Microbiome is not linked to clinical disease severity of familial Mediterranean fever in an international cohort of children

S. Ozen[1], H.L. Lutz[2], V.M. Rivera[3], A. Reiff[3], E.D. Batu[1], E. Anderson[4], M. Salas Garcia[2,3], G. Aldrovandi[3], T.H. Akbaba[6], İ. Pazarbasi[7], Y. Bilginer[1], A. Balat[8], B. Balci-Peynircioglu[6], J.A. Gilbert[2,3], F. Dedeoglu[4], J.S. Hausmann[4,9]

[1]Dept. of Paediatric Rheumatology, Hacettepe University Faculty of Medicine in Ankara, Turkey; [2]Dept. of Paediatrics, University of California San Diego, La Jolla, CA, and Scripps Institution of Oceanography, University of California San Diego, La Jolla, CA, USA; [3]Division of Rheumatology at CHLA, Los Angeles, CA, USA; [4]Program in Rheumatology, Division of Immunology, Boston Children's Hospital, Boston, MA, USA; [6]Dept. of Medical Biology, Hacettepe University, Ankara, Turkey; [7]Dept. of Nutrition and Dietetics, Hacettepe University, Ankara, Turkey; [8]Division of Nephrology and Rheumatology, Istanbul Aydin University, Istanbul, Turkey; [9]Division of Rheumatology and Clinical Immunology, Beth Israel Deaconess Medical Center, Boston, MA, USA.

*Seza Ozen, MD**
*Holly L. Lutz, PhD**
Vanessa M. Rivera, MD
Andreas Reiff, MD
Ezgi D. Batu, MD, MSc
Edwin Anderson, MSc
Mariana Salas Garcia, MPM
Grace Aldrovandi, MD, PhD
Tayfun H. Akbaba, MSc
İlker Pazarbasi, MSc
Yelda Bilginer, MD
Ayse Balat, MD
Banu Balci-Peynircioglu, PhD
Jack A. Gilbert, PhD
Fatma Dedeoglu, PhD
Jonathan S. Hausmann, MD

**These authors contributed equally.*

Please address correspondence to:
Seza Ozen,
Department of Paediatric Rheumatology,
Hacettepe University,
06100 Ankara, Turkey.
E-mail: sezaozen@gmail.com

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ABSTRACT

Objective. *The severity of familial Mediterranean fever (FMF) may vary in different areas, suggesting a role for environmental factors. We analysed the composition of gut microbiota among children with FMF and healthy controls from Turkey and the USA and determined its effect on disease severity.*

Methods. *Children with FMF with pathogenic MEFV mutations and healthy controls from Turkey and the USA were enrolled. FMF disease activity was evaluated with the Autoinflammatory Disease Activity Index (AIDAI). Gut bacterial diversity was assessed by sequencing 16S rRNA gene libraries.*

Results. *We included 36 children from Turkey (28 patients with FMF, 8 healthy controls), and 21 patients and 6 controls from the USA. In the Turkish group, 28.6% of patients had severe disease, while 13.3% of US group patients had severe disease. As expected, we observed substantial differences between the gut microbiota of children from the two geographic regions, with Turkish patients and controls exhibiting higher relative abundances of Bacteriodia, while US patients and controls exhibited higher relative abundances of Clostridia. Alpha- and betadiversity did not differ significantly between FMF patients and controls, and neither was predictive of disease severity within each geographic region. We observed differences between FMF patients and controls in the relative abundance of some bacterial taxa at the amplicon sequence variant (ASV) level, but these differences received mixed statistical support.*

Conclusion. *Among an international cohort of children with FMF, we did not find a strong effect of gut microbiota composition on disease severity. Other environmental or epigenetic factors may be operative.*

Introduction

Autoinflammatory diseases are a group of disorders characterised by inappropriate activation of the innate immune system. Identification of single-gene defects in various pathways of the innate immune system has led to the discovery of dozens of autoinflammatory diseases and an improved understanding of the inflammatory pathways that underlie many conditions (1, 2). Independent of geography, familial Mediterranean fever (FMF) remains the most common autoinflammatory disease worldwide (3). The pathogenesis of FMF is associated with mutations in the *MEFV* gene coding for the protein pyrin (4, 5) which leads to exaggerated interleukin-1β (IL-1β) production, which is the main inflammatory cytokine in FMF. FMF is characterised by recurrent, short-lived (1-3 days) episodes of fever, serositis, and systemic inflammation. FMF occurs most commonly in eastern Mediterranean populations (3).

