

**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

41 The application of sensitive, culture-independent methods for detecting microbes, based
42 on detection and identification of “signature” sequences of DNA or RNA in clinical
43 samples, is transforming concepts of the dimensions and nature of the microbial world
44 and of the intimacy of its association with human health and disease. Studies applying
45 these techniques have begun to describe the extraordinary richness and diversity of the
46 microbial populations in the mouth, sinuses, gastrointestinal tract, skin, vagina, and
47 tracheobronchial tree and have begun as well to suggest relationships between the
48 composition and function of a site or organ’s microbiome and that site or organ’s
49 function. Animal studies using these techniques have also established relationships
50 between the bacterial populations in the gastrointestinal tract and the numbers, activity,
51 and function of specific immune cells, especially T cells, not just in the gut, but also in
52 the circulation and in distant organs, including the lungs and airways, and
53 epidemiological studies have demonstrated relationships between the G-I microbiome
54 and clinical manifestations of immune-mediated disease¹⁻⁵.

55
56 Taken together, the findings suggesting that the composition and function of the
57 gastrointestinal microbiome shapes systemic immune function, and the findings
58 suggesting that the composition and function of a local microbiome can affect local
59 function, have clear implications for concepts of the pathogenesis of allergic diseases,
60 including allergic asthma. One implication is that some feature of the gastrointestinal
61 microbiome may underlie the disorder or imbalance in systemic immune function that is
62 thought to account for the predisposition to allergic sensitization and thus to allergic
63 disease. This disorder in immune function is now thought to consist of an imbalance in
64 the function of effector Th2 cells and suppressive regulatory T cells, so that T cell
65 responses are skewed toward a proinflammatory Th2-mediated pattern. There is also
66 intense interest in more recently identified T cell subsets, such as Th17 cells, which are
67 clearly regulated by gastrointestinal microbiota, although their roles in asthma remain
68 unclear. So, too, does the role of other T cells, including natural killer T cells, gamma-
69 delta T cells, and CD8 cells^{6,7}. Insofar as the circulating populations of these cells are
70 shaped by bacterial populations in the gastrointestinal tract, the gastrointestinal
71 microbiome of people with allergic disease may differ in composition from the
72 gastrointestinal microbiome of healthy, non-allergic controls. It is also possible that
73 asthma is a function not just of differences in systemic or local immune function, but
74 also of differences in the composition and function of bacterial populations in the
75 tracheobronchial tree⁸⁻¹⁰.

76
77 The findings that the G-I microbiome may shape systemic immune function and that a
78 local, site or organ-specific microbiome might affect local, site or organ-specific immune
79 function also have implications for our understanding of the pathophysiologic
80 mechanisms underlying different phenotypic forms of asthma. Evidence supporting the
81 idea that differences in local immune function may account for different subtypes or
82 “phenotypes” of asthma has been provided by the NHLBI’s Severe Asthma Research
83 Program. This research group reported that unsupervised clustering of the
84 concentrations of cytokines in bronchoalveolar lavage fluid from asthmatic patients with

85 a range of asthma severities identified four distinct groups – or “intermediate
86 phenotypes” - of asthma. One group, enriched in patients with severe asthma, showed
87 differences in BAL cellular content, reductions in pulmonary function, and enhanced
88 responsiveness to inhaled methacholine. The differences in the proportions of patients
89 with severe asthma in the different subgroups suggests that they may well differ in
90 responsiveness to inhaled corticosteroid therapy¹¹⁻¹³. Whether these differences in the
91 patterns of activation of immune response in BAL fluid are related to differences in the
92 numbers of functional status of systemic or local T-cell populations is not known.

93
94 While most attention has focused on cellular mediators of allergic inflammation in
95 asthma, it is also possible that the microbial populations in the lungs and airways may
96 be importantly involved in the pathogenesis of asthma or in its particular phenotypic
97 expression. Our own previous work¹⁴ and that of Hilty et al. in Oxford¹⁵, have reported
98 that application of 16S-based, culture-independent methods to examine samples
99 collected from the bronchial mucosa showed greater bacterial richness and diversity in
100 the samples obtained from the asthmatic subjects than healthy subjects, with significant
101 differences in the abundance of numerous specific taxa in their samples. We found, for
102 example, differences in the relative abundance of certain bacterial taxa to be associated
103 with greater bronchial responsiveness to methacholine and with clinical responsiveness
104 to 16 weeks of treatment with clarithromycin, a macrolide antibiotic. The demonstration
105 of differences in the bronchial microbiome in patients with different phenotypic features
106 of asthma would prompt reconsideration of our current concepts of asthma and could
107 lead to changes in approaches to prevention and treatment. Studies so far have been
108 limited by the potential confounding effect of treatment with an inhaled corticosteroid on
109 the microbiome. It is thus not clear whether the differences that have been
110 demonstrated in the bronchial microbiome of asthmatic vs. healthy subjects were a
111 function of their disease, or of its treatment.

112
113 This protocol proposes to apply recently developed methods to examine the
114 relationships among gut microbiome, systemic immune function, bronchopulmonary
115 inflammatory response, bronchial microbiome, and detailed assessments of clinical and
116 physiologic features of pulmonary function in three populations: (1.) inhaled
117 corticosteroid-naïve allergic asthmatic adults (2.) allergic but non-asthmatic adults; and
118 (3.) non-allergic, non-asthmatic healthy adults. For identification and characterization of
119 gastrointestinal and bronchial microbiomes, we propose to apply a sensitive, broadly
120 parallel method, the 16S-rRNA PhyloChip¹⁶, to stool samples and to samples obtained
121 by protected bronchial brushings from these three groups of subjects. To assess
122 systemic and broncho-pulmonary immune function, we will apply 11 color FACS
123 analysis to enumerate innate T cells, regulatory T cells, and other CD4 T cell subsets in
124 samples of blood and bronchial lavage fluid. CD4 T cells will be characterized for their
125 pattern of expression of three chemokine receptors (CXCR3, CCR6, and CCR4) that we
126 and others have shown correlate with ability of those cells to produce the signature
127 cytokines IFN- γ (Th1), IL-17 (Th17), and IL-4 and IL-13 (Th2)¹⁷⁻¹⁹. FACS will also be
128 used to enumerate other relevant cells, including CD8 T cells, NK cells, B cells,
129 monocytes, basophils, eosinophils, and neutrophils. The same methods will be applied
130 to assess the composition of inflammatory cells and T cell subsets in bronchial lavage

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

133
134 We also propose to examine in the asthmatic subjects the effects of inhaled
135 corticosteroid (ICS) treatment on the bronchial microbiome and pulmonary immune
136 function by comparing the changes associated with six weeks of treatment with an ICS
137 vs. 6 weeks of treatment with a placebo inhaler.

138
139 The primary purpose of our analysis will be to determine whether the composition of the
140 microbial communities in the airways differs in allergic asthmatic, allergic non-asthmatic,
141 and non-allergic, non-asthmatic adults. We will also examine whether among the
142 asthmatic subjects, differences in bronchial microbial composition are associated with
143 differences in phenotypic features of asthma such as proneness to exacerbations,
144 irreversible airflow obstruction, presence of eosinophils or neutrophils in airway
145 secretions, expression of Th2-dependent genes in the bronchial epithelium²⁰, and
146 responsiveness to inhaled corticosteroid treatment. Finally, we will examine whether the
147 bronchial microbiome is altered by inhaled corticosteroid treatment.

148
149 Data collected for these analyses will enable examination of the association of features
150 of the intestinal microbiome (gross community metrics and specific taxonomic
151 composition) with differences in the numbers and proportions of T cell subsets and
152 other inflammatory cells in the circulation and airways. They will also make possible
153 examination of whether the features of the intestinal microbiome – and of circulating
154 inflammatory cells - differ between the allergic subjects (including asthmatic and non-
155 asthmatics) and the non-allergic healthy subjects, but do not differ between the allergic
156 asthmatic and allergic non-asthmatic subjects. If so, this would suggest the possibility
157 that the immune dysregulation underlying “allergy” may be shaped by the composition
158 of the intestinal microbiome, but that asthma is a function of some other determinant,
159 possibly limited to the lungs and airways, that affects some, but not all allergic subjects.
160 A candidate for this other determinant might be the microbiome colonizing the bronchial
161 tree.

162
163 The addition of the simple, well-tolerated procedure of nasal brushing to this protocol
164 will enable collection and storage of samples permitting later study of questions that
165 may become salient depending on the findings of this and other on-going clinical
166 research studies. Should, for example, this study’s findings suggest a difference in the
167 bronchial microbiome of allergic asthmatic vs non-allergic, non-asthmatic subjects, then
168 it will become interesting to examine whether these differences in the bronchial
169 microbiome are reflected by similar differences in the nasal microbiome of subjects with
170 allergic rhinitis vs subjects without allergy. Comparison could also be done of the nasal
171 and bronchial microbiome to determine whether analysis of microbial community
172 composition of the nose permits inferences about the microbial community composition
173 of the bronchial airways. Along these lines, should studies now examining the
174 epigenome in nasal epithelial cells obtained from asthmatic and non-asthmatic children
175 show potentially important differences, then study of epigenetic changes in the DNA
176 extracted from nasal and bronchial cells obtained at the same time from the same adult

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

186
187 **In summary, this protocol will enable examination of possible relationships**
188 **between stool microbiome, systemic immune function, pulmonary immune**
189 **function, bronchial microbiome, and pulmonary function and inflammation across**
190 **three populations: allergic asthmatics, allergic non-asthmatics, and non-allergic,**
191 **non-asthmatics. Such analysis is unprecedented in depth and scope and may**
192 **enable potentially transformative insights into the relationships between the**
193 **human microbiome and pulmonary health and disease.**

194 **I. Hypotheses and Specific Aims**

195 **A. Primary Research Hypotheses**

- 197 1. The microbiota of the bronchial airways of allergic asthmatic, allergic non-
198 asthmatic, and non-allergic, non-asthmatic healthy subjects differ in diversity,
199 richness, evenness, and/or taxonomic composition.
200
- 201 2. Clinical, physiologic, and inflammatory phenotypic features of asthma (including
202 “Th2- vs. non-Th2” pattern of gene expression in bronchial epithelial cells, and
203 cluster by BAL cytokine pattern) are associated with characteristic bronchial
204 microbial community compositions.
205
- 206 3. Inhaled corticosteroid (ICS) treatment alters bronchial microbial community
207 composition in asthmatic subjects.
208
- 209 4. In the absence of respiratory infection, antibiotic treatment, or change in inhaled
210 therapy, bronchial microbial community composition is stable over six weeks.
211
- 212 5. Differences in bronchial microbial community composition at baseline or after ICS
213 treatment are associated with differences in responsiveness to ICS treatment.
214

215 **B. Secondary Research Hypotheses – Related to asthma**

- 216 6. Differences in bronchial microbial community composition, as revealed by
217 exploratory methods such as cluster analysis, are associated with differences in
218 clinical, physiologic, and inflammatory phenotypic features of asthma.
219

- 220 7. The relative distribution of inflammatory cells and T cell subsets in bronchial
221 lavage fluid are associated with differences in clinical, physiologic, and
222 inflammatory phenotypic features of asthma.
223
- 224 8. The relative distribution of inflammatory cells and T cell subsets in bronchial
225 lavage fluid from asthmatic subjects are associated with differences in bronchial
226 microbial community composition.
227
- 228 9. The composition of the microbial community of induced sputum closely
229 resembles that of bronchial brushings.
230

231 **C. Secondary Research Hypotheses – Related to allergy**

- 232 10. Richness, evenness, diversity, or composition of stool microbiota differs between:
233 a. Allergic and non-allergic subjects.
234 b. Allergic asthmatic and allergic non-asthmatic subjects.
235
- 236 11. The numbers and relative distribution of circulating innate T cells and CD4 T cell
237 subsets differs between allergic and non-allergic subjects.
238
- 239 12. Differences in the numbers and relative distribution of innate T cells and CD4 T
240 cell subsets are associated with differences in the richness, evenness, diversity,
241 or composition of stool microbiota.
242
- 243 13. Differences in stool microbial community composition, as revealed by exploratory
244 methods such as cluster analysis, are associated with relative distribution of
245 innate T cells and CD4 T cell subsets
246
247
248
249

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

251 Specific Aim 1: To evaluate whether the microbiota of the bronchial airways in allergic
252 asthmatic, allergic non-asthmatic, and non-allergic, non-asthmatic healthy subjects
253 differ in microbial diversity, richness, evenness, or composition of specific bacterial taxa.
254

255 Specific Aim 2: To determine whether ICS treatment alters bronchial microbial
256 community composition in allergic asthmatic subjects.
257

258 Specific Aim 3: To determine whether characteristics of bronchial microbial community
259 composition at baseline or after ICS treatment, are associated with differences in
260 responsiveness to the treatment as measured by change in FEV₁ and PC₂₀Mch
261

262 Specific Aim 4: To evaluate whether clinical, physiologic, and inflammatory phenotypic
263 features of asthma (including “Th2- vs. non-Th2-molecular phenotype”) are associated
264 with characteristics of bronchial microbial community composition.
265

266 Specific Aim 5: To examine whether there are associations between bronchial microbial
267 community composition and
268 a. Pulmonary immune function as inferred from the relative distribution of innate
269 T cells, CD4 T cell subsets, and other inflammatory cells (eosinophils,
270 monocytes, basophils) in bronchial lavage fluid.
271 b. Pulmonary immune activation state as inferred from measurement of selected
272 cytokines in bronchial lavage fluid.
273

274 Specific Aim 6: To evaluate the concordance of the airway microbiota detected in
275 induced sputum and bronchial brushings from a subset of ten healthy and ten asthmatic
276 subjects participating in this study.
277

278 **E. Specific Aims – Related to Stool Microbiome, Systemic Immune Function, 279 Allergy, and Asthma.**

280 Specific Aim 7: To examine whether the metrics of gross microbial community
281 composition (richness, evenness, and diversity) or discrete taxonomic members of
282 these communities in **stool samples** differ:
283

- 284 a. Among allergic asthmatic, allergic but otherwise healthy non-asthmatic, and non-
285 allergic, non-asthmatic adults.
- 286 b. Among different phenotypic subgroups of allergic asthmatic subjects (eg. Th2 vs.
287 non-Th2, eosinophilic vs. non-eosinophilic, ICS-responsive vs. non-ICS
288 responsive, exacerbation-prone vs. exacerbation resistant, etc).
289

290 Specific Aim 8: To examine whether the numbers and relative distribution of circulating
291 innate T cells, CD4 T cell subsets, and other inflammatory cells (eosinophils,
292 monocytes, basophils) differ between allergic and non-allergic subjects.
293

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

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

298 **II. Background and Significance**

299 **A. Bronchial Microbiome and Asthma**

301 **1. Introduction.**

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

310 mechanism or factor is suggested by the finding of specific bacteria, particularly
311 *Mycoplasma pneumoniae* and *Chlamydia pneumoniae*, in bronchial biopsies from
312 some asthmatic patients^{21,22}. Links have also been suggested between the presence of
313 *S. pneumoniae*, *M. catarrhalis*, and *H. influenzae* in hypopharyngeal cultures from 1-
314 month-old infants and their risk of asthma in early life⁹ and in hypopharyngeal cultures
315 from children presenting with acute wheezing illnesses⁸. That perturbations from a
316 normal “bronchial microbiome” might be important has been suggested by recent
317 culture-independent studies of bronchial samples^{14,15}. These have shown that the
318 airway microbiota of asthmatic and healthy individuals differ in diversity and in the
319 relative abundance of specific bacterial species. These findings could be interpreted as
320 suggesting that a perturbation of normal bronchial microbiota is causally associated with
321 asthma, or at least some forms of asthma, but such a suggestion would be grossly
322 premature, not least because all of the asthmatic subjects examined were on inhaled
323 corticosteroid therapy, making it unclear whether the differences found are associated
324 with asthma or with ICS treatment. Because some of the bacteria identified in asthmatic
325 subjects, like the Comamonadaceae, can express genes involved in steroid degradation
326 pathways^{23,24}, it can even be hypothesized that differences in bronchial microbial
327 community composition may influence corticosteroid-responsiveness. Taken together,
328 these observations suggest that study of microbial community composition in the
329 airways of carefully characterized (or “phenotyped”) healthy and asthmatic subjects may
330 provide findings that could foster development of anti- or pro-biotic treatments for
331 asthma.

