Airway and Gut Microbiome in Allergy and Asthma: Relationships to Immune and Clinical Phenotype. Effects of Inhaled Corticosteroid Treatment. A Proof of Concept Study

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Abstract

The application of sensitive, culture-independent methods for detecting microbes, based on detection and identification of "signature" sequences of DNA or RNA in clinical samples, is transforming concepts of the dimensions and nature of the microbial world and of the intimacy of its association with human health and disease. Studies applying these techniques have begun to describe the extraordinary richness and diversity of the microbial populations in the mouth, sinuses, gastrointestinal tract, skin, vagina, and tracheobronchial tree and have begun as well to suggest relationships between the composition and function of a site or organ's microbiome and that site or organ's function. Animal studies using these techniques have also established relationships between the bacterial populations in the gastrointestinal tract and the numbers, activity, and function of specific immune cells, especially T cells, not just in the gut, but also in the circulation and in distant organs, including the lungs and airways, and epidemiological studies have demonstrated relationships between the G-I microbiome and clinical manifestations of immune-mediated disease[1-5].

Taken together, the findings suggesting that the composition and function of the gastrointestinal microbiome shapes systemic immune function, and the findings suggesting that the composition and function of a local microbiome can affect local function, have clear implications for concepts of the pathogenesis of allergic diseases, including allergic asthma. One implication is that some feature of the gastrointestinal microbiome may underlie the disorder or imbalance in systemic immune function that is thought to account for the predisposition to allergic sensitization and thus to allergic disease, This disorder in immune function is now thought to consist of an imbalance in the function of effector Th2 cells and suppressive regulatory T cells, so that T cell responses are skewed toward a proinflammatory Th2-mediated pattern. There is also intense interest in more recently identified T cell subsets, such as Th17 cells, which are clearly regulated by gastrointestinal microbiota, although their roles in asthma remain unclear. So, too, does the role of other T cells, including natural killer T cells, gamma-delta T cells, and CD8 cells[6,7]. Insofar as the circulating populations of these cells are shaped by bacterial populations in the gastrointestinal tract, the gastrointestinal microbiome of people with allergic disease may differ in composition from the gastrointestinal microbiome of healthy, non-allergic controls. It is also possible that asthma is a function not just of differences in systemic or local immune function, but also of differences in the composition and function of bacterial populations in the tracheobronchial tree[8-10].

The findings that the G-I microbiome may shape systemic immune function and that a local, site or organ-specific microbiome might affect local, site or organ-specific immune function also have implications for our understanding of the pathophysiologic mechanisms underlying different phenotypic forms of asthma. Evidence supporting the idea that differences in local immune function may account for different subtypes or "phenotypes" of asthma has been provided by the NHLBI's Severe Asthma Research Program. This research group reported that unsupervised clustering of the concentrations of cytokines in bronchoalveolar lavage fluid from asthmatic patients with

a range of asthma severities identified four distinct groups – or "intermediate phenotypes" - of asthma. One group, enriched in patients with severe asthma, showed differences in BAL cellular content, reductions in pulmonary function, and enhanced responsiveness to inhaled methacholine. The differences in the proportions of patients with severe asthma in the different subgroups suggests that they may well differ in responsiveness to inhaled corticosteroid therapy[11-13]. Whether these differences in the patterns of activation of immune response in BAL fluid are related to differences in the numbers of functional status of systemic or local T-cell populations is not known.

While most attention has focused on cellular mediators of allergic inflammation in asthma, it is also possible that the microbial populations in the lungs and airways may be importantly involved in the pathogenesis of asthma or in its particular phenotypic expression. Our own previous work[14] and that of Hilty et al. in Oxford[15], have reported that application of 16S-based, culture-independent methods to examine samples collected from the bronchial mucosa showed greater bacterial richness and diversity in the samples obtained from the asthmatic subjects than healthy subjects, with significant differences in the abundance of numerous specific taxa in their samples. We found, for example, differences in the relative abundance of certain bacterial taxa to be associated with greater bronchial responsiveness to methacholine and with clinical responsiveness to 16 weeks of treatment with clarithromycin, a macrolide antibiotic. The demonstration of differences in the bronchial microbiome in patients with different phenotypic features of asthma would prompt reconsideration of our current concepts of asthma and could lead to changes in approaches to prevention and treatment. Studies so far have been limited by the potential confounding effect of treatment with an inhaled corticosteroid on the microbiome. It is thus not clear whether the differences that have been demonstrated in the bronchial microbiome of asthmatic vs. healthy subjects were a function of their disease, or of its treatment.

This protocol proposes to apply recently developed methods to examine the relationships among gut microbiome, systemic immune function, bronchopulmonary inflammatory response, bronchial microbiome, and detailed assessments of clinical and physiologic features of pulmonary function in three populations: (1.) inhaled corticosteroid-naïve allergic asthmatic adults (2.) allergic but non-asthmatic adults; and (3.) non-allergic, non-asthmatic healthy adults. For identification and characterization of gastrointestinal and bronchial microbiomes, we propose to apply a sensitive, broadly parallel method, the 16S-rRNA PhyloChip[16], to stool samples and to samples obtained by protected bronchial brushings from these three groups of subjects. To assess systemic and broncho-pulmonary immune function, we will apply 11 color FACS analysis to enumerate innate T cells, regulatory T cells, and other CD4 T cell subsets in samples of blood and bronchial lavage fluid. CD4 T cells will be characterized for their pattern of expression of three chemokine receptors (CXCR3, CCR6, and CCR4) that we and others have shown correlate with ability of those cells to produce the signature cytokines IFN-γ (Th1), IL-17 (Th17), and IL-4 and IL-13 (Th2)[17-19]. FACS will also be used to enumerate other relevant cells, including CD8 T cells, NK cells, B cells, monocytes, basophils, eosinophils, and neutrophils. The same methods will be applied to assess the composition of inflammatory cells and T cell subsets in bronchial lavage

fluid. In addition, we will assess ongoing pulmonary immune responses by measuring a panel of 23 cytokines in bronchial lavage fluid using multiplex magnetic bead ELISA.

We also propose to examine in the asthmatic subjects the effects of inhaled corticosteroid (ICS) treatment on the bronchial microbiome and pulmonary immune function by comparing the changes associated with six weeks of treatment with an ICS vs. 6 weeks of treatment with a placebo inhaler.

The primary purpose of our analysis will be to determine whether the composition of the microbial communities in the airways differs in allergic asthmatic, allergic non-asthmatic, and non-allergic, non-asthmatic adults. We will also examine whether among the asthmatic subjects, differences in bronchial microbial composition are associated with differences in phenotypic features of asthma such as proneness to exacerbations, irreversible airflow obstruction, presence of eosinophils or neutrophils in airway secretions, expression of Th2-dpendent genes in the bronchial epithelium[20], and responsiveness to inhaled corticosteroid treatment. Finally, we will examine whether the bronchial microbiome is altered by inhaled corticosteroid treatment.

Data collected for these analyses will enable examination of the association of features of the intestinal microbiome (gross community metrics and specific taxonomic composition) with differences in the numbers and proportions of T cell subsets and other inflammatory cells in the circulation and airways. They will also make possible examination of whether the features of the intestinal microbiome – and of circulating inflammatory cells - differ between the allergic subjects (including asthmatic and non-asthmatics) and the non-allergic healthy subjects, but do not differ between the allergic asthmatic and allergic non-asthmatic subjects. If so, this would suggest the possibility that the immune dysregulation underlying “allergy” may be shaped by the composition of the intestinal microbiome, but that asthma is a function of some other determinant, possibly limited to the lungs and airways, that affects some, but not all allergic subjects. A candidate for this other determinant might be the microbiome colonizing the bronchial tree.

The addition of the simple, well-tolerated procedure of nasal brushing to this protocol will enable collection and storage of samples permitting later study of questions that may become salient depending on the findings of this and other on-going clinical research studies. Should, for example, this study’s findings suggest a difference in the bronchial microbiome of allergic asthmatic vs non-allergic, non-asthmatic subjects, then it will become interesting to examine whether these differences in the bronchial microbiome are reflected by similar differences in the nasal microbiome of subjects with allergic rhinitis vs subjects without allergy. Comparison could also be done of the nasal and bronchial microbiome to determine whether analysis of microbial community composition of the nose permits inferences about the microbial community composition of the bronchial airways. Along these lines, should studies now examining the epigenome in nasal epithelial cells obtained from asthmatic and non-asthmatic children show potentially important differences, then study of epigenetic changes in the DNA extracted from nasal and bronchial cells obtained at the same time from the same adult

subjects will allow determination as to whether description of the epigenome of the nasal epithelium allows inferences as to the epigenome of the bronchial epithelium. Finally, study of these samples could allow analysis of possible relationships between the microbiome and epigenome at two epithelial surfaces. These studies are not included in this protocol proposal. Simply the collection and storage of nasal brushings for later analysis, possibly by other or collaborating investigators, as approved by the NHLBI and AsthmaNet Steering Committee is proposed. These samples will be stored initially at the UCSF Airway Sample Bio-Bank and then transferred to the AsthmaNet Biologic Specimen and Data Repository Information Coordinating Center (BioLINCC).

In summary, this protocol will enable examination of possible relationships between stool microbiome, systemic immune function, pulmonary immune function, bronchial microbiome, and pulmonary function and inflammation across three populations: allergic asthmatics, allergic non-asthmatics, and non-allergic, non-asthmatics. Such analysis is unprecedented in depth and scope and may enable potentially transformative insights into the relationships between the human microbiome and pulmonary health and disease.

I. Hypotheses and Specific Aims

A. Primary Research Hypotheses

1. The microbiota of the bronchial airways of allergic asthmatic, allergic non-asthmatic, and non-allergic, non-asthmatic healthy subjects differ in diversity, richness, evenness, and/or taxanomic composition.

2. Clinical, physiologic, and inflammatory phenotypic features of asthma (including "Th2- vs. non-Th2" pattern of gene expression in bronchial epithelial cells, and cluster by BAL cytokine pattern) are associated with characteristic bronchial microbial community compositions.

3. Inhaled corticosteroid (ICS) treatment alters bronchial microbial community composition in asthmatic subjects.

4. In the absence of respiratory infection, antibiotic treatment, or change in inhaled therapy, bronchial microbial community composition is stable over six weeks.

5. Differences in bronchial microbial community composition at baseline or after ICS treatment are associated with differences in responsiveness to ICS treatment.

B. Secondary Research Hypotheses – Related to asthma

6. Differences in bronchial microbial community composition, as revealed by exploratory methods such as cluster analysis, are associated with differences in clinical, physiologic, and inflammatory phenotypic features of asthma.

7. The relative distribution of inflammatory cells and T cell subsets in bronchial lavage fluid are associated with differences in clinical, physiologic, and inflammatory phenotypic features of asthma.

8. The relative distribution of inflammatory cells and T cell subsets in bronchial lavage fluid from asthmatic subjects are associated with differences in bronchial microbial community composition.

9. The composition of the microbial community of induced sputum closely resembles that of bronchial brushings.

C. Secondary Research Hypotheses – Related to allergy

10. Richness, evenness, diversity, or composition of stool microbiota differs between:

    a. Allergic and non-allergic subjects.

    b. Allergic asthmatic and allergic non-asthmatic subjects.

11. The numbers and relative distribution of circulating innate T cells and CD4 T cell subsets differs between allergic and non-allergic subjects.

12. Differences in the numbers and relative distribution of innate T cells and CD4 T cell subsets are associated with differences in the richness, evenness, diversity, or composition of stool microbiota.

13. Differences in stool microbial community composition, as revealed by exploratory methods such as cluster analysis, are associated with relative distribution of innate T cells and CD4 T cell subsets

D. Specific Aims – Related to Bronchial Microbiome and Asthma.

Specific Aim 1: To evaluate whether the microbiota of the bronchial airways in allergic asthmatic, allergic non-asthmatic, and non-allergic, non-asthmatic healthy subjects differ in microbial diversity, richness, evenness, or composition of specific bacterial taxa.

Specific Aim 2: To determine whether ICS treatment alters bronchial microbial community composition in allergic asthmatic subjects.

Specific Aim 3: To determine whether characteristics of bronchial microbial community composition at baseline or after ICS treatment, are associated with differences in responsiveness to the treatment as measured by change in FEV_1 and PC_{20}Mch

Specific Aim 4: To evaluate whether clinical, physiologic, and inflammatory phenotypic features of asthma (including “Th2- vs. non-Th2-molecular phenotype”) are associated with characteristics of bronchial microbial community composition.

Specific Aim 5: To examine whether there are associations between bronchial microbial community composition and

a. Pulmonary immune function as inferred from the relative distribution of innate T cells, CD4 T cell subsets, and other inflammatory cells (eosinophils, monocytes, basophils) in bronchial lavage fluid.
b. Pulmonary immune activation state as inferred from measurement of selected cytokines in bronchial lavage fluid.

Specific Aim 6: To evaluate the concordance of the airway microbiota detected in induced sputum and bronchial brushings from a subset of ten healthy and ten asthmatic subjects participating in this study.

E. Specific Aims – Related to Stool Microbiome, Systemic Immune Function, Allergy, and Asthma.

Specific Aim 7: To examine whether the metrics of gross microbial community composition (richness, evenness, and diversity) or discrete taxonomic members of these communities in **stool samples** differ:

a. Among allergic asthmatic, allergic but otherwise healthy non-asthmatic, and non-allergic, non-asthmatic adults.
b. Among different phenotypic subgroups of allergic asthmatic subjects (eg. Th2 vs. non-Th2, eosinophilic vs. non-eosinophilic, ICS-responsive vs. non-ICS responsive, exacerbation-prone vs. exacerbation resistant, etc).

Specific Aim 8: To examine whether the numbers and relative distribution of circulating innate T cells, CD4 T cell subsets, and other inflammatory cells (eosinophils, monocytes, basophils) differ between allergic and non-allergic subjects.

Specific Aim 9: To examine whether there are associations between **stool microbial community composition** and

c. the numbers and relative distribution of circulating innate T cells, CD4 T cell subsets, and other inflammatory cells (eosinophils, monocytes, basophils):.

