Alterations in the gut microbiome implicate key taxa and metabolic pathways across inflammatory arthritis phenotypes

One sentence summary: Gut microbiome configurations and activity exhibit similarities across distinct types of inflammatory arthritis.

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Abstract

Musculoskeletal diseases affect up to 20% of adults worldwide. The gut microbiome has been implicated in inflammatory conditions, but large-scale metagenomic evaluations have not yet traced the routes by which immunity in the gut affects inflammatory arthritis. To characterize the community structure and associated functional processes driving gut microbial involvement in arthritis, the Inflammatory Arthritis Microbiome Consortium investigated 440 stool shotgun metagenomes comprising 221 adults diagnosed with rheumatoid arthritis, ankylosing spondylitis, or psoriatic arthritis, and 219 healthy controls and individuals with joint pain without an underlying inflammatory cause. Diagnosis explained about 2% of gut taxonomic variability, which is comparable in magnitude to inflammatory bowel disease. We identified several candidate microbes with differential carriage patterns in patients with elevated blood markers for inflammation. Our results confirm and extend previous findings of increased carriage of typically oral and inflammatory taxa, and decreased abundance and prevalence of typical gut clades,

indicating that distal inflammatory conditions, as well as local conditions, correspond to alterations to the gut microbial composition. We identified several differentially encoded pathways in the gut microbiome of patients with inflammatory arthritis, including changes in vitamin B salvage and biosynthesis and enrichment of iron sequestration. Although several of these changes characteristic of inflammation could have causal roles, we hypothesize that they are mainly positive feedback responses to changes in host physiology and immune homeostasis. By connecting taxonomic alternations to functional alterations, this work expands our understanding of the shifts in the gut ecosystem that occur in response to systemic inflammation during arthritis.

Introduction

Alterations to the gut microbiome have been implicated in several inflammatory diseases, particularly in the gastrointestinal tract, including the inflammatory bowel diseases (IBD) and colorectal cancer (CRC)(*1, 2*). Although the role of gut microbes in other inflammatory conditions such as type 1 diabetes (T1D) and metabolic syndrome have recently come under investigation(*3, 4*), their influences on or responses to systemic inflammation or disease progression remain poorly elucidated. Inflammatory musculoskeletal arthropathies stand to benefit from a better understanding of gut microbial ecology, both as an early biomarker for diagnosis of these conditions, and as a potential new route for therapy. Arthropathies, including rheumatoid arthritis (RA), ankylosing spondylitis (AS), and psoriatic arthritis (PsA), affect over 50 million adults worldwide(*5-7*), who currently have no curative treatment options. Thus, understanding their corresponding alterations within the gut microbiome is essential to both the underlying basic biology driving systemic inflammation and clinical routes of arthritis treatment.

The etiology of many of the subtypes of arthritis can be traced back to aberrant immune responses, which may be triggered or sustained by acute or long-term interactions with gut microbial populations(*8*). This is true over and above human genetic contributions, which include variants of the human leukocyte antigen (HLA) family(*9*). In RA, heritability is estimated at 60%, although without clearly-resolved causal loci(*10-13*). Conversely, the heritability of AS approached 90%, with HLA-B27 carriage the strongest genetic risk factor(*14-16*). Additionally, smoking has been established as a likely trigger of RA, representing at least one specific interaction between environmental and genetic factors in arthritis etiology(*13, 17*). The microbiome is one of the most proximal forms of “environment”, and indeed many arthritis risk alleles such as HLA are, like those of IBD, known to be involved in microbial interactions or immune sensing(*18*).

Since arthritis pathology is localized in the periphery, all of these arthropathies represent cases in which any involvement of the gut microbiome would be “transmitted” systemically through biochemical and immune-mediated signals. Research on this so-called “gut-joint-axis” dates back to the 1890s, when researchers hypothesized that arthritic conditions could be caused by *Mycobacterium* infections(*19*). Murine models have furthered this hypothesis by showing that microbial disease triggers are required for SpA type arthritis to develop(*20-22*) and that gut microbial colonization is necessary for Th17 differentiation (protecting germ-free mice from disease)(*23*). Several strong indicators of the “gut-joint-axis” exist in humans as well, including subsets of patients with chronic IBD exhibiting increased risk of peripheral arthritis(*24, 25*), reactive arthritis occurring after pathogen infections(*26*), and the induction of autoreactive cartilage degradation by specific bacterial strains(*25*). Several studies in smaller human populations, primarily studied using 16S rRNA gene amplicon (16S) sequencing, found compositional alterations of the gut microbiome in patients with RA, PsA, and AS(*27-41*). These included the presence of clades that are frequently pathogenic, increased abundance of

typically oral microbes in the gut, and altered abundance of typical human gut clades(*39-42*). However, there is no substantial agreement on which dysbioses are hallmarks of systemic inflammation in arthritis. Additionally, 16S-based profiles do not provide direct insight into the functional implications of microbial compositional changes, and thus far the agreement in functional changes from the few shotgun studies is limited but have identified sweeping changes(*34, 36, 39-41, 43*). Therefore, a comprehensive understanding of the role of the gut microbiome in arthritis development and persistence is still lacking, which has the potential to better-support early disease detection, prevention, or later-stage therapy.

Here, we introduce the work of the Inflammatory Arthritis Microbiome Consortium (IAMC), which includes analysis of shotgun metagenome profiles spanning 440 participants with RA, AS, PsA, and controls without inflammatory arthritis. We assessed the taxonomic and functional landscape of the resulting gut microbiomes to elucidate key ecological and biochemical shifts linked to host inflammatory responses and clinical arthritis phenotypes. In patients with inflammatory arthritis, the overall compositional and functional profiles of the gut microbiome were substantially altered. We identified enrichment of typically oral, pro-inflammatory, and mucin-degrading microbes, with a corresponding decrease in several typical human gut-resident clades. Notably, several strains of *Ruminococcus gnavus* isolated from human patients induced more severe phenotypes when inoculated into mice. Further, several alterations in microbial community function were identified, including the differential encoding of vitamin B salvage and biosynthesis and the encoding of folic acid metabolism pathways. Similar to other local and distal inflammatory diseases, iron scavenging was enriched in patients with current inflammation across heme, non-heme, and siderophore-based mechanisms. Although these findings point to pathways and molecules of interest and will serve as an important resource for hypothesis generation, future work will be required to determine if these consistent functional changes occur causally, in response to inflammatory arthritis, or both. At the least, our findings of community level taxonomic and functional alterations in the gut microbiome implicate an interplay between host genetics, immune system, and gut microbiome over the course of initiation, progression, and severity of arthritis.

Results

Patient Cohort Characteristics

We recruited 440 adults (ages 20 to 93) from different clinical locations in the United Kingdom, Oxford (primarily AS patients), Birmingham (primarily RA patients), and Newcastle (primarily RA patients), who met classification criteria for one of three arthritis subtypes or were included in the non-inflammatory joint pain control group (**Fig. 1A-C**). Patient diagnoses included primarily treatment naïve rheumatoid arthritis (RA, n=119), axial spondyloarthritis/ankylosing spondylitis (AS, n=67), psoriatic arthritis (PsA, n=35), and non-inflammatory joint pain (typically fibromyalgia [NIJP], n=54), as well as age-matched healthy controls (HC, n=165) (**Fig. 1A, table S1**). Although some members of the NIJP category had low-titer autoantibodies for either rheumatoid factor (RF) or anti-cyclic citrullinated peptide (anti-CCP), none were considered by consulting rheumatologists to have clinically suspect arthralgia with respect to RA(*44*).

Earlier studies have identified alterations in gut microbial taxonomic profiles with both arthritis diagnosis and local and distal inflammation(*27-33*). To expand these results, we focused on patient diagnosis and current degree of disease activity as primary outcomes while adjusting for relevant clinical covariates including patient age, current arthritis-related drug use, and disease duration, as well as technical confounders such as sequencing batch and clinical site (**fig. S1A to I**). Only a small subset of patients (58/440) reported use of antibiotics in the last six months,

~half (22) within the previous two months and none at the time of sampling. These were equally spread across all patients and HCs (**table S1**); such non-recent antibiotics use corresponded with very little overall variation (PERMANOVA R^2=0.0049), and no individual features were associated with antibiotics use (MaAsLin 2). Antibiotic covariates were thus omitted from further analyses. We defined disease activity using two variables: (1) discretized C-reactive protein values (CRP) as a marker of current systemic inflammation (**Fig. 1D**), and (2) serum hemoglobin concentrations, as many patients with inflammatory arthritis also experience anemia as a feature of chronic disease (**fig. S1I**).

