Specific changes in fecal microbiota are associated with familial Mediterranean fever

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Abstract

Objectives: Familial Mediterranean fever (FMF) can be complicated by AA amyloidosis, though it remains unclear why only some patients develop amyloidosis. We examined the gut microbiota composition and inflammatory markers in patients with FMF complicated or not by AA amyloidosis.

Methods: We analyzed the gut microbiota of 34 FMF patients without AA amyloidosis, 7 FMF patients with AA amyloidosis, 19 patients with AA amyloidosis of another origin, and 26 controls using 16S ribosomal ribonucleic acid gene sequencing with the Illumina MiSeq platform. Associations between bacterial taxa and clinical phenotypes were evaluated using the multivariate association with linear models (MaAsLin) statistical method. Blood levels of interleukin (IL)-1β, IL-6, tumor necrosis factor-α and adipokines were assessed by enzyme-linked immunosorbent assay; indoleamine 2,3-dioxygenase (IDO) activity was determined by high-performance liquid chromatography.

Results: Compared to healthy subjects, specific changes in fecal microbiota was observed in FMF and AA amyloidosis groups. Several operational taxonomic units were associated with FMF. Moreover, 2 operational taxonomic units were overrepresented in FMF-related AA amyloidosis compared to FMF without AA amyloidosis. Additionally, higher adiponectin levels and IDO activity were observed in FMF-related AA amyloidosis compared to FMF without AA amyloidosis ($p<0.05$).

Conclusions: The presence of specific changes in fecal microbiota in FMF and in FMF-related AA amyloidosis suggest that intestinal microorganisms may play a role in the pathogenesis of these diseases. These findings may offer an opportunity to utilize techniques for gut microbiota manipulation.

Key messages

What is already known about this subject?

- Several risk factors for AA amyloidosis in familial Mediterranean fever have previously been found, including environmental factors.
- Some data point toward the influence of the environment on AA amyloidosis occurrence, particularly the gut microbiota.

What does this study add?

- Familial Mediterranean fever was associated with specific changes in fecal microbiota, suggesting that the gut microbiota might be involved in the clinical expression of familial Mediterranean fever.
- In familial Mediterranean fever patients, amyloidosis was independently associated with a specific alteration in the microbiota composition, suggesting that intestinal microorganisms may play a role in AA amyloidosis pathogenesis.

How might this impact on clinical practice or future developments?

- The presence of specific changes in fecal microbiota in familial Mediterranean fever and AA amyloidosis patients may allow for the use of techniques for gut microbiota manipulation to prevent inflammation and amyloidosis occurrence.

Key words: Familial Mediterranean Fever; AA Amyloidosis; Microbiota; Adipokines; Indoleamine 2,3-dioxygenase

INTRODUCTION

Familial Mediterranean fever (FMF) is the most common monogenic autoinflammatory disease, secondary to mutations in the *MEFV* gene, which encodes the protein pyrin [1]. AA amyloidosis is the most serious complication of FMF and is characterized by the presence of extracellular deposits of an amorphous substance, including an amyloidogenic derivative of the serum amyloid associated (SAA) protein [2]. Other diseases, mainly chronic idiopathic inflammatory diseases (such as chronic inflammatory rheumatic diseases or inflammatory bowel diseases), autoinflammatory diseases other than FMF, chronic infections and obesity, can be complicated by AA amyloidosis [3]. The alarmins S100A8 and A9 have been studied as inflammatory biomarkers for many of these diseases [4], whereas changes in adipokine levels are observed in obesity, including pro-inflammatory leptin and adiponectin, which has an anti-inflammatory effect on atherosclerosis [5,6].

The gut microbiota has a key role in several physiological functions, particularly in host energetic and vitamin metabolism and protection against pathogens, due to components of these bacteria and/or the production or processing of metabolites [7–9]. For example, tryptophan metabolism represents a significant pathway by which the gut microbiota influences the host. These metabolites can be of microbial or endogenous origin and are mainly metabolized by indoleamine 2,3-dioxygenase (IDO, EC 1.13.11.52), the activity of which is stimulated by several pro-inflammatory components, such as lipopolysaccharide, tumor necrosis factor (TNF)-α, and interleukins (ILs)-1 and -6. Thus, a pro-inflammatory environment will induce overactivation of IDO, which is responsible for many immunomodulatory activities and a shortage of tryptophan [10]. Nevertheless, overactivation of IDO can also have pro-inflammatory effects under certain conditions [11–14].

To date, it remains unclear why only some FMF patients develop AA amyloidosis. Several risk factors have been found, such as male sex and genetic factors such as specific mutations in the *MEFV* gene (M694V homozygosity, associated with a more severe phenotype, including more frequent and long attacks and higher dose of colchicine needed [15,16]) or in the gene encoding SAA [17–19]. More interestingly, some risk factors are environmental, such as the country of residence [17,20]. Association between the single-nucleotide polymorphism Arg753Gln in Toll-like receptor 2, which belongs to the microbe recognition pathway, has also been reported [21]. Other data highlight the influence of the environment on AA amyloidosis occurrence, particularly the gut microbiota. Indeed, SAA acts as an opsonin, and intestinal epithelial cells can synthesize it in response to the gut microbiota [22,23]. Moreover, a high-fat diet can induce AA amyloidosis in a mouse model overexpressing hepatic SAA [24], and

several bacteria can produce amyloid-enhancing factors that can cross species barriers and may be transmitted by ingestion [2,25–27]. Digestive tract involvement was found in nearly all cases of amyloidosis in autopsy series [28], and evidence of gut microbiota involvement in the pathogenesis of another disease involving amyloid plaques, Alzheimer's disease, is accumulating, both *in vitro* and *in vivo*, in animal models as well as in humans [29–32].

Based on these findings, the gut microbiota appears to be a credible candidate for both the overproduction of the SAA protein and synthesis of amyloid-enhancing factors in FMF. Here, we investigate the gut microbiota and several pro-inflammatory markers, cytokines, adipokines, and tryptophan metabolites in FMF patients, with or without AA amyloidosis, in non-FMF-related AA amyloidosis patients and in healthy controls. We tested the hypothesis of specific dysbiosis in patients with FMF and in those with FMF complicated by AA amyloidosis.

