Inflammation and Intestinal Metaplasia of the Distal Esophagus Are Associated With Alterations in the Microbiome

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BACKGROUND & AIMS: Gastroesophageal reflux causes inflammation and intestinal metaplasia and its downstream sequela adenocarcinoma in the distal esophagus. The incidence of esophageal adenocarcinoma has increased approximately 6-fold in the United States since the 1970s following a significant increase in prevalence of gastroesophageal reflux disease (GERD). Although specific host factors might predispose to disease risk, such rapid increase in incidence must be predominantly environmental. Among the environmental agents that have been considered are microbes. The human body can be viewed as a superorganism composed of an amalgam of both microbial and human cells. Our relationships with bacteria can be considered to span a broad spectrum, from mutualism to pathogenicity. Currently, 2 theories explain bacterial diseases. The classic pathogen theory, attributed to Koch, requires the presence of specific pathogens, ie, Mycobacterium tuberculosis and Bacillus anthracis. Alternatively, the microecologic disease or “pathogenic microbial community” theory is a new concept in which the entire community contributes to pathogenicity, although no individual community members can be categorized as classic pathogens. In mouse models mimicking inflammatory bowel diseases, development of mucosal inflammation and adenocarcinoma requires both a trigger (chemical or genetic) and presence of commensal bacteria.

Dysbiosis refers to an abnormal state of the microbial ecosystem in a host. It further divides commensal bacteria into “protective” and “harmful” species, attributing the causes of certain chronic diseases to alterations of balance between the 2 species. Gut microbiome in ob/ob mice (which have a mutation in the leptin gene causing obesity), for example, has an increased capacity to harvest energy from the diet and might contribute to pathophysiology of obesity.

Recent studies of a small number of hosts have shown that nearly 100 commensal bacterial species reside in the normal distal esophagus. Although human exposure to many exogenous pathogens has been monitored, little attention has been paid to changes in the indigenous microbiome where gastric acid reflux causes reflux esophagitis, Barrett’s esophagus (BE), and its downstream sequelae esophageal adenocarcinoma. Incidence of the adenocarcinoma has increased approximately 6-fold in the United States since the 1970s following a significant increase in prevalence of gastroesophageal reflux disease (GERD). Dysbiosis refers to an abnormal state of the microbial ecosystem in a host. It further divides commensal bacteria into “protective” and “harmful” species, attributing the causes of certain chronic diseases to alterations of balance between the 2 species. Gut microbiome in ob/ob mice (which have a mutation in the leptin gene causing obesity), for example, has an increased capacity to harvest energy from the diet and might contribute to pathophysiology of obesity.

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Abbreviations used in this paper: BE, Barrett’s esophagus; GERD, gastroesophageal reflux disease; NRR, normal reference range; OTU, operational taxonomic units; SLOTU, species-level taxonomic unit.

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microbiome, partly because of complexity and difficulties in culture and analysis. This is exemplified by the number of attempts to date in studying the esophageal microbiome with only limited success (Supplementary Table 1). In the present study, we examined whether pathologic findings in the esophageal mucosa are related to changes in the overlying microbiome (Supplementary Figure 1).

