Microbiome Analytics of the Gut Microbiota in Patients With Juvenile Idiopathic Arthritis: A Longitudinal Observational Cohort Study

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Objective. To assess the composition of gut microbiota in Italian and Dutch patients with juvenile idiopathic arthritis (JIA) at baseline, with inactive disease, and with persistent activity compared to healthy controls.

Methods. In a multicenter, prospective, observational cohort study, fecal samples were collected at baseline from 78 Italian and 21 Dutch treatment-naive JIA patients with <6 months of disease duration and compared to 107 geographically matched samples from healthy children. Forty-four follow-up samples from patients with inactive disease and 25 follow-up samples from patients with persistent activity were analyzed. Gut microbiota composition was determined by 16S ribosomal RNA–based metagenomics. Alpha- and β-diversity were computed, and log ratios of relative abundance were compared between patients and healthy controls using random forest models and logistic regression.

Results. Baseline samples from Italian patients showed reduced richness compared to healthy controls (P < 0.001). Random forest models distinguished between Italian patient baseline samples and healthy controls and suggested differences between Dutch patient samples and healthy controls (areas under the curve >0.99 and 0.71, respectively). The operational taxonomic units (OTUs) of Erysipelotrichaceae (increased in patients), Allobaculum (decreased in patients), and Faecalibacterium prausnitzii (increased in patients) showed different relative abundance in Italian patient baseline samples compared to controls after controlling for multiple comparisons. Some OTUs differed between Dutch patient samples and healthy controls, but no evidence remained after controlling for multiple comparisons. No differences were found in paired analysis between Italian patient baseline and inactive disease samples.

Conclusion. Our findings show evidence for dysbiosis in JIA patients. Only patient/control status, age, and geographic origin appear to be drivers of the microbiota profiles, regardless of disease activity stage, inflammation, and markers of autoimmunity.

INTRODUCTION

The human body is host to myriad nonpathogenic microorganisms, the so-called microbiota. Many factors contribute to its composition, among which are modes of delivery, diet, and drug use (1). The interaction between host and microbiota is complex and not yet fully elucidated. What is known is that microbiota play a role in host defense against pathogens and provide the host with nutrients and vitamins (1,2).

Recent studies have demonstrated alterations in the composition of gut microbiota in patients with autoimmune disorders such as rheumatoid arthritis and type 1 diabetes mellitus (1,3–8). These alterations of the microbial profile induce a dysbiotic state of the microbiota that also affects functional...
activities of the gut (5,9). It is still unclear if there is any causal relationship underlying the association between dysbiosis and autoimmune disorders. However, various observations in germ-free mice are compatible with such a pathogenic role of gut dysbiosis (1,10,11).

Preliminary observations have suggested the presence of dysbiosis also in pediatric patients with juvenile idiopathic arthritis (JIA) (12–15). JIA is defined as any arthritis of unknown etiology, lasting for more than 6 weeks, with an onset before age 16 years (16). The study findings in JIA are interesting because of the hypothesized pathogenic role of the gut microbiota and potential therapeutic ramifications. However, these findings of microbiota in JIA were inconclusive, as the studies were small and cross-sectional, with some including patients with longstanding disease treated with disease-modifying antirheumatic drugs (DMARDs). The differences between patients with active and those with inactive disease have only been explored within small groups. Also, the statistical analyses used in these studies can lead to a marked increase, up to 68%, of the false discovery rate (FDR) (17).

Therefore, the hypothesis of whether there is dysbiosis in JIA patients needs to be explored further. We addressed the aforementioned issues in a large, multicenter longitudinal study of the gut microbiota composition in a well-defined cohort of JIA patients at baseline and during inactive disease compared to healthy children.

