The faecal microbiota is distinct in HLA-B27+ ankylosing spondylitis patients *versus* HLA-B27+ healthy controls

M.L. Stoll[1], H. Sawhney[2], P.M. Wells[3], P.R. Sternes[4], J.D. Reveille[5], C.D. Morrow[6], C.J. Steves[3], M.A. Brown[7], L.S. Gensler[2]

[1]Department of Pediatrics, University of Alabama at Birmingham (UAB), AL, USA; [2]Department of Medicine, University of California at San Francisco, CA, USA; [3]Department of Twin Research, King's College London (KCL), UK; [4]Centre for Microbiome Research, Queensland University of Technology, Brisbane, Australia; [5]Department of Internal Medicine, University of Texas at Houston, TX, USA; [6]Department of Cell, Developmental and Integrative Biology, UAB, Birmingham, AL, USA; [7]Department of Medical and Molecular Genetics, Faculty of Life Sciences and Medicine, King's College London, UK; Genomics England Ltd, London, UK.

Abstract

Objective

Spondyloarthritis (SpA) results from the interplay between genetic and environmental factors. An emerging modifiable factor is the human intestinal microbiota, which multiple studies in children and adults have shown to be abnormal in SpA patients, including enthesitis related arthritis and ankylosing spondylitis (AS). However, HLA-B27 itself appears to impact the contents of the microbiota and is more common in SpA patients versus controls, thus serving as a confounding factor in most comparative studies.

Methods

This was a cross-sectional study that evaluated the contents of the faecal microbiota among 29 patients with HLA-B27+ AS and 43 healthy adults who underwent 16S sequencing and genotyping as part of the TwinsUK Programme.

Results

HLA-B27 positive+ patients and healthy controls demonstrated substantial clustering based upon diagnosis. Decreased richness was observed among the AS patients, although measures of evenness were similar. After correction for multiple comparisons, several taxa – including Faecalibacterium prausnitzii and Coprococcus – were elevated in AS patients compared to controls, even when restricted to female subjects, while Bacteroides fragilis, Ruminococcus, and Akkermansia muciniphila were depleted in AS patients.

Conclusion

Consistent with some previous studies, our study demonstrates in patients with AS associations with Coprococcus, Bacteroides, and Ruminococcus. Other findings, including increased Faecalibacterium, are inconsistent with previous studies and thus potentially underscore the necessity of evaluating HLA-B27 positive controls in studies evaluating the impact of the intestinal microbiota on SpA.

Key words

microbiota, ankylosing spondylitis

Matthew L. Stoll, MD, PhD
Henna Sawhney, MD
Philippa M. Wells, PhD
Peter R. Sternes, PhD
John D. Reveille, MD
Casey D. Morrow, PhD
Claire J. Steves, PhD
Matthew A. Brown, MD
Lianne S. Gensler, MD

The work should be attributed to the Department of Pediatrics, University of Alabama at Birmingham.

Please address correspondence to:
Matthew Stoll
University of Alabama at Birmingham, 1601 4th Ave. S / CPP Suite G10, 35233 Birmingham (AL), USA.
E-mail: mstoll@uabmc.edu

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Introduction

Spondyloarthritis (SpA) impacts about 1–2% of the adult population (1) and can result in significant disability and reduced quality of life (2). The cause appears to be multifactorial, with both genetic and environmental contributing factors. The best-characterised genetic risk factor is the HLA-B27 allele, which is present in 80–95% of white ankylosing spondylitis (AS) patients (3) compared to about 7.5% of the U.S. white population (4). However, carriage of HLA-B27 is insufficient by itself to cause AS, which develops in <5% of people with this allele (5). While other genetic factors are clearly involved (6), there has been substantial interest in environmental factors, particularly the human intestinal microbiota. Specifically, multiple studies have demonstrated alterations in the contents and function of the intestinal microbiota in patients with paediatric and adult SpA (7-11).