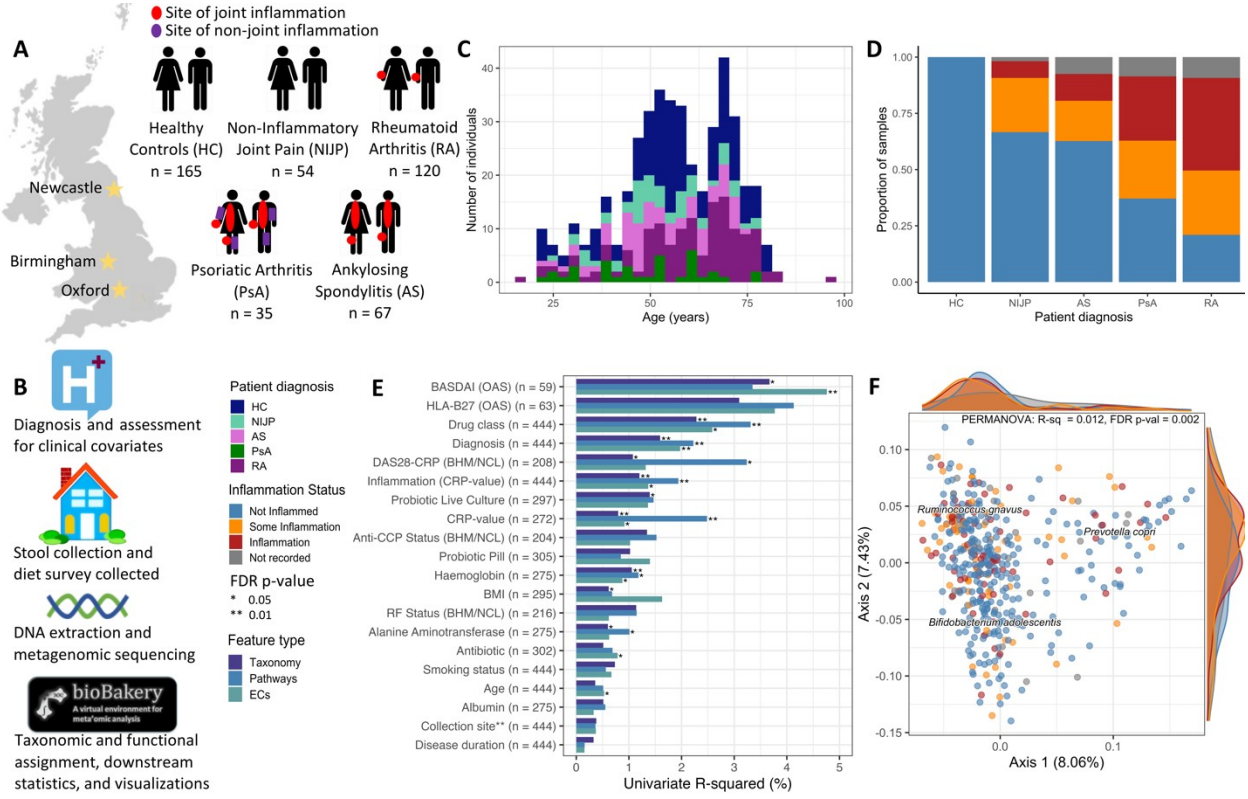

Figure 1: The gut microbiome is altered in patients with inflammatory arthritis. (A) Overview of the participants and samples collected from each subtype of arthritis and the **(B)** collection schema. **(C)** Prevalence of arthritis subtypes by age collected under this cross-sectional study by the Inflammatory Arthritis Microbiome Consortium (IAMC). **(D)** Proportion of patients with overt inflammation, some inflammation and no inflammation defined by tertiles of circulating serum concentrations of CRP by diagnosis (0 to 4mg/L, Not inflamed; 4 to 10mg/L Some inflammation; 10 to 167mg/L Inflammation). **(E)** Univariate PERMANOVA of Bray-Curtis dissimilarity by demographic and clinical measures. Color indicates the features assessed (microbial taxonomy, metagenomic pathways, and metagenomic Enzyme Commission (ECs) numbers). Tests are batch adjusted (**Methods**). **The effect of the collection site was also adjusted for by diagnosis to account for the fact that some sites only enrolled individuals with a particular disease subtype. **(F)** Bray-Curtis principal coordinates analysis of all 440 taxonomic profiles. Species are labeled using weighted averages and limited to just species whose abundance explains samples that fall outside of 0.04 distance from origin.

Individuals' disease activities varied from low to high disease activity [Bath Ankylosing Spondylitis Disease Activity Index (BASDAI) = 0.08 to 8.4 (AS-specific measure); Disease Activity Score 28 for RA with CRP (DAS28-CRP) = 1.54 to 8.01 (RA/PsA-specific measure)] (**Fig. 1C**, **table S1, fig. S1C and D**). Although not ideal measures of inflammation, CRP concentrations for each participant provide a measure that was collected consistently across all population cohorts and ranged from 0 to 167 mg/L (**fig. S1C and D**). Thus, as the most consistently collected measure, we used CRP as a proxy for systemic inflammation; when

available, it compared favorably to more direct measures such as BASDAI for AS or DAS28 for RA. Due to the asymmetric nature of these data, we categorized these patients using tertiles into three categories: not inflamed (0 to 4 mg/L), some inflammation (4 to 10 mg/L), and inflammation (greater than 10 mg/L). Healthy controls only contributed fecal samples, not blood, and we could thus not quantify CRP concentrations from these individuals and categorized all controls into the "not inflamed" group **(Fig. 1D)**. From here on, we refer to the discretized CRP-value for systemic inflammation as simply "inflammation." Anemia was also quantified in this population by current hemoglobin concentrations, with anemia called when hemoglobin was less than 120g/L or 135g/L for females and males, respectively (**fig. S1I**). Human leukocyte antigen B27 (HLA-B27) status was quantified as either negative or positive, but only for the patients from Oxford (AS patients, n=67). In addition, RF and anti-CCP status was categorized as negative or positive for RA patients (n=113) **(table S1, fig. S1F to H**).

The human gut microbiome is altered in inflammatory arthritis.

Alterations in the overall composition of the gut microbiome were identified in patients with inflammatory arthritis. Patient diagnosis explained a maximum of 1.6% and 2.3% of the compositional differences in the taxonomic and functional microbial profiles, respectively, after adjusting for the sequencing batch [Bray-Curtis PERMANOVA; false discovery rate (FDR) p-value = 0.003 and 0.006]. In pairwise comparisons, these results were driven largely by differences in the RA patients (**fig. S2**). Categorized CRP values, which represent the current amount of inflammation a patient is experiencing, accounted for a maximum of 1.2% (FDR p-value = 0.003) and 2.0% (FDR p-value = 0.006) of the variation in the composition of the taxonomic and functional profiles, respectively (**Fig. 1E, fig. S2**). Inflammation thus explained a small but notable shift in the overall gut microbial composition, not greatly below the amount often observed in IBD(*1*) (**Fig. 1F**). Clinical measures of inflammation, such as the patients' DAS28-CRP and BASDAI also explained similar amounts of variation within the gut ecology (**Fig. 1E)**. This indicates that systemic inflammation during arthritis, as characterized by either disease-specific markers or circulating measures in all patients, corresponds with a substantial amount of variation in the patients' gut microbiomes. Intriguingly, similar amounts of variation were also explained by a patient's hemoglobin concentrations (g/L) (Taxonomy; R^2 = 1.1% and FDR p-value = 0.003, Pathway; R^2 = 1.2% and FDR p-value = 0.009, **Fig. 1E**). Similar effect sizes also demonstrate a consistent, but diverse, coupling of taxonomic and functional aspects of the gut microbiome, as expected. HLA-B27, anti-CCP and RF status all did not induce alterations in the overall composition of the gut microbiome (Bray-Curtis PERMANOVA taxonomy; FDR p-value >0.01). Taken together, these results indicate that patients with inflammatory arthritis do harbor broadly different configurations of microbes within their gut when compared to similarly-aged healthy controls, consistent with previous studies(*28, 34, 42*).

