Fecal metabolomics in pediatric spondyloarthritis implicate decreased metabolic diversity and altered tryptophan metabolism as pathogenic factors

ML Stoll¹, R Kumar², EJ Lefkowitz³, RQ Cron¹, CD Morrow⁴ and S Barnes⁵

We have previously shown alterations in the composition of the gut microbiota in children with enthesitis-related arthritis (ERA). To explore the mechanisms by which an altered microbiota might predispose to arthritis, we performed metabolomic profiling of fecal samples of children with ERA. Fecal samples were collected from two cohorts of children with ERA and healthy control subjects. Nano-liquid chromatography—mass spectroscopy (LC-MS) was performed on the fecal water homogenates with identification based upon mass: charge ratios. Sequencing of the 16S ribosomal DNA (rDNA) on the same stool specimens was performed. In both sets of subjects, patients demonstrated lower diversity of ions and under-representation of multiple metabolic pathways, including the tryptophan metabolism pathway. For example, in the first cohort, out of 1500 negatively charged ions, 154 were lower in ERA patients, compared with only one that was higher. Imputed functional annotation of the 16S ribosomal DNA sequence data demonstrated significantly fewer microbial genes associated with metabolic processes in the patients compared with the controls (77 million versus 58 million, P = 0.050). Diminished metabolic diversity and alterations in the tryptophan metabolism pathway may be a feature of ERA.

ORIGINAL ARTICLE

INTRODUCTION

Interest in the composition of the gut microbiota in subjects with spondyloarthritis (SpA), like enthesitis-related arthritis (ERA)/juvenile idiopathic arthritis (JIA), has been accumulating.¹ We and others have identified taxonomic differences in fecal bacteria between pediatric or adult SpA subjects and healthy controls.²⁻⁴ However, these studies have not provided a mechanism whereby dysbiosis can result in arthritis. One possible mechanism is through alterations in metabolic pathways. The metabolic capacity of bacteria is an under-appreciated aspect of the human microbiome. In total, bacteria contain over 3 million genes, 100 times the human host;⁵ they perform a variety of metabolic functions including metabolism of dietary components, drug detoxification and synthesis of vitamins and essential amino acids.⁵ Bacteria are not created equal in their capacity to perform these functions, and thus a particular microbiome may be more or less effective than another at carrying out certain activities. To evaluate the functional potential of the microbiome, fecal water metabolomics on children with ERA and controls was performed. The aim of metabolomics is to conduct a comprehensive analysis on the identities of the low molecular weight ions (in our facility, < 1000 Da) present in a sample, so as to obtain insight into function.⁶ Metabolomics of fecal water, the supernatant obtained following high-speed centrifugation of feces, can discriminate between inflammatory bowel disease (IBD) patients and healthy individuals, with some studies showing elevated levels of amino acids and decreased short chain fatty acids such as butyrate.⁷,⁸

Results

Our intent was to identify mechanisms by which the microbiota might predispose or contribute to arthritis in children with ERA.

Subjects

Two cohorts were used in this study (Table 1). Cohort 1 consisted of children with relatively newly diagnosed arthritis (JIA/ERA); five were naïve to treatment at the time of stool collection, while the remainder had been exposed to one or more immunosuppressive medications for no more than two months. Cohort 2 included JIA/ERA children with more long-standing disease. None of the controls had ever been exposed to chronic immunosuppressive therapy or were taking corticosteroids at the time of the study.

Metabolomics analysis

Fecal specimens were subjected to metabolomics analysis with nano-liquid chromatography—mass spectroscopy (LC-MS). Out of nearly 1500 negatively charged ions identified in the derivation run, 154 were significantly less abundant in patients, while only one was significantly higher in patients. In this analysis, differentially present negatively charged ions revealed 21 pathways that were under-abundant in ERA patients (Table 2 and Supplementary Table S3). Similar findings were observed with positively charged ions, where 107 were lower in ERA patients, yielding five pathways, compared with only 17 that were higher.
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Table 1. Subjects included in the study

| Feature                  | Cohort 1 | Cohort 2 |
|--------------------------|----------|----------|
|                          | ERA      | Control  | ERA      | Control  |
| N                        | 14       | 10       | 10       | 10       |
| Age (years)*             | 14; 7–17 | 10; 7–18 | 14; 8–16 | 12; 9–17 |
| Male: female             | 5: 9     | 2: 7     | 7: 3     | 5: 5     |
| BMI*                     | 26; 17–35| 19; 14–24| 20; 15–27| 19; 15–32|
| HLA-B27†                 | 2/13     | ND       | 4/10     | ND       |
| Duration of drug therapy (months)* | 0:0–2 | NA       | 40:27    | NA       |
| Meds                     | None     | 5        | 11       | 0        |
| MTX alone                | 6        | 0        | 3        | 0        |
| MTX, anti-TNF            | 2        | 0        | 4        | 0        |
| Anti-TNF alone           | 1        | 0        | 3        | 0        |