332
333 Methods for identifying airway microbiota in most prior studies of asthma have had
334 significant limitations. The vast majority of bacteria are non-culturable²⁵, and the utility of
335 targeted PCR to identify species is limited by its ability to detect unanticipated bacteria.
336 The application of more recently developed, high resolution, culture-independent
337 methods for microbial detection has demonstrated a great diversity of airway microbiota
338 in airway disease, including cystic fibrosis^{26,27}, ventilator-associated pneumonia²⁸,
339 COPD^{27,29}, and also asthma^{14,15}. In a recently published study by Huang et al.¹⁴,
340 conducted by the Asthma Clinical Research Network (ACRN), bronchial brushings from
341 suboptimally controlled asthmatic subjects (all taking ICS) were analyzed using the 16S
342 ribosomal RNA PhyloChip, developed by collaborators at Lawrence Berkeley National
343 Laboratory. This microarray-based method exploits sequence polymorphisms in the
344 broadly conserved, ubiquitous prokaryotic 16S rRNA gene to identify and classify
345 species, and is able to distinguish in a single assay ~ 8,500 bacterial taxa (taxa are
346 defined as groups of species with $\geq 97\%$ homology in their 16S rRNA gene sequence)¹⁶.
347 ³⁰. The PhyloChip permits rapid bacterial community profiling of many sample types with
348 significantly higher resolution than traditional clone library-sequencing analysis of the
349 same amplicon pools^{28,30}, and detects species present in low abundance as efficiently
350 as those in higher abundance in a given community³⁰ and is ideal for high-resolution
351 comparative analyses of treatment groups.

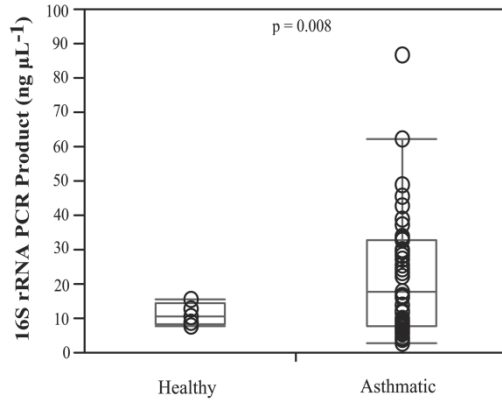
352
353 Comprehensive profiling of the microbiota by high-resolution, molecular approaches
354 permits complementary study of the potential functional effects of a microbial
355 community. Recent research findings on the human microbiome collectively have

356 outlined two concepts: (1) the structural complexity of microbial communities at a given
357 host site, rather than simply the presence of individual species, can be important in
358 determining states of health vs. disease³¹, and (2) differences in the structure or
359 composition of a microbial community underlie the collective functional effects exerted
360 by the community, including potential influences on host responses^{31,32}. Studies
361 highlighting these notions include the demonstration that differences in the gut
362 microbiota between obese and lean phenotypes³³ are associated with different
363 functional capacities for energy harvest, and that the presence of gut microbiota
364 influences the severity of induced type-1 diabetes in MyD88-deficient mice³⁴. These
365 examples emphasize that advancements in knowledge about the airway microbiota
366 could yield important insights into polymicrobial-host interactions relevant to the
367 pathogenesis or course of asthma. Indeed, in the “Add-On” study to the ACRN’s
368 Macrolides in Asthma trial, significant relationships were identified between features of
369 the airway microbiota, asthma and airway hyperresponsiveness¹⁴ (see preliminary
370 data), suggesting potential pathophysiologic links between the airway microbiota and
371 this clinical-pathophysiologic feature of asthma.
372

373 **2. Previously Published Data.**

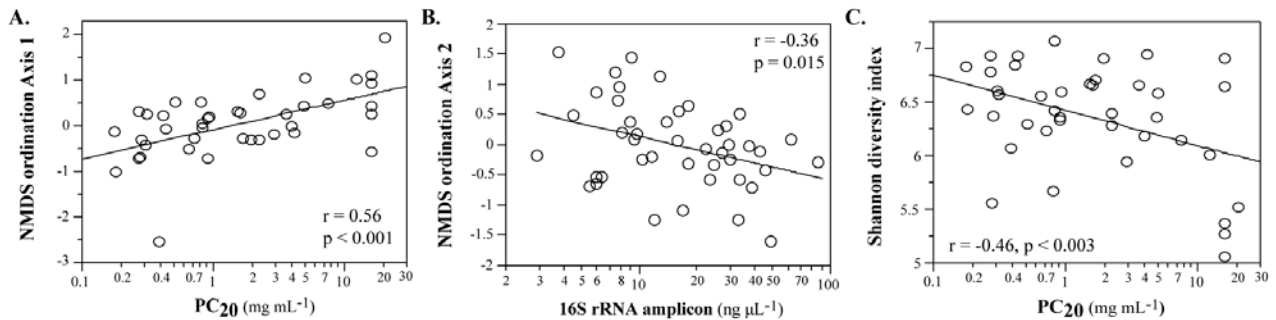
374 In the “Add-On” study to the ACRN’s Macrolides in Asthma trial¹⁴, an early observation
375 was that bacterial burden in bronchial epithelial samples (using measurements of 16S
376 rRNA amplicon concentrations as a proxy, and confirmed by quantitative PCR) was
377 significantly higher among the asthmatic group than healthy controls (Fig. 1).
378 Furthermore, as mentioned above, significant relationships between airway
379 hyperresponsiveness and characteristics of the airway microbiota profiled by PhyloChip
380 were observed using several different, independent analysis approaches. These
381 included a distance measure-based analysis of dissimilarity in bacterial community
382 composition among samples using a statistical ordination method (non-metric
383 multidimensional scaling, or NMDS). This approach also enabled assessment of which
384 variables may be most contributory to observed dissimilarities (or variability) in bacterial
385 community composition. As shown in Fig. 2A and 2B, this revealed that measurements
386 of PC₂₀Mch measurements and bacterial burden were most strongly correlated with
387 community variability. In a separate analysis based on calculations for each sample of a
388 commonly used measure of bacterial diversity (Shannon index), we found that diversity
389 indices were significantly and inversely correlated with PC₂₀Mch (Fig. 2C), suggesting
390 increasing bronchial bacterial diversity with greater airway hyperresponsiveness.
391 Finally, we examined for linear relationships between the relative abundance of all taxa
392 detected by PhyloChip across samples (~1,900) and PC₂₀Mch. After corrections for
393 false discovery and the application of fairly conservative significance criteria, we found
394 that the relative abundance of ~100 specific bacterial phylotypes profiled by the array
395 had the most significant correlations with greater airway hyperresponsiveness.

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



397
398

399 **Figure 2.** Panels A-B. NMDS analysis showing that variability in bronchial bacterial
400 community composition across samples is strongly correlated with PC₂₀Mch and
401 bacterial burden (circles represent the total community present in a single subject
402 sample). Panel C. Shannon indices of bacterial diversity increase with lower PC₂₀Mch
403 values. (Ref. 6)



404
405

406 3. Inhaled Corticosteroid treatment - Rationale and Duration of Treatment

407 To our knowledge, only two studies have been published to on the airway microbiota in
408 chronic asthma^{14,15}. As all asthmatic subjects in both studies were taking ICS therapies,
409 it remains unclear whether differences found in the airway microbiota are related to ICS
410 treatment or to asthma itself. Several different inhaled corticosteroids delivered from a
411 dry-powder inhaler are approved as maintenance treatment for asthma (budesonide,
412 fluticasone, mometasone). In this study, we wish to compare the effects of inhalation of
413 an ICS to inhalation of placebo. We propose to have the subjects inhale 250 mcg of
414 fluticasone by Diskus inhaler twice daily for six weeks in this protocol. This duration of
415 treatment was selected because a previous study conducted by the Asthma Clinical
416 Research Network, the “PRICE” study showed that six weeks of inhaled corticosteroid
417 treatment was sufficient to identify patients as “responders” or “non-responders” as
418 judged by a greater than 5% increase in FEV1 or a greater than 1 doubling dose of
419 methacholine in PC₂₀, without further change in FEV1 after 16 additional weeks of
420 continued ICS therapy³⁵.
421

422 **4. Selection of allergic subjects.**

423 As noted above, it has not been possible to interpret the differences in the bronchial
424 microbiome of the asthmatic and healthy subjects studied so far as necessarily
425 suggesting a relationship between bronchial microbiota and asthma, for all of the
426 asthmatic subjects were under treatment with an inhaled corticosteroid at the time of
427 bronchoscopy. That is why we now propose to study asthmatic subjects who have taken
428 no ICS treatment for 6 months. Another possible confounding determinant of bronchial
429 microbiota is allergy, for allergy is associated with demonstrably different patterns of
430 immune function in the airways, and these differences could plausibly account for
431 differences in the microbes allowed to colonize or infect airway mucosal surfaces. Since
432 the primary research question of this study is whether the bronchial microbiome differs
433 in people with and without asthma, we think it necessary to enroll allergic non-asthmatic
434 adult subjects, as nearly similar as possible in all other regards as a control group.
435 Because >80% of the asthmatic subjects participating in ACRN and CARE network
436 studies have been allergic, we know that recruitment of allergic asthmatic subjects is far
437 easier than recruitment of non-allergic asthmatic subjects. It follows that the healthy
438 subjects enrolled in this study must be allergic as well. We thus think it necessary for
439 this study that at least one “control” group for comparison is made up of non-asthmatic
440 subjects who, like the asthmatic subjects, have a positive Phadiatop test result.
441 Whether allergy, as distinct from asthma, is associated with differences in
442 gastrointestinal or bronchial microbial community composition is an interesting and
443 potentially important question that will be addressed by the additional enrollment of a
444 second control group, of non-allergic, non-asthmatic healthy adults (see section B.,
445 below). For subjects identified as allergic by Phadiatop testing, we will additionally
446 measure specific IgE antibodies directed against a standard panel of aeroallergens,
447 including cat, dog, mouse, mold mix, cockroach (German), mixes of grass, tree (2
448 mixes), weed (2 mixes), and mite (2: Der f and Der p) . This may permit analysis of
449 whether exposure to allergens to which the subject is sensitized affects burden or
450 composition of the bronchial microbiome.
451

452 **5. Bronchoscopic vs. non-bronchoscopic airway specimens.**

453 The primary specimens to be analyzed for differences or changes in the bronchial
454 microbiome are protected bronchial epithelial brushings. From prior experience in the
455 MIA Add-On study¹⁴, three bronchial brushings per subject provided greater pooled
456 yield than bronchial biopsies in terms of total DNA recovered and bacterial 16S rRNA
457 PCR product. While bronchial brushings are a preferred specimen type for analysis of
458 the bronchial airway microbiome, the potential utility of non-bronchoscopically collected
459 specimens for inferring bronchial microbial community composition is not clear. Of
460 potential options, microbiome analysis of induced sputum may be useful, given
461 precedence for analysis of sputum inflammation in asthma studies³⁶. Preliminary
462 PhyloChip analysis of paired induced sputum and bronchial brushings from 6 subjects
463 (3 healthy, 3 asthmatics not taking ICS) found 80-97% concordance in the specific
464 bacterial taxa detected. For two of the three healthy subjects, all taxa identified in the
465 bronchial brushings were detected also in the paired sputum. However, there was
466 greater variability in the ICS-naïve asthmatic subject specimens, where 8.9% – 19% of

467 detected taxa (or 67 – 240 taxa) were identified in the brushings only. Since many taxa
468 can comprise a given bacterial subfamily, this translated into 9 to 23 bacterial
469 subfamilies that were identified from brushings only. Comparison of the microbiome
470 revealed by analysis of induced sputum and bronchial brush samples in this study will
471 enable us to determine if induced sputum samples reflect >90% of all microbial taxa
472 detected in bronchial brush samples.
473

474 **6. Anticipated Significance.**

475 A resident microbial community has been identified in the bronchial airways of asthmatic
476 subjects in two recent studies^{14,15}, and specific features of the microbiota have been
477 found to correlate with bronchial hyperresponsiveness¹⁴, an important
478 pathophysiological feature of asthma. In addition, known functional properties of specific
479 organisms associated with this clinical feature may potentially contribute to asthma
480 pathogenesis or prognosis (e.g. organisms with steroid-degradation capacity).
481 Collectively, these recent findings could lead to the development of novel therapeutic
482 approaches for asthma, including pro-biotic, anti-biotic, or other specific mechanistic
483 targets directed at the microbiota. To pursue this further, however, it is necessary to
484 establish whether these findings are confounded by the use of ICS therapies in all
485 asthmatics examined in these two prior studies. If differences in the bronchial
486 microbiome are discovered between healthy and ICS-naïve asthmatics in this study, this
487 would have important implications for further research on the role of microbiota in
488 asthma pathogenesis. If specific microbial community features are found to be
489 associated with responsiveness to ICS treatment or related changes in other phenotypic
490 variables, this could provide a microbial signature for prognosticating response to ICS
491 therapy, which may be evaluated in future studies with other asthmatic populations.

