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Three clinically distinct chronic pediatric airway infections share a common core microbiota

Running head: Common pediatric core respiratory microbiota

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31

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39

40

41 **Abstract:**

42 **Rationale:** DNA-based microbiological studies are moving beyond studying healthy human microbiota to

43 investigate diverse infectious diseases, including chronic respiratory infections such as those in the airways of

44 people with cystic fibrosis (CF) and non-CF bronchiectasis (BE). The species identified in the respiratory

45 secretion microbiota from such patients can be classified into those that are common and abundant among

46 similar subjects (core) versus those that are infrequent and rare (satellite). This categorization provides a vital

47 foundation for investigating disease pathogenesis and improving therapy. However, whether the core microbiota

48 of people with different respiratory diseases, which are traditionally associated with specific culturable

49 pathogens, are unique or shared with other chronic infections of the lower airways is not well studied. Little is

50 also known about how these chronic infection microbiota change from childhood into adulthood.

51

52 **Objectives:** We sought to compare the core microbiota in respiratory specimens from children and adults with

53 different chronic lung infections.

54

55 **Methods:** We used bacterial 16S rRNA gene pyrosequencing, phylogenetic analysis, and ecological statistical

56 tools to compare the core microbiota in respiratory samples from three cohorts of symptomatic children with

57 clinically distinct airway diseases (protracted bacterial bronchitis, BE, CF), and four healthy children. We then

58 compared the core pediatric respiratory microbiota with those in samples from adults with BE and CF.

59

60 **Measurements and Main Results:** All three pediatric disease cohorts shared strikingly similar core respiratory

61 microbiota that differed from adult CF and BE microbiota. The most common species in pediatric disease cohort

62 samples were also detected in those from healthy children. The adult CF and BE microbiota also differed from

63 each other, suggesting common early infection airway microbiota that diverge by adulthood. The shared core

64 pediatric microbiota included both traditional pathogens and many species not routinely identified by standard

65 culture.

66

67 **Conclusions:** Our results indicate that these clinically distinct chronic airway infections share common early

68 core microbiota, which are likely shaped by natural aspiration and impaired clearance of the same airway

69 microbes, but that disease-specific characteristics select for divergent microbiota by adulthood. Longitudinal and

70 interventional studies will be required to define the relationships between microbiota, treatments, and disease
71 progression.

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Introduction

A growing body of evidence indicates that the airways of even healthy people contain detectable microbes (1–4), and that these microbiota are altered in many chronic lung diseases. For example, culture-independent analyses of cystic fibrosis (CF) respiratory samples have revealed remarkably diverse microbial communities compared with culture results. Among the bacteria detected most frequently, and at highest abundance, in those CF samples were anaerobic species not usually detected by standard laboratory methods (5). Both cross-sectional and longitudinal studies indicate that CF airway microbiota generally decrease in diversity over time, concurrent with a decrease in average lung function (6–9). Whether these ecological changes are the cause of lung function decline, or whether they are the result of increasing antibiotic exposure or changing properties of the infected airways, are not understood (9). Furthermore, exactly where the species that comprise CF secretion microbiota come from, and whether features unique to the CF airway or its secretions select for CF-specific microbiota, are unknown.

Several other childhood lung diseases also involve chronic airway infection, including non-CF bronchiectasis (BE) and protracted bacterial bronchitis (PBB) (10). At least some underlying factors that predispose to infection in CF, BE, and PBB may be shared (e.g., mucus hypersecretion or stasis, impaired airway clearance), while others vary (e.g., inflammatory response, intensity of antibiotic use, physicochemical mucus characteristics), and the associated infectious microbiota could therefore differ at the beginning or throughout these diseases. In support of this concept, specific microbes have been associated with the pathogenesis of each of these diseases in culture-based studies. For example, *Staphylococcus aureus* and *Pseudomonas aeruginosa* are traditionally associated with CF lung disease (11). In contrast, *Streptococcus pneumoniae*, *Moraxella catarrhalis*, and *Haemophilus influenzae* are traditionally associated with both PBB and early BE (10), with *P. aeruginosa* associated with more advanced BE disease (12). Treatment regimens therefore tend to differ for each disease, designed to target specific pathogens. Moreover, it has been suggested that these bacteria play primary roles in initiating or driving associated symptoms (13), which could imply disease-specific infection pathogenesis. However, it is now known that children with CF have lung disease without detectable infection by *P. aeruginosa* or *S. aureus* (14, 15). The identification of diverse microbiota in even healthy airways further complicates the task of delineating “infected” and/or “pathogenic” from “normal” conditions. Therefore, comparing the earliest stages of chronic infection for diverse airways diseases could be helpful in understanding whether infection begins (and drives disease) in distinct or similar ways in different conditions.

106 An analytical approach that has been particularly useful in understanding environmental and infectious
107 microbiota involves partitioning the constituent microbes into core and satellite species (7). In cross-sectional
108 studies, core species are those that are detected in the majority of samples from subjects with a specific
109 disease, while satellite species are those that are infrequent. While this categorization does not necessarily
110 identify which species are pathogens, it provides a useful framework within which to compare microbiota from
111 different subject groups, and the resulting similarities and differences have important implications for infection
112 pathogenesis. Here, we compared the core and satellite microbiota in three very different cohorts of children with
113 PBB, BE, and CF using next-generation sequencing and ecological analytical approaches. Because these study
114 subjects were children, and therefore at relatively early stages of disease, we hypothesized that these cohorts
115 would have similar, rather than disease-specific, early core respiratory sample microbiota. We then compared
116 pediatric and adult CF and BE respiratory metacommunities, using data from two of our recent adult studies (16,
117 17) to identify evidence of disease-specific changes in microbiota as patients with each disease progress to
118 adulthood.

119

120

121 **Materials and methods**

122 *Patients and clinical samples*

123 All children in this study were participants in previous studies of CF, PBB, and BE, respectively (18–20).
124 Inclusion and exclusion criteria and study details are listed in the Online Supplement. Sputum and
125 bronchoalveolar lavage (BAL) fluid, as well as clinical data, were collected during those studies as described
126 (18–20). The study was approved by the institutional review boards at Seattle Children's Hospital (SCH) for the
127 CF samples, and at the Royal Children's Hospital (RCH, Brisbane, Australia) for the PBB, BE and control
128 pediatric samples. Data from two adult BE ($n = 38$ samples, age 37 - 74 years) and CF ($n = 30$, 18 - 55 years)
129 cohorts from previous studies were used for metacommunity comparisons with the microbiota from the pediatric
130 cohorts (16, 17).