Although FMF was initially considered a simple autosomal recessive disease, there is a variety of disease expression that cannot be predicted just based on the genotype. Mutations in the *MEFV* gene are now considered to be gain-of-function with a gene-dosage effect so that patients with only one mutation in *MEFV* sometimes develop symptoms of FMF (6, 7). Even in patients homozygous for M694V, the most common mutation to cause severe FMF attacks (8), disease severity may vary among individuals, and it appears to be modulated by epigenetic factors.

For example, the risk of developing amyloidosis, the major complication of FMF, varies between patients living in different countries, the risk of amyloidosis is lower in Armenian patients with FMF living in the US compared to Armenian patients with FMF living in Armenia (9). In an international study of FMF, the risk of renal amyloidosis was most closely related to the country of residence, being more common in the eastern Mediterranean countries, and less common in Western countries (10). Studies have shown that Turkish patients with FMF living in Germany have milder disease than Turks with FMF residing in Turkey (11). This observation was confirmed through the Eurofever registry, which demonstrated that patients with FMF in Western Europe had less severe disease than those in the Eastern Mediterranean (12). The geographic differences between FMF disease severity suggest that environmental factors, possibly in connection with the microbiome, may alter the genetic expression of FMF.

There is a growing interest in the role of the microbiome and its effect on inflammatory diseases through its influence on the host immune system. In mouse models of autoinflammatory diseases, the microbiome has been shown to play an essential role in disease expression. For example, *Pstpip2cmo* mice spontaneously develop chronic multifocal osteomyelitis due to homozygous mutations in *Pstpip2* (13). When placed on a regular low-fat diet, all *Pstpip2cmo* mice develop inflammatory bone disease. However, mice fed a high-fat diet developed a different gut microbiome composition which protected them from inflammatory bone disease. Direct modulation of the microbiome through the use of antibiotics or fecal transplants was also able to alter disease expression.

The primary aim of this study was to identify differences in gut-associated microbiota in children with FMF living in Turkey as compared to children with FMF living in the United States. Our secondary aim was to test the hypothesis that gut-associated microbiota correlates with disease severity in children with FMF.

Methods

Patient sampling

This is a cross-sectional study of children with FMF and healthy controls from two countries. Children were included from two centers in Turkey (Department of Paediatric Rheumatology in Hacettepe University, Ankara, the capital of Turkey, and the Children's Hospital of Gaziantep University, Gaziantep, in the southeast region of Turkey) and from two centres in the USA (Boston Children's Hospital (BCH), Boston, MA, and Children's Hospital of Los Angeles (CHLA), Los Angeles, CA). All children with FMF were younger than 18 years of age, and all met both the PRINTO classification criteria for FMF (14) and the proposed Yalcinkaya and Ozen criteria for children (15). Inclusion criteria required having two pathogenic *MEFV* mutations, as analysed by Sanger sequencing, and clinical evidence of FMF. Clinical evidence was defined as having attacks of fever and serositis lasting less than 3 days. Most children had abdominal pain, while some had arthritis or chest pain as well.

FMF disease activity was evaluated with the validated AIDAI (Autoinflammatory Diseases Activity Index) (16). Scores over 6 defined severe disease whereas scores less than 2 defined mild or responsive disease. The FMF patients who had scores over 6 also had elevated CRP levels despite appropriate colchicine doses (17). All patients were receiving colchicine, and none had received antibiotics in the previous 6 months.

This study was approved by the Institutional Review Boards of all participating institutions (Hacettepe Ethical board: GO 15/115-15, Boston Children's Hospital: IRB protocol number at BCH is: P00016846, CHLA IRB approval no 16-00383). All patients provided informed consent to publish study results.

