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Exploratory Findings From the FLORA Trial

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Small Intestinal Permeability and Metabolomic Profiles in Feces and Plasma Associate With Clinical Response in Patients With Active Psoriatic Arthritis Participating in a Fecal Microbiota Transplantation Trial: Exploratory Findings From the FLORA Trial

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Objective. We investigated intestinal permeability and fecal, plasma, and urine metabolomic profiles in methotrexate-treated active psoriatic arthritis (PsA) and how this related to clinical response following one sham or fecal microbiota transplantation (FMT).

Methods. This exploratory study is based on the FLORA trial cohort, in which 31 patients with moderate-to-high peripheral PsA disease activity, despite at least 3 months of methotrexate-treatment, were included in a 26-week, double-blind, 1:1 randomized, sham-controlled trial. Participants were randomly allocated to receive either one healthy donor FMT (n = 15) or sham (n = 16) via gastroscopy. The primary trial end point was the proportion of treatment failures through 26 weeks. We performed a lactulose-to-mannitol ratio (LMR) test at baseline (n = 31) and at week 26 (n = 26) to assess small intestinal permeability. Metabolomic profiles in fecal, plasma, and urine samples collected at baseline, weeks 4, 12, and 26 were measured using ^{1}H Nuclear Magnetic Resonance.

Results. Trial failures (n = 7) had significantly higher LMR compared with responders (n = 19) at week 26 (0.027 [0.017-0.33]) vs. 0.012 [0-0.064], $P = 0.013$), indicating increased intestinal permeability. Multivariate analysis revealed a significant model for responders (n = 19) versus failures (n = 12) at all time points based on their fecal ($P < 0.0001$) and plasma ($P = 0.005$) metabolomic profiles, whereas urine metabolomic profiles did not differ between groups ($P = 1$). Fecal N-acetyl glycoprotein GlycA correlated with Health Assessment Questionnaire Disability Index (coefficient = 0.50; $P = 0.03$) and fecal propionate correlated with American College of Rheumatology 20 response at week 26 (coefficient = 27, $P = 0.02$).

Conclusion. Intestinal permeability and fecal and plasma metabolomic profiles of patients with PsA were associated with the primary clinical trial end point, failure versus responder.

INTRODUCTION

Encouraged by the field of pharmacomicrobiomics, proposing how gut permeability (1,2) and microbial enzymatic products may affect the bioavailability, clinical efficacy, and toxicity of disease-modifying antirheumatic drugs (DMARDs), the clinical prospects of microbiota-based therapies (including fecal microbiota transplantation [FMT]) in disease management of immune-

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[Correction added on 28 September 2023, after first online publication: The surname of the fourth author has been corrected to Serrano-Contreras in this version.]

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mediated arthritis have attracted attention within recent years (3). Some early, yet consistent, evidence has coupled abnormalities within the intestinal microbial environment with psoriatic arthritis (PsA) (4). Compared with healthy individuals, patients with PsA have decreased stool bacterial diversity (5) with a distinct bacterial compositional signature (6) and subclinical gut inflammation characterized by increased expression of antimicrobial peptides and up-regulation of interleukin-17 (IL-17), IL-22, and IL-9 (7). However, at present, the underlying mechanisms linking these structural and compositional changes with PsA pathogenesis and its functional implications for disease severity and response to therapy remain to be investigated (8).

Microbiota-derived metabolites and cometabolites, such as short chain fatty acids (SCFAs), amino acids (AAs), and secondary bile acids, are important players in the complex host-microbiota crosstalk serving as both products and reactants to myriad reactions. In the context of the gut-joint axis, these metabolites are capable of modulating gut barrier integrity and function and exert direct immunomodulatory activities on immune cell subsets (9). Microbial antigens and metabolites may also be transported across the gut barrier in an uncontrolled fashion (10), especially if there is an alteration in intestinal permeability due to local inflammation (a "leaky" gut) (2), and act on distant tissues and organs (11).

Previous studies have demonstrated distinct fecal (12) and serum metabolomic signatures in PsA compared with both rheumatoid arthritis (RA) and healthy individuals (13,14), which correlated to specific cytokines and clinical features. Hence, assessment of intestinal permeability and metabolic profiling of biofluids has an emergent role in exploring the contribution of host-microbiota interactions to the pathogenesis and treatment response of immune-mediated arthritis (15). The lactulose-to-mannitol ratio (LMR) assay is an established test for evaluating gut barrier integrity, whereas ^{1}H nuclear magnetic resonance (NMR) is a high-throughput, reproducible tool for "global metabolomic profiling," assessing a broad range of host and microbial metabolites across a range of biofluids.

We have previously reported the clinical results of the 26-week, double-blind, randomized FLORA trial exploring if one FMT, in which gut microbiota donated from healthy screened individuals is transferred to the recipient's intestine, could be a favorable treatment supplement in methotrexate (MTX) nonresponder patients with active peripheral PsA (16). In this trial, a sufficient reduction in disease severity was observed in 40% of FMT-treated patients for 26 weeks, whereas this was the case in 80% of patients receiving sham (saline) transplantation. Exploring changes in the metabolism of the intestinal milieu associated with beneficial responses may therefore help guide future interventional studies within this field. Thus, we sought to investigate how functional permeability of the small intestine in addition to fecal, plasma, and urine metabolomic profiles related to the primary and key secondary outcomes of the FLORA trial.