PATIENTS AND METHODS

We conducted a multicenter, observational, prospective cohort study in Italy (Istituto Giannina Gaslini and Ospedale Pediatrico Bambino Gesù [OPBG]) and The Netherlands (Wilhelmina Children’s Hospital). All consecutive patients were enrolled who presented between October 2013 and December 2015 with new-onset JIA according to the International League Against Rheumatism criteria (18) and with <6 months of disease duration since the first symptoms. Patients with systemic JIA and patients treated previously with DMARDs, intraarticular steroid injections, or systemic steroids were excluded. No patients were excluded for recent antibiotic exposure. Patients were included at the time of diagnosis and followed up every 6 months for 24 months. Collected clinical data included patient demographics, JIA category, a full joint count, the Juvenile Arthritis Disease Activity Score in 71 joints (JADAS-71) (19), physician’s global assessment of disease activity, parent’s or patient’s global assessment of well-being, the presence of active uveitis, antinuclear antibody (ANA) positivity, rheumatoid factor positivity, erythrocyte sedimentation rate, C-reactive protein level, and the use of nonsteroidal antiinflammatory drugs (NSAIDs). At the follow-up visits, data were collected regarding treatment and disease activity status, defined according to the American College of Rheumatology (ACR) provisional criteria for inactive disease (20).

The study was approved by the local ethics committees and was conducted in accordance with the Principles of Good Clinical Practice and the Declaration of Helsinki. Written informed consent was obtained from all participants.

Fecal samples. Fecal samples were collected at baseline and during follow-up in cases of inactive disease (defined according to the ACR provisional criteria), persistent activity (i.e., no achievement of inactive disease), or disease flare (defined as the return of any symptom of disease activity after a period of inactive disease). We attempted to obtain samples as soon as inactive disease or disease flare was noted. Details of sample collection and analysis are specified in the Supplementary Methods, available on the Arthritis & Rheumatology web site at http://onlinelibrary.wiley.com/doi/10.1002/art.40827/abstract.

At the time of fecal sample collection, patient information was collected regarding diet; previous antibiotic, prebiotic, and probiotic use; and gastrointestinal infections and diagnosis of inflammatory bowel disorder. Also, fecal samples from healthy children, geographically matched with the patient baseline samples, were collected.

Italian healthy children were recruited at primary and secondary schools, from among the children of health workers and researchers at OPBG, or through collaboration with primary pediatricians who enlisted children at regular check-ups. The inclusion criteria for Italian healthy controls were absence of chronic diseases, absence of gastrointestinal infections, no antibiotic and preprobiotic intake in the 2 months prior to sample collection, and an omnivorous diet. Samples from Dutch healthy controls were collected at primary and secondary schools in the vicinity of the hospital.

Statistical analysis. To assess the overall differences in microbial community structure between patient groups and healthy controls, we calculated measures of α-diversity with the Chao1 and Shannon indices using the “alpha_rarefaction.py” script of the QIIME software on the entire operational taxonomic unit (OTU) matrix (21). The Chao1 index summarizes microbial diversity (richness) per sample, accounting for rare species that might have been missed due to under-sampling. The Shannon index is a measure of entropy, taking into account species richness and evenness in the sample. These indices were compared between patients and controls in linear regression models using age, sex, and geography as covariates. Moreover, to estimate the dissimilarity of microbial communities among groups, measures of β-diversity were calculated on the entire OTU matrix by unweighted UniFrac (22) using the scripts “beta_diversity_through_plots.py” and “compare_categories.py” of the QIIME software (21). Beta-diversity measures were compared across groups using principal coordinates analysis (PCoA) and permutational multivariate analysis of variance (PERMANOVA). PCoA is a tech-
nique to visualize distances (dissimilarities) between samples in a lower-dimensional space. PERMANOVA compares groups and assesses whether the centroids or dispersion of points are similar between 2 groups, basing its significance test on random permutation of the points in the groups. Measures of β-diversity, as opposed to α-diversity, take into account the distance of OTUs along the phylogenetic tree.

After α- and β-diversity analyses, OTUs present in <20% of the samples were removed from the analysis (12,23–26). Since microbiota data are expressed as relative abundances of the OTUs, the sum of which equals 100% for each sample, they cannot be analyzed using standard statistical techniques (17,27,28). Rather, the analysis should be based on log ratios of the individual parts with the geometric mean of the sample, according to the framework of compositional data analysis (27,28). This analysis was performed using the package “compositions” in R statistical software. Since it is impossible to take the logarithm of zero, zeros were imputed by means of robust imputation using the package “robCompositions,” as specified in the literature (29,30). Next, the log ratio–transformed OTU relative abundances were analyzed using principal components analysis (PCA), showing differences between Italian and Dutch patient samples, but not between Roman and Genovese patient samples (see Supplementary Figure 1, available on the Arthritis & Rheumatology web site at http://onlinelibrary.wiley.com/doi/10.1002/art.40827/abstract). Therefore, samples from Italian and Dutch patients were analyzed separately. Likewise, baseline, inactive disease, and persistent activity samples were analyzed separately. A random forest classification model was utilized to classify Italian patient samples taken at baseline and control samples, using the microbiota composition, age, and sex as covariates. To this end, the data were split at random into 70% for model training and 30% for model testing. The best model was selected in the training set, using 10-fold cross-validation repeated 10 times. This model was then applied