METHODS

Study design and control groups

This cross-sectional study included FMF patients followed in the national reference center for FMF and inflammatory amyloidosis in Tenon hospital, Paris, and fulfilling the Tel Hashomer criteria with at least one *MEFV* mutation [33]. Patients were diagnosed as having AA amyloidosis-complicating FMF in the presence of proteinuria with biopsy-proven AA amyloidosis (positive immunohistochemistry with an anti-SAA antibody or proteomics). The inclusion period ranged from April 2016 to March 2018. Demographic and clinical data were retrieved from the patients' clinical files.

Two other control groups were assembled: patients with AA amyloidosis secondary to a cause other than FMF and healthy controls; the latter did not have a chronic disease that required systemic treatment. None of the patients took antibiotics in the 6 weeks preceding stool sampling. Stool and blood samples were collected at the same time.

All patients provided their informed consent. The study was approved by the ethics committee (Comité de Protection des Personnes – Ile de France VI, n°DC-2015-2586) and was conducted in compliance with good clinical practices and the Declaration of Helsinki principles.

Patient and Public Involvement

This research was conducted without patient involvement.

MEFV genotyping

Genomic DNA was isolated from peripheral leukocytes using standard procedures. Direct sequencing of the *MEFV* gene was performed using the Sanger method. Exons and flanking intronic sequences (NM_000243) were PCR-amplified from genomic DNA. The primer sequences used are available upon request. The PCR products were sequenced using the Big Dye Terminator reaction kit (Applied Biosystems, Foster City, CA, USA) and a 96-capillary ABI Prism sequencer and were then analyzed using SeqScape v2.6 software (Applied Biosystems, CA, USA).

Gut microbiota analysis

Stool samples were immediately cooled at 4°C and then stored at −80°C within 6 hours until DNA extraction. Following a previously described method [11], the stool samples were resuspended in 250 µL of 4M guanidine thiocyanate and 40 µL of 10% N-lauroyl sarcosine; 500 µL of 5% N-lauroyl sarcosine was then added. DNA was extracted by mechanical disruption of the microbial cells in a FastPrep instrument (MP Biomedicals) after the addition

of 500 mg of 0.1-mm-diameter glass beads. The nucleic acids were recovered from the clear lysate by alcohol precipitation. The DNA was stored at −20°C until 16S ribosomal ribonucleic acid (rRNA) gene sequencing.

Microbial diversity was determined for each sample by amplifying the V3 and V4 hypervariable regions of the 16S rRNA gene (5′-TACGGRAGGCAGCAG-3′ (sense) and 5′-CTACCNGGGTATCTAAT-3′ (antisense)) using a 16S-amplicon-library preparation protocol (Metabiote, GenoScreen, Lille, France). According to the manufacturer's protocol, 16S rRNA gene polymerase chain reaction (PCR) was performed using 5 ng of genomic DNA with 192 bar-coded primers (Metabiote MiSeq Primers) at final concentrations of 0.2 μM and an annealing temperature of 50°C for 30 cycles. The PCR products were purified using an Agencourt AMPure XP-PCR Purification system (Beckman Coulter, Brea, CA, USA), quantified according to the manufacturer's protocol, and multiplexed at equal concentrations. A 300-bp paired-end sequencing protocol with the Illumina MiSeq sequencing platform (Illumina, San Diego, CA, USA) was applied for 16S rRNA gene sequencing at GenoScreen, Lille, France. Raw paired-end reads were quality-filtered using the PRINSEQ-lite PERL script and assembled using FLASH (fast length adjustment of short reads to improve genome assemblies) with a minimum overlap of 30 bases and 97% overlap identity; primer sequences were removed using CutAdapt.

The sequences were then demultiplexed and quality-filtered using the "quantitative insights into microbial ecology" (QIIME, version 1.9.1) software package, and Illumina reads were joined using the fastq-join method (https://expressionanalysis.github.io/ea-utils/). We utilized the UCLUST algorithm to assign operational taxonomic units (OTUs) to sequences with a 97% threshold of pairwise identity, and the sequences were classified taxonomically using the Greengenes reference database. Rarefaction was performed (13,000 reads per sample) and used to compare the abundances of OTUs across samples.

Serum levels of inflammatory proteins, cytokines, and adipokines and IDO activity

C-reactive protein (CRP) and SAA protein levels were determined by immunonephelometry using an IMMAGE® analyzer (Beckman-Coulter, Villepinte, France). The IL-6 level was assessed by chemiluminescence enzyme immunoassay using a LUMIPULSE® analyzer (Fujirebio, Tokyo, Japan). The blood levels of other inflammatory cytokines (IL-1β and TNF-α), adipokines (leptin, adiponectin) (Quantikine, R&D Systems, Oxford, UK) and S100A8/A9 proteins (Bühlmann, Amherst, USA) were measured by enzyme-linked immunosorbent assay according to the manufacturers' instructions. For these circulating biomarkers, the control group was formed in part by 17 healthy male volunteers from the French

study METASPERME (N°IDRCB: 2011-A01052-39) and 7 healthy women volunteers presented in a previous publication [34]. None of the healthy controls were diabetic or obese.

Tryptophan and kynurenine levels were evaluated by high-performance liquid chromatography with a coulometric detection system (ESA Coultronics, ESA Laboratories, Chelsford, MA, USA). The kynurenine/tryptophan ratio was determined from the kynurenine and tryptophan absolute concentrations and used as a marker for IDO activity. The values for our cohort were compared to 48 age- and sex-matched healthy controls.

Statistical analyses

The Bray-Curtis index was used for β-diversity analysis, and the results are presented in the form of principal coordinate analysis, where each sample is colored according to the disease. Statistical significance between the studied groups was evaluated using the nonparametric analysis of similarities (ANOSIM) test with 9,999 permutations. Shannon and Chao1 indexes were applied to assess α-diversity, and statistical significance was evaluated using the nonparametric Mann-Whitney test. Relative abundances and associations between bacterial taxa and clinical and biological data were assessed using the multivariate association with linear models (MaAsLin) statistical method [35], taking into consideration the effects of potential confounding factors such as age, sex, body mass index (defined as the weight in kg divided by the height squared), smoking status and treatment. Only OTUs present in more than 50% of the samples were considered in the analysis.

