Gut microbiota between environment and genetic background in Familial Mediterranean Fever (FMF)

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Review

Gut microbiota between environment and genetic background in