HLA-B27 status explained a relatively large amount of variation in the composition of the gut microbiome. However, none of these associations were significant after FDR-correction (Bray-Curtis PERMANOVA taxonomy; FDR p-value = 0.605, pathways; FDR p-value = 0.381, ECs; FDR p-value = 0.691), likely due to reduced sample numbers, as only the samples from patients with AS and controls from Oxford had this information available (n = 135) **(Fig. 1E)**. Previous studies have identified per-feature and overall compositional differences in the gut microbiome of patients with HLA-B27(*45, 46*). Those genetic loci that do explain compositional shifts typically impact the immune system, which in turn shapes (and is shaped by) the microbiome(*8*). Additionally, both the anti-CCP antibody and the RF-status of the RA patients explained less than 2% of the variation in the gut microbiomes and were not statistically significant (**Fig. 1E**). Taken together, these results indicate that patients with inflammatory arthritis do harbor broadly

different configurations of microbes within their gut when compared to similarly-aged healthy controls, consistent with previous studies(*28, 34, 42*).

Microbial taxonomic alterations occur in rheumatoid arthritis and ankylosing spondylitis.

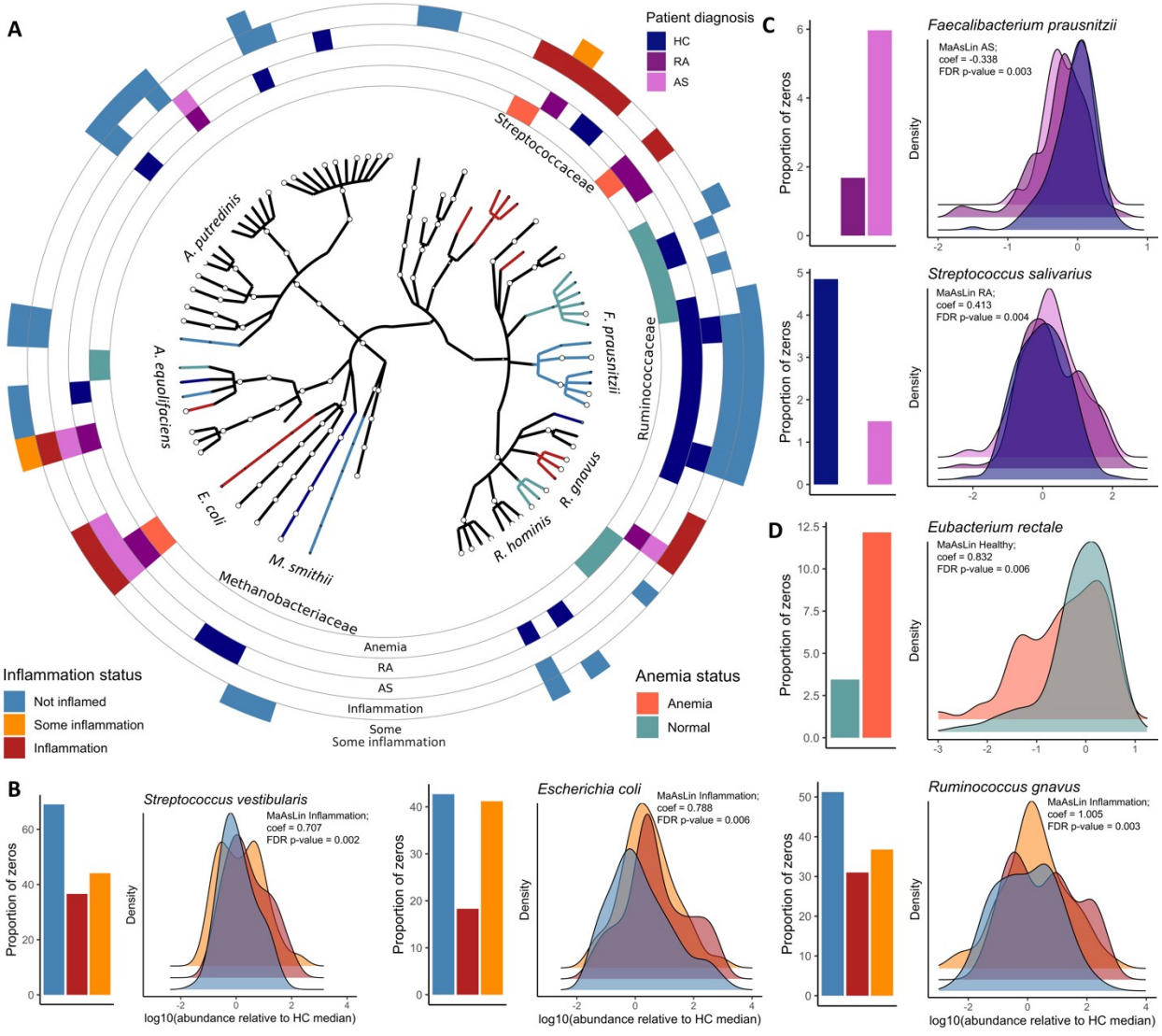

Figure 2: Taxonomic features differ in both a diagnosis- and severity-specific manner. (A) Shown are clade-specific taxonomic alterations of the gut microbiome by inflammation (inflamed versus not inflamed, some inflammation versus not inflamed), diagnosis (RA versus HC, AS versus HC), and hemoglobin concentrations (Anemia versus Normal). All associations were identified using MaAsLin 2. Internal branches are colored by strongest association, while outer rings summarize all associations. Leaf size was set as -log(pval)*sign(coef). These variables have been reduced for clarity; all results are presented in **fig. S4. (B)** Microbial species associated with inflammation status were determined based on serum CRP concentrations. Changes were observed in both the profiles of each clade's relative abundance within the gut community and their prevalence, here represented by the total number of zero abundance samples present for each condition (proportion of zeros). Increased abundance and prevalence were observed in three previously inflammation-associated species; *Streptococcus vestibularis, Escherichia coli,* and *Ruminococcus gnavus*. **(C)** Disease-specific alterations in the gut microbiomes were correlated with patients' inflammatory arthritis diagnoses. *Faecalibacterium prausnitzii* exhibits a lower abundance and prevalence in patients with RA, and to a lesser extent, AS, while *Streptococcus salivarius* had a higher prevalence and abundance especially in RA patients. **(D)** Several gut microbes exhibited strong correlations between several typical human gut residents and hemoglobin concentrations (d/L), with the highest effect size observed in *Eubacterium rectale.*

We identified several taxa associated with inflammation, diagnosis, and anemia or more disease-specific markers of inflammation (e.g. BASDAI or DAS28-CRP) that paralleled changes previously observed in dysbiotic individuals with IBD(*1*), including the clades *Streptococcus sp*., *Escherichia coli*, and *Ruminococcus gnavus*(*47*) (**Fig. 2A, fig. S3 to 7**). Examining the prevalence of these organisms across patients, it appears that *E. coli* and *R. gnavus* may exhibit a high abundance but low prevalence phenotype, in which a small number of patients had substantially higher abundances of these taxa. Previously, this pattern was observed with *Prevotella copri* in patients with treatment-naïve RA (*29, 34, 48*), but that was not the case in this cohort (**fig. S8**). Several of the clades that increased during inflammation are more commonly identified in the oral cavity(*49-51*) than in the gut, including *Streptococcus mutans*, *S. vestibularis*, *S. salivarius*, and *Bifidobacterium dentium* (**Fig. 2B and C, fig. S4, 5, and 7**). For these tests, we were careful to adjust for proton pump inhibitor (PPI) usage, which has been hypothesized to facilitate the transversion of oral taxa into the gut(*52, 53*). However, only 11 patients out of 275 were documented to be actively taking PPIs at the time of sample collection, and we thus do not believe this to be the mechanism. We do not have information on the oral health status (e.g. periodontal disease) of these patients, although previous studies have found that the patients with RA were four times as likely to have poor dental health(*54*). This strengthens the association of these different types of microbial disruption, but leaves their respective causalities unclear.