| Table 1. Clinical features of the patients and healthy controls at baseline* |
|----------------------------------|-----------------|-----------------|-----------------|-----------------|
|                                  | Italian Patients (n = 78) | Controls Patients (n = 79) | Dutch Patients (n = 21) | Controls Patients (n = 28) |
| Female sex                       | 61 (78.2)          | 38 (48.1)        | 16 (76.2)        | 20 (71.4)        |
| Age, median (IQR) years          | 3.9 (2.3–6.7)      | 8 (5–11)         | 8.5 (4.8–11.7)   | 8.0 (4.8–11.2)   |
| JIA category at onset            |                  |                  |                  |                  |
| Oligoarticular                   | 47 (60.3)         | –                | 14 (66.7)        | –                |
| Polyarticular RF positive        | 2 (2.6)           | –                | 0 (0)            | –                |
| Polyarticular RF negative        | 24 (30.8)         | –                | 4 (19.0)         | –                |
| Enthesitis-related               | 0 (0)             | –                | 2 (9.5)          | –                |
| Psoriatic                        | 3 (3.8)           | –                | 1 (4.8)          | –                |
| Undifferentiated                 | 2 (2.6)           | –                | 0 (0)            | –                |
| Number of active joints, median (IQR) | 3 (1–5)        | –                | 2 (1–4)          | –                |
| PhGA, median (IQR)               | 6 (4–8)           | 2.0 (1.5–3.0)    | 5.7 (2.0–8.1)    | –                |
| PGA, median (IQR)                | 3.1 (1.0–5.2)     | 5.7 (2.0–8.1)    | 9.5 (6.6–13.5)   | –                |
| JADAS-71, median (IQR)           | 13.2 (8.6–19.0)   | 9.5 (6.6–13.5)   | 3.2 (1.0–14.0)   | –                |
| ESR, median (IQR) mm/hour        | 23 (14–43)        | 11 (6–31)        | 8 (38.1)         | –                |
| ANA                              | 51 (65.4)         | –                | 8 (38.1)         | –                |
| RF                               | 2 (2.6)           | –                | 0 (0)            | –                |
| NSAIDs                           | 62 (79.5)         | –                | 17 (81.0)        | –                |
| Antibiotics                      | 13 (16.7)         | 0 (0)            | 0 (0)            | 0 (0)            |
| Prebiotics                       | 14 (17.9)         | 0 (0)            | 8 (38.1)         | 9 (32.1)         |
| Probiotics                       | 14 (17.9)         | 0 (0)            | 0 (0)            | 3 (10.7)         |
| GI infections                    | 11 (14.1)         | 3 (14.3)         | 1 (3.6)          | –                |
| GI disorders†                    | 6 (7.7)           | 2 (9.5)          | 0 (0)            | –                |

* Except where indicated otherwise, values are the number (%). IQR = interquartile range; JIA = juvenile idiopathic arthritis; RF = rheumatoid factor; PhGA = physician’s global assessment of disease activity; PGA = parent’s global assessment of disease activity; JADAS-71 = Juvenile Arthritis Disease Activity Score in 71 joints; ESR = erythrocyte sedimentation rate; ANA = antinuclear antibody; NSAIDs = nonsteroidal antiinflammatory drugs. † Gastrointestinal (GI) disorders were constipation (n = 3), celiac disease (n = 2), milk and egg allergy (n = 1), abdominal bloating (n = 1), and irritable bowel syndrome and lactose intolerance (n = 1).
to the test set to assess its performance. The same procedure was used for the Dutch samples.