PATIENTS AND METHODS

Study setting and participants. Thirty-one patients with PsA with moderate-to-high peripheral disease activity, despite ongoing MTX treatment, were included in this 26-week, double-blind, parallel-group, 1:1 randomized, sham-controlled trial (FLORA; NCT03058900) (16). The study was approved by the Regional Committees on Health Research Ethics for Southern Denmark (DK-S-20150080) and the Danish Data Protection Agency (15/41684). Patients fulfilled the Classification for Psoriatic Arthritis (CASPAR) criteria and had received treatment with steady state dose MTX (monotherapy) administered as subcutaneous injections (n = 25) or oral tablets (n = 6) for at least 3 months prior to trial inclusion (16). They all continued with this treatment throughout the trial. We asked patients not to take nonsteroidal antiinflammatory drugs (NSAIDs) while participating in the trial. Patients were randomly allocated to receive either one gastroscopic-guided single-donor FMT or sham transplantation (brown-colored saline). The fecal donations came from four healthy and thoroughly screened, unrelated, lean individuals (two men and two women with an age between 26 and 54 years) (16). Each FMT product consisted of 50 g processed feces from one of the donors, prepared using standard Danish protocols (16). Products were stored at –80°C and thawed to 36°C just before clinical application.

The primary efficacy end point of the trial was the proportion of treatment failures through 26 weeks, defined as need for treatment escalation because of insufficient improvement of current

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Drs. Marchesi and Ellingsen contributed equally to this work.

Metabolite data from the ^{1}H nuclear magnetic resonance analysis will be uploaded to an online repository and will be available upon reasonable request after publication of the main results in a peer-reviewed journal. Requests on data sharing can be made by contacting Drs. Marchesi and Ellingsen. Data access and subsequent data handling must be in agreement with the European General Data Protection Regulation. Data will be shared after review and approval by the ethical and scientific board of the study. Terms of collaboration will be reached together with a signed data access agreement.

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symptoms or disease worsening (16). In the current report, this primary trial outcome will be referred to as trial failure versus responder. At time of enrollment, all patients were candidates for starting infusions with biological DMARDs according to national guidelines, resulting in the instigation of tumor necrosis factor inhibitors (TNFi) in all trial failure cases, but one, during the trial. Hence, five stool/blood/urine samples collected at week 12, and seven stool/blood/urine samples collected at week 26 came from patients who had started treatment with TNFi. The three key secondary end points of the trial were evaluated at week 26: Health Assessment Questionnaire Disability Index (HAQ-DI) score, the American College of Rheumatology (ACR) 20 response, and the Spondyloarthritis Research Consortium of Canada (SPARCC) Enthesitis Index (16).

Trial sample collection, storage, and shipping. We collected biological specimens at baseline, week 4, week 12, and week 26. Further details on patient sample collection, storage, and shipping are provided in the Supplementary Methods.

Sample preparation for ^{1}H NMR spectroscopy. Sample preparation protocols are described in the Supplementary Methods.

^{1}H NMR analysis. Fecal, urine, and plasma metabolomic profiles were measured on a Bruker 600 MHz spectrometer. Plasma samples were measured at a constant temperature of 310 K, and fecal and urine samples were measured at 300 K. Standard one-dimensional Nuclear Overhauser Effect Spectroscopy (1D) ^{1}H NMR experiment with water suppression was performed for each sample using the following NMR pulse sequence: relaxation delay (RD)-90°-t_1-90°-tm-90°-acquisition where t_1 (short delay between the two 90° pulses) = 4 μs, t_m (mixing time) = 100 μs, and 90°pulse = ∼10 μs. For each experiment, a total of 32 scans were obtained. Carr – Purcell – Meiboom – Gill (CPMG) spin-echo sequence was used to edit peak intensity based on NMR relaxation times in plasma and serum to resolve broad peaks produce because of the presence of lipoproteins and other macromolecules. Baseline correction, automatic phasing, and spectral calibration to trimethylsilylpropanoic acid (TSP) (0 parts per million [ppm]) were performed in Topspin 4.1.3 software. All the sample extractions, preparation, and experimentation were randomized.

Preprocessing of ^{1}H NMR spectra. The NMR raw spectra were imported into MATLAB software (R2014a), and in-house scripts were integrated to continue the preprocessing of the data. First, the obtained NMR raw spectra were phased and referenced as follows: plasma and fecal water spectra was referenced to 0 ppm using TSP signal. Baseline was corrected to produce 10 ppm spectra that were digitized into 20,000 data points with a resolution of 0.0005 ppm. Next, water, urea (just in urine samples), and TSP peaks were removed. The alignment of the spectra was resolved using the recursive segment-wise peak alignment algorithm. All NMR data sets were normalized by a probabilistic quotient normalization.

^{1}H NMR metabolite identification. Metabolites present signals at specific positions in the 1D 1H NMR frequency domain spectrum. The chemical shift, peak area integration, and multiplicities were used for structural metabolite identification. Selected metabolites were identified with Initially Statistical Total Correlation Spectroscopy using a previously published in-house MATLAB script in which a relative index was integrated from the area under the peak for each metabolite (17). To avoid peaks overlapping, the peak integration was carefully selected. Furthermore, variation in integral width and baseline between samples was also avoided. Annotations were confirmed using 2D J-resolved NMR spectra from a selected number of samples. Internal and external databases, such as the Human Metabolome Data Base, were used for confirmation of assignments.

LMR test. We performed the LMR test at baseline (n = 31) and at the final 26-week visit (n = 26) to assess the functional permeability of the small intestinal wall (higher LMR indicates higher permeability) (18). After an overnight fast, patients provided a urine sample before ingesting 100 mL water containing 10 g of lactulose (Pharmanovia A/S) and 5 g of D-mannitol (Fresenius Kabi). We collected all the urine passed in the subsequent 3 hours. No food or drinking (except for water) was allowed during the test. We measured LMR in urine using an LC-MS method developed on a Waters Acquity Ultra-Performance Liquid Chromatography system coupled to a high-resolution mass spectrometer Waters Xevo G2-S QTof (Waters Corp.). Chromatographic separation of urinary sugars was achieved using an ACQUITY UPLC BEH Amide 1.7 μm, 2.1 × 150 mm column (Waters Corporation) operating at 0.4 mL/min flow rate (45°C). Mobile phase A used was 10 mM ammonium acetate at pH 9.8 in water and mobile phase B was neat acetonitrile. More details are available in the Supplemental Methods.