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

493 **1. Introduction.**

494 While analysis of relationships between features of the bronchial microbiome and the
495 clinical, physiological, and inflammatory features of airway function in allergic asthmatic
496 and allergic non-asthmatic adults holds promise for shedding new light on
497 pathophysiologic mechanisms of asthma, we have recognized that more could be
498 learned by coincidentally assessing the microbial composition of stool samples and the
499 circulating cell populations that mediate immune function.
500

501 The rationale underlying these additional analyses rests first on evidence that allergic
502 sensitization and response reflect a disorder in systemic immune function,
503 predominantly expressed at mucosal surfaces, consisting of an imbalance between
504 effector Th2 cells and suppressive regulatory T cells, so that T cell responses are
505 skewed toward a pro-inflammatory Th2-mediated pattern. Other more recently
506 described T cell subsets such as Th17 cells may play a role in asthma pathogenesis as
507 well. So, too, may other T cells, including natural killer T cells, gamma-delta T cells, and
508 CD8 cells^{6,7}.
509

510 Also underlying our interest in these additional analyses are the findings of studies
511 showing relationships between the composition of the microbial community in the
512 gastrointestinal tract and clinical manifestations of immune-mediated disease
513 (epidemiologic studies) and in the numbers, activity, and function of specific immune
514 cells, especially T cells (clinical and murine studies)^{1-3,37,38}
515

516 We thus propose to examine relationships among stool microbiome, systemic immune
517 function, and pulmonary immune response in healthy and asthmatic subjects by also
518 obtaining and analyzing samples of stool (for characterization of fecal microbiome), of
519 blood (for “immunophenotyping” of T cell and inflammatory cell populations), and of
520 bronchial lavage fluid (for concentrations of selected cytokines and of T-cell
521 populations) from allergic asthmatic and allergic non-asthmatic, and non-allergic, non-
522 asthmatic healthy subjects..
523

524 The enrollment of a group of **non-allergic, non-asthmatic healthy subjects** will permit
525 assessment of the possibility that the disturbances in immune function underlying
526 **allergy** are associated with differences in the intestinal microbiome, whereas the
527 disturbances in function underlying **allergic asthma** are associated with differences in
528 the microbial populations resident in the airways. It is of course also possible that the
529 gut microbiota shape not only the imbalance in immune function that underlies allergic
530 sensitization and response but also the difference in immune function that permit a
531 distinct pattern of bacterial colonization of the bronchial mucosa. It is not inconceivable
532 even that particular patterns of gut microbial community composition may be associated
533 with particular phenotypes of asthma.
534

535 **2. Background Data.**

536 **Blood cell composition correlates with immune function:** The cellular composition
537 of circulating white blood cells reflects immune status and can be used to assess
538 immune function and disposition to inflammatory and/or allergic responses. For
539 example, blood eosinophilia is a standard biomarker of allergy. Helper T cells are
540 central coordinators of immune responses, and the prevalence of different functional
541 subsets (e.g. Th1, Th2, and Th17 cells) correlates with the type of responses that each
542 subset directs. Allergic responses are driven by Th2 cells that produce the signature
543 cytokines IL-4 and IL-13, and T cells expressing the Th2-associated cell surface protein
544 CCR4 are also present at higher frequency in the peripheral blood of patients with
545 allergic diseases including atopic dermatitis and asthma^{19,39}.
546

547 **3. Subject selection.**

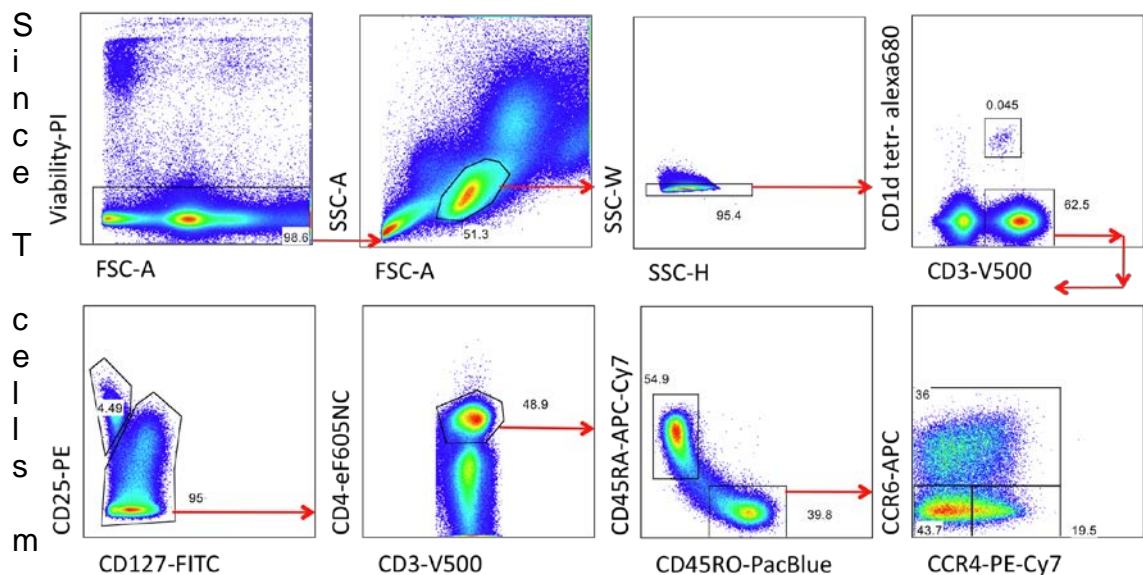
548 Enrollment of three group of subjects – allergic asthmatic, allergic non-asthmatic, and
549 non-allergic, non-asthmatic adults, all otherwise healthy – will enable determination not
550 only of the relationship of the bronchial microbiome to asthma and its phenotypic
551 features but also of whether allergy, as distinct from asthma, is associated with
552 differences in gastrointestinal or bronchial microbial community composition.
553

554 **4. Methods and previous findings.**

555 **a. Characterization of systemic immune function and of pulmonary immune**
556 **response.**

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

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585 **Figure 3.** Representative FACS staining for T cell subsets.

587 T cells must be restimulated through their antigen receptor to reveal which cytokines
588 they will produce. Therefore, extensive efforts have been made to uncover patterns of
589 expression cell surface proteins that correlate with cytokine production capability. We
590 have adopted this strategy and now routinely characterize helper T cell subsets in blood
591 and bronchial lavage fluid by their pattern of expression of three chemokine receptors:
592 CXCR3, CCR6, and CCR4 (**Figure 4**). CCR6 expression correlates closely with the
593 ability to make IL-17^{17,40}, whereas IFN- γ -producing cells generally coexpress CXCR3¹⁸.
594 CCR4 is expressed by Th2 cells and by many Th17 cells, but the vast majority Th2 cells
595 can be captured within the CCR4+CCR6- population by co-staining for both markers.
596 Co-staining for all three markers also allows us to track T cell populations expressing
597 combinations of CXCR3, CCR4, and CCR6, which can include cells that make more
598 than one of the signature helper T cell cytokines.

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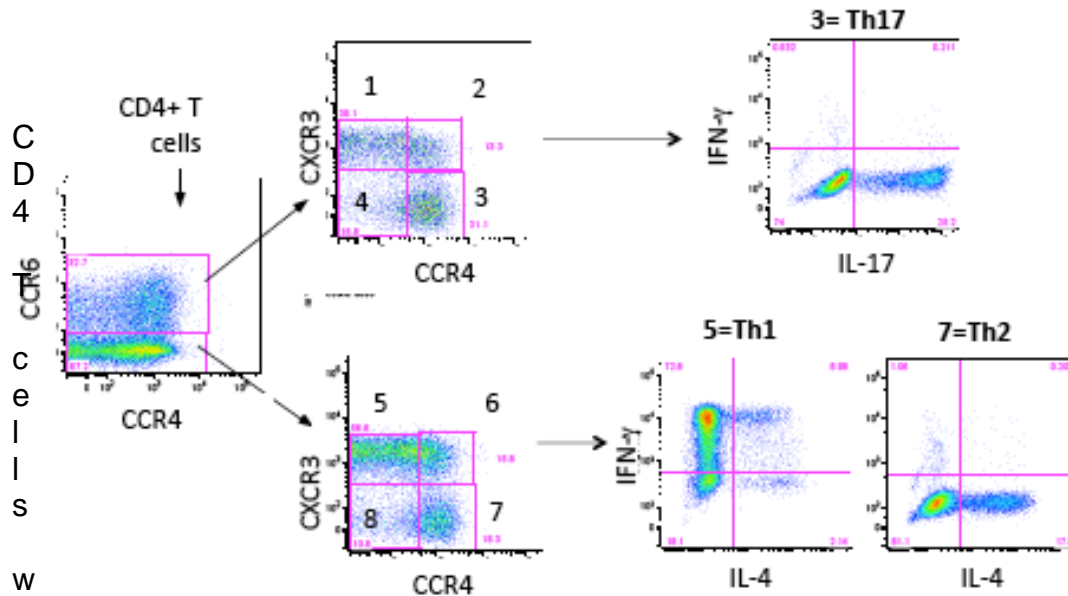


Figure 4. CD4+CD45RO+ T cells were FACS sorted into 8 populations defined by expression of CCR6, CCR4, and CXCR3 (*left panels*). Each population was subsequently stimulated *in vitro* and stained intracellularly to reveal cytokine production (*right panels*; numbers refer to corresponding quadrants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

F
F

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

630
631 Ongoing pulmonary immune responses will be assessed by measuring a panel of 23
632 cytokines in bronchial lavage fluid using multiplex fluorescent magnetic bead ELISA
633 according to the manufacturer's instructions (Millipore). These analyses will be
634 conducted at the Blood Systems Research Institute core facility, which has experience
635 with cytokine measurements in bronchial lavage fluid⁴¹. Patterns of cytokine expression
636 relative to each other will be compared as described^{11,12}.

637 638 **b. Detection and characterization of stool microbiome**

639 The diverse ecosystem of the human gut microbiome houses the greatest burden of
640 microbes, members of which encode genes for essential functions that the human host
641 is incapable of performing, such as vitamin production and metabolism of indigestible
642 dietary polysaccharides⁴²⁻⁴⁴. Thus, the host immune system must strike a balance
643 between providing a favorable environment for this vital community while protecting
644 against invasion or outgrowth of pathogenic species. Enteric microbes constantly prime

645 the innate immune system, thus facilitating a rapid response to pathogens⁴⁵.
646 Appropriate microbial colonization also plays a key role in the development of the gut-
647 associated lymphoid tissue (GALT), a primary mechanism of defense against enteric
648 pathogens^{46,47}. Moreover, intestinal microbial colonization stimulates the production of
649 effector molecules such as secretory IgA⁴⁸, the differentiation of TH17 cells⁴⁹, and the
650 development and activation of regulatory T (T-Reg) cells^{50,51}. Significantly, it has also
651 been demonstrated that the presence of a GI microbiota and early stimulation of the
652 immature immune system by a diversity of commensal microbes is fundamental to
653 establishing and maintaining the essential balance between Th1, Th2, or Th17 cytokine
654 expressing T-cells⁵²⁻⁵⁵.

656 Increasing interest is focused on the complex interplay between initial events in the
657 assembly of the GI microbiota and the development and maintenance of the host's
658 immune system homeostasis and whether manipulation of microbiota during this key
659 developmental stage (or indeed in adulthood) can impact inflammatory disease
660 outcomes in both the gastrointestinal and pulmonary tracts⁵⁶⁻⁵⁸. This interest is partly
661 driven by the findings of epidemiologic studies linking GI pediatric gastrointestinal
662 microbiome dysbiosis to the development of childhood asthma and allergy. For
663 example, microbiological examination of almost 1,000 stool samples from 1-month-old
664 infants demonstrated that high abundance of *Escherichia coli* was associated with the
665 subsequent development of eczema, while high abundance of *Clostridium difficile* was
666 associated with development of eczema, recurrent wheeze, allergic sensitization and
667 allergic dermatitis⁵⁷. Such findings have demonstrated a clear link between GI
668 microbiome composition and allergic disease, and that, at least in pediatric patients,
669 overgrowth of specific bacterial species predisposes to inflammatory disorders.
670 Significantly, we have previously demonstrated that a specific murine GI bacterial
671 species, segmented filamentous bacteria, can promote proliferation of Th17 cells⁴⁹, a
672 relatively recently described subset of T-cells whose proliferation is associated with a
673 number of chronic inflammatory diseases. This significant finding reinforces the concept
674 that enrichment of particular microbial species in the complex community present in the
675 GI microbiome can drive specific pro-inflammatory responses. Moreover, Kwon and
676 colleagues recently demonstrated that feeding a mix of bacterial species resulted not
677 only in local promotion of CD4+ FoxP3 T-reg cells in a murine model of colitis, but also
678 trafficking of this T-cell subtype to affected sites remote from the GI tract including the
679 skin in an animal model of dermatitis⁵⁹. More recently, it has been demonstrated that the
680 composition of the gastrointestinal microbiome governs host response to viral
681 infection⁶⁰. Using respiratory influenza virus as the model infectious agent, investigators
682 demonstrated that gastrointestinal microbiota composition critically regulates the
683 generation of virus-specific CD4 and CD8 T cells and antibody responses to this viral
684 respiratory pathogen. To confirm these observations, the authors also demonstrated
685 that oral treatment with a non-absorbed antibiotic, neomycin, dramatically altered the
686 pulmonary response to influenza infection, indicating that neomycin-sensitive bacteria
687 are associated with the induction of productive immune responses in the lung. Thus
688 given the depth of the proposed study, which includes airway and GI microbiome
689 profiling and immune phenotyping of subjects, study of relationships between

690 microbiome membership and the ability of specific species identified in the study to
691 prime local and remote responses associated with asthma and allergy will be possible.
692

693 We propose to use the G3 16S rRNA PhyloChip, a high-density, culture-independent
694 microarray-based assay designed in 2010, based on publicly available 16S rRNA
695 sequence databases (which includes all of the human microbiome 16S rRNA
696 sequences deposited by this date), to generate a high-resolution bacterial community
697 profile of samples collected in this proposed study. This array, housing 1,000,000
698 oligonucleotide probes can detect ~60,000 bacterial taxa (defined as species or strains
699 sharing $\geq 99\%$ 16S rRNA sequence identity). Briefly, The 16S rRNA gene will be
700 amplified, purified, labeled and quantified. A standardized concentration of labeled 16S
701 rRNA will be spiked with known concentrations of control oligonucleotides that act as
702 internal standards for data normalization. PhyloChip processing will be performed as
703 previously reported⁶¹.
704

705 While 454-sequencing has been the mainstay of microbiota profiling for several years,
706 and has a number of clear advantages, e.g. acquisition of an actual sequence read,
707 ability to determine relative abundance within a single sample, drawbacks include the
708 potential for relatively poor community coverage of complex and diverse assemblages.
709 This could potentially lead to overlooking key species involved in, for example specific
710 disease states, since the species in question do not necessarily represent the dominant
711 members of the community. Indeed, this has recently been highlighted in a study of the
712 oral microbiome in which a low abundance member of the community, *Porphyromonas*
713 *gingivalis*, drives periodontal inflammatory disease via immune and microbiome
714 composition manipulation without ever becoming a dominant community member⁶².
715 Such studies underline the need for high-resolution profiling approaches, particularly in
716 comparative studies where identification of lower abundance species may prove key to
717 the disease or disorder in question.
718

719 The G3 PhyloChip provides such coverage, and can detect low abundance, rarer
720 community members in parallel with high abundance organisms, thus producing a high-
721 resolution profile of the community members in a relatively economical assay, ideal for
722 comparative or correlative statistical analyses to identify key species associated with the
723 phenotype in question. We have directly compared 454-pyrosequencing to PhyloChip
724 for profiling microbiota in house dust samples. Using quality-filtered 454-sequence
725 reads ranging from 11,994-31,982 per sample, as many as 8,027 unique sequence
726 reads were identified in one sample suggesting the presence of substantial diversity in
727 these communities. This was confirmed by rarefaction analysis of the sequence data
728 **(Fig. 5.A)**, which indicated that that the depth of sequence performed permitted
729 sampling of only a very small subset of the complex community present. To compare
730 454 data to that of PhyloChip, we first reclassified the PhyloChip data using the RDP
731 classifier. Aligned representative sequences were downloaded from Greengenes and
732 the V4-V5 sequence regions were extracted and provided to the Ribosomal Database
733 Project (RDP) classifier. Comparison of parallel PhyloChip data generated from the
734 same extracted DNA revealed that while the large majority of organisms detected by
735 454-sequencing were also detected by PhyloChip (at higher levels of classification

736 concordance was 100%). In all samples the array consistently detected substantially
737 more community members (**Fig. 5.B**).
738

739 To further illustrate that these findings are due to improved community coverage by the
740 parallel nature of the array, we performed resampling of the sample with the greatest
741 sequence reads at different sequencing depths (1,000, 5,000, 10,000, 20,000 and
742 31,982 sequence reads from the sample with greatest read depth). Classified genera at
743 each sequencing effort level
744 were compared to the 419
745 classified genera derived
746 from representative
747 sequences of the taxa
748 detected by PhyloChip.