131

132 *DNA extraction and Q-PCR*

133 Full details of DNA extraction from BAL and sputum samples, and of the use of quantitative PCR to
134 determine total bacterial abundance using universal eubacterial primers, are in the Online Supplement.

135

136 *Bacterial Tag-Encoded FLX Amplicon Pyrosequencing (bTEFAP) and sequence analyses*

137 The bacterial taxonomy of each sample was evaluated using bacterial tag-encoded FLX-titanium
138 pyrosequencing (bTEFAP) of a fragment of the 16S rRNA gene covering the V1-V3 variable regions, as
139 described previously (21). Details of amplification, sequencing, and sequence processing and analysis are in the
140 Online Supplement.

141

142 *Statistical analyses*

143 To avoid potential biases in comparisons of diversity between local communities due to varying number
144 of sequences per sample, a randomised re-sampling method, using three indices of diversity (S^* , H' , and $1-D$),
145 were employed as previously described (7). Bacterial species within each metacommunity were partitioned into
146 core and satellite species groups using the Poisson distribution test as previously described (7) (Online
147 Supplement).

148

149 Results

150 *Pediatric study cohorts*

151 We analysed the microbiota in respiratory samples (sputum and BAL fluid) from 3 cohorts of children
152 with BE, PBB, and CF, respectively. All of these children either underwent bronchoscopy for chronic wet cough
153 or were expectorating, indicative of symptomatic disease. As shown in Table 1, the cohorts were substantially
154 different in terms of demographic and treatment features that could play important roles in determining the
155 airway microbiota, including age, antibiotic exposure, and *P. aeruginosa* culture-positivity, which have each been
156 shown to correlate inversely with airway microbiota diversity in CF, and FEV₁% predicted, which correlates
157 directly with diversity (9). The children with CF also had higher rates of inhaled corticosteroid use, a treatment
158 that could conceivably alter airway microbiota constituency (2). The CF cohort originated entirely from a single
159 hospital in the US, whereas all children with BE and PBB were from a single hospital in Australia. Each of these
160 differences, if they were to influence airway microbiota, would be unlikely to bias our analyses towards finding
161 microbial similarities between the three cohorts.

163 *Pediatric community diversity and composition*

164 Bacterial pyrosequencing data were used to assess and compare diversity and composition of
165 microbiota from respiratory samples within and across the 3 pediatric cohorts. A total of 408,335 bacterial
166 sequence reads (mean \pm SD per sample, 5041 \pm 3557), identifying 104 genera and 225 distinct operational
167 taxonomic units (OTUs) classified to species level (Table S1), were generated from all samples combined. The
168 average numbers of bacterial sequence reads per sample were similar among the three cohorts (mean \pm SD:
169 BE, 4284 \pm 3476; PBB, 4471 \pm 3718; and CF, 4785 \pm 2702). Estimates of total bacterial abundances were also
170 similar when quantified by qPCR (for all samples, including sputum and BAL cell pellets, from which sufficient
171 DNA remained after sequencing analysis, mean cfu/mL equivalents/sample \pm SD: BE, 1.47 $\times 10^7 \pm 2.32 \times 10^7$
172 $N=18$ samples; PBB, 9.89 $\times 10^6 \pm 1.38 \times 10^7$, $N=10$; and CF, 5.82 $\times 10^7 \pm 6.28 \times 10^7$, $N=21$).

173 Bacterial diversity within and between cohorts was compared using three indices of diversity: species
174 richness (S^* , the total number of species), Shannon-Wiener (H' , a metric that accounts for both number and
175 relative abundance of species), and Simpson's (1- D , a measure of the probability that two species randomly
176 selected from a sample will differ). Bacterial diversity between BE sputum and BAL samples was compared
177 using all three measures and found not to be significantly different ($P > 0.05$, Fig. S1), and hence those samples

178 were combined for subsequent analyses. S^* was found to vary substantially within cohorts, with means of $30.6 \pm$
179 10.5 , 30.9 ± 11.5 , and 19.5 ± 10.0 for the BE, PBB, and CF cohorts, respectively (Fig. 1); similar results were
180 obtained for H' and $1-D$ (Fig. 1 and Table S2). Diversity between pediatric cohorts was not significantly different
181 ($P > 0.05$) in all instances except for one measure (H') between the PBB and CF cohorts (Table S2).

182 Similarities and differences in community membership and structure for microbiota samples both within
183 and between disease cohorts were assayed with two different indices: Sørensen (S_{SOR} , which accounts for the
184 number of species present in each community and those that are shared) and Bray-Curtis quantitative (S_{BC} ,
185 similar to S_{SOR} but also accounts for abundance of each species). Each index varies in value from 0 to 1, with
186 higher values indicating greater similarity. It has been previously observed that community composition is highly
187 variable between CF patients (7, 22). Here, we found that observation held regardless of underlying pediatric
188 lung disease, with mean similarities in bacterial community membership taken pair-wise for samples within
189 pediatric cohorts of $S_{SOR} = 0.44 \pm 0.14$ for BE ($n = 171$ pair wise comparisons); $S_{SOR} = 0.51 \pm 0.12$ for PBB ($n =$
190 66); and $S_{SOR} = 0.50 \pm 0.12$ for CF ($n = 300$), with a mean similarity of the entire pool of samples of $S_{SOR} = 0.46 \pm$
191 0.13 ($n = 1540$) (Fig. 2). For S_{BC} , the mean between all samples was $S_{BC} = 0.19 \pm 0.15$ ($n = 1540$), while that
192 within cohorts was $S_{BC} = 0.18 \pm 0.14$ for BE ($n = 171$); $S_{BC} = 0.27 \pm 0.12$ for PBB ($n = 66$); and $S_{BC} = 0.27 \pm 0.17$
193 for CF ($n = 300$). Therefore, individual sample microbiota differed within each pediatric disease group to a similar
194 degree, and the microbiota identified within each pediatric cohort's samples were pooled together into disease-
195 specific metacommunities (defined as the set of all microbiota identified in samples from a given cohort) for
196 subsequent comparisons.