Dietary data collection and analysis

A questionnaire evaluating dietary habits, including the consumption of fat, gluten, and history and duration of breastfeeding, was obtained from most patients and controls from the Turkish and BCH groups. A dietician (IP) evaluated these records and tabulated nutritional intake. To assess dietary differences, we compared the protein, carbohydrate, and fat intake of healthy children in these two countries. For comparing daily calorie and carbohydrate intakes, we used the Mann Whitney U-test, whereas an independent samples t-test was used for comparing daily fat and protein intakes, and the percentages in daily energy intake.

DNA isolation, microbiome library preparation and sequencing

Faecal samples were collected both from FMF patients and healthy individuals using faecal sample collection tubes. A minimum of 5 ml fresh stool sample was collected in a 15 ml Falcon tube or sterile urine container without liquid medium, and rapidly transferred to -80 C to be stored in an upright position until DNA extraction. DNA extraction was performed by using the PowerFecal® DNA Isolation Kit (Qiagen, Catalog No. 12830). DNA concentrations and quailties were measured using the NanoDrop spectrophotometer (Thermo Scientific). Samples from Hacettepe University, Department of Medical Biology were shipped to the Gilbert Lab, UC San Diego for continuing 16s rRNA processing. Stool samples from Children's Hospital, Los Angeles were shipped to the Aldrovandi Lab at the University of California, Los Angeles for 16s rRNA processing. This was followed by 23 µl PCR reaction contained the following mixture: 1 µl of template DNA, 9.5 µl of MoBio PCR Water (Certified DNA-Free; Mo Bio Laboratories), 12.5 µl of 5-Prime HotMasterMix (1×), 1 µl of forward primer (5 µM concentration, 200 pM final), 1 µl of Golay Barcode Tagged Reverse Primer (5 µM concentration, 200 pM final). The conditions for PCR were as follows: 94°C for 3 min to denature the DNA, with 35 cycles at 94°C for 45 s, 50°C for 60 s, and 72°C for 90 s, with a final extension of 10 min at 72 °C to ensure complete amplification. Amplicons were quantified using PicoGreen (Invitrogen, Grand Island, NY, USA) assays and a plate reader SpectraMax iD3, normalisation using epMotion®

5070, clean-up using UltraClean® PCR Clean-Up Kit (Mo Bio Laboratories) and then quantification using Qubit readings (Invitrogen). The V4 region of the 16S rRNA gene (515F-806R) was amplified with region-specific primers that included the Illumina flow cell adapter sequences and a 12-base barcode sequence. The 16S rRNA sequencing on an Illumina MiSeq platform (2×150 paired-end sequencing, V3 chemistry) was conducted at the IGM Genomics Center, University of California, San Diego, La Jolla, CA. according to Earth Microbiome Project (EMP) standard protocols.

2.4 16S rRNA microbial analyses

Amplicon sequence variants (ASVS) were identified using the Deblur method (18) following standard demultiplexing and quality filtering using the Quantitative Insights Into Microbial Ecology pipeline (QIIME2) (19). Taxonomy was assigned using the Greengenes Database (May 2013 release, http://greengenes.lbl.gov). Analyses were performed on non-rarefied data that was transformed to library read depth (20) for detailed methods on library filtering and transformation). Alphadiversity was assessed both as observed richness (raw ASV counts) and using the Shannon index. The significance of differences in microbial alphadiversity between regional groupings of disease severity and healthy *versus* sick patients were assessed using the Kruskal-Wallis rank-sum test with Bonferroni-corrected *p*-values. Betadiversity was measured using three separate metrics: weighted UniFrac, unweighted UniFrac, and the Jaccard similarity index. Permutational analysis of variance (PERMANOVA) using the adonis2 function (R package vegan2.4-2) (21) was performed for each of the three betadiversity metrics to assess the effect of disease severity on variance within each geographic region. We assessed the relative abundance of the top ten most abundant microbial classes (as a function of bacterial read count over total library read depth), faceted by geographic region and FMF status (healthy individual versus FMF patient). Lastly, we statistically assessed relative differential abundance of individual ASVs between FMF patients and healthy controls within each country (US and Turkey) using two independent methods of analysis that attempt to account for the compositional nature of 16s rRNA data produced via high throughput sequencing. First, we performed an analysis of microbial community composition (ANCOM) (22), which assesses the significance of log2 fold changes in the relative abundance of ASVs. Second, we implemented the programme Songbird, which relies on relative rankings of log2 fold changes in abundance of ASVs (23). Both ANCOM and Songbird were performed independently for Turkish and US patient populations, controlling for regional variation between Boston and Los Angeles patients within the US. Songbird parameters included a differential prior of 0.50 and running test and null models for 100,000 epochs, with a learning rate of 1e-5.