**Materials and Methods**

DNA extraction, bacterial 16S ribosomal RNA gene amplification, cloning, and sequencing of the polymerase chain reaction (PCR) products were performed as previously described. In brief, the distal esophageal microbiome was sampled by endoscopic biopsy and DNA extracted from the biopsy specimens. From each sample, a 16S ribosomal RNA (rRNA) gene cloning library was constructed with 16S rRNA genes amplified using broad range primers 8F and 1510R, and 200 clones were sequenced by single-pass Sanger sequencing. The average length of the sequencing reads were 912 nucleotides, ranging between 813 and 970 nucleotides. For assignment of species-level taxonomic unit (SLOTU), each sequence was analyzed using Sequence Match at Ribosomal Database Project II (RDP II, release 9.39, http://rdp.cme.msu.edu), as previously described. Compared with the RDP database of 16S rRNA genes, a sequence that had a similarity score >0.8725 (equivalent to 97% sequence identity) with a best matched sequence in the database was assigned to the species assigned by RDP II, whereas a sequence that had a best similarity score <0.8725 was assigned as unclassified species. We used the experimentally defined 97% sequence identity as the species boundary to minimize subjective influence on defining a species. Because no single identity cutoff can reliably classify all natural bacterial species, 97% threshold used in this study was for operational purpose to approximate species diversity. Ranks at genus or above were defined by using CLASSIFIER at RDP II, with a confidence threshold of 80%, which has been established as one of the most suitable methods for taxonomic assignment of 16S rRNA genes of human gastrointestinal microbiome. The classification was verified by phylogenetic analysis (see Supplementary Data). The genus level assignment for a sequence was confirmed by inferring from its species assignment if a discrepancy occurred between the assignments by CLASSIFIER and phylogenetic analysis. Each of the species identified was assigned into the anaerobic, microaerophilic, or aerobic group or the gram-positive or gram-negative group by empirically inferring from their taxonomic identity and known culture conditions as described in the American Type Culture Collection instruction and Bergey’s manual as well as original publications describing the species. A sequence was assigned to unclassified if knowledge about it was unavailable or the taxon it belongs to was heterogeneous in these properties. The total number of SLOTUs that could be present in the 16S rRNA gene data sets from the distal esophageal microbiome, as a whole or within phenotypic groups, was predicted by a nonparametric richness estimator, Chao1. The Shannon–Wiener diversity index and Shannon–Wiener index of evenness were calculated by using EstimateS at http://purl.oclc.org/estimatet. To avoid a type 1 error in multiple comparisons on a single data set, an Omnibus test was first performed to determine whether there is an overall among-group difference. The Omnibus test was performed for categorical data using the Fisher exact R × C frequency table and for continuous data using the 1-way analysis of variance (ANOVA), with the statistical significance level set at P < .05, for 2-tailed analysis. Follow-up analyses for between-group differences were performed using Fisher exact tests or t tests with the false discovery rate controlled at 5%. Data used in the t test were examined for distribution relative to a normal curve by Kolmogorov-Smirnoff goodness-of-fit test, with critical value set at 0.05. Fisher exact test was performed using StatXact 8 (Cytel Inc., Cambridge, MA), and ANOVA and t test were performed using SPSS 13.0 and SigmaPlot 8.0 (SPSS Inc., Chicago, IL). Regression and correlation were performed using the online statistical tools hosted at http://fonsg3.let.uva.nl/Service/Statistics.html.

**Results**

**Esophageal Microbiome Differs in Health and Diseases**

We obtained esophageal samples from 34 subjects and classified them to 1 of 3 histologic phenotypes based on histopathologic changes in the human tissue (Supplementary Figure 1): normal (n = 12), esophagitis (n = 12), or Barrett’s esophagus (BE) (n = 10) (Supplementary Table 2). In total, 6800 (200/sample) 16S rRNA gene sequences were analyzed. We started with an unsupervised approach by asking whether samples of the microbiome form natural groups, independent of histopathologic phenotypes associated with each sample. Hierarchical clustering analysis using combined genetic distance between samples revealed 2 distinct clusters (Figure 1A) that we designated as 2 microbiome types. We then asked whether the naturally occurring microbiome types correlate with host phenotypes. Although none of the microbiome types exclusively correlated with the 3 phenotypes, nearly all normal samples (11/12) were located in one cluster, whereas the majority of abnormal samples (13/22) was located in another cluster. This type of distribution suggests that the association between the host phenotypes and microbiome types is nonrandom. To validate this association, we calculated the normal reference range (NRR) (equations 1–4 in Supplementary Materials and Methods) based on the mean genetic distance among phenotypically normal samples. The 11 normal samples (with normal distribution after exclusion...
of 1 outlier) had a mean distance of 0.1170 between themselves and a 95% NRR (mean ± 1.96 SD) between 0.0648 and 0.1693 (Figure 1B). The mean distance between each of the other 22 samples and the 11 normal samples was calculated. A single outlier was identified, and the mean distance for each remaining 11 normal samples was recalculated after excluding the outlier. The 95% NRR was defined as mean distance ± 1.96 SD based on the 11 normal samples. The mean distance for each sample in the esophagitis and BE groups is the mean distance between the sample and the 11 normal samples. The dotted line (0.1693) is the upper limit of the 95% NRR, which separates the 34 samples into the normal (inside the NRR) and abnormal microbiome (outside the NRR). (C) Double principal coordinate analysis (DPCoA) of the microbiome. Samples are represented by circles. Microbiome types are indicated by fill colors (blue for type I and brown for type II). Host phenotypes are indicated by edge colors (green for normal, red for esophagitis, and black for Barrett’s esophagus). Within-sample diversity is proportional to circle size, determined by Rao’s analysis. The location of a sample in the plot was determined by the first 2 orthogonal principal axes. The percentages shown for each axis represent the percent of total dissimilarity captured by the axis. The samples from the 2 types of microbiome are separable along the first principal coordinate, as indicated by the dividing line at x = 0.015.