197

198 *Microbiota within a metacommunity framework*

199 Previously, we established that the categorization of component species in CF microbiota into core and
200 satellite species revealed important aspects of species-abundance distributions within a metacommunity that
201 would be neglected without such a distinction (7). A coherent metacommunity could be expected to exhibit a
202 direct relationship between prevalence and abundance of individual species within the constituent communities.
203 Consistent with this prediction, the abundance of species in each pediatric study cohort was significantly
204 correlated with the number of individual respiratory sample communities those species occupied (Fig. 3A).

205 The species identified in each pediatric cohort metacommunity were then classified as core or satellite
206 based upon their distributions (Fig. 3B). Of the 177 species that comprised the BE metacommunity, 88 were core

207 and 89 were satellite species. The PBB metacommunity (130 species) was comprised of 63 core and 67 satellite
208 species, and the CF metacommunity (143 species) comprised of 66 and 77 species, respectively.

209 Similarities between the three pediatric metacommunities were assayed using S_{SOR} and S_{BC} indices for
210 all species, and for the core and satellite groups. The resulting cluster analysis revealed community membership
211 to be relatively well-conserved between cohorts for all species ($S_{SOR} = 0.74 \pm 0.02$ [$n = 3$ pair-wise
212 comparisons]), but much more highly conserved between the core groups ($S_{SOR} = 0.93 \pm 0.02$) (Fig. 4). The
213 satellite groups, comprised of randomly distributed species, were highly divergent ($S_{SOR} = 0.35 \pm 0.01$). Results
214 from Bray-Curtis quantitative analyses were similar: All $S_{BC} = 0.50 \pm 0.10$; core $S_{BC} = 0.51 \pm 0.10$; and satellite
215 $S_{BC} = 0.10 \pm 0.02$. These results indicate that the high level of similarity between the three pediatric cohort
216 metacommunities is attributable to core species.

217 To determine whether disease-specific microbiota emerge with disease progression into adulthood for
218 the two chronic diseases, BE and CF (PBB usually improves long-term with antibiotic treatment (10)), we
219 compared the microbiota from our previous studies of adult CF (16) and BE (17) cohorts with the pediatric cohort
220 microbiota. The adult BE cohort metacommunity ($n = 38$ patients) was comprised of 86 core and 54 satellite
221 species, while the adult CF cohort ($n = 30$) metacommunity was comprised of 68 core and 81 satellite species.
222 The resulting analyses revealed adult community membership to be highly divergent in all cases (whole
223 microbiota $S_{SOR} = 0.36 \pm 0.03$; core $S_{SOR} = 0.46 \pm 0.06$; and satellite $S_{SOR} = 0.09 \pm 0.03$ [$n = 6$ pairwise
224 comparisons in all cases]) (Fig. 4). When relative abundances of constituent taxa were included to examine
225 differences in community structure, an even more pronounced divergence was observed than for membership
226 (whole $S_{BC} = 0.07 \pm 0.03$; core $S_{BC} = 0.07 \pm 0.04$; and satellite $S_{BC} = 0.01 \pm 0.01$ [$n = 6$ in all cases]). In addition,
227 comparisons between the adult CF and BE metacommunities also revealed a high degree of divergence both in
228 terms of membership (whole $S_{SOR} = 0.38$; core $S_{SOR} = 0.48$; and satellite $S_{SOR} = 0.16$) (Fig. 4) and structure
229 (whole $S_{BC} = 0.05$; core $S_{BC} = 0.05$; and satellite $S_{BC} = 0.02$). Therefore, the adult CF and BE microbiota differed
230 substantially from each other, contrasting with the similarities among the pediatric cohort microbiota.

231 To test whether the adult and pediatric metacommunities were significantly similar or dissimilar, the
232 whole, core, and satellite microbiota were compared between cohorts using the Raup and Crick probability-
233 based similarity index (S_{RC}) to determine whether compositional similarities in the microbiota were more or less
234 significantly similar than expected by chance (Fig. 5). The resulting cluster analysis revealed the pediatric whole,
235 core, and satellite microbiota to be significantly dissimilar ($S_{RC} < 0.05$) from the corresponding adult CF and BE

236 microbiota. Likewise, the whole and core microbiota from the pediatric cohorts were significantly similar ($S_{RC} >$
 237 0.95), adding further weight to the observations of pediatric community membership (S_{SOR}) and structure (S_{BC})
 238 (Fig. 5). In contrast, the pediatric satellite microbiota were not significantly similar or dissimilar to each other
 239 ($S_{RC} > 0.05$ and < 0.95), which would be expected given that satellite microbiota in a given cohort represent rare
 240 and randomly distributed species. Furthermore, the adult CF and BE microbiota were significantly dissimilar to
 241 each other ($S_{RC} < 0.05$) (Fig. 5).

242 Similarity of percentages (SIMPER) analysis of the metacommunities was used to identify those species
 243 that contributed most to the observed similarity between the three pediatric cohorts. These species, all core
 244 group members, are listed in decreasing order of contribution in Table 2. *H. influenzae* contributed the greatest
 245 amount to the observed similarity between pediatric samples. Most of the major contributors have previously
 246 been associated with respiratory tract infections and other opportunistic infections, and many are known
 247 inhabitants of the oral cavity, including both aerobic and anaerobic species (Table S1). These findings
 248 contrasted with the SIMPER analysis of the adult CF cohort, wherein *P. aeruginosa* contributed over 83% to the
 249 observed similarity between samples (Table S3a) in addition to 3 other species: *Streptococcus pneumoniae*,
 250 *Prevotella melaninogenica*, and *Veillonella parvula*. Within the adult BE cohort, the main contributors to the
 251 overall similarity between samples were *H. influenzae* (50.4%), *P. aeruginosa* (8.62 %) and *V. dispar* (8.11%)
 252 (Table S3b) along with *S. pneumoniae* and, as for the pediatric cohorts, other species associated with
 253 respiratory infections, many of which are known oral microbiota members. As also observed for the pediatric
 254 cohorts, the species identified from the SIMPER analyses for both adult cohorts were all core species in their
 255 respective metacommunities. Although there were some species identified in common for the adult and pediatric
 256 cohorts by the SIMPER analyses, these similarities were greatly outweighed by the differences in species
 257 content, accounting for the observed differences in whole and core microbiota within each adult-pediatric
 258 disease cohort pair.