II. Background and Significance

A. Bronchial Microbiome and Asthma

1. Introduction.

The nosologic term “asthma” encompasses a heterogeneous collection of disorders sharing the features of airflow obstruction and bronchial hyperreactivity, but differing in important features, such as pattern of bronchial inflammation (e.g. eosinophilic vs. neutrophilic), responsiveness to beta-agonist and corticosteroid treatment, and susceptibility to exacerbation. Thus, asthmatic patients are now often clustered into groups of “asthma phenotypes”, but it is not established whether different phenotypes reflect different pathophysiologic mechanisms or the influence of different modifying factors on a common underlying mechanism. That bacterial infection might be one such

mechanism or factor is suggested by the finding of specific bacteria, particularly Mycoplasma pneumoniae and Chlamydophila pneumoniae, in bronchial biopsies from some asthmatic patients[21,22]. Links have also been suggested between the presence of S. pneumoniae, M. catarrhalis, and H. influenzae in hypopharyngeal cultures from 1-month-old infants and their risk of asthma in early life[9] and in hypopharyngeal cultures from children presenting with acute wheezing illnesses[8]. That perturbations from a normal "bronchial microbiome" might be important has been suggested by recent culture-independent studies of bronchial samples[14,15]. These have shown that the airway microbiota of asthmatic and healthy individuals differ in diversity and in the relative abundance of specific bacterial species. These findings could be interpreted as suggesting that a perturbation of normal bronchial microbiota is causally associated with asthma, or at least some forms of asthma, but such a suggestion would be grossly premature, not least because all of the asthmatic subjects examined were on inhaled corticosteroid therapy, making it unclear whether the differences found are associated with asthma or with ICS treatment. Because some of the bacteria identified in asthmatic subjects, like the Comamonadaceae, can express genes involved in steroid degradation pathways[23,24], it can even be hypothesized that differences in bronchial microbial community composition may influence corticosteroid-responsiveness. Taken together, these observations suggest that study of microbial community composition in the airways of carefully characterized (or "phenotyped") healthy and asthmatic subjects may provide findings that could foster development of anti- or pro-biotic treatments for asthma.

Methods for identifying airway microbiota in most prior studies of asthma have had significant limitations. The vast majority of bacteria are non-culturable[25]**,** and the utility of targeted PCR to identify species is limited by its ability to detect unanticipated bacteria. The application of more recently developed, high resolution, culture-independent methods for microbial detection has demonstrated a great diversity of airway microbiota in airway disease, including cystic fibrosis[26,27], ventilator-associated pneumonia[28], COPD[27,29], and also asthma[14,15]. In a recently published study by Huang et al.[14], conducted by the Asthma Clinical Research Network (ACRN), bronchial brushings from suboptimally controlled asthmatic subjects (all taking ICS) were analyzed using the 16S ribosomal RNA PhyloChip, developed by collaborators at Lawrence Berkeley National Laboratory. This microarray-based method exploits sequence polymorphisms in the broadly conserved, ubiquitous prokaryotic 16S rRNA gene to identify and classify species, and is able to distinguish in a single assay ~ 8,500 bacterial taxa (taxa are defined as groups of species with $\geq$ 97% homology in their 16S rRNA gene sequence)[16,30]. The PhyloChip permits rapid bacterial community profiling of many sample types with significantly higher resolution than traditional clone library-sequencing analysis of the same amplicon pools[28,30], and detects species present in low abundance as efficiently as those in higher abundance in a given community[30] and is ideal for high-resolution comparative analyses of treatment groups.

Comprehensive profiling of the microbiota by high-resolution, molecular approaches permits complementary study of the potential functional effects of a microbial community. Recent research findings on the human microbiome collectively have

outlined two concepts: (1) the structural complexity of microbial communities at a given host site, rather than simply the presence of individual species, can be important in determining states of health vs. disease[31], and (2) differences in the structure or composition of a microbial community underlie the collective functional effects exerted by the community, including potential influences on host responses[31,32]. Studies highlighting these notions include the demonstration that differences in the gut microbiota between obese and lean phenotypes[33] are associated with different functional capacities for energy harvest, and that the presence of gut microbiota influences the severity of induced type-1 diabetes in MyD88-deficient mice[34]. These examples emphasize that advancements in knowledge about the airway microbiota could yield important insights into polymicrobial-host interactions relevant to the pathogenesis or course of asthma. Indeed, in the "Add-On" study to the ACRN's Macrolides in Asthma trial, significant relationships were identified between features of the airway microbiota, asthma and airway hyperresponsiveness[14] (see preliminary data), suggesting potential pathophysiologic links between the airway microbiota and this clinical-pathophysiologic feature of asthma.

2. Previously Published Data.

In the "Add-On" study to the ACRN's Macrolides in Asthma trial[14], an early observation was that bacterial burden in bronchial epithelial samples (using measurements of 16S rRNA amplicon concentrations as a proxy, and confirmed by quantitative PCR) was significantly higher among the asthmatic group than healthy controls (Fig. 1). Furthermore, as mentioned above, significant relationships between airway hyperresponsiveness and characteristics of the airway microbiota profiled by PhyloChip were observed using several different, independent analysis approaches. These included a distance measure-based analysis of dissimilarity in bacterial community composition among samples using a statistical ordination method (non-metric multidimensional scaling, or NMDS). This approach also enabled assessment of which variables may be most contributory to observed dissimilarities (or variability) in bacterial community composition. As shown in Fig. 2A and 2B, this revealed that measurements of PC_{20}Mch measurements and bacterial burden were most strongly correlated with community variability. In a separate analysis based on calculations for each sample of a commonly used measure of bacterial diversity (Shannon index), we found that diversity indices were significantly and inversely correlated with PC_{20}Mch (Fig. 2C), suggesting increasing bronchial bacterial diversity with greater airway hyperresponsiveness. Finally, we examined for linear relationships between the relative abundance of all taxa detected by PhyloChip across samples (~1,900) and PC_{20}Mch. After corrections for false discovery and the application of fairly conservative significance criteria, we found that the relative abundance of ~100 specific bacterial phylotypes profiled by the array had the most significant correlations with greater airway hyperresponsiveness.

Figure 1. Bronchial bacterial burden by study group (Ref. 6).

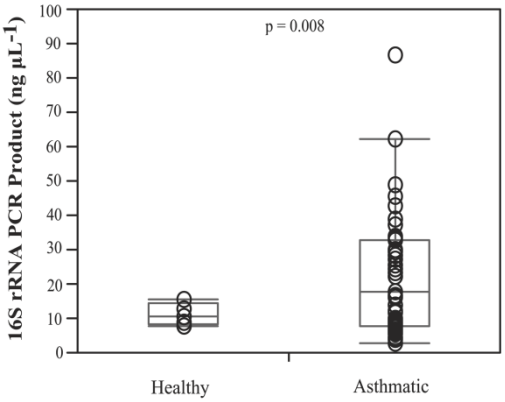
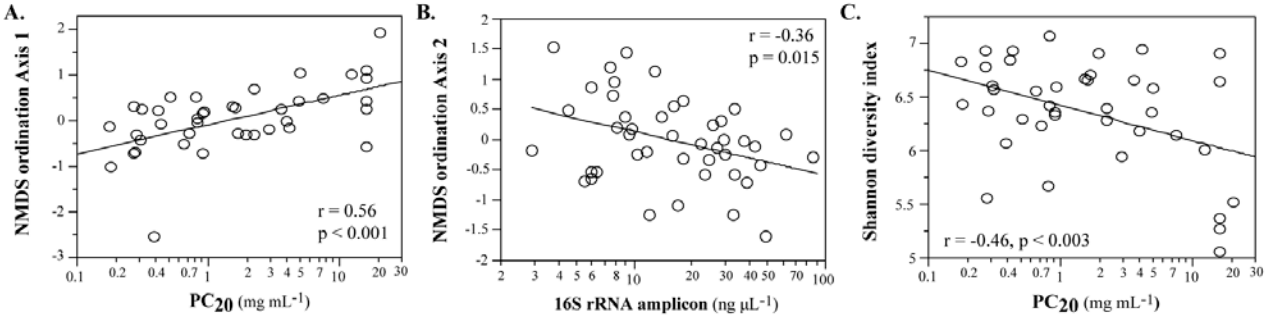


Figure 2. Panels A-B. NMDS analysis showing that variability in bronchial bacterial community composition across samples is strongly correlated with PC_{20}Mch and bacterial burden (circles represent the total community present in a single subject sample). Panel C. Shannon indices of bacterial diversity increase with lower PC_{20}Mch values. (Ref. 6)



3. Inhaled Corticosteroid treatment - Rationale and Duration of Treatment

To our knowledge, only two studies have been published to on the airway microbiota in chronic asthma[14,15]. As all asthmatic subjects in both studies were taking ICS therapies, it remains unclear whether differences found in the airway microbiota are related to ICS treatment or to asthma itself. Several different inhaled corticosteroids delivered from a dry-powder inhaler are approved as maintenance treatment for asthma (budesonide, fluticasone, mometasone). In this study, we wish to compare the effects of inhalation of an ICS to inhalation of placebo. We propose to have the subjects inhale 250 mcg of fluticasone by Diskus inhaler twice daily for six weeks in this protocol. This duration of treatment was selected because a previous study conducted by the Asthma Clinical Research Network, the "PRICE" study showed that six weeks of inhaled corticosteroid treatment was sufficient to identify patients as "responders" or "non-responders" as judged by a greater than 5% increase in FEV1 or a greater than 1 doubling dose of methacholine in PC_{20}, without further change in FEV1 after 16 additional weeks of continued ICS therapy[35].

4. Selection of allergic subjects.

As noted above, it has not been possible to interpret the differences in the bronchial microbiome of the asthmatic and healthy subjects studied so far as necessarily suggesting a relationship between bronchial microbiota and asthma, for all of the asthmatic subjects were under treatment with an inhaled corticosteroid at the time of bronchoscopy. That is why we now propose to study asthmatic subjects who have taken no ICS treatment for 6 months. Another possible confounding determinant of bronchial microbiota is allergy, for allergy is associated with demonstrably different patterns of immune function in the airways, and these differences could plausibly account for differences in the microbes allowed to colonize or infect airway mucosal surfaces. Since the primary research question of this study is whether the bronchial microbiome differs in people with and without asthma, we think it necessary to enroll allergic non-asthmatic adult subjects, as nearly similar as possible in all other regards as a control group. Because >80% of the asthmatic subjects participating in ACRN and CARE network studies have been allergic, we know that recruitment of allergic asthmatic subjects is far easier than recruitment of non-allergic asthmatic subjects. It follows that the healthy subjects enrolled in this study must be allergic as well. We thus think it necessary for this study that at least one "control" group for comparison is made up of non-asthmatic subjects who, like the asthmatic subjects, have a positive Phadiatop test result. Whether allergy, as distinct from asthma, is associated with differences in gastrointestinal or bronchial microbial community composition is an interesting and potentially important question that will be addressed by the additional enrollment of a second control group, of non-allergic, non-asthmatic healthy adults (see section B., below). For subjects identified as allergic by Phadiatop testing, we will additionally measure specific IgE antibodies directed against a standard panel of aeroallergens, including cat, dog, mouse, mold mix, cockroach (German), mixes of grass, tree (2 mixes), weed (2 mixes), and mite (2: Der f and Der p) . This may permit analysis of whether exposure to allergens to which the subject is sensitized affects burden or composition of the bronchial microbiome.

5. Bronchoscopic vs. non-bronchoscopic airway specimens.

The primary specimens to be analyzed for differences or changes in the bronchial microbiome are protected bronchial epithelial brushings. From prior experience in the MIA Add-On study14, three bronchial brushings per subject provided greater pooled yield than bronchial biopsies in terms of total DNA recovered and bacterial 16S rRNA PCR product. While bronchial brushings are a preferred specimen type for analysis of the bronchial airway microbiome, the potential utility of non-bronchoscopically collected specimens for inferring bronchial microbial community composition is not clear. Of potential options, microbiome analysis of induced sputum may be useful, given precedence for analysis of sputum inflammation in asthma studies[36]. Preliminary PhyloChip analysis of paired induced sputum and bronchial brushings from 6 subjects (3 healthy, 3 asthmatics not taking ICS) found 80-97% concordance in the specific bacterial taxa detected. For two of the three healthy subjects, all taxa identified in the bronchial brushings were detected also in the paired sputum. However, there was greater variability in the ICS-naïve asthmatic subject specimens, where 8.9% – 19% of

detected taxa (or 67 – 240 taxa) were identified in the brushings only. Since many taxa can comprise a given bacterial subfamily, this translated into 9 to 23 bacterial subfamilies that were identified from brushings only. Comparison of the microbiome revealed by analysis of induced sputum and bronchial brush samples in this study will enable us to determine if induced sputum samples reflect >90% of all microbial taxa detected in bronchial brush samples.

6. Anticipated Significance.

A resident microbial community has been identified in the bronchial airways of asthmatic subjects in two recent studies[14,15], and specific features of the microbiota have been found to correlate with bronchial hyperresponsiveness[14], an important pathophysiological feature of asthma. In addition, known functional properties of specific organisms associated with this clinical feature may potentially contribute to asthma pathogenesis or prognosis (e.g. organisms with steroid-degradation capacity). Collectively, these recent findings could lead to the development of novel therapeutic approaches for asthma, including pro-biotic, anti-biotic, or other specific mechanistic targets directed at the microbiota. To pursue this further, however, it is necessary to establish whether these findings are confounded by the use of ICS therapies in all asthmatics examined in these two prior studies. If differences in the bronchial microbiome are discovered between healthy and ICS-naïve asthmatics in this study, this would have important implications for further research on the role of microbiota in asthma pathogenesis. If specific microbial community features are found to be associated with responsiveness to ICS treatment or related changes in other phenotypic variables, this could provide a microbial signature for prognosticating response to ICS therapy, which may be evaluated in future studies with other asthmatic populations.

B. G-I Microbiome, Systemic Immune Function, Allergy, and Asthma.

1. Introduction.

While analysis of relationships between features of the bronchial microbiome and the clinical, physiological, and inflammatory features of airway function in allergic asthmatic and allergic non-asthmatic adults holds promise for shedding new light on pathophysiologic mechanisms of asthma, we have recognized that more could be learned by coincidentally assessing the microbial composition of stool samples and the circulating cell populations that mediate immune function.

The rationale underlying these additional analyses rests first on evidence that allergic sensitization and response reflect a disorder in systemic immune function, predominantly expressed at mucosal surfaces, consisting of an imbalance between effector Th2 cells and suppressive regulatory T cells, so that T cell responses are skewed toward a pro-inflammatory Th2-mediated pattern. Other more recently described T cell subsets such as Th17 cells may play a role in asthma pathogenesis as well. So, too, may other T cells, including natural killer T cells, gamma-delta T cells, and CD8 cells[6,7].