Table 1. Association Between Host Phenotypes and Microbiome Types in the Distal Esophagus

<table>
<thead>
<tr>
<th>Groups compared</th>
<th>Normal</th>
<th>Esophagitis</th>
<th>BE</th>
<th>P value</th>
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<tr>
<td>Microbiome type</td>
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<td>BE</td>
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<td>4</td>
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Follow-up tests

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<th>Normal</th>
<th>BE</th>
<th>Esophagitis</th>
<th>BE</th>
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<tbody>
<tr>
<td>Microbiome type</td>
<td>Normal</td>
<td>Esophagitis</td>
<td>Normal</td>
<td>BE</td>
<td>Esophagitis</td>
<td>BE</td>
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<td>7</td>
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<td>6</td>
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<tr>
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<td>.020</td>
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<td>Odds ratio (95% CI)</td>
<td>15.4 (1.5–161.0)</td>
<td>16.5 (1.5–183.1)</td>
<td>1.1 (0.2–5.9)</td>
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BE, Barrett’s esophagus.

*The Omnibus test was performed using the 2-tailed Fisher-Freeman-Halton 3 × 2 probability test.

*The follow-up tests were performed with the 2-tailed Fisher exact 2 × 2 probability test.

*Tests that are statistically different at the false discovery rate <5%.

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mal samples (1/12) to exhibit the type II microbiome (odds ratio [OR], 15.4; 95% confidence interval [CI]: 1.5–161.0, and OR, 16.5; 95% CI: 1.5–183.1, respectively) but were indistinguishable from one another. These data indicated that, although the histologically defined phenotypic groups were heterogeneous in microbiome types, strong associations were nevertheless present.

We also examined the relationship between microbiome types and host phenotypes, using the UniFrac significance test, with 1000 permutations and Bonferroni correction for multiple comparisons.22,23 UniFrac tests the hypothesis that there has been more phylogenetic diversity unique to a single environment than would be expected if the sequences were randomly distributed among environments. UniFrac revealed across the board differences between the 2 microbiome types (sequences pooled by type: 4000 for type I and 2400 for type II), among and between microbiomes associated with the 3 phenotypes (sequences pooled by phenotype: 2400 for normal, 2400 for esophagitis, and 2000 for BE), as well as among samples (n = 34, 200 sequences per sample) (Supplementary Table 3). Further analyses for between-samples difference were not informative because it requires more than 10,000 permutations to reveal any difference, which exceeds the software’s limit of 1000 permutations. To assess the effect of the intracommunity diversity on the difference between the 2 types of microbiomes, we performed FST test and found that the average within-community diversity is significantly less than the diversity when the 2 types of microbiomes are combined (P < .001). Analyses of the lineage through time curves in the data set revealed constant rates of birth and extinction of operational taxonomic units (OTUs) since the bacterial domain, identity level at 46% (ID46) was formed through the species level (ID97) when the rates abruptly accelerate, with the result that a limited number of lineage ancestors burst into numerous, closely related OTUs (Figure 2A and B), suggesting that the majority of differences observed was concentrated below the species level.