259 To investigate whether the core microbiota shared between the three pediatric disease groups were
 260 unique to children with airways disease, we identified the microbiota in BALF from four children who underwent
 261 bronchoscopy for reasons other than suspected infection (described in the Online Supplement and ¹⁶). Of the 10
 262 shared core species that contributed the most to similarity between disease cohorts from Table 2, six were
 263 detected in all four “control” samples, and the other four species were detected in three of the four samples,
 264 suggesting that the shared core microbiota are commonly found in healthy, as well as diseased, children’s

265 airways (Table S4), consistent with prior airway microbiota studies that included samples from healthy
266 subjects^{3,21}.

267

Discussion

We found that cohorts of symptomatic children with three clinically distinct airways diseases- CF, BE, and PBB- shared remarkably similar respiratory sample core microbiota, the most common of which were also found in airway samples from children without clinical evidence of infection. The shared core microbiota included both traditional pathogens and many bacteria that are either not identified by routine clinical laboratory methods or are generally classified as “oral flora” and are routinely ignored with respect to pathophysiology or treatment. In contrast, the respiratory sample microbiota from adults with CF and BE differed significantly from each other and from those of children with the same disease. These results indicate that the core microbes in different chronic respiratory infection types likely begin similarly, yet are divergent by adulthood.

While these observations provide valuable insight into the microbiota in diseased airways, they also identify next steps to take in understanding the pathogenesis of diverse airway infections. Given the high abundance and prevalence of the core species among pediatric patients, one interpretation of these results could be that the core species represent those that contribute the most to airway inflammation and disease progression. Alternatively, it could be that the satellite species play key pathogenic roles in specific contexts or stages of infection; for example, infection at any stage with specific low-abundance or infrequent bacteria could lead to increased inflammation, airway damage, mucus hypersecretion, and/or clinical deterioration. While these questions are not fully answered by the current study, the relative rarity of any specific species in each satellite group may indicate a consistent, general role for the core groups in pathogenesis. Future studies will be required to longitudinally compare the changes over time in airway microbiota in health and disease to better understand the microbial contribution to pathogenesis, and the best therapeutic approach to infection.

These findings provide valuable perspective for results from studies of many respiratory diseases, usually at more advanced stages (2). A study using a relatively low-depth sequencing technique, 16S rRNA gene clone sequencing, comparing microbiota in explanted lungs from adults with end-stage BE and CF identified diverse communities often dominated by *Pseudomonas* in both sample sets (23). Likewise, two studies using the same technique applied to sputa from patients with CF and BE found some similarities and some differences between the subject groups, but no formal comparisons were made (24, 25). In support of our results (17), a recent pyrosequencing study of 21 older adults with non-CF bronchiectasis showed their sputum to contain diverse microbiota (26). Studies of sputum, BAL fluid, and explanted lung tissue from patients at various stages of disease due to chronic obstructive pulmonary disease (COPD) and asthma, sometimes comparing with

297 healthy subjects, have yielded varying and often conflicting results, in some cases demonstrating disease-
298 specific microbiota and in others identifying substantial overlap (1, 2, 27–29). In all of these studies, the effects
299 of therapies that could conceivably impact airway microbiota, such as intubation, steroids and antibiotics, were
300 difficult to control for. Our results suggest that the microbiota observed in those older patients, at later stages of
301 disease, likely had similar (or identical) beginnings and diverged over time. Whether those changes resulted
302 from features of the underlying disease or from differences in treatment are not yet clear. While the differences in
303 antibiotic use among study cohorts support the idea that antibiotics did not have a profound, lasting impact on
304 airway microbiota (similar to observations from CF (8, 30)), and there was no evidence that differences in steroid
305 use resulted in differences in microbiota in these children, chronic or repetitive drug exposure could have
306 gradual effects (as indicated by longitudinal studies in CF (9)). Regardless, these findings suggest that factors
307 intrinsic to airways predominate in establishing the early microbiota in these chronic respiratory infections.

308 Several observations support these conclusions. Culture-based studies of children with CF
309 demonstrated that radiographic lung disease, respiratory symptoms, and inflammation all precede the detection
310 of “standard CF pathogens” (14, 15, 31, 32). All of the shared core microbes identified in the current study, many
311 of which would traditionally be considered “oral flora”, were also commonly identified in oropharyngeal swabs
312 from infants with CF by pyrosequencing (33). The identification of such “oral flora” has been associated with both
313 inflammation (32) and structural lung disease (15) in children with CF. Similarly, piglets with engineered CFTR
314 mutations (34) concurrently develop diverse airway microbiota and lung disease similar to those observed in
315 children with CF. The cultured microbiota of CF pigs were similar in constituency to those of wild-type pigs, but
316 more persistent and higher in abundance. Therefore, the requirement for traditional pathogens for CF lung
317 disease or symptom development is questionable.

318 Both cross-sectional and longitudinal studies of CF respiratory samples using molecular methods have
319 demonstrated correlations between decreasing microbiota diversity, age, and disease progression (3, 8, 9, 30).
320 Interestingly, some organisms traditionally associated with chronic airways disease were classified as core
321 species in some disease cohorts but not others in our study (for example, *P. aeruginosa*, *S. aureus*, and several
322 species each of *Veillonella* and *Prevotella* were core members in both the CF and BE metacommunities, but
323 satellite for PBB, Table S1). Collectively, these results could indicate that particular microbes are associated with
324 and/or accelerate specific lung diseases. Alternatively, changes over time in airway microbiota, including the
325 gradual decrease in community diversity observed in CF and in COPD (28), and the gradual identification of *P.*

326 *aeruginosa* in people with CF and non-CF bronchiectasis (23), could all result from intensified antibiotic
327 treatment and/or underlying disease, but this concept would not require the microbes that eventually dominate to
328 be particularly pathogenic. Similarly, changes in the respiratory microbiota could result from evolution (therapy-
329 driven or otherwise) in another reservoir that seeds the airways, such as the oropharynx (as suggested by
330 abundance in our study of microbes traditionally associated with this space (35)) or the GI tract (which a recent
331 longitudinal study of infants with CF suggested could be a source for respiratory microbiota (33)).