Differences in the log ratio–transformed relative abundances of all OTUs between baseline samples and healthy controls, inactive disease samples and healthy controls, and persistent activity samples and healthy controls were assessed with logistic regression, using age and sex as covariates and patient/control status as a dependent variable. A separate model was fitted for each of the OTUs and each of the disease states. As mentioned above, Italian patients and healthy controls were analyzed separately from Dutch patients and healthy controls. The FDR was controlled by the Benjamini-Hochberg procedure (an FDR threshold of 0.05 was considered significant).

Finally, associations between the gut microbiota compositions and various clinical variables were explored using PCA, principal components regression, K-means clustering, and the Kruskal-Wallis test. Statistical analysis was carried out using R Statistics, version 3.3.2.

RESULTS

A total of 169 patients were initially included. Fecal samples were collected at baseline from 99 patients (78 Italian and 21 Dutch). Samples from the remaining patients were not collected due to difficulties collecting or shipping the samples. There were no differences between patients who provided a sample and patients who did not provide a sample (all variables in Table 1 were compared; data not shown). Baseline characteristics of the analyzed patients are shown in Table 1 and reflect characteristics common to a population with recent-onset JIA. The majority of participants were female and most had oligoarticular JIA. Disease activity at baseline was moderate-to-high according to the JADAS-71 (19,31). Corroborating clinical observations, the age at disease onset was higher in Dutch patients when compared to Italian patients.

The baseline samples were matched with 107 samples from healthy children (Table 1). Furthermore, 44 patients contributed at least 1 sample obtained during inactive disease (39 Italian and 5 Dutch) (see Supplementary Table 1, available on the Arthritis & Rheumatology web site at http://onlinelibrary.wiley.com/doi/10.1002/art.40827/abstract). These samples were collected at a median time of 1 year after diagnosis (range 0.3–2.0 years).

Persistent activity or flare samples were collected from 25 patients (24 Italian and 1 Dutch) (see Supplementary Table 2, available at http://onlinelibrary.wiley.com/doi/10.1002/art.40827/abstract) at a median of 0.5 years after diagnosis (range 0.1–1.1 years). After data filtering, a total of 998,583 sequence reads of 16S ribosomal RNA gene amplicons were obtained with an average of 3,210 reads per sample and an average length of 487 basepairs, calculated after primer removal. In all samples collectively, 321 different OTUs were discovered. After removal of those OTUs present in <20% of the samples, 57 remained for analysis (see Supplementary Table 3, available at http://onlinelibrary.wiley.com/doi/10.1002/art.40827/abstract). All raw 454 sequencing reads and the associated metadata are available at NCBI: Bioprojects: PRJNA379123 and PRJNA280490 (http://www.ncbi.nlm.nih.gov/bioproject/?term=).

Baseline samples. With age, sex, and geography as covariates, linear regression models showed strong evidence for a reduced richness, as indicated by a lower Chao1 index score, in baseline samples from Italian patients as compared to healthy controls (β = −158 [95% confidence interval (95% CI) −219, −97], P < 0.001). There was weak evidence that Dutch baseline samples had an increased richness (β = 76 [95% CI 8, 145], P = 0.03). In both the Italian and Dutch groups, the results remained the same regardless of whether age was introduced as a continuous variable or a categorical variable. Moreover, the results did not change when administration of antibiotics, prebiotics, or probiotics, or the presence of gastrointestinal disturbances were adjusted for as potential confounders simultaneously. Dutch female controls showed lower richness than the other control groups (Figure 1A). There was no evidence of differences in the Shannon index scores. β-diversity differed between baseline samples from Italian patients and healthy controls (P = 0.001) (Figure 1B), as well as between baseline samples from Dutch patients and healthy controls (P = 0.013) (Figure 1C).

The random forest classification algorithm separated Italian patients at baseline from healthy controls almost perfectly, with receiver operating characteristic (ROC) curve analysis showing the area under the curve (AUC) as >0.99 in both training and test data. This was mainly due to the OTUs *Allobaculum*, *Erysipelotrichaceae*, *Propionibacterium acnes*, and *Barnesiellaceae*.