GraphPad Prism 7 software (GraphPad Software Inc., San Diego, CA, USA) was used for statistical analyses of biological parameters. Categorical variables are reported as percentages and were compared using the χ^2 or Fisher's tests, according to expected frequencies. Continuous variables are expressed as medians and quartile 1-quartile 3 values, and were analyzed using the bilateral Student t-test for data normally distributed or the nonparametric Mann–Whitney test when 2 groups were compared. When more than 2 groups were compared, one-way analysis of variance (ANOVA) with Tukey's *post hoc* test or the nonparametric Kruskal-Wallis test followed by Dunn's *post hoc* test was employed. The Spearman correlation coefficient was calculated to determine correlations between 2 continuous variables. Associations were considered significant if the p-value was <0.05 and the q-value (i.e., the false discovery rate using the Benjamini-Hochberg correction method) was <0.25.

RESULTS

Study populations

We performed gut microbiota analysis on FMF patients without (n=34, including 29 in FMF remission) or with (n=7) AA amyloidosis, 19 patients with AA amyloidosis secondary to another hereditary disease (n=4, i.e., pyrin-associated autoinflammation with neutrophilic dermatosis, Fabry disease, tumor necrosis receptor-associated periodic syndrome and other genetic etiology), to inflammatory rheumatic diseases (n=4, i.e., synovitis, acne, pustulosis, hyperostosis and osteitis syndrome, rhupus syndrome, psoriatic rheumatism and rheumatoid arthritis), to obesity (n=3), to an unknown reason (n=4) or to miscellaneous causes (n=4, i.e., Crohn's disease, Waldenstrom's macroglobulinemia with *MyD88* mutation, human immunodeficiency virus infection and Schnitzler syndrome), and 26 healthy controls (Table 1).

Table 1. Clinical, demographic, and renal function data for the study population

	FMF without AA amyloidosis (n=34)	FMF with AA amyloidosis (n=7)	Non-FMF-related AA amyloidosis (n=19)	Controls (n=26)	p-value
Female sex, n (%)	15 (44%)	4 (57%)	8 (42%)	12 (46%)	0.87
Median age at sampling [Q1-Q3]	45.5 [31.5-56]	54 [46-60.5]	64 [47-68.5]	30 [28-41]	<0.001
Body mass index (kg/m²), median [Q1-Q3]	24 [21.5-27] *(n=33)*	22.5 [21-26]	23.5 [22-27]	21 [20-23] *(n=21)*	0.02
Current smoker, n (%)	9 (29%) *(n=31)*	2 (29%)	5 (26%)	0 *(n=21)*	<0.01
Creatininemia (µmol/L), median [Q1-Q3]	71 [60-79]	165 [119-220.5]	166 [99.5-324]	ND	<0.001
eGFR > 60 mL/min/1.73 m², n (%)	33 (97%)	0	7 (37%)	ND	<0.001
eGFR < 60 mL/min/1.73 m², n (%)	1 (3%)	7 (100%)	12 (63%)		

Q: quartile; FMF: familial Mediterranean fever; eGFR: estimated glomerular filtration rate, by the MDRD formula; ND: not determined.

Regarding the FMF patients without AA amyloidosis, 31 (91%) carried homozygous or compound heterozygous mutations in the *MEFV* gene. Conversely, all FMF patients with AA amyloidosis were homozygous (M694V/M694V, n=6, or M680I/M680I, n=1). The treatments that each group of patients underwent are described in online supplementary Tables S1 and S2.

Biological data, including acute phase proteins (CRP, SAA and S100A8/A9), pro-inflammatory cytokines (IL-1β, -6 and TNF-α), adipokines (leptin, adiponectin) and IDO activity, are depicted in Figure 1. All patient groups had higher blood levels of IDO, S100A8/A9, IL-1β, IL-6 and TNF-α than did the controls. The patients with FMF complicated by AA amyloidosis had higher adiponectin levels and IDO activity than did FMF patients without AA amyloidosis ($p<0.05$). No correlation was found between the glomerular filtration rate estimated by the MDRD formula and IDO activity or adiponectin in FMF patients with AA amyloidosis (Spearman correlation test, $p=0.33$ and 0.32, respectively). When pooling data, IDO activity positively correlated with creatininemia, IL-6, TNF-α, adiponectin and age. In addition, adiponectin was correlated with SAA and CRP (online supplementary Figure S2).

Bacterial dysbiosis in FMF and AA amyloidosis

Compared to healthy subjects, significant decreases in α-diversity were observed in FMF patients globally (online supplementary Figure S1) and specifically in those without amyloidosis and in patients with non-FMF-related AA amyloidosis, as assessed by the Shannon and Chao1 indexes ($p<0.05$, Figure 2A). In addition, β-diversity analysis showed significant differences between the healthy controls and these two groups, suggesting a significantly different microbiota composition ($p<0.05$ and <0.001, respectively, Figure 2B). Although no significant difference was observed regarding FMF patients with AA amyloidosis, the statistical power was limited by the small number of subjects in this group.

As expected, most bacteria identified belong to the phyla Firmicutes, Bacteroidetes, Proteobacteria and Actinobacteria and to the families *Lachnospiraceae* and *Ruminococcaceae* (Figure 2C-D) [36,37].

Differential bacterial composition in FMF patients with and without AA amyloidosis

After multivariate association testing with the MaAsLin statistical method to control for the effects of potential confounding factors, several OTUs belonging to the order Clostridiales were specifically associated with FMF (Figure 2E). Nonetheless, no bacterial taxon was independently associated with AA amyloidosis, likely because the different types of underlying disease that led to AA amyloidosis have specific impacts on the gut microbiota, thus increasing

heterogeneity among patients with amyloidosis. When restricting the analysis to FMF patients only, 2 OTUs belonging to Clostridiales were specifically associated with AA amyloidosis (Figure 2F).