Patients with IBD and T1D have both showed reduced abundance of the species *Faecalibacterium prausnitzii, Roseburia intestinalis, Eubacterium rectale* and *Alistipes putredinis*(*1, 55-57*), and we observed a similar decrease in our study of patients with AS and RA, either by our proxy marker for inflammation or by disease phenotype or disease-specific markers (**Fig. 2C and D, fig. S3, 4, and 7**)(*57-63*). In particular, *F. prausnitzii* and *R. intestinalis* lost both abundance and prevalence in patients with current inflammation, whereas *E. rectale* abundance was observed to be tightly coupled with hemoglobin concentrations. These microbes are generally considered to be both highly responsive to inflammation and themselves anti-inflammatory by routes such as short chain fatty acid (SCFA) production(*64-66*), making the causality of these changes difficult to untangle observationally. However, it is striking that gut microbial changes observed here for systemic inflammation during arthritis were both taxonomically and functionally similar to those occurring during gastrointestinal inflammation, which has been hypothesized to occur due to changes in oxygen availability in the gut ecosystem(*67, 68*).

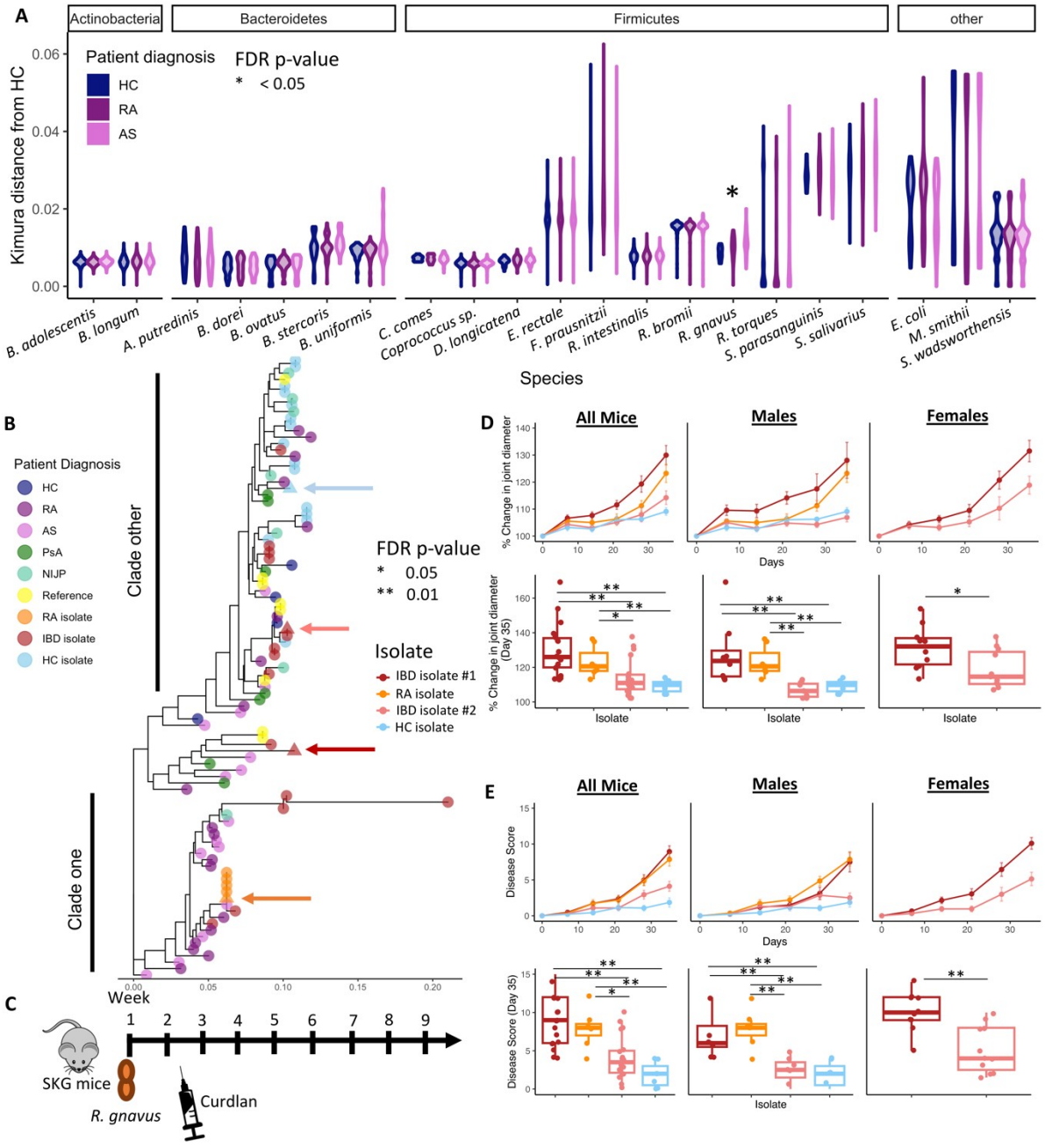

Figure 3: A *Ruminococcus gnavus* sub-species clade is enriched in patients with inflammatory arthritis. (A) Species were tested for sub-species phylogenetic structure associated with diagnosis and inflammation. Violin plots represent the spread and density of the pairwise 2-parameter Kimura distances between dominate strains within each sample compared only to the healthy control samples. Wilcoxon tests were used to determine significant changes in the pairwise distances between conditions and p-values were FDR corrected. **(B)** Phylogeny of *R. gnavus* strains from each individual's gut microbiomes. Isolates from NCBI and isolates cultured as part of this study (**Methods**) were included to add context to the subclade groupings. Triangles with arrows pointing at them indicate isolated strains used in (D) and (E). **(C)** Previously germ-free SKG mice were inoculated with a monoculture of a single *R. gnavus* strain (arrows on **Fig. 3B**) at week 1. Two to three weeks later curdlan was injected to simulate arthritis. (**D and E**) Male and female mice were then followed for joint diameter changes (**D**) and disease score (**E**). Longitudinal data are presented as mean disease score or joint diameter +/- the standard error. Box and whisker plots indicate the 25th, median, 75th and 1.5 times the interquartile range.

Within the ruminococci, a well characterized group of mucus-degrading bacteria(*69, 70*), *R. gnavus* has been implicated in many inflammatory conditions(*71-74*) and has been researched more extensively in IBD(*75-77*). Again, taxonomic associations with arthritis strikingly mimicked those of patients with IBD, though at a lower magnitude (**Fig. 2B; fig. S4-5, 7**). The abundance of *R. gnavus* was significantly increased in several patients with current high levels of CRP (Linear model; Not inflamed vs. Inflammation; coef = 1.005, FDR p-value = 0.003, **Fig. 2B, fig. S9-10**), interestingly including several NIJP subjects. Additionally, using both single nucleotide variants (SNVs, using StrainPhlAn(*78*)) and differences in pangenome-wide gene content (using PanPhlAn(*79*)), we identified phylogenetic structures that were significantly enriched in AS and RA patients (denoted Clade One; Kimura 2-parameter distance, PERMANOVA; $R^2 = 0.18$, FDR p-value = 0.01, **Fig. 3A**) when compared the NIJP and HC individuals (which tended to carry members of Clade Other; **Fig. 3B** and **fig. S11 to 13**). To strengthen these results, an isolate from one RA patient also fell into Clade One, along with several isolates from a previously published IBD cohort(*75*). These results indicate that the presence of inflammation both locally and at distal locations in the host can correlate with structural, and potentially functional, changes in the gut microbiome. Other species tested did not exhibit the same subclade structuring as *R. gnavus* in this population (**fig. S9**).

Isolates from Clade One specifically enhanced inflammatory phenotypes when introduced into a mouse model of arthritis. New isolates (**Fig. 3B**) were derived from participant fecal samples and inoculated into previously germ-free SKG mice. Two to three weeks after the introduction of these monocultures of *R. gnavus*, curdlan was injected to induce arthritis symptomology. The presence of isolates from Clade One in the gut were able to potentiate the severity of arthritis-like symptoms in the SKG mouse, using both joint diameter and disease score as indicators of severity (**Fig. 3C to E**). This showed that the presence of these strains of *R. gnavus* is sufficient to induce a more severe phenotype, supporting its likely interaction with the immune system, as previously postulated(*76, 80*), and furthering arthritis symptoms.

Functional profiling reveals consistent functional alterations across all subtypes of arthritis.