In the comparison of Italian patient samples at baseline and healthy controls, *Erysipelotrichaceae*, *Faecalibacterium prausnitzii*, *Parabacteroides*, *Enterococcus*, and *Ruminococcaceae* were increased, whereas *Allobaculum*, *Gemellaceae*, *P. acnes*, and *Turicibacter* were less abundant, in patients compared to healthy controls (FDR-corrected P < 0.05). Table 2 shows OTUs with crude P values of <0.05 (see Supplementary Table 4 for full list, available at http://onlinelibrary.wiley.com/doi/10.1002/art.40827/abstract). Figure 2 shows the relative abundances of the 4 OTUs with the strongest evidence of differences between patients and healthy controls.

Baseline samples from Dutch patients were separated well from healthy control samples by the random forest algorithm in the training set (AUC ROC >0.99). The most important OTUs for this separation were *Eggerthella lenta*, *Rikenellaceae*, *Coprobacillus*, and *Mogibacteriaceae*. In the test data set (n = 14), the AUC ROC was 0.71 (95% CI 0.41, >0.99). Various OTUs showed

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Figure 1. Plots of $\alpha$- and $\beta$-diversity. A, Box plots of the Chao1 index of microbial diversity ($\alpha$-diversity) in samples from Italian subjects (n = 58 male and 99 female) and Dutch subjects (n = 13 male and 36 female). Distributions of median indices in healthy controls, baseline samples, inactive disease (ID), and persistent activity (PA) are shown. Linear regression models of the Chao1 index with age, sex, and geography as covariates show evidence of reduced richness in baseline samples, inactive disease, and persistent activity as compared to healthy controls, except for samples from Dutch patients at baseline. Data are shown as box plots, where lines inside the boxes represent the median. Each box represents the 25th to 75th percentiles. Lines outside the boxes show the highest and lowest observations still within 1.5 times the interquartile range removed from the upper and lower quartile, respectively. Circles indicate outliers. B and C, Principal coordinates analysis (PCoA) of measures of $\beta$-diversity, calculated by unweighted UniFrac in samples from Italian subjects (n = 157) (B) and Dutch subjects (n = 49) (C). Patients at baseline and controls (left), and baseline, inactive disease, persistent activity, and healthy controls (right) are shown. Permutational multivariate analysis of variance shows evidence of pairwise differences between all subgroups, except for persistent activity versus inactive disease samples. PC = principal component.
a difference in relative abundance in logistic regression models, but no evidence of differences was maintained after FDR correction (Figure 3) (also see Supplementary Table 5, available at http://onlinelibrary.wiley.com/doi/10.1002/art.40827/abstract).

**Inactive disease samples.** There was strong evidence of reduced richness, as shown by a lower Chao1 index score, in samples from patients with inactive disease versus healthy controls using a linear regression model with age, sex, and geography.

**Figure 2.** Relative abundance of the 4 operational taxonomic units with the strongest evidence of differences between Italian patient baseline samples (n = 78) and healthy controls (n = 79). The age of the subjects confounded the relationship between patient/control status and the relative abundance. Therefore, the plots were divided into patients older (top) and younger (bottom) than the median age of 6 years. The relative abundances are shown, and the analysis was performed on the log ratio of the relative abundances. The broken lines show the limit of detection at a relative abundance of $4.5 \times 10^{-5}$. Relative abundances below the broken line were not observed, but were the results of imputation. Note the log_{10} transformation of the y-axis. Data are shown as box plots, where the lines inside the boxes represent the median. Each box represent the 25th to 75th percentiles. Lines outside the boxes show the highest and lowest observations still within 1.5 times the interquartile range removed from the upper and lower quartile, respectively. Circles indicate outliers.
Figure 3. Relative abundance of the 4 operational taxonomic units with the strongest evidence of differences between Dutch patient baseline samples (n = 21) and healthy controls (n = 28). The age of the subjects confounded the relationship between patient/control status and the relative abundance. Therefore, the plots were divided into patients older (top) and younger (bottom) than the median age of 8 years. No evidence of differences was maintained after false discovery rate control by the Benjamini-Hochberg procedure. The relative abundances are shown, and the analysis was performed on the log ratio of the relative abundances. The broken lines show the limit of detection at a relative abundance of $4.5 \times 10^{-5}$. Relative abundances below the broken line were not observed, but were the results of imputation. Note the log$_{10}$ transformation of the y-axis. Data are shown as box plots, where the lines inside the boxes represent the median. Each box represents the 25th and 75th percentiles. Lines outside the boxes show the highest and lowest observations still within 1.5 times the interquartile range removed from the upper and lower quartile, respectively. Circles indicate outliers.