DISCUSSION

In this study, we compared the microbiota and several biological markers of FMF patients with or without AA amyloidosis, non-FMF-related AA amyloidosis patients and healthy controls. FMF was associated with gut microbiota dysbiosis characterized by a decrease in α-diversity and a significantly altered composition. Moreover, among FMF patients, AA amyloidosis was associated with some alteration in the gut microbiota with 2 increased OTUs as well as higher adiponectin levels and IDO activity.

Environmental factors have long been recognized as influencing the disease phenotype of FMF [17,20,38]. Moreover, small intestinal bacterial overgrowth (SIBO) has been associated with unresponsiveness to colchicine, and decontamination therapy by rifaximin leads to a decrease in FMF attacks [39]. Apart from abdominal pain during FMF attacks, none of our FMF patients had clinical features suggestive of SIBO. To our knowledge, the only previous study of the gut microbiota in FMF patients reported increases in Enterobacteriaceae, Acidaminococcaceae, *Ruminococcus* and *Megasphaera* and a decrease in *Roseburia* in 12 FMF patients in remission compared to healthy controls [40]. We did not confirm these results, which may be due to the use of different statistical methods, such as the lack of multivariate analysis or adjustment for multiple comparisons in the study by Khachatryan *et al*., to fewer patients recruited (with 13/19 being homozygous and only from Armenia), or to other environmental or genetic factors. Among the OTUs we found, the genera *Blautia* and *Coprococcus* are short-chain fatty acid-producing bacteria that are decreased in Crohn's disease [41]. Interestingly, an increase in systemic concentrations of short- and long-chain fatty acids, produced by the gut microbiota, has also been observed in FMF, suggesting a leaky gut [42,43]. As already described in other diseases [44], we may assume that a leaky gut in FMF, with the entry of environmental components into the systemic circulation, may exacerbate inflammation or even trigger amyloid nucleation.

The gut microbiota has never been studied in AA amyloidosis. In our study, 2 OTUs belonging to Clostridiales including one from the genus *Blautia* were overrepresented in patients with AA amyloidosis secondary to FMF compared to FMF patients without amyloidosis. Interestingly, although its exact role has not been explored, *Blautia* has also been associated with Alzheimer's disease [45]. In contrast, no bacterial taxon was associated with non-FMF-related AA amyloidosis, a result that might be explained by several reasons.

(1) The great heterogeneity in AA amyloidosis patients in terms of underlying inflammatory disease and of ongoing treatments, and these treatments might have modified their microbiota. However, colchicine used in FMF patients does not appear to influence the gut microbiota, at

least in *in vitro* experiments [46]. Moreover, dysbiosis has been shown to be disease specific, such as in the different phenotypes of rheumatic inflammatory diseases or inflammatory bowel diseases [47,48]. Therefore, it is possible that the occurrence of AA amyloidosis is promoted by the disappearance or emergence of certain bacterial taxa, differing for each underlying inflammatory disease but with common features. It would be very interesting to compare the gut microbiota between patients suffering from a specific disease (other than FMF) with or without AA amyloidosis.

(2) Transient involvement of the gut microbiota in AA amyloidosis pathogenesis. It is conceivable that the transient presence of bacteria producing amyloid-enhancing factors is sufficient, within the context of an inflammatory disease, to generate nucleation and to induce a vicious circle. Indeed, SAA protein synthesis is dependent on pro-inflammatory cytokines; in turn, SAA can induce production of IL-1β, IL-6, TNF-α and IL-17A in several cell types [49].

(3) Analysis of the gut microbiota from stool samples.

(4) A difference in bacterial functions and not in bacterial concentrations. Meta-omics studies would be a very interesting approach to address this possibility.

(5) The preponderance of other environmental and/or genetic factors.

Previous studies on cytokines in FMF found increases in IL-6, IL-12, IL-17, IL-18, soluble IL-2 receptor, interferon-γ and TNF-α during an attack, as well as ongoing subclinical inflammation during remission [50–52]. We confirmed that IL-6 and TNF-α but also IL-1β levels were significantly higher in FMF patients than in controls. To our knowledge, no cytokine studies have been performed on AA amyloidosis patients. Although CRP and SAA protein levels were similar between FMF patients without AA amyloidosis and non-FMF-related AA amyloidosis patients, AA amyloidosis patients exhibited higher IL-6 and TNF-α levels. This is consistent with the fact that AA amyloidosis is secondary to chronic inflammation and explains the efficacy of anti-cytokine therapies for this disease [2].

A significant increase in IDO activity was observed in AA amyloidosis patients. This might be secondary to the kidney failure associated with AA amyloidosis, as IDO activity is increased in kidney failure [53]. This may also be explained by the fact that IDO activity is dependent on pro-inflammatory cytokines. Moreover, the tryptophan shortage induced by increased IDO activity enhances the synthesis of pro-inflammatory cytokines by macrophages, possibly inducing a vicious circle in AA amyloidosis [10,12].

Similarly, adiponectin levels were higher in AA amyloidosis patients than in FMF patients without AA amyloidosis, despite the fact that levels of TNF-α and IL-6, which inhibit adiponectin synthesis, are increased in these patients [6]. These differences might be explained in part by kidney involvement [6]. There are reports of adiponectin exerting pro-inflammatory

activities in various tissues and contexts, and it thus may also be involved in AA amyloidosis pathogenesis [54].

The high levels of adiponectin and IDO activity in AA amyloidosis patients may be evaluated as diagnostic biomarkers to identify those at risk for AA amyloidosis among patients with an inflammatory disease. Furthermore, in addition to proteinuria and creatininemia, they might be useful as intermediate markers in therapeutic follow-up for AA amyloidosis. Finally, if the involvement of IDO in AA amyloidosis is confirmed, the use of IDO inhibitors, currently in clinical trials in oncology, may be implemented [55].