A limitation of most of these studies is an imbalance in the frequency of HLA-B27 positivity between the patient and control subjects, as this allele is present more frequently in patients with SpA as compared to the general population (4). This issue is particularly relevant given potential impacts of this allele on the microbiota. Specifically, data from the HLA-B27 transgenic rat model of SpA demonstrated that in multiple different genetic backgrounds, this allele resulted in substantial impacts of the microbiota (12). Likewise, studies of healthy human subjects have demonstrated altered faecal and intestinal microbiota populations between HLA-B27 positive and negative subjects (13). Indeed, among the theories accounting for the associations between the HLA-B27 allele and SpA is that the allele mediates the disease directly by impacting the microbiota (14). However, we have recently published data in paediatric offspring of AS patients that HLA-B27 did not have substantial impacts on the microbiota, and the primary organism impacted by the HLA-B27 allele was *Faecalibacterium prausnitzii*, which the above and other studies had demonstrated to be depleted in children with SpA (7, 15, 16). This paediatric study also demonstrated differences between HLA-B27+ SpA patients and HLA-B27 positive offspring, indicating a potential role for the gut microbiota even regardless of HLA-B27 status (16).

In this study, we set out to compare the faecal microbiota of adult HLA-B27 positive patients with ankylosing spondylitis (AS) to that of HLA-B27 positive controls. To do so, we used the TwinsUK programme, in which approximately 2500 healthy adults have provided stool specimens for 16S analysis as well as saliva for genotyping. Given the large number of participants, we were able to limit to those who are imputed to be positive for HLA-B27. To our knowledge, this is the first comparison of adult AS patients and adult healthy controls limited to HLA-B27 positive patients and controls.

Patients and methods

Overview

This was a cross-sectional study that compared the contents of the faecal microbiota among adults with HLA-B27 positive AS and healthy HLA-B27 positive controls from the TwinsUK programme. Additional comparisons were subsequently performed exclusively involving HLA-B27 TwinsUK subjects. The study was reviewed by the Flora Twins Study National Research Ethics Service London – Westminster REC as well as by the Institutional Review Boards at the University of California in San Francisco (UCSF) and the University of Alabama at Birmingham (UAB). All subjects provided informed consent.

Ankylosing spondylitis subjects

Patients with AS were identified by a single rheumatologist (LSG) at UCSF. They were diagnosed with AS and classified by the modified New York criteria (17). Subjects with exposure to antibiotics or sulfasalazine within three months prior to the sample collection were excluded.

TwinsUK subjects

TwinsUK is a registry of monozygotic and dizygotic twins established in 1992 to examine environmental and genetic predictors of a range of rheumatolo-

gic diseases. It presently includes over 15,000 twins, of whom over 3,000 have undergone evaluation of the faecal microbiota. Subjects who have had both sequencing of the 16S ribosomal RNA and genotyping were eligible for inclusion in the current study. The following potential control subjects were excluded from the study: (1) Those reporting current or recent (one month) antibiotic usage; (2) those reporting use of immunomodulatory therapy, due to the presumption that such individuals had an inflammatory condition that could impact the faecal microbiota; (3) those older than 70 years of age, as aged 70–80 appears to be an inflection point with respect to changes in the faecal microbiota (18, 19). For HLA-B27 positive participants, if two twins of a pair met the eligibility criteria, then in the case of dizygotic twins, the one with the higher posterior probability for carriage of HLA-B27 was selected, while in the case of monozygotic twins, the one labelled as "Twin-1" was arbitrarily selected prior to any assessment of the microbiota. The rationale for using only one twin is the known similarity between the faecal microbiota of twins compared to unrelated individuals (20). Analyses of HLA-B27 negative TwinsUK subjects were limited to subjects aged 40–60, and only one member of a sib set was included.

Processing and sequencing of faecal samples

For the AS patients, this was done as previously reported (15). Briefly, subjects collected the samples at home and immediately placed them in a container filled with Cary-Blair media (21) and shipped them overnight with an ice pack to the Microbiota Core at UAB. Microbial DNA was isolated with the Zymo MiniPrep kit (Irvine, CA) as per the manufacturer's instructions. For TwinsUK, faecal samples were generally collected at home during the two days prior to a clinic visit and stored in a refrigerator until the visit. When this was not performed, specimens were likewise shipped overnight with an ice pack to the Department of Twin Research. Upon receipt at both centers, they were stored at -80°C. Purification of DNA was performed with the PowerSoil® - htp DNA isolation kit (MoBio Laboratories Ltd, Carlsbad, CA). Both studies used ~100 ng of DNA to perform PCR amplication of the V4 region of the 16S ribosomal DNA gene as per Caporoso *et al*. (22) on the Illumina MiSeq platform, generating 250 base-pair paired-end reads. The sequence reads of the AS subjects were deposited into the Sequence Read Archive (23).