We observed increased carriage of folate metabolism pathway and enzymes in individuals with arthritis and in those individuals with current high degrees of systemic inflammation (e.g. EC. 1.5.1.3 Dihydrofolate reductase; linear model not inflamed vs. inflammation; coef = 0.180, p-value = 0.028; **Fig. 4A to C, fig. S14 and 15**). However, carriage of the methionine cycle, which typically includes production of putrescine and homocysteine, was not consistently changed across both RA and inflammation (e.g. PWY-6151: S-adenosyl-L-methionine cycle I; linear model not inflamed vs. inflammation; coef = -0.03, FDR p-value = 0.17) (**Fig. 4A to C**). Alterations to nucleotide and amino acid pathways downstream of these processes are enumerated in **fig. S16** These lines of evidence suggest a dysregulation of folate metabolism in the gut ecosystem during arthritis, although this was distributed among a variety of different potential encoding organisms in different participants (**fig. S15**).

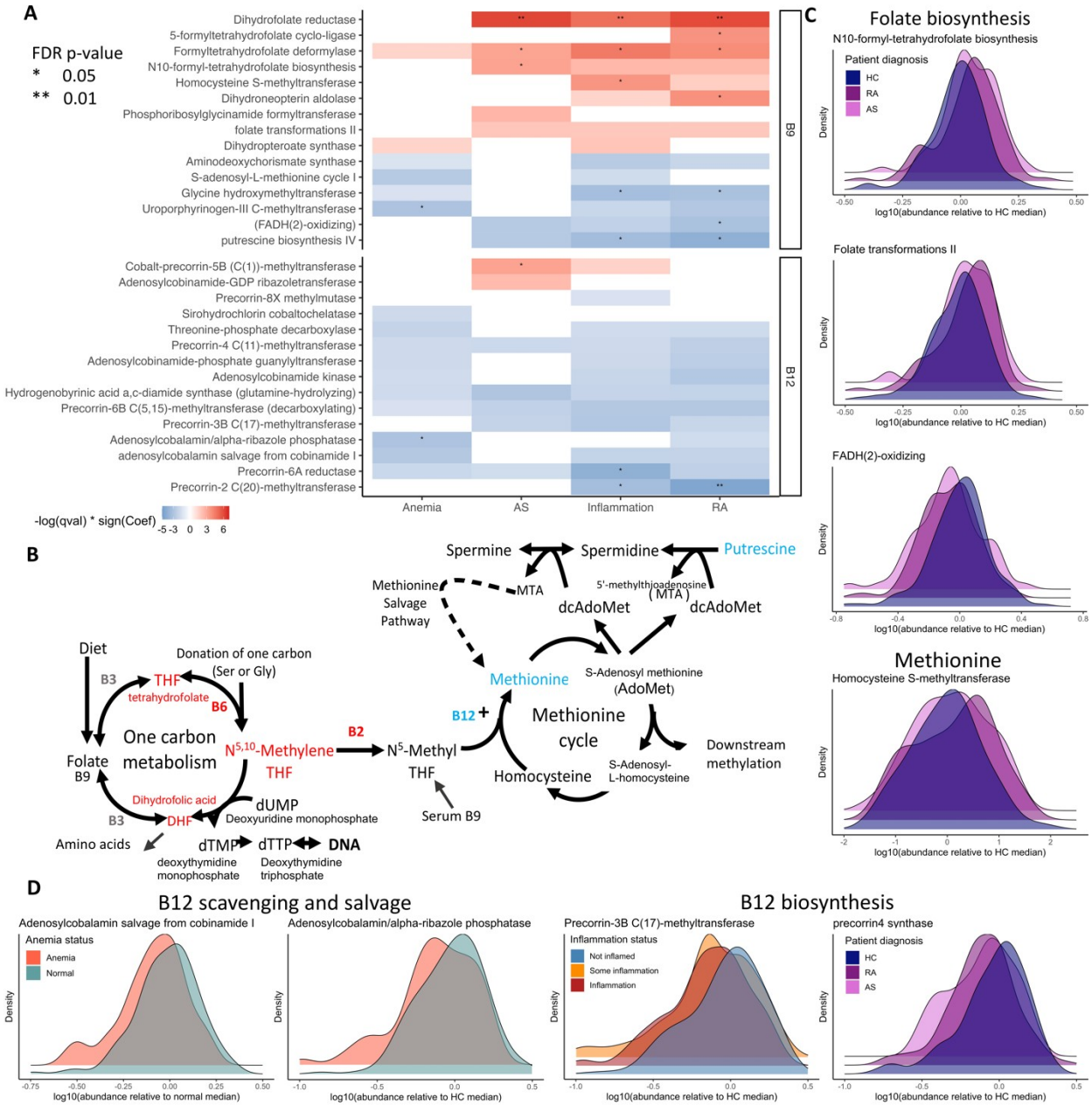

Figure 4: Differential encoding of vitamin B metabolism and processing were observed in the gut microbiomes of patients with inflammatory arthritis. (A) Shown are associations between patient diagnosis, inflammation status, and hemoglobin levels with folic acid metabolism, vitamin B12 salvage and biosynthesis and methionine biosynthesis and cycling. These associations were quantified using MaAsLin 2. **(B)** The folic acid metabolism pathway components carried by gut microbes. Enzymes in the one-carbon metabolism cycle that were enriched in gut metagenomes during inflammatory arthritis are shown in red, whereas enzymes decreased in the methionine cycle are shown in blue. **(C)** Encoding of folate metabolism cycle components within the gut microbiomes of patients was associated with a diagnosis of inflammatory arthritis. **(D)** Encoding of both the salvage and biosynthesis of vitamin B12 was correlated with anemia status, inflammation status, and patient diagnosis.

Patient hemoglobin concentrations and inflammation status were also associated with decreases in gut microbial carriage of vitamin B12 salvage and biosynthesis pathways (e.g. EC 1.2.1.54: Precorrin-6A reductase; linear model Normal vs. Anemia; coef = -0.17, FDR p-value = 0.14), in tandem with disruptions in vitamin B9 (folate) and its interconnected pathways (**Fig.**

4A, B, and D). Vitamin B12 is a required cofactor in the link between the folate and methionine cycles(*81*) (**Fig. 4B**). These alterations in the encoding of B12 salvage appear to be due to mainly (but not entirely) to shifts in the relative abundance of *Eubacterium rectale* as noted above (**fig. S17**). Additionally, the metagenomic abundances of several other enzymes associated with vitamin B metabolism were also linked to patient diagnosis, inflammation status, or anemia status, including vitamin B1 (thiamin/thiazole), vitamin B2 (flavin), vitamin B6 (pyridoxine), and vitamin B7 (biotin) (**fig. S14, 18, and 19**).