749 This approach
750 demonstrated that
751 increasingly deeper
752 sequencing efforts validated
753 greater numbers of
754 PhyloChip detected genera
755 (**Fig. 5.C**), suggesting that in
756 the complex communities of
757 the GI microbiota, this tool
758 represents a standardized
759 economical approach to
760 high-resolution profiling of
761 bacterial community
762 composition. The data
763 presented are not exclusive
764 to dust samples, we recently
765 performed a study of
766 pediatric patients with
767 irritable bowel syndrome
768 involving 454-sequencing
769 and PhyloChip profiling of
770 stool which demonstrated
771 excellent concordance⁶³. We
772 and others have also
773 previously compared the
774 PhyloChip to both traditional
775 clone library and relatively
776 high numbers of next
777 generation 454
778 pyrosequencing reads
779 generated in parallel from
780 clinical and environmental
781 samples and have

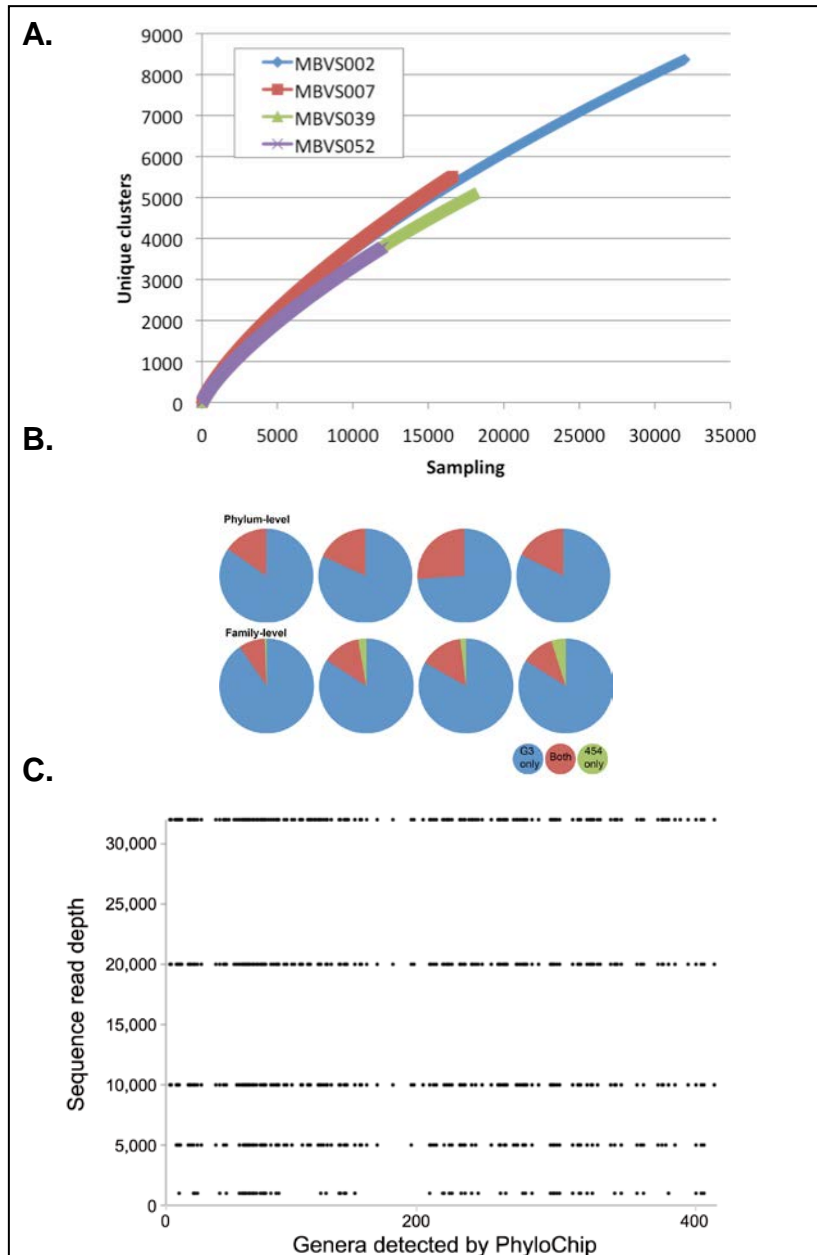


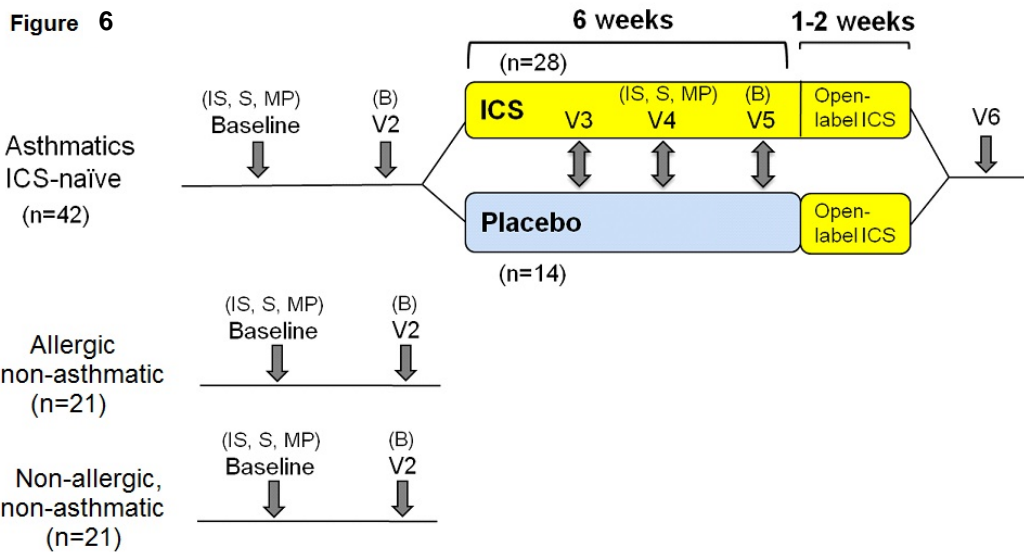
Fig. 5A. Rarefaction curves at 0.03 distance clustering showing exponential increase in number of new sequence clusters detected (even at ~30,000 sequences), indicating highly rich microbiota. **B.** Stool bacterial community members detected by both array and sequencing (red), exclusively by either 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).

782 consistently demonstrated that while the array detected the majority of genera identified
 783 by sequencing (>97% of those detected by sequencing), it also detected several
 784 hundred additional taxa providing a substantially higher-resolution profile of these
 785 communities^{28,30}. Because of these advantages and because the normalized datasets
 786 generated by the array permit application of robust statistical analyses, we propose to
 787 use the G3 PhyloChip to profile bacterial communities present in samples collected in
 788 this study.
 789

790 **III. Protocol**

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

804



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

805
806

807 Forty-two ICS-naïve allergic asthmatic subjects (7 visits, pre- and post-ICS intervention
808 assessments), 21 allergic, non-asthmatic subjects, and 21 non-allergic, non-asthmatic
809 healthy subjects (3 visits, for baseline assessments only) will be studied. The methods
810 for clinical assessment and “phenotyping” are those used in previous ACRN studies.
811 These include standardized questionnaires to characterize asthma onset, severity,
812 treatment, exacerbation history, and current control. Baseline measurements include
813 spirometry with bronchodilator reversibility, bronchial reactivity (PC₂₀ Mch), Phadiatop
814 test, serum IgE, and sputum eosinophil and neutrophil percentages. Methods for
815 sputum induction and bronchoscopy will again be those used in previous ACRN studies.
816 Oral saline rinse will be performed prior to sputum induction and bronchoscopy, to
817 reduce contamination of these samples by oral secretions. Subjects will be given
818 standard kits for collection of a first morning stool sample to bring to the center on the
819 day of bronchoscopy, and will have blood drawn from the intravenous line placed at the
820 time of bronchoscopy for analysis of cell populations.

821
822 The specimens for microbiome analysis by 16S rRNA PhyloChip are protected
823 bronchial brushings and first morning stool samples. Induced sputum samples will also
824 be analyzed by 16S rRNA PhyloChip in a subset of subjects, to examine whether airway
825 sampling by this less invasive, approach permits inference about the bronchial
826 microbiome. For microbial analysis of these samples, total DNA and RNA will be
827 extracted by the combined protocol optimized for bacterial nucleic acid extraction used
828 in prior studies¹⁴. DNA will be processed for 16S RNA PhyloChip and related microbial
829 community analyses. In addition, as part of the secondary hypotheses and analyses,
830 aliquots of the RNA extracted from the bronchial brush samples will be processed for
831 QT-PCR analysis of Serpin-B2, CLCA-1, Periostin, and other genes related to the “TH2-
832 molecular phenotype” of asthma, using methods previously described by Woodruff et
833 al⁶⁴. The remaining RNA will be stored at -80° C for future functional/metatranscriptomic
834 analyses.

835
836 Other analyses of the samples obtained include FACS (10 color panel) analysis of the
837 numbers of innate T cells and CD4 T cell subsets further divided by expression of
838 chemokine receptors correlating with ability to produce IFN- γ (Th1), CCR6 IL-17A
839 (Th17), and IL-4 and IL-13 (Th2). FACS analysis will also enumerate other relevant
840 cells, including CD8 T cells, NK cells, B cells, monocytes, basophils, eosinophils, and
841 neutrophils. For assessment of pulmonary immune function and response, we will
842 measure cytokines of interest and the numbers of inflammatory cells and T cells and
843 their subsets in the bronchoalveolar lavage fluid obtained at bronchoscopy.

844
845 Other analyses beyond the scope of this proposal could be performed on the samples
846 collected in this study and stored in the planned AsthmaNet Sample bank. For
847 example, the RNA remaining after analysis of expression of the genes of the “TH2
848 phenotype” will be stored at -80° C for possible future functional/metatranscriptomic
849 analyses. The DNA remaining after removal of aliquots for amplification of 16S-rRNA
850 for PhyloChip analysis will be stored as well, and would be available for study by
851 sequencing or array-based methods for detection of fungal organisms (e.g., by
852 “MycChip”). Aliquots of bronchoalveolar lavage fluid will also be stored at -80° C, and

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

860
861

A. Subjects

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

874

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

876

B. Inclusion Criteria – Asthmatic subjects

- 877
- 878 1. Men and women, 18-60 years of age.
 - 879 2. History of physician-diagnosed asthma.
 - 880 3. Methacholine PC₂₀ ≤ 8 mg/ml and/or FEV₁ improvement ≥ 12% in response to 4
881 puffs albuterol.
 - 882 4. FEV₁ ≥ 70% of predicted after 4 puffs albuterol.
 - 883 5. Nonsmoker (less than 5 pack-year lifetime smoking history and no smoking
884 within the previous year).
 - 885 6. Stable asthma for ≥ 3 months prior to enrollment (no urgent care visits, no
886 systemic corticosteroid treatment).
 - 887 7. Asthma Control Questionnaire 6 Score (i.e., without score for FEV₁ or PEF) ≤1.5
888 at Visit 0.
 - 889 8. Able to provide informed consent.
 - 890 9. Able to perform spirometry as per ATS criteria.
 - 891 10. Evidence by Phadiatop testing of sensitivity to an aeroallergen in blood sample
892 drawn at Visit 0.
 - 893 11. Willingness, if female and able to conceive, to utilize one medically-acceptable
894 form of contraception.

895

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

898

899 **C. Exclusion Criteria – Asthmatic subjects**

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

922
923 **D. Inclusion Criteria for randomization – Asthmatic subjects**

- 924 1. Absence of respiratory infection since Visit 0.
- 925 2. No corticosteroid use since Visit 0.
- 926 3. No antibiotic use since Visit 0.
- 927 4. No significant asthma exacerbation since Visit 0.
- 928 5. Asthma Control Questionnaire 6 Score (i.e., without score for FEV_1 or PEF) ≤ 1.5 .
- 929 6. Continued absence of exclusion criteria described above.

930
931 Intention-to-treat principles will apply following randomization. Thus, subjects will be
932 dropped after randomization for safety reasons only. These may include pregnancy or
933 the development of a significant asthma exacerbation (as defined in section O.1,
934 "Asthma Exacerbations") found not to be, in the opinion of the investigator, responsive
935 to protocol treatment as defined in section O.

