minutes, 40 cycles of 92°C for 10 seconds, 57°C for 20 seconds, 68°C for 30 seconds, and final extension at 68°C for 10 minutes. We used AcycloPrime-FP kits for enzymatic cleanup and single base extension genotyping reactions. Plates were read on an EnVision fluorescence polarization plate reader (PerkinElmer). PCR primers and allele-specific FP primers and Fluorescent tagged terminator nucleotides are listed in Section 3.2.

SDS polyacrylamide gel electrophoresis for AMCase in BAL

To detect AMCase in BAL, we used a polyclonal anti-mouse YM1 antibody (Stem Cell Technologies), because this antibody was generated against a peptide sequence that is also present in human AMCase and there is no other orthologue to YM1 in humans [110]. Using purified human recombinant AMCase as a control, we confirmed that the YM1 antibody detects human AMCase.

Statistical Analysis

All two-way subject group comparisons of gene expression data and chitinase activity were performed by using the Mann-Whitney rank-sum test to account for the non-normally distributed nature of the tested variables. Correlations between chitinase activity at pH 4.6 and 7.0, and between chitinase activity and gene expression data was performed by Spearman's rank correlation test. Multiple linear regression analysis was used to test association of the CHIT1 duplication genotype on chitinase activity at pH 4.6. Chitinase activity was log transformed for use in the regression model. In the habitual smoker and asthmatic subgroups, linear regression was also used to test associations between measures of airflow obstruction (FEV1 % predicted) and chitinase activity (at pH 4.6). These models included potential confounders such as age, gender, and CHIT1 genotype. All statistical analysis was performed using STATA 8.0 S/E statistical software (College Station, TX).

4.3 Results

Lung chitinase activity is modulated by pH and smoking habit.

We measured total chitinase activity in the bronchoalveolar lavage (BAL) of 77 subjects including 31 asthmatics, 24 healthy subjects, and 22 habitual smokers using a synthetic chitin substrate, 4-MU-(4-deoxy)chitobiose [88]. This substrate is digested by both AMCase and CHIT1, but the pH profile for the activity of these two chitinolytic enzymes is distinct and can be used to infer whether CHIT1 or AMCase is the responsible enzyme for chitinase activity in a biological sample. For example, previous studies have shown that optimal enzymatic activity of AMCase and CHIT1 occurs at a pH of 4.6, but that AMCase enzyme retains 30% of its activity at pH of 2.2, whereas CHIT1 is inactive at this pH [79, 103]. In contrast, CHIT1 retains 75% of its enzymatic activity at pH of 7.0, whereas AMCase is only 30% active [79, 103]. We found that chitinase activity in BAL was undetectable at pH 2.2 in the large majority of samples; lavage from only one subject (a smoker) had activity above the lower assay limits (Figure 4.1A). In contrast, chitinase activity was easily detectable in BAL from all subject groups at pH's of 4.6 and 7.0, although values at pH 7.0 were ~20% lower than at pH 4.6 (Figure 4.1A). In addition, chitinase activity at pH 4.6 and pH 7.0 were strongly correlated (rho=0.99, p <.0.0001). This pH profile for chitinase activity in lavage is consistent with the activity of CHIT1 in lung secretions and inconsistent with significant AMCase activity [79, 103].

Figure 4.1
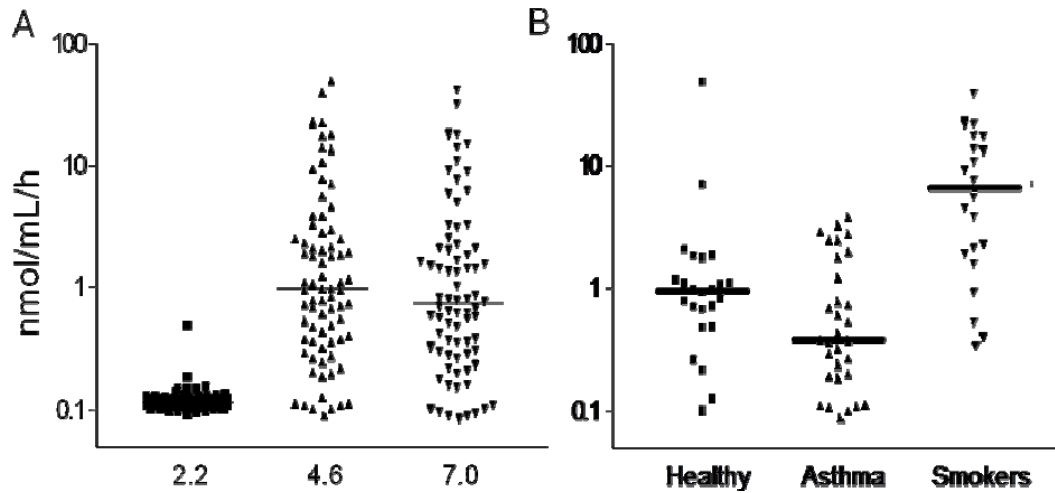
Fig. 4.1 Effect of pH and disease status on chitinase activity in 77 bronchoalveolar lavage (BAL) samples. Panel A – Points represent chitinase activity measured for each BAL sample using the synthetic chitin substrate 4-MU-(4-deoxy)chitobiose in McIlvaine's buffer at pH 2.2, pH 4.6, and pH 7.0. Line represents the median value. Panel B – Points represent chitinase activity measured for each BAL sample (at pH 4.6) stratified by disease status. Line represents the median value. *Indicates significantly greater than healthy, p<0.0001.

Comparison of chitinase activity at pH 4.6 between subject groups revealed that activity was lower than normal in the asthma subgroup and markedly higher than normal among habitual smokers(Figure 4.1B). Specifically, median chitinase activity was 60% lower than normal in the asthma subgroup (0.38 nmoles/ml/hr [interquartile range 0.2-1.77] vs 0.95 nmoles/ml/hr [0.57 – 1.47], p=0.077)(Figure 4.1B). In contrast, median chitinase activity was 7-fold higher than normal in the habitual smoker subgroup (6.64 [1.90 – 17.48], p<0.0001)(Figure 4.1B). The increase in chitinase activity was apparent in smokers with and without COPD with activity levels in both subgroups of smokers being significantly higher than normal (both p<0.006). The median chitinase activity in the smokers with COPD

(7 available samples) was 38% higher than in the smokers without COPD (15 available samples) although the numbers available for this subgroup comparison were small and the differences were not statistically significant (7.7 vs. 5.6 nmole/ml/hr, p=0.65). Similarly, the expression of CHIT1 in epithelial cells and in macrophages was higher in the smokers with COPD than without, but this difference was not statistically significant (data not shown). In the habitual smoker and asthmatic subgroups, we formally tested for associations between measures of airflow obstruction (FEV1 % predicted) and chitinase activity (at pH 4.6), and we did not find any significant associations.

CHIT1 is the primary active lung chitinase

The absence of BAL chitinase activity at pH 2.2 in nearly all subjects coupled with high activity at pH 7.0 suggested that CHIT1 was the primary source of chitinase activity in the lungs. To examine this hypothesis further, we exploited knowledge of a common null genetic variant in the CHIT1 gene. Namely, there is a well-characterized 24bp duplication polymorphism in exon 10 of the CHIT1 gene, resulting in abnormally spliced mRNA and completely inactive CHIT1 protein [63]. Subjects homozygous for this duplication should not display significant chitinase activity at either pH 4.6 or 7.0 if CHIT1 is the only source of chitinase activity in the BAL fluid. DNA was available on 58 of the original 77 subjects used to measure chitinase activity, including 25 asthmatics, 19 healthy subjects, and 14 habitual smokers. Genotyping the 24bp duplication revealed 2 of the healthy subjects (10.5%), 4 of the asthmatic subjects (16%), and none of the habitual

smokers were homozygous for the duplication. Strikingly, all six subjects

homozygous for the CHIT1 duplication displayed no chitinase activity at either pH

4.6 or pH 7.0 (Figure 4.2), a result which strongly implicates CHIT1 as the

primary source of chitinase activity in human lung secretions. Indeed, no subjects

genotyped lacked chitinase activity at either pH 4.6 or pH 7.0, other than those

homozygous for the CHIT1 duplication polymorphism. Furthermore, we found

median chitinase activity levels to be higher in subjects not carrying the

duplication versus subjects heterozygous for the duplication, implying a gene

dosage effect (Figure 4.2). This additive genotypic effect of the CHIT1 duplication

on chitinase activity was tested by linear regression and persisted after correcting

for age, gender, and race ($P < 0.0001$).