Also underlying our interest in these additional analyses are the findings of studies showing relationships between the composition of the microbial community in the gastrointestinal tract and clinical manifestations of immune-mediated disease (epidemiologic studies) and in the numbers, activity, and function of specific immune cells, especially T cells (clinical and murine studies)[1-3,37,38]

We thus propose to examine relationships among stool microbiome, systemic immune function, and pulmonary immune response in healthy and asthmatic subjects by also obtaining and analyzing samples of stool (for characterization of fecal microbiome), of blood (for "immunophenotyping" of T cell and inflammatory cell populations), and of bronchial lavage fluid (for concentrations of selected cytokines and of T-cell populations) from allergic asthmatic and allergic non-asthmatic, and non-allergic, non-asthmatic healthy subjects..

The enrollment of a group of **non-allergic, non-asthmatic healthy subjects** will permit assessment of the possibility that the disturbances in immune function underlying ***allergy*** are associated with differences in the intestinal microbiome, whereas the disturbances in function underlying ***allergic asthma*** are associated with differences in the microbial populations resident in the airways. It is of course also possible that the gut microbiota shape not only the imbalance in immune function that underlies allergic sensitization and response but also the difference in immune function that permit a distinct pattern of bacterial colonization of the bronchial mucosa. It is not inconceivable even that particular patterns of gut microbial community composition may be associated with particular phenotypes of asthma.

2. Background Data.

Blood cell composition correlates with immune function: The cellular composition of circulating white blood cells reflects immune status and can be used to assess immune function and disposition to inflammatory and/or allergic responses. For example, blood eosinophilia is a standard biomarker of allergy. Helper T cells are central coordinators of immune responses, and the prevalence of different functional subsets (e.g. Th1, Th2, and Th17 cells) correlates with the type of responses that each subset directs. Allergic responses are driven by Th2 cells that produce the signature cytokines IL-4 and IL-13, and T cells expressing the Th2-associated cell surface protein CCR4 are also present at higher frequency in the peripheral blood of patients with allergic diseases including atopic dermatitis and asthma[19,39].

3. Subject selection.

Enrollment of three group of subjects – allergic asthmatic, allergic non-asthmatic, and non-allergic, non-asthmatic adults, all otherwise healthy – will enable determination not only of the relationship of the bronchial microbiome to asthma and its phenotypic features but also of whether allergy, as distinct from asthma, is associated with differences in gastrointestinal or bronchial microbial community composition.

4. Methods and previous findings.

a. Characterization of systemic immune function and of pulmonary immune response.

We will apply 11 color FACS analysis to assess systemic immune function. One panel of antibodies will focus on CD3+ T cells **(Figure 3)**. Invariant NK T cells are identified using CD1d tetramers. Regulatory T cells will be distinguished as CD4+ cells with high expression of CD25 and low expression of CD127. Among the remaining CD4+ T cells, naïve cells that have not been involved in an immune response express CD45RA, while activated and memory T cells instead expressed the CD45RO isoform. The CD45RO+ cell population contains the functionally relevant helper T cell subsets that are primed for cytokine production upon secondary antigen encounter. Despite some overlap in these subsets and evidence for functional plasticity in some conditions, at least three main subsets can be defined by the expression of their signature cytokines (Th1, IFN-γ; Th2, IL-4 and IL-13; Th17, IL-17A).

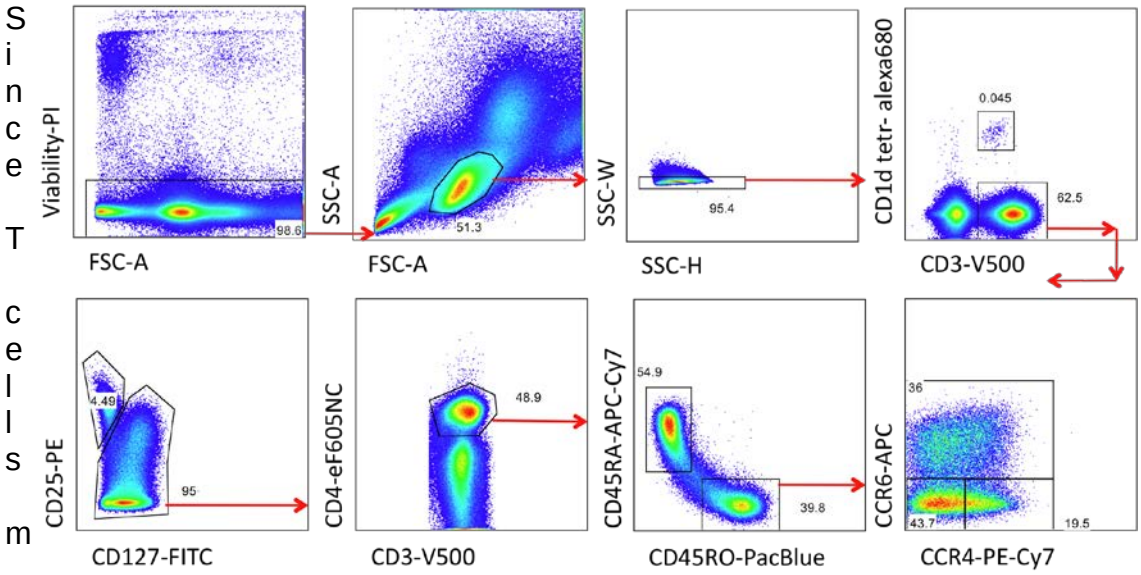


Figure 3. Representative FACS staining for T cell subsets.

T cells must be restimulated through their antigen receptor to reveal which cytokines they will produce. Therefore, extensive efforts have been made to uncover patterns of expression cell surface proteins that correlate with cytokine production capability. We have adopted this strategy and now routinely characterize helper T cell subsets in blood and bronchial lavage fluid by their pattern of expression of three chemokine receptors: CXCR3, CCR6, and CCR4 **(Figure 4)**. CCR6 expression correlates closely with the ability to make IL-17[17,40]**,** whereas IFN-γ-producing cells generally coexpress CXCR3[18]. CCR4 is expressed by Th2 cells and by many Th17 cells, but the vast majority Th2 cells can be captured within the CCR4+CCR6- population by co-staining for both markers. Co-staining for all three markers also allows us to track T cell populations expressing combinations of CXCR3, CCR4, and CCR6, which can include cells that make more than one of the signature helper T cell cytokines.

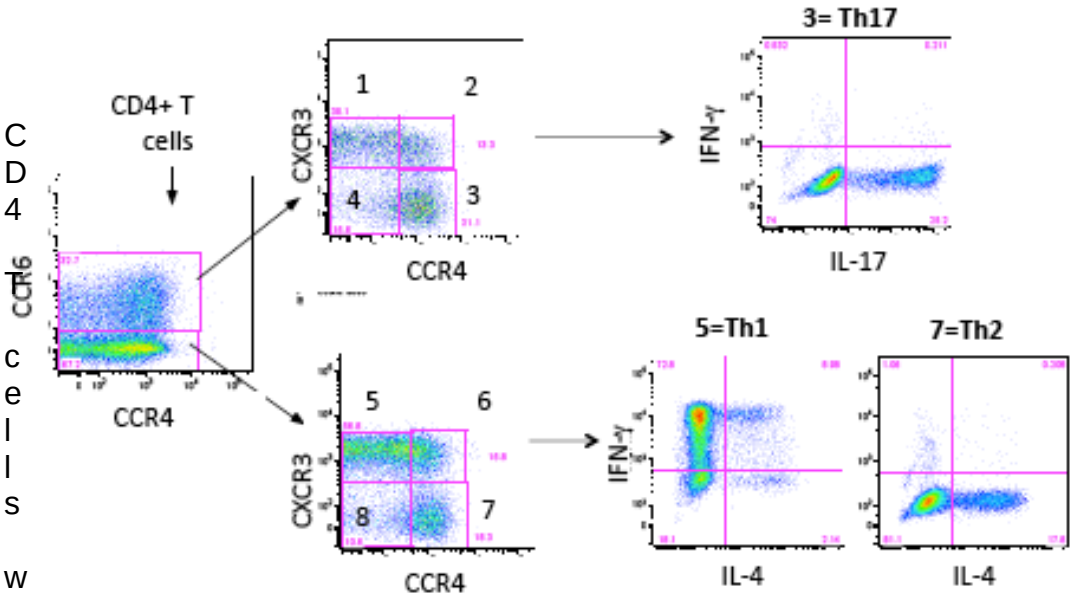


Figure 4. CD4+CD45RO+ T cells were FACS sorted into 8 populations defined by expression of CCR6, CCR4, and CXCR3 (*left panels*). Each population was subsequently stimulated in vitro and stained intracellularly to reveal cytokine production (*right panels;* numbers refer to corresponding quadrants at left).

A second panel of staining antibodies will be used to enumerate CD8 T cells, NK cells, B cells, monocytes, basophils, eosinophils, and neutrophils. Importantly, we have found that both blood and bronchial lavage cells can be preserved overnight at room temperature using Streck BCT reagent and subsequently stained for these FACS analyses on the following day (i.e. following courier shipment to the flow cytometry site at UCSF).

Ongoing pulmonary immune responses will be assessed by measuring a panel of 23 cytokines in bronchial lavage fluid using multiplex fluorescent magnetic bead ELISA according to the manufacturer's instructions (Millipore). These analyses will be conducted at the Blood Systems Research Institute core facility, which has experience with cytokine measurements in bronchial lavage fluid[41]. Patterns of cytokine expression relative to each other will be compared as described[11,12].

b. Detection and characterization of stool microbiome

The diverse ecosystem of the human gut microbiome houses the greatest burden of microbes, members of which encode genes for essential functions that the human host is incapable of performing, such as vitamin production and metabolism of indigestible dietary polysaccharides[42-44]. Thus, the host immune system must strike a balance between providing a favorable environment for this vital community while protecting against invasion or outgrowth of pathogenic species. Enteric microbes constantly prime

the innate immune system, thus facilitating a rapid response to pathogens[45]. Appropriate microbial colonization also plays a key role in the development of the gut-associated lymphoid tissue (GALT), a primary mechanism of defense against enteric pathogens[46,47]**.** Moreover, intestinal microbial colonization stimulates the production of effector molecules such as secretory IgA[48], the differentiation of TH17 cells[49], and the development and activation of regulatory T (T-Reg) cells[50,51]. Significantly, it has also been demonstrated that the presence of a GI microbiota and early stimulation of the immature immune system by a diversity of commensal microbes is fundamental to establishing and maintaining the essential balance between Th1, Th2, or Th17 cytokine expressing T-cells[52-55]**.**

Increasing interest is focused on the complex interplay between initial events in the assembly of the GI microbiota and the development and maintenance of the host's immune system homeostasis and whether manipulation of microbiota during this key developmental stage (or indeed in adulthood) can impact inflammatory disease outcomes in both the gastrointestinal and pulmonary tracts[56-58]. This interest is partly driven by the findings of epidemiologic studies linking GI pediatric gastrointestinal microbiome dysbiosis to the development of childhood asthma and allergy. For example, microbiological examination of almost 1,000 stool samples from 1-month-old infants demonstrated that high abundance of *Escherichia coli* was associated with the subsequent development of eczema, while high abundance of *Clostridium difficile* was associated with development of eczema, recurrent wheeze, allergic sensitization and allergic dermatitis[57]**.** Such findings have demonstrated a clear link between GI microbiome composition and allergic disease, and that, at least in pediatric patients, overgrowth of specific bacterial species predisposes to inflammatory disorders. Significantly, we have previously demonstrated that a specific murine GI bacterial species, segmented filamentous bacteria, can promote proliferation of Th17 cells[49], a relatively recently described subset of T-cells whose proliferation is associated with a number of chronic inflammatory diseases. This significant finding reinforces the concept that enrichment of particular microbial species in the complex community present in the GI microbiome can drive specific pro-inflammatory responses. Moreover, Kwon and colleagues recently demonstrated that feeding a mix of bacterial species resulted not only in local promotion of CD4+ FoxP3 T-reg cells in a murine model of colitis, but also trafficking of this T-cell subtype to affected sites remote from the GI tract including the skin in an animal model of dermatitis[59]**.** More recently, it has been demonstrated that the composition of the gastrointestinal microbiome governs host response to viral infection[60]**.** Using respiratory influenza virus as the model infectious agent, investigators demonstrated that gastrointestinal microbiota composition critically regulates the generation of virus-specific CD4 and CD8 T cells and antibody responses to this viral respiratory pathogen. To confirm these observations, the authors also demonstrated that oral treatment with a non-absorbed antibiotic, neomycin, dramatically altered the pulmonary response to influenza infection, indicating that neomycin-sensitive bacteria are associated with the induction of productive immune responses in the lung. Thus given the depth of the proposed study, which includes airway and GI microbiome profiling and immune phenotyping of subjects, study of relationships between

microbiome membership and the ability of specific species identified in the study to prime local and remote responses associated with asthma and allergy will be possible.

We propose to use the G3 16S rRNA PhyloChip, a high-density, culture-independent microarray-based assay designed in 2010, based on publicly available 16S rRNA sequence databases (which includes all of the human microbiome 16S rRNA sequences deposited by this date), to generate a high-resolution bacterial community profile of samples collected in this proposed study. This array, housing 1,000,000 oligonucleotide probes can detect ~60,000 bacterial taxa (defined as species or strains sharing ≥ 99% 16S rRNA sequence identity). Briefly, The 16S rRNA gene will be amplified, purified, labeled and quantified. A standardized concentration of labeled 16S rRNA will be spiked with known concentrations of control oligonucleotides that act as internal standards for data normalization. PhyloChip processing will be performed as previously reported[61].

While 454-sequencing has been the mainstay of microbiota profiling for several years, and has a number of clear advantages, e.g. acquisition of an actual sequence read, ability to determine relative abundance within a single sample, drawbacks include the potential for relatively poor community coverage of complex and diverse assemblages. This could potentially lead to overlooking key species involved in, for example specific disease states, since the species in question do not necessarily represent the dominant members of the community. Indeed, this has recently been highlighted in a study of the oral microbiome in which a low abundance member of the community, *Porphyromonas gingivalis*, drives periodontal inflammatory disease via immune and microbiome composition manipulation without ever becoming a dominant community member[62]. Such studies underline the need for high-resolution profiling approaches, particularly in comparative studies where identification of lower abundance species may prove key to the disease or disorder in question.