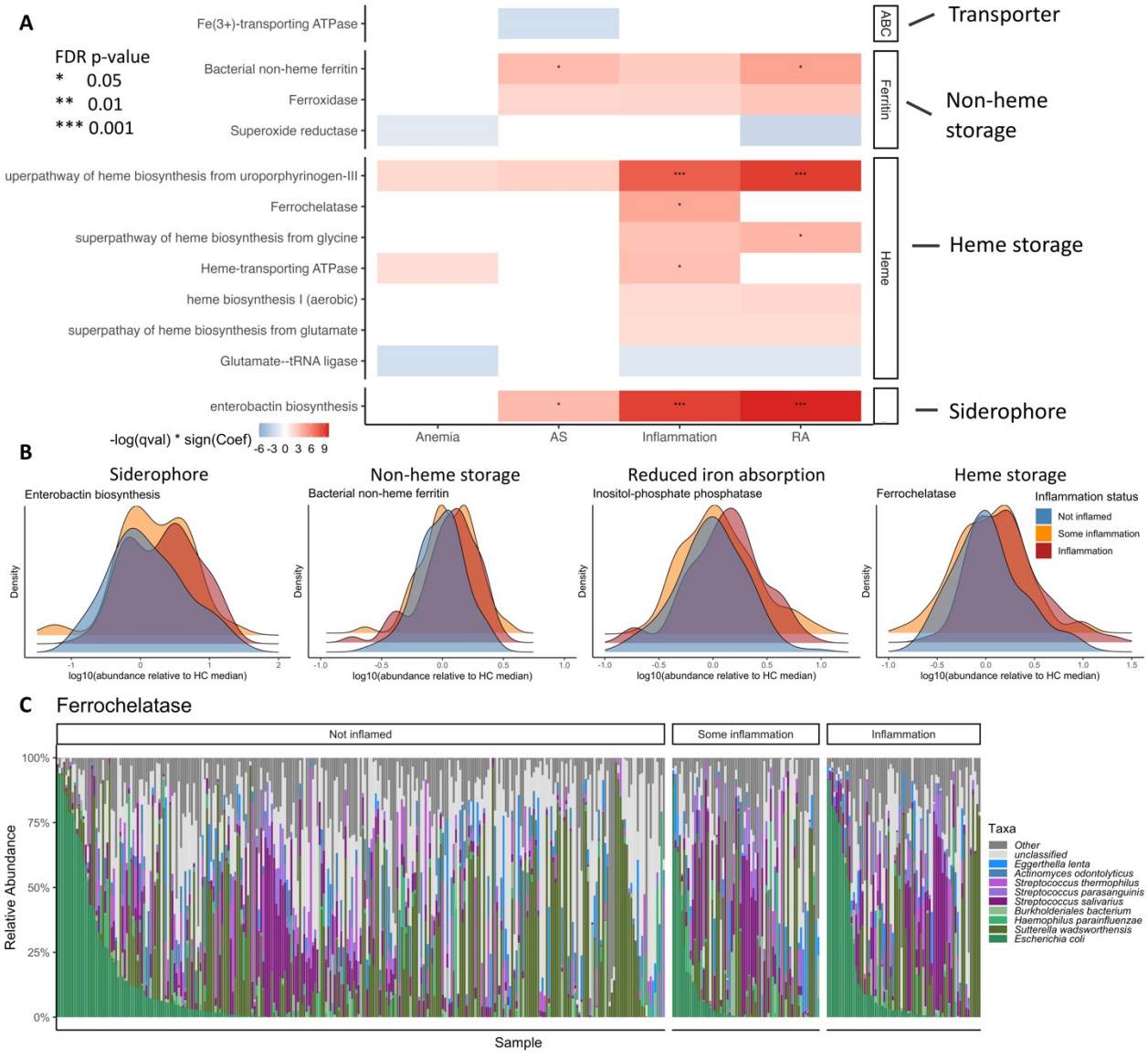

Figure 5: Increased metagenomic carriage of pathways and enzymes involved in iron sequestration across several distinct mechanisms were observed in individuals with arthritis with high serum CRP. (A) Metagenomic functional features (pathways and enzymes) associated with iron metabolism were correlated with the gut ecosystems of patients AS, RA, evidence of anemia and elevated CRP values. Mechanisms of microbial iron sequestration included heme and non-heme (ferritin) based storage, and high affinity siderophores. These associations were quantified using MaAsLin 2. **(B)** Enzymes from several of the sequestration mechanisms were correlated with inflammation status. **(C)** Species that most contribute to carriage of the ferrochelatase iron sequestration gene family are shown based on inflammation status.

Alterations of other gut metagenomic pathways regulating metabolic cofactors were also identified among inflammatory arthritis patients. Genes encoding several enzymes linked with microbial iron sequestration were enriched in inflamed patients, as well as in RA and AS patients specifically (e.g. ENTBACSYN-PWY: Enterobactin biosynthesis; linear model not inflamed vs. inflammation; coef = 0.62, p-value = 0.00014). These included genes encoding ferrochelatase, bacterial non-heme ferritin, ferroxidase, and heme biosynthesis (at the pathway level) (**Fig. 5A and B**). This agrees with results in an earlier, smaller RA cohort in which oral and gut capacity for iron transport was disrupted(*34*). However, this was previously attributed specifically to *Klebsiella* spp.; in contrast, as with disruptions in folate metabolism, we found contributions to iron sequestration to be encoded by diverse taxa (**Fig. 5C** and **fig. S20**), and for non-heme mechanisms, no one clade was individually associated with their differential carriage. This again indicates that dysbiosis of these processes can be distributed among different microbes in different people, or that there are strain-level differential carriage within taxa, such as with ABC transporters within *R. gnavus* (**Fig. 3C**). However, heme-related mechanisms appear to be driven mainly by the high abundance phenotype described above for *E.coli* in this population, as it is a major contributor to these functions (**Fig. 5C** and **fig. S20**).

Several other functional classes, including both pathways and enzymes, exhibited differential metagenomic carriage either by inflammation status or patient diagnosis (**fig. S21 to 25**). The gene classes most highly associated with both RA and inflammation were those relating to the production of isoprenoids or volatile hydrocarbons (VOCs) (**fig. S21**). Microbial gene families that explicitly interact with host immunity were also differentially carried during arthritis, such as cell wall remodeling proteins and oxidative stress response (**fig. S22 to 25**). Finally, enzymes involved in the oxidative stress response, including methanogenesis, glutathione, and peroxiredoxin (**fig. S22**) were differentially carried in inflammation. Also of current interest to the field, enzymes involved in SCFA metabolism (*82*), 3-hydroxybutyryl-CoA dehydrogenases and short-chain acyl-CoA dehydrogenase enzymes were also found to be differentially carried by the gut ecosystem in inflammation (**fig. S23**). Overall, alterations to the functional landscape of the gut ecosystem indicated disruptions in several key metabolism pathways during inflammatory arthritis.

Several other functional classes, including both pathways and enzymes, exhibited differential metagenomic carriage either by inflammation status or patient diagnosis (**fig. S21-25**). The gene classes most highly associated with both RA and inflammation were those relating to the production of isoprenoids or volatile hydrocarbons (VOCs). Interestingly, the production of VOCs under inflammatory conditions has been previously noted(*83, 84*), but not the involvement of the gut microbiome in its upregulation. Several studies have also indicated anti-inflammatory properties of isoprenoids, especially geraniol, farnesol, and geranylgeraniol(*85*). Here, we observed that many gut microbial pathways with greater carriage during arthritis were involved in geranylgeraniol biosynthesis (**fig. S21**). Thus, microbes within the gut ecosystem could be increasing production of these small molecules if they are less bioavailable from the host or diet.

Microbial gene families that explicitly interact with host immunity were also differentially carried during arthritis, such as cell wall remodeling proteins and oxidative stress response (**fig. S22-25**). This trend was observed in relatively few genes and was especially true for patients with RA and not those with AS. Enzymes involved in the oxidative stress response including methanogenesis, glutathione, and peroxiredoxin were differentially abundant in these patients' gut metagenomes (**fig. S22**). Finally, and of interest to the current short-chain fatty acid literature(*82*), we observed a few select genes involved in butyrate metabolism to be differential, with the majority less abundant in patients with RA or with higher circulating markers of inflammation. These included the 3-hydroxybutyryl-CoA dehydrogenases and short-chain acyl-

CoA dehydrogenase enzymes (**fig. S23**). Overall, alterations to the functional landscape of the gut ecosystem indicated disruptions in several key metabolism pathways during inflammatory arthritis.

Discussion

Here, we present the findings of a large cross-sectional study of adults with inflammatory arthritis diagnosis (and control participants), investigating alterations in gut microbiome composition and function associated with disease status and inflammation. The signals we detected associated with this family of systemic inflammatory conditions largely paralleled those identified in diseases defined by gastrointestinal inflammation, such as IBD. Changes in microbial taxa, functions (pathways and individual gene families), and in some cases even strains (such as those within the species R*uminococcus gnavus*) were shared between arthritis patients and other inflammatory diseases such as IBD, T1D, and other metabolic disorders. These changes were largely consistent among individuals with RA, AS, and PsA. In addition to the initial results presented here, the corresponding large shotgun metagenomic and clinical dataset offers the ability for further hypothesis generation and testing, including the potential for identification of additional arthritis therapeutic targets.

In particular, several previously-suggested “pro-inflammatory” microbes were enriched here during arthritis(*1*). This was particularly true for *E. coli*, which had an especially unique influence on the corresponding community functional potential, and has been previously shown to be enriched in many conditions including RA(*43, 86*). Mucin-degrading microbes such as *R. gnavus*(*68, 69*) were also differentially carried and functional during disease, down to the subspecies level. Recently, a substantial number of studies have found direct associations between *R. gnavus* and inflammation, including in arthritis(*84, 85*). Potential mechanisms include direct interaction of *R. gnavus* with the host immune system through extracellular proteins(*86*). Notably, a subset of phylogenetically distinct *R. gnavus* isolates from an individual in this cohort with RA and isolates from a healthy control and two IBD individuals were sufficient to increase arthritis severity when introduced into SKG, both supporting their causality and agreeing with previous subclade results. Finally, the presence of characteristically oral taxa in the gut microbiome of patients with chronic inflammation has been well documented, including in patients with IBD, UC, CRC, and metabolic disorders(*1, 48, 87, 88*). A few studies on patients with both AS and RA have also identified increases in streptococci in the gut(*21, 42*), similar to what was observed in our population.