Figure 4.2
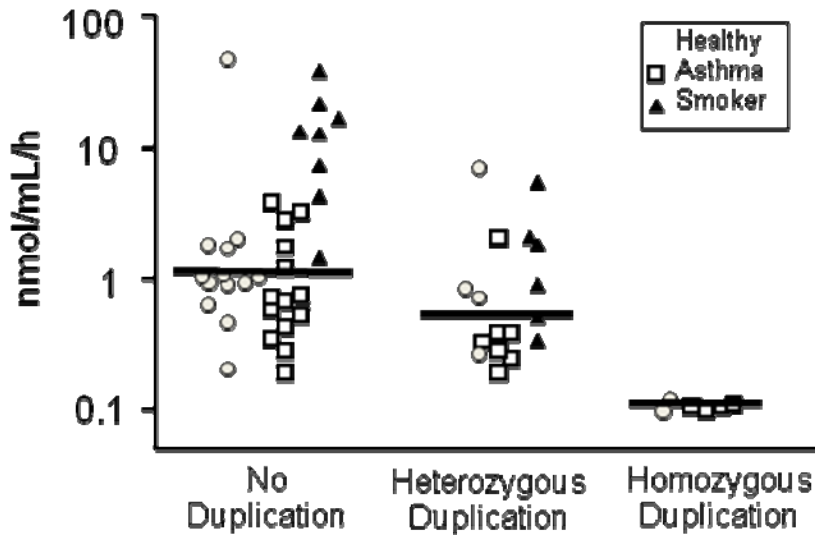
Fig. 4.2 Effect of CHIT1 24bp duplication genotype status on chitnase activity in BAL. Points represent chitinase activity measured at pH 4.6 for subjects with a no duplication CHIT1 genotype and for subjects heterozygous and homozygous for the CHIT1 duplication. Chitinase activity was undetectable in subjects homozygous for the duplication and reduced in subjects heterozygous for the duplication.

Absence of AMCase enzymatic activity is due to inactive splice variant

The pH profile of chitinase activity in BAL samples and the effect of the CHIT1 duplication on BAL chitinase activity argue against AMCase activity in human lung secretions. However, previous reports have found AMCase mRNA expression in both lung epithelial and macrophage cells [50]. Moreover, another study found expression of multiple splice variants of the AMCase mRNA in human lungs [52]. Interestingly, one of these splice variants (TSA1902S) results in the removal of the sixth exon which contains the conserved active site residues required for enzymatic activity of the AMCase protein (Figure 4.3A) [52]. Based on

this report, we hypothesized that the TSA1902S transcript was the primary splice variant of AMCase mRNA expressed in the lung. To test this hypothesis, we designed two sets of quantitative RT-PCR primers. The first primer set was designed against a portion of the AMCase mRNA contained in all known transcripts (Figure 4.3A). The second primer set was designed against a region within the spliced out Exon 6 of the inactive TSA1902S variant (Figure 4.3A). We used these two primer sets to measure AMCase transcripts in the two main cell types with the potential to secrete chitinase proteins into the airway; epithelial cells from 76 bronchial brushing samples and airway macrophages purifed from a subset (n=45) of the subjects. As a positive control, we also measured AMCase transcripts in human stomach poly A RNA (pooled from 7 subjects, Clontech, Mountain View, CA). Epithelial gene expression of AMCase examined with either primer was low or absent (data not shown); this was not a result of assay failure, because abundant AMCase transcripts were detected in stomach tissue using both primer types. Using the first primer set, we detected "total" AMCase expression in macrophages, but we found no difference in expression between subject groups (Figure 4.3B). Using the second primer set, we found absent or very low expression of AMCase in macrophages from all subject groups (Figure 4.3B). These data indicate that the AMCase mRNA expressed in lung macrophages is primarily a splice variant lacking exon 6 and thus enzymatically inactive.

Figure 4.3
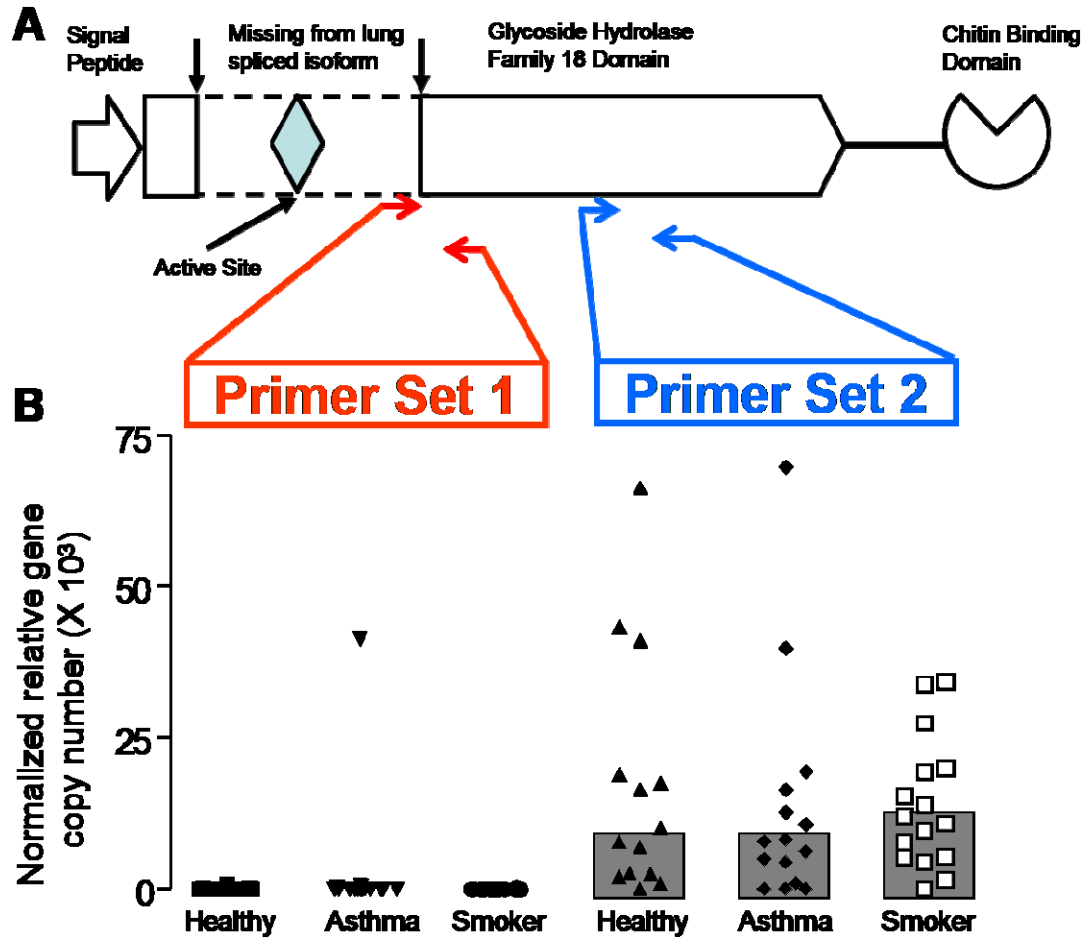
Fig. 4.3 AMCase gene expression in lavage macrophages from healthy subjects, asthmatics, and habitual smokers. Panel A - AMCase protein domain structure, with region spliced out in TSA1902S transcript denoted by arrows and dotted lines. Red arrows denote region amplified by RT-PCR primer set 1, and blue arrows denote region amplified by RT-PCR primer set 2. Panel B - AMCase gene expression stratified by red primer set 1 (active site-containing transcripts) and blue primer set 2 (all AMCase transcripts).

To examine AMCase at the protein level, we probed BAL from a subset of subjects for AMCase using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Figure 4.4). Semi-quantitative densitometry analysis of the protein blots showed that AMCase protein levels were lower than normal in

asthmatic subjects (P = 0.023) (Figure 4.4). BAL AMCase protein data was available for three of the six subjects homozygous for the null CHIT1 duplication. AMCase protein was easily detectable in BAL from these three subjects, even though, as described above, all three had no chitinase activity in their BAL. Taken together, these results suggest that the AMCase protein detectable in lung secretions is an isoform lacking enzymatic activity.

Figure 4.4
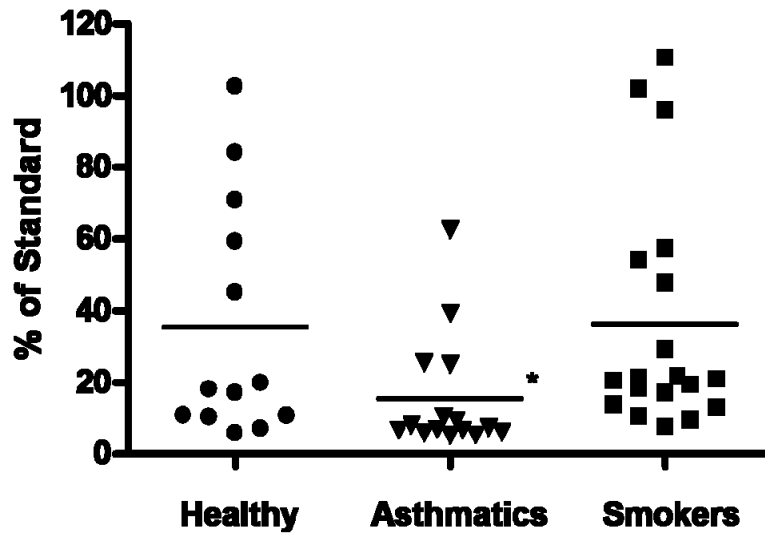
Fig. 4.4 AMCase protein in BAL from healthy subjects (n = 13), asthmatics (n = 16), and habitual smokers (n = 19). Y-axis values generated from densitometry analysis of protein bands relative to intensity of control AMCase protein. Line represents the median value. *Indicates significantly less than healthy, p=0.023.