332 Together with the above earlier studies, our results support a unified model of early airway infections, in
333 which a defect in the clearance of otherwise normal airway microbiota (for example, due to early airway injury,
334 altered mucus, defective ciliary motility, mechanical obstruction, or immunodeficiency) contributes to progressive
335 inflammation and airway damage. In this model, an extension of the “vicious cycle hypothesis” (13), either
336 medical treatment or the physical effects of underlying disease (such as diseased tissue or accumulated
337 secretions) could lead to progressive change in the microbiota. These microbiological changes (including
338 eventual selection for *P. aeruginosa* in CF or BE) could in turn accelerate lung disease, or they could be
339 consequences rather than causes of this progression. Future studies, either interventional or longitudinal, may
340 be able to refine this model.

341 Antibiotics play important roles in the management of all three of these symptomatic chronic infections.
342 For example, inhaled and oral antibiotics are commonly used in CF and BE both for maintenance (11, 36) and
343 exacerbation treatments (11, 13). For PBB, a prolonged course of antibiotics usually leads to resolution of
344 symptoms (18). These observations underscore the importance of bacteria in the pathogenesis of each disease;
345 the current findings provide a rationale for reexamining and comparing the culture-based antibiotic approaches
346 to each of these airway infections, particularly during early stages.

347 Our study was limited by the small sizes of each subject group. The groups were not matched for age,
348 treatment, disease severity, or demographics, limiting our ability to determine relationships between microbiota
349 and these factors. However, the similarity in microbiota among demographically and clinically divergent pediatric
350 groups could be interpreted to further support the concept of a common early pediatric respiratory microbiota;
351 additional study with demographically well-matched cohorts could strengthen this argument. We did not
352 distinguish between viable and nonviable cells in the current analysis due to the small volumes of many of the
353 samples analyzed, and thus the detection of a microbe cannot definitively be said to indicate its persistence
354 (although even transient or nonviable bacterial cells could potentially contribute to airway inflammation).

355 Similarly, it could be proposed that the microbial diversity found in our study and others is due to contamination
356 by upper airway bacteria during sample collection. However, the significant similarity in microbiota among
357 sputum and BAL samples from the children with BE in our study, as observed in studies of children with CF (5,
358 22), as well as the high abundance of microbes in our samples, argue against this issue significantly altering our
359 findings.

360 In conclusion, children with early chronic lung infections due to three distinct diseases shared strikingly
361 similar core airway microbiota that included many bacteria not generally identified by clinical laboratory culture.
362 These similarities were independent of underlying diagnosis or disease severity, geography, antibiotic and
363 steroid use, and age. These results indicate that different chronic airway infections begin similarly, remaining
364 similar even after symptoms have begun, but diverging by adulthood. Our findings provide a baseline to
365 compare with in future, longitudinal microbiota studies of diverse chronic airway infections, which would provide
366 vital insight into the microbial determinants of chronic lung disease progression and could lead to improved
367 treatments.

368

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377 Data deposition: The sequence data reported in this paper have been submitted to the NCBI Short Read
378 Archive database (Bioproject accession number: PRJNA200702).

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Figure legends

Fig. 1. Box plot comparisons of bacterial diversity between the BE, PBB, and CF cohorts. Given are three measures of diversity: species richness (S^*), Simpson's complement index ($1-D$), and Shannon-Wiener index (H'). The top and bottom boundaries of each box indicate the 75th and 25th quartile values, respectively, and lines within each box represent the 50th quartile (median) values. Ends of whiskers mark the lowest and highest diversity values in each instance. One-way ANOVA summary statistics are given in Table S2. The only significant difference found was for H' between the CF and PBB cohorts ($P=0.027$, Table S2).

Fig. 2. Dendrograms of bacterial community membership for all patients from the BE, PBB, and CF cohorts. Black, grey, and white shaded boxes are given to show positions of BE, PBB, and CF samples, respectively. Patient species profiles were compared using the Sørensen index of similarity and un-weighted pair-group averages (UPGMA).

Fig. 3. Distribution and dispersal of bacterial species among BE, PBB, and CF pediatric cohorts. (A) The number of samples for which each detected bacterial species (open circles) was observed, plotted against the abundance (\log_{10} scale) of that species among all samples within each cohort (BE, $r^2 = 0.64$, $F_{1,175} = 311.5$, $P < 0.0001$; PBB, $r^2 = 0.72$, $F_{1,128} = 321.1$, $P < 0.0001$; and CF, $r^2 = 0.75$, $F_{1,141} = 418.4$, $P < 0.0001$). (B) A dispersal plot to identify which bacterial species are randomly distributed within each cohort, a measure used to assign core versus satellite status. Index of dispersion was calculated as the ratio of variance to mean of abundance for each species within each cohort and plotted for each sample. The line depicts the 2.5 % confidence limit for the χ^2 distribution. Species that fall below this line are randomly distributed and were considered satellite species, whereas those that are above the line are non-randomly distributed and were considered core species. The 97.5 % confidence limit was not plotted, as no species fell below that line.

Fig. 4. Dendrograms of community membership similarity between the pediatric BE, PBB, and CF bacterial metacommunities and compared with adult CF and BE metacommunities. Given are whole, core and satellite microbiota. Metacommunity profiles were compared using the Sørensen index of similarity and UPGMA. Similarities between the microbiota from different cohorts are read as the location of the horizontal line

522 connecting those cohorts (the “node”) on the Y-axis; for example, the similarity between pediatric core microbiota
523 was $\geq 90\%$, while that between adult core microbiota was $\geq 40\%$.

524

525 **Fig. 5.** Dendrograms of Raup and Crick (S_{RC}) probability-based index of similarity between the pediatric BE,
526 PBB, and CF bacterial metacommunities and compared with adult CF and BE metacommunities. Given are the
527 whole, core and satellite microbiota. $S_{RC} < 0.95$ and $S_{RC} > 0.05$ denote similarity no greater than expected by
528 chance. $S_{RC} < 0.05$ denotes significant dissimilarity and $S_{RC} > 0.95$ significant similarity. The 0.05 and 0.95
529 thresholds are depicted with dashed lines in each instance. Dendrograms were constructed using UPGMA.