Analysis of the 16S ribosomal DNA faecal specimens

All of the sequence files were analysed identically and simultaneously, including removal of sequences with low quality scores and merger of the paired reads. Clustering was performed with the quantitative insight into microbial ecology (QIIME) suite (v. 1.9) (24), using uclust (25) as implemented by the pick_open_reference_otus.py script in QIIME using the Silva database at 97% similarity for reference (26). Rare (<0.1%) operational taxonomic units (OTUs) were removed, and the resulting Biom file was uploaded into R (v. 3.6.0) for further processing with Phyloseq (27), including transformation. A distance matrix generated using the Bray-Curtis (28) test and principal coordinates analysis was used to visualise the clustering. Permutational Analysis of Variance (PermANOVA) testing (29) as administered by the Adonis test in the Vegan package (30) was used to test the significance of subject clustering by group (beta diversity). The Chao1 measure was used to assess richness, while the Shannon and inverse Simpson measures were used to assess evenness.

To evaluate which taxa were different between the two groups, we used the DeSeq2 programme (31). Although initially designed for analysis of RNAseq data, this is well-suited for the analysis of microbiota data, which is also characterised by large numbers of comparators many of which are present at near-zero levels, thus imposing often impossibly high barriers for corrected statistical significance. DeSeq2 normalises for total read counts; filters out very low abundant features, which have a very low a priori likelihood of being informative; flags outliers based upon the Cook's distance for either removal or imputation depending on the group sample size; and calculates the $\log_2$ fold change between the two groups, here AS *versus* healthy controls, while correcting for multiple comparisons with the Benjamini-Hochberg false discovery rate (FDR) test (32) with a corrected significance threshold of 0.05.

Genotyping

This was performed as previously reported (13). Single nucleotide polymorphisms were imputed across the MHC using the Michigan Imputation Server (33) and the 1000 Genomes Phase 3 reference data set, as previously described (34). HLA types and their amino acid composition were imputed using SNP2HLA (35) against the Haplotype Reference Consortium panel (36).

Statistical analysis

Differences in alpha diversity were evaluated using the student's t-test, with an alpha of 0.05.

Results

Subjects

29 patients with AS and 43 unrelated HLA-B27 positive controls from the TwinsUK programme were included in this study. The controls were older than the patients (60.7±7.9 *vs*. 45.3±7.1 years) and were almost uniformly female (42/43 *vs*. 7/29.) Information on demographics and medication use is included in Table I.

16S rRNA gene sequencing

Following filtering, the sequencing depths in the two cohorts were similar: 67,642±83,495 reads in the AS patients *versus* 55,890±34,174 reads in the healthy controls. There was decreased richness (Chao1 test) in the microbiota of the AS patients (104±17 *vs*. 122±11, $p<0.001$), while measures of evenness (Shannon [3.45±0.33 *vs*. 3.30±0.40, $p=0.104$], inverse Simpson [19.9±7.3 *vs*. 17.3±8.6, $p=0.197$]) were similar (Fig. 1) A comparison of the overall structure of the microbiota using the Bray-Curtis distance matrix (28) dem-

Table I. Demographic and clinical variables of the study population.

Feature	TwinsUK	Ankylosing spondylitis
n	43	29
Demographics		
Male sex	1 (2.3%)	22 (75.9%)
Age (years)	60.7 ± 7.9	45.3 ± 7.1
Treatment		
NSAIDs	0	16 (55.2%)
Corticosteroids	0	3 (10.3%)
Any DMARD		16 (55.2%)
Methotrexate	0	1 (3.4%)
Etanercept	0	5 (17.2%)
TNFi mAb	0	11 (37.9%)
Secukinumab	0	1 (3.4%)
Body mass index	26.6 ± 4.5	25.5 ± 4.1
ASDAS-CRP	Not collected	1.8 ± 1.0

DMARD: disease-modifying anti-rheumatic drug; NSAIDs: non-steroidal anti-inflammatory drugs; TNFi mAb: tumour necrosis factor inhibitor (monoclonal antibody).

Table II. Summary of univariate predictors of the microbiota.