CHIT1 gene expression is strongly correlated with lung chitinase activity

CHIT1 gene expression was most abundant in macrophages but was also observed in epithelial cells (Figure 4.5A & 4.5B). Expression was markedly increased in both epithelial cells and in macrophages from the smoker subgroup (Figure 4.5A & 4.5B). Moreover, CHIT1 gene expression in macrophages was positively correlated with lavage chitinase activity (rho = 0.68, P < 0.0001) (Figure 4.5C). We also found a positive correlation between CHIT1 gene expression in epithelial cells and lavage chitinase activity, although the association was not as strong as in macrophages (rho = 0.52, P < 0.0001) (data not shown).

Figure 4.5
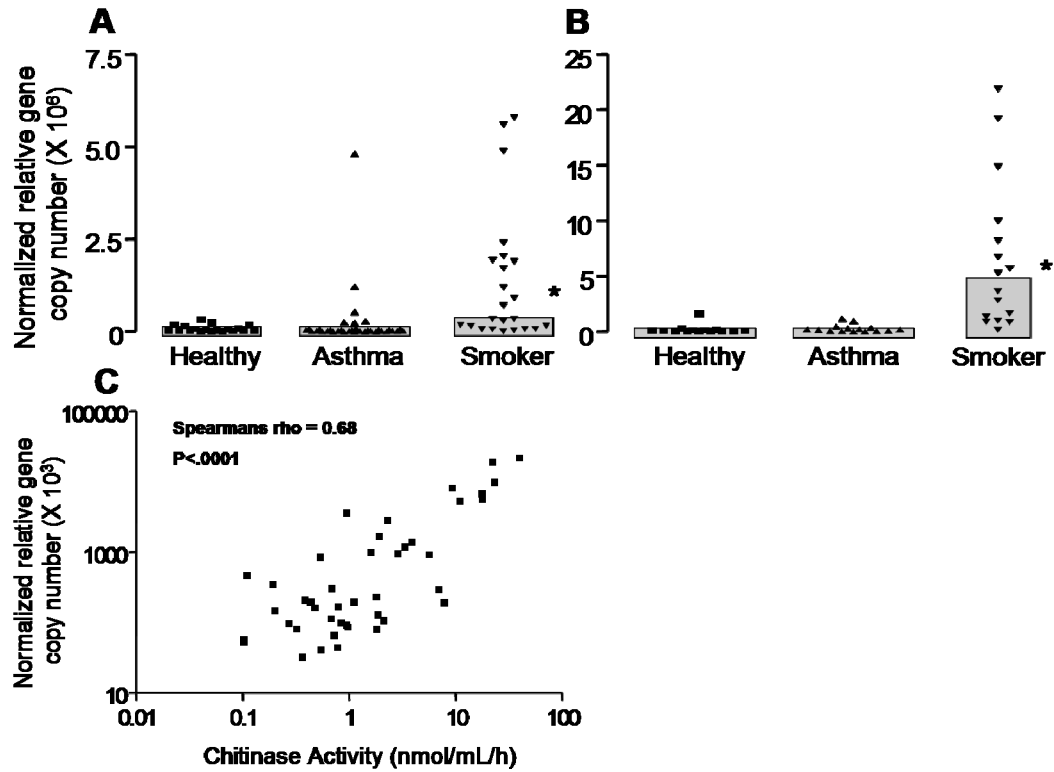
Fig. 4.5 CHIT1 gene expression and correlation with BAL chitinase activity**.** Panel A – CHIT1 gene expression in bronchial brushings stratified by disease status. *Indicates significantly greater than healthy, p<0.05. Panel B – CHIT1 gene expression in lavage macrophages stratified by disease status. *Indicates significantly greater than healthy, p<0.05. Panel C– Scatterplot of CHIT1 gene expression by BAL chitinase activity at pH 4.6.

4.4 Discussion

We characterized the relative contribution of both active human chitinases, CHIT1 and AMCase, to chitinase activity in the lung from healthy subjects, asthmatics, and habitual smokers. We determined CHIT1 to be the primary active chitinase in the lung, and we found that it's expression is strongly dependent on genetics and on smoking habit.

We used a multi-faceted approach to establish that CHIT1, not AMCase, is the principal active chitinase in the human lung, including determination of enzymatic activity, gene expression, and protein expression. We found that the pH profile for lung chitinase activity was consistent with the activity of CHIT1, not AMCase, showing high activity at near neutral pHs values and a complete absence of activity at low pH values [79, 103]. In addition, we found a lack of chitinase activity in BAL from all six subjects genetically deficient in CHIT1 activity. Indeed, there appeared to be an additive effect of the CHIT1 duplication on lung chitinase activity, with carriers of the duplication variant having reduced chitinase activity compared to non-carriers. In addition, CHIT1 gene expression in both macrophages and epithelial cells was strongly correlated with chitinase activity in lung secretions, further solidifying the primacy of CHIT1 in contribution to lung chitinase activity.

AMCase transcript numbers have been reported to be increased in asthmatic airways [50], but our data do not confirm this. Rather, we find that inactive AMCase variants are expressed in the lung and that these variants are not differentially

expressed in asthmatics or in smokers. Specifically, our data show that the numbers of "total" AMCase trancripts are higher than the numbers of active AMCase transcripts in epithelial cells and macrophages in all subject groups. This finding agrees with a previous report showing expression of splice variants of AMCase in the lung, variants which lack regions of exon-six containing the conserved active site residues required for enzymatic activity [52]. Consistent with this is our finding that BAL samples which had no chitinase activity still had detectable AMCase protein, indicating that the protein lacks chitinase activity.

Our results have important implications for the role of chitinases in asthma. Paradoxically, mouse studies have implicated lung chitinase activity in both the promotion of allergen-induced airway inflammation and in reduction of chitin-induced airway eosinophilia [50, 51]. Our results indicate that the upregulation in lung chitinase activity in mouse models of asthma does not extend to human asthmatics. In fact, our results do not support a pro-inflammatory role for lung chitinase activity in asthma pathology; rather, we find that chitinase activity tends to be lower than normal in asthmatics. Furthermore, we find that AMCase protein levels in BAL are lower than normal in asthma. These results are more supportive of the proposed protective role of chitinase activity and chitin-binding proteins in asthma and allergy, as evidenced by the inhibition of polymeric chitin's Th2 inflammatory effects after digestion with AMCase [51]. However, the isoform of AMCase expressed in the lung is likely devoid of enzymatic activity. Thus, if AMCase activity modulates human asthma, degradation of chitin by this enzyme would be most likely occuring at extra-pulmonary sites. It is important to

note several limitations with regard to interperation of this data. First, our observations were restricted to stable mild-moderate adult asthmatics. It will be important to determine whether our observations extend to subjects with more severe disease or in response to acute allergen challenge. Second, although the lung expressed AMCase is inactive, it does retain an intact chitin-binding domain, and as such it could bind chitin-containing environmental allergens and affect airway inflammation. The function of chi-lectins is unknown but these molecules have been implicated in mechanisms of tissue remodeling and inflammation [111-114]. Most notably, serum levels of CHI3L1 protein are increased in asthma, especially in patients with more severe disease [57], and genetic variants in CHI3L1 which result in increased protein levels have also been associated with airway obstruction and asthma [104].

Chitinase activity was markedly higher than normal in bronchoalveolar lavage from the subgroup of habitual smokers. To our knowledge, this is the first report of increased chitinase activity in airway secretions from smokers. CHIT1 gene expression was much higher in macrophages than in epithelial brushings, and we found a positive correlation between lavage chitinase activity and CHIT1 gene expression in macrophages, findings which point to macrophages as the main cellular source of the chitinase activity in these subjects. The mechanism by which cigarette smoke induces CHIT1 expression remains to be determined. CHIT1 upregulation occurred in habitual smokers with and without COPD and CHIT1 gene expression was higher than normal in both epithelial brushings and macrophages. The mechanism by which cigarette smoke causes CHIT1

upregulation in the airway and the consequences of smoke-induced CHIT1

upregulation in the airway remain to be determined, as does the more general

question of the role of lung chitinase activity in health and disease. Plant

chitinases have well defined roles in pathogen response [115] and the role of

human chitinases has generally been assumed to be in defense against chitin

containing pathogens [116, 117]. One possibility is that there are chitin particles in

inhaled tobacco smoke, which occur as a consequence of fungal infection of

tobacco leaf. It is estimated that as many as 270 fungal spores may be present in

a single cigarette, and fungal spores from *A. alternata* have been detected in

cigarette ash [118-120]. Polymeric chitin is known to be pro-inflammatory [51], and if

present in cigarette smoke, may activate macrophages and airway epithelial cells

to increase chitinase expression and/or secretion. Other fungal-derived

compounds such as beta glucans can also induce chitinase production from

plants and are potent macrophage stimulators [121].