936
937 **E. Inclusion Criteria – Allergic, non-asthmatic Healthy subjects**

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

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

946
947 **F. Inclusion Criteria – Non-allergic, non-asthmatic Healthy subjects**

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

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

952
953 Healthy participants will be excluded if they meet ANY of the following exclusion criteria:
954

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

- 956 1. Any history of asthma.
957 2. Presence of significant medical illness or other chronic diseases whose treatment
958 could affect the clinical features measured, or risks of participating in the study
959 (see Appendix).
960 3. History of atrial or ventricular tachyarrhythmia.
961 4. Changes suggestive of cardiac ischemia on ECG at baseline.
962 5. Smoking ≥ 5 pack-years, or within the past year
963 6. FEV₁ or FVC < 80% predicted.
964 7. Methacholine PC₂₀ ≤ 16 mg/ml and/or FEV₁ improvement $\geq 12\%$ in response to
965 albuterol.
966 8. History of upper respiratory infection in the previous 6 weeks.
967 9. History of sinusitis, bronchitis, or antibiotic use in the previous 3 months.
968 10. Use of ≥ 10 doses of a nasal corticosteroid preparation in the previous 3 months
969 11. Evidence of chronic sinusitis.
970 12. Change in bowel function (e.g., diarrheal illness) in the previous 4 weeks.
971 13. Inability or unwillingness to perform required study procedures.
972 14. History of bleeding disorder.
973 15. Reduced creatinine clearance.
974 16. Contraindication to bronchoscopy on history or examination.

975
976 **H. Active Treatment Medication (for Asthmatic Subjects Only)**

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

983
984 **I. Outcome Variables**

985 Primary outcome variables

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

- 988 - Richness (number of different bacterial taxa identified)
- 989 - Evenness (distribution of the relative abundance of the taxa identified)
- 990 - Diversity (a function of richness and evenness)
- 991 - Presence and relative abundance of specific bacterial taxa
- 992
- 993 2. Descriptors of stool microbial community composition at baseline (see above).
- 994 3. Numbers of innate T cells and CD4 T cell subsets identified by surface receptor
- 995 expression as Th1, Th17, and Th2, and numbers of CD8 T cells, NK cells, B cells,
- 996 monocytes, basophils, eosinophils, and neutrophils in blood and in BAL fluid.
- 997
- 998 Secondary outcome variables
- 999 1. Clinical, physiologic, and inflammatory phenotypic features of asthma:
- 1000 - FEV₁ % predicted pre-albuterol
- 1001 - FEV₁ % predicted post-albuterol
- 1002 - Change in FEV₁ % predicted pre- to post-albuterol
- 1003 - Asthma Control Questionnaire-6 score
- 1004 - PC₂₀ Mch
- 1005 - % eosinophils and neutrophils in induced sputum sample
- 1006 - Serum IgE level
- 1007 - Blood eosinophil %
- 1008 - Number of positive Phadiatop results to testing with common aero-allergens.
- 1009 - Age of onset of asthma
- 1010 - BMI
- 1011 - Number of exacerbations requiring oral corticosteroid treatment in the past 5 years.
- 1012 - History of cough productive of mucus
- 1013 - Cold questionnaire response of viral “colds” as being “usually” or “always” associated
- 1014 with worsening of asthma (on four point Likert scale of “rarely, sometimes, usually,
- 1015 and always”)
- 1016
- 1017 2. Levels of cytokines in BAL fluid (L-1beta, 2, 3, 4, 5, 6, 8, 9, 10, 13, 17, IFN-gamma,
- 1018 TNF-alpha, Eotaxin, GM-CSF, IL-21, 23, 33, TSLP), as assessed by Luminex
- 1019 multiplex cytokine ELISAs.
- 1020
- 1021 3. “Molecular Phenotype,” as inferred from expression level of IL-13-dependent genes
- 1022 in bronchial epithelial cells (Periostin, CLCA-1, Serpin-B2) and classified as “TH2”
- 1023 and “non-TH2” molecular phenotypes. Measured at baseline only.
- 1024 4. In response to 6 weeks of inhaled corticosteroid (fluticasone 250 mcg twice daily), or
- 1025 dry-powder placebo inhaler:
- 1026 - Change in FEV₁ % predicted
- 1027 - Change in PC₂₀ Mch
- 1028 - Change in sputum eosinophil %
- 1029 - Change in ACQ-6 score (i.e., without score for FEV₁ or PEF).
- 1030
- 1031 5. History of exposure to household pet dog, cat, or other furred animal within the past
- 1032 year.

1033

1034 **J. Description of Study Visits and Periods**

1035 Specific elements for each study visit are provided in **Table 1**, below.

1036

1037 ***Baseline:***

1038 Visit 0.

1039 Subjects will first be told the purposes, risks, and alternatives to participation and will
1040 sign an IRB-approved document for informed consent. Asthmatics will complete the
1041 Asthma Control Questionnaire. Blood will be drawn for Phadiatop and IgE testing.
1042 Specific IgE to a panel of aeroallergens will be measured only in Phadiatop positive
1043 subjects.

1044

1045 Visit 1. Participants who meet allergen sensitivity requirement will complete an asthma
1046 characterization visit. If female and of reproductive age, a urine pregnancy test must be
1047 negative, and if asthmatic, must report use of an appropriate method of contraception
1048 for the duration of the study. Standardized questionnaires used in prior ACRN and
1049 AsthmaNet studies will be administered to characterize asthma onset, severity,
1050 treatment, exacerbation history, and current control. Baseline measurements will
1051 include an EKG, spirometry, bronchial reactivity (PC₂₀ Mch), and collection of induced
1052 sputum, all by methods used in previous ACRN and other AsthmaNet studies;
1053 procedural details are specified in the Microbiome Manual of Procedures (MOP) and
1054 AsthmaNet Spirometry, Methacholine (including medication and dosing) and Sputum
1055 MOPs, respectively. Blood will be drawn for genetic analysis and for measurement of
1056 BUN, creatinine and eosinophil number. Oral saline rinse will be performed prior to
1057 sputum induction to reduce contamination of the samples by oral secretions.

1058

1059 If tests show that a person presenting as a healthy subject is not eligible to participate in
1060 the study (because of electrocardiographic abnormalities, abnormal pulmonary function,
1061 or bronchial hyper-reactivity (PC₂₀ ≤ 16 mg/ml), a study physician will perform a brief
1062 medical history and physical exam, will advise the subject as to the possible clinical
1063 significance of the test finding, and will offer to communicate the finding to the subject's
1064 primary physician or to refer the subject to a physician if the subject wishes. The same
1065 basic procedure will be followed for people presenting as an asthmatic subject who
1066 have abnormal EKG findings, severe airflow obstruction (FEV₁<55% predicted), or
1067 absence of bronchial hyper-reactivity.

1068

1069 For asthmatic subjects, albuterol will be prescribed for as-needed rescue use. Subjects
1070 will be allowed to continue other chronic medications, as long as they are not in conflict
1071 with the inclusion/exclusion criteria. Subjects with clinically-significant allergic rhinitis
1072 treated with oral antihistamines but not nasal steroids will be allowed to continue the
1073 former. Subjects with symptomatic allergic conjunctivitis using ophthalmic
1074 antihistamines or mast cell stabilizers will be allowed to continue these agents. Subjects
1075 who meet the inclusion/exclusion criteria will be allowed to proceed to Visit 2, at which
1076 bronchoscopy will be performed. On discharge from visit 1, they will be given a stool
1077 sample collection kit, with instructions to bring in a sample from the first morning bowel
1078 movement on the day of bronchoscopy.

1079
1080 Visit 2. Subjects who meet safety criteria for bronchoscopy will return 1 week \pm 3 days
1081 after enrollment for the second study visit. Pregnancy test will be repeated in women of
1082 child-bearing potential. Spirometry will be performed before and after inhalation of 4
1083 puffs of albuterol. Intravenous access will be secured prior to bronchoscopy and a
1084 sample of 10 ml of blood will be placed into two labeled 5 mL Cyto-Chex BCT blood
1085 collection tubes (2 x 5mL) for shipping to UCSF for FACS analysis on the following day.
1086 Subjects will then undergo fiberoptic bronchoscopy with five protected bronchial
1087 brushings and a bronchial lavage (instillation and recovery by suction of 200 ml of
1088 warmed saline instilled in four boluses of 50 ml through a bronchoscope wedged into a
1089 segmental bronchus); procedural details, including medication and dosing, are specified
1090 in the Microbiome Bronchoscopy MOP. Of the five brushings, four will be stored in
1091 RNALater and one will be stored in 35% glycerol. Brushings will be shipped in batches
1092 at -80° C to UCSF for microbiome analysis (See Section I, Microbiologic Variables).
1093 These will serve as baseline samples for analysis prior to ICS or placebo intervention in
1094 the asthmatic group. An aliquot of BAL fluid will be taken and processed at the center
1095 for total and differential cell count. Two additional aliquots of BAL fluid will be added to
1096 RNALater for microbiome analysis. The balance of BAL fluid will be centrifuged, the cell
1097 button resuspended in labeled BAL immunophenotyping collection vials containing 1 mL
1098 Streck Cell Preservative and shipped to UCSF for FACS analysis on the following day.
1099 Five 10 mL aliquots of BAL supernatant, 2 into tubes containing RLT (lysis) buffer (for
1100 Viral RNA preservation), will be stored at -80° C and later batched for shipment to UCSF
1101 for cytokine analysis and for forwarding to AsthmaNet sample biobank.

1102
1103 Immediately after the bronchoscopy has been completed, each nostril will be sprayed
1104 with a small volume of nebulized 2% lidocaine solution, and the surface of the floor and
1105 inferior nasal turbinate of both nares will be brushed with a standard cytology brush and
1106 the brushes placed in RNALater for 24h before storage at -80° C. These brushes will be
1107 stored in RNALater and labeled, processed, and shipped as are the bronchial brushes.

1108
1109 Albuterol MDI (2-4 puffs) or nebulizer (2.5 mg premix solution) will be administered to
1110 participants with dyspnea, wheeze, chest tightness, or hypoxia post-bronchoscopy and
1111 as needed. All subjects will be observed for 2-4 hours after bronchoscopy and
1112 discharged home if their FEV₁ has returned to within $\geq 90\%$ of their baseline on arrival. If
1113 this criterion is not met, a physician must evaluate the subject to decide if they are
1114 stable for discharge. All subjects will be contacted the evening and day after
1115 bronchoscopy and will return for evaluation if any significant adverse events are
1116 reported. Healthy subjects will be discharged from the study at this point. At the end of
1117 the visit, asthmatic subjects will be assigned at random (in a 2:1 ratio) to receive a
1118 Diskus inhaler delivering 250 mcg of fluticasone per puff or an identical-appearing
1119 placebo inhaler, with instructions to take one puff twice daily for six weeks.

1120
1121 Visit 3. Subjects will return to the study center 14-21 days after randomization for repeat
1122 spirometry, for analysis of the number of inhalations taken from the Diskus inhaler (from
1123 the dose counter on each Diskus device) and for reinforcement of the importance of
1124 adhering to treatment. Subjects who demonstrate $> 75\%$ adherence with inhaler use will

1125 continue per the study schedule. Subjects who do not will be instructed to improve
 1126 adherence and return for a repeat Visit 3 10-14 days later. At this visit, inquiry will also
 1127 be made as to whether subjects have had any symptoms of worsening asthma control,
 1128 difficulties with inhaler use, or adverse events. Twelve to 18 days after Visit 3
 1129 (approximately four weeks after randomization), the subjects will be contacted by
 1130 telephone for the same inquiries made at the two week visit.

1131
 1132 Visit 4 (study week 6). Five weeks after randomization, and while still receiving the
 1133 treatment intervention, asthmatic subjects will return for measurement of spirometry and
 1134 bronchial reactivity (PC₂₀ Mch), and collection of induced sputum preceded by oral
 1135 saline rinse. Bronchoscopy will be scheduled for the following week in subjects who
 1136 meet safety criteria for the procedure. Subjects will continue on their assigned study
 1137 inhaler, 1 puff twice daily.

1138
 1139 Visit 5. An interval history will be taken and a brief examination performed. Spirometry
 1140 before and after administration of 4 puffs of albuterol will be performed. A sample of 10
 1141 ml of blood will be collected same as at visit 2 and sent to UCSF for FACS analysis on
 1142 the following day. Fiberoptic bronchoscopy with five protected bronchial brushings and
 1143 a 200 ml bronchial lavage will then be performed. Bronchoscopy procedures will be
 1144 identical to those of the first, baseline bronchoscopy. The samples collected will be
 1145 compared to those collected prior to intervention for changes in the bronchial
 1146 microbiome, and in the cell and cytokine content of BAL fluid. At completion of the
 1147 bronchoscopy, nasal brushings will be obtained as they were at visit 2. All subjects will
 1148 be given open fluticasone treatment (250 mcg/puff) to take twice daily after
 1149 bronchoscopy up until returning for Visit 6.

1150
 1151 Visit 6. Subjects will return to the study center for brief history and physical examination,
 1152 inquiry as to adverse effects from participation, and measurement of FEV₁ and FVC
 1153 prior to discharge from the study. At the end of this visit, the subject will be informed of
 1154 the degree of severity and control of their asthma, as inferred from their examination by
 1155 a study physician and from their pulmonary function test results, and will be advised as
 1156 to the recommended level of treatment. The subject will be given a letter summarizing
 1157 this information and, if the subject wishes, a prescription for the recommended
 1158 treatment. This information will also be given at the final visit of healthy subjects and
 1159 asthmatic subjects who do not complete the study.