Statistical analysis. Statistical analyses were conducted using the primary efficacy end point of the trial (responder vs. failure) as the primary clinical variable of this exploratory study. Because this was an exploratory study confined by the design of and number of patients included in the original clinical trial, no power calculation was performed. Processing and analysis were executed with in-house scripts (19) for MATLAB and SIMCA 17.0 software. Pareto scaling was used for the ^{1}H NMR data, which scales the data by dividing each variable by the square root

of the standard deviation. For the LMR data, we used StataSE-64 version 17 to perform the Wilcoxon rank sum and the matched-pairs signed-rank test. LMR data are presented as median (min, max).

The main study objective was to investigate if metabolomic profiles measured in 1) fecal samples, 2) plasma samples, and 3) urine samples were associated with the primary trial outcome, both for all patients regardless of the experimental treatment received, as well as for patients allocated to each intervention group (FMT vs. sham). Unsupervised principal component analysis (PCA) was used for dimensionality reduction and visualization of intrinsic variation between the sample's spectra. After the PCA analysis, we used a supervised multivariate orthogonal partial least squares-discriminant analysis (OPLS-DA) to model metabolic differences among groups. Orthogonal-Signal-Correction was used to filter the variations in the spectrometric data of the samples (predictor matrix or X) that are uncorrelated with the selected sample class (clinical data or Y). To assess the quality of the models, K-fold cross-validation was used. The results of the cross-validation were summarized by the value of R2 and Q2 parameters; the first one summarizes the goodness of the fit (R^2 = 1 would indicate perfect fit), and Q2 the predictability of the model (Q2 = 1 would indicate perfect predictability). A *P* value was estimated from the K-fold cross-validation, and default SIMCA software 7-fold cross-validation software was used. To obtain further validation of the model, a permutation test was used (1000 permutations) regarding a *P* value less than or equal to 0.05 as a valid model. For the significant models, we used p(corr) parameter, which represents the loadings of the model scaled as a correlation coefficient, thus standardizing the range from −1.0 to 1.0. p(corr) cutoff was set at 0.5.

The integrals of significant metabolites were statistically analyzed with Wilcoxon signed-rank test and Kruskal–Wallis one-way analysis of variance. The post hoc analysis after the Kruskal–Wallis test was Dunn's Multiple Comparison Test. *P* values were adjusted for multiple hypothesis testing corrections with Benjamini-Hochberg method for false discovery rate (FDR) calculation with an FDR cutoff set at 0.05. Aforementioned tests were performed with rstatix package for *R*. Visualizations were produced with ggplot2 and ComplexHeatmap packages.

Secondly, we wanted to investigate how FMT affected individual fecal metabolites. We used the 2-way analysis of variance (ANOVA) to study differences in the temporal dynamics of identified fecal metabolites between the FMT and sham group. Finally, we correlated each of the fecal metabolites with the three key secondary end points of the trial in all patients, as well as in FMT-treated patients. To assess these correlations, we used mixed effect models. These models were fitted with lme4 R package. The model was adjusted for sex, age, and body mass index, and the subject ID was introduced as a random effect to account for the longitudinal nature of the data. *P* values were adjusted with FDR using the Benjamini-Hochberg correction, and the cutoff was set at 0.10.

RESULTS

Patient characteristics. Thirty-one patients were included in the trial. A summary of the key baseline demographics and disease characteristics is presented in Table 1. Detailed demographics have been published elsewhere (16).

Increased small intestinal permeability in the failure group. We observed no significant difference in the LMR between the four healthy FMT donors and the 31 patients at baseline (0.0065 [0-0.063] vs. 0.014 [0-0.28]; *P* = 0.50). The LMR increased significantly from baseline to week 26 in the 16 sham-treated patients (0.0046 [–0.012 to 0.088]; *P* = 0.032). This difference was not the case in the ten FMT-treated patients (0.0020 [–0.27 to 0.32]; *P* = 0.92). However, we did not observe any significant differences in LMR at week 26 between FMT-treated (n = 10) versus sham-treated patients (n = 16) (Figure 1A). Interestingly, failures (n = 7) had a significantly higher LMR compared with responders (n = 19) at week 26 (0.027 [0.017-0.33] vs. 0.012 [0-0.064]; *P* = 0.013), indicating a higher small intestinal permeability in the failure group (Figure 1B).

Healthy donor metabolomic profiles differ markedly from those of patients with PsA. We aimed to assess the degree to which the overall global metabolomic profile of different biofluids differed between healthy stool donors and patients with PsA. In the baseline samples collected from the 31 patients with PsA and the four FMT donors, we were able to quantify 29 fecal metabolites (Figure 2A), 35 plasma metabolites (Figure 2B), and 43 urine metabolites (Supplementary Table 1). The multivariate OPLS-DA analysis of all metabolites generated a model, which could significantly differentiate metabolomic profiles of FMT donations from the patients' fecal samples at baseline (OPLS-DA model; R2 = 0.87, Q2 = 0.565; *P* < 0.0001). While butyrate (*r* = 0.56) and valine (*r* = 0.51) correlated with donor samples, glutamate (*r* = –0.64), tyrosine (*r* = –0.63), alanine (*r* = –0.62), and tryptophan (*r* = –0.53) were associated with patient samples. We did not observe any differences in baseline fecal metabolomic profiles (OPLS-DA model; R2 = 0.196, Q2 = –0.444, *P* = 1), plasma profiles (OPLS-DA model; R2 = 0.174, Q2 = –0.452, *P* = 1) nor urine profiles (OPLS-DA model; R2 = 0.182, Q2 = –0.140, *P* = 1) between patients allocated to FMT versus sham transplantation.