The G3 PhyloChip provides such coverage, and can detect low abundance, rarer community members in parallel with high abundance organisms, thus producing a high-resolution profile of the community members in a relatively economical assay, ideal for comparative or correlative statistical analyses to identify key species associated with the phenotype in question. We have directly compared 454-pyrosequencing to PhyloChip for profiling microbiota in house dust samples. Using quality-filtered 454-sequence reads ranging from 11,994-31,982 per sample, as many as 8,027 unique sequence reads were identified in one sample suggesting the presence of substantial diversity in these communities. This was confirmed by rarefaction analysis of the sequence data **(Fig. 5.A),** which indicated that that the depth of sequence performed permitted sampling of only a very small subset of the complex community present. To compare 454 data to that of PhyloChip, we first reclassified the PhyloChip data using the RDP classifier. Aligned representative sequences were downloaded from Greengenes and the V4-V5 sequence regions were extracted and provided to the Ribosomal Database Project (RDP) classifier. Comparison of parallel PhyloChip data generated from the same extracted DNA revealed that while the large majority of organisms detected by 454-sequencing were also detected by PhyloChip (at higher levels of classification

concordance was 100%). In all samples the array consistently detected substantially more community members **(Fig. 5.B).**

To further illustrate that these findings are due to improved community coverage by the parallel nature of the array, we performed resampling of the sample with the greatest sequence reads at different sequencing depths (1,000, 5,000, 10,000, 20,000 and 31,982 sequence reads from the sample with greatest read depth). Classified genera at each sequencing effort level were compared to the 419 classified genera derived from representative sequences of the taxa detected by PhyloChip. This approach demonstrated that increasingly deeper sequencing efforts validated greater numbers of PhyloChip detected genera **(Fig. 5.C),** suggesting that in the complex communities of the GI microbiota, this tool represents a standardized economical approach to high-resolution profiling of bacterial community composition. The data presented are not exclusive to dust samples, we recently performed a study of pediatric patients with irritable bowel syndrome involving 454-sequencing and PhyloChip profiling of stool which demonstrated excellent concordance[63]**.** We and others have also previously compared the PhyloChip to both traditional clone library and relatively high numbers of next generation 454 pyrosequencing reads generated in parallel from clinical and environmental samples and have

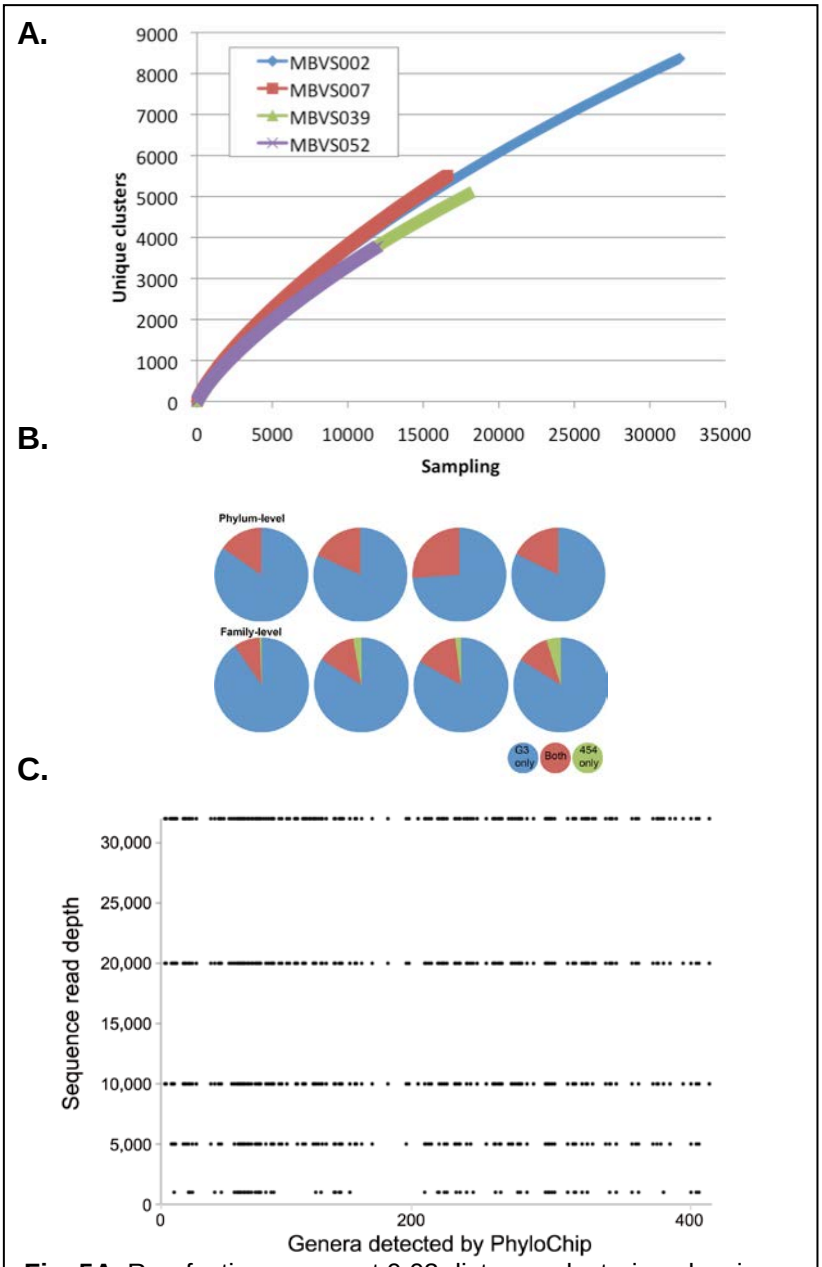


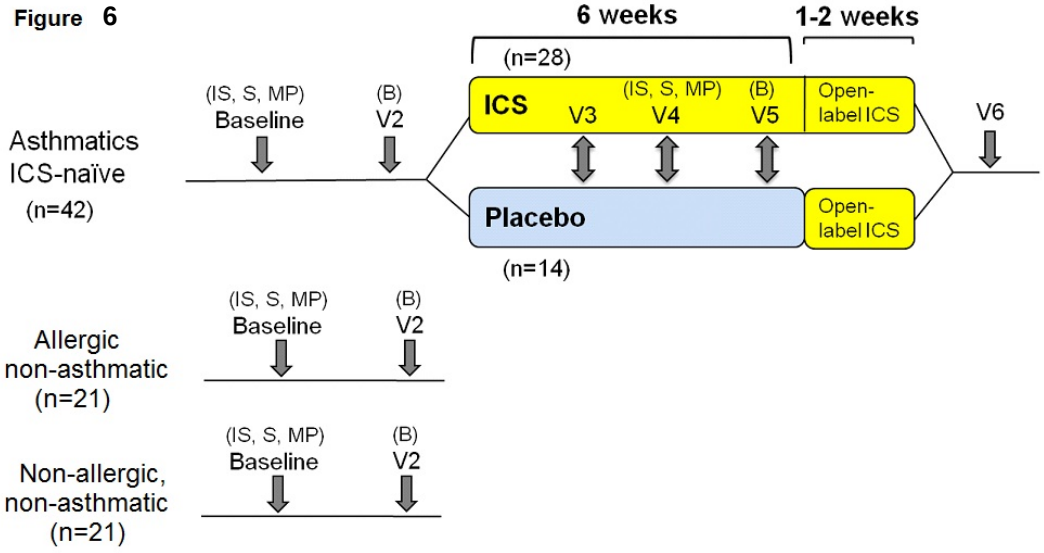
Fig. 5A. Rarefaction curves at 0.03 distance clustering showing exponential increase in number of new sequence clusters detected (even at ~30,000 sequences), indicating highly rich microbiota. **B.** Stool bacterial community members detected by both array and sequencing (red), exclusively by either sequencing (green) or array (blue) at both the phylum and family level, illustrate the increased community coverage afforded by the parallel sampling nature of the array. **C.** Increasing sequence read depth results in identification of more genera detected by the PhyloChip (each line plots represent the number of genera detected by both methods).

consistently demonstrated that while the array detected the majority of genera identified by sequencing (>97% of those detected by sequencing), it also detected several hundred additional taxa providing a substantially higher-resolution profile of these communities[28,30]. Because of these advantages and because the normalized datasets generated by the array permit application of robust statistical analyses, we propose to use the G3 PhyloChip to profile bacterial communities present in samples collected in this study.

III. Protocol

This study is best regarded as a combination of a cross-sectional study and a double-blind, placebo-controlled study. The purposes of the cross-sectional study are to compare the bronchial microbiome, the stool microbiome, and the cellular mediators of immune function in blood and BAL fluid in three groups of subjects: allergic asthmatic, allergic non-asthmatic, and non-allergic non-asthmatic adults. An additional purpose is to examine within the asthmatic subjects the relationships between bronchial microbiome community composition and clinical and inflammatory phenotypic features of asthma. The purposes of the prospective, double-blind, placebo controlled study are to determine the effects of inhaled corticosteroid therapy on the bronchial microbiome in asthmatic subjects, and to determine whether responsiveness to ICS treatment is related to the bronchial microbiome community composition at baseline or to changes in composition with ICS treatment. An overview of the protocol is shown in Figure 6, and details of individual study visits may be found in Table 1 below.

Figure 6



ICS, inhaled corticosteroid; IS, induced sputum; S, spirometry; MP, methacholine provocation; B, bronchoscopy. See Table 1 for procedures at each visit.

Forty-two ICS-naïve allergic asthmatic subjects (7 visits, pre- and post-ICS intervention assessments), 21 allergic, non-asthmatic subjects, and 21 non-allergic, non-asthmatic healthy subjects (3 visits, for baseline assessments only) will be studied. The methods for clinical assessment and "phenotyping" are those used in previous ACRN studies. These include standardized questionnaires to characterize asthma onset, severity, treatment, exacerbation history, and current control. Baseline measurements include spirometry with bronchodilator reversibility, bronchial reactivity (PC_{20} Mch), Phadiatop test, serum IgE, and sputum eosinophil and neutrophil percentages. Methods for sputum induction and bronchoscopy will again be those used in previous ACRN studies. Oral saline rinse will be performed prior to sputum induction and bronchoscopy, to reduce contamination of these samples by oral secretions. Subjects will be given standard kits for collection of a first morning stool sample to bring to the center on the day of bronchoscopy, and will have blood drawn from the intravenous line placed at the time of bronchoscopy for analysis of cell populations.

The specimens for microbiome analysis by 16S rRNA PhyloChip are protected bronchial brushings and first morning stool samples. Induced sputum samples will also be analyzed by 16S rRNA PhyloChip in a subset of subjects, to examine whether airway sampling by this less invasive, approach permits inference about the bronchial microbiome. For microbial analysis of these samples, total DNA and RNA will be extracted by the combined protocol optimized for bacterial nucleic acid extraction used in prior studies[14]. DNA will be processed for 16S RNA PhyloChip and related microbial community analyses. In addition, as part of the secondary hypotheses and analyses, aliquots of the RNA extracted from the bronchial brush samples will be processed for QT-PCR analysis of Serpin-B2, CLCA-1, Periostin, and other genes related to the "TH2-molecular phenotype" of asthma, using methods previously described by Woodruff et al[64]. The remaining RNA will be stored at -80° C for future functional/metatranscriptomic analyses.

Other analyses of the samples obtained include FACS (10 color panel) analysis of the numbers of innate T cells and CD4 T cell subsets further divided by expression of chemokine receptors correlating with ability to produce IFN-y (Th1), CCR6 IL-17A (Th17), and IL-4 and IL-13 (Th2). FACS analysis will also enumerate other relevant cells, including CD8 T cells, NK cells, B cells, monocytes, basophils, eosinophils, and neutrophils. For assessment of pulmonary immune function and response, we will measure cytokines of interest and the numbers of inflammatory cells and T cells and their subsets in the bronchoalveolar lavage fluid obtained at bronchoscopy.

Other analyses beyond the scope of this proposal could be performed on the samples collected in this study and stored in the planned AsthmaNet Sample bank. For example, the RNA remaining after analysis of expression of the genes of the "TH2 phenotype" will be stored at -80° C for possible future functional/metatranscriptomic analyses. The DNA remaining after removal of aliquots for amplification of 16S-rRNA for PhyloChip analysis will be stored as well, and would be available for study by sequencing or array-based methods for detection of fungal organisms (e.g., by "MycoChip"). Aliquots of bronchoalveolar lavage fluid will also be stored at -80° C, and

will be available for study by sequencing or array-based methods (e.g., the "Virochip"65) for detection of viral organisms. Thus, the culture-independent detection of bacteria in this study, and application of similar culture-independent methods for detection of fungi and viruses in samples collected and stored in this study could enable complete characterization of the bacterial, fungal, and viral microbiome of the bronchial airways of healthy and asthmatic subjects. Similar additional analyses could be done on aliquots of the stool samples, which will also be stored at -80° C.

A. Subjects

A total of 84 adult subjects, 42 allergic asthmatic, 21 allergic non-asthmatic, and 21 non-allergic, non-asthmatic subjects will be enrolled at participating AsthmaNet partnerships. We will target enrollment at 50% female and 33% of minority race or ethnicity. Asthmatic subjects will have prior MD-diagnosed asthma without treatment other than "as needed" short-acting beta-agonist treatment for at least the previous 6 months. All subjects will be non-smoking adults (<5 pack-years, no tobacco smoking in past year). All asthmatic subjects and 21 non-asthmatic subjects will be allergic, as shown by a positive Phadiatop test result. The 21 non-allergic, non-asthmatic subjects will have no history of allergic rhinitis, conjunctivitis, or dermatitis and negative Phadiatop test result. Subjects will be recruited from established cohorts, by advertisement, and by physician referral, by the recruitment methods and procedures found effective at the various participating AsthmaNet Centers.

All asthmatic participants will meet ALL of the following inclusion criteria:

B. Inclusion Criteria – Asthmatic subjects

1. Men and women, 18-60 years of age.
2. History of physician-diagnosed asthma.
3. Methacholine PC_{20} ≤ 8 mg/ml and/or FEV_1 improvement ≥ 12% in response to 4 puffs albuterol.
4. FEV_1 ≥ 70% of predicted after 4 puffs albuterol.
5. Nonsmoker (less than 5 pack-year lifetime smoking history and no smoking within the previous year).
6. Stable asthma for ≥ 3 months prior to enrollment (no urgent care visits, no systemic corticosteroid treatment).
7. Asthma Control Questionnaire 6 Score (i.e., without score for FEV_1 or PEF) $\leq$1.5 at Visit 0.
8. Able to provide informed consent.
9. Able to perform spirometry as per ATS criteria.
10. Evidence by Phadiatop testing of sensitivity to an aeroallergen in blood sample drawn at Visit 0.
11. Willingness, if female and able to conceive, to utilize one medically-acceptable form of contraception.