Figure 4. Comparison of the differences in relative abundance in Italian patients between baseline patient samples and healthy controls (black), inactive disease (ID) samples and healthy controls (dark gray), and persistent activity samples and healthy controls (light gray). Values are the regression coefficients (odds ratio [OR]) and false coverage rate–corrected confidence intervals for the depicted operational taxonomic units (OTUs), obtained in a separate multivariable logistic regression analysis for each OTU, with age and sex as covariates and patient/control status as dependent variable. Only OTUs with an adjusted \( P \) value of <0.05 for at least 1 of the baseline, the inactive disease, or the persistent activity analyses are shown. Regression coefficients and confidence intervals to the left of the vertical broken line (at \( x = 1 \)) denote decreased relative abundance in patients; values to the right of the broken line denote increased relative abundance in patients.
as covariates ($\beta = 141$ [95% CI $-202$ to $-81$], $P < 0.001$) (Figure 1A). There was no evidence of differences in the Shannon index score. The analysis of $\beta$-diversity showed evidence of differences between samples from patients with inactive disease and healthy controls ($P = 0.001$), as well as between samples from patients with inactive disease and baseline samples ($P = 0.035$) (Figure 1).

Various OTUs in Italian patient inactive disease samples were differently abundant compared to control samples (see Supplementary Table 6, available at http://onlinelibrary.wiley.com/doi/10.1002/art.40827/abstract). Figure 4 shows the regression coefficients and false coverage rate–corrected CIs in comparison with those obtained in the analysis of baseline samples. Erysipelotrichaceae and F. prausnitzii were increased in both baseline and inactive disease samples compared to healthy controls, whereas Allobaculum and P. acnes were decreased. Enterococcus, Gemellaceae, Parabacteroides, Ruminococcaceae, and Turicibacter showed differences at baseline, but not in inactive disease, whereas Bacteroides caccae, Barnesiellaceae, and Dorea showed differences in inactive disease samples, but not at baseline. A paired analysis of selected OTUs was performed in 24 Italian patients who contributed a sample at baseline and a follow-up sample during inactive disease, showing no differences between baseline and inactive disease (see Supplementary Figure 2, available at http://onlinelibrary.wiley.com/doi/10.1002/art.40827/abstract). However, $\alpha$-diversity was lower in inactive disease in this subset of patients ($P = 0.007$ by Wilcoxon’s signed rank test) (see Supplementary Figure 3, available at http://onlinelibrary.wiley.com/doi/10.1002/art.40827/abstract). No evidence for differences between samples from Dutch patients with inactive disease and healthy controls was found (see Supplementary Table 7, available at http://onlinelibrary.wiley.com/doi/10.1002/art.40827/abstract).

**Persistent disease activity samples.** Analysis of $\alpha$-diversity showed decreased richness in samples from patients with persistent disease activity compared to healthy controls according to the Chao1 index ($P = 0.004$) (Figure 1). There was no evidence of differences according to the Shannon index. Analysis of $\beta$-diversity showed evidence of differences between samples from patients with persistent activity and healthy controls ($P = 0.001$). There was weak evidence of differences between samples from patients with persistent activity and baseline samples ($P = 0.02$), and very weak evidence of differences between samples from patients with persistent activity and samples from those with inactive disease ($P = 0.07$) (Figure 1).

Results of the logistic regressions comparing samples from Italian patients with persistent activity and samples from healthy controls are reported in Supplementary Table 8 (available at http://onlinelibrary.wiley.com/doi/10.1002/art.40827/abstract). Evidence of differences was found for Allobaculum and Erysipelotrichaceae only. Figure 4 compares the results with those obtained in the baseline and inactive disease analyses.