**Streptococcus Determines Microbiome Types**

Further analysis using double principal coordinate analysis24 indicated that the maximal separation of the phenotypically normal samples from the abnormal ones could be obtained along the first principal coordinate (PC1) (69.2% of total diversity) by an empirical dividing line (x = 0.015) (Figure 1C), which assigned the 34 samples to 2 groups, completely in agreement with the 2 types of microbiome. The reducibility of the complex microbiome typing scheme to PC1 by double principal coordinate analysis suggests that the microbiome types might be primarily determined by specific bacterial subpopulations within the whole microbiome. This finding led us to examine whether the microbiome types are determined by the relative abundance of 1 or a few specific taxa (Supplementary Figure 1). We approached this question by classifying the 16S rRNA sequences at various taxonomic ranks, correlating the relative abundances of all main taxonomic groups with
Figure 3. Differential representation of genera between the 2 types of microbiome. Pooled 16S rRNA gene sequences from type I samples were compared at the genus level (or the lowest classifiable rank above genus) with those from type II samples using LIBRARY COMPARE in RDP II. Relative abundances is shown in the table on the right and by the horizontal bars, with genera that are significantly different between the 2 types of microbiome highlighted in red. Taxa unclassified at the genus level are marked with a paragraph symbol (¶). Distribution of the genera in the taxonomic hierarchy of domain bacteria is shown in the phylogenetic tree, with alternating black and green brackets to contrast neighboring phyla. Bootstrap values were based on 500 replicates.
PC1, defining normal and abnormal taxonomic types by using abundance-based NRR of the bacterial groups, and validating the taxonomic types by comparing them with microbiome types in assignment of individual samples.

First, we binned the 6800 PCR clones into taxonomic groups at the phylum, genus, and species levels. In total, 9 phyla, 70 genera, and 166 SLOTUs were represented (Figure 3 and Supplementary Figure 2). We used Chao1 estimation to assess the relative depth of coverage. The analysis indicates that the human distal esophagus may harbor \( \sim 213 \) (95% CI: 191–254) SLOTUs and suggests that 77.9% of the SLOTUs (166/213) have been identified in this study (Supplementary Figure 3). Firmicutes (4868 clones) was the only phylum consistently detected in all 34 samples, whereas the other 8 phyla, Bacteroidetes (720, 33/34 samples), Proteobacteria (843, 31/34), Actinobacteria (240, 28/34), Fusobacteria (92, 26/34), TM7 (32, 13/34), Spirochaetes (3, 2/14), Cyanobacteria (1, 1/34), and unclassified bacteria (1, 1/34) were less common. The 6 most abundant phyla were shared by the 2 types of microbiome.

Streptococcus was the predominant (3989 clones) genus, represented by 17 SLOTUs, in particular, S. mitis (2173) and S. pseudopneumoniae (1119), both being members of the mitis group. Streptococcus and S. mitis were the only taxa found in all 34 samples at the genus and species levels, respectively.

Next, we designed an analysis, which we called microbiome-abundance correlation (MAC) to facilitate identifying taxonomic groups whose relative abundance in the 34 samples significantly correlated with their PC1 in the double principal coordinate analysis (Figure 1C) (equation 6 in Supplementary Materials and Methods). In the direction from the type I to type II microbiome, the PC1 significantly correlated with decreasing abundance of Firmicutes \( r = -0.97, P = 9.6 \times 10^{-14} \), Streptococcus \( r = -0.99, P = 4.5 \times 10^{-15} \), and S. mitis \( r = -0.73, P = 1.9 \times 10^{-3} \) (Figure 4A).

Because of its stronger correlation with PC1, we considered that the relative abundance of the genus Streptococcus probably determined the 2 types of microbiome. To test this hypothesis, we calculated a 95% NRR based on the relative abundance of Streptococcus. After excluding 1 outlier, the remaining 11 samples from phenotypically normal esophagus had a mean ± SD of 75.9% ± 12.8% Streptococcus and a 95% NRR (mean ± 1.96 SD) of 50.8%–100%. Use of the lower limit of the NRR (50.8%) as a threshold separated the 24 samples from the esophagitis and BE groups into 2 taxonomic types (Figure 4B). All 13 cases classified as abnormal by the NRR corresponded to the type II microbiome, whereas all 9 samples classified as normal (n = 9) belonged to the type I microbiome, without ambiguity. These 9 samples had a mean Streptococcus abundance similar to that of the 11 normal samples (82.2% vs 75.9%, respectively, \( P = 0.238 \)), whereas the outlier sample from the normal esophagus group that was categorized as type II microbiome had a low Streptococcus abundance (13.5%). Overall, the 20 type I samples had a mean of 78.8% Streptococcus (range, 60.5%–97.0%), whereas the 14 type II samples had a mean of 30.0% (range, 8.0%–46.5%) \( (P < 1 \times 10^{-13}, t \text{ test}) \). The mean of relative abundance of Streptococcus in the normal esophagus group (75.9%, n = 11) was significantly higher than that in the esophagitis (50.5%, n = 12) and BE (54.1%, n = 10) groups (Supplementary Table 4).