Abbreviations: BMI, body mass index; ERA, enthesitis-related arthritis; HLA, human leukocyte antigen; NA, not applicable; ND, not done; TNF, tumor necrosis factor. *Continuous variables are shown as median; range.

Table 2. Pathways under-represented among patients in cohort 1

| Pathway                          | Overlap size | Pathway size | P-value |
|----------------------------------|--------------|--------------|---------|
| Positively charged ions          |              |              |         |
| Tryptophan metabolism            | 9            | 37           | 0.0038  |
| Xenobiotics metabolism           | 8            | 36           | 0.00544 |
| Selenoamino acid metabolism      | 3            | 12           | 0.0042  |
| Vitamin B6 (pyridoxine) metabolism | 2        | 6            | 0.0405  |
| Purine metabolism                | 4            | 24           | 0.05128 |
| Negatively charged ions          |              |              |         |
| Glycosphingolipid biosynthesis—gangliosides | 5 | 7 | 0.00091 |
| Tryptophan metabolism            | 13           | 46           | 0.00106 |
| Glycosphingolipid biosynthesis—gobiosides | 3 | 3 | 0.00122 |
| N-glycan biosynthesis            | 3            | 6            | 0.00328 |
| Tyrosine metabolism              | 14           | 68           | 0.00349 |
| Glycolysis and gluconeogenesis   | 6            | 23           | 0.00425 |
| Butanoate metabolism             | 4            | 12           | 0.00431 |
| Bipterin metabolism              | 4            | 13           | 0.00565 |
| Fructose and mannose metabolism  | 4            | 16           | 0.01275 |
| Ubiquinone biosynthesis          | 2            | 4            | 0.01946 |
| Heparan sulfate degradation       | 2            | 4            | 0.01948 |
| Keratan sulfate degradation       | 2            | 4            | 0.01948 |
| Chondroitin sulfate degradation   | 2            | 4            | 0.01948 |
| Linoleate metabolism             | 3            | 11           | 0.01895 |
| Drug metabolism—other enzymes     | 4            | 18           | 0.0213  |
| N-glycan degradation              | 2            | 5            | 0.02444 |
| Pyrimidine metabolism            | 5            | 27           | 0.03493 |
| De novo fatty acid biosynthesis   | 2            | 6            | 0.03734 |
| Purine metabolism                | 6            | 35           | 0.04268 |
| Histidine metabolism             | 3            | 14           | 0.04941 |

Overlap size indicates the number of metabolites present in a particular pathway; for example, among negatively charged ions, 13 metabolites associated with tryptophan metabolism were identified in the original run as being significantly higher in controls as compared with patients. Pathway size is the total number of metabolites in the pathway that were present in the input files.

Data was available on 22 out of 23 subjects, yielding a total of 1 435 797 unique sequences; one control subject was excluded from the analysis due to a sequencing depth of < 500 reads. Principal coordinates analysis (Figure 2) revealed partial clustering with the Unweighted UniFrac test, indicating differences in rare bacteria. The observations shown in Figure 2 were supported by the Permanova test, which showed that the presence versus absence of JIA/ERA affected the overall community structure (F = 1.67, P = 0.050). There was also an

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with psoriatic arthritis and IBD. Literature showing decreased gut microbial diversity in patients not statistically significant. Differences in the overall community structure (Figure 2); significantly, these differences resulted in higher functional potential in the ERA patients, while very few were more abundant in the patients. This appears to be a unique finding with respect to arthritis, although similar findings of decreased metabolic diversity were reported in salivary samples of patients with Sjogren Syndrome. In addition, these findings are consistent with the literature showing decreased gut microbial diversity in patients with psoriatic arthritis and IBD. Despite differences in gut metabolic diversity, there were not any striking differences at the taxonomic level in the microbiota between patients and controls in this cohort of largely newly diagnosed patients; this stands in contrast to our previous findings in established JIA/ERA patients. The significance of this finding will bear further study. However, it is of interest that despite the modest differences at the taxonomic level, there were differences in the overall community structure (Figure 2); significantly, clustering present with the Unweighted Unifrac test indicate that there are likely differences in the presence versus the absence of several bacteria, although none of these pairwise differences on their own attained statistical significance. Importantly, these differences resulted in higher functional potential in the controls as indicated by the PICRUSt analysis, and thus likely accounted for the metabolomics differences. Thus, the microbiota may have an influence on disease, even without substantial differences in the levels of any single organism.