Predictor	TwinsUK	Ankylosing spondylitis	Full cohort
Diagnosis	NA	N/A	F=8.2, p=0.001
Sex	F=1.5, p=0.098	F=1.2, p=0.221	F=6.6, p=0.001
Age	F=1.4, p=0.119	F=0.69, p=0.816	F=5.9, p=0.001
NSAIDs	NA	F=0.75, p=0.808	NA
Any DMARD	NA	F=0.93, p=0.502	NA
Etanercept	NA	F=0.86, p=0.617	NA
Anti-TNF monoclonal antibody	NA	F=1.6, p p=0.071	NA
BMI	F=0.83, p=0.670	F=1.2, p=0.258	F=0.56, p=0.967
ASDAS-CRP*	NA	F=1.1, p=0.277	NA

AS: ankylosing spondylitis; ASDAS-CRP: ankylosing spondylitis disease activity sacale with CRP; DMARD: disease-modifying anti-rheumatic drug; NA: not applicable; NSAIDs: non-steroidal anti-inflammatory drugs. The ASDAS-CRP was only performed on the AS cohort; three patients had missing ASDAS-CRP scores and were excluded from this portion of the Univariate analysis. The impact of therapeutics was only evaluated on the AS cohort.

Table III. Summary of DeSeq2 findings.

Organism	Comparison				
	TwinsUK *vs*. AS	TwinsUK *vs*. AS; females	TwinsUK *vs*. AS; 40–59 yrs	TwinsUK *vs*. AS; Tx_naive	TwinsUK males *vs*. females
F. prausnitzii	AS	AS	AS	AS	No difference
Coprococcus	AS	AS	AS*	AS	Females
Bacteroides	TwinsUK	TwinsUK	TwinsUK	TwinsUK	No difference
Ruminococcus	TwinsUK	TwinsUK	TwinsUK	TwinsUK	No difference
A. muciniphila	TwinsUK	No difference	TwinsUK	TwinsUK	Females

AS: ankylosing spondylitis; Tx_naive: treatment-naive.
*Not statistically significant following correction for multiple comparisons.

onstrated substantial clustering (Fig. 2; p<0.001). To sort out the varying factors that may have contributed to the observed clustering, we performed a series of Univariate tests on each of the clinical and demographic variables listed in Table I on the group as a whole as well as separately on the AS and control subjects, when appropriate (Table II). The disease activity metric used in the adult subjects was the Ankylosing Spondylitis Disease Activity Score with the C-reactive protein (ASDAS-CRP), which takes into account patient reported outcomes, the physician global assessment of disease activity and the CRP (37). Three of the AS patients were missing ASDAS scores, so that evaluation was only performed on 26 patients. Consistent with the data depicted in the PCoA plot, subject group was highly associated with the clustering of the microbiota (F=8.2, p=0.001). In light of the demographic differences between the AS patients and controls, sex and age also strongly predicted clustering (Table II). However, when each subject group was evaluated individually, neither sex nor age affected the contents of the microbiota. Likewise, disease-specific factors among the AS patients, such as treatment and the ASDAS-CRP, were also not significantly associated with the microbiota contents. To tease out the relative influence of age and diagnosis on clustering, we used the PermANOVA test to perform multivariable analysis, adjusting for age (Distance matrix ~ Age + Diagnosis). Here, diagnosis remained statistically significantly associated with the structure of the microbiota (F=3.2, p=0.001). However, when we modelled on age using diagnosis as a predictor, age lost its statistical significance (F=1.1, p=0.252), indicating that within the subject age ranges included in the study, age was not independently associated with the structure of the microbiota.

Next, we sought to identify which of the bacteria were responsible for this clustering. As discussed in the methods section, we used the DeSeq2 programme (31) for this analysis. Using an FDR-corrected significance threshold of 0.05, we found 70 operational taxonomic units (OTUs) to be significantly different between the two groups (Supplementary Table S1, ranked according to decreasing mean abundance; Table III). Among the organisms previously linked to SpA, *Faecalibacterium prausnitzii* (log2fold change 2.6, p<0.001; Fig. 3A) and *Coprococcus* (log2fold change 2.5, p<0.001; Fig. 3B) were significantly elevated in the AS patients, while taxa of interest that were depleted in AS included *B. fragilis* (log2fold change -1.3, p=0.004; Fig. 3C), *Ruminococcus* (log2fold change -4.8, p<0.001; Fig. 3D) and *Akkermansia muciniphila* (log2fold change -3.6, p<0.001; Fig. 3E).