Table 1. Baseline demographics and disease characteristics

Characteristic	FMT (n = 15)	Sham (n = 16)	Total (n = 31)
Female sex, n (%)	8 (53)	12 (75)	20 (65)
Age, y	48.9 (16.1)	52.4 (11.0)	50.7 (13.6)
Height, cm	175.2 (7.0)	169.8 (8.6)	172.4 (8.2)
Weight, kg	93.6 (15.4)	92.4 (24.8)	93.0 (20.5)
Time since diagnosis, y[a]	2.6 (0.3-5.8)	5.6 (0.5-8.8)	3.7 (0.5-8.3)
Rheumatoid factor IgM negative, n (%)[b]	13 (93)	15 (94)	28 (93)
Anticitrullinated peptide antibody negative, n (%)[b]	14 (100)	16 (100)	30 (100)
HLA-B27 negative, n (%)	15 (100)	13 (81)	28 (90)
C-reactive protein, mg/L	4.98 (7.18)	5.54 (5.87)	5.27 (6.43)
HAQ-DI[c]	0.89 (0.51)	0.78 (0.50)	0.83 (0.50)
Swollen joint 66 count	7.5 (3.0)	6.7 (2.7)	7.1 (2.8)
Tender joint 68 count	14.9 (8.9)	17.3 (8.8)	16.1 (8.8)
SPARCC enthesitis index[d]			
Score ≥1, n (%)	13 (87)	15 (94)	28 (90)
Score in patients with a score ≥1	8.1 (4.3)	7.2 (3.3)	7.6 (3.8)
Antibiotics within one year of inclusion, no. (%)	4 (27)	6 (38)	10 (32)
Smoking status, n (%)			
Current	5 (33)	4 (25)	9 (29)
Previous	4 (27)	8 (50)	12 (39)
Never	6 (40)	4 (25)	10 (32)
Alcohol consumption, units per week	0.9 (0.9)	0.8 (0.6)	0.8 (0.7)

Data are mean (SD) or n (%) unless otherwise stated.
Abbreviations: DMARD, disease-modifying antirheumatic drug; FMT, fecal microbiota transplantation; HAQ-DI, Health Assessment Questionnaire Disability Index; HLA-B27, human leukocyte antigens B27; IgM, immunoglobulin M; SPARCC, Spondyloarthritis Research Consortium of Canada.
[a]Time since diagnosis of psoriatic arthritis is presented as median and interquartile range.
[b]Presence of rheumatoid factor (IgM) and anticitrullinated peptide antibody was not accessed in one patient from the FMT group.
[c]Scores on the HAQ-DI range from 0 to 3, with higher scores indicating greater disability.
[d]SPARCC Enthesitis Index range from 0 to 16, with higher scores indicating more severe disease.

Fecal metabolomic profiles differentiate between responders and failures. We observed a clear difference in the fecal metabolomic profiles between trial responders (n = 19) and failures (n = 12) among all patients at all time points (R2 = 0.613, Q2 = 0.220, $P < 0.0001$). This OPLS-DA model and the correlations between metabolites and the primary trial

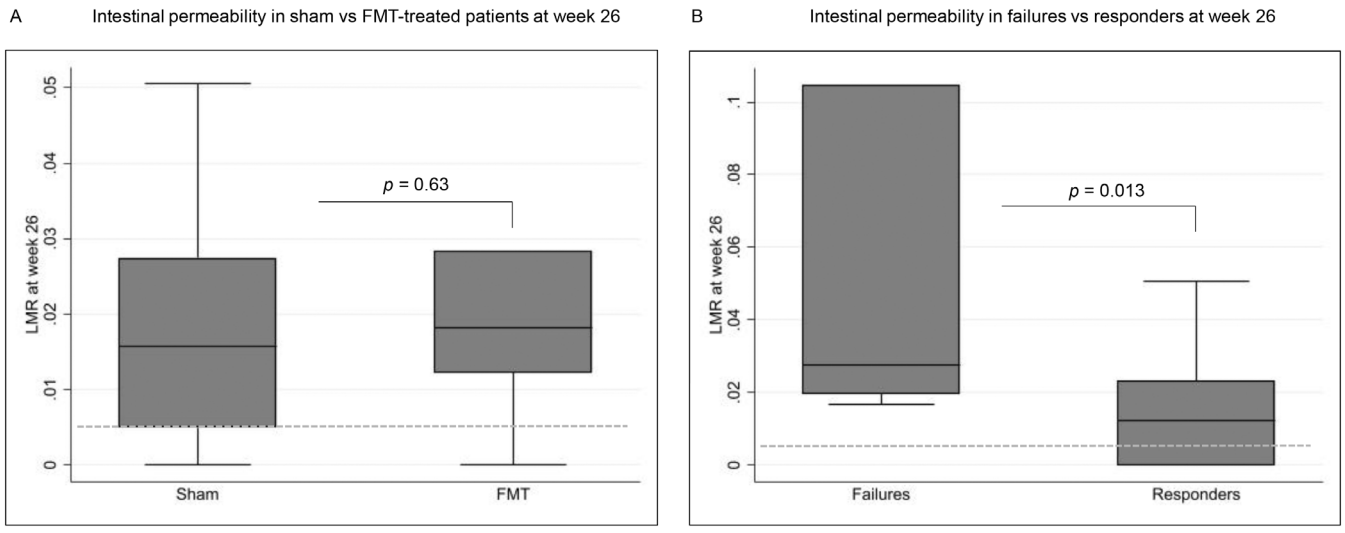

Figure 1. Small intestinal permeability evaluated by LMR test. **A,** LMR in sham-treated patients (n = 16) versus FMT-treated patients (n = 10) at week 26, and **(B)** LMR in trial failures (n = 7) versus responders (n = 19) at week 26. Higher LMR indicates higher small intestinal permeability. Median (black line) and IQR (box). Outside values were excluded in this figure but included in the statistical analysis. The dotted line represents the median LMR of the four healthy FMT donors (LMR = 0.0065). *P* values were calculated using the Wilcoxon rank sum test. FMT, fecal microbiota transplant. IQR, interquartile range; LMR, lactulose-to mannitol ratio.

