BRIEF REPORT

Intestinal Dysbiosis in Ankylosing Spondylitis

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Objective. Ankylosing spondylitis (AS) is a common, highly heritable immune-mediated arthropathy that occurs in genetically susceptible individuals exposed to an unknown but likely ubiquitous environmental trigger. There is a close relationship between the gut and spondyloarthritis, as exemplified in patients with reactive arthritis, in whom a typically self-limiting arthropathy follows either a gastrointestinal or urogenital infection. Microbial involvement in AS has been suggested; however, no definitive link has been established. The aim of this study was to determine whether the gut in patients with AS carries a distinct microbial signature compared with that in the gut of healthy control subjects.

Methods. Microbial profiles for terminal ileum biopsy specimens obtained from patients with recent-onset tumor necrosis factor antagonist–naive AS and from healthy control subjects were generated using culture-independent 16S ribosomal RNA gene sequencing and analysis techniques.

Results. Our results showed that the terminal ileum microbial communities in patients with AS differ significantly (P < 0.001) from those in healthy control subjects, driven by a higher abundance of 5 families of bacteria (Lachnospiraceae [P = 0.001], Ruminococcaceae [P = 0.012], Rikenellaceae [P = 0.004], Porphyromonadaceae [P = 0.001], and Bacteroidaceae [P = 0.001]) and a decrease in the abundance of 2 families of bacteria (Veillonellaceae [P = 0.01] and Prevotellaceae [P = 0.004]).

Conclusion. We show evidence for a discrete microbial signature in the terminal ileum of patients with AS compared with healthy control subjects. The microbial composition was demonstrated to correlate with disease status, and greater differences were observed between disease groups than within disease groups. These results are consistent with the hypothesis that genes associated with AS act, at least in part, through effects on the gut microbiome.

Intestinal microbiome dysbiosis and microbial infections have been implicated in several immune-mediated diseases, including multiple sclerosis, inflammatory bowel disease (IBD), and type 1 diabetes. Bacterial infection in the gut and urogenital tract is known to trigger episodes of reactive arthritis, a form of spondyloarthropathy (SpA) belonging to a group of related inflammatory arthropathies, of which ankylosing spondylitis (AS) is the prototypic disease. The close relationship between the gut and SpA is exemplified in patients with reactive arthritis, in whom a typically self-limiting arthropathy follows either gastrointestinal infection with Campylobacter, Salmonella, Shigella, or Yersinia, or urogenital infection with Chlamydia. Microbial involvement in AS has been suggested; however, no definitive link has been established (1).

Up to 70% of patients with AS have subclinical gut inflammation, and 5–10% of these patients have more severe intestinal inflammation that progresses to clinically defined IBD resembling Crohn’s disease (CD) (2). The high heritability of AS, the global disease distribution, and the absence of outbreaks of the disease suggest that AS is triggered by a common environmental agent in genetically susceptible individuals (3). Multiple
genes associated with AS also play a role in gut immunity, such as genes involved in the interleukin-23 (IL-23) pathway (4), which are important regulators of intestinal “health.” Marked overrepresentation of genes that are associated with CD is also associated with AS (5); this suggests that the 2 diseases may have similar etiologic mechanisms, possibly involving gut dysbiosis.

Several studies have shown that patients with AS and their first-degree relatives have increased intestinal permeability relative to unrelated healthy control subjects, which, again, is consistent with a role for the gut microbiome in AS (6). To date, no comprehensive characterization of intestinal microbiota in patients with AS has been performed. We therefore performed culture-independent microbial community profiling of terminal ileum biopsy specimens to characterize and investigate differences in the gut microbiome between patients with AS and healthy control subjects.

**PATIENTS AND METHODS**

Biopsy specimens from the terminal ileum were obtained at the time of colonoscopy from 9 consecutively enrolled tumor necrosis factor inhibitor-naive patients with recent-onset (duration from symptom onset ≤48 months) AS (defined according to the modified New York classification criteria for AS [7]) and 9 healthy control subjects (Table 1) (see also Supplementary Table 1, available on the Arthritis & Rheumatology web site at http://onlinelibrary.wiley.com/doi/10.1002/art.38967/abstract). All patients gave written informed consent, and the study protocol was approved by the relevant University of Palermo and University of Queensland research ethics committees. One patient with AS was receiving nonsteroidal antiinflammatory drugs (NSAIDs) at the time of biopsy. AS patients 3, 4, and 10 had reported occasional use of NSAIDs, which was interrupted due to gastrointestinal upset. Other patients with AS did not report use of NSAIDs but were receiving either acetaminophen and/or tramadol.