Due to the demographic and treatment differences between the AS patients

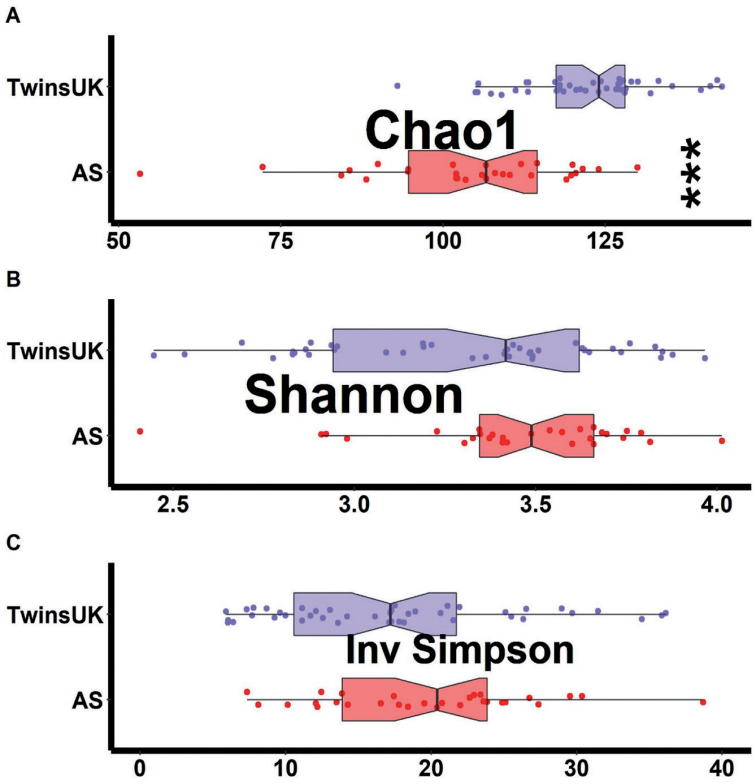

Fig. 1. Alpha diversity analyses of the microbiota of AS and TwinsUK subjects. The chao1 metric of richness (**A**) and Shannon (**B**) and inverse Simpson (**C**) metrices of evenness are displayed. ***Statistically significant at $p<0.05$.

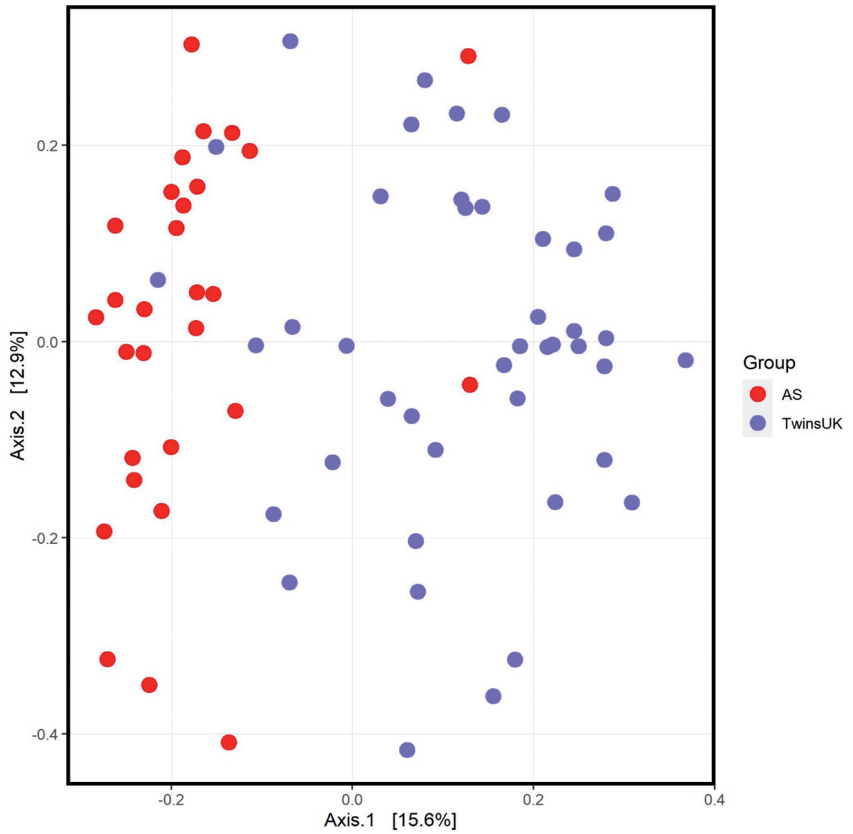

Fig. 2. Principal coordinates analysis of the microbiota of subjects included in the study. Red and purple dots reflect AS patients and TwinsUK subjects, respectively.