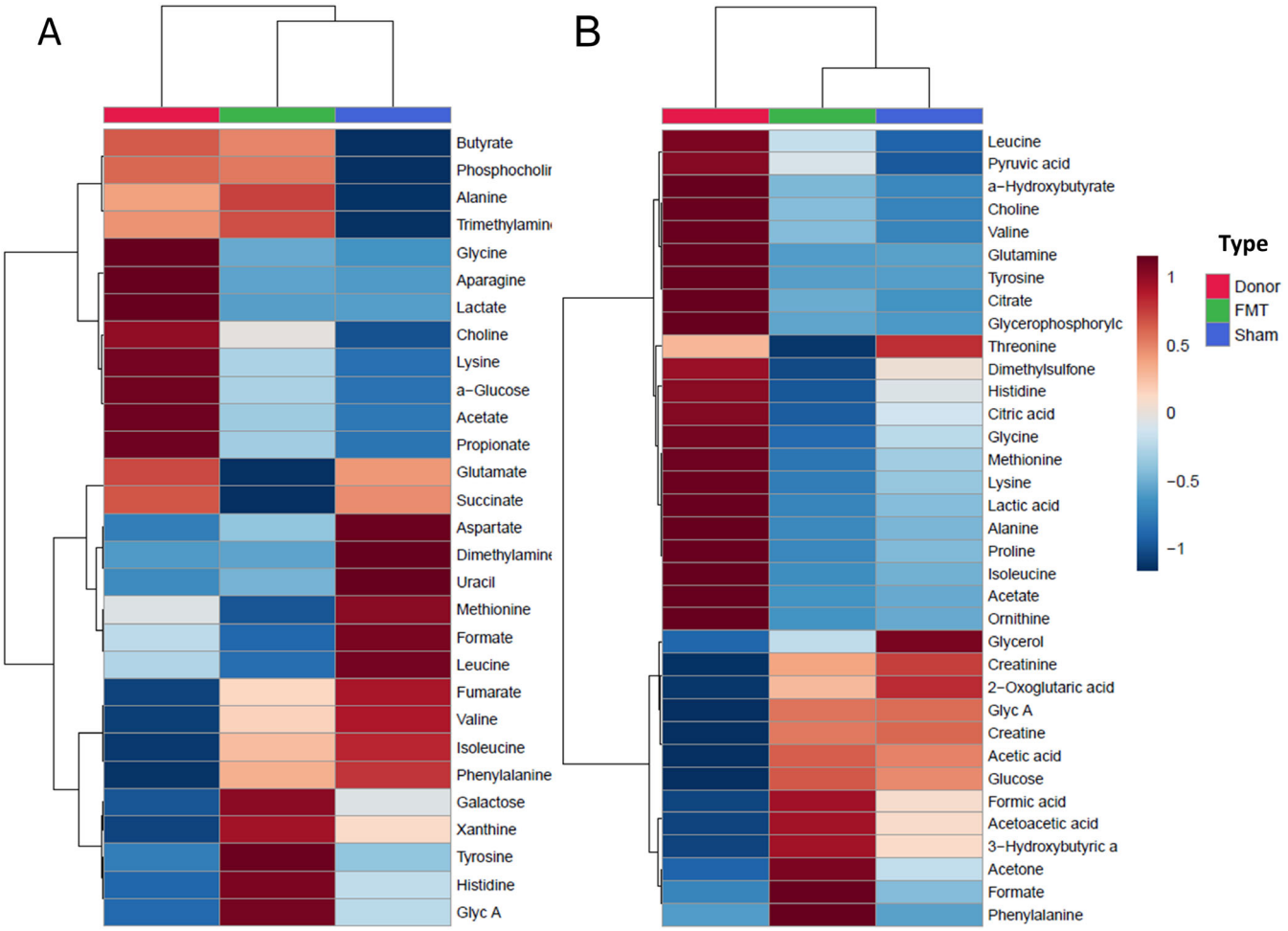

Figure 2. Baseline metabolomic patterns of donors and patients. Heatmap presenting the different metabolites quantified in baseline fecal (**A**) and plasma (**B**) samples from healthy donors (n = 4) and patients with psoriatic arthritis allocated to FMT (n = 15) and sham (n = 16). Data were scaled using the mean-centering method. Euclidean distance and Ward clustering method were used. Each colored cell on the map corresponds to a scaled concentration value. FMT, fecal microbiota transplantation.

outcome with a correlation coefficient (r) greater than or equal to 0.50 are presented in Figure 3A. Subgrouping patients by the received experimental treatment resulted in a strong model in sham-treated patients that was able to differentiate responders and failures based on their metabolomic profiles (R2 = 0.622, Q2 = 0.280, $P = 0.0008$), and a weaker one in FMT-treated patients (R2 = 0.211, Q2 = 149, $P = 0.082$). In the model of sham-treated patients, tyrosine ($r = 0.67$), leucine ($r = 0.67$), and phenylalanine ($r = 0.57$) correlated with failure. We did not assess metabolite correlations in the FMT model because this model was not statistically significant.

Defined plasma metabolites differ between responders and failures. The supervised multivariate OPLS-DA analysis of plasma metabolites revealed a significant model for trial responders (n = 19) versus failures (n = 12) among all patients at all time points (R2 = 0.514; Q2 = 0.121; $P = 0.005$). Correlations between each metabolite and the primary trial outcome with a correlation coefficient (r) greater than or equal to 0.50 are presented in Figure 3B. When patients were stratified for intervention type, the models continued to show significant separation (FMT arm: R2 = 0.766, Q2 = 0.383, $P < 0.0001$; sham arm: R2 = 0.790, Q2 = 0.260, $P = 0.002$). In both the FMT and sham models, plasma glucose ($r = 0.67$ and $r = 0.63$) correlated with failure, whereas lysine ($r = –0.83$ and $r = –0.65$) correlated with responder.