Table I. Sampling by sex and geographic region. Homozygous individuals include female and male children who possess two copies of the M694V allele, which has been linked with an increased disease severity in FMF patients.

Region	Control		Patients with FMF	
	F	M	F	M
Turkey	4	4	15	13
US	3	3	9	12

Table II. Permutational analysis of variance (PERMANOVA) of disease severity within each geographic region, using three betadiversity metrics (unweighted UniFrac, weighted UniFrac, and the Jaccard Index).

Metric	Region	Df	Sum of Sqs	R2	F	Pr(>F)
Unweighted UniFrac	Boston	3	0.40	0.36	1.12	0.38
	LA	1	0.13	0.05	0.97	0.50
	Turkey	2	0.26	0.05	0.90	0.65
Weighted UniFrac	Boston	3	0.07	0.17	0.41	0.99
	LA	1	0.06	0.05	0.92	0.42
	Turkey	2	0.04	0.03	0.48	0.93
Jaccard Index	Boston	3	0.69	0.24	0.65	1.00
	LA	1	0.31	0.04	0.88	0.70
	Turkey	2	0.69	0.05	0.86	0.88

Results

Patient sampling and demographics

Sampling of Turkish FMF patients included 15 female and 13 male children, all of whom carried two pathogenic mutations linked to FMF; the US cohort of patients with FMF included 9 females and 12 males (total of 21) (Table I). Samples were obtained from age and sex-matched healthy controls (8 from Turkey and 6 from Boston). The gender distribution was 1:1 in the control groups. The median age of patients from Turkey and the US were 11 and 8.5 years, respectively. The median age of healthy controls from Turkey and the US were 11.25 and 12 years, respectively. The median age of FMF patients with severe and mild disease was 10.25 and 13 years, respectively. In the Turkish group, 28.6% of patients with FMF had severe disease and it was similar between the two Turkish centres. In contrast, only 13.3% of patients in the US group had severe disease (Supplementary Table S1).

Analysis of dietary differences between geographic regions

Although the carbohydrate intake was not significantly different between US and Turkish patients, the percentage of carbohydrates for the total energy intake was higher in Turkish children (59±10.9 in Turkish children compared to 45±4.6 in the American children) ($p<0.05$).

On the other hand, the percentage of fat intake for the total energy intake was higher in the US children (38±4.1) as compared to the Turkish children

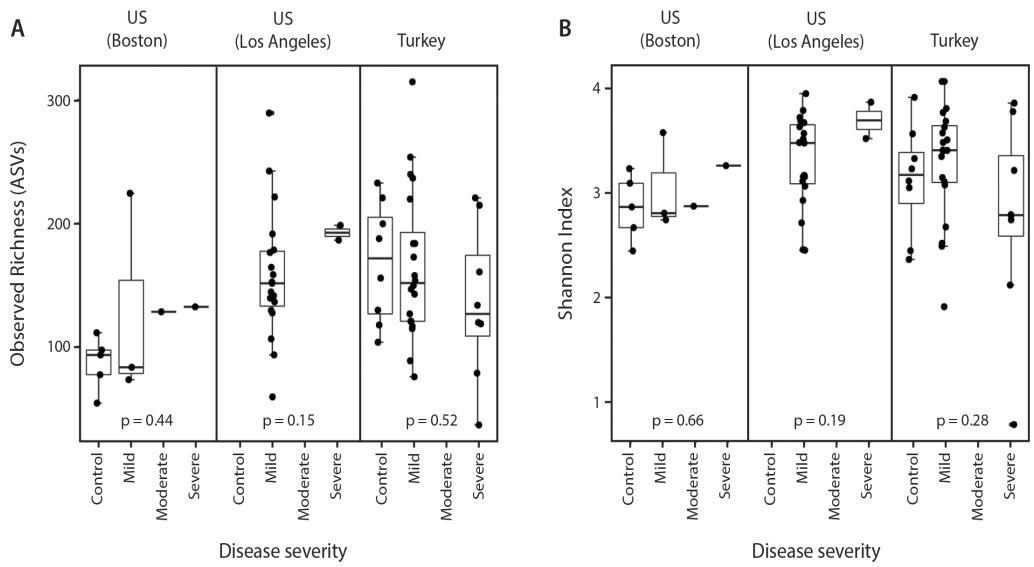

Fig. 1. Alphadiversity of healthy controls *versus* FMF patients grouped by geographic region and disease severity, measured as: **A**) observed ASV richness, and **B**) Shannon Index.