and healthy controls (Table I), we performed stratified analyses to evaluate whether any of these variables could have accounted for the observed findings (Table III) As all but one of the controls was female, we performed an analysis limited to female patients (Twins UK, n=42; AS, n=7). Despite having substantially lower statistical power, diagnosis still predicted clustering of the subjects (F=2.1, p=0.009). Additionally, *F. prausnitzii* (log2fold change 2.9, p=0.003) and *Coprococcus* (log2fold change 2.4, p=0.023) remained significantly more abundant in the AS patients, while *Bacteroides* and *Ruminococcus* species remained significantly lower in the AS patients; there were no longer differences in *A. muciniphila*. Due to the age difference between patients and controls, we performed a sensitivity analysis limited to subjects between the ages of 40–59 (Twins UK, n=16; AS, n=22.) Here, despite lower numbers, all of the findings reported in the main analysis were sustained, although the difference in the abundance of *Coprococcus* lost its statistical significance following correction (log2fold change 1.4, p=0.091). Finally, we performed a sensitivity analysis in which the 16 AS patients exposed to immunomodulatory therapy were excluded. As with the age-stratified analysis, all of the differentially present taxa present in the primary analysis again showed statistically significant differences between the two groups.

As a complementary means to determine whether sex might have accounted for the differences between the AS patients and TwinsUK controls, we also compared the microbiota of unrelated HLA-B27 negative females aged 40–59 to that of unrelated HLA-B27 negative males the same age range. Although some visually evident clustering was observed (Fig. 4), this was not statistically significant by the PermANOVA test (F=1.26, p=0.134). Furthermore, DeSeq2 data do not generally support the possibility that the observed differences between AS patients and controls were due to sex. Specifically, of the two genera that were increased in the largely male AS

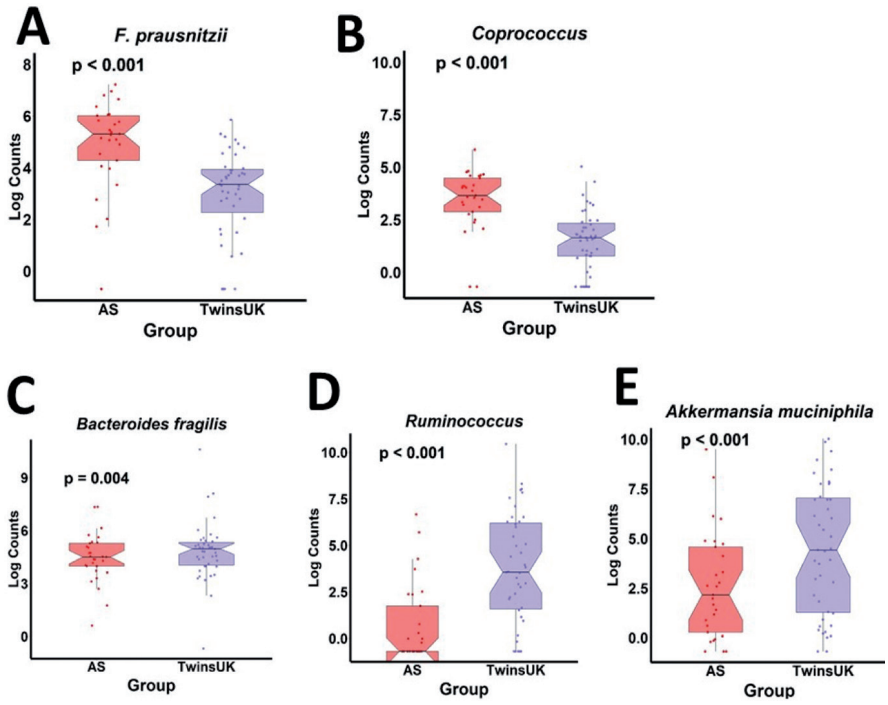

Fig. 3. Comparison of the faecal abundance of specific taxa. *Faecalibacterium prausnitzii* (**A**) and *Coprococcus* (**B**) were present at increased abundance in the patients, while *Bacteroides fragilis* (**C**), *Ruminococcus* (**D**), and *Akkermansia muciniphila* (**E**) were present at lower abundance in the patients. All differences were significant at adjusted *p*-values of <0.05.