1160

1161 **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

1162
1163 Actual visit times may vary slightly. *Asthmatic subject visits only. ¹Phadiatop and total
1164 IgE at V0, allergen-specific IgE at V1 for participants with positive Phadiatop;
1165 ²Spirometry pre- and post-bronchodilator (4 puffs albuterol); ³Healthy Controls only;
1166 ⁴Methacholine challenge procedure details, including medication and dosing, found in
1167 AsthmaNet Methacoline MOP; ⁵Bronchoscopy procedure details, including medication
1168 and dosing, found in Microbiome Bronchoscopy MOP; ACQ: Asthma Control
1169 Questionnaire, IgE: immunoglobulin E; PC₂₀: methacholine challenge, CBC: complete
1170 blood count and differential, BUN: blood urea nitrogen, ECG: electrocardiogram (for
1171 patients >45 y.o.)
1172

1173 K. Rationale for Data Collection and Procedures

1174 Clinical and Biologic Variables:

- 1176 1. History and physical exam will establish safety for entry into the study and for
- 1177 participation in study-related treatments and procedures.
- 1178 2. Standardized questionnaires used in previous ACRN and AsthmaNet studies will
- 1179 provide information on age of onset of asthma, prior treatments, exacerbation

- 1180 history, response to viral respiratory infections, sputum/phlegm production and
1181 other historical features of asthma, sometimes used in defining possible asthma
1182 phenotypes.
- 1183 3. Pregnancy test. To eliminate risk of teratogenicity, pregnancy testing will be used
1184 throughout the study due to the use of methacholine (pregnancy class C), and
1185 the performance of bronchoscopy with the use of midazolam (class D) and
1186 fentanyl (class C). A medically-acceptable form of contraception will be required
1187 of asthmatics throughout the study.
 - 1188 4. Asthma Control Questionnaire (ACQ) will be used in the asthmatic subjects to
1189 monitor asthma control during the run-in and throughout the period of ICS vs.
1190 placebo inhalation treatments. ACQ-6 scores (i.e., without scoring for PEF) will
1191 be used for exploratory analysis of associations between microbial community
1192 composition and ACQ score at baseline and the change in ACQ with ICS
1193 treatment.
 - 1194 5. IgE and Phadiatop test will be obtained as a baseline phenotypic variable to
1195 characterize atopy. Specific IgE will be measured to a panel of aeroallergens in
1196 Phadiatop-positive subjects.
 - 1197 6. Plasma and serum separated from a 10 ml sample of venous blood from each
1198 subject will be stored at -80°C for later analysis of potential biomarkers
1199 associated with asthma phenotypes (e.g., the “TH2” and “non-TH2” molecular
1200 phenotypes of asthma.”
 - 1201 7. Induced sputum. A cell count and differential will be measured as baseline
1202 phenotypic variables, allowing classification of the asthmatic subjects as
1203 “eosinophilic” (>2% sputum eosinophils), “neutrophilic” (>60% sputum
1204 neutrophils), or pauci-granulocytic (<2% eos; <60% neutrophils). Analysis of
1205 induced sputum samples from over 1,000 asthmatic subjects enrolled in ACRN
1206 studies has shown that the proportions of these “inflammatory phenotypes” are
1207 roughly 25%, 20%, and 50%, respectively, in both ICS-treated and ICS-naïve
1208 subjects⁶⁶. DNA will be extracted from induced sputum cell pellets for PhyloChip
1209 analysis for comparison of the microbial community composition of induced
1210 sputum to the microbial community composition of bronchial brushings in 10
1211 healthy and 10 asthmatic subjects. The DNA from these samples will be stored at
1212 -80°C , as will the cell pellets from the other sputum samples collected, as a
1213 resource for later analysis.
 - 1214 8. Circulating immune cells: The blood sample obtained at the time of insertion of
1215 intravenous access immediately prior to bronchoscopy will be assayed by 11-
1216 color FACS analysis for enumeration of innate T cells and CD4 T cell subsets
1217 identified by surface receptor expression as Th1, Th17, and Th2, and numbers of
1218 CD8 T cells, NK cells, B cells, monocytes, basophils, eosinophils, and
1219 neutrophils (see section B.4.a, above). The absolute number and relative
1220 distribution of these cells types will be analyzed for associations with clinical
1221 classification (allergic asthmatic; allergic non-asthmatic; non-allergic, non-
1222 asthmatic) and for associations with stool microbial community composition.
 - 1223 9. Bronchial epithelial cell gene expression: the RNA extracted from the cells
1224 recovered from the protected bronchial brushings (95-97% epithelial cells) will be
1225 analyzed by QT-PCR for expression of Serpin B2, CLCA-1, and Periostin, the trio

1226 of genes upregulated in the bronchial epithelium of the TH2-molecular phenotype
1227 of asthma, described by Woodruff and Fahy⁶⁴. This will be analyzed for
1228 associations with microbial community composition in the bronchial epithelial
1229 brushing and in stool samples,, for associations with the number and distribution
1230 of circulating immune cells (see #8, above) and for associations with the immune
1231 response state of the lung, as reflected by BAL fluid content of inflammatory cells
1232 and cytokines (see #10, below)
1233 10. BAL cells and cytokines: The inflammatory cell content of BAL fluid will be
1234 measured by FACS analysis by the method described in #8, above. The levels of
1235 cytokines in BAL fluid (L-1beta, 2, 3, 4, 5, 6, 8, 9, 10, 13, 17, IFN-gamma, TNF-
1236 alpha, Eotaxin, GM-CSF, IL-21, 23, 33, TSLP), will be measured by Luminex
1237 multiplex cytokine ELISAs. The cytokine levels will be used for constructing
1238 logistic regression models predicting distinct asthma phenotypes, as was done in
1239 a previous study by the Severe Asthma Research Program¹². This will in turn
1240 enable analysis as to whether those phenotypes are associated with differences
1241 in bronchial microbiome, circulating and/or BAL immune cell populations, and
1242 stool microbiome.
1243 11. Nasal Brushings: No analysis of these samples is proposed in this protocol.
1244 They are obtained for storage to be available for later study of the relationships
1245 among the nasal and bronchial microbiomes and epigenomes, as described
1246 above (see lines 163-185).

1247
1248
1249

Physiologic Variables

1250 1. Spirometry and bronchodilator response. These standard physiologic parameters
1251 will be collected to characterize subjects at baseline and throughout the study.
1252 The change in pre-bronchodilator FEV₁ from baseline to the value measured
1253 after 6 weeks of ICS therapy will be used to classify subjects assigned to ICS
1254 treatment as “ICS-responsive” or “ICS non-responsive” based on a $\geq 5\%$ or $< 5\%$
1255 improvement in FEV₁.
1256 2. Methacholine PC₂₀. This physiologic variable will be utilized as an entry criterion
1257 to confirm the diagnosis of asthma at the time of screening. A threshold PC₂₀
1258 value of ≤ 8 mg/mL will be used for asthmatics. This parameter will also be
1259 measured at the end of the active treatment periods and will be used as a
1260 second, although exploratory marker for examination of possible differences in
1261 the bronchial microbiome of ICS “responders” vs. “non-responders,” as reflected
1262 by a \geq one step increase in PC₂₀Mch (a doubling dose).

1263
1264

Microbiologic Variables

1265 1. Stool sample collection: for analysis of microbial community composition.
1266
1267 2. Bronchoscopy: All subjects will undergo bronchoscopy for 5 protected bronchial
1268 brushings and a bronchial lavage. Two of the brushings will be analyzed for total
1269 and differential cell count; three will be processed for extraction of DNA and
1270 RNA. The DNA will be processed further for 16S rRNA gene amplification and
1271 analysis of bacterial taxa by PhyloChip (see above). RNA will be analyzed for

1272 quantification of IL13-dependent genes (CICA-1, Serpin B2, Periostin) by QT-
1273 PCR for classification of each subject as of the “TH2” vs. “non-TH2” molecular
1274 phenotype. The balance of the extracted DNA will be stored at -80°C for possible
1275 later detection of fungal organisms (by ARISA, sequencing, or “MycoChip”); RNA
1276 will be similarly stored for possible future metatranscriptomic analysis to permit
1277 inferences about pathways activated at the interface between the airway
1278 microbiome and the cells of the airway epithelium.
1279

1280 The bronchial lavage sample (collected by instilling and suctioning back 4 x 50 ml
1281 of warmed normal saline through a wedged bronchoscope) will be aliquotted into
1282 tubes prepared by addition of RLT (lysis) buffer, and stored at -80°C for possible
1283 later analysis for detection and characterization of viruses, as by “ViroChip” or
1284 deep sequencing methods.
1285

1286 **Environmental Variables**

1288 To assess activities or exposures that might alter bacterial colonization of the
1289 airways, we will modify an existing questionnaire developed by Michael Cabana,
1290 MD for his study of probiotic supplementation of neonates at high risk for asthma.
1291 This questionnaire inquires about activities that might affect bacterial exposures,
1292 such as household or occupational exposure to infants (<3 yrs) or young children
1293 (<12 yrs), household exposure to pets or other animals, exposure to soil (e.g.,
1294 gardening), caring for livestock, chickens, or other domesticated animals,
1295 woodburning (in household fireplace), and environmental tobacco smoke.
1296 Similarly, to assess dietary habits that might alter bacterial colonization of the
1297 gastro-intestinal tract, a standard questionnaire on dietary habits will be
1298 administered, with particular attention to consumption of fermented foods (e.g.,
1299 “live culture” yogurts). Subjects will be instructed not to modify their dietary habits
1300 while participating in this study, and will be asked specifically about recent
1301 ingestion of live-culture yogurts at study visits.
1302

1303 **Safety Variables:**

- 1304 1. CBC with differential cell count. To collect baseline phenotypic data with regard
1305 to eosinophil and neutrophil counts.
- 1306 2. Renal function (BUN and creatinine). Impaired creatinine clearance is associated
1307 with an increased risk of bleeding at the time of bronchoscopy and is an
1308 exclusion criterion. Serum creatinine will be measured at baseline.
- 1309 3. Electrocardiogram. An ECG will be performed at visit 1 in all subjects > 45 yrs of
1310 age, to allow identification of ischemic changes, an exclusion criterion for
1311 research bronchoscopy.
1312

1313 **L. Adherence monitoring**

1314 The number on the dose counter on the DPI ICS/placebo delivery device will be noted
1315 at each visit as a way of tracking adherence with the twice daily use required of the
1316 asthmatic subjects enrolled in this study. Adherence also will be determined (and

1317 encouraged) at each visit by direct inquiry.

1318

1319 **M. Recruitment and Feasibility**

1320 Based on the experience of the AsthmaNet Centers that participated in the ACRN
1321 “Macrolides in Asthma” protocol, and/or in other studies involving bronchoscopy, we
1322 believe that we will be able to identify subjects who meet the enrollment criteria for this
1323 study. The ACRN experience with the 83 subjects in the PRICE study suggests that at
1324 least 1/3 of steroid-naïve asthmatics will fail to show a $\geq 5\%$ improvement or \geq a one
1325 step increase in their PC₂₀ Methacholine.

1326

1327 **N. Statistical Analysis**

1328 Primary outcome variables include both gross descriptive metrics of microbial
1329 community composition (richness, evenness, diversity) and relative abundance of
1330 bacterial taxa of the bronchial and stool microbiome. These metrics are obtained by
1331 reduction of the complex data sets generated by microbiota profiling for the purposes of
1332 analysis. Each sample will be represented by three calculated indices (richness,
1333 evenness and diversity) that reflect the gross composition of each bacterial community.
1334 For every sample analyzed by PhyloChip, community richness - the number of taxa
1335 detected in the sample - will be determined by using a positive fraction cut off of > 0.9 ;
1336 data matrices including only taxa that fulfill this criterion will be imported into the “R”
1337 statistical environment and metrics calculated for each sample. In particular:

1338

- Richness is the total number of taxa detected.
- Shannon diversity²⁹ (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 index⁶⁷ (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.

1349

1350 All indices will be calculated using the Vegan package in the “R” statistical environment.
1351 The relative abundance of discrete taxa detected will be assessed by measurement of
1352 fluorescence intensity, and will be compared among groups by ANOVA.

1353

1354 Secondary outcome variables, including measures of phenotypic features of asthma,
1355 are described earlier (see section H. above). Other second outcome variables are the
1356 measures of systemic immune function, as reflected by FACS enumeration of the
1357 numbers of innate T cells and CD4 T cell subsets identified by surface receptor
1358 expression as Th1, Th17, and Th2, and numbers of CD8 T cells, NK cells, B cells,
1359 monocytes, basophils, eosinophils, and neutrophils in blood and in BAL fluid.

1360

1361 Addressing the **Primary Research Hypotheses** (and corresponding Specific Aims) will
1362 entail the evaluation of differences in bronchial microbial community composition in
1363 relation to allergic asthmatic vs. allergic non-asthmatic, and non-asthmatic vs. non-
1364 allergic non-asthmatic status. Additional research hypotheses to be addressed include
1365 evaluation of differences in stool microbial community composition in the groups,
1366 differences in bronchial microbial community composition after treatment with an
1367 inhaled corticosteroid, and evaluation of the relationship of bronchial or stool
1368 microbiome to the collected metadata, such as measures of phenotypic features of
1369 asthma (clinical, physiologic and inflammatory), measures of systemic immune function,
1370 measures of pulmonary immune response.
1371

1372 We will evaluate differences in bronchial microbial communities first by comparing the
1373 values for community **structure** – richness, evenness, and diversity (see above).
1374 Differences in these values for the groups being compared (allergic asthmatic vs.
1375 allergic non-asthmatic, and allergic non-asthmatic vs. non-allergic non-asthmatic
1376 subjects at baseline for **Specific Aim 1**) will be tested for significance by pairwise
1377 comparison within the ANOVA framework, or Kruskal-Wallis if there is marked
1378 maldistribution. These two comparisons will be made at the 0.025 significance level. We
1379 will additionally assess possible differences in bacterial community **composition** by
1380 testing for differences in the relative abundance of discrete taxa between the groups,
1381 i.e. identifying specific taxa that characterize each group. We will do this by comparing
1382 the mean fluorescence intensity for each detected taxon in the three groups, using log-
1383 transformed array data and a row-based custom script in R. The output from this
1384 multiple testing will then be corrected for false discovery using the q-values approach⁶⁸.
1385 Developed for genome-wide tests of significance to correct for multiple comparisons, q-
1386 values indicate the likelihood that a significant finding arose by chance, reducing the
1387 incidence of Type I error (false positives) in the dataset. In contrast to reporting a false
1388 positive rate based on the total number of tests, q-values permit determination of a false
1389 discovery rate only among the significant tests. A less conservative approach than, for
1390 example, Bonferroni correction, in determining truly significant tests, the use of q-values
1391 to control for false discovery has been effectively applied in prior PhyloChip-based
1392 metagenomic studies^{14, 29, 69}.
1393

1394 In comparing the bronchial microbiome of asthmatic, allergic, and healthy subjects, we
1395 will need to consider the possible confounding effects of differences in environmental
1396 microbial exposures. Examples might include exposure to differences in the microbial
1397 content of outdoor atmospheric aerosols⁷⁰ or of indoor aerosols that might be related to
1398 pet ownership. We have demonstrated differences in the microbial community
1399 composition in house dust obtained from pet-keeping vs. pet-free households⁷¹ and pet
1400 ownership has been associated with a decreased risk of development of childhood
1401 asthma⁷². Other investigators also have shown that exposure to a microbe-rich farming
1402 environment is associated with a decreased prevalence or risk of asthma⁷³. However,
1403 whether differences in microbial exposure to any environmental factor (other than
1404 tobacco smoke) affect the bronchial microbiome has not been studied. We therefore
1405 propose to apply methods such as hierarchical cluster analysis or non-metric
1406 dimensional scaling, and multivariate regression to reveal characteristics of bronchial

1407 microbial community composition that are associated with activities or exposures such
1408 as household or occupational exposure to infants (< 3yrs) or young children (< 12 yrs),
1409 household exposure to pets or other animals, soil (e.g., gardening), caring for livestock,
1410 chickens, or other domesticated animals, wood burning (household fireplace),
1411 environmental tobacco smoke, and consumption of fermented foods (e.g., “live culture”
1412 yogurts), that are related to specific bacterial colonization patterns in the airways.

1413
1414 Given the very high rates of pet-keeping among U.S. households, exclusion of all pet-
1415 exposed subjects would impair recruitment into this study. In addition, limiting the study
1416 population to those without pets, might reduce variability in the bronchial microbiome
1417 and therefore, might obscure differences in the microbiome which we hypothesize is
1418 associated with asthma status. A similar argument can be made for why there should
1419 be no limitation on other environmental exposures or activities (other than smoking) –
1420 their exclusion could limit variability.