Urine metabolites. The supervised OPLS-DA analysis did not reveal any significant differences in metabolomic profiles of urine samples between responders (n = 19) and failures (n = 12) (R2 = 0.115, Q2 = –0.274, $P = 1$).

Increased fecal butyrate levels following FMT. When comparing effects of FMT versus sham upon levels of fecal metabolites, we observed significantly increased levels of fecal butyrate in the FMT arm ($P = 0.026$), which was most pronounced in the first weeks after the intervention (Figure 4). Additional results

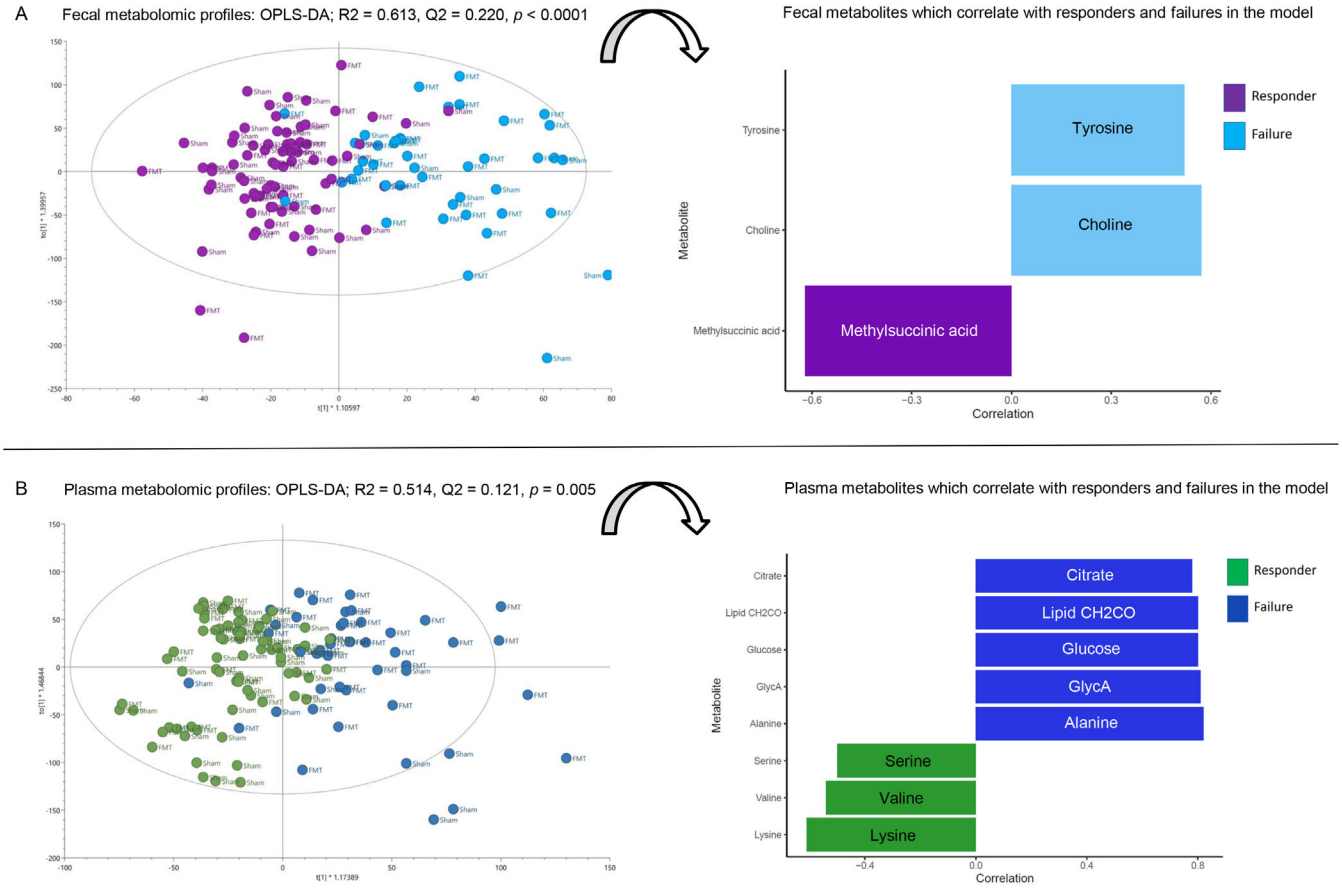

Figure 3. OPLS-DA models that significantly separate trial responders (n = 19) from failures (n = 12) based on their fecal (**A**) and plasma (**B**) metabolomic profiles. Plots include 1H-NMR profiles (each patient at each time point visualized by a single dot) for all patients at all time points (ie, each patient is represented by up to four dots). Number of predictive components = 1, number of orthogonal components = 4. *P* values calculated using CV-ANOVA. The box plots show the metabolites (with a correlation coefficient [*r*] ≥ 0.50), which are associated with being failure and responder in each of the two models. CV-ANOVA, analysis of variance of the cross-validated residuals; FMT, fecal microbiota transplantation; OPLS-DA, orthogonal partial least squares-discriminant analysis.

from the two-way ANOVA exploring differences in levels of individual metabolites across time between patients allocated to FMT and sham are presented in Supplementary Figure 2 in the Supplementary Results.

Specific fecal metabolites associate with key secondary arthritis disease activity end points. After adjusting for multiple testing, fecal N-acetyl glycoprotein GlycA (r = 0.50; P = 0.03) was positively associated with HAQ-DI for all patients (n = 31) at all time points. Another six fecal metabolites were significantly associated with being ACR20 responder in the FMT group at week 26 (n = 15) (Table 2). These significant associations with ACR20 were not observed in the sham group. No metabolites correlated significantly with the SPARCC enthesitis index at week 26.