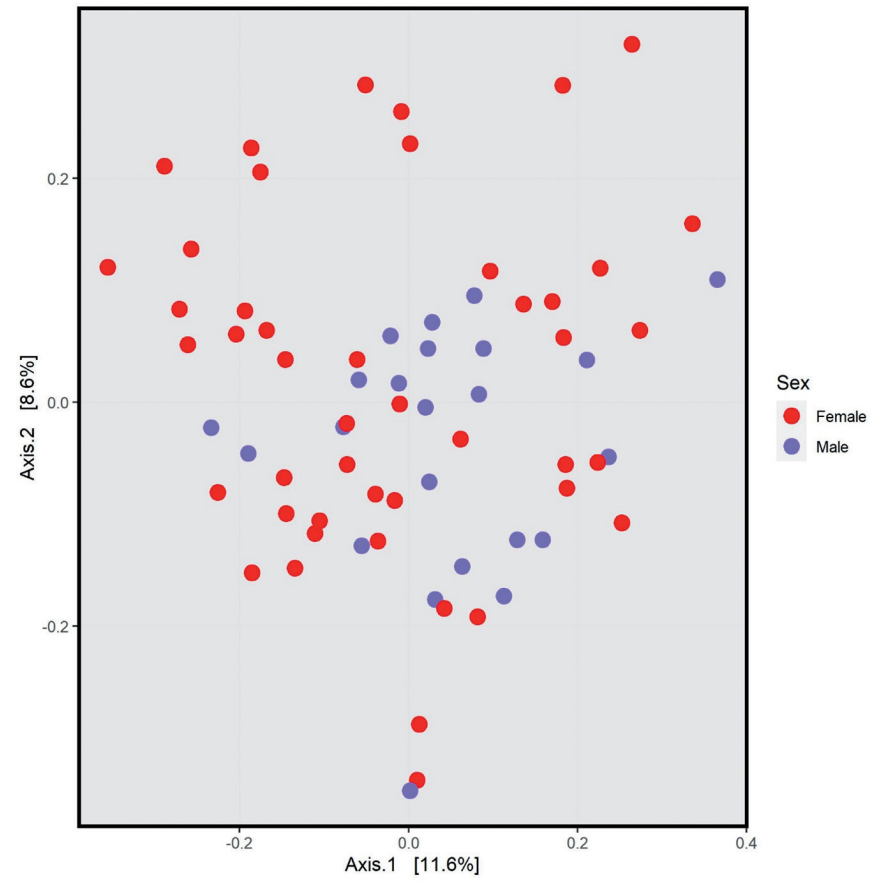

Fig. 4. Principal coordinates analysis of the microbiota of HLA-B27 negative TwinsUK subjects clustered by sex. Blue AND red dots reflect male and female TwinsUK subjects, respectively.

cohort, *Faecalibacterium prausnitzii* was not impacted by the sex of the HLA-B27 negative TwinsUK participants, while *Coprococcus* was actually higher in the female TwinsUK controls (Suppl. Table S2, 3.) Likewise, of the three genera that were depleted in the AS patients, *Ruminococcus bromii* was increased in male TwinsUK participants, the abundance of some members of the *Bacteroides* genus were higher in females while one was higher in males; and *Akkermansia muciniphila* was also higher in the female TwinsUK participants. Thus, taken together, the analyses indicate that of the five organisms listed in Table III as differentially abundant between the AS patients and TwinsUK controls, the only one that may represent confounding by sex is *Akkermansia muciniphila*.

Discussion

We evaluated two cohorts of HLA-B27 positive subjects: a cohort of patients with AS and a cohort of healthy adults. Previous studies have provided contradictory findings with respect to both *Coprococcus* and *Ruminococcus* in patients with SpA (8, 38-42). Like *A. muciniphila*, *Ruminococcus* species are mucin-degrading organisms (43), and we previously reported increased *A. muciniphila* in children with SpA, hypothesising that injury to the intestinal barrier secondary to mucin degradation may contribute to the disease (7, 44). While work in inflammatory bowel disease has shown decreased abundance of *A. muciniphila* (45, 46), it has been speculated that this is due to loss of the mucin substrate secondary to the inflammatory process (45). This hypothesis could potentially account for findings reported herein with respect to both *Ruminococcus* and *A. muciniphila*.