In conclusion, we have used multiple lines of evidence including biochemical,

genetic, and gene and protein expression data in determining that CHIT1, not

acidic mammalian chitinase, is the primary chitinase responsible for chitinase

activity in the lung. We found that chitinase activity tended to be lower than

normal in asthma, a finding which supports a protective role for chitinolytic

activity in allergic inflammation. In contrast, chitinase activity and CHIT1 gene

expression are increased in habitual smokers, probably because cigarette smoke

induces activation of pulmonary macrophages. Taken together, our findings show

that CHIT1 activity in lung disease is modulated in ways which reflect underlying

disease susceptibilities and specific environmental exposures.

Chapter 5. Discussion and Future Directions

The seminal work completed by Zhu et al. strongly implicated the acidic mammalian chitinase (AMCase) in Th2-driven inflammation of mouse lungs. In this work AMCase was reported as a downstream effector of IL-13, which was capable of driving much of the bronchial inflammation and hyperresponsiveness observed in the lungs of allergen challenged mice [50]. However, this work conflicted with another high-profile mouse study which reported that no inflammatory phenotype was observed in transgenic AMCase over-expressing mice [51]. Rather it was observed that the substrate chitin could act as a trigger of Th2 inflammation in the mouse lung, and that AMCase was a negative regulator of this process [51]. These studies resulted in two very different hypotheses of the role of chitinases in asthma, one pro-inflammatory and the other as a negative regulator of inflammation in mice. To gain insight into the role of chitinases in human asthma we have conducted studies of chitinases using human genetics, human molecular genetics, and the characterization of human biological samples.

Based on the importance of AMCase's enzymatic activity in mouse models of asthma, we focused our genetic analysis on amino acid changing variants in the AMCase gene. Our approach resulted in the identification of a coding AMCase haplotype that was strongly associated with asthma in the African American population, and determined to significantly increase enzymatic activity of the resultant protein isoform. Clearly, the association of a gain-of-function AMCase

haplotype with asthma protection is consistent with the work of Reese et al., showing AMCase could act as a negative regulator of Th2 inflammation. However, further work will be needed to prove this association is the result of increased degradation of inhaled or ingested environmental chitin. Namely, epidemiological studies will need to be conducted examining the effects of chitin exposure on asthma traits, and their possible modification by chitinase genetics. These studies would allow us to determine whether differential chitin exposure is involved in the positive and negative results we observed for the protective haplotype in Latino Americans and foreign Latinos, respectively. Therefore, methods will have to be developed to sample, extract, and quantify environmental chitin in the homes of subjects involved in chitinase genetic studies. Studies of populations in diverse environments may be needed to achieve significant variation in environmental chitin exposure, as has been observed for other microbial exposures such as endotoxin [122]. Although the associated AMCase haplotype has not been tested by other groups, a prior study has found both other single SNPs and haplotypes in the AMCase gene to be strongly associated with asthma in a German cohort [61], providing replication at the gene level.

In contrast we did not observe association between a null duplication variant in the CHIT1 gene and asthma in either African Americans or Latinos, replicating the lack of association priorly observed in a German asthma cohort [62]. However, in subsequent resequencing of the CHIT1 gene we identified other indel polymorphisms likely to result in no protein expression. Therefore, more

comprehensive CHIT1 genetic analyses are needed in this and other populations including full haplotype analysis and analysis of the risk conferred by pooled null variants. It will also be interesting to conduct gene-gene interaction analyses with both AMCase and CHIT1 since they are functionally redundant.

Despite the genetic results implicating AMCase rather than CHIT1 in human asthma, we found the latter rather than the former was primarily responsible for lung lavage chitinase activity. Although we observed AMCase protein expression in the lavage, we determined the majority of the protein expressed was translated from an inactive splice variant of the AMCase transcript. This result raises the question of whether chitinase activity is required by AMCase to affect asthma pathophysiology. Although AMCase observed in the lung would not be able to digest chitin it still contains a functional chitin-binding domain. Therefore AMCase would retain the ability to bind environmental chitin in the airway. Recent work has shown that genetic variants in the enzymatically inactive chi-lectin, YKL-40 (or CHI3L1), are associated with asthma [104]. Also higher YKL-40 protein expression was associated with airway remodeling and a decrement in lung function [57]. Supporting this possibility, we did observe a significant decrease in AMCase protein in the lavage of asthmatics. Genetically engineered mice with an active site mutation in AMCase would be helpful to delineate the role of chitin-binding versus chitin cleavage in airway biology.

Another possibility is that the expression and splicing of the AMCase gene in the lung may vary in a temporal fashion. Previous studies have observed levels of

the chi-lectin YKL-40 to decrease significantly from 1 to 2 years of age. Therefore measurements of AMCase activity in adults may not accurately reflect early life activity, which could be more relevant to development of the immune system and allergic responses [104]. Therefore, detailed characterization of lung chitinase activity and expression are needed at early life time points.

Another hypothesis to be explored is whether AMCase activity in the stomach can modulate the development of immune responses. We did find the protective AMCase isoform was significantly more active than the non-protective isoform at low pH values corresponding to the stomach. In fact we found the active AMCase transcript to be the primary form expressed in the stomach, and a prior study has observed stomach AMCase activity [123]. AMCase activity in the stomach may be important to immune responses developed in the gut and influenced by gut flora, especially in early life. Studies in mice have found differential gut colonization can polarize development of the immune system towards either Th1 or Th2 immunity [96, 124]. AMCase could affect gut colonization of chitin-containing fungi, as CHIT1 has been observed to act in a fungistatic manner in the mouse [116]. Additionally, polymeric chitin but not N-acetyl-glucosamine monomers have been shown to disrupt interactions between bacteria and host bacterial adherence proteins expressed in the gut [94]. Tissue-specific knockout mouse models will be needed to help define the precise roles of lung and stomach AMCase in developing immune responses. Studies observing chitinase genetics, stomach chitinase activity, coupled with metagenomic studies of the early life bowel flora would aide greatly in testing these ideas.

Although we did not find CHIT1 genetics to be associated with asthma traits, it was the primary chitinase expressed in lavage. However, expression and activity of CHIT1 was low in both healthy subjects and asthmatics compared to smokers, who experienced a dramatic upregulation. The source of the abundant CHIT1 expression appears to be alternatively activated macrophages. These results are interesting in that they point to a possible role of CHIT1 in a more severe sub-phenotype of asthma where alternatively activated macrophages are known to be abundant in the lung. Moreover, expression of the chi-lectin, YKL-40 was strongly linked with airway remodeling in severe asthma [57]. Both our genetic and lavage characterization studies were conducted in mild-moderate asthmatics, future studies will be needed in moderate-severe populations to test this hypothesis. Additionally, the effects of long-term, high expression of CHIT1 will need to be determined in the lungs of patients with chronic obstructive pulmonary disease (COPD). An initial report showed significantly elevated CHIT1 activity in the lungs of smokers with COPD compared to smokers without COPD [125]. Basic studies looking at the effect of CHIT1 and other chi-lectins on airway biology will be needed to elucidate the possible mechanisms for these observations in human populations.

In conclusion, our work examined in the context of current literature suggests chitinases may play key roles in airway biology and asthma, and has generated exciting new hypotheses for the involvement of chitinases in these processes. Cross-fertilization between studies of chitinase biology in model organisms, human genetics, epidemiology, and clinical characterization of biological samples

from diverse human populations will be needed to further clarify the roles of chitin and chitinases in human asthma. *–M.A.S.*