(25±9.3, $p<0.05$). The protein intake between the children from Turkey and the US were not significantly different.

16S rRNA microbial results

A total of 76 16S rRNA sequencing libraries were generated, consisting of an average read depth of 35,827 reads (SD ± 25,361 reads). Across all libraries, we identified 1,352 unique amplicon sequence variants (ASVs) using Deblur (18). No significant differences were observed in alpha-diversity (observed richness and Shannon index) between healthy controls and FMF patients exhibiting differing degrees of clinical severity from any location (Kruskal-Wallis p-value >0.15) (Fig. 1). PERMANOVA analysis of weighted UniFrac, unweighted UniFrac, and Jaccard beta diversity metrics found that disease severity did not significantly explain variation (Table II), suggesting that FMF disease severity does not correlate with differences in host-associated microbiota.

Regional differences in the microbiome appeared to be the most influential driving factor of variation of betadiversity (Fig. 2). The relative abundance of the top 10 bacterial classes did not appear to differ between healthy and FMF patients but did vary between regions. Turkish patients exhibited a much higher abundance of Bacteriodia than their US counterparts, and US patients both from Boston and Los Angeles showed a higher abundance

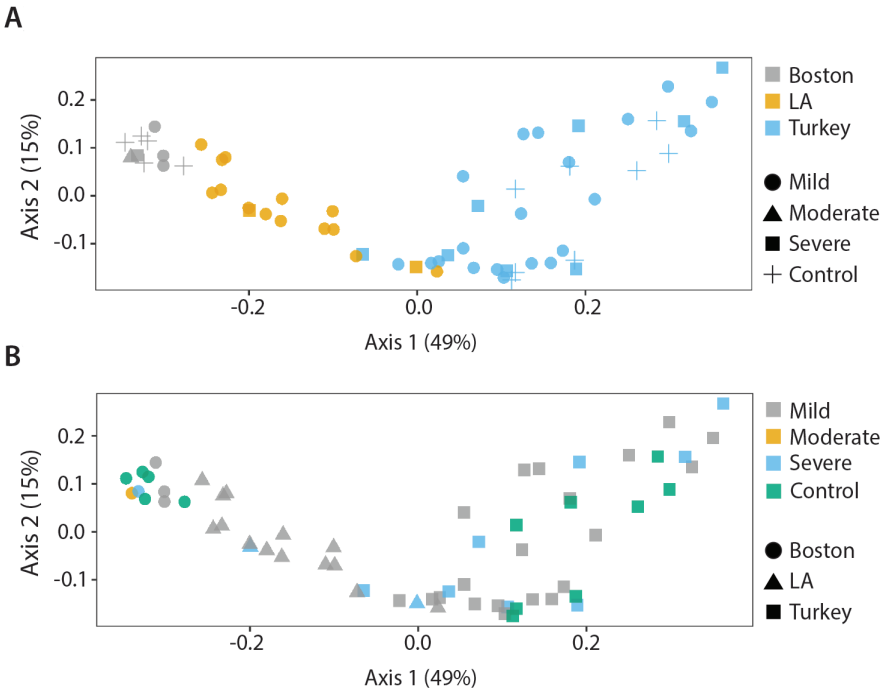

Fig. 2. PCoA plot of weighted UniFrac beta diversity across all regions and samples, showing: **A**) samples coloured by geographic region and disease severity designated by shapes, and **B**) samples coloured by disease severity and geographic region designated by shapes.

of Clostridia. Patients in the US also exhibited the presence of bacteria in the class 4C0d-2, which was rarely observed in Turkish patients (Fig. 3).