The terminal ileum microbial and mock communities were profiled by high-throughput amplicon sequencing of the 16S ribosomal RNA (rRNA) gene (250-bp barcode) on an Illumina MiSeq sequencer using dual-indexed v4-region forward primer 517F (5′-GCCAGAGGTTTACGACTT-3′) and reverse primer 803R (5′-CTACCRGGGTATCTAATCC-3′). In addition to exploring community-level differences between disease states, we evaluated technical and biologic replication. Biologic replication was examined by halving the biopsy specimens and performing all subsequent studies in parallel to assess the reproducibility of findings from the time of biopsy forward. Technical replication was performed by analyzing forward and reverse sequencing reads separately and then comparing them. The resulting sequence libraries were analyzed using the Quantitative Insights Into Microbial Ecology (QIIME) pipeline (8).

Alpha diversity metrics, which are used to examine the diversity within a sample, were generated using the QIIME workflow. Hierarchical clustering was performed, using the unweighted pair group method with arithmetic mean (including both the weighted and unweighted UniFrac distance) to detect significant differences within microbial communities between patients with AS and healthy controls. The weighted UniFrac distance metric detects changes in the number of organisms present in a community, taking into account the relative abundance of microbes present. The unweighted UniFrac distance metric describes community membership. UniFrac, enabled in QIIME, was used to generate sample distance metrics.

**Table 1. Characteristics of the patients with AS and the healthy control subjects at the time of biopsy**

<table>
<thead>
<tr>
<th>Subject ID</th>
<th>Age, years</th>
<th>Sex</th>
<th>Disease duration, years†</th>
<th>ESR, mm/hour</th>
<th>CRP, mg/liter</th>
<th>BASDAI</th>
<th>HLA-B27 status</th>
<th>NSAID treatment</th>
<th>Histologic inflammation</th>
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<tr>
<td>AS01</td>
<td>56 M</td>
<td>7</td>
<td>34</td>
<td>18</td>
<td>7</td>
<td>Positive</td>
<td>Current</td>
<td>Chronic</td>
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<tr>
<td>AS02</td>
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<td>4</td>
<td>22</td>
<td>1</td>
<td>5.5</td>
<td>Positive</td>
<td>None</td>
<td>Acute</td>
<td></td>
</tr>
<tr>
<td>AS03</td>
<td>24 F</td>
<td>5</td>
<td>18</td>
<td>8</td>
<td>4.8</td>
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<td>33</td>
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<td></td>
</tr>
<tr>
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<td></td>
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<tr>
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<td>28</td>
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<tr>
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<td>45</td>
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<tr>
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<td>0.8</td>
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<td>–</td>
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</table>

* In all subjects, the ileum was the biopsy site. AS = ankylosing spondylitis; ESR = erythrocyte sedimentation rate; CRP = C-reactive protein; BASDAI = Bath AS Disease Activity Index; NSAID = nonsteroidal antiinflammatory drug; HC = healthy control.
† Beginning at the onset of symptoms.
metrics as well as principal coordinate analysis (PCoA). Core microbiome analysis as well as supervised learning was performed to further characterize the intestinal microbial signature, using the workflow pipelines in QIIME (8). The function of bacteria and communities was predicted using PICRUSt (9).

To determine differences in the microbial load, the total microbial biomass in biopsy samples was quantified using real-time quantitative polymerase chain reaction (PCR) analysis of the 16S rRNA gene, as described by Willner et al (10). PERMANOVA analysis was conducted at the genus level using the vegan package in R to test the relationship between the whole microbial community and disease. Indicator species analysis was performed using the labdsv package in R. Co-occurrence network analysis was conducted using the spaa package in R.

RESULTS

The microbial communities in the terminal ileum of patients with AS were significantly different (Figure 1A) and more diverse (Figure 1C) compared with those in healthy control subjects (P < 0.001), as determined using PERMANOVA. PCoA showed that AS samples, including biologic replicates, grouped separately from control samples, indicating that disease is the primary factor influencing the community differences (Figure 1B) (see also Supplementary Figure 1, available on the Arthritis & Rheumatology web site at http://onlinelibrary.wiley.com/doi/10.1002/art.38967/abstract).