There are likewise contradictory data with respect to the *Bacteroides* genus in patients with SpA, with decreased abundance previously reported in adult subjects despite increased abundance in paediatric subjects (7, 11, 15, 16, 47). *B. fragilis* is generally considered to have regulatory properties (48), although increased abundance in paediatric subjects with autoimmune disease may reflect differential impacts of mi-

crobial-derived lipopolysaccharide on long-term immunomodulatory mechanisms (49).

Finally, it is of interest that the taxa identified herein as elevated in the AS patients, namely *Faecalibacterium* and *Coprococcus*, fall within the family of butyrate-producing bacteria (50) and are generally considered to be anti-inflammatory organisms (51). This could potentially represent a compensatory increase, underscoring the importance of studying disease populations at an earlier stage. Indeed, we have recently demonstrated that HLA-B27 positive juvenile SpA patients carried reduced abundance of *F. prausnitzii* as compared to HLA-B27+ offspring of AS patients (16). Alternatively, as a previous study demonstrated decreased abundance of *F. prausnitzii* in patients with AS (52), this could reflect strain level differences that could better be evaluated with shotgun sequencing.

This study has limitations, including the potential for differences due to the age gap between the AS patients and TwinsUK controls. We believe this to be unlikely. Several studies have demonstrated that most rapid changes occurred prior to age 3, with long-term stability of the microbiota believed to occur among humans ranging from mid-childhood through approximately age 70 (53, 54). We excluded subjects above 70 due to scholarship indicating changes in the faecal microbiota after aged 70–80 (18, 19). Age did not impact clustering in either group, and the differences in the contents of the microbiota remained significant after adjustments were made for age. Finally, limiting the study to subjects between the ages of 40–59 did not impact the principal findings. Another factor potentially impacting the differences observed between the AS patients and TwinsUK cohort is sex, as most of the SpA patients were male, while the TwinsUK cohort is predominantly female. Sex does appear to impact the contents of the intestinal microbiota (55, 56), so could be a confounding factor in this study, although clustering by sex among the TwinsUK subjects was not observed (Fig. 4). When we restricted the study to female subjects, the diagnosis continued to be associated with the overall structure of the microbiota and findings of increased abundance of all of the key distinguishing taxa except for *Akkermansia muciniphila* were sustained, and likewise the only taxonomic difference between the male and female control subjects that was consistent with the observed differences between the patients and controls was increased abundance of *Akkermansia muciniphila* among the female controls (Table III). It is also possible that the observed differences represented geographic variation. Our previous (15) work did not reveal significant geographic variation of the intestinal microbiota among children with SpA, and among inhabitants of developed nations, the microbiota may not vary widely even across countries (57). However, further studies in which the subjects are better balanced by age, sex, and geographic location would be required to validate these findings. Finally, the patients differed by use of immunomodulatory treatment. No single therapeutic clearly impacted the contents of the microbiota in this study. While cross-sectional studies have suggested that TNF inhibition impacts the microbiota (39), longitudinal studies did not substantiate that finding (58-60). Additionally, a placebo-controlled study of infliximab in patients with bipolar disease identified no clear impact of the study drug on the microbiota (61). While secukinumab does appear to impact the microbiota (58), this was used only by one subject. Importantly, restriction of the study to the 13 AS subjects unexposed to immunomodulatory therapy did not alter the principal findings. Additionally, the faecal DNA extraction method could have impacted the findings. However, both kits used to prepare the samples studied herein used bead-beating to lyse bacteria, and therefore it is unlikely that the specific kit would have a meaningful impact on the findings (62-64). The amplification and sequencing steps were identical. Finally, we acknowledge the relatively low number of subjects in this study, impacting the statistical power.

A novel aspect of this work is that of comparing HLA-B27 positive AS patients to HLA-B27 positive healthy controls. Prior studies of SpA patients (15, 47, 65, 66) had compared SpA patients to healthy controls, the majority of whom are HLA-B27 negative. However, HLA-B27 influences the contents of the microbiota in human subjects (13). Ours is the first study in adults limited to individuals with this risk allele, with the results partially confirming and partially contradicting previous studies. Future studies of SpA patients should take into account the risk allele, and additional studies involving both males and females are required to validate the findings reported herein. Longitudinal studies of SpA patients may also shed light on the changes that occur prior to *versus* as a result of this condition.

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