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532 **Tables:**

533 **Table 1.** Demographic characteristics of the pediatric study groups.
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Clinical Characteristic	BE ¹	PBB	CF
Number of subjects	19	12	25
Geographic origin	Australia (Brisbane)	Australia (Brisbane)	US (Seattle)
Sample type	9 BAL & 10 Sputum	All BAL	All Sputum
Age (y; Mean \pm SD, range)	8.9 \pm 4.7, 1.8-16.3 ⁶	2.3 \pm 1.7, 0.6-5.5 ^{4, 5}	12.5 \pm 3.5, 2.3-17.7
Gender (% female)	36%	25%	68%
FEV ₁ % predicted (Mean \pm SD, range, no. subjects with measurements)	85.7 \pm 21%, 41-120%, 15 subjects	117 \pm 1.4%, 116 and 118%, 2 subjects ^{4, 5}	77.9 \pm 24%, 22.2-116.2%, 21 subjects
Number (%) of subjects on antibiotics at sampling ²	8 of 19 (42%)	1 of 12 (8%)	7 of 25 (28%)
Number (%) of subjects on corticosteroids at sampling	6 of 19 (32%), one oral, 5 inhaled	2 of 12 (16%), all inhaled	11 of 25 (48%), all inhaled
Number (%) culture-positive for <i>P. aeruginosa</i> ³	0	0	8 (34%)
Number (%) culture-positive for <i>S. aureus</i> ³	2 (11%)	2 (16%)	22 (96%)
Number (%) culture-positive for <i>H. influenzae</i> ³	4 (19%)	6 (50%)	7 (30%)

535 ¹ Of the 10 children with BE with additional diagnoses available, 5/10 (50%) had idiopathic bronchiectasis, one
536 had previously had an airway foreign body, one had primary ciliary dyskinesia, one had chronic aspiration due to
537 a tracheoesophageal fistula, one had Mounier-Kuhn syndrome, and one had common variable
538 immunodeficiency.
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540 ² Antibiotics for children with CF included tobramycin (inhaled), linezolid, levofloxacin, ciprofloxacin, trimethoprim-
541 sulfamethoxazole, ticarcillin-clavulanate, and amoxicillin-clavulanate. Antibiotics for children with PBB included
542 only erythromycin. Antibiotics for children with BE included erythromycin, azithromycin, roxithromycin,
543 clarithromycin, amoxicillin-clavulanate, cefotaxime, ceftriaxone, and ticarcillin-clavulanate.

544 ³ Of 23 subjects with CF with culture data available; all subjects with PBB and BE had available culture data.

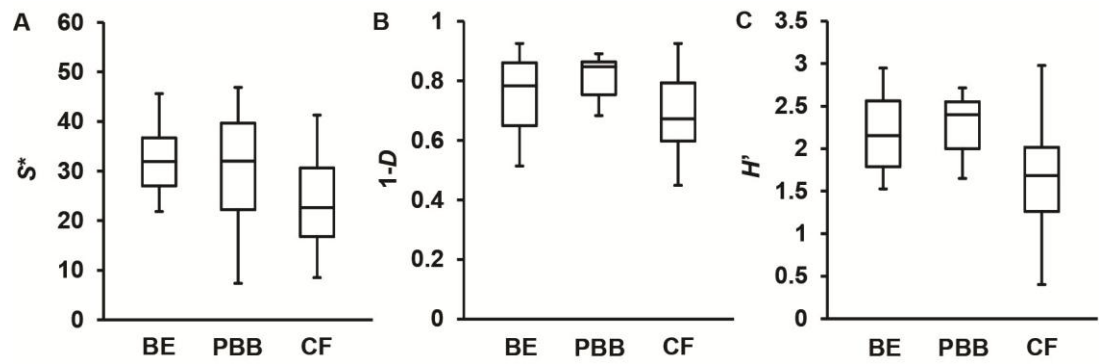
545 ⁴ $P < 0.001$ compared with CF; ⁵ $P < 0.001$ compared with BE; ⁶ $P < 0.01$ compared with CF.
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Table 2. Similarity of Percentages (SIMPER) analysis of bacterial community similarity (Bray-Curtis) between pediatric metacomunities. Given is occupancy, or the number of patients for each disease group a given species was detected in. Next is mean % abundance of sequences for a species across the samples it was observed to occupy. Mean contribution represents the average contribution of a given species to the average similarity between samples (overall mean = 50.2%). Percentage contribution is the mean contribution divided by mean similarity across samples. The list of species is not exhaustive, so cumulative % value does not sum to 100%. Species level identities of detected taxa are reported here. However, given the length of the ribosomal sequences analysed, these identities should be considered putative.