To further demonstrate the distinct groupings of AS samples and control samples, supervised learning was conducted to test the predictive capacity of the microbiome differences, and all 9 AS samples obtained from patients with AS had been predicted to be AS samples (Figure 1B). PERMANOVA analysis further confirmed the presence of a significant relationship between disease status and microbial community composition (P < 0.001). This was not due to heterogeneity in biopsy samples, because biologic replicates showed no significant differences between each other. Quantitative PCR analysis of biomass showed no significant differences, on average, in the 16S rRNA copy number between AS samples and control samples, indicating that the observed differences were not attributable to overgrowth or dominance of bacteria driving community differences (see Supplementary Figure 2, available on the Arthritis & Rheumatology web site at http://onlinelibrary.wiley.com/doi/10.1002/art.38967/abstract). The average UniFrac distances demonstrated that differences between both

**Figure 1.** A, Difference in taxonomy at the phylum level between patients with ankylosing spondylitis (AS) and healthy control subjects (HCs), showing the increase in *Bacteroides* and the change in the *Bacteroides*-to-*Firmicutes* ratio in the AS samples. B, Distinct clustering of AS samples compared with control samples, as determined by weighted principal coordinate analysis (PCoA). Supervised learning showed that all 9 AS samples were predicted to be AS. C, Alpha diversity box plot showing increased microbial diversity (observed species) in AS samples compared with control samples. Data are presented as box plots, where the boxes represent the 25th to 75th percentiles, the lines within the boxes represent the median, and the lines outside the boxes represent the 10th and 90th percentiles. D, Comparison of variation within and between disease status, showing greater significance between and within disease status than between technical replicates and biologic replicates. Values are the mean ± SD. *** = P < 0.001 by Mann-Whitney test. NS = not significant.
biologic and technical replicates were significantly less than differences between individuals and disease states \((P < 0.001, \text{ by Mann-Whitney test})\) (Figure 1D).

Community profiling showed 51 genera across all biopsy specimens, with major differences observed at the phylum level between AS and control specimens (Figure 1A). Indicator species analysis was performed to determine whether alterations in specific species were driving the differences observed between communities in patients with AS and healthy control subjects. This analysis showed that, compared with the microbial communities in healthy control subjects, those in patients with AS were characterized by a higher abundance of \textit{Lachnospiraceae}, including \textit{Coprococcus} species and \textit{Roseburia} species \((P = 0.001)\), \textit{Ruminococcaceae} \((P = 0.012)\), \textit{Rikenellaceae} \((P = 0.004)\), \textit{Porphyromonadaceae} including \textit{Parabacteroides} species \((P = 0.001)\), and \textit{Bacteroidaceae} \((P = 0.001)\). Decreases in the abundance of \textit{Veillonellaceae} \((P = 0.01)\) and \textit{Prevotellaceae} \((P = 0.004)\) were observed (Figure 2A).

Further drilling down into the AS microbiome signature using co-occurrence analysis, which examines the correlation between microbes, showed that bacterial interactions further shape the AS microbial community signature, with positive correlations observed between the indicator species \textit{Lachnospiraceae} and \textit{Ruminococcaceae} and negative correlations observed between \textit{Veillonella} and \textit{Prevotella} (Figure 2B). Microbial functional prediction using PICRUSt indicated 31 significant pathways with differential representation in patients with AS compared with healthy controls \((P < 0.02, \text{ with Bonferroni correction})\) (see Supplementary Table 2, available on the \textit{Arthritis & Rheumatology} web site at http://onlinelibrary.wiley.com/doi/10.1002/art.38967/abstract). These pathways included a decrease in bacterial invasion of epithelial cells \((P = 4.33 \times 10^{-5})\), an increase in antimicrobial production in the butirosin and neomycin biosynthesis pathway \((P = 0.002)\), which is consistent with an increase in \textit{bacteria} from the \textit{Bacteroidaceae} family, and an increase in the secondary bile acid biosynthesis pathway \((P = 0.004)\), which is consistent with an increase in \textit{Clostridia} and \textit{Ruminococcaceae} species.

We were interested in further interrogating the AS microbiome signature, which is the overall combination of microbes that distinguishes patients with AS from healthy control subjects, by investigating whether an assemblage or “core” set of microbes was present in all AS and control samples at varying levels, and to probe their functional capacity. Exploring the core microbiome is imperative to better understand the stable and consistent components across complex microbial communities, given the distinct AS microbial signature. The Core 100 species (microbes present in all samples) included the \textit{Clostridium}, \textit{Actinomycetaceae}, \textit{Bacteroidaceae}, \textit{Lachnospiraceae}, \textit{Porphyromonadaceae}, \textit{Rikenellaceae}, \textit{Ruminococcaceae}, and \textit{Veillonellaceae} families of bacteria. These families of bacteria are also AS indicator species, suggesting that the core microbiome is driving the AS microbial signature (Figure 2A).