Asthmatic participants will be excluded if they meet ANY of the following exclusion criteria:

C. Exclusion Criteria – Asthmatic subjects

1. Presence of lung disease other than asthma.
2. Use of $\geq$ 10 doses of nasal corticosteroids in the previous 3 months.
3. Presence of significant medical illness or other chronic diseases whose treatment could affect the clinical features measured, responses to the therapies to be given in this study, or risks of participating in the study (see Appendix).
4. History of atrial or ventricular tachyarrhythmia.
5. Changes suggestive of cardiac ischemia on ECG at baseline.
6. History of upper respiratory infection in the previous 6 weeks.
7. History of sinusitis, bronchitis, or antibiotic use in the previous 3 months.
8. Evidence of chronic sinusitis.
9. History of long-term controller medication use for asthma (inhaled or oral corticosteroid, leukotriene pathway antagonist, cromolyn, or theophylline within the preceding 6 months.
10. FEV_1 < 70% of predicted after 4 puffs albuterol.
11. Asthma Control Questionnaire 6 Score (i.e., without score for FEV_1 or PEF) >1.5.
12. Inability, in the opinion of the Study Investigator, to coordinate use of inhaler or otherwise comply with medication regimens.
13. Change in bowel function (e.g., diarrheal illness) in the previous four weeks.
14. Inability or unwillingness to perform required study procedures.
15. History of bleeding disorder.
16. Reduced creatinine clearance.
17. Contraindication to bronchoscopy on history or examination.

D. Inclusion Criteria for randomization – Asthmatic subjects

1. Absence of respiratory infection since Visit 0.
2. No corticosteroid use since Visit 0.
3. No antibiotic use since Visit 0.
4. No significant asthma exacerbation since Visit 0.
5. Asthma Control Questionnaire 6 Score (i.e., without score for FEV_1 or PEF) $\leq$1.5.
6. Continued absence of exclusion criteria described above.

Intention-to-treat principles will apply following randomization. Thus, subjects will be dropped after randomization for safety reasons only. These may include pregnancy or the development of a significant asthma exacerbation (as defined in section O.1, "Asthma Exacerbations") found not to be, in the opinion of the investigator, responsive to protocol treatment as defined in section O.

E. Inclusion Criteria – Allergic, non-asthmatic Healthy subjects

1. Men and women, 18-60 years of age.
2. No history of chronic respiratory disease including asthma.
3. Nonsmoker (less than 5 pack-year lifetime smoking history and no smoking within the previous year).
4. Evidence by Phadiatop testing of sensitivity to an aeroallergen in blood sample drawn at Visit 0.

5. Able to provide informed consent.
6. Able to perform spirometry as per ATS criteria.

F. Inclusion Criteria – Non-allergic, non-asthmatic Healthy subjects

Identical to criteria for allergic non-asthmatic healthy subjects except for

1. No history of allergic disease, including allergic rhinitis, conjunctivitis, dermatitis, or food allergy.
2. Negative Phadiatop test result in blood sample drawn at Visit 0.

Healthy participants will be excluded if they meet ANY of the following exclusion criteria:

G. Exclusion Criteria – Allergic and non-allergic healthy subjects

1. Any history of asthma.
2. Presence of significant medical illness or other chronic diseases whose treatment could affect the clinical features measured, or risks of participating in the study (see Appendix).
3. History of atrial or ventricular tachyarrhythmia.
4. Changes suggestive of cardiac ischemia on ECG at baseline.
5. Smoking $\geq$ 5 pack-years, or within the past year
6. FEV_1 or FVC < 80% predicted.
7. Methacholine PC_{20} ≤ 16 mg/ml and/or FEV_1 improvement ≥ 12% in response to albuterol.
8. History of upper respiratory infection in the previous 6 weeks.
9. History of sinusitis, bronchitis, or antibiotic use in the previous 3 months.
10. Use of $\geq$ 10 doses of a nasal corticosteroid preparation in the previous 3 months
11. Evidence of chronic sinusitis.
12. Change in bowel function (e.g., diarrheal illness) in the previous 4 weeks.
13. Inability or unwillingness to perform required study procedures.
14. History of bleeding disorder.
15. Reduced creatinine clearance.
16. Contraindication to bronchoscopy on history or examination.

H. Active Treatment Medication (for Asthmatic Subjects Only)

1. Randomly-allocated (in 2:1 ratio) to twice daily inhalation of 250 mcg fluticasone from Diskus DPI inhaler or matched placebo. This drug will be administered in a double-blind fashion to asthmatic subjects for 6 weeks and as “open-label’ treatment after the second bronchoscopy, performed at the end of 6 weeks of blinded treatment, until the final visit.
2. As-needed albuterol for relief of acute symptoms.

I. Outcome Variables

Primary outcome variables

1. Descriptors of bronchial microbial community composition at baseline, and before and after ICS treatment intervention:

- Richness (number of different bacterial taxa identified)
- Evenness (distribution of the relative abundance of the taxa identified)
- Diversity (a function of richness and evenness)
- Presence and relative abundance of specific bacterial taxa

2. Descriptors of stool microbial community composition at baseline (see above).

3. Numbers of innate T cells and CD4 T cell subsets identified by surface receptor expression as Th1, Th17, and Th2, and numbers of CD8 T cells, NK cells, B cells, monocytes, basophils, eosinophils, and neutrophils in blood and in BAL fluid.

Secondary outcome variables

1. Clinical, physiologic, and inflammatory phenotypic features of asthma:
- FEV_1 % predicted pre-albuterol
- FEV_1 % predicted post-albuterol
- Change in FEV_1 % predicted pre- to post-albuterol
- Asthma Control Questionnaire-6 score
- PC_{20} Mch
- % eosinophils and neutrophils in induced sputum sample
- Serum IgE level
- Blood eosinophil %
- Number of positive Phadiatop results to testing with common aero-allergens.
- Age of onset of asthma
- BMI
- Number of exacerbations requiring oral corticosteroid treatment in the past 5 years.
- History of cough productive of mucus
- Cold questionnaire response of viral "colds" as being "usually" or "always" associated with worsening of asthma (on four point Likert scale of "rarely, sometimes, usually, and always")

2. Levels of cytokines in BAL fluid (L-1beta, 2, 3, 4, 5, 6, 8, 9, 10, 13, 17, IFN-gamma, TNF-alpha, Eotaxin, GM-CSF, IL-21, 23, 33, TSLP), as assessed by Luminex multiplex cytokine ELISAs.

3. "Molecular Phenotype," as inferred from expression level of IL-13-dependent genes in bronchial epithelial cells (Periostin, CLCA-1, Serpin-B2) and classified as "TH2" and "non-TH2" molecular phenotypes. Measured at baseline only.

4. In response to 6 weeks of inhaled corticosteroid (fluticasone 250 mcg twice daily), or dry-powder placebo inhaler:
- Change in FEV_1 % predicted
- Change in PC_{20} Mch
- Change in sputum eosinophil %
- Change in ACQ-6 score (i.e., without score for FEV_1 or PEF).

5. History of exposure to household pet dog, cat, or other furred animal within the past year.

J. Description of Study Visits and Periods

Specific elements for each study visit are provided in **Table 1**, below.

Baseline:

Visit 0.
Subjects will first be told the purposes, risks, and alternatives to participation and will sign an IRB-approved document for informed consent. Asthmatics will complete the Asthma Control Questionnaire. Blood will be drawn for Phadiatop and IgE testing. Specific IgE to a panel of aeroallergens will be measured only in Phadiatop positive subjects.

Visit 1. Participants who meet allergen sensitivity requirement will complete an asthma characterization visit. If female and of reproductive age, a urine pregnancy test must be negative, and if asthmatic, must report use of an appropriate method of contraception for the duration of the study. Standardized questionnaires used in prior ACRN and AsthmaNet studies will be administered to characterize asthma onset, severity, treatment, exacerbation history, and current control. Baseline measurements will include an EKG, spirometry, bronchial reactivity (PC_{20} Mch), and collection of induced sputum, all by methods used in previous ACRN and other AsthmaNet studies; procedural details are specified in the Microbiome Manual of Procedures (MOP) and AsthmaNet Spirometry, Methacholine (including medication and dosing) and Sputum MOPs, respectively. Blood will be drawn for genetic analysis and for measurement of BUN, creatinine and eosinophil number. Oral saline rinse will be performed prior to sputum induction to reduce contamination of the samples by oral secretions.

If tests show that a person presenting as a healthy subject is not eligible to participate in the study (because of electrocardiographic abnormalities, abnormal pulmonary function, or bronchial hyper-reactivity (PC20 ≤ 16 mg/ml), a study physician will perform a brief medical history and physical exam, will advise the subject as to the possible clinical significance of the test finding, and will offer to communicate the finding to the subject's primary physician or to refer the subject to a physician if the subject wishes. The same basic procedure will be followed for people presenting as an asthmatic subject who have abnormal EKG findings, severe airflow obstruction (FEV1<55% predicted), or absence of bronchial hyper-reactivity.

For asthmatic subjects, albuterol will be prescribed for as-needed rescue use. Subjects will be allowed to continue other chronic medications, as long as they are not in conflict with the inclusion/exclusion criteria. Subjects with clinically-significant allergic rhinitis treated with oral antihistamines but not nasal steroids will be allowed to continue the former. Subjects with symptomatic allergic conjunctivitis using ophthalmic antihistamines or mast cell stabilizers will be allowed to continue these agents. Subjects who meet the inclusion/exclusion criteria will be allowed to proceed to Visit 2, at which bronchoscopy will be performed. On discharge from visit 1, they will be given a stool sample collection kit, with instructions to bring in a sample from the first morning bowel movement on the day of bronchoscopy.

Visit 2. Subjects who meet safety criteria for bronchoscopy will return 1 week ± 3 days after enrollment for the second study visit. Pregnancy test will be repeated in women of child-bearing potential. Spirometry will be performed before and after inhalation of 4 puffs of albuterol. Intravenous access will be secured prior to bronchoscopy and a sample of 10 ml of blood will be placed into two labeled 5 mL Cyto-Chex BCT blood collection tubes (2 x 5mL) for shipping to UCSF for FACS analysis on the following day. Subjects will then undergo fiberoptic bronchoscopy with five protected bronchial brushings and a bronchial lavage (instillation and recovery by suction of 200 ml of warmed saline instilled in four boluses of 50 ml through a bronchoscope wedged into a segmental bronchus); procedural details, including medication and dosing, are specified in the Microbiome Bronchoscopy MOP. Of the five brushings, four will be stored in RNALater and one will be stored in 35% glycerol. Brushings will be shipped in batches at -80° C to UCSF for microbiome analysis (See Section I, Microbiologic Variables). These will serve as baseline samples for analysis prior to ICS or placebo intervention in the asthmatic group. An aliquot of BAL fluid will be taken and processed at the center for total and differential cell count. Two additional aliquots of BAL fluid will be added to RNALater for microbiome analysis. The balance of BAL fluid will be centrifuged, the cell button resuspended in labeled BAL immunophenotyping collection vials containing 1 mL Streck Cell Preservative and shipped to UCSF for FACS analysis on the following day. Five 10 mL aliquots of BAL supernatant, 2 into tubes containing RLT (lysis) buffer (for Viral RNA preservation),will be stored at -80° C and later batched for shipment to UCSF for cytokine analysis and for forwarding to AsthmaNet sample biobank.

Immediately after the bronchoscopy has been completed, each nostril will be sprayed with a small volume of nebulized 2% lidocaine solution, and the surface of the floor and inferior nasal turbinate of both nares will be brushed with a standard cytology brush and the brushes placed in RNALater for 24h before storage at -80°C. These brushes will be stored in RNALater and labeled, processed, and shipped as are the bronchial brushes.

Albuterol MDI (2-4 puffs) or nebulizer (2.5 mg premix solution) will be administered to participants with dyspnea, wheeze, chest tightness, or hypoxia post-bronchoscopy and as needed. All subjects will be observed for 2-4 hours after bronchoscopy and discharged home if their FEV_1 has returned to within ≥90% of their baseline on arrival. If this criterion is not met, a physician must evaluate the subject to decide if they are stable for discharge. All subjects will be contacted the evening and day after bronchoscopy and will return for evaluation if any significant adverse events are reported. Healthy subjects will be discharged from the study at this point. At the end of the visit, asthmatic subjects will be assigned at random (in a 2:1 ratio) to receive a Diskus inhaler delivering 250 mcg of fluticasone per puff or an identical-appearing placebo inhaler, with instructions to take one puff twice daily for six weeks.

Visit 3. Subjects will return to the study center 14-21 days after randomization for repeat spirometry, for analysis of the number of inhalations taken from the Diskus inhaler (from the dose counter on each Diskus device) and for reinforcement of the importance of adhering to treatment. Subjects who demonstrate > 75% adherence with inhaler use will

continue per the study schedule. Subjects who do not will be instructed to improve adherence and return for a repeat Visit 3 10-14 days later. At this visit, inquiry will also be made as to whether subjects have had any symptoms of worsening asthma control, difficulties with inhaler use, or adverse events. Twelve to 18 days after Visit 3 (approximately four weeks after randomization), the subjects will be contacted by telephone for the same inquiries made at the two week visit.

Visit 4 (study week 6). Five weeks after randomization, and while still receiving the treatment intervention, asthmatic subjects will return for measurement of spirometry and bronchial reactivity (PC_{20} Mch), and collection of induced sputum preceded by oral saline rinse. Bronchoscopy will be scheduled for the following week in subjects who meet safety criteria for the procedure. Subjects will continue on their assigned study inhaler, 1 puff twice daily.

Visit 5. An interval history will be taken and a brief examination performed. Spirometry before and after administration of 4 puffs of albuterol will be performed. A sample of 10 ml of blood will be collected same as at visit 2 and sent to UCSF for FACS analysis on the following day. Fiberoptic bronchoscopy with five protected bronchial brushings and a 200 ml bronchial lavage will then be performed. Bronchoscopy procedures will be identical to those of the first, baseline bronchoscopy. The samples collected will be compared to those collected prior to intervention for changes in the bronchial microbiome, and in the cell and cytokine content of BAL fluid. At completion of the bronchoscopy, nasal brushings will be obtained as they were at visit 2. All subjects will be given open fluticasone treatment (250 mcg/puff) to take twice daily after bronchoscopy up until returning for Visit 6.