CONCLUSION

This study emphasizes the association between FMF and gut microbiota dysbiosis characterized by a decrease in α-diversity and a significant alteration in composition. Although these results are descriptive, they suggest that the gut microbiota might be involved in the clinical expression of FMF. In FMF patients, amyloidosis was independently associated with a specific alteration in the microbiota composition, suggesting that the gut microbiota may play a role in AA amyloidosis pathogenesis. These data need to be further consolidated in mechanistic and interventional studies.

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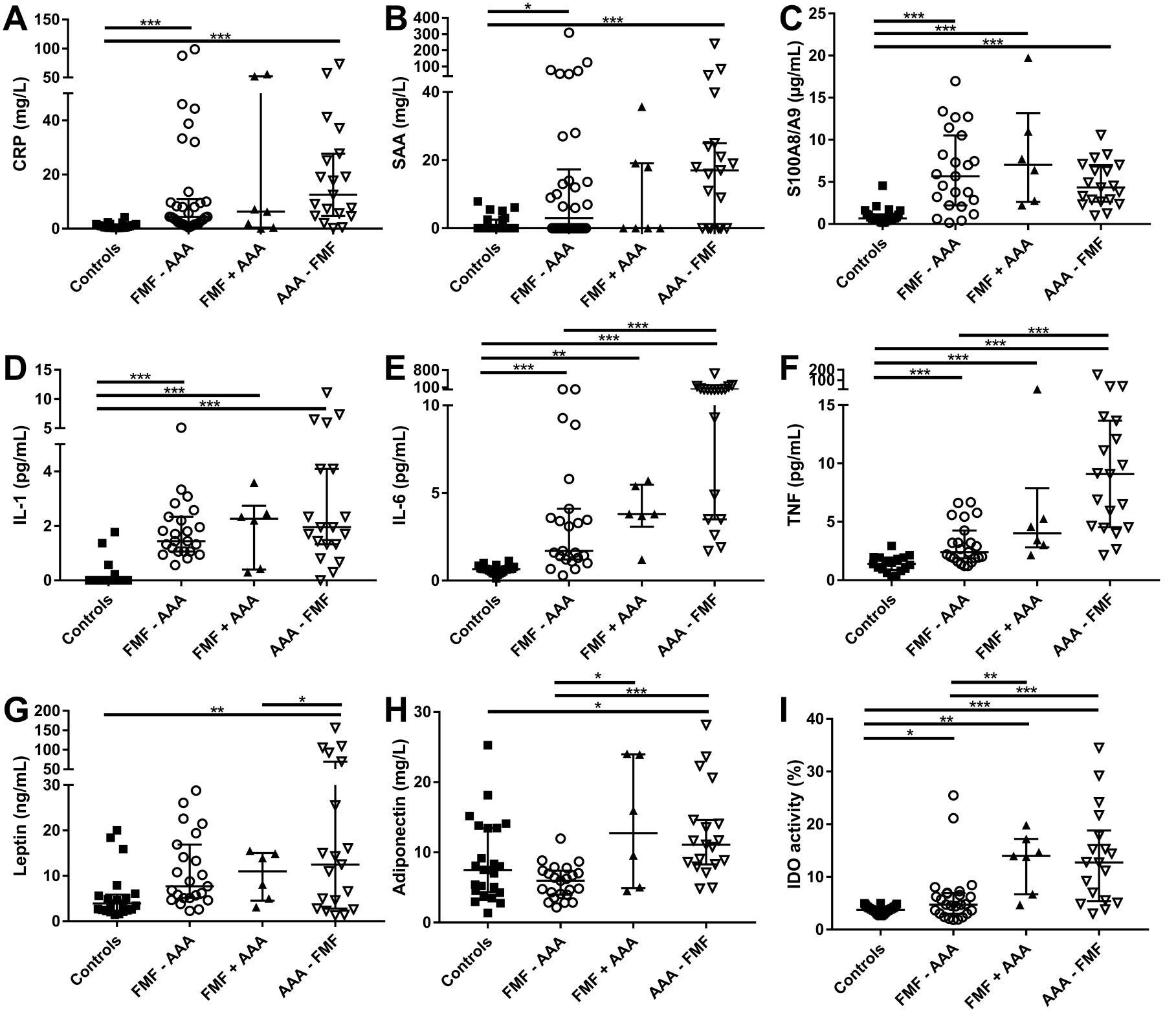
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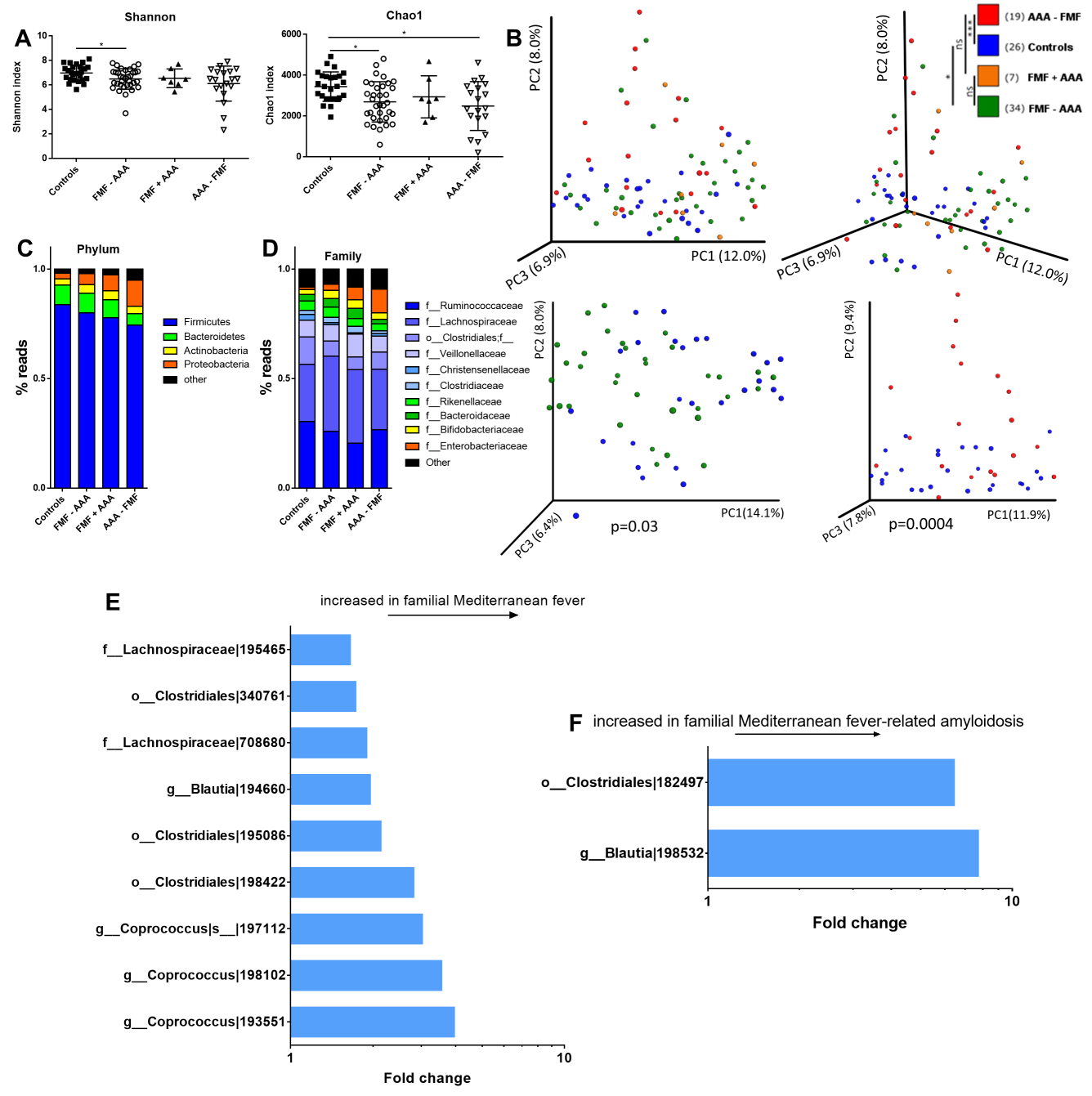
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FIGURE LEGENDS