We shall not cease from exploration

And the end of all our exploring

Will be to arrive where we started

And know the place for the first time

-T.S. Eliot

6. Bibliography

1.   Burchard EG, et al. The importance of race and ethnic background in biomedical research and clinical practice. N Engl J Med 2003; 348:1170-5.
2.   Choudhry S, et al. Dissecting complex diseases in complex populations: asthma in latino americans. Proc Am Thorac Soc 2007; 4:226-33.
3.   U.S. Census Bureau. 2000.
4.   Parra EJ, et al. Estimating African American admixture proportions by use of population-specific alleles. Am J Hum Genet 1998; 63:1839-51.
5.   Lara M, et al. Heterogeneity of childhood asthma among Hispanic children: Puerto Rican children bear a disproportionate burden. Pediatrics 2006; 117:43-53.
6.   Drake KA, et al. Race, ethnicity and social class and the complex etiologies of asthma. Pharmacogenomics 2008; 9:453-62.
7.   Homa DM, et al. Asthma mortality in U.S. Hispanics of Mexican, Puerto Rican, and Cuban heritage, 1990-1995. Am J Respir Crit Care Med 2000; 161:504-9.
8.   Burchard EG, et al. Lower bronchodilator responsiveness in Puerto Rican than in Mexican subjects with asthma. Am J Respir Crit Care Med 2004; 169:386-92.
9.   Naqvi M, et al. Ethnic-specific differences in bronchodilator responsiveness among African Americans, Puerto Ricans, and Mexicans with asthma. J Asthma 2007; 44:639-48.
10.   Feng Y, et al. G protein-coupled receptor 154 gene polymorphism is associated with airway hyperresponsiveness to methacholine in a Chinese population. J Allergy Clin Immunol 2006; 117:612-7.
11.   Kormann MS, et al. G-Protein-coupled receptor polymorphisms are associated with asthma in a large German population. Am J Respir Crit Care Med 2005; 171:1358-62.
12.   Laitinen T, et al. Characterization of a common susceptibility locus for asthma-related traits. Science 2004; 304:300-4.
13.   Malerba G, et al. Chromosome 7p linkage and GPR154 gene association in Italian families with allergic asthma. Clin Exp Allergy 2007; 37:83-9.
14.   Melen E, et al. Haplotypes of G protein-coupled receptor 154 are associated with childhood allergy and asthma. Am J Respir Crit Care Med 2005; 171:1089-95.
15.   Seibold M.A. CS, Beckman K.B., Burchard E.G., Meade K., Michael Lenoir M., Watson H.G., Thyne S., Rodriguez-Santana J., Rodriguez-Cintron W., Burchard E.G. . Non-synonymous GPRA polymorphisms are not associated with asthma in diverse U.S. asthma populations. . Unpublished Observations 2008.
16.   Wu H, et al. Lack of association between genetic variation in G-protein-coupled receptor for asthma susceptibility and childhood asthma and atopy. Genes Immun 2008; 9:224-30.
17.   Choudhry S, et al. Pharmacogenetic differences in response to albuterol between Puerto Ricans and Mexicans with asthma. Am J Respir Crit Care Med 2005; 171:563-70.
18.   Tsai HJ, et al. Beta 2-adrenergic receptor polymorphisms: pharmacogenetic response to bronchodilator among African American asthmatics. Hum Genet 2006; 119:547-57.
19.   Baldini M, et al. A Polymorphism* in the 5' flanking region of the CD14 gene is associated with circulating soluble CD14 levels and with total serum immunoglobulin E. Am J Respir Cell Mol Biol 1999; 20:976-83.

20. Buckova D, et al. Two CD14 promoter polymorphisms and atopic phenotypes in Czech patients with IgE-mediated allergy. Allergy 2003; 58:1023-6.
21. Koppelman GH, et al. Association of a promoter polymorphism of the CD14 gene and atopy. Am J Respir Crit Care Med 2001; 163:965-9.
22. Takeuchi K, et al. A CD14 gene polymorphism is associated with the IgE level for Dermatophagoides pteronyssinus. Acta Otolaryngol 2005; 125:966-71.
23. Kabesch M, et al. A promoter polymorphism in the CD14 gene is associated with elevated levels of soluble CD14 but not with IgE or atopic diseases. Allergy 2004; 59:520-5.
24. Sengler C, et al. Evaluation of the CD14 C-159 T polymorphism in the German Multicenter Allergy Study cohort. Clin Exp Allergy 2003; 33:166-9.
25. Ober C, et al. A second-generation genomewide screen for asthma-susceptibility alleles in a founder population. Am J Hum Genet 2000; 67:1154-62.
26. Simpson A, et al. Endotoxin exposure, CD14, and allergic disease: an interaction between genes and the environment. Am J Respir Crit Care Med 2006; 174:386-92.
27. Williams LK, et al. Gene-environment interactions with CD14 C-260T and their relationship to total serum IgE levels in adults. J Allergy Clin Immunol 2006; 118:851-7.
28. Stephens JC, et al. Haplotype variation and linkage disequilibrium in 313 human genes. Science 2001; 293:489-93.
29. Seibold MA, et al. An African-specific functional polymorphism in KCNMB1 shows sex-specific association with asthma severity. Hum Mol Genet 2008.
30. Choudhry S, et al. Population stratification confounds genetic association studies among Latinos. Hum Genet 2006; 118:652-64.
31. Gonzalez Burchard E, et al. Latino populations: a unique opportunity for the study of race, genetics, and social environment in epidemiological research. Am J Public Health 2005; 95:2161-8.
32. Pritchard JK, et al. Inference of population structure using multilocus genotype data. Genetics 2000; 155:945-59.
33. Parra EJ, et al. Ancestral proportions and admixture dynamics in geographically defined African Americans living in South Carolina. Am J Phys Anthropol 2001; 114:18-29.
34. Salari K, et al. Genetic admixture and asthma-related phenotypes in Mexican American and Puerto Rican asthmatics. Genet Epidemiol 2005; 29:76-86.
35. Reich D, et al. Admixture mapping of an allele affecting interleukin 6 soluble receptor and interleukin 6 levels. Am J Hum Genet 2007; 80:716-26.
36. Reiner AP, et al. Genetic ancestry, population sub-structure, and cardiovascular disease-related traits among African-American participants in the CARDIA Study. Hum Genet 2007; 121:565-75.
37. Stephens JC, et al. Mapping by admixture linkage disequilibrium in human populations: limits and guidelines. Am J Hum Genet 1994; 55:809-24.
38. Bonilla C, et al. Admixture in the Hispanics of the San Luis Valley, Colorado, and its implications for complex trait gene mapping. Ann Hum Genet 2004; 68:139-53.
39. Zhang Q, et al. Genome-wide admixture mapping for coronary artery calcification in African Americans: the NHLBI Family Heart Study. Genet Epidemiol 2008; 32:264-72.
40. Freedman ML, et al. Admixture mapping identifies 8q24 as a prostate cancer risk locus in African-American men. Proc Natl Acad Sci U S A 2006; 103:14068-73.

41. Nalls MA, et al. Admixture mapping of white cell count: genetic locus responsible for lower white blood cell count in the Health ABC and Jackson Heart studies. Am J Hum Genet 2008; 82:81-7.

42. Ziv E, et al. Human population structure and genetic association studies. Pharmacogenomics 2003; 4:431-41.

43. Grunig G, et al. Requirement for IL-13 independently of IL-4 in experimental asthma. Science 1998; 282:2261-3.

44. Wills-Karp M, et al. Interleukin-13: central mediator of allergic asthma. Science 1998; 282:2258-61.

45. Kuperman DA, et al. Direct effects of interleukin-13 on epithelial cells cause airway hyperreactivity and mucus overproduction in asthma. Nat Med 2002; 8:885-9.

46. Christodoulopoulos P, et al. TH2 cytokine-associated transcription factors in atopic and nonatopic asthma: evidence for differential signal transducer and activator of transcription 6 expression. J Allergy Clin Immunol 2001; 107:586-91.

47. Humbert M, et al. Elevated expression of messenger ribonucleic acid encoding IL-13 in the bronchial mucosa of atopic and nonatopic subjects with asthma. J Allergy Clin Immunol 1997; 99:657-65.

48. Hoshino A, et al. STAT6-mediated signaling in Th2-dependent allergic asthma: critical role for the development of eosinophilia, airway hyper-responsiveness and mucus hypersecretion, distinct from its role in Th2 differentiation. Int Immunol 2004; 16:1497-505.

49. Kuperman DA, et al. Dissecting asthma using focused transgenic modeling and functional genomics. J Allergy Clin Immunol 2005; 116:305-11.

50. Zhu Z, et al. Acidic mammalian chitinase in asthmatic Th2 inflammation and IL-13 pathway activation. Science 2004; 304:1678-82.

51. Reese TA, et al. Chitin induces accumulation in tissue of innate immune cells associated with allergy. Nature 2007; 447:92-6.

52. Saito A, et al. Isolation and mapping of a human lung-specific gene, TSA1902, encoding a novel chitinase family member. Gene 1999; 239:325-31.

53. Bargagli E, et al. Chitotriosidase analysis in bronchoalveolar lavage of patients with sarcoidosis. Sarcoidosis Vasc Diffuse Lung Dis 2007; 24:59-64.

54. Bargagli E, et al. Chitotriosidase activity in patients with interstitial lung diseases. Respir Med 2007; 101:2176-81.

55. Song HM, et al. Ym1 and Ym2 expression in a mouse model exposed to diesel exhaust particles. Environ Toxicol 2008; 23:110-6.

56. Webb DC, et al. Expression of the Ym2 lectin-binding protein is dependent on interleukin (IL)-4 and IL-13 signal transduction: identification of a novel allergy-associated protein. J Biol Chem 2001; 276:41969-76.

57. Chupp GL, et al. A chitinase-like protein in the lung and circulation of patients with severe asthma. N Engl J Med 2007; 357:2016-27.

58. Ward C, et al. Airway inflammation, basement membrane thickening and bronchial hyperresponsiveness in asthma. Thorax 2002; 57:309-16.

59. Kuepper M, et al. Chitinase-like protein and asthma. N Engl J Med 2008; 358:1073-5; author reply 5.

60. Funkhouser JD, et al. Chitinase family GH18: evolutionary insights from the genomic history of a diverse protein family. BMC Evol Biol 2007; 7:96.