Analysis of bacterial community composition identified a small number of ASVs that were similarly over- or under-represented in Turkish and US patients (Fig. 4). In both US and Turkish patients, we observed an increase of *Bifidobacterium adolescentis* (ASV 1) and Clostridiales (ASV 13) in FMF patients relative to healthy controls. An additional ASV in the genus *Bifidobacterium* (ASV 16) was increased in Turkish patients as well. Other similarities between US and Turkish FMF patients included a decreased abundance of *Streptococcus* sp. (ASV 2), *Faecalibacterium prausnitzii* (ASV 10), and Erysipelotrichaceae (ASV 15) relative to healthy controls in both geographic locations. At a broader taxonomic level, many bacteria showed paradoxical as-

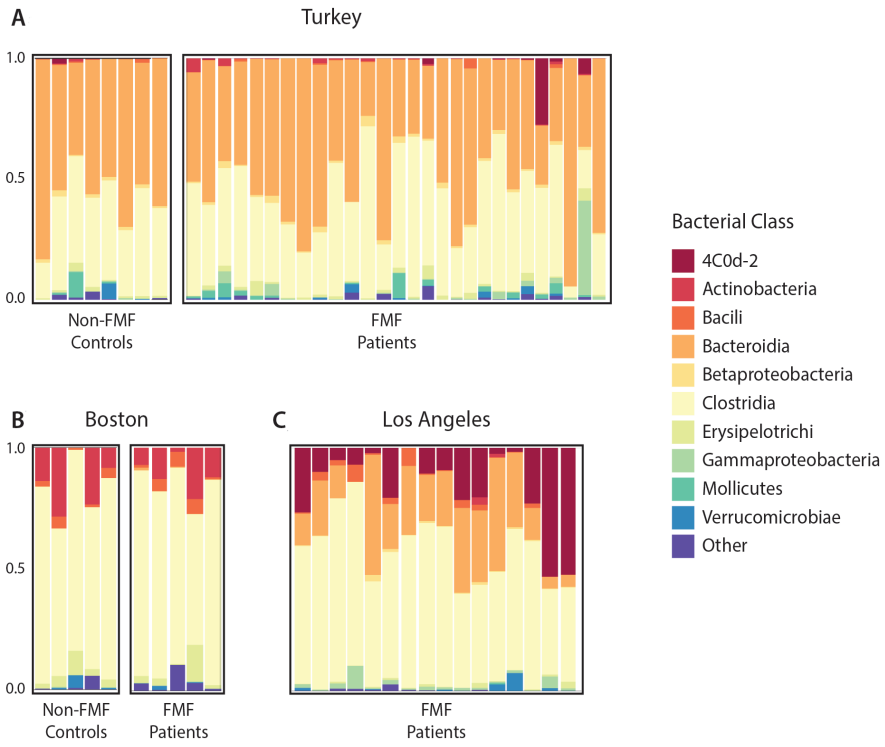

Fig. 3. Relative abundance of top ten bacterial taxa (at the class level) among individual patients in **A**) Turkey, **B**) Boston, and **C**) Los Angeles.

sociations between FMF patients and healthy controls (*e.g.* Ruminococcaceae, Lachnospiraceae, Clostridiales), suggesting a lack of importance in associated FMF phenotypes. Songbird analyses of Turkish patient data found that models seeking to associate patient disease status (FMF or control) with the microbiome did not differ significantly from null models generated from the same data for either Turkish patients (Q^2 = -0.094) or US patients (Q^2 = -0.557) (Suppl. Fig. S1), meaning that the disease status variable essentially exhibits no explanatory power.

Discussion

As expected, we observed significant differences between the microbiota of Turkish children as compared to those from the US. However, within each geographic region, there was no difference for alpha- or beta-diversity between controls and FMF patients, and neither diversity metric was predictive of disease severity within each country. Similarly, there was no evidence that genotype influenced the microbiome. Thus, although there were significant differences in the microbiota of children with FMF between the two regions, we could not attribute these differences to the severity of the disease.