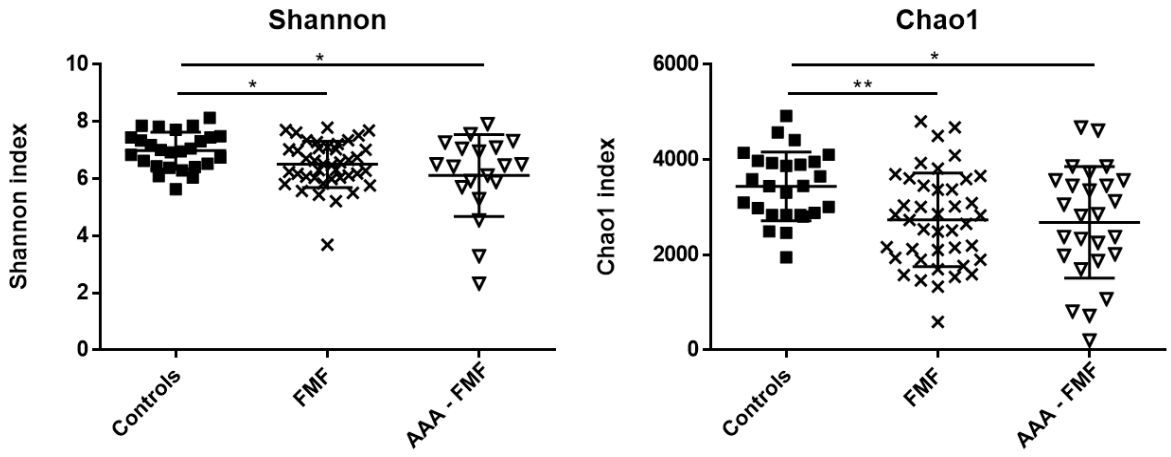
Figure 1. Biological data among the different studied groups. Blood dosages of C-reactive protein (A), serum amyloid A (B), S100A8/A9 proteins (C), interleukin-1β (D), interleukin-6 (E), tumor necrosis factor α (F), leptin (G), adiponectin (H) and indoleamine 2,3-dixoygenase activity (I) are shown as medians and quartiles 1-3 among familial Mediterranean fever patients without (FMF – AAA) or with (FMF + AAA) AA amyloidosis, patients with AA amyloidosis of another cause (AAA – FMF) and healthy controls. Differences between each group were evaluated by Student's t-test for data normally distributed or the nonparametric Mann–Whitney test. The association was considered significant if the p-value was less than 0.05 and if the q-value (i.e., the false discovery rate using the Benjamini-Hochberg correction) was less than 0.25 [* = p <0.05, ** = p <0.01, *** = p <0.001]. *CRP: C-reactive protein; SAA: serum amyloid A; IL: interleukin; TNF: tumor necrosis factor; IDO: indoleamine 2,3-dioxygenase.*

Figure 2. Altered bacterial microbiota composition and diversity in FMF and AA amyloidosis patients. (A) Microbial richness and evenness were calculated based on the Shannon index and microbial richness based on the Chao1 index, and statistical significance was evaluated using the nonparametric Mann-Whitney test. (B) β-diversity. Principal coordinate analysis of the Bray-Curtis distance with each sample colored according to the study population. PC1, PC2 and PC3 represent the 3 principal coordinates that captured most of the diversity, with the fraction of the diversity captured given as a percentage. Differences between the study groups were evaluated by the nonparametric analysis of similarity test (ANOSIM, 9,999 permutations). The global composition of the bacterial microbiota at the phylum (C) and family (D) levels, as expressed as the relative abundance for each group. Differences in bacterial taxon abundance between FMF patients without AA amyloidosis and healthy controls (E) and between FMF patients with or without AA amyloidosis (F) were calculated using the multivariate association with linear models (MaAsLin) statistical method. The fold change was calculated by dividing the mean abundance in each studied group. Only taxa present in more than 50% of the samples were used. The association was considered significant if the p-value was less than 0.05 and if the q-value (i.e., the false discovery rate using the Benjamini-Hochberg correction) was less than 0.25 [* = p <0.05, ** = p <0.01, *** = p<0.001]. *FMF: familial Mediterranean fever; AAA: AA amyloidosis.*