In addition to the relatively decreased gut microbial diversity in JIA/ERA patients, in both cohorts, evidence of altered tryptophan metabolism in children with ERA was also observed, with lower levels of tryptophan metabolites in patients compared with controls. The finding of decreased tryptophan metabolites is consistent with a similar finding in the synovial fluid of subjects with rheumatoid arthritis, as compared with controls with osteoarthritis. It is also consistent with data from a mouse model of colitis caused by deficiency in caspase recruitment domain 9, in which levels of fecal indole-3-acetic were reduced; in this model, transfer of the microbiota to wild-type mice also resulted in colitis. The same study also showed decreased fecal levels of indole-3-acetic in human subjects with IBD. In addition, a study of mice with dextran-sulfate induced colitis also showed decreased fecal indole-3-acetic. Importantly, we excluded children who had received antibiotics within 3 months before collection of the sample, as antibiotic usage can result in long-lasting effects on the microbiota and can also impact the metabolome, including the tryptophan pathway. As recently reviewed, antibiotic usage may also increase the future risk of both JIA and IBD, although distant antibiotic usage was not explored in our subjects.

The metabolomics data, and confirmed to a lesser extent with the 16S data, suggest differences in tryptophan metabolism in children with ERA attributable to the gut microbiota. The essential amino acid tryptophan can be metabolized to a variety of different byproducts (Figure 1), several of which are immunologically relevant. When exposed to indoleamine-2,3-dioxigenase and tryptophan dioxigenase, tryptophan forms l-kynurenine and subsequently 3-hydroxy-kynurenine; it is estimated that ~95% of tryptophan metabolism is generated via this route. Kynurenine appears to favor the development of regulatory T cells. In addition, metabolism of tryptophan by tryptophanase into indole, by aromatic amino acid decarboxylase into tryptamine, and by tryptophan hydroxylase into 5-hydroxy-tryptophan, all result in the generation of ligands of the aryl hydrocarbon receptor (AhR). Ligand binding of the AhR results in attenuation of inflammation in vitro results in attenuation of cytokine production normally induced by pro-inflammatory mediators, such as interleukin-1 and tumor necrosis factor. However, AhR are not all created equally with respect to pro-inflammatory potential, and 5-hydroxy tryptophan appears to be anti-inflammatory, whereas indole itself appears to be pro-inflammatory.

### DISCUSSION

This is the first study to apply metabolomics to query the function of the gut microbiome communities in SpA. In two separate cohorts, despite phenotypic differences as well as differences in treatment durations, ERA patients had substantially lower diversity of ions present in their fecal water. Specifically, in cohorts 1 and 2, 8.6% and 6.3%, respectively, of the identified ions were less abundant in the ERA patients, while very few were more abundant in the patients. This appears to be a unique finding with respect to arthritis, although similar findings of decreased metabolic diversity were reported in salivary samples of patients with Sjogren Syndrome. In addition, these findings are consistent with the literature showing decreased gut microbial diversity in patients with psoriatic arthritis and IBD.

### Table 3. Pathways under-represented among patients in cohort 2

| Pathway                          | Overlap size | Pathway size | P-value  |
|----------------------------------|--------------|--------------|----------|
| **Negatively charged ions**      |              |              |          |
| Urea cycle/amo acid metabolism   | 3            | 21           | 0.0106   |
| Bioperin metabolism             | 2            | 7            | 0.01162  |
| Tryptophan metabolism           | 3            | 37           | 0.03769  |
| Glycerophospholipid metabolism  | 2            | 16           | 0.03784  |
| **Positively charged ions**      |              |              |          |
| N-glycan biosynthesis           | 2            | 3            | 2.00E–05 |
| Drug metabolism—cytochrome P450  | 5            | 30           | 2.00E–05 |
| Ubiquinone biosynthesis         | 2            | 5            | 9.00E–05 |
| Hexose phosphorylation          | 2            | 7            | 0.0003   |
| Linoate metabolism              | 2            | 7            | 0.0003   |
| Histidine metabolism            | 2            | 11           | 0.00218  |
| Galactose metabolism            | 2            | 15           | 0.00922  |
| Squalene and cholesterol biosynthesis | 2        | 15           | 0.00922  |
| Glycerophospholipid metabolism  | 2            | 18           | 0.02106  |