Relatedly, *Prevotella copri* has been implicated as a potential disease trigger in RA, both epidemiologically(*35*) and in studies linking the HLA-DR-presenting peptide of certain strains of *P. copri* and the stimulation of a Th1 response in the onset of RA(*47*). However, across the microbiome studies conducted in primarily treatment naïve arthritis patients, there are conflicting reports regarding its role: some studies indicating increased burden of *P. copri* in the gut microbiomes of RA patients(*35, 47*), while others have found no link(*42, 61*). Additionally, there is evidence suggesting that treatment of RA patients with methotrexate (MTX) may revert the *P. copri* abundance back to normal levels(*89, 90*). In the current UK based study, we did not observe any evidence for increased *P. copri* abundance or prevalence in this cohort (**Fig. 1F** and **Fig. S8**). The RA patients included in this study were all disease modifying anti-rheumatic drug (DMARD) naive, thus none had yet been exposed to MTX or any other DMARD at the time of sample collection. Other studies with shotgun sequencing have also found other *Prevotella* species with increased abundance, which we also did not observe in our population(*39*). *P. copri* carriage has been shown to differ by both country of origin and diet(*91*), which could explain some of these differences.

These examples represent two ways in which our results generally agree with previous studies of the gut microbiome in inflammatory arthritis(*41, 43, 86*). We also observed similar broad patterns, such as the loss of typical gut consortia and increasing abundance of oral taxa and clades associated with gastrointestinal inflammation(*34-41, 43*). Although we were slightly hindered by differences in collection targets within our disease subtypes (e.g. individuals with treatment-naive early RA versus individuals with AS with predominantly controlled disease, as well as the larger sample size in our RA group), microbiome alterations across different inflammatory arthritis phenotypes were, when detectable, largely shared among such subpopulations. We found many of the same microbes associated with either AS or BASDAI as we identified within our RA individuals. We hypothesize this is most likely due to the consistent collection, sequencing, and analysis methods applied throughout our cohort, as well as its relative geographical and environmental homogeneity, any of which can otherwise cause inter-study differences(*87, 88*).

The observation of consistent shifts in the functional capacity of gut microbial communities in patients with inflammatory arthritis provides the opportunity to explain their potential chemical and regulatory consequences. These include changes in folic acid metabolism, iron sequestration, metabolism of broad classes of B vitamins, and production of isoprenoids. Folic acid metabolism in particular (microbial processing of folate to downstream compounds) was more abundant in arthritis patients with higher circulating CRP. Methotrexate (MTX) is a dihydrofolate antagonist, which competitively binds to and blocks several folate pathway enzymes. In patients with treatment-naïve RA, higher basal folate metabolism has been documented in the peripheral serum, and MTX treatment was shown to normalize that degree of folate metabolism(*89*). Treatment with MTX often relieves arthritis patients of many of their joint inflammation symptoms, indicating a potential role of folic acid metabolism in the disease etiology (although it is not clear that this is the mechanism of action for MTX in this case). Almost no patients in this study were currently taking MTX (specifically none of the RA patients), and increased microbial folic acid metabolism thus appears independent of MTX exposure. Further, several studies have implicated the role of well-regulated folate metabolism in the appropriate functioning of the host's immune system(*90-93*), including natural killer (NK) cells(*90*), the proliferation of $CD8^+$ T lymphocytes(*92*), the survival of $FOXP3^+$ regulatory T cells(*93*). One previous study in a smaller cohort has also identified changes in folic acid metabolism pathways associated with disease improvement within the gut ecosystem of patients with RA(*41*). Thus, folic acid metabolism within the gut microbiome is a potential player in the etiology of arthritis, and warrants further mechanistic validation both linked to and independently of MTX usage.

In an even clearer example of this causal vs. responsive dichotomy, increased carriage of microbial iron sequestration via non-heme, heme, and ferroxide related mechanisms (**Fig. 5**) could occur due to i) changes in the gut environment during disease that favor microbes sequestering iron, or ii) greater microbial sequestration of iron as a contributing risk factor in disease (or both). Notably, many different clades encoding iron sequestration systems were enriched during arthritis, with no one primary driver taxon. Potentially relatedly, in RA, the immune system has been shown to sequester iron away from other cell types, often resulting in anemia(*94*). Previous studies in murine models have indicated that in response to iron, GIT microbes are capable of both secreting small molecules that inhibit the transcription of HIF-2α, which is responsible for the uptake of iron in the intestines, and concurrently upregulating their own iron sequestration mechanisms resulting in decreased iron absorption in the host(*95, 96*). A similar enrichment of iron sequestration genes was observed in patients with IBD, although with a clearer corresponding hypothesis that it may be due to increased presence of blood within the

GIT(*97*). Even if true in IBD, this is unlikely to be the case in arthritis, where increased microbial iron sequestration might instead result from anemic conditions within the host(*98, 99*).

Our study has limitations, despite increasing both the sample size and depth of microbial data compared to previous studies, the inter-individual diversity of the human gut microbiome means that our results are still derived from a relatively small sample size - notably from a single country and dominated by a single ethnicity. This is especially true with the confounding nature of clinical data, including site specific collection of distinct diagnoses, a large age range, inherent differences in the sex distribution and uneven loading of arthritis subtypes across sequencing batches. Further, since we only used sequencing data, especially since these data are based on DNA profiles only, we do not have a true functional profile. Thus, as noted several times above, it is impossible to establish the causality or mechanism of these gut microbial changes from an observational human study, and we fully expect our own and others' longitudinal human and model system research to clarify these.

However, this study comprehensively evaluates functional changes within the gut microbiome of patients with RA and AS at scale. We found what are becoming canonical shifts in the distribution of several microbial processes in the gut during inflammation, including for both local gastrointestinal conditions and systemic inflammatory disease. Our study contributes to the growing body of evidence that the gut microbiome and inflammation throughout the body are tightly coupled, likely both casually and responsively, as the gut microbiome serves as a mediator of environmental triggers and then also changes in response to immune activity. We hypothesize that this occurs in part due to a functional "echo" of systemic inflammation in the gut microbiome, due to the similarity in the specific processes that are altered in IBD and in arthritis. Some of these alterations, such as those for B vitamin metabolism, including both B9 and B12, could represent mechanisms for long-term prevention, risk reduction, or treatment, as could microbial iron sequestration during arthritis-linked anemia. We thus expect these results and resources to represent the next step in understanding and managing inflammatory arthritis through its interplay with the gut microbiome.

Methods

Study Design

Participants were recruited for this multi-center study in Birmingham, UK (primarily RA and HC patients, exact numbers in **table S1**), Newcastle, UK (RA, PsA, NIJP, AS, and HC), and Oxford, UK (AS and HC only) from June 2015 until March 2020, samples were accepted until the last year of the grant period. Patients enrolled for this study were aged 17 to 97 years. As expected, based on disease epidemiology(*100, 101*), diagnoses were skewed by female sex, comprising 63%, 30%, 40%, 85%, and 58% of the patients with RA, AS, PsA, NIJP, and HC, respectively. The majority of participants reported non-Hispanic white ethnicity (74%). Approximately 50% of our population had never smoked cigarettes, and this was generally lower among cases than controls (**table S1**). A simple power calculation based on the human microbiome project data(*102*), indicated that at 400 samples (150 healthy controls and 250 cases and adjusting for 15 covariates, we were well powered to quantify changes in both abundant and rare taxa, power = 1).

This observational cross-sectional study was designed as a sub-project within the Inflammatory Arthritis Microbiome Consortium (IAMC). All samples were collected under the IAMC umbrella biospecimen protocol from one of the three main collection sites. For this study we focused on adults with clearly defined arthritis (corresponding to collection sites in Oxford, Birmingham, and

Newcastle, UK) to robustly establish the associations between inflammatory arthritis and the gut microbial composition. Patients were consented by their treating physician to have blood and stool collected. Other important clinical metadata captured by the treating physician included disease-specific measures of BASDAI (for AS patients), DAS28 (for RA and PsA), and swollen/ active joint counts. All clinical and demographic information was curated and securely housed in REDCap(*103, 104*). Participants provided written informed consent. The study was approved by the Newcastle and North Tyneside Regional (REC 12/NE/0251), Oxfordshire (REC 06/Q1606/139) and West Midlands-Back Country (REC 12/WM/0258) Research Ethics Committees.