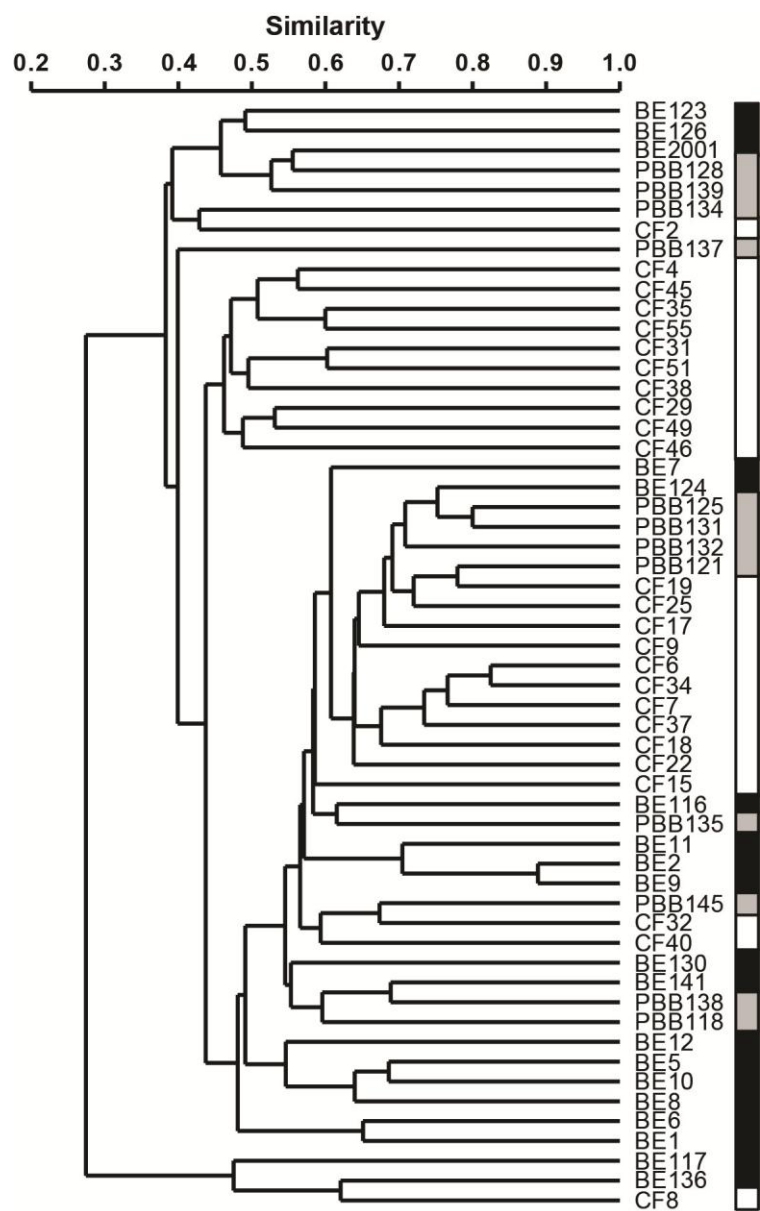
Species	Occupancy ¹			Mean Abund.	Mean Cont.	% Cont.	Cum. %
	BE	PBB	CF				
<i>Haemophilus influenzae</i>	12	11	19	23.22	18.79	37.44	37.44
<i>Streptococcus mitis</i>	18	12	25	14.59	8.45	16.84	54.28
<i>Prevotella melaninogenica</i>	16	11	24	9.16	4.59	9.15	63.43
<i>Veillonella dispar</i>	19	12	24	4.48	3.90	7.77	71.20
<i>Fusobacterium nucleatum</i>	17	11	21	2.63	1.99	3.97	75.17
<i>Neisseria flavescens</i>	12	11	19	5.04	1.73	3.44	78.61
<i>Porphyromonas catoniae</i>	17	11	18	1.66	1.05	2.09	80.70
<i>Haemophilus parainfluenzae</i>	15	10	16	1.30	1.04	2.06	82.76
<i>Porphyromonas gingivalis</i>	17	9	17	1.60	0.97	1.92	84.69
<i>Prevotella nanceiensis</i>	13	11	15	0.85	0.54	1.08	85.77
<i>Prevotella histicola</i>	12	7	19	0.81	0.46	0.92	86.69
<i>Granulicatella adiacens</i>	15	10	19	0.53	0.44	0.87	87.57
<i>Prevotella oris</i>	10	4	13	1.07	0.37	0.73	88.30
<i>Sphingomonas echinoides</i>	9	7	3	0.73	0.36	0.71	89.02
<i>Gemella morbillorum</i>	13	11	21	0.86	0.35	0.70	89.71
<i>Prevotella pallens</i>	16	8	13	0.63	0.32	0.63	90.35

¹Total N for each pediatric disease cohort: BE, 19; PBB, 12; CF, 25.