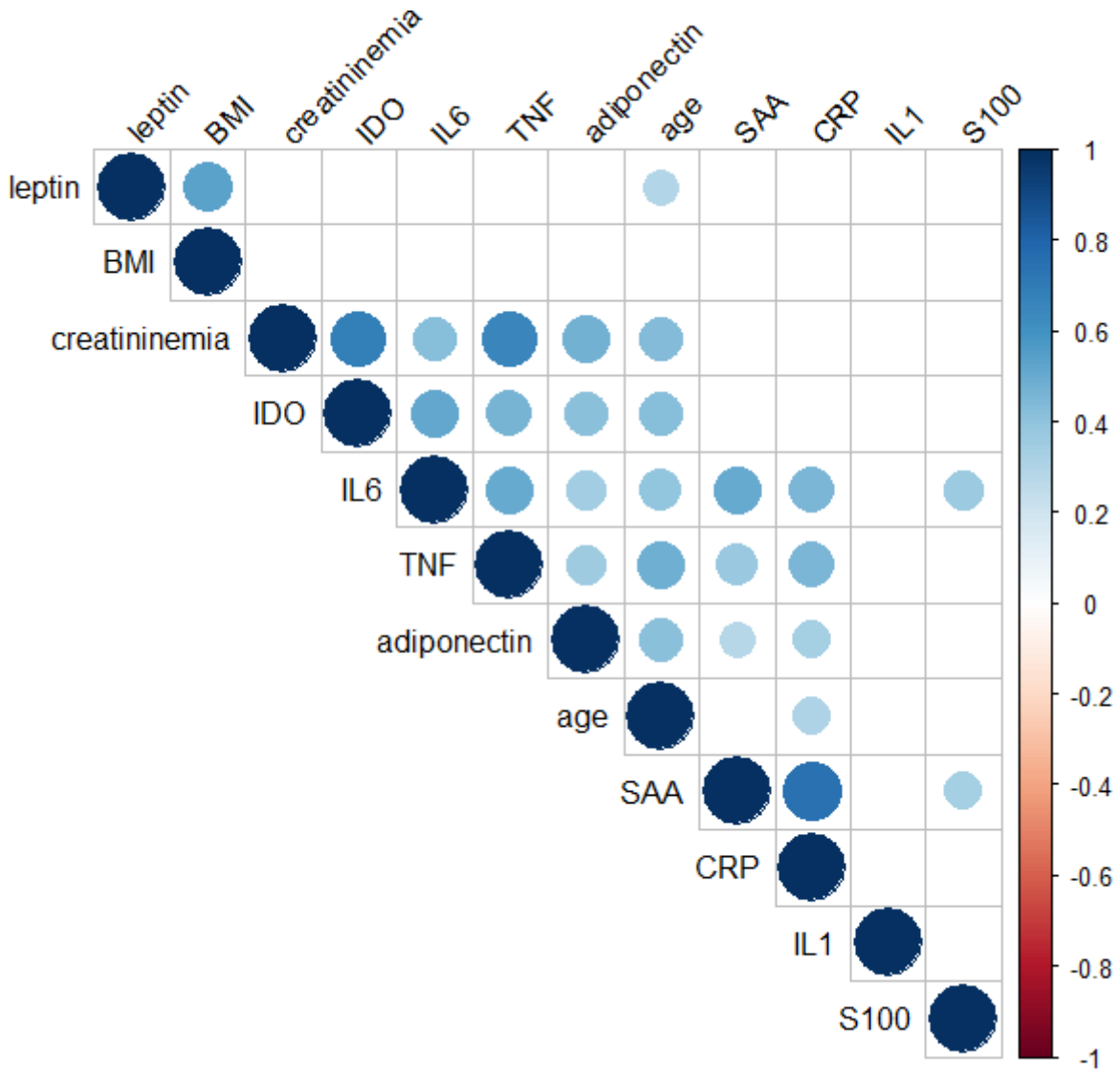

A
CRP (mg/L)
B
SAA (mg/L)
C
S100A8/A9 (µg/mL)
D
IL-1 (pg/mL)
E
IL-6 (pg/mL)
F
TNF (pg/mL)
G
Leptin (ng/mL)
H
Adiponectin (mg/L)
I
IDO activity (%)
Controls
FMF - AAA
FMF + AAA
AAA - FMF


A
Shannon
Shannon Index
Controls
FMF - AAA
FMF + AAA
AAA - FMF
Chao1
Chao1 index
B
PC2 (8.0%)
PC3 (6.9%)
PC1 (12.0%)
(19) AAA - FMF
(26) Controls
(7) FMF + AAA
(34) FMF - AAA
ns
C
Phylum
% reads
Firmicutes
Bacteroidetes
Actinobacteria
Proteobacteria
other
D
Family
f__Ruminococcaceae
f__Lachnospiraceae
o__Clostridiales;f__
f__Veillonellaceae
f__Christensenellaceae
f__Clostridiaceae
f__Rikenellaceae
f__Bacteroidaceae
f__Bifidobacteriaceae
f__Enterobacteriaceae
Other
PC2 (8.0%)
PC3 (6.4%)
PC1(14.1%)
p=0.03
PC2 (9.4%)
PC3 (7.8%)
PC1(11.9%)
p=0.0004
E
increased in familial Mediterranean fever
f__Lachnospiraceae|195465
o__Clostridiales|340761
f__Lachnospiraceae|708680
g__Blautia|194660
o__Clostridiales|195086
o__Clostridiales|198422
g__Coprococcus|s__|197112
g__Coprococcus|198102
g__Coprococcus|193551
Fold change
F
increased in familial Mediterranean fever-related amyloidosis
o__Clostridiales|182497
g__Blautia|198532
Fold change

Supplementary Figure S1. Microbial richness and evenness in healthy controls, familial Mediterranean fever patients and non-familial Mediterranean fever-related AA amyloidosis patients were calculated based on the Shannon index and microbial richness based on the Chao1 index, and statistical significance was evaluated using the nonparametric Mann-Whitney test [* = p <0.05, ** = p <0.01]. *FMF: familial Mediterranean fever; AAA: AA amyloidosis.*



Supplementary Figure S2. Correlations between biological data, age at sampling and body mass index in all patients. The size and color intensity of the circles are proportional to the correlation coefficient, calculated with the nonparametric Spearman correlation test. Only statistically significant correlations ($p<0.05$ and q-value, i.e., the false discovery rate using the Benjamini-Hochberg correction was less than 0.25) are indicated. *TNF: tumor necrosis factor; SAA: serum amyloid A; CRP: C-reactive protein; BMI: body mass index; IDO: indoleamine 2,3-dioxygenase; S100: S100 A8/A9; IL: interleukin*.