61. Bierbaum S, et al. Polymorphisms and haplotypes of acid mammalian chitinase are associated with bronchial asthma. Am J Respir Crit Care Med 2005; 172:1505-9.
62. Bierbaum S, et al. Genetic polymorphisms of chitotriosidase in Caucasian children with bronchial asthma. Int J Immunogenet 2006; 33:201-4.
63. Boot RG, et al. The human chitotriosidase gene. Nature of inherited enzyme deficiency. J Biol Chem 1998; 273:25680-5.
64. Shibata Y, et al. Oral administration of chitin down-regulates serum IgE levels and lung eosinophilia in the allergic mouse. J Immunol 2000; 164:1314-21.
65. Holt PG. Infections and the development of allergy. Toxicol Lett 1996; 86:205-10.
66. Shibata Y, et al. Alveolar macrophage priming by intravenous administration of chitin particles, polymers of N-acetyl-D-glucosamine, in mice. Infect Immun 1997; 65:1734-41.
67. Shibata Y, et al. Th1 adjuvant N-acetyl-D-glucosamine polymer up-regulates Th1 immunity but down-regulates Th2 immunity against a mycobacterial protein (MPB-59) in interleukin-10-knockout and wild-type mice. Infect Immun 2001; 69:6123-30.
68. Reece JJ, et al. Innate immune responses to lung-stage helminth infection induce alternatively activated alveolar macrophages. Infect Immun 2006; 74:4970-81.
69. Martinez FD. Gene-environment interactions in asthma and allergies: a new paradigm to understand disease causation. Immunol Allergy Clin North Am 2005; 25:709-21.
70. CDC. Surveillance for Asthma --United States, 1960-1995. MMWR 1998; 47.
71. Celedon JC, et al. Exposure to dust mite allergen and endotoxin in early life and asthma and atopy in childhood. J Allergy Clin Immunol 2007; 120:144-9.
72. von Mutius E. Allergies, infections and the hygiene hypothesis--the epidemiological evidence. Immunobiology 2007; 212:433-9.
73. Araujo AC, et al. Cytochemical localization of carbohydrate residues in microfilariae of Wuchereria bancrofti and Brugia malayi. J Histochem Cytochem 1993; 41:571-8.
74. Debono M, et al. Antibiotics that inhibit fungal cell wall development. Annu Rev Microbiol 1994; 48:471-97.
75. Fuhrman JA, et al. Chitin synthesis and sheath morphogenesis in Brugia malayi microfilariae. Mol Biochem Parasitol 1985; 17:93-104.
76. Neville AC, et al. The chitin crystallite in arthropod cuticle. J Cell Sci 1976; 21:73-82.
77. Shahabuddin M, et al. Plasmodium: parasite chitinase and its role in malaria transmission. Exp Parasitol 1994; 79:85-8.
78. Bussink AP, et al. Evolution of mammalian chitinase(-like) members of family 18 glycosyl hydrolases. Genetics 2007.
79. Chou YT, et al. Kinetic characterization of recombinant human acidic mammalian chitinase. Biochemistry 2006; 45:4444-54.
80. Bussink AP, et al. The biology of the Gaucher cell: the cradle of human chitinases. Int Rev Cytol 2006; 252:71-128.
81. Boot RG, et al. Marked differences in tissue-specific expression of chitinases in mouse and man. J Histochem Cytochem 2005; 53:1283-92.
82. Ferris BG. Epidemiology Standardization Project (American Thoracic Society). Am Rev Respir Dis 1978; 118:1-120.
83. Denlinger LC, et al. Guideline-defining asthma clinical trials of the National Heart, Lung, and Blood Institute's Asthma Clinical Research Network and Childhood Asthma Research and Education Network. J Allergy Clin Immunol 2007; 119:3-11; quiz 2-3.
84. Chen X, et al. Fluorescence polarization in homogeneous nucleic acid analysis. Genome Res 1999; 9:492-8.

85. Tsai HJ, et al. Admixture-matched case-control study: a practical approach for genetic association studies in admixed populations. Hum Genet 2006; 118:626-39.

86. Laird NM, et al. Implementing a unified approach to family-based tests of association. Genet Epidemiol 2000; 19 Suppl 1:S36-42.

87. Lin DY, et al. Maximum likelihood estimation of haplotype effects and haplotype-environment interactions in association studies. Genet Epidemiol 2005; 29:299-312.

88. Aguilera B, et al. Transglycosidase activity of chitotriosidase: improved enzymatic assay for the human macrophage chitinase. J Biol Chem 2003; 278:40911-6.

89. Robert M. Naclerio SRD, Niels Mygind. Rhinitis : Mechanisms and Management. Lung Biology in Health and Disease 1999; 123.

90. Tovey ER, et al. Mite faeces are a major source of house dust allergens. Nature 1981; 289:592-3.

91. Li X, et al. The chitinolytic cascade in Vibrios is regulated by chitin oligosaccharides and a two-component chitin catabolic sensor/kinase. Proc Natl Acad Sci U S A 2004; 101:627-31.

92. Epidemiologic Notes and Reports Asthma-Like Illness among Crab-Processing Workers -- Alaska MMWR 1982; 31:95-6.

93. Kitch BT, et al. Socioeconomic predictors of high allergen levels in homes in the greater Boston area. Environ Health Perspect 2000; 108:301-7.

94. Cash HL, et al. Symbiotic bacteria direct expression of an intestinal bactericidal lectin. Science 2006; 313:1126-30.

95. Chen HC, et al. Evaluation of N-acetylchitooligosaccharides as the main carbon sources for the growth of intestinal bacteria. FEMS Microbiol Lett 2002; 209:53-6.

96. Noverr MC, et al. Development of allergic airway disease in mice following antibiotic therapy and fungal microbiota increase: role of host genetics, antigen, and interleukin-13. Infect Immun 2005; 73:30-8.

97. Bussink AP, et al. Evolution of mammalian chitinase(-like) members of family 18 glycosyl hydrolases. Genetics 2007; 177:959-70.

98. Lee P, et al. Human chitotriosidase polymorphisms G354R and A442V associated with reduced enzyme activity. Blood Cells Mol Dis 2007; 39:353-60.

99. Piras I, et al. 24 bp duplication of CHIT1 gene is not correlated with coronary artery disease in Corsica Island (France). Exp Mol Pathol 2007; 83:490-2.

100. Naclerio RM, Durham SR, Mygind N. 1999; 123. Rhinitis : Mechanisms and Management. . New York; 1999.

101. Aerts JM, et al. Plasma and metabolic abnormalities in Gaucher's disease. Baillieres Clin Haematol 1997; 10:691-709.

102. Barone R, et al. Plasma chitotriosidase activity in patients with beta-thalassemia. Am J Hematol 2003; 72:285-6.

103. Boot RG, et al. Identification of a novel acidic mammalian chitinase distinct from chitotriosidase. J Biol Chem 2001; 276:6770-8.

104. Ober C, et al. Effect of variation in CHI3L1 on serum YKL-40 level, risk of asthma, and lung function. N Engl J Med 2008; 358:1682-91.

105. Innes AL, et al. Epithelial mucin stores are increased in the large airways of smokers with airflow obstruction. Chest 2006; 130:1102-8.

106. Woodruff PG, et al. Genome-wide profiling identifies epithelial cell genes associated with asthma and with treatment response to corticosteroids. Proc Natl Acad Sci U S A 2007; 104:15858-63.

107. Woodruff PG, et al. Hyperplasia of smooth muscle in mild to moderate asthma without changes in cell size or gene expression. Am J Respir Crit Care Med 2004; 169:1001-6.

108. Woodruff PG, et al. A distinctive alveolar macrophage activation state induced by cigarette smoking. Am J Respir Crit Care Med 2005; 172:1383-92.

109. Dolganov GM, et al. A novel method of gene transcript profiling in airway biopsy homogenates reveals increased expression of a Na+-K+-Cl- cotransporter (NKCC1) in asthmatic subjects. Genome Res 2001; 11:1473-83.

110. Boot RG, et al. Human acidic mammalian chitinase erroneously known as eosinophil chemotactic cytokine is not the ortholog of mouse YM1. J Immunol 2005; 175:2041-2.

111. De Ceuninck F, et al. YKL-40 (cartilage gp-39) induces proliferative events in cultured chondrocytes and synoviocytes and increases glycosaminoglycan synthesis in chondrocytes. Biochem Biophys Res Commun 2001; 285:926-31.

112. Ling H, et al. The chitinase 3-like protein human cartilage glycoprotein 39 inhibits cellular responses to the inflammatory cytokines interleukin-1 and tumour necrosis factor-alpha. Biochem J 2004; 380:651-9.

113. Malinda KM, et al. Gp38k, a protein synthesized by vascular smooth muscle cells, stimulates directional migration of human umbilical vein endothelial cells. Exp Cell Res 1999; 250:168-73.

114. Recklies AD, et al. The chitinase 3-like protein human cartilage glycoprotein 39 (HC-gp39) stimulates proliferation of human connective-tissue cells and activates both extracellular signal-regulated kinase- and protein kinase B-mediated signalling pathways. Biochem J 2002; 365:119-26.

115. Kasprzewska A. Plant chitinases--regulation and function. Cell Mol Biol Lett 2003; 8:809-24.

116. van Eijk M, et al. Characterization of human phagocyte-derived chitotriosidase, a component of innate immunity. Int Immunol 2005; 17:1505-12.

117. Labadaridis I, et al. Chitotriosidase in neonates with fungal and bacterial infections. Arch Dis Child Fetal Neonatal Ed 2005; 90:F531-2.