1421
1422 Accordingly, we propose that the first step is to analyze the richness, evenness,
1423 diversity and relative abundance of bacterial taxa in the bronchial microbiome and their
1424 relationship to allergic asthmatic, allergic non-asthmatic, or non-allergic healthy status.
1425 A subsequent step will be to examine factors associated with these measures of the
1426 bronchial microbiome, including pet ownership and clinical and inflammatory phenotypic
1427 features of asthma. This can be accomplished by distance-based permutational
1428 multivariate analysis of variance (R package *vegan*, function *adonis*⁷⁴), or regression-
1429 based canonical correspondence analysis⁷⁵, two approaches commonly applied in
1430 ecological studies to determine relationships between community composition and
1431 environmental variables.

1432
1433 To evaluate whether treatment of asthmatic subjects with inhaled corticosteroid alters
1434 the bronchial microbial community in **Specific Aim 2**, we will compare the calculated
1435 microbial community metrics (richness, evenness, diversity) in the ICS and placebo
1436 treated groups at the end of the intervention period by analysis of covariance using
1437 treatment assignment as the factor and baseline microbial community metrics as
1438 covariates. We will also examine interaction effects between treatment assignment and
1439 covariates to explore whether the baseline microbial community might modify any ICS
1440 effect. In the event that the outcomes are mal-distributed beyond what can be corrected
1441 via transformation, we will compare treatment groups using the Wilcoxon rank-sum test.

1442
1443 We will also compare the calculated microbial community metrics before and after ICS
1444 intervention by paired t-test or alternatively, Wilcoxon signed rank test. We will also
1445 evaluate for differences in the bacterial taxa present and changes in the relative
1446 abundance of all detected taxa before and after ICS treatment. As described for Specific
1447 Aim 1, we will compare the mean relative abundance of each detected taxon before and
1448 after treatment by paired t-test, followed by correction for false discovery using the q-
1449 value approach. Finally, we will evaluate whether ICS treatment affects the phylogenetic
1450 relatedness of bacterial communities by comparing the calculated community metrics,
1451 Nearest-taxon and Net-relatedness indices (NTI and NRI; R package *picante*)⁷⁶. These
1452 indices provide continuous measures of how closely related detected communities are

1453 and at what phylogenetic level of similarity, and will be compared by paired t-test or
1454 Wilcoxon signed rank-sum test. This allows for assessment of whether ICS might
1455 promote the appearance or increased abundance of closely-related bacterial organisms,
1456 a pattern that has been described in response to treatment interventions or in pathogen
1457 colonization of a given niche^{69, 77, 78}.

1458
1459 For **Specific Aim 3**, to determine whether differences in bronchial microbial community
1460 composition at baseline or after ICS treatment are associated with differences in
1461 responsiveness to the treatment, we first will consider the primary response variables as
1462 physiologic measures, such as change in post-bronchodilator FEV₁ % predicted,
1463 change in PC₂₀ Mch, and change in sputum eosinophil percentage. Then we will
1464 evaluate by two approaches whether the pre- or post-treatment bronchial microbial
1465 community is associated with changes in these response variables. In the first
1466 approach, we will determine correlations (Pearson or Kendall) between the relative
1467 abundance of each taxa found at baseline and the change in each of the above
1468 response variables. This will be performed also using abundance data for the taxa
1469 detected post-ICS treatment, as well as using the change in relative abundance with
1470 treatment. Similar to as described for Specific Aim 1, a row-based custom script for
1471 correlation testing with each detected taxon will be performed (R package *multtest*),
1472 followed by false discovery correction using q-values.

1473
1474 The second approach by which we will assess relationships between bronchial
1475 microbiota composition and treatment response will be in the following manner. First,
1476 the microbial dataset will be reduced to include only those taxa that demonstrate a
1477 significant change in relative abundance with ICS treatment (specific significance
1478 criteria to be determined based on the p- and q-values indicating a reasonably low false
1479 discovery rate, typically 5% or less based on recent studies^{14, 29, 69}). Second, Bray-Curtis
1480 distance matrices, a measure commonly used in ecological analyses, will then be
1481 constructed from this reduced dataset⁷⁹. These will then be used for non-metric
1482 multidimensional scaling (NMDS)^{79, 80}, a distance-based ordination method that, as
1483 applied here, essentially will “map” the relatedness of samples, based on how dissimilar
1484 the microbial community composition of each is relative to all other samples. Based on
1485 the resulting distance matrices, permutational multivariate analysis of variance (R
1486 package *vegan*, function *adonis*)⁷⁴ will then be conducted to evaluate whether the
1487 response variables of interest are associated with the observed changes in microbial
1488 community composition with treatment.

1489 For **Specific Aim 4**, to evaluate whether phenotypic features of asthma are associated
1490 with differences in bronchial microbial community composition. The phenotypic features
1491 of interest include clinical features, such as history of exacerbations, response to the
1492 “cold questionnaire” on frequency of worsening of asthma with viral URI’s, and ACQ
1493 score. Other phenotypic features of interest are physiologic measures (e.g. FEV₁ %
1494 predicted, FEV₁ reversibility, PC₂₀ Mch), inflammatory markers (e.g. sputum eosinophil
1495 and neutrophil %), and bronchial epithelial cell expression of the genes of the “Th2
1496 molecular phenotype.” We will apply both correlation analysis and ordination methods,
1497 as described above for Specific Aim 3. The former will entail comparisons between

1498 continuous phenotypic variables and continuous microbial community metrics (richness,
1499 evenness, diversity, and species relative abundance). Ordination is widely applied in
1500 ecological analyses to reveal relationships between communities and environmental
1501 characteristics. We will apply NMDS ordination and permutational multivariate analysis
1502 of variance⁷⁴ as described for Specific Aim 3. This model will also allow us to test
1503 dichotomous data or continuous data parsed by relevant cutoffs for certain variables,
1504 such as PC₂₀ Mch < 2 mg/ml vs. ≥ 2 mg/ml, or sputum eosinophils < 2% vs. ≥ 2%.

1505 Conversely, for **Specific Aim 5**, we will explore whether there are natural grouping
1506 patterns in the bronchial microbial community data, and if so, evaluate how these
1507 patterns relate to phenotypic features of asthma. To do this, we will use the entire
1508 microbial community dataset (after filtering for taxa that are present in at least one
1509 subject) and compute Bray-Curtis distance measures for hierarchical cluster analysis
1510 and canonical correspondence analysis or nonmetric multidimensional scaling. If distinct
1511 microbial community group patterns are revealed, between-group differences in the
1512 phenotypic data associated with samples in the groups will be assessed. This will
1513 include using multivariate analysis of variance (function *adonis*⁷⁴) and least squares
1514 fitting of phenotypic variables (function *envfit*) to the ordination models. We will use
1515 very similar methods to explore whether natural grouping patterns in bronchial microbial
1516 community composition are related to features of environmental exposure, such as
1517 exposure to infants, children, household pets, wood-burning fires, to activities such as
1518 gardening, or to consumption of fermented foods (see “Environmental Variables,
1519 Section I, above).

1520
1521 As a secondary Research Hypothesis (Specific Aim 6), we will assess the concordance
1522 of the microbial community composition detected in induced sputum vs. bronchial
1523 brushings in a subset of healthy and asthmatic subjects. In baseline (pre-treatment)
1524 samples, we will evaluate both the community richness and specific composition
1525 profiled by microarray. Based on preliminary data on comparison of sputum and
1526 bronchial brushings (see section D), we will consider 90% or greater within-subject
1527 overlap in the specific taxa detected in both specimen types as highly concordant.

1528
1529 Our general approach to analysis of the additional aims of this expanded study,
1530 especially determination of whether relationships exist between datasets (e.g. airway
1531 microbiome composition and cytokine profile, or stool microbiome composition and
1532 numbers and distribution of inflammatory cells) will be to construct non-metric
1533 dimensional scaling plots based on distance matrices for each dataset and to examine
1534 them using the Mantel test, which permits determination of statistical correlations
1535 between two sets of data matrices. This will provide initial indications that community
1536 composition is correlated with other metadata sets generated in the course of this study.

1537
1538
1539 Our approach to analysis of specific Aim 7a, which compares stool microbial
1540 composition among allergic asthmatic, allergic non-asthmatic, and non-allergic non-
1541 asthmatic subjects, we will make use of the same approaches and statistical tools as

1542 outlined above for specific aims 1 and 2, For analysis of specific aims 7b, 8, and 9, we
1543 will use methods similar to those proposed for analysis of specific aims 3-6.

1544
1545 **O. Sample Size**

1546 For this study, the total number of proposed subjects to be studied is 84 (42 allergic
1547 asthmatics, 21 allergic non-asthmatic and 21 non-allergic non-asthmatic healthy
1548 subjects). The primary hypotheses involve the comparison of bronchial microbial
1549 community composition (as sampled by protected bronchial brushings) among the three
1550 subject groups at baseline (allergic asthmatic vs. allergic non-asthmatic and allergic
1551 non-asthmatic vs. non-allergic non-asthmatic), and evaluation for alterations in bronchial
1552 microbial community composition by ICS treatment. In the “Add-On” study to the MIA
1553 trial¹⁴, a significant difference in bronchial bacterial diversity was observed between
1554 asthmatic and healthy subjects (difference in mean Shannon diversity index of 0.6
1555 units). A sample size of 42 allergic asthmatic and 21 allergic non-asthmatic subjects will
1556 provide >90% power to detect a difference of 0.6 units in mean diversity. This
1557 calculation is based on two, 2-sided tests with $\alpha=0.025$, a common standard
1558 deviation for the diversity index of 0.47 (based on data from asthmatic and healthy
1559 groups in the MIA “Add-On” study).

1560
1561 The effect of ICS use on bronchial microbial community composition has not been
1562 previously examined. We hypothesize that ICS use will alter microbial community
1563 diversity and composition, and reason that a 0.4 unit difference in the mean Shannon
1564 diversity index may be important. This value is extrapolated from the observation in the
1565 MIA Add-On study¹⁴ that among asthmatics treated with clarithromycin, there was a
1566 significant difference of 0.4 units in bacterial diversity between subjects who did or did
1567 not demonstrate an improvement in bronchial reactivity (defined as at least a doubling in
1568 the dose of Mch PC₂₀). We reason therefore that this difference in bronchial microbial
1569 diversity may also be clinically meaningful in examining the effect of ICS use. The
1570 proposed sample size of 28 asthmatics treated with ICS and 14 asthmatics treated with
1571 placebo will provide 84% power to detect a difference of 0.4 units in the mean Shannon
1572 diversity index. This calculation is based on assuming a 2-side test with $\alpha=0.05$, a
1573 common standard deviation for the diversity index of 0.37 (based on data from the MIA
1574 “Add-On” study; asthmatic, clarithromycin-treated subgroup), and allowing for a 15%
1575 dropout rate.

1576
1577 For analysis of the effect of ICS on bronchial microbial diversity before and after
1578 treatment, the proposed sample size of 28 asthmatic subjects will provide $\geq 95\%$ power
1579 to detect a change in the diversity index of 0.4 units with ICS treatment (same effect
1580 size as assumed for the ICS vs. placebo analysis above). This calculation assumes a
1581 paired test with $\alpha=0.05$, common standard deviation in the diversity index of 0.37,
1582 and a 15 or 20% dropout rate.

1583
1584 The power analyses above are based on best-available data and experience. It is
1585 noted, however, that for metagenomic microbial studies, there are no validated
1586 approaches for sample size calculation, related in part to the unclear biological
1587 significance of, for instance, a unit change in community diversity (or in community

1588 richness or evenness). Nonetheless, for the purposes of this study, effect sizes based
1589 on community diversity were chosen, as this feature was noted in the study by Huang et
1590 al¹⁴ to differ significantly between asthmatic and healthy subjects, as well as to correlate
1591 positively with methacholine PC₂₀ measures of bronchial hyperresponsiveness, a
1592 clinically relevant, pathophysiologic feature of asthma.
1593

1594 Additional studies involving relatively small sample sizes and utilizing high-resolution
1595 tools for bacterial community profiling, have identified significant relationships between
1596 the microbiota in a given niche (e.g. the airway or gut) and clinical/phenotypic features
1597 of disease. For example, in an age-stratified cross-sectional study of 51 subjects with
1598 cystic fibrosis, microarray analysis illustrated that the airway microbial community
1599 become less even and less diverse with increasing patient age and impairment of
1600 pulmonary function⁷⁷. Longitudinal sample analysis from 13 of the patients found initial
1601 diversification of the bacterial community among younger CF patients compared to a
1602 progressive loss of diversity over time among older patients. In a preliminary analysis of
1603 25 sputum samples from COPD patients, the significant changes in bacterial diversity
1604 that occurred over time correlated with clinical symptom scores (Y. Huang, unpublished
1605 data). Another illustration of the findings made possible by this approach to microbial
1606 detection is Dr. Lynch's recent study of chronic rhinosinusitis (CRS) involving 14
1607 subjects. The seven with CRS, compared to seven healthy patients, exhibited significant
1608 decreases in sinus microbiota diversity and a coincident significant increase in the
1609 relative abundance of a single *Corynebacterium* species. Subsequent modeling in a
1610 murine model confirmed that reduced microbiota diversity was essential to susceptibility
1611 to inoculation with this species of *Corynebacterium*, with the resulting infection
1612 replicating the pathophysiological and immunological features of the disease in the
1613 infected mice (S. Lynch, personal communication, manuscript submitted 2012). Finally,
1614 in a recent study evaluating statistical approaches for analyzing metagenomic microbial
1615 community data, even undersampled communities demonstrated differential diversity
1616 patterns, which were significantly associated with relevant environmental variable
1617 gradients⁸¹. This indicates that even with relatively small sample sizes, relevant
1618 relationships between the microbial community and phenotypic variables can be
1619 revealed. Collectively, the above examples coupled with the power analyses provided,
1620 support the likelihood that the planned sample size for this study will be able to
1621 successfully evaluate the hypotheses.
1622

1623 **P. Risks**

1624 **1. Asthma Exacerbations**

1625 This study will enroll asthmatics who are not taking controller maintenance therapy at
1626 entry and whose ACQ-6 (i.e., without score for FEV₁ or PEF) is <1.5, so – even though
1627 these subjects will have a history of asthma of no greater than mild-moderate severity,
1628 the possibility exists that subjects may experience one or more exacerbations during the
1629 study period, especially those randomized to placebo inhaler treatment. Asthma
1630 exacerbations will be defined as the development of an increase in symptoms of cough,
1631 chest tightness, and/or wheezing in association with one or more of the following: (1) an
1632 increase in rescue albuterol of ≥ 8 inhalations/day over baseline use for a period of 48
1633 hours or ≥ 16 actuations per 24 hours, with baseline defined as average daily use

1634 during the week prior to randomization; (2) a fall in FEV₁ to < 80% of baseline (visit 1);
1635 (3) FEV₁ < 50% predicted, or (4) if a subject receives systemic corticosteroids for an
1636 exacerbation from a non-study-related clinician. Subjects who are potentially
1637 experiencing an exacerbation will be instructed to contact the clinic coordinator and/or
1638 be evaluated at the study site or the nearest medical emergency facility as rapidly as
1639 possible. Subjects will be given handouts outlining what to do and who to call in the
1640 event of an asthma exacerbation.