Supplementary Table 1. Characteristics of familial Mediterranean fever patients.

Patient	Sex	Age at stool sampling	Parental country of origin	*MEFV* genotype	Current treatment
1	Male	27	Armenia	M694V	Colchicine
2	Male	30	Morocco/Tunisia	M694V / M694V	Colchicine
3	Female	48	Algeria/Tunisia	M694V / M694V	Colchicine
4	Female	67	Morocco	M694V / M694V	Colchicine
5	Male	25	Armenia	M694V / M694V	Colchicine
6	Male	29	Algeria	M694V / M694V	Colchicine
7	Female	39	Tunisia/Morocco	M694V / M694V	Colchicine
8	Female	29	Tunisia/Morocco	M694V / M694V	Colchicine
9	Female	21	Tunisia/Turkey	M694V / V726A	Colchicine
10	Male	76	Algeria	M694V / M694V	Colchicine
11	Female	41	Algeria	M694V / M694V	Colchicine
12	Male	69	Algeria	M694V / M694V	Colchicine
13	Male	25	Armenia	M680I / V726A	Colchicine
14	Female	55	Armenia	M694V / M694V	Colchicine
15	Male	51	Turkey	M694V / M694V	Colchicine
16	Female	31	Armenia	V726A / F479L / E148Q	Colchicine
17	Male	53	Morocco	M694V / M694V	Colchicine + anti-interleukin-1
18	Male	46	Egypt	M694V	Colchicine
19	Male	76	Tunisia	M694V / M694V	Colchicine
20	Male	52	Morocco	M694V / V726A	Colchicine
21	Male	43	Morocco	M694V / M694V	Colchicine
22	Female	58	Morocco/Algeria	M694V / M694V	Colchicine + anti-tumor necrosis factor-α
23	Male	45	Armenia	M694V / M694V	Colchicine
24	Female	56	Armenia	M694V / M694V	Colchicine + anti-interleukin-1
25	Female	33	Tunisia	M694V / M694V	Colchicine
26	Male	34	Morocco	M694V / L695R	Colchicine
27	Female	41	Armenia	M694V / V726A	Colchicine
28	Female	60	Syria	M694V / M694V	Colchicine
29	Male	56	Syria	M694V / M694V	Colchicine
30	Male	54	Morocco/Algeria	M694V / M694V	Colchicine
31	Female	39	Tunisia	M694V / M694V	Colchicine
32	Female	78	Tunisia	M694V / M694V	Colchicine
33	Male	18	Algeria/Israel	M694V	Colchicine
34	Male	56	Israel	M694V / M694V	Colchicine

Supplementary Table 2. Characteristics of AA amyloidosis patients.

Patient	Sex	Age at stool sampling	Cause of AA amyloidosis	Tissues biopsied for AA amyloidosis diagnosis	Current treatment
1	Male	73	Familial Mediterranean fever M694V/M694V	Minor salivary glands	Colchicine + anti-interleukin-1
2	Female	56	Familial Mediterranean fever M694V/M694V	Minor salivary glands	Anti-interleukin-1
3	Female	65	Familial Mediterranean fever M694V/M694V	Kidney	Colchicine
4	Female	45	Familial Mediterranean fever M694I/M694I	Kidney	Colchicine
5	Male	42	Familial Mediterranean fever M694V/M694V	Kidney	Anti-interleukin-1
6	Female	47	Familial Mediterranean fever M694V/M694V	Kidney	Colchicine
7	Male	54	Familial Mediterranean fever M694V/M694V	Minor salivary glands and duodenum	Colchicine + anti-interleukin-1
8	Male	40	Pyrin-Associated Autoinflammation with Neutrophilic Dermatosis	Colon	Anti-tumor necrosis factor-α
9	Female	69	Rheumatoid arthritis	Kidney	None
10	Female	54	Fabry disease	Kidney	Colchicine + anti-interleukin-1 + enzyme replacement therapy
11	Female	68	Obesity	Kidney	None
12	Female	64	Psoriatic rheumatism	Kidney	Anti-tumor necrosis factor-α + steroids + sulfasalazine
13	Male	50	Unknown	Minor salivary glands and colon	Colchicine
14	Female	66	Obesity	Kidney	None
15	Male	47	Crohn's disease	Kidney	None
16	Female	67	Rhupus syndrome	Kidney, minor salivary glands,	Anti-interleukin-6

				digestive tract, skin and bladder	
17	Male	47	Unknown	Kidney and spleen	Anti-interleukin-6
18	Male	74	Waldenstrom's macroglobulinemia with *MyD88* mutation	Kidney, minor salivary glands and rectum	Anti-interleukin-1
19	Male	67	Unknown	Kidney	None
20	Female	60	Human immunodeficiency virus infection	Kidney and liver	Anti-retroviral therapy
21	Male	74	Schnitzler syndrome	Kidney	Anti-interleukin-1
22	Male	33	Other genetic etiology	Minor salivary glands and skin	None
23	Male	77	Unknown	Minor salivary glands	None
24	Female	76	Obesity	Kidney	None
25	Male	40	Synovitis, Acne, Pustulosis, Hyperostosis and Osteitis syndrome	Kidney	Anti-interleukin-6
26	Male	43	Tumor necrosis Receptor Associated Periodic Syndrome	Digestive tract	Anti-interleukin-6