118. Verweij PE, et al. Fungal contamination of tobacco and marijuana. Jama 2000; 284:2875.

119. Salo PM, et al. Dustborne Alternaria alternata allergens in US beds; results from the first National Survey of Lead and Allergens in Housing. American Journal of Respiratory and Critical Care Medicine 2004; 169(suppl 7):A 368.

120. Bogden JD, et al. Composition of tobaccos from countries with high and low incidences of lung cancer. I. Selenium, polonium-210, Alternaria, tar, and nicotine. J Natl Cancer Inst 1981; 66:27-31.

121. Jamois F, et al. Glucan-like synthetic oligosaccharides: iterative synthesis of linear oligo-beta-(1,3)-glucans and immunostimulatory effects. Glycobiology 2005; 15:393-407.

122. Martinez FD. CD14, endotoxin, and asthma risk: actions and interactions. Proc Am Thorac Soc 2007; 4:221-5.

123. Paoletti MG, et al. Human gastric juice contains chitinase that can degrade chitin. Ann Nutr Metab 2007; 51:244-51.

124. Mazmanian SK, et al. An immunomodulatory molecule of symbiotic bacteria directs maturation of the host immune system. Cell 2005; 122:107-18.

125. S. Letuve AK, N. Arouche, M. Grandsaigne, J. Reed, M.C. Dombret, P.A. Kiener, M. Aubier, A.J. Coyle, M. Pretolani. Elevated Lung Chitinolytic Activity and Chitotriosidase in Chronic Obstructive Pulmonary Disease. American Thoracic Society 2008 International Conference 2008; Meeting Abstract.

7. Appendix

Appendix Note 2.1 Cloning of the AMCase-Fc expression construct

The *AMCase* cDNA was received as a gift cloned into an expression vector. The AMCase cDNA sequence contained in this vector is identical to cDNA AF290004 contained in the NCBI database and described previously [110]. The AMCase cDNA, minus the signal sequence [110], was PCR cloned out of this vector. The two exons encoding the IgG Fc region were PCR cloned out of a vector and fused by PCR to use as a tag for protein purification. We did not clone the hinge region of IgG to prevent dimerization of the AMCase proteins. The Fc cDNA was then fused to the AMCase cDNA and the entire AMCase-Fc cDNA was cloned into the pMIB-V5-His-(A) vector (Invitrogen) using the SpeI and XhoI restriction sites in the vector. PCR primers for all of these cloning steps are contained in Appendix Table 2.3. Successive rounds of in-vitro mutagenesis were conducted on the AMCase expression construct using the Stratagene Quik-Change Kit, to generate both the protective and non-protective AMCase haplotypes. In vitro mutagenesis primers are listed in Appendix Table 2.4.

Appendix Note 2.2 AMCase Purification Protocol

AMCase expressing supernatants and mock transfected supernatants were harvested after 5 days of expression, then immediately put on ice and EDTA was added to a final concentration of 2mM in the supernatant. Supernatants were immediately loaded into Snakeskin Dialysis tubing (3.5 Kd cut-off) (Pierce Biotechnology) and concentrated using polyethylene glycol. Concentrated samples were dialyzed against 1X PBS overnight. Dialyzed samples were then purified by batch purification using the Immobilized Protein G Resin (Pierce Biotechnology). Briefly, samples were incubated with 0.75 ml resin at 4 degrees for 4 hours. Resin was then loaded onto 10ml columns and washed with 10ml fractions of PBS, until no more protein eluted. AMCase protein was then eluted in 0.5ml fractions with 0.1 M glycine pH 3.0 into tubes containing 1 M Tris-HCl pH 10.0. Gels were stained with Silver Stain Plus (Bio-rad) and showed the presence of only one band corresponding to the AMCase-Fc protein of 75 Kd. Western blots using the anti-Fc antibody of the same eluted fractions revealed the same band observed in the SDS-PAGE gel at 75 Kd. The eluted fractions were immediately mixed and loaded onto Slide-a-Lyzer dialysis cassettes (10 Kd cut-off) (Pierce Biotechnology) and then dialyzed overnight against 1X PBS. Samples were stored at 4 degrees Celsius until assayed.

Appendix Table 2.1 Primers for resequencing of the AMCase gene

Amplicon	Primer Direction	Primer Sequence
Exon1	Forward	5' GCGTTCATCCAGCACTGTT 3'
Exon1	Reverse	5' GATGTGTGAGGGGAGGAGAA 3'
Exon2	Forward	5' TTATCAGCCCTTGGAACTGC 3'
Exon2	Reverse	5' TTCAGAGCCTGTGAGAGAAAAA 3'
Exon3	Forward	5' CGAAGGTACCACGGTCATTT 3'
Exon3	Reverse	5' ACAGCGGCTCTCTTTCCATA 3'
Exon4	Forward	5' AAGGCCAAAACTCACAGCAC 3'
Exon4	Reverse	5' GAAGTACTGAAAAAGGGGATGC 3'
Exon5	Forward	5' TGCATTCATGACAGGAACAA 3'
Exon5	Reverse	5' GAGGTTGTTTGGGAACCTCA 3'
Exon6	Forward	5' AGGGCTCTGAGGCAGGAAT 3'
Exon6	Reverse	5' CGCACGATATATAAGACTCTTTGC 3'
Exon7 & 8	Forward	5' TTAAGAGAGAGAAGCATTATACAGACA 3'
Exon7 & 8	Reverse	5' TTTGTGCCTGAATCTCTATGTGA 3'
Exon9	Forward	5' ACCTGTCGGAATAGGGGACT 3'
Exon9	Reverse	5' TGTGAAGGGATATTATGCATTGTT 3'
Exon10 & 11	Forward	5' ATTGAGCAAAACCCCAACTG 3'
Exon10 & 11	Reverse	5' GTGGGAAGACATCAGGGTTG 3'
Exon12	Forward	5' CACCTCCACACTTCCACTCC 3'
Exon12	Reverse	5' CACACTCTACTCCACCACTCCA 3'

.

Appendix Table 2.2 FP-TDI AMCase genotyping primers and terminator dye combinations

SNP	Primer	FP Dye	Sequence
G339T	PCR LP	-	5' AAGGCCAAAACTCACAGCAC 3'
	PCR RP	-	5' GAAGTACTGAAAAAGGGGATGC 3'
	FP1	G/T	5' GATCTACGCCTTTGCTGGGA 3'
	FP2	C/A	5' GTGGTGATCTCGTTGTTCTGC 3'
G461A	PCR LP	-	5' TTTCAGTAGGGGAGGAAAATT 3'
	PCR RP	-	5' CCTCAATACCCTGTCTTAGATTGT 3'
	FP1	G/A	5' GCCATTGGAGGCTGGAACTTC 3'
A531G	PCR LP	-	5' AGGTGTTGGGACCACCAAG 3'
	PCR RP	-	5' ACCCTAGAGCCACCCAATTC 3'
	FP2	T/C	5' AACTCATACTGGCGCAGGAAT3'

Appendix Table 2.3 Primers for construction of AMCase-Fc expression construct

Cloning Step	Primer Sequence
Cloning Fc Exon 3 FP	5' GTGATTGCTGCAACTGG GCAATCGAAGGCAGAGCA 3'
Cloning Fc Exon 3 RP	5' TGTGGTTCTCGGGGCTGCCCTTT GGCTTTGGAGATGGTTTTC 3'
Cloning Fc Exon 4 FP	5' AAACCATCTCCAAAGCCAAA GGGCAGCCCCGAGAACCACAGG 3'
Cloning Fc Exon 4 RP	5' CCTGGAACTCGAGTCATTTA CCCGGAGACAGGGAGAGG 3'
Cloning AMCase cDNA FP	5' CGTGCATACTAGTCTACCA GCTGACATGCTACTTC 3'
Cloning AMCase cDNA RP	5' CCCCCCAGGAGTTCAGGTGCTCTGCCT TCGATTGCCCAGTTGCAGCAATCACAG 3'
Fusion of Fc Exon 3&4 FP	5' GTGATTGCTGCAACTGGGCAATCGAAGG CAGAGCACCTGAACTCCTGGGGGGAC 3'
Fusion of Fc Exon 3&4 RP	5' CCTGGAACTCGAGTCATT TACCCGGAGACAGGGAGAGG 3'
Fusion of AMCase and Fc FP	5' CGTGCATACTAGTCTA CCAGCTGACATGCTACTTC 3'
Fusion of AMCase and Fc RP	5' CCTGGAACTCGAGTCAT TTACCCGGAGACAGGGAGAGG 3'