Associations between intestinal permeability and fecal metabolites. Fecal hypoxanthine was positively associated with LMR for all patients at baseline (r = 0.00013,

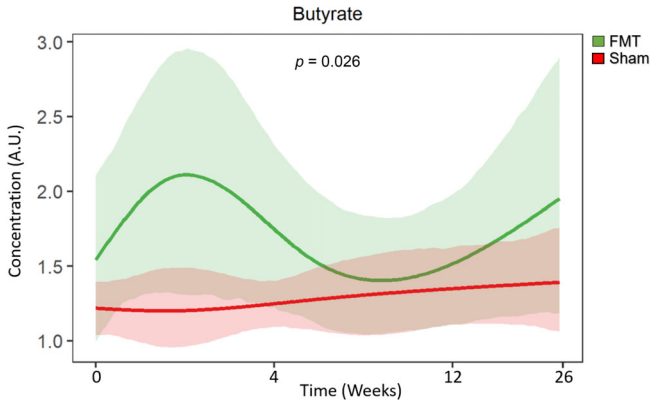

Figure 4. Differences in fecal butyrate levels after treatment with FMT and sham, respectively. Time trajectory plot was generated with short asynchronous time-series analysis (santa)R package. *P* value is obtained from the two-way ANOVA analysis across all time points (baseline, week 4, week 12, and week 26). ANOVA, analysis of variance; A.U., arbitrary units; FMT, fecal microbiota transplantation.

Table 2. Associations between fecal metabolites and being ACR20 responder in the FMT group at week 26

Metabolite	Coefficient (95% CI)	*P* value
Propionate	27 (4.7 to 49)	0.02
Leucine	0.54 (0.14 to 0.94)	0.01
Lysine	0.41 (0.17 to 0.64)	0.004
Alanine	0.24 (0.05 to 0.43)	0.02
Fumarate	−0.012 (−0.020 to −0.0036)	0.01
Histidine	−0.015 (−0.024 to −0.0059)	0.005

Only metabolites with a *P* value below 0.10 after adjusting for multiple testing using the Benjamini-Hochberg method are presented in the table.
Abbreviations: ACR20, American College of Rheumatology 20; CI, confidence interval; FMT, fecal microbiota transplantation.

$P < 0.0001$). However, this relation was not observed at week 26 ($r = -0.00022$, $P = 0.42$).

DISCUSSION

Being the principal site of interaction between gut microbes and the host immune system, the intestinal wall may hold the key to advance our understanding of the gut-joint axis (20). Results from several small studies have demonstrated increased intestinal permeability in patients with ankylosing spondylitis (2), and a recent study has provided evidence for altered colonic tight junction proteins and increased serum biomarkers of intestinal permeability in RA (1). A significant reduction in intestinal permeability has also been observed 6 weeks after FMT in patients with nonalcoholic fatty liver disease (21), which is a condition associated with psoriatic disease and leaky gut (22). Although our study did not reveal any significant changes in small intestinal permeability 26 weeks after FMT, our findings imply that increased permeability assessed by the LMR test may be associated with disease activity and/or treatment with TNFi in patients with PsA.

The supervised multivariate OPLS-DA analysis of plasma, as well as fecal metabolites, were a robust and significant model for discriminating responders versus failures among all patients at all time points. The fecal metabolite model revealed that tyrosine and choline correlated with the metabolomic profiles of patients in the failure group. The correlation between tyrosine and failure was also seen when only looking at the sham-treated patients. In addition, tyrosine was significantly associated with patients with PsA samples versus healthy donor samples. In line with this, we observed a slight decrease in tyrosine levels from baseline to week 4 in the FMT-treated patients (Supplementary Figure 1, Supplementary Results) but not in the sham-treated patients. Consequently, the FMT treatment was not likely the cause of the higher tyrosine levels in the failure group. Tyrosine is a nonessential aromatic AA that functions as a precursor of neurotransmitters and catecholamines in addition to occurring in signal transduction proteins. Our observations may suggest a potential role of tyrosine pathways in moderate-to-high PsA disease activity. Interestingly, an increased abundance of gut microbial genes encoding pathways of tyrosine degradation has been associated with IBD-associated arthropathy (23).

The other fecal metabolite associated with insufficient improvement in disease activity was choline, which is a methyl donor involved in many physiological processes, including epigenetic regulation. The main dietary sources of choline in Western Countries are animal-based products. Importantly, bacteria can also produce choline to avoid desiccation and to maintain turgor in the cell. Serum choline and its gut microbiota-dependent metabolite, trimethylamine N-oxide, have been implicated in inflammatory arthritis disease progression (24) and have also been associated with tender and swollen joint count in PsA (25). Brain choline also seems to be positively related to the severity of fatigue in RA (26). Findings from studies in vitro and in vivo further suggest that choline metabolism is activated in RA fibroblast-like synoviocytes under proinflammatory conditions and that selective blocking of choline kinase might be beneficial in inflammatory arthritis (27). Contrasting these observations, levels of serum choline have been reported to be inversely related with RA disease activity defined by DAS28 (28) and associated with beneficial response to MTX (29).

In our study, the only fecal metabolite associated with a beneficial response was methylsuccinic acid, which is an intermediate in the tricarboxylic acid cycle that provides energy for human cells (30). In high-fat diet-induced hypercholesterolemic rats, supplementation of Bacillus sp. DU-106 has proven to induce remarkable changes of the gut metabolomic profile, including elevation of methylsuccinic acid, which was associated with amelioration of hypercholesterolemia (31). From this, higher levels of fecal methylsuccinic acid could be a marker of normalization of fatty acid metabolism, which has been coupled to beneficial effect of MTX therapy in skin psoriasis (32).