Visit 6. Subjects will return to the study center for brief history and physical examination, inquiry as to adverse effects from participation, and measurement of FEV_1 and FVC prior to discharge from the study. At the end of this visit, the subject will be informed of the degree of severity and control of their asthma, as inferred from their examination by a study physician and from their pulmonary function test results, and will be advised as to the recommended level of treatment. The subject will be given a letter summarizing this information and, if the subject wishes, a prescription for the recommended treatment. This information will also be given at the final visit of healthy subjects and asthmatic subjects who do not complete the study.

Table 1. Study visit schedule.

Visit	0	1	2	3*	Telephone Call	4*	5*	6*
Study week	-1	0	1	3	5	6	7	8-9
Characterize		X						
Randomize			X					
Clinical								
History		X					X	
Asthma/general questionnaires		X						
Long Exam		X						

Short Exam			X			X	X	X
Urine Pregnancy Test		X	X			X	X	
ACQ	X	X	X	X		X	X	X
Sputum induction with oral rinse		X				X		
ECG		X						
Phadiatop, IgE	X[1]	X[1]						
Genetics blood draw		X						
Physiologic								
Spirometry		X	X[2]	X		X	X[2]	X
PC_{20}[4]		X				X		
Microbiologic								
Stool sample collection			X					
Oral rinse/tongue scraping		X	X			X	X	
Sputum induction		X				X		
Bronchoscopy[5]			X				X	
Nasal brushing			X				X	
Safety								
CBC		X						
BUN/Creatinine		X						
ECG		X						
Adverse Event query		X	X	X	X	X	X	X
Adherence								
Dispense study DPI			X	X		X		
Dispense open ICS							X	
Record doses taken				X	X	X	X	
Immunophenotyping								
Blood draw for FACS analysis			X				X	
Other								
Satisfaction Questionnaire			X[3]					X

Actual visit times may vary slightly. *Asthmatic subject visits only. [1]Phadiatop and total IgE at V0, allergen-specific IgE at V1 for participants with positive Phadiatop; [2]Spirometry pre- and post-bronchodilator (4 puffs albuterol); [3]Healthy Controls only; [4]Methacholine challenge procedure details, including medication and dosing, found in AsthmaNet Methacoline MOP; [5]Bronchoscopy procedure details, including medication and dosing, found in Microbiome Bronchoscopy MOP; ACQ: Asthma Control Questionnaire, IgE: immunoglobulin E; PC_{20}: methacholine challenge, CBC: complete blood count and differential, BUN: blood urea nitrogen, ECG: electrocardiogram (for patients >45 y.o.)

K. Rationale for Data Collection and Procedures

Clinical and Biologic Variables:

1. History and physical exam will establish safety for entry into the study and for participation in study-related treatments and procedures.
2. Standardized questionnaires used in previous ACRN and AsthmaNet studies will provide information on age of onset of asthma, prior treatments, exacerbation

history, response to viral respiratory infections, sputum/phlegm production and other historical features of asthma, sometimes used in defining possible asthma phenotypes.

3. Pregnancy test. To eliminate risk of teratogenicity, pregnancy testing will be used throughout the study due to the use of methacholine (pregnancy class C), and the performance of bronchoscopy with the use of midazolam (class D) and fentanyl (class C). A medically-acceptable form of contraception will be required of asthmatics throughout the study.
4. Asthma Control Questionnaire (ACQ) will be used in the asthmatic subjects to monitor asthma control during the run-in and throughout the period of ICS vs. placebo inhalation treatments. ACQ-6 scores (i.e., without scoring for PEF) will be used for exploratory analysis of associations between microbial community composition and ACQ score at baseline and the change in ACQ with ICS treatment.
5. IgE and Phadiatop test will be obtained as a baseline phenotypic variable to characterize atopy. Specific IgE will be measured to a panel of aeroallergens in Phadiatop-positive subjects.
6. Plasma and serum separated from a 10 ml sample of venous blood from each subject will be stored at -80^{0}C for later analysis of potential biomarkers associated with asthma phenotypes (e.g., the "TH2" and "non-TH2" molecular phenotypes of asthma."
7. Induced sputum. A cell count and differential will be measured as baseline phenotypic variables, allowing classification of the asthmatic subjects as "eosinophilic" (>2% sputum eosinophils), "neutrophilic" (>60% sputum neutrophils), or pauci-granulocytic (<2% eos; <60% neutrophils). Analysis of induced sputum samples from over 1,000 asthmatic subjects enrolled in ACRN studies has shown that the proportions of these "inflammatory phenotypes" are roughly 25%, 20%, and 50%, respectively, in both ICS-treated and ICS-naïve subjects[66]. DNA will be extracted from induced sputum cell pellets for PhyloChip analysis for comparison of the microbial community composition of induced sputum to the microbial community composition of bronchial brushings in 10 healthy and 10 asthmatic subjects. The DNA from these samples will be stored at -80^{0}C, as will the cell pellets from the other sputum samples collected, as a resource for later analysis.
8. Circulating immune cells: The blood sample obtained at the time of insertion of intravenous access immediately prior to bronchoscopy will be assayed by 11-color FACS analysis for enumeration of innate T cells and CD4 T cell subsets identified by surface receptor expression as Th1, Th17, and Th2, and numbers of CD8 T cells, NK cells, B cells, monocytes, basophils, eosinophils, and neutrophils (see section B.4.a, above). The absolute number and relative distribution of these cells types will be analyzed for associations with clinical classification (allergic asthmatic; allergic non-asthmatic; non-allergic, non-asthmatic) and for associations with stool microbial community composition.
9. Bronchial epithelial cell gene expression: the RNA extracted from the cells recovered from the protected bronchial brushings (95-97% epithelial cells) will be analyzed by QT-PCR for expression of Serpin B2, CLCA-1, and Periostin, the trio

of genes upregulated in the bronchial epithelium of the TH2-molcular phenotype of asthma, described by Woodruff and Fahy[64]**.** This will be analyzed for associations with microbial community composition in the bronchial epithelial brushing and in stool samples,, for associations with the number and distribution of circulating immune cells (see #8, above) and for associations with the immune response state of the lung, as reflected by BAL fluid content of inflammatory cells and cytokines (see #10, below)

10. BAL cells and cytokines: The inflammatory cell content of BAL fluid will be measured by FACS analysis by the method described in #8, above. The levels of cytokines in BAL fluid (L-1beta, 2, 3, 4, 5, 6, 8, 9, 10, 13, 17, IFN-gamma, TNF-alpha, Eotaxin, GM-CSF, IL-21, 23, 33, TSLP), will be measured by Luminex multiplex cytokine ELISAs. The cytokine levels will be used for constructing logistic regression models predicting distinct asthma phenotypes, as was done in a previous study by the Severe Asthma Research Program[12]. This will in turn enable analysis as to whether those phenotypes are associated with differences in bronchial microbiome, circulating and/or BAL immune cell populations, and stool microbiome.
11. Nasal Brushings: No analysis of these samples is proposed in this protocol. They are obtained for storage to be available for later study of the relationships among the nasal and bronchial microbiomes and epigenomes, as described above (see lines 163-185).

Physiologic Variables

1. Spirometry and bronchodilator response. These standard physiologic parameters will be collected to characterize subjects at baseline and throughout the study. The change in pre-bronchodilator FEV_1 from baseline to the value measured after 6 weeks of ICS therapy will be used to classify subjects assigned to ICS treatment as “ICS-responsive” or “ICS non-responsive” based on a $\geq$5% or <5% improvement in FEV_1.
2. Methacholine PC_{20}. This physiologic variable will be utilized as an entry criterion to confirm the diagnosis of asthma at the time of screening. A threshold PC_{20} value of ≤ 8 mg/mL will be used for asthmatics. This parameter will also be measured at the end of the active treatment periods and will be used as a second, although exploratory marker for examination of possible differences in the bronchial microbiome of ICS “responders” vs. “non-responders,” as reflected by a $\geq$ one step increase in PC_{20}Mch (a doubling dose).

Microbiologic Variables

1. Stool sample collection: for analysis of microbial community composition.

2. Bronchoscopy: All subjects will undergo bronchoscopy for 5 protected bronchial brushings and a bronchial lavage. Two of the brushings will be analyzed for total and differential cell count; three will be processed for extraction of DNA and RNA. The DNA will be processed further for 16S rRNA gene amplification and analysis of bacterial taxa by PhyloChip (see above). RNA will be analyzed for

quantification of IL13-dependent genes (ClCA-1, Serpin B2, Periostin) by QT-PCR for classification of each subject as of the "TH2" vs. "non-TH2" molecular phenotype. The balance of the extracted DNA will be stored at -80^{0}C for possible later detection of fungal organisms (by ARISA, sequencing, or "MycoChip"); RNA will be similarly stored for possible future metatranscriptomic analysis to permit inferences about pathways activated at the interface between the airway microbiome and the cells of the airway epithelium.

The bronchial lavage sample (collected by instilling and suctioning back 4 x 50 ml of warmed normal saline through a wedged bronchoscope) will be aliquotted into tubes prepared by addition of RLT (lysis) buffer, and stored at -80^{0}C for possible later analysis for detection and characterization of viruses, as by "ViroChip" or deep sequencing methods.

Environmental Variables

To assess activities or exposures that might alter bacterial colonization of the airways, we will modify an existing questionnaire developed by Michael Cabana, MD for his study of probiotic supplementation of neonates at high risk for asthma. This questionnaire inquires about activities that might affect bacterial exposures, such as household or occupational exposure to infants (<3 yrs) or young children (<12 yrs), household exposure to pets or other animals, exposure to soil (e.g., gardening), caring for livestock, chickens, or other domesticated animals, woodburning (in household fireplace), and environmental tobacco smoke. Similarly, to assess dietary habits that might alter bacterial colonization of the gastro-intestinal tract, a standard questionnaire on dietary habits will be administered, with particular attention to consumption of fermented foods (e.g., "live culture" yogurts). Subjects will be instructed not to modify their dietary habits while participating in this study, and will be asked specifically about recent ingestion of live-culture yogurts at study visits.

Safety Variables:

1. CBC with differential cell count. To collect baseline phenotypic data with regard to eosinophil and neutrophil counts.
2. Renal function (BUN and creatinine). Impaired creatinine clearance is associated with an increased risk of bleeding at the time of bronchoscopy and is an exclusion criterion. Serum creatinine will be measured at baseline.
3. Electrocardiogram. An ECG will be performed at visit 1 in all subjects > 45 yrs of age, to allow identification of ischemic changes, an exclusion criterion for research bronchoscopy.

L. Adherence monitoring

The number on the dose counter on the DPI ICS/placebo delivery device will be noted at each visit as a way of tracking adherence with the twice daily use required of the asthmatic subjects enrolled in this study. Adherence also will be determined (and

encouraged) at each visit by direct inquiry.

M. Recruitment and Feasibility

Based on the experience of the AsthmaNet Centers that participated in the ACRN "Macrolides in Asthma" protocol, and/or in other studies involving bronchoscopy, we believe that we will be able to identify subjects who meet the enrollment criteria for this study. The ACRN experience with the 83 subjects in the PRICE study suggests that at least 1/3 of steroid-naïve asthmatics will fail to show a $\geq$ 5% improvement or $\geq$ a one step increase in their PC_{20} Methacholine.

N. Statistical Analysis

Primary outcome variables include both gross descriptive metrics of microbial community composition (richness, evenness, diversity) and relative abundance of bacterial taxa of the bronchial and stool microbiome. These metrics are obtained by reduction of the complex data sets generated by microbiota profiling for the purposes of analysis. Each sample will be represented by three calculated indices (richness, evenness and diversity) that reflect the gross composition of each bacterial community. For every sample analyzed by PhyloChip, community richness - the number of taxa detected in the sample - will be determined by using a positive fraction cut off of > 0.9; data matrices including only taxa that fulfill this criterion will be imported into the "R" statistical environment and metrics calculated for each sample. In particular:

- Richness is the total number of taxa detected.
- Shannon diversity[29] (D) is a function of the distribution of the total number of organisms across all of the species. If S is the total number of species in the sample and p_i = the number of organisms in the i^{th} species divided by the total number of organisms, then $D = -\sum_{i=1}^{S} p_i \ln(p_i)$. Shannon diversity is a type of entropy measure.
- Pielou's evenness index[67] (E) is a scaled measure of biodiversity and is equal to the observed Shannon diversity index divided by the maximum possible Shannon diversity index, which would occur if all of the species in the sample were equally abundant. $E = \frac{D}{\ln(S)}$, where D is the Shannon Diversity index and ln(S) is the maximum diversity of the sample.

All indices will be calculated using the Vegan package in the "R" statistical environment. The relative abundance of discrete taxa detected will be assessed by measurement of fluorescence intensity, and will be compared among groups by ANOVA.

Secondary outcome variables, including measures of phenotypic features of asthma, are described earlier (see section H. above). Other second outcome variables are the measures of systemic immune function, as reflected by FACS enumeration of the numbers of innate T cells and CD4 T cell subsets identified by surface receptor expression as Th1, Th17, and Th2, and numbers of CD8 T cells, NK cells, B cells, monocytes, basophils, eosinophils, and neutrophils in blood and in BAL fluid.

Addressing the **Primary Research Hypotheses** (and corresponding Specific Aims) will entail the evaluation of differences in bronchial microbial community composition in relation to allergic asthmatic vs. allergic non-asthmatic, and non-asthmatic vs. non-allergic non-asthmatic status. Additional research hypotheses to be addressed include evaluation of differences in stool microbial community composition in the groups, differences in bronchial microbial community composition after treatment with an inhaled corticosteroid, and evaluation of the relationship of bronchial or stool microbiome to the collected metadata, such as measures of phenotypic features of asthma (clinical, physiologic and inflammatory), measures of systemic immune function, measures of pulmonary immune response.

We will evaluate differences in bronchial microbial communities first by comparing the values for community **structure** – richness, evenness, and diversity (see above). Differences in these values for the groups being compared (allergic asthmatic vs. allergic non-asthmatic, and allergic non-asthmatic vs. non-allergic non-asthmatic subjects at baseline for **Specific Aim 1**) will be tested for significance by pairwise comparison within the ANOVA framework, or Kruskal-Wallis if there is marked maldistribution. These two comparisons will be made at the 0.025 significance level. We will additionally assess possible differences in bacterial community **composition** by testing for differences in the relative abundance of discrete taxa between the groups, i.e. identifying specific taxa that characterize each group. We will do this by comparing the mean fluorescence intensity for each detected taxon in the three groups, using log-transformed array data and a row-based custom script in R. The output from this multiple testing will then be corrected for false discovery using the q-values approach[68]. Developed for genome-wide tests of significance to correct for multiple comparisons, q-values indicate the likelihood that a significant finding arose by chance, reducing the incidence of Type I error (false positives) in the dataset. In contrast to reporting a false positive rate based on the total number of tests, q-values permit determination of a false discovery rate only among the significant tests. A less conservative approach than, for example, Bonferroni correction, in determining truly significant tests, the use of q-values to control for false discovery has been effectively applied in prior PhyloChip-based metagenomic studies[14, 29, 69].