Appendix Table 2.4 Mutagenesis primers for the AMCase expression construct

SNP	Primer Sequence
A290G & G296A FP	5'GGCGCTTCATGCCTGACGACATCAACCCCTGCC3'
A290G & G296A RP	5'GGCAGGGGTTGATGTCGTCAGGCATGAAGCGCC3'
G339T FP	5'CGCCTTTGCTGGGATGCAGAACAACGAGATCACC3'
G339T RP	5'GGTGATCTCGTTGTTCTGCATCCCAGCAAAGGCG3'
G1172A FP	5'GGCAATGTGTGGGTTGGCTATGACAACGTCAAGAGCTTCG3'
G1172A RP	5'CGAAGCTCTTGACGTTGTCATAGCCAACCCACACATTGCC3'
T1218C FP	5'GCTTAAGCACAACAAATCTGGAGGCGCCATGGTCTGGG3'
T1218C RP	5'CCCAGACCATGGCGCCTCCAGATTTGTTGTGCTTAAGC3'
G1452T FP	5'GGCAGTGGATTCTGTGCTGGCAGAGCCAACGGCC3'
G1452T RP	5'GGCCGTTGGCTCTGCCAGCACAGAATCCACTGCC3'

Appendix Table 2.5 AMCase genetic variants identified in SNP Discovery

dbSNP rs#	Variants / Position	Alleles	Amino Acid	Exon /Intron	Allele	Frequency (%)		
						AA	MX	PR
rs34698010	Insertion +6	CT	5'UTR	E1	Ins	28	54	60
Novel	12	G/A	5'UTR	E1	A	2	NA	NA
rs12026825	34	G/A	5'UTR	E1	A	29	54	60
Novel	Insertion +129/130	CCAAT CTAGG	5'UTR	E2	Ins	26	9	13
Novel	260	C/T	R35W	E4	T	4	NA	NA
Novel	SNP1 +290	A/G	N45D	E4	G	26	9	13
Novel	SNP2 +296	G/A	D47N	E4	A	26	9	13
Novel	319	G/C	L54L	E4	C	4	NA	NA
Novel	SNP3 +339	G/T	R61M	E4	T	26	9	13
rs3818820	111658643	C/A	-	I4	A	15	6	15
rs2786152	111658677	C/T	-	I4	T	23	50	44
rs3818822	SNP4 +461	G/A	G102R	E5	A	15	4	10
Novel	SNP5 +531	A/G	R125K	E6	G	5	6	10
rs2786161	111663237	A/T	-	I9	T	42	57	50
rs17027410	1153	G/A	V332V	E10	A	9	7	5
rs2275253	SNP6 +1172	A/G	I339V	E10	A	21	64	45
rs2275254	SNP7 +1218	T/C	S354F	E11	C	39	30	39
rs2820092	1318	C/T	L387L	E11	T	31	11	12
rs12094378	1322	C/T	L389L	E11	T	17	8	9
rs2256721	SNP8 +1452	G/T	V432G	E12	T	22	64	45
Novel	1610	C/T	3'UTR	E12	T	2	NA	NA

Appendix Table 3.1 Primers for resequencing of the CHIT1 gene

Exon	Direction	Orientation	Sequence	Orientation
1	Forward	5'	TCATGCTGCTTGACATCTTACC	3'
1	Reverse	5'	AAAATGACCCTCTGAAGTTCTCC	3'
2	Forward	5'	CTGAAAGGAGAAAGCTTGATTTG	3'
2	Reverse	5'	TTGGAAGAGAGTCCCCACTG	3'
3 & 4	Forward	5'	GTCCCTCCACTGACTCCAGG	3'
3 & 4	Reverse	5'	AGAAGGAAATTCAGCCCTCAG	3'
5 & 6	Forward	5'	CCATAACTCACCAGCAAATATCC	3'
5 & 6	Reverse	5'	TAAGGATGGGACTTTCCAAATG	3'
7	Forward	5'	CTGTCTGGGTCACCTTCTGC	3'
7	Reverse	5'	AAGCTTCTTGTTTCTCAGTGCC	3'
8 & 9	Forward	5'	AGGCTCTAAAGAAGATGGGGTAG	3'
8 & 9	Reverse	5'	CATTTTGTCTTTTCTGAGTCAATAGG	3'
10 & 11	Forward	5'	CAGAATCTACAGCCACTCACAGG	3'
10 & 11	Reverse	5'	AGACAGTGAAGATTCAACCAAGG	3'
12	Forward	5'	GGCTGTACTCAGCCTGTAGGG	3'
12	Reverse	5'	ACCTGTGTACTGGAAACTGCC	3'

Appendix Table 4.1 Additional characterization data for asthma subgroup

IgE levels (n=39)	458 ± 530
	(Median 246; Range 19-2627)
Positive Skin Test Reactions*	(n=38)[†]
≥ 1+ skin test response	36 (95)
D *farinae*	32 (84)
Dog	8 (21)
Alternaria	13 (34)
Aspergillus	6 (16)
Ragweed	10 (26)
Oak	10 (26)
D *pteronyssinus*	30 (79)
Cat	25 (66)
Roach	15 (39)
Cladosporium	4 (11)
Sorel	7 (18)
Grasses	20 (53)

Appendix Table 4.2 Primers for Real-time PCR experiments

Chitinases	Name	Sequence 5'-3'
AMCase (Primer 1)	Outflanking primer, forward	CTGCAGTAGCTGCTGGCATCT
	Outflanking primer, reverse	GCCATGGAGGTCGTAGGTCAT
	Forward Taqman primer	TGCAGTAGCTGCTGGCATCT
	Taqman Probe	AGATCCCCCAACTGTCACAGTACCTGGA
	Reverse Taqman primer	GCCATGGAGGTCGTAGGTCAT
AMCase (Primer 2)	Outflanking primer, forward	TGGTGCAGGAAATGCGTG
	Outflanking primer, reverse	GATGCCAGCAGCTACTGCAG
	Forward Taqman primer	GGAAATGCGTGAAGCTTTTGA
	Taqman Probe	AGGCCAAGCAGATCAACAAGCCCA
	Reverse Taqman primer	CAGCTACTGCAGCAGTGACCAT
CHIT1	Outflanking primer, forward	TAGATGACTTTGCCGGCTTCTC
	Outflanking primer, reverse	CCTGCCGTAGCGTCTGGAT
	Forward Taqman primer	GATGACTTTGCCGGCTTCTC
	Taqman Probe	TGCAACCAGGGCCGATACCCC
	Reverse Taqman primer	TGCCGTAGCGTCTGGATGA

Housekeeping	Name	Sequence 5'-3'
GAPDH/NM_002046	GAPDH-RTF	CAATGACCCCTTCATTGACCTC
	GAPDH-RTR	CTCGCTCCTGGAAGATGGTGAT
	GAPDH-TMF	GATTCCACCCATGGCAAATTC
	GAPDH-TMP	CGTTCTCAGCCTTGACGGTGCCA
	GAPDH-TMR	GGGATTTCCATTGATGACAAGC
UBIQUITIN/NM_018955	UBIQUITIN-RTF	GGGCGGTTGGCTTTGTT
	UBIQUITIN-RTR	CCTGTTAGCGGATACCAGGATC
	UBIQUITIN-TMF	GGGCGGTTGGCTTTGTT
	UBIQUITIN-TMP	TGAGCTTGTTTGTGTCCCTGTGGGTG
	UBIQUITIN-TMR	TGCCAATCACCAACCACGT
PP2A/NM_002715	PP2A-RTF	ACATGGTGGTCTCTCGCCA
	PP2A-RTR	GATATACCCCAACCACCACGG
	PP2A-TMF	CGCCTACAAGAAGTTCCCCA
	PP2A-TMP	ACCACAGCAAGTCACACATTGGACCCT
	PP2A-TMR	CCACCACGGTCATCTGGATC
PPIA/NM_021130	PPIA-RTF	ATGAGAACTTCATCCTAAAGCATACG
	PPIA-RTR	TTGGCAGTGCAGATGAAAAACT
	PPIA-TMF	ACGGGTCCTGGCATCTTGT
	PPIA-TMP	ATGGCAAATGCTGGACCCAACACA
	PPIA-TMR	GCAGATGAAAAACTGGGAACCA

Housekeeping (cont.)	Name	Sequence 5'-3'
B2M/NM_004048	B2M-RTF	GCCGTGTGAACCATGTGACT
	B2M-RTR	CAATCCAAATGCGGCATCT
	B2M-TMF	TGACTTTGTCACAGCCCAAGATA
	B2M-TMP	CATGATGCTGCTTACATGTCTCGATCC
	B2M-TMR	AAATGCGGCATCTTCAAACC
EEF1A/NM_001402	EEF1A-RTF	TGCTAACATGCCTTGGTTCAAG
	EEF1A-RTR	TTGGACGAGTTGGTGGTAGGAT
	EEF1A-TMF	CCTTGGTTCAAGGGATGGAA
	EEF1A-TMP	CACTGGCATTGCCATCCTTACGGG
	EEF1A-TMR	GCCTCAAGCAGCGTGGTT

Publishing Agreement

It is the policy of the University to encourage the distribution of all theses and dissertations. Copies of all UCSF theses and dissertations will be routed to the library via the Graduate Division. The library will make all theses and dissertations accessible to the public and will preserve these to the best of their abilities, in perpetuity.

I hereby grant permission to the Graduate Division of the University of California, San Francisco to release copies of my thesis or dissertation to the Campus Library to provide access and preservation, in whole or in part, in perpetuity.

_____ 7 - 25 - 8
Author Signature   Date