**Gram-Negative Anaerobes Prevail in the Type II Microbiome**

In addition to Streptococcus, MAC analyses also revealed significant, but weaker, correlations of PC1 with
the relative abundances of Bacteroidetes \( r = 0.81, P = 4.0 \times 10^{-8} \), Proteobacteria \( r = 0.65, P = 3.7 \times 10^{-5} \), and Fusobacteria \( r = 0.63, P = 8.2 \times 10^{-5} \). Unlike for \textit{Streptococcus}, further analyses using abundance-based 95% NRR for each of these phyla or their predominant genera could not clearly assign all samples into the 2 types of microbiome. Because the majority of PCR clones from these 3 phyla was gram negative and/or anaerobic/microaerophilic bacteria, we hypothesized that these broad properties also could be used to determine the microbiome types.

MAC analysis showed a strong correlation of PC1 with the relative abundance of anaerobic/microaerophilic bacteria \( r = 0.98, P = 2.3 \times 10^{-14} \) (Figure 5A). Anaerobic (type I: 11.0% vs type II: 38.2%, \( P = 1.2 \times 10^{-5} \), t test) and microaerophilic bacteria (5.4% vs 23.0%, respectively, \( P = 1.1 \times 10^{-7} \)) were more abundant in the 14 type II samples than in the 20 type I samples (Figure 5E). In combination, anaerobic and microaerophilic bacteria comprised an average of 61.1% of the sampled clones in type II samples but only 16.3% in type I samples \( (P < 1 \times 10^{-10}, \text{t test}) \). The 95% NRR based on the relative abundance of aerobic/microaerophilic bacteria in the phenotypically normal samples correctly identified the microbiome types for all but 1 of the 34 samples (Supplementary Figure 4A).

MAC analysis also showed a significant correlation of PC1 with the relative abundance of gram-negative bacteria \( r = 0.97, P = 5.8 \times 10^{-14} \) (Figure 5B). Gram-negative bacteria comprised an average of 53.4% of sampled clones in the 20 type II samples but only 14.9% in the 14 type I samples \( (P = 8.0 \times 10^{-10}, \text{t test}) \) (Figure 5F). The 95% NRR based on the relative abundance of gram-negative bacteria in the phenotypically normal samples correctly identified the microbiome types of all but 3 of the 34 samples (Supplementary Figure 4B).

The strong correlations between PC1 and the relative abundance of the predominant bacterial groups in the type I (\textit{Streptococcus}) and type II microbiome (gram-negative bacteria and anaerobes/microaerophils) suggest an
The present study has provided 2 new contributions to the field of human microbial ecology. First, we have performed a comprehensive study of the human distal esophagus microbiome and demonstrated the presence of a complex microbiome about which little prior knowledge was available. The esophageal microbiome is comparable in complexity with those found in the mouth, stomach, colon, vagina, and skin. Collectively, 9 phyla were observed, represented by 166 species. The distal esophagus could harbor >200 species, as predicted by the Chao1 richness estimator. Second, we have demonstrated by both unsupervised and phenotype-directed analyses that the esophageal microbiome can be classified into 2 types and that the type II microbiome is the strongest (OR, >15) among all known environmental factors that are associated with the pathologic changes related to GERD (Supplementary Table 5). Overall, the findings have opened a new approach to understanding the recent surge in the incidence/prevalence of GERD and esophageal adenocarcinoma and suggest the possible role of dysbiosis in their pathogenesis.