Figure 1. Abbreviated version of the KEGG tryptophan map. Asterisks depict locations in metabolic pathways of metabolites differentially present in controls versus ERA patients. The increased thickness size of the down arrow reflects that most of the metabolism of tryptophan is through IDO. AAD, aromatic amino acid decarboxylase; IDO, indoleamine-2,3-dioxigenase; TDO, tryptophan dioxygenase; TRPase, tryptophanase; TRP OHase, tryptophan hydroxylase.

(713 868 versus 533 826, P = 0.070), but not in tryptophan metabolism (82 540 versus 70 962, P = 0.365). Similar trends were observed in cohort 2, although the differences were modest and not statistically significant (not shown). Thus, the 16S fecal microbiome sequencing data confirms the relative decrease in tryptophan metabolism in JIA/ERA patients, relative to controls, as noted in metabolomics analyses.

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inflammatory and is also a particularly strong AhR agonist.29 Thus, it is of interest that patients had lower levels of metabolites that served as cofactors for 5-hydroxy tryptophan synthesis, as well as its downstream products, as well as higher levels of metabolites of kynurenine. None of the metabolites higher in controls appeared to be associated with indole production. Thus, alterations in tryptophan metabolism may affect the plastic nature of effector CD4 T cells away from regulatory and towards pro-inflammatory based on the altered tryptophan catabolism. Importantly, this is an associative study, and no definitive causal conclusions can be drawn. In addition, the low numbers of patients in this study, as well as the need for validation of the specific metabolic findings, are acknowledged. However, the replication of the observations that controls had a much greater variety of metabolites in general, as well as specifically within the tryptophan pathway, represents important internal validation. In addition, the two cohorts of ERA subjects were dissimilar from one another with respect to disease duration, medications used, and demographic features (Table 1), yet the findings were similar, speaking to the generalizability of these results. Finally, the possibility of the immunomodulatory therapies themselves resulting in some of the findings, such as the highly significant association with drug metabolism pathways in the second cohort, must be considered. However, the extent of the differences was far more striking among the cohort that was largely naive to therapy. Further work is required to validate our findings and also to assess the metagenomics and metabolic effects of immuno-suppressive therapy. We are just beginning to understand the functional implications of these alterations, with much more work to be done exploring potential clinical significance and therapeutic implications of abnormalities in the production of important metabolites, including, but surely not limited to the tryptophan pathway.

PATIENTS AND METHODS

Subjects

Patients were children with JIA/ERA evaluated at a single rheumatology center, Children’s of Alabama, while the controls consisted of children aged 7–18 in the community recruited through advertisements. These control children were negative by questionnaire for symptoms suggestive of IBD, psoriasis, arthritis or inflammatory back pain. Subjects were excluded if they used antibiotics within 3 months of the collection of the fecal specimen.

Stool collection and processing

Stool samples were immediately placed by the subject in Cary-Blair media, which is a low-nutrient medium that prevents overgrowth of bacteria.32 Samples were shipped overnight to our laboratory, aliquoted, and stored in 10% glycerol as a cryopreservative at −80 °C until analyzed.