The Core 100 species belonged to 22 significant pathways \((P < 0.02, \text{ with Bonferroni correction})\) (see Supplementary Table 3, available on the \textit{Arthritis & Rheumatology} web site at http://onlinelibrary.wiley.com/doi/10.1002/art.38967/abstract) that again included bacterial invasion of epithelial cells \((P = 0.004)\), antimicro-
bial production in the butirosin and neomycin biosynthesis pathway \((P = 0.007)\), and the secondary bile acid biosynthesis pathway \((P = 0.013)\). Members of the *Bacteroides*, *Clostridium*, and *Ruminococcus* groups are known to be involved in cholesterol metabolism and secondary bile synthesis; disordered bile acid synthesis due to intestinal dysbiosis has previously been implicated in the pathogenesis of IBD \((11)\).

As the threshold for core membership was reduced to 90%, 75%, and 50%, the families of bacteria present remained the same; however, the number of genus and species within these families increased, especially in *Clostridia*, *Lachnospiraceae*, and *Ruminococcus*. This demonstrates that the structure of the core microbiome is robust, and that decreasing the threshold expands the number of genera and species of bacteria only within these families. As the core threshold decreased, however, the number of significant pathways also decreased, suggesting that as the threshold is relaxed, the expansion of genera and operational taxonomic units dilutes pathway signals.

Indicator species analysis also determined that healthy control subjects had an increased abundance of *Streptococcus* and *Actinomyces* compared with patients with AS. No difference was noted between patients with AS and control subjects in the presence of either bacteria known to be associated with reactive arthritis or *Klebsiella* species, which have been proposed to play a role in triggering AS \((12)\).

**DISCUSSION**

Here, we present the first characterization and identification of intestinal dysbiosis in the AS microbiome, using 16S rRNA community profiling of terminal ileum biopsy specimens. We show evidence for a discrete microbial signature in the terminal ileum of patients with AS compared with healthy control subjects. The microbial profile differences are not attributable to differences in overall bacterial quantity between patients with different diseases but are qualitative. PCoA was able to show the distinct grouping of patients with AS versus healthy control subjects; larger studies will be required to define the individual bacterial species involved. Statistically, the relationship between disease status and microbial community composition was confirmed, indicating that the differences in composition of the microbial community were not due to heterogeneity in the biopsy specimens or a lack of technical reproducibility, but that the driving force is disease state.

Of the 7 families of microbes with differences in abundance within the AS microbiome, *Lachnospiraceae*, *Ruminococcaceae*, and *Prevotellaceae* are strongly associated with colitis and CD, with *Prevotellaceae* especially known to elicit a strong inflammatory response in the gut \((13)\). Further investigations showed that correlations between these families of bacteria further shape the AS microbial signature, and that these microbes were present in all AS samples studied, suggesting that they are not only driving the microbial signature but also are at the core of the AS microbial signature. Increases in *Prevotellaceae* and decreases in *Rikenellaceae* in the intestinal microbiome have also recently been observed in the HLA–B27–transgenic rat model of spondyloarthritis, suggesting that underlying host genetics may play a role in sculpting the gut microbiome in this animal model \((14)\).

The increased diversity of the AS community without an overall change in microbial load shows that no overgrowth or dominance of a particular microbe drives the signature. However, murine experiments demonstrate that both the overall composition of the intestinal microbiome and the presences and/or absence of specific microbes can have a substantial impact on host response, regulation of inflammation, and development of intestinal cells. In the K/BxN mouse model of arthritis, it was shown that the introduction of a single gut-residing species, segmented filamentous bacteria, into germ-free mice was sufficient to reinstate Th17 cells, leading to the production of autoantibodies and arthritis. When IL-17 was neutralized in specific pathogen–free K/BxN mice, development of arthritis was prevented. Thus, a single commensal microbe, via its ability to promote a specific T helper cell subset, can drive immune-mediated disease \((15)\).

Further studies are needed to investigate whether the changes in intestinal microbial composition are due to host genetics and how this affects the overall function of the gut microbiome in AS patients, including how the microbiome then goes on to shape the immune response and influence inflammation. In particular, given the strong association of HLA–B27 with AS, it has been hypothesized that HLA–B27 induces AS by effects on the gut microbiome, in turn driving spondyloarthritis-inducing immunologic processes such as IL-23 production \((16)\). Our data showing intestinal dysbiosis in patients with AS is consistent with this hypothesis, but further studies are clearly required to distinguish cause-and-effect interactions between the host genome and immune system and the gut microbiome. These investigations will provide new insights and help us to better understand the pathogenesis of AS.
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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Brown had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.


Acquisition of data. Costello, Ciccia, Gardiner, Marshall, Triolo, Brown.


REFERENCES