The need to compare with risk factors identified by conventional studies prompted us to design a dual assignment scheme for the esophageal samples. This approach enabled testing the independence of 2 categorical variables: host phenotype and microbiome type. The microbiome types determined from the present study are relevant to pathology in the distal esophagus. There are 2 possible explanations for the significant association between the type II microbiome and the abnormal histologic phenotype. First, the type II microbiome might play a causative role in GERD, which has a complex and not yet completely understood pathophysiology. Abnormal lower esophageal sphincter pressure and esophageal acidification during transient lower esophageal sphincter relaxation are believed to be critical, but the etiology of the abnormal lower esophageal sphincter function is unknown. One possibility is that the esophageal microbiome could be intrinsic: each individual might harbor either a stable type I or type II microbiome. A distinct microbiome can be inherited via kinship from mother or caregivers, as suggested in studies of the mouse colonic

Discussion

The present study has provided 2 new contributions to the field of human microbial ecology. First, we have performed a comprehensive study of the human distal esophagus microbiome and demonstrated the presence of a complex microbiome about which little prior knowledge was available. The esophageal microbiome is comparable in complexity with those found in the mouth, stomach, colon, vagina, and skin. Collectively, 9 phyla were observed, represented by 166 species. The distal esophagus could harbor >200 species, as predicted by the Chao1 richness estimator. Second, we have demonstrated by both unsupervised and phenotype-directed analyses that the esophageal microbiome can be classified into 2 types and that the type II microbiome is the strongest (OR, >15) among all known environmental factors that are associated with the pathologic changes related to GERD (Supplementary Table 5). Overall, the findings have opened a new approach to understanding the recent surge in the incidence/prevalence of GERD and esophageal adenocarcinoma and suggest the possible role of dysbiosis in their pathogenesis.

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microbiome, which can be modified by exposure to antibiotics during or after the postnatal development of the microbiome is complete. The gram-negative predominant type II microbiome could serve as a primary or synergistic mechanism in promoting gastric reflux because lipopolysaccharides, mainly produced by gram-negative bacteria, induce abnormal relaxation of the lower esophageal sphincter via activation of the inducible nitric oxide synthase pathway. Second, the type II microbiome might be secondary to changes caused by gastric reflux. The esophageal microbiome could be transitory: the type I microbiome could represent a direct extension of the normal oral flora via saliva, whereas the type II microbiome could represent regurgitated bacteria in gastric juice. Alternatively, gastroesophageal reflux might modify the esophageal microbiome by selecting against acid-sensitive bacteria in the esophagus. Testing these hypotheses might shed light on the pathogenesis of GERD and lead to new biomarkers for GERD.

Although the present study yielded one of the largest data sets from a single study of microbiome in human diseases, our understanding of the esophageal microbiome is far from complete. The power of this study might be limited by unrecognized factors that are unrelated to GERD but may affect the bacterial microbiome, including diet, medications, and oral and gastric diseases. Similarly, any interpretation of our data in relation to GERD might be limited by unrecognized factors that are unresolved by the single factors. Significant acid reflux can occur in 19% of (normal) subjects without reflux-related symptoms. Not all patients with abnormal acid exposure have esophagitis, as defined by histology, which can present in subjects without reflux; a substantial proportion of patients with BE lack reflux symptoms. The significant but nonexclusive association of the type II microbiome with histologically defined changes related to GERD is consistent with the complex GERD phenotype. Notably, our study subjects were generally elderly male veterans. This relatively homogeneous cohort helped limit confounding but also might limit the application of our findings to a more general population. Despite the complex host and environmental factors, the findings in the present analyses make possible design of further studies to directly examine possible causal roles of the type II microbiome in GERD development. If GERD represents a microecologic disease, a new type of treatment for reflux might become possible, for example, by converting the type II to type I microbiome through use of antibiotics, probiotics, or prebiotics.

Supplementary Data

Note: To access the supplementary material accompanying this article, visit the online version of Gastroenterology at www.gastrojournal.org, and at doi: 10.1053/j.gastro.2009.04.046.

References


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Conflicts of interest
The authors disclose no conflicts.

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