Upon further analysis of the microbial community composition and differential abundance of specific bacterial ASVs using ANCOM, we did observe a small number of bacteria that were either over- or underrepresented in FMF patients relative to controls within both countries. Most notably, we observed an increased relative abundance of bacteria belonging to the genus *Bifidobacterium*. This relationship is counterintuitive, as *Bifidobacterium* are typically associated with anti-inflammatory responses in healthy humans (24). We also observed differential abundance of bacteria in the orders Bacteroides and Clostridiales, families Lachnospiraceae and Ruminococcaceae, and at the species level, multiple ASVs belonging to the species group *Faecalibacterium prausnitizii*. These bacteria did not follow any readily interpretable trend, and ASVs from each of these bacterial groups could be found to be over- or underrepresented in FMF patients, suggesting that they may not necessarily be linked to FMF. Further investigation at a finer taxonomic and perhaps specific bacterial strain level is needed to elucidate the possible role of such bacteria in influencing the course of FMF phenotypes.

To our knowledge, this is the first international study that examined the role of the microbiome in children with FMF. Three other studies have examined the gut microbiome of patients with FMF compared with healthy controls (25-27). Khachatryan *et al*. (25) suggested that the gut microbiota of Armenian (mostly adult) patients with FMF differed from that of healthy controls. However, we found no such patterns in our international study of children with FMF. The differences observed in the relative abundance of specific bacterial ASVs between FMF patients and healthy controls across countries are likely artefactual, representing the limitations of microbial analyses due to the inherent compositional nature of 16s rRNA data from high throughput sequencing platforms. While further studies may identify bacteria associated with changes in the severity of the FMF clinical phenotype, our results suggest that a combination of genetic and environmental effects, other than the patient's gut microbiome, likely play a more important role in disease severity in FMF patients. A second study of patients with FMF showed that administration of colchicine did not normalise the microbiome but shifted the composition toward a new equilibrium (26).

A more recent study explored the microbiome of adults with and without FMF, as well as those with and without AA amyloidosis (27). Compared with healthy controls, patients with FMF exhibited significant decreases in alpha diversity and increased relative abundance of several bacteria. Although we observed no differences in alpha- or beta-diversity between our FMF cohorts and controls, we did identify increased relative abundances of ASVs in the Lachnospiraceae and Clostridiales among FMF patients similar to the observations of Deshayes *et al*. (27). The fact that our results did not support similar findings in either country (US or Turkey) is intriguing and warrants further study. In our study, we applied