Another interesting observation was that the time trajectory plot of the fecal SCFA, butyrate, indicated a short-term rapid rise within the first weeks of FMT. Similar metabolic effects following FMT have been reported in patients with systemic lupus erythematosus and individuals with the metabolic syndrome (33,34). Moreover, patients with psoriasis who progress to PsA have depressed serum butyrate levels compared with those who do not (14). Butyrate is derived from intestinal microbial fermentation of dietary fiber and is important for improving tight junctions and gut barrier function (35). It also possesses epigenetic potential by inhibition of histone deacetylase and has been attributed anti-inflammatory properties by induction of regulatory T-cells and suppression of IL-17 production (36). Consistent with these assets, increased levels of butyrate following FMT have been associated with a beneficial response in patients with inflammatory bowel disease (37).

Intriguingly, we identified another fecal SCFA, propionate, as a potential clinically relevant metabolite in PSA because of its strong association with ACR20 response in the FMT group at week 26. Propionate serves several functions in the human body,

including promotion of enteric smooth muscle contractions and stimulation of host defense peptide expression (38). In RA, propionate is believed to induce systemic effects on adaptive immune cells and modulate the functional phenotype of arthritogenic synovial fibroblasts (39). Supplementation of propionate in the drinking water in mice with collagen induced arthritis (a preclinical RA model) has been shown to result in reduced frequency of bone marrow megakaryocyte erythrocyte progenitor population (40), which may support inflammatory arthritis attenuating effects (41). Finally, in a prospective observational study, a high-fiber low-meat diet (providing the fuel for SCFA biosynthesis) was associated with better odds for responding to biological treatment in RA (42).

In the present study, the discriminatory power of the plasma metabolomic profiles was the case for the entire trial population, as well as for each of the two treatment arms (FMT vs. sham). In all three models, plasma glucose correlated with failure, whereas lysine, an essential AA, correlated with successful response. In line with this, a recent study of patients with RA reported that plasma lysine was significantly elevated in 6-month responders to tocilizumab (43). We also observed that fecal lysine was positively associated with ACR20 response in the FMT group at week 26. Synthesis of lysine by intestinal microbiota with absorption by the host has been suggested as a potentially vital source of this metabolite, but the absolute contribution remains to be determined (44). In addition to its importance as a precursor for protein synthesis, lysine is a precursor for the biosynthesis of carnitine, which plays an important role in β-oxidation.

Both treatment with MTX and TNFi have previously been linked to improved insulin sensitivity (45,46). Moreover, because no patients were treated with systemic corticosteroids during the trial, we do not think that DMARDs are the likely cause of the elevated plasma glucose levels observed in the failure group. On the contrary, we speculate if higher plasma levels of glucose could negatively affect response to DMARD treatment. In support of this hypothesis, previous studies have reported that presence of type 2 diabetes in patients with RA is associated with insufficient response to MTX (47). The metabolic syndrome has also been associated with a lower probability of achieving minimal disease activity in patients with PsA in therapy with TNFi (48). Hence, plasma glucose might be a marker of treatment effect and/or be directly involved in the (lack of) effectiveness of MTX and other DMARDs in immune-mediated arthritis. Consistent with this, we observed that plasma GlycA also correlated with failure. This emerging serological biomarker of systemic inflammation and cardiometabolic risk has previously been linked to both RA and psoriatic disease activity (49). However, this report is the first to suggest a possible correlation between fecal GlycA and PsA disease severity (HAQ-DI).

The limitations of this study include the limited number of participants and, in the case of the LMR test, the long interval from the experimental intervention to follow-up (26 weeks). Although the LMR test is the most commonly used method to assess functional permeability of the gut, we might therefore have missed early short-term changes in intestinal permeability in response to FMT. Moreover, we cannot completely rule out that unreported use of NSAIDs during the trial could have affected the intestinal permeability in the treatment failure group.

The fact that fecal hypoxanthine was significantly associated with small intestinal permeability (LMR) at baseline is an interesting finding because this metabolite has previously been linked to intestinal barrier function, energy metabolism in gut epithelial cells, and intestinal inflammation (50). Still, the small effect size and the lack of association at week 26 imply that other factors may be of equal or greater importance. Identifying both intrinsic and extrinsic modifiers of gut barrier integrity will therefore be important work for future studies.

Next, the longitudinal data included in the OPLS-DA models were based on samples collected at four time points from 31 patients. The limited subject number did not allow us to adjust for repeated measurements. We acknowledge that the purest form of OPLS-DA is only one time point/sample for each patient. Nevertheless, because we used the same number of time points for each patient in both the FMT and sham group, we consider risk of bias low. The benefits of our approach using repetitive measurements with OPLS-DA have previously been discussed (51).

Finally, we observed that the response rate differed between FMT products that derived from different donors varying from 0% to 100% success. However, the small numbers (four donors and only 15 patients treated) restricted our ability to robustly divide our donors into "good" and "bad" donors. Taken together, more and larger cohorts are needed to explore the clinical implication of donation variability on FMT outcome in immune-mediated arthritis, reveal the clinical importance of intestinal permeability dynamics in the disease course of PsA, and to confirm our findings from the OPLS-DA models.

In conclusion, increased small intestinal permeability, as well as fecal and plasma metabolomic profiles of patients with active peripheral PsA, was associated with the primary clinical end point (failure vs. responder) of the first randomized trial evaluating safety and efficacy of one single-donor FMT. The results of this exploratory study reinforce the relationship between PsA and host-microbiota cross-talk and the possibilities for therapies aimed at restoring the microbiota ecosystem and/or modulating specific metabolites/microbiota-host pathways in PsA. More randomized controlled trials are needed to determine the clinical implications of our findings.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be submitted. Miguens Blanco and Kragsnaes 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.

Study conception and design. Kragsnaes, Miguens Blanco, Marchesi, Ellingsen.

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