1641
1642

1643 AsthmaNet rescue algorithms for subjects with exacerbations of asthma are based on
1644 recommendations from the NAEPP Guidelines for Diagnosis and Management of
1645 Asthma⁸²:

1646

1647 *Home care of exacerbations:* Asthma exacerbations will be identified by the criteria
1648 described above. Patients will be educated to recognize exacerbations as early as
1649 possible to facilitate prompt treatment and to lessen morbidity. Patients who recognize
1650 an exacerbation will be instructed to use albuterol by MDI, 2-4 puffs, every 20 min for
1651 60-90 min if needed. If symptoms do not improve after the first 60-90 min of therapy, the
1652 patient should contact the study coordinator, investigator, their primary physician, or
1653 seek care in the emergency department.

1654

1655 *Physician's Office or Emergency Room Treatment of exacerbations:* Patients will be
1656 assessed by history, physical examination, and by physiological monitoring including
1657 spirometry or PEF. If the patient's PEF or FEV₁ are less than 25% predicted or if the
1658 patient shows evidence of altered mental status, cyanosis, labored breathing, or use of
1659 accessory muscles, sampling of arterial blood for respiratory gas analysis is indicated,
1660 with appropriate action taken depending on the results obtained. When treated in the
1661 physician's office or the hospital emergency room, patients should initially be given
1662 albuterol by nebulization (0.5 cc of 0.5% solution) every 20 min over the first 60-90 min.

1663

1664 If the PEF increases to >65% of baseline after the first 60-90 min, the patient can be
1665 discharged to continue treatment at home. Prednisone may be administered at the
1666 discretion of the physician to augment therapy. If symptoms persist and PEF remains
1667 ≤65% baseline, nebulized albuterol should be continued as often as every hour and
1668 further treatment with oral or parenteral corticosteroids should be considered (e.g.
1669 prednisone 40 mg orally; methylprednisolone 40 mg IV bolus). Monitoring of PEF or
1670 spirometry should continue every hour. Within 4 hours of treatment, a decision should
1671 be made regarding patient disposition. If PEF increases to >65% baseline within 4
1672 hours, the patient can be discharged to continue treatment at home. Home treatment
1673 should include a 5-day course of prednisone (see below). If PEF remains >40% but
1674 ≤65%, an individualized decision should be made to hospitalize the patient for more
1675 aggressive therapy or to continue therapy at home with a course of prednisone. If PEF
1676 is ≤40% baseline after repeated albuterol treatments, the patient should be admitted to
1677 the hospital unless in the physician's best judgment alternative treatment could suffice.

1678

1679 *Prednisone Treatment:* In this protocol, prednisone will be used when acute
1680 exacerbations cannot be controlled by increased albuterol therapy alone. The dose of
1681 prednisone used during an acute exacerbation shall consist of 40 mg as a single oral
1682 dose every day for 5 days. The decision to initiate or to continue a course of prednisone
1683 beyond 5 days is left to the discretion of the physician.
1684

1685 *Exacerbations induced by bronchoscopy:* In rare cases, fiberoptic bronchoscopy may
1686 induce an asthma exacerbation. All cases of bronchoscopy-induced exacerbations will
1687 be treated with prednisone 40 mg po daily for 5 days. Should an exacerbation occur
1688 after bronchoscopy, a two-week recovery period will be imposed following the
1689 completion of prednisone therapy. Bronchoscopy will not be performed at Visit 5 if the
1690 participant experienced an exacerbation after bronchoscopy at Visit 2.
1691

1692 Bronchoscopy is associated with risks of the procedure and of conscious sedation. In
1693 this study, bronchoscopy will include five protected bronchial brushings and a standard
1694 200 ml (4 x 50 ml of warmed, normal saline solution) bronchial lavage. Bronchial
1695 brushing is associated with a low risk of minimal bleeding, and platelet count data will
1696 be available to the investigator prior to the procedure. Bronchial lavage is associated
1697 with the risks of coughing and slight worsening of asthma symptoms after the
1698 procedure. Conscious sedation poses risks of over-sedation and hypoventilation.
1699 Standard monitoring protocols will be used, and reversal agents will be readily available
1700 to reduce this small risk. The risks of worsening asthma symptoms will be minimized by
1701 pre-treatment with albuterol before bronchoscopy and by “as needed” administration of
1702 albuterol aerosol after the procedure.
1703

1704 Inhaled corticosteroids can cause dysphonia and oral pharyngeal candidiasis, but
1705 systemic side effects are not anticipated during a study of this duration. Subjects will be
1706 instructed in proper inhaler use including rinsing of the mouth afterwards.
1707

1708 There are no direct benefits to individual subjects, although it is possible that some
1709 asthmatic subjects may experience an improvement in symptoms if they receive ICS
1710 study drug. There is a potential benefit to patients with asthma in general as new bases
1711 for therapy may develop from this study.
1712

1713 **2. Bronchoscopy Safety**

1714 Bronchoscopy with five bronchial brushings and a bronchial lavage will be performed
1715 according to standard AsthmaNet procedures, with safety strategies similar to those
1716 previously employed by the NHLBI-sponsored Asthma Clinical Research Network.
1717

1718 Subjects must demonstrate a post-bronchodilator FEV₁ of $\geq 70\%$ to be eligible to
1719 undergo bronchoscopy. An upper age limit of 60 for this study has been selected as
1720 conservative and biased in the direction of subject safety. In addition to safety criteria
1721 outlined below, subjects must additionally be judged otherwise to be clinically
1722 appropriate for bronchoscopy by the bronchoscopist at the time of the procedure. Safety
1723 of the subject is the overriding concern in making this determination.

1724
1725 The presence of any of the following characteristics will exclude a subject from
1726 participating as a bronchoscopy volunteer:

1727
1728 *Events occurring within 6 months of bronchoscopy:* ED visit or hospitalization for
1729 asthma, or more than 2 exacerbations requiring systemic corticosteroid treatment.

1730
1731 *Events occurring within 48 hours of bronchoscopy:* pulse oximetry demonstrating
1732 oxygen saturation < 90% on room air, use of more than 8 puffs of a short acting
1733 beta-agonist per day for significant increase in asthma symptoms

1734
1735 *Events on day of bronchoscopy:* ACQ-6 score of >1.50.

1736
1737 **Hospitalization Indicators**

1738 For any subjects who exhibit any of the following characteristics during or after
1739 bronchoscopy, overnight hospitalization should be provided: significant cough persisting
1740 beyond 2 hours after completion of procedure, failure of PFTs after bronchodilator
1741 administration to return to within 15% of prebronchodilator FEV₁ at end of monitoring
1742 time, persistent hypoxia < 90% at end of monitoring time, persistent tachycardia > 130
1743 bpm at end of monitoring time, unexpected altered mental status during or after
1744 procedure, significant hemoptysis > 50 ml, or requirement for bronchodilator every 2
1745 hours on more than 3 occasions.

1746
1747 Treatment should be directed towards resolving underlying airway obstruction and
1748 symptoms, based on the best clinical judgment of the physicians involved. Follow-up
1749 telephone contact should be made for all subjects in the evening following
1750 bronchoscopy and 24 hours after the procedure is completed. If issues have not
1751 resolved in either group at the time of the last scheduled contact, additional contact and
1752 necessary medical care should be arranged.

1753
1754 **Q. Recruitment**

1755 Recruitment is performed by accessing established subject data banks, obtaining
1756 referrals, and local advertising. The Recruitment and Retention Committee will facilitate
1757 this process. However, what works for a given site may or may not work in a different
1758 geographic/population make-up. Standard print and radio advertisements will be geared to
1759 age groups and population characteristics. Notices will be placed in newspapers and as
1760 fliers at stores, student lounges, and hospital clinics. For all AsthmaNet protocols, at
1761 least 50% women and 33% minorities are required in the population of subjects
1762 enrolled. This was consistently met in ACRN and CARE trials. The clinical centers
1763 involved in AsthmaNet were chosen based in part on documentation of their capacity for
1764 enrollment of appropriate subjects.

1765
1766

1767 **IV. Human Subjects**

1768 **A. Subjects**

- 1769 1. General Description: study population and inclusion/exclusion criteria are as
1770 described in the protocol above.
1771
- 1772 2. Gender/Minority Inclusion: as stated above, at least 33% of subjects will be
1773 minority and 50% female. No subject will be excluded based on gender or ethnicity.
1774
- 1775 3. Exclusion of children: Because this study involves an invasive procedure,
1776 bronchoscopy, performed only for research purposes, children (<18 yrs old) will be
1777 excluded from participation.
1778

1779

1780 **B. Potential Risks and Procedures for Minimizing Risks**

- 1781 1. Pain and/or hematoma formation may occur at an intravenous puncture site.
1782 This is not a serious complication.
- 1783 2. Dizziness during blood sampling may occur. Subjects will be supine during blood
1784 sampling to avoid this problem.
- 1785 3. Spirometry may exacerbate bronchospasm, but in previous ACRN studies this
1786 has not been a serious problem. Subjects will be monitored closely during the
1787 procedure and an inhaled β -2 agonist will be administered if needed.
- 1788 4. Methacholine challenge causes bronchospasm, but subjects are monitored and
1789 testing stopped when the FEV₁ falls 20% from baseline and/or at the subject's
1790 request. An inhaled β -2 agonist is always administered after the procedure and
1791 response measured by spirometry.
- 1792 5. Induced sputum technique can cause bronchospasm. Standard AsthmaNet
1793 MOP for sputum induction, based on prior experience in the ACRN, extensively
1794 covers safety precautions for this technique, which we have used in multiple
1795 protocols without untoward problems. The precautions involve pre-treatment with
1796 4 puffs of albuterol and close monitoring of PEF and FEV₁ at intervals throughout
1797 the procedure.
- 1798 6. Nasal brushing causes transient itching or minor pain in the nose of 3-5 seconds
1799 duration. In about 5% of cases, it causes transient oozing of blood over the nasal
1800 mucosal surface and may result in blood-tinged nasal secretions. Actual epistaxis
1801 is unusual (< 1%) (personal communication, Pedro Avila, MD, Northwestern
1802 University)
- 1803 7. Bronchoscopy is associated with risks of the procedure and of conscious
1804 sedation. Bronchial brushing is associated with a minimal risk of bleeding, and
1805 platelet count data will be available to the investigator prior to the procedure.
1806 Conscious sedation poses risks of over-sedation and hypoventilation, and
1807 standard monitoring protocols will be used and reversal agents will be readily
1808 available.
- 1809 8. Inhaled Fluticasone treatment: inhalation of fluticasone is associated with the
1810 minor risks of hoarseness and of oropharyngeal candidiasis. The first resolves
1811 simply by stopping the inhaled corticosteroid treatment; resolution for the second

1812 can be accelerated by topical treatment with cotrimazole lozenges or oral rinse
1813 with nystatin. The risks of systemic absorption are minimal in this study because
1814 of the use of a moderate dose of an inhaled corticosteroid (500 mcg/d of
1815 fluticasone) for the short period of 6 weeks.

1816 9. Participation in research may involve a loss of privacy, but information will be
1817 handled as confidentially as possible. All records related to the study will be kept
1818 in a secure area in the investigators' office space and only research personnel
1819 will have access to the records. Computerized data will be password protected
1820 with access limited to the study personnel. Data will be released if it will help the
1821 urgent treatment of a subject.

1822
1823 **C. Adverse Events**

1824 An adverse event shall be defined as any detrimental change in the subject's condition,
1825 whether it is related to an exacerbation of asthma or to another unrelated illness.
1826 Adverse events related to asthma exacerbations will be managed according to rescue
1827 algorithms outlined above. Subjects will have handouts outlining what to do and who to
1828 call in the event of an asthma exacerbation.

1829
1830 An adverse event is deemed serious if it suggests a significant hazard, contraindication,
1831 side effect, or precaution. Serious adverse events include any experience that is fatal
1832 or life-threatening, is permanently disabling, requires or prolongs inpatient
1833 hospitalization, or is a congenital anomaly, cancer, or overdose. Serious adverse
1834 events must be reported to the DCC and the National Institutes of Health Project
1835 Scientist within 72 hours of notification. Once notified, the DCC will disseminate
1836 information about the event to the Data Safety and Monitoring Board and to the Steering
1837 Committee.

1838
1839 Adverse events due to therapy or concurrent illnesses other than asthma may be
1840 grounds for withdrawal if the illness is considered significant by the study investigator or
1841 if the subject is no longer able to effectively participate in the study. Subjects
1842 experiencing minor intercurrent illnesses may continue in the study provided that the
1843 nature, severity, and duration of the illness are recorded and that any unscheduled
1844 medications required to treat the illness are also recorded. Examples of minor
1845 intercurrent illnesses include acute rhinitis, sinusitis, upper respiratory infections, urinary
1846 tract infections, and gastroenteritis. Medications are allowed for treatment of these
1847 conditions in accordance with the judgment of the responsible study physician.

1848
1849 Documentation of adverse events will be recorded on an Adverse Event Report Form
1850 and will include the following information: description of the illness, dates of illness,
1851 treatment of illness and dates (medications, doses, and dose frequency), whether
1852 emergency treatment or hospitalization was required, and treatment outcome.

1853
1854 **D. Potential Benefits Gained From Data**

1855 The benefits resulting from this research include an improved understanding of the link
1856 between chronic airway colonization or infection by a consortium of microbial organisms
1857 and chronic asthma, including the impact of commonly prescribed inhaled corticosteroid

1858 therapy. This study may also suggest a relationship between gut microbial community
1859 composition with allergy, and with the circulating immune cells thought to mediate
1860 allergic disease, but no further, specific relationship with allergic asthma. This finding
1861 would increase interest in the possibility of manipulation of the gut microbiome as an
1862 approach to the treatment or prevention of allergic disease. This finding would also
1863 heighten interest in the role of local pulmonary events, possibly directly or indirectly
1864 mediated by the functional activity of microbial populations in the bronchial tree. Another
1865 possible finding of this study is of a difference in bronchial microbiome in different
1866 phenotypic subgroups of asthmatic subjects, such as eosinophilic or non-eosinophilic
1867 asthma, Th2 or non-TH2 molecular phenotypes of asthma, or corticosteroid-responsive
1868 vs. non-CS-responsive asthma. Any of these findings would represent a major shift in
1869 current conceptions of the pathogenesis of asthma and of its clinical expression.
1870
1871

1872 **V. Literature Cited**

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- 2084 **VI. Appendices**
- 2085
- 2086 **A. Appendix 1. Exclusionary Medical Conditions (may not be inclusive)**
- 2087
- 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 \geq 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)

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