In comparing the bronchial microbiome of asthmatic, allergic, and healthy subjects, we will need to consider the possible confounding effects of differences in environmental microbial exposures. Examples might include exposure to differences in the microbial content of outdoor atmospheric aerosols[70] or of indoor aerosols that might be related to pet ownership. We have demonstrated differences in the microbial community composition in house dust obtained from pet-keeping vs. pet-free households[71] and pet ownership has been associated with a decreased risk of development of childhood asthma[72]. Other investigators also have shown that exposure to a microbe-rich farming environment is associated with a decreased prevalence or risk of asthma[73]. However, whether differences in microbial exposure to any environmental factor (other than tobacco smoke) affect the bronchial microbiome has not been studied. We therefore propose to apply methods such as hierarchical cluster analysis or non-metric dimensional scaling, and multivariate regression to reveal characteristics of bronchial

microbial community composition that are associated with activities or exposures such as household or occupational exposure to infants (< 3yrs) or young children (< 12 yrs), household exposure to pets or other animals, soil (e.g., gardening), caring for livestock, chickens, or other domesticated animals, wood burning (household fireplace), environmental tobacco smoke, and consumption of fermented foods (e.g., "live culture" yogurts), that are related to specific bacterial colonization patterns in the airways.

Given the very high rates of pet-keeping among U.S. households, exclusion of all pet-exposed subjects would impair recruitment into this study. In addition, limiting the study population to those without pets, might reduce variability in the bronchial microbiome and therefore, might obscure differences in the microbiome which we hypothesize is associated with asthma status. A similar argument can be made for why there should be no limitation on other environmental exposures or activities (other than smoking) – their exclusion could limit variability.

Accordingly, we propose that the first step is to analyze the richness, evenness, diversity and relative abundance of bacterial taxa in the bronchial microbiome and their relationship to allergic asthmatic, allergic non-asthmatic, or non-allergic healthy status. A subsequent step will be to examine factors associated with these measures of the bronchial microbiome, including pet ownership and clinical and inflammatory phenotypic features of asthma. This can be accomplished by distance-based permutational multivariate analysis of variance (R package *vegan,* function *adonis*[74]), or regression-based canonical correspondence analysis[75], two approaches commonly applied in ecological studies to determine relationships between community composition and environmental variables.

To evaluate whether treatment of asthmatic subjects with inhaled corticosteroid alters the bronchial microbial community in **Specific Aim 2**, we will compare the calculated microbial community metrics (richness, evenness, diversity) in the ICS and placebo treated groups at the end of the intervention period by analysis of covariance using treatment assignment as the factor and baseline microbial community metrics as covariates. We will also examine interaction effects between treatment assignment and covariates to explore whether the baseline microbial community might modify any ICS effect. In the event that the outcomes are mal-distributed beyond what can be corrected via transformation, we will compare treatment groups using the Wilcoxon rank-sum test.

We will also compare the calculated microbial community metrics before and after ICS intervention by paired t-test or alternatively, Wilcoxon signed rank test. We will also evaluate for differences in the bacterial taxa present and changes in the relative abundance of all detected taxa before and after ICS treatment. As described for Specific Aim 1, we will compare the mean relative abundance of each detected taxon before and after treatment by paired t-test, followed by correction for false discovery using the q-value approach. Finally, we will evaluate whether ICS treatment affects the phylogenetic relatedness of bacterial communities by comparing the calculated community metrics, Nearest-taxon and Net-relatedness indices (NTI and NRI; R package *picante*)[76]. These indices provide continuous measures of how closely related detected communities are

and at what phylogenetic level of similarity, and will be compared by paired t-test or Wilcoxon signed rank-sum test. This allows for assessment of whether ICS might promote the appearance or increased abundance of closely-related bacterial organisms, a pattern that has been described in response to treatment interventions or in pathogen colonization of a given niche[69, 77, 78].

For **Specific Aim 3**, to determine whether differences in bronchial microbial community composition at baseline or after ICS treatment are associated with differences in responsiveness to the treatment, we first will consider the primary response variables as physiologic measures, such as change in post-bronchodilator FEV_1 % predicted, change in PC_{20} Mch, and change in sputum eosinophil percentage. Then we will evaluate by two approaches whether the pre- or post-treatment bronchial microbial community is associated with changes in these response variables. In the first approach, we will determine correlations (Pearson or Kendall) between the relative abundance of each taxa found at baseline and the change in each of the above response variables. This will be performed also using abundance data for the taxa detected post-ICS treatment, as well as using the change in relative abundance with treatment. Similar to as described for Specific Aim 1, a row-based custom script for correlation testing with each detected taxon will be performed (R package *multtest*), followed by false discovery correction using q-values.

The second approach by which we will assess relationships between bronchial microbiota composition and treatment response will be in the following manner. First, the microbial dataset will be reduced to include only those taxa that demonstrate a significant change in relative abundance with ICS treatment (specific significance criteria to be determined based on the p- and q-values indicating a reasonably low false discovery rate, typically 5% or less based on recent studies[14, 29, 69]). Second, Bray-Curtis distance matrices, a measure commonly used in ecological analyses, will then be constructed from this reduced dataset[79]. These will then be used for non-metric multidimensional scaling (NMDS)[79, 80], a distance-based ordination method that, as applied here, essentially will "map" the relatedness of samples, based on how dissimilar the microbial community composition of each is relative to all other samples. Based on the resulting distance matrices, permutational multivariate analysis of variance (R package *vegan,* function *adonis*)[74] will then be conducted to evaluate whether the response variables of interest are associated with the observed changes in microbial community composition with treatment.

For **Specific Aim 4,** to evaluate whether phenotypic features of asthma are associated with differences in bronchial microbial community composition. The phenotypic features of interest include clinical features, such as history of exacerbations, response to the "cold questionnaire" on frequency of worsening of asthma with viral URI's, and ACQ score. Other phenotypic features of interest are physiologic measures (e.g. FEV_1 % predicted, FEV_1 reversibility, PC_{20} Mch), inflammatory markers (e.g. sputum eosinophil and neutrophil %), and bronchial epithelial cell expression of the genes of the "Th2 molecular phenotype." We will apply both correlation analysis and ordination methods, as described above for Specific Aim 3. The former will entail comparisons between

continuous phenotypic variables and continuous microbial community metrics (richness, evenness, diversity, and species relative abundance). Ordination is widely applied in ecological analyses to reveal relationships between communities and environmental characteristics. We will apply NMDS ordination and permutational multivariate analysis of variance[74] as described for Specific Aim 3. This model will also allow us to test dichotomous data or continuous data parsed by relevant cutoffs for certain variables, such as PC_{20} Mch < 2 mg/ml vs. $\geq$ 2 mg/ml, or sputum eosinophils < 2% vs. $\geq$ 2%.

Conversely, for **Specific Aim 5**, we will explore whether there are natural grouping patterns in the bronchial microbial community data, and if so, evaluate how these patterns relate to phenotypic features of asthma. To do this, we will use the entire microbial community dataset (after filtering for taxa that are present in at least one subject) and compute Bray-Curtis distance measures for hierarchical cluster analysis and canonical correspondence analysis or nonmetric multidimensional scaling. If distinct microbial community group patterns are revealed, between-group differences in the phenotypic data associated with samples in the groups will be assessed. This will include using multivariate analysis of variance (function *adonis*[74]) and least squares fitting of phenotypic variables (function *envfit*) to the ordination models. We will use very similar methods to explore whether natural grouping patterns in bronchial microbial community composition are related to features of environmental exposure, such as exposure to infants, children, household pets, wood-burning fires, to activities such as gardening, or to consumption of fermented foods (see "Environmental Variables, Section I, above).

As a secondary Research Hypothesis (Specific Aim 6), we will assess the concordance of the microbial community composition detected in induced sputum vs. bronchial brushings in a subset of healthy and asthmatic subjects. In baseline (pre-treatment) samples, we will evaluate both the community richness and specific composition profiled by microarray. Based on preliminary data on comparison of sputum and bronchial brushings (see section D), we will consider 90% or greater within-subject overlap in the specific taxa detected in both specimen types as highly concordant.

Our general approach to analysis of the additional aims of this expanded study, especially determination of whether relationships exist between datasets (e.g. airway microbiome composition and cytokine profile, or stool microbiome composition and numbers and distribution of inflammatory cells) will be to construct non-metric dimensional scaling plots based on distance matrices for each dataset and to examine them using the Mantel test, which permits determination of statistical correlations between two sets of data matrices. This will provide initial indications that community composition is correlated with other metadata sets generated in the course of this study.

Our approach to analysis of specific Aim 7a, which compares stool microbial composition among allergic asthmatic, allergic non-asthmatic, and non-allergic non-asthmatic subjects, we will make use of the same approaches and statistical tools as

outlined above for specific aims 1 and 2, For analysis of specific aims 7b, 8, and 9, we will use methods similar to those proposed for analysis of specific aims 3-6.

O. Sample Size

For this study, the total number of proposed subjects to be studied is 84 (42 allergic asthmatics, 21 allergic non-asthmatic and 21 non-allergic non-asthmatic healthy subjects). The primary hypotheses involve the comparison of bronchial microbial community composition (as sampled by protected bronchial brushings) among the three subject groups at baseline (allergic asthmatic vs. allergic non-asthmatic and allergic non-asthmatic vs. non-allergic non-asthmatic), and evaluation for alterations in bronchial microbial community composition by ICS treatment. In the "Add-On" study to the MIA trial[14], a significant difference in bronchial bacterial diversity was observed between asthmatic and healthy subjects (difference in mean Shannon diversity index of 0.6 units). A sample size of 42 allergic asthmatic and 21 allergic non-asthmatic subjects will provide >90% power to detect a difference of 0.6 units in mean diversity. This calculation is based on two, 2-sided tests with alpha=0.025, a common standard deviation for the diversity index of 0.47 (based on data from asthmatic and healthy groups in the MIA "Add-On" study).

The effect of ICS use on bronchial microbial community composition has not been previously examined. We hypothesize that ICS use will alter microbial community diversity and composition, and reason that a 0.4 unit difference in the mean Shannon diversity index may be important. This value is extrapolated from the observation in the MIA Add-On study[14] that among asthmatics treated with clarithromycin, there was a significant difference of 0.4 units in bacterial diversity between subjects who did or did not demonstrate an improvement in bronchial reactivity (defined as at least a doubling in the dose of Mch PC_{20}). We reason therefore that this difference in bronchial microbial diversity may also be clinically meaningful in examining the effect of ICS use. The proposed sample size of 28 asthmatics treated with ICS and 14 asthmatics treated with placebo will provide 84% power to detect a difference of 0.4 units in the mean Shannon diversity index. This calculation is based on assuming a 2-side test with alpha=0.05, a common standard deviation for the diversity index of 0.37 (based on data from the MIA "Add-On" study; asthmatic, clarithromycin-treated subgroup), and allowing for a 15% dropout rate.

For analysis of the effect of ICS on bronchial microbial diversity before and after treatment, the proposed sample size of 28 asthmatic subjects will provide $\geq$ 95% power to detect a change in the diversity index of 0.4 units with ICS treatment (same effect size as assumed for the ICS vs. placebo analysis above). This calculation assumes a paired test with alpha=0.05, common standard deviation in the diversity index of 0.37, and a 15 or 20% dropout rate.

The power analyses above are based on best-available data and experience. It is noted, however, that for metagenomic microbial studies, there are no validated approaches for sample size calculation, related in part to the unclear biological significance of, for instance, a unit change in community diversity (or in community

richness or evenness). Nonetheless, for the purposes of this study, effect sizes based on community diversity were chosen, as this feature was noted in the study by Huang et al[14] to differ significantly between asthmatic and healthy subjects, as well as to correlate positively with methacholine PC_{20} measures of bronchial hyperresponsiveness, a clinically relevant, pathophysiologic feature of asthma.

Additional studies involving relatively small sample sizes and utilizing high-resolution tools for bacterial community profiling, have identified significant relationships between the microbiota in a given niche (e.g. the airway or gut) and clinical/phenotypic features of disease. For example, in an age-stratified cross-sectional study of 51 subjects with cystic fibrosis, microarray analysis illustrated that the airway microbial community become less even and less diverse with increasing patient age and impairment of pulmonary function[77]. Longitudinal sample analysis from 13 of the patients found initial diversification of the bacterial community among younger CF patients compared to a progressive loss of diversity over time among older patients. In a preliminary analysis of 25 sputum samples from COPD patients, the significant changes in bacterial diversity that occurred over time correlated with clinical symptom scores (Y. Huang, unpublished data). Another illustration of the findings made possible by this approach to microbial detection is Dr. Lynch's recent study of chronic rhinosinusitis (CRS) involving 14 subjects. The seven with CRS, compared to seven healthy patients, exhibited significant decreases in sinus microbiota diversity and a coincident significant increase in the relative abundance of a single *Corynebacterium* species. Subsequent modeling in a murine model confirmed that reduced microbiota diversity was essential to susceptibility to inoculation with this species of *Corynebacterium*, with the resulting infection replicating the pathophysiological and immunological features of the disease in the infected mice (S. Lynch, personal communication, manuscript submitted 2012). Finally, in a recent study evaluating statistical approaches for analyzing metagenomic microbial community data, even undersampled communities demonstrated differential diversity patterns, which were significantly associated with relevant environmental variable gradients[81]. This indicates that even with relatively small sample sizes, relevant relationships between the microbial community and phenotypic variables can be revealed. Collectively, the above examples coupled with the power analyses provided, support the likelihood that the planned sample size for this study will be able to successfully evaluate the hypotheses.