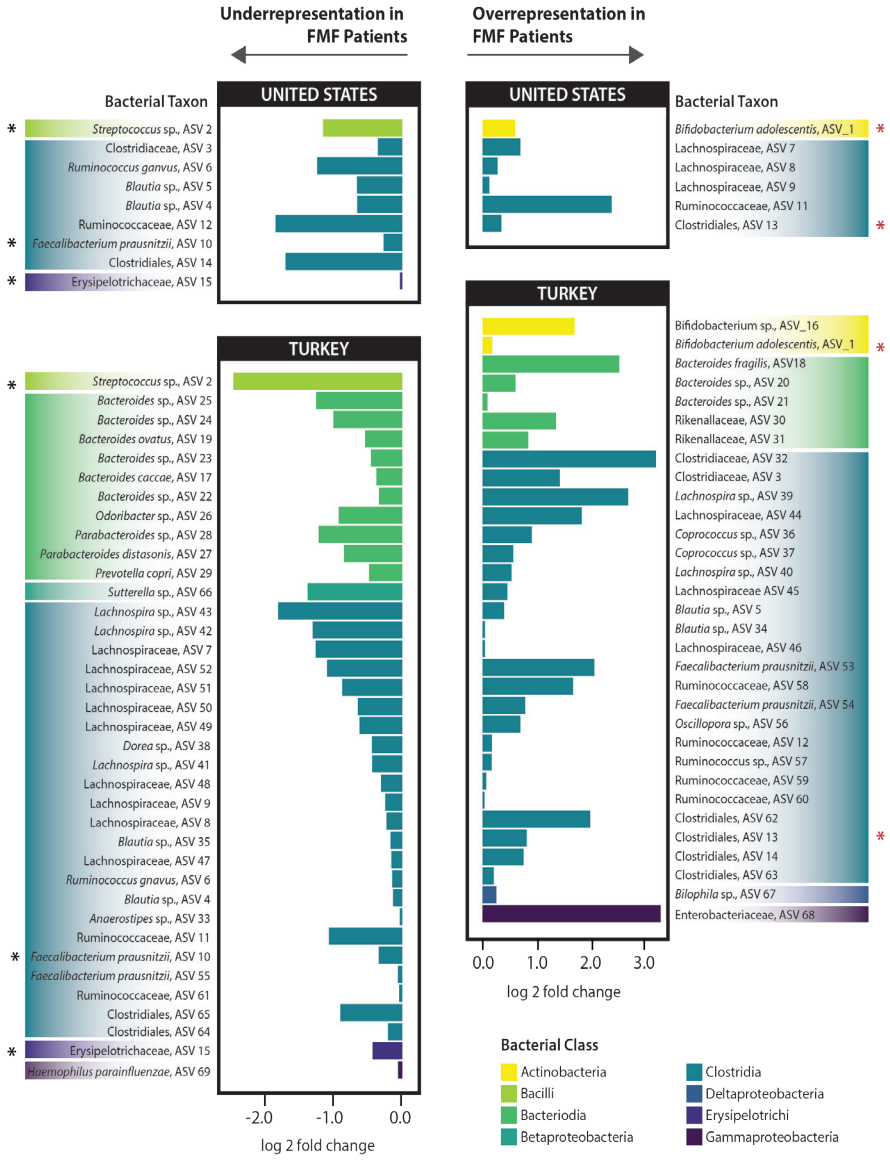

Fig. 4. Log2 fold change in relative abundance of bacterial ASVs identified via ANCOM. Results are grouped by country and ASVs are coloured by bacterial class. Black and red asterisks denote ASVs found in both US and Turkish patients to be significantly under- or overrepresented, respectively.

recently developed analytical methods for identifying differential abundances of microbiota between patients and controls. These more sophisticated methods take into account the sparse nature of microbial data, and are less likely to erroneously identify ASVs (or features, generally) as differing significantly between groups. Whether our findings are the result of small sample sizes or the identification of microbial differences observed in previous studies are reflective of true differences will require additional research to elucidate. Patients with the same genotype in this monogenic disease may indeed have different phenotypes. The causes of regional variation in disease severity, and the fact that severity can differ among related individuals, remain enigmatic. Our analyses suggest that the microbiome does not have a significant effect on disease severity. We speculate that the SNPs in other associated genes (*e.g.* in molecules involved in the innate immune system) may affect the clinical presentation of FMF disease. Further studies comparing these patients will undoubtedly shed light on the missing parts of the puzzle in FMF.

Our study has some limitations. Due to the relative rarity of symptomatic FMF in children in the US, our study was small. Some samples in the final analyses were bioinformatically filtered out due to poor quality. We also faced challenges in recruiting age- and sex-matched controls who had not had recent antibiotic use and were willing to provide stool samples, so we had fewer controls than patients with FMF, although they were all sex- and age-matched. No controls were enrolled from the Los Angeles cohort due to differences in IRB protocols. Due to the complexity of obtaining biosamples internationally, we used two different laboratories for 16s rRNA processing, which may have introduced unintended variance between the samples, although we used identical techniques and the same reagents in both laboratories.

Conclusion

In conclusion, although we hypothesised that the microbiota was one of the factors affecting the severity of FMF, we were unable to show any differences in the microbiome between patients with mild *versus* severe disease. Furthermore, there was no difference in alpha- or beta diversity between the microbiota of healthy controls and that of patients with FMF. We did observe geographic differences in the microbiota of patients and controls between the two countries, with Turkish children exhibiting a much higher abundance of Bacteriodia than their US counterparts, and US children showing a higher abundance of Clostridia. Future studies involving larger numbers of patients with FMF may identify geographically localised patterns of microbial associations with disease severity; however, our findings do not support the hypothesis that disease severity in patients with FMF varies with microbial community composition or diversity in the gut.

Take home messages

- Although the gut microbiota differed between that of children in Turkey and children in the US, there were no significant differences between children with FMF and healthy controls within each country.
- We found no correlation between the gut microbiome and severity of disease in FMF patients and controls in either US or Turkish cohorts.

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Competing interests
None declared for the present work.
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