**Clinical features.** Principal components regression showed strong evidence that the microbiota composition differed with age (association with the first and fifth principal components [$P < 0.001$ for both]) (see Supplementary Table 9, available at http://onlinelibrary.wiley.com/doi/10.1002/art.40827/abstract). A weak association was found with the presence of uveitis at baseline. Differently abundant OTUs were Blautia ($P = 0.003$), Coriobacteriaceae ($P = 0.009$), Coprococcus ($P = 0.03$), and Lachnospiraceae ($P = 0.04$) (all $P$ values unadjusted; no differences remained after FDR correction). However, only 7 patients had uveitis at baseline. No or weak associations were found with sex, JIA subtype, physician’s global assessment of disease activity, number of active joints, ANA positivity, use of NSAIDs, use of prebiotics, or prior gastrointestinal infections (Table 1 shows gastrointestinal disorders in the cohort) (also see Supplementary Table 9, available at http://onlinelibrary.wiley.com/doi/10.1002/art.40827/abstract).

**Sensitivity analyses.** The administration of antibiotics or probiotics in the month prior to sample collection was associated with the gut microbiota composition (see Supplementary Table 9). Therefore, the abovementioned analysis of Italian JIA patients at baseline was repeated after the exclusion of subjects who had taken either antibiotics or probiotics (sensitivity analysis A), and showed no substantial differences. Likewise, a sensitivity analysis was performed after excluding patients with a gastrointestinal disorder (sensitivity analysis B), and showed no substantial differences. To explore potential confounding even further, we selected all samples within the same age range from among Italian patients at baseline and healthy controls (age range 2–15 years), simultaneously excluding those who took antibiotics, prebiotics or probiotics, or who experienced gastrointestinal disorders (sensitivity analysis C), and this analysis showed no substantial differences (see Supplementary Tables 10–13, available at http://onlinelibrary.wiley.com/doi/10.1002/art.40827/abstract). A further analysis was performed calculating log ratios using simple multiplicative replacement of zeros in the data (29) instead of robust imputation, without major differences (data not shown). Finally, for completeness, a comparison of all OTUs (including those present in $<20\%$ of samples) between Italian patients at baseline and healthy controls was performed; the results are reported in Supplementary Table 3 (available at http://onlinelibrary.wiley.com/doi/10.1002/art.40827/abstract).

**DISCUSSION**

This is the first study of microbiota composition in a prospective inception cohort of JIA patients, taking into account JIA disease activity state (12,14,15). The results of our study...
support the hypothesis that the composition of microbiota is different in JIA patients compared to healthy children. In our study, evidence was found that microbiota richness, in terms of rare OTUs as measured by the Chao1 index, was reduced in JIA patients compared to healthy controls, at baseline, during inactive disease, and during persistent disease activity, especially in Italian subjects. Dutch female patients showed an increased richness compared to Dutch female healthy controls, who had a decreased richness compared to all other control groups (Figure 1A). Measures of β-diversity were different in all subgroups of patient samples compared to healthy controls, demonstrating the variability of microbial communities between groups. Differences in gut microbiota composition were observed between JIA patients at baseline and healthy children, both when classifying samples using the random forest algorithm (AUCs in test data >0.99 for Italian samples and 0.71 for Dutch samples, despite the Dutch samples not reaching traditional levels of significance in test data) and when analyzing the OTUs individually. Most notably, Erysipelotrichaceae and F prausnitzii were increased, while Allobaculum was decreased, in samples from Italian patients (Table 2 and Figure 4). Dutch patient samples were dissimilar to Italian patient samples, confirming the prior belief that the gut microbiota composition differs between these populations, due potentially to diet and other environmental factors. However, it could not be ruled out that the observed differences were due to different sample collection procedures used in the Italian and Dutch cohorts.

Few OTUs were differently abundant between controls and patients across all disease activity states. Nonetheless, when comparing baseline samples with paired inactive disease samples, no differences in relative abundance were found (Supplementary Figure 2, available at http://onlinelibrary.wiley.com/doi/10.1002/art.40827/abstract). This finding may indicate that the gut microbiota profile is specific to the individuals with JIA, rather than disease activity status. However, it should be taken into account that JIA is very heterogeneous clinically, and different diseases may be underlying the clinical symptoms. Therefore, this conclusion is still preliminary. Finally, the results also indicated a strong relationship of the microbiota composition with age, as found previously (32,33).