P. Risks

1. Asthma Exacerbations

This study will enroll asthmatics who are not taking controller maintenance therapy at entry and whose ACQ-6 (i.e., without score for FEV_1 or PEF) is <1.5, so – even though these subjects will have a history of asthma of no greater than mild-moderate severity, the possibility exists that subjects may experience one or more exacerbations during the study period, especially those randomized to placebo inhaler treatment. Asthma exacerbations will be defined as the development of an increase in symptoms of cough, chest tightness, and/or wheezing in association with one or more of the following: (1) an increase in rescue albuterol of ≥ 8 inhalations/day over baseline use for a period of 48 hours or ≥ 16 actuations per 24 hours, with baseline defined as average daily use

during the week prior to randomization; (2) a fall in FEV_1 to < 80% of baseline (visit 1); (3) FEV_1 < 50% predicted, or (4) if a subject receives systemic corticosteroids for an exacerbation from a non-study-related clinician. Subjects who are potentially experiencing an exacerbation will be instructed to contact the clinic coordinator and/or be evaluated at the study site or the nearest medical emergency facility as rapidly as possible. Subjects will be given handouts outlining what to do and who to call in the event of an asthma exacerbation.

AsthmaNet rescue algorithms for subjects with exacerbations of asthma are based on recommendations from the NAEPP Guidelines for Diagnosis and Management of Asthma[82]:

Home care of exacerbations: Asthma exacerbations will be identified by the criteria described above. Patients will be educated to recognize exacerbations as early as possible to facilitate prompt treatment and to lessen morbidity. Patients who recognize an exacerbation will be instructed to use albuterol by MDI, 2-4 puffs, every 20 min for 60-90 min if needed. If symptoms do not improve after the first 60-90 min of therapy, the patient should contact the study coordinator, investigator, their primary physician, or seek care in the emergency department.

*Physician's Office or Emergency Room Treatment of exacerbations***:** Patients will be assessed by history, physical examination, and by physiological monitoring including spirometry or PEF. If the patient's PEF or FEV_1 are less than 25% predicted or if the patient shows evidence of altered mental status, cyanosis, labored breathing, or use of accessory muscles, sampling of arterial blood for respiratory gas analysis is indicated, with appropriate action taken depending on the results obtained. When treated in the physician's office or the hospital emergency room, patients should initially be given albuterol by nebulization (0.5 cc of 0.5% solution) every 20 min over the first 60-90 min.

If the PEF increases to >65% of baseline after the first 60-90 min, the patient can be discharged to continue treatment at home. Prednisone may be administered at the discretion of the physician to augment therapy. If symptoms persist and PEF remains ≤65% baseline, nebulized albuterol should be continued as often as every hour and further treatment with oral or parenteral corticosteroids should be considered (e.g. prednisone 40 mg orally; methylprednisolone 40 mg IV bolus). Monitoring of PEF or spirometry should continue every hour. Within 4 hours of treatment, a decision should be made regarding patient disposition. If PEF increases to >65% baseline within 4 hours, the patient can be discharged to continue treatment at home. Home treatment should include a 5-day course of prednisone (see below). If PEF remains >40% but ≤65%, an individualized decision should be made to hospitalize the patient for more aggressive therapy or to continue therapy at home with a course of prednisone. If PEF is ≤40% baseline after repeated albuterol treatments, the patient should be admitted to the hospital unless in the physician's best judgment alternative treatment could suffice.

Prednisone Treatment: In this protocol, prednisone will be used when acute exacerbations cannot be controlled by increased albuterol therapy alone. The dose of prednisone used during an acute exacerbation shall consist of 40 mg as a single oral dose every day for 5 days. The decision to initiate or to continue a course of prednisone beyond 5 days is left to the discretion of the physician.

Exacerbations induced by bronchoscopy: In rare cases, fiberoptic bronchoscopy may induce an asthma exacerbation. All cases of bronchoscopy-induced exacerbations will be treated with prednisone 40 mg po daily for 5 days. Should an exacerbation occur after bronchoscopy, a two-week recovery period will be imposed following the completion of prednisone therapy. Bronchoscopy will not be performed at Visit 5 if the participant experienced an exacerbation after bronchoscopy at Visit 2.

Bronchoscopy is associated with risks of the procedure and of conscious sedation. In this study, bronchoscopy will include five protected bronchial brushings and a standard 200 ml (4 x 50 ml of warmed, normal saline solution) bronchial lavage. Bronchial brushing is associated with a low risk of minimal bleeding, and platelet count data will be available to the investigator prior to the procedure. Bronchial lavage is associated with the risks of coughing and slight worsening of asthma symptoms after the procedure. Conscious sedation poses risks of over-sedation and hypoventilation. Standard monitoring protocols will be used, and reversal agents will be readily available to reduce this small risk. The risks of worsening asthma symptoms will be minimized by pre-treatment with albuterol before bronchoscopy and by “as needed” administration of albuterol aerosol after the procedure.

Inhaled corticosteroids can cause dysphonia and oral pharyngeal candidiasis, but systemic side effects are not anticipated during a study of this duration. Subjects will be instructed in proper inhaler use including rinsing of the mouth afterwards.

There are no direct benefits to individual subjects, although it is possible that some asthmatic subjects may experience an improvement in symptoms if they receive ICS study drug. There is a potential benefit to patients with asthma in general as new bases for therapy may develop from this study.

2. Bronchoscopy Safety

Bronchoscopy with five bronchial brushings and a bronchial lavage will be performed according to standard AsthmaNet procedures, with safety strategies similar to those previously employed by the NHLBI-sponsored Asthma Clinical Research Network.

Subjects must demonstrate a post-bronchodilator FEV_1 of $\geq$ 70% to be eligible to undergo bronchoscopy. An upper age limit of 60 for this study has been selected as conservative and biased in the direction of subject safety. In addition to safety criteria outlined below, subjects must additionally be judged otherwise to be clinically appropriate for bronchoscopy by the bronchoscopist at the time of the procedure. Safety of the subject is the overriding concern in making this determination.

The presence of any of the following characteristics will exclude a subject from participating as a bronchoscopy volunteer:

Events occurring within 6 months of bronchoscopy: ED visit or hospitalization for asthma, or more than 2 exacerbations requiring systemic corticosteroid treatment.

Events occurring within 48 hours of bronchoscopy: pulse oximetry demonstrating oxygen saturation < 90% on room air, use of more than 8 puffs of a short acting beta-agonist per day for significant increase in asthma symptoms

Events on day of bronchoscopy: ACQ-6 score of >1.50.

Hospitalization Indicators

For any subjects who exhibit any of the following characteristics during or after bronchoscopy, overnight hospitalization should be provided: significant cough persisting beyond 2 hours after completion of procedure, failure of PFTs after bronchodilator administration to return to within 15% of prebronchodilator FEV_1 at end of monitoring time, persistent hypoxia < 90% at end of monitoring time, persistent tachycardia > 130 bpm at end of monitoring time, unexpected altered mental status during or after procedure, significant hemoptysis > 50 ml, or requirement for bronchodilator every 2 hours on more than 3 occasions.

Treatment should be directed towards resolving underlying airway obstruction and symptoms, based on the best clinical judgment of the physicians involved. Follow-up telephone contact should be made for all subjects in the evening following bronchoscopy and 24 hours after the procedure is completed. If issues have not resolved in either group at the time of the last scheduled contact, additional contact and necessary medical care should be arranged.

Q. Recruitment

Recruitment is performed by accessing established subject data banks, obtaining referrals, and local advertising. The Recruitment and Retention Committee will facilitate this process. However, what works for a given site may or may not work in a different geographic/population make-up. Standard print and radio advertisements will geared to age groups and population characteristics. Notices will be placed in newspapers and as fliers at stores, student lounges, and hospital clinics. For all AsthmaNet protocols, at least 50% women and 33% minorities are required in the population of subjects enrolled. This was consistently met in ACRN and CARE trials. The clinical centers involved in AsthmaNet were chosen based in part on documentation of their capacity for enrollment of appropriate subjects.

IV. Human Subjects

A. Subjects

1. General Description: study population and inclusion/exclusion criteria are as described in the protocol above.

2. Gender/Minority Inclusion: as stated above, at least 33% of subjects will be minority and 50% female. No subject will be excluded based on gender or ethnicity.

3. Exclusion of children: Because this study involves an invasive procedure, bronchoscopy, performed only for research purposes, children (<18 yrs old) will be excluded from participation.

B. Potential Risks and Procedures for Minimizing Risks

1. Pain and/or hematoma formation may occur at an intravenous puncture site. This is not a serious complication.
2. Dizziness during blood sampling may occur. Subjects will be supine during blood sampling to avoid this problem.
3. Spirometry may exacerbate bronchospasm, but in previous ACRN studies this has not been a serious problem. Subjects will be monitored closely during the procedure and an inhaled ß-2 agonist will be administered if needed.
4. Methacholine challenge causes bronchospasm, but subjects are monitored and testing stopped when the FEV_1 falls 20% from baseline and/or at the subject's request. An inhaled ß-2 agonist is always administered after the procedure and response measured by spirometry.
5. Induced sputum technique can cause bronchospasm. Standard AsthmaNet MOP for sputum induction, based on prior experience in the ACRN, extensively covers safety precautions for this technique, which we have used in multiple protocols without untoward problems. The precautions involve pre-treatment with 4 puffs of albuterol and close monitoring of PEF and FEV_1 at intervals throughout the procedure.
6. Nasal brushing causes transient itching or minor pain in the nose of 3-5 seconds duration. In about 5% of cases, it causes transient oozing of blood over the nasal mucosal surface and may result in blood-tinged nasal secretions. Actual epistaxis is unusual (< 1%) (personal communication, Pedro Avila, MD, Northwestern University)
7. Bronchoscopy is associated with risks of the procedure and of conscious sedation. Bronchial brushing is associated with a minimal risk of bleeding, and platelet count data will be available to the investigator prior to the procedure. Conscious sedation poses risks of over-sedation and hypoventilation, and standard monitoring protocols will be used and reversal agents will be readily available.
8. Inhaled Fluticasone treatment: inhalation of fluticasone is associated with the minor risks of hoarseness and of oropharyngeal candidiasis. The first resolves simply by stopping the inhaled corticosteroid treatment; resolution for the second

can be accelerated by topical treatment with cotrimazole lozenges or oral rinse with nystatin. The risks of systemic absorption are minimal in this study because of the use of a moderate dose of an inhaled corticosteroid (500 mcg/d of fluticasone) for the short period of 6 weeks.

9. Participation in research may involve a loss of privacy, but information will be handled as confidentially as possible. All records related to the study will be kept in a secure area in the investigators' office space and only research personnel will have access to the records. Computerized data will be password protected with access limited to the study personnel. Data will be released if it will help the urgent treatment of a subject.

C. Adverse Events

An adverse event shall be defined as any detrimental change in the subject's condition, whether it is related to an exacerbation of asthma or to another unrelated illness. Adverse events related to asthma exacerbations will be managed according to rescue algorithms outlined above. Subjects will have handouts outlining what to do and who to call in the event of an asthma exacerbation.

An adverse event is deemed serious if it suggests a significant hazard, contraindication, side effect, or precaution. Serious adverse events include any experience that is fatal or life-threatening, is permanently disabling, requires or prolongs inpatient hospitalization, or is a congenital anomaly, cancer, or overdose. Serious adverse events must be reported to the DCC and the National Institutes of Health Project Scientist within 72 hours of notification. Once notified, the DCC will disseminate information about the event to the Data Safety and Monitoring Board and to the Steering Committee.

Adverse events due to therapy or concurrent illnesses other than asthma may be grounds for withdrawal if the illness is considered significant by the study investigator or if the subject is no longer able to effectively participate in the study. Subjects experiencing minor intercurrent illnesses may continue in the study provided that the nature, severity, and duration of the illness are recorded and that any unscheduled medications required to treat the illness are also recorded. Examples of minor intercurrent illnesses include acute rhinitis, sinusitis, upper respiratory infections, urinary tract infections, and gastroenteritis. Medications are allowed for treatment of these conditions in accordance with the judgment of the responsible study physician.

Documentation of adverse events will be recorded on an Adverse Event Report Form and will include the following information: description of the illness, dates of illness, treatment of illness and dates (medications, doses, and dose frequency), whether emergency treatment or hospitalization was required, and treatment outcome.

D. Potential Benefits Gained From Data

The benefits resulting from this research include an improved understanding of the link between chronic airway colonization or infection by a consortium of microbial organisms and chronic asthma, including the impact of commonly prescribed inhaled corticosteroid

therapy. This study may also suggest a relationship between gut microbial community composition with allergy, and with the circulating immune cells thought to mediate allergic disease, but no further, specific relationship with allergic asthma. This finding would increase interest in the possibility of manipulation of the gut microbiome as an approach to the treatment or prevention of allergic disease. This finding would also heighten interest in the role of local pulmonary events, possibly directly or indirectly mediated by the functional activity of microbial populations in the bronchial tree. Another possible finding of this study is of a difference in bronchial microbiome in different phenotypic subgroups of asthmatic subjects, such as eosinophilic or non-eosinophilic asthma, Th2 or non-TH2 molecular phenotypes of asthma, or corticosteroid-responsive vs. non-CS-responsive asthma. Any of these findings would represent a major shift in current conceptions of the pathogenesis of asthma and of its clinical expression.

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VI. Appendices

A. Appendix 1. Exclusionary Medical Conditions (may not be inclusive)

Addison's disease
AIDS
Bleeding disorder (history of)
Cardiac arrhythmias (clinically significant)
Cardiac ischemia
Congenital anomaly, including growth abnormalities (clinically significant)
Congestive heart failure
Coronary artery disease (unstable or severe)
Cushing's disease
Diabetes mellitus (poorly controlled)
Dyspnea by any cause other than asthma
Eating disorder (e.g. anorexia or bulimia (active disease)
Hematologic disease (unstable, e.g. severe anemia)
Hepatic disease
Hypertension (poorly controlled)
Hyperthyroidism
Immunologic compromise
Chronic kidney disease (glomerulonephritis, polycystic kidney disease, etc.)
Lactation
Lidocaine allergy
Lung disease other than asthma (COPD, emphysema, chronic bronchitis, pulmonary embolism, malignancy, cystic fibrosis, among others)
Lupus (active disease requiring immunosuppressant)
Any malignancy other than basal cell skin cancers
Mental illness (uncontrolled)
Mental retardation
Morbid obesity (BMI ≥ 35)
Neurologic disease (including epilepsy requiring treatment)
Peptic ulcer disease (active)
Pregnancy
Renal insufficiency (creatinine > 1.2 mg/dl)
Schizophrenia
Skeletal disorders, including osteoporosis and rheumatoid arthritis
Sleep apnea (untreated)
Sleep disorder (history of)
Substance abuse (including active drug or alcohol abuse)
Tachyarrhythmia (atrial or ventricular, history of)
Tuberculosis (history of positive skin test with negative chest x-ray allowed)
Urinary retention (active symptoms within last 6 months)
Vocal cord dysfunction (diagnosis of)