Metabolomics

Fecal water was processed at 4 °C via acid extraction as modified from Marchesi et al.3 as follows: double-distilled H2O was added 1:1 to the fecal suspension, followed by addition of 98% formic acid (1 μl ml−1; Sigma: St Louis, MO, USA). The fecal homogenates were ultracentrifuged (14 000g for 15 min at 4 °C), and the supernatants extracted with 2 × 2 volumes of ethyl acetate. The solvent extracts were combined and evaporated under N2. The supernatants were analyzed by nano-liquid chromatography—mass spectrometry (LC-MS) on a silica CHIP C18 reverse-phase column (200 μm id × 15 cm) as our group has performed previously.23 Specifically, the dried residues were reconstituted in 100 μl 0.1% formic acid and 5 μl aliquots were loaded onto a 0.5 cm × 200 μm ID silica CHIP C18 reverse-phase cartridge, which was washed for 5 min with 0.1% formic acid. Trapped metabolites were separated by nano-LC-MS on a 15 cm × 200 μm ID silica CHIP C18 reverse-phase column using a 20 min, 0–95% linear gradient of acetonitrile containing 0.1% formic acid at 1 μl min−1, followed by a 1 min wash at 100% acetonitrile and column re-equilibration for 4 min with 0.1% formic acid. An Eksigent 415 (SCIEX, Concord, ON, Canada) nano-LC pump was used to provide the gradient. The Chip LC column was contained in an Eksigent Nanoflex (SCIEX), which maintained it at 45 °C. Column eluate was passed through the nanoelectrospray ionization interface of a SCIEX 5600 TripleTOF (SCIEX). Using a duty cycle of 1.2 s, first, a high-resolution time-of-flight—mass spectrometry spectrum was collected for 100 ms, followed by 50 ms tandem mass spectrometry spectra of the 20 most intense precursor ions. Once an adequate tandem mass spectrometry spectrum had been collected for a given precursor ion, it was placed on an exclusion list for the next 30 s. Mass calibration using SCIEX APCI standards (APCI Positive and Negative Calibration Solutions, SCIEX) for both positive and negative mode was run between each unknown sample in order to maintain mass calibration of the mass spectrometer (typically 2–3 p.p.m.). Mean retention time variation for all metabolites eluting between 5 and 25 min using the nano-LC-MS system was 0.26%. Each sample was subjected to two separate metabolomics analyses: one to identify negatively charged ions; and the other to identify positively charged ions. The collected LC-MS data (.wiff and.wiffscan files) were analyzed by XCMSonline (https://xcmsonline.scripps.edu) for retention time alignment and initial statistical analysis. Data (mass to charge (m/z) values, retention times, and ion intensities) from the aligned ions for each sample were submitted for statistical analysis using MetaBolAnalyst (http://www.metaboanalyst.ca). To control for differences between samples, the data were normalized to the total ion current. Data were mean centered and subjected to Pareto scaling.

To identify the ions based upon this information and to make inferences about function, Li et al.34 generated a program (mummichog version 1.0.7) that generates metabolic networks consisting of modules (sets of ions that are chemically related) and pathways (for example, folic acid metabolism). The power behind this statistical package is its ability to evaluate all combinations of potential biochemical assignments for ions determined to be statistically different in order to identify network modules or pathways. Mummichog, by using all ions including those that are not changed significantly, establishes expected network/pathways associations under the null hypothesis, which is that there are no differences in pathway representation among the two inputted groups (in this case, ERA patients and controls). Over-representation of statistically significant ions in modules and pathways can then be identified as those higher than would be predicted by chance. Thus, the output is modules and pathways that are over- or under-represented in one group relative to another. The input to mummichog was the full set of ions, as well as those that were differentially present between the two groups (1.5-fold or higher difference with P-values of 0.05 or less). The mummichog program uses the Fisher exact test to compare pathway utilization between the list of select ions versus the full list of ions, removing one hit from each identified pathway in turn until the best pathway set of hits that are differentially present between the two groups were identified.
pathway so as to penalize pathways with very few hits that might have arisen by chance. Mummiouch analysis was performed four times, twice for ions higher in patients (once each for negatively and positively charged ions), and twice for ions higher in controls. Assignment of metabolites to pathways is based upon the KEGG database[10] and their own curation,[5] which also included the Edinburgh Human Metabolic Network Reconstruction.[11] Sample size was based upon the study by Marchesi et al.[5]

Sequencing of 16S ribosomal DNA
Amplification of the variable V4 region of the 16S ribosomal DNA was performed as previously described and sequenced with the MiSeq (Illumina, San Diego, CA, USA).[12] Following quality-control steps, sequence data were processed with the Quantitative Insight Into Microbial Ecology tool suite.[36] Sequences were grouped into groups of similar sequences (known as operational taxonomic units) using uclust.[37] The resulting operational taxonomic unit table was imported into PICRUSt.[12] PICRUSt is an open-source bioinformatics software program that predicts metabolic functions based upon 16S ribosomal DNA sequence data. Assessment of between-sample diversity (similarity between the sequences of each subject) was performed with the Unweighted UniFrac test[38] and visualized with principal coordinates analysis. The Permanova test evaluated whether a categorical variable (JIA/ERA versus control) predicted the sample clustering obtained from the UniFrac test. Pairwise comparisons of abundance of metabolic functions were performed with the Mann–Whitney U-test.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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