In light of these results, we ask whether the microbiota composition is involved in the pathogenesis of JIA. Observations compatible with a pathogenic role of microbiota include a reduced number of Th17- and FoxP3-positive regulatory T cells in germ-free mice (1,2,10,11). Administration of stools from healthy mice to these germ-free mice led to a reconstitution of Th17 numbers (34), suggesting that microbiota influence the development of the immune system. Conversely, inoculation of fecal samples from rheumatoid arthritis patients into mice led to an increase of Th17 cells and development of severe arthritis (3). These cell populations are of critical importance in immune homeostasis and play a key role in the pathogenesis of autoimmune diseases, including JIA (2,35). Additionally, in a cohort of patients with the enthesitis-related arthritis (ERA) subtype of JIA compared to healthy controls, differences in community structure were predicted to decrease metabolic functions. In the same cohort, a lower diversity of ions and underrepresentation of the tryptophan metabolic pathway was observed in patients (36). Tryptophan metabolites can lead to the production of proinflammatory cytokines, thus potentially linking the gut microbiota with inflammation (36). In another cohort of ERA patients, predicted functions of the gut microbiota were related to cell motility and chemotaxis (12). Furthermore, the risk of developing autoimmune disorders was increased following the use of antibiotics in early childhood. This risk was dependent on the number, dose, and timing of the antibiotic prescriptions (37,38). Our finding of a weak association with uveitis is consistent with the previous finding in mouse models that activation of retina-specific T cells was dependent on gut commensal microbiota (39).

Fecal microbiota transplantation has led to promising, though varying, results for inflammatory bowel disorders (40–43), and an exploratory analysis in JIA patients showed that exclusive enteral feeding had an antiinflammatory effect (44). Finally, our finding of reduced diversity in inactive disease samples compared to baseline samples (see Supplementary Figure 3, available at http://onlinelibrary.wiley.com/doi/10.1002/art.40827/abstract) supports the idea that reduced microbiota diversity is not a consequence of inflammation. Nevertheless, these are preliminary findings, and a causal pathway has yet to be demonstrated.

The OTUs Erysipelotrichaceae, F prausnitzii, and Allobaculum have been associated previously with human disease. Erysipelotrichaceae appears to be highly immunogenic (45,46) and can potentially overgrow in the gut after treatment with broad-spectrum antibiotics (47). F prausnitzii was identified to have antiinflammatory properties, and a decreased abundance of this microorganism in the gut has been associated with Crohn's disease (48) and JIA (12,14). Yet in our study it was increased in Italian patients at baseline. However, previous studies examined ERA only, whereas our cohort contained a majority of patients with oligoarticular and polyarticular disease. Furthermore, previous studies did not use compositional data analysis when comparing OTUs between groups. Finally, the balance among different subspecies of F prausnitzii can result in changes in short-chain fatty acid production; therefore, a fine-level characterization of F prausnitzii strains is important to better understand their real function in the microbiome (49). Such a fine-level characterization was not available in our study. Another possibility is that F prausnitzii was increased as an effect, rather than a cause, of inflammation. Allobaculum is associated with obesity. Several studies have demonstrated that its abundance was lower in the gut of obese mice (50,51) and increased in weight-reduced mice (52). Furthermore,
Allobaculum was positively correlated with plasma high-density lipoprotein concentrations in hamsters (53) and negatively correlated with leptin concentration (52).

Our study has several strengths. We studied a large cohort of treatment-naive JIA patients compared to healthy controls. Patients were followed up longitudinally, allowing for the comparison of microbiota between various disease activity states. Finally, we used compositional data analysis to assess data expressed as proportions of a whole, such as microbiota relative abundances (27,28). Failure to do so can lead to an increased FDR, up to 68% (17).

Despite this being the largest study of gut microbiota composition in JIA patients, one limitation is the relatively small number of Dutch patient inactive disease and persistent disease activity samples, as well as the number of paired samples, which re-emphasizes the need for replication in future cohorts. Moreover, age and potentially sex should be matched more closely between patients and controls to allow for a better comparison. Finally, future studies should examine the mechanistic implications of gut dysbiosis in JIA.

In conclusion, our results support the hypothesis of gut dysbiosis in JIA patients in terms of richness and compositional deviation from healthy subjects. Clearly, future investigations should be aimed at replicating these results in other populations, elucidating the potential causal role of gut microbiota in the pathogenesis of JIA, and investigating the interaction among genetics, microbiota, and environmental factors in the development of JIA.

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

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