Biological material was obtained from consenting patients referred from primary care with suspected arthritis and seen in either the Newcastle Early Arthritis Clinic (NEAC), UK [Newcastle upon Tyne NHS Foundation Trust], the Birmingham Early Arthritis Cohort from Sandwell and West Birmingham NHS Trust, University Hospitals Birmingham NHS Foundation Trust, and the Nuffield Orthopaedic Center (NOC) and Oxford biobank during 2017-2019. The recruitment strategy was designed to minimize enrollment of individuals exposed to systemic corticosteroids or disease-modifying anti-rheumatic drugs (DMARDs) prior to biological sampling. Clinical diagnoses were ascertained by board-certified rheumatologists in accordance with standard and appropriate classification criteria where available. RA was assigned only where 1987 American College of Rheumatology or 2010 European League Against Rheumatism/American College of Rheumatology classification criteria were fulfilled. All axial spondyloarthritis patients met the Assessment of Spondyloarthritis International Society (ASAS) criteria for axial spondyloarthritis(*105*). When plain radiographs were performed, the vast majority (>90%) additionally met modified New York Criteria for Ankylosing Spondylitis and we have hence used AS. A diagnosis of non-inflammatory joint pain (NIJP) was assigned when the consulting rheumatologist considered the presentation neither attributable to an inflammatory arthritis, nor to osteoarthritis. For the HC's from the Oxford biobank, samples were selected to enrich for HLA-B27 positive individuals, specifically selecting for about 50% of the controls being positive for the HLA-B27 allele.

Sample collection

Blood samples were collected at routine clinical visits. Whole blood was drawn and stored at -80°C. For serum, blood was drawn into a 5-ml SST tube and allowed to clot at room temperature after centrifugation for 15 min at 1000G. Serum supernatants were aliquoted and stored at -80°C and in accordance with approved protocols. Blood samples were assayed for CRP, RF, anti-CCP, HLA-B27 genotype, full blood count, and liver function by UK certified labs. RF and anti-CCP were classified as positive or negative according to local laboratory cut-offs.

Those enrolled were provided with a previously-validated stool collection kit designed to maximize ease of participation and to impart a minimum perturbative effect on downstream extraction and computational protocols(*106*). They were also furnished with a brief dietary inventory modeled after prior investigations and a questionnaire surveying various microbiome-relevant exposures, such as the recent use of antibiotics, each completed at the time of collection. All EtOH-fixed kits used for metagenomic sequencing were returned within 1 to 3 days of a matched blood sample by Royal Mail Response Service Delivery. Participants used a FecesCatcher (Tag Hemi) to collect a stool aliquot into a Sarstedt Feces container containing 100% molecular biology grade ethanol (Merck Life Sciences) to facilitate preservation at more ambient temperatures. Participants returned the samples enclosed in UN3373 Category B Postal kit boxes (Air Sea Containers) to the Kennedy Institute of Rheumatology by Royal Mail delivery. Samples were immediately stored at -80°C upon arrival until processed for DNA and RNA extractions. For fresh-frozen stool used for bacterial isolation, participants were provided

with stool collection kits during clinic visits. Samples were collected at home using a FecesCatcher (Tag Hemi) and a feces container (Starstedt) and placed inside a disposable styrofoam container with frozen ice packs. Participants returned the kits to the clinic in person and samples were frozen at -80°C immediately upon arrival. Fresh-frozen stool was pulverized into a homogenous mixture utilizing a Biopulverizer (Stratech) cooled in liquid nitrogen prior to aliquoting.

Statistical analysis

All raw, individual-level data for experiments where n<20 are presented in data file S2. Two primary classes of statistical testing were used throughout this analysis, omnibus tests and per-feature tests. The former assessed whether whole microbial community structure was significantly different based on phenotype, whereas the latter assessed this for each individual feature (e.g., taxon, pathway, etc.). Omnibus tests were generally carried out using Bray-Curtis-based PERMANOVA for the taxonomic, pathway and, enzyme-based feature tables using the vegan v2.5-6 package(*107*) in R. With one expectation, we ran all models in an adjusted univariate format, consisting of sequencing batch followed by the variable of interest [adonis(bray ≈ batch + x)] with 1,000 permutations. However, to test the collection center variable, we added diagnosis to the adjustments to the model, since diagnosis was substantially confounded with clinical site (patients carrying certain diagnoses were only seen at certain centers). All p-values that are presented are FDR corrected using the *p.adjust* function unless otherwise stated.

Additionally, for diagnosis and inflammation status, we used the package default pairwise.adonis v0.0.1(*108*) to identify which of the diagnoses or inflammation status categories might be driving the overall results. The same method PERMANOVA model was used for strain testing, but with Kimura 2-parameter distances as input (ape::dist.dna). For our PanPhlAn presence/absence data, we tested for significantly different presence or absence of genes using a chi-squared test by leveraging the Gtest function in the DescTools package(*109*) in R. Difference in means for both Kimura 2-parameter distances and changes in disease score and joint diameter were quantified using Wilcoxon tests.

For parametric feature-wise multivariable testing we used MaAsLin 2 v1.4.0(*110*) in R, which finds associations between microbial features and metadata of interest. MaAsLin uses a transformed generalized linear model to associate each feature iteratively with covariates of interest, here using a variance-stabilizing log transformation plus a small pseudocount of half the minimum feature value for microbial relative abundances (total sum scaling). It then models each microbial feature as a function of the patient's age and adjusts the resulting p-values for multiple hypothesis tests, using a Benjamini–Hochberg correction. As noted above for different analyses, we used several variants of the main feature-covariate model, where Feature ≈ batch + drug + age + inflammation status OR patient diagnosis OR anemia status: first, a fully multivariable model that was the most conservative adjusting for the most patient information; second, a reduced model accounting only for technical sequencing batch; and finally, the least conservative model only adjusting for age with the metadata of interest (inflammation status or patient diagnosis or anemia status). In general, the model without sequencing batch was not used, since there was little evidence of technical batch effects and since inflammation and diagnosis were imbalanced across our sequencing batches. Further we compared the categorized way of processing CRP data to other disease specific markers including BASDAI and the DAS28 metrics and they identified many of the same taxonomic features and importantly in the same direction (**fig. S6**).

Most visualizations were carried out using standard methods in R ggplot2 v3.3.2(*111*), ggridges v0.5.2(*112*), ggthemes v4.2.0(*111*), gridExtra v2.3(*113*), gtools v3.81(*114*), and ggtree v2.0.2(*115, 116*). The principal coordinate analysis was done using the capscale function in vegan(*107*). Additionally, we used GraPhlAn v0.9.7(*117*) to construct the cladogram in **Fig. 2**. Additional R packages used for data manipulation and processing include pylr v1.8.6(*118*), dpylr v1.0.2(*119*), scales v1.1.0(*119*), mgsub v1.71(*120*), and RColorBrewer v1.1.2(*121*).

Supplemental Material

Fig. S1 to S26
Table S1 and S2
MDAR Reproducibility Checklist
Data file S1 and S2
References (122-146)

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Author contributions

This study was conceptualized and designed by FP, CH, KR, PB, AGP, DRL, SPY, JJF, LRW and PCT. Data and specimen collection was completed by PC, SJB, AF, CP, SK, AGP, PB, and KR. Molecular methods prior to sequencing were done by PC, IB, CP, CM, VK, and LHL. Computational storage of clinical and sequence data was designed, maintained, and organized by KNT, KSB, NEI, PC, LJM, MAJ, and BM. Analysis was completed by KNT, KSB, NEI, GJB, SM, and LHN. Feedback and directions were guided by AGP, PB, and KR. Mouse work was completed by GJB and JJF. Manuscript was written by KNT, LHN, AP and CH. Editing was completed by all authors.

Competing interests

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Data availability

All data associated with this study are in the paper or supplementary materials. Sequence data and metadata are available for approved user to download through the EGA (https://ega-archive.org/), study accession number EGAS00001005525. Processed taxonomic and functional tables are available in data file S1. Bioinformatic workflows for metagenomic processing are available at https://huttenhower.sph.harvard.edu/biobakery_workflows, these include some basic statistical and visualization scripts. Custom analysis scripts are available at http://huttenhower.sph.harvard.edu/Adult_cross-sectional_IAMC

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