558 Figure 1



582 Figure 2



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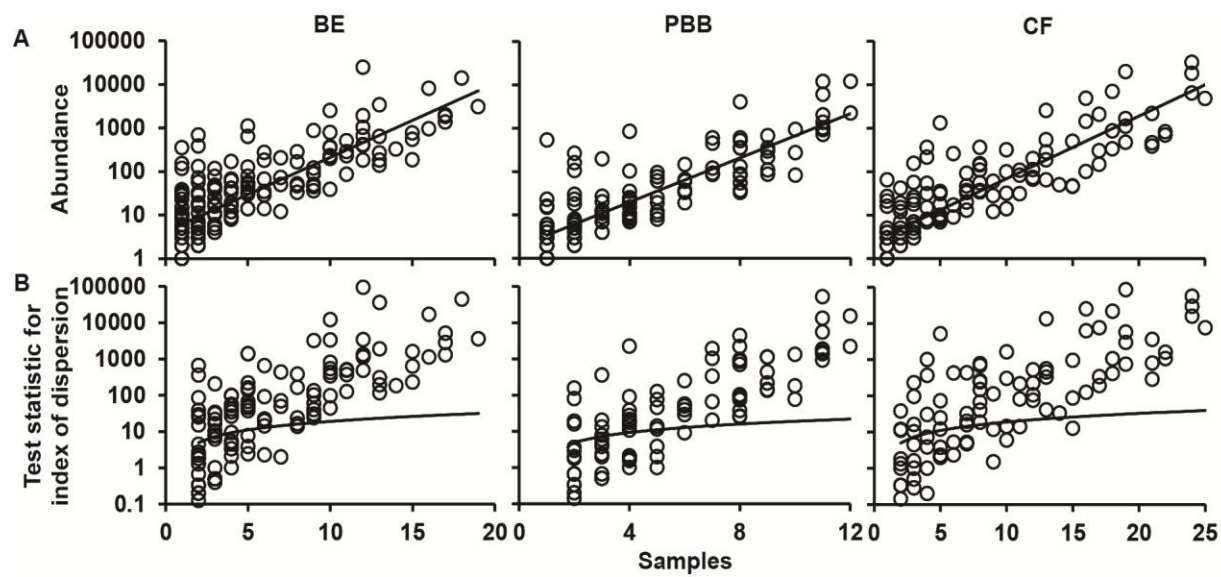
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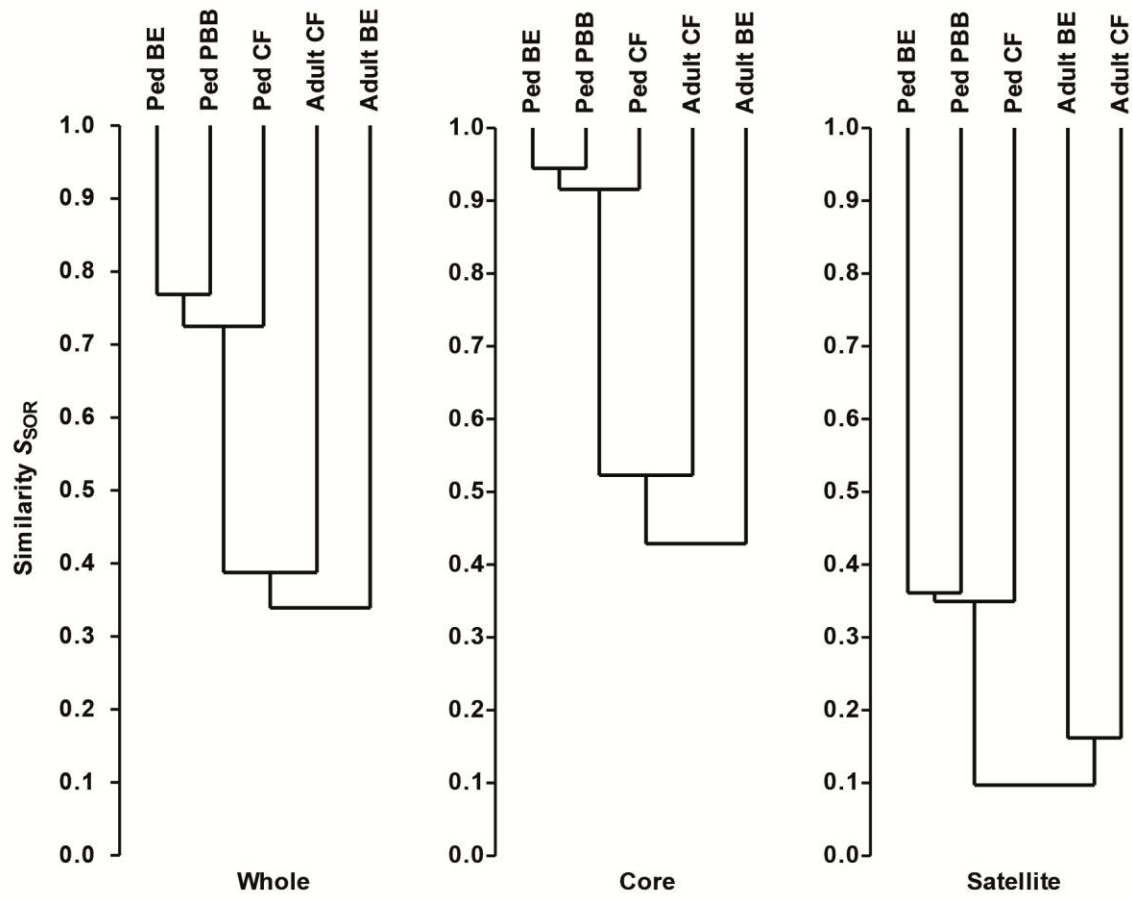
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592 Figure 3

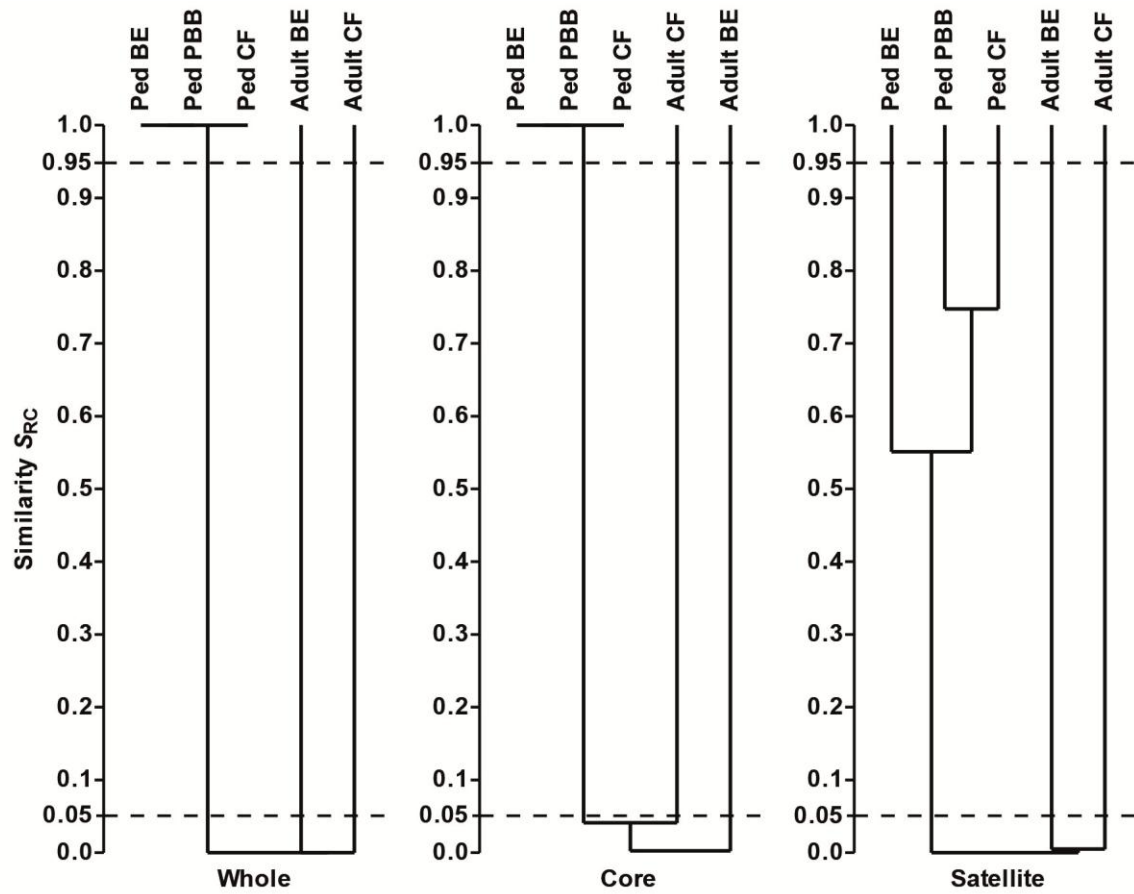


613 Figure 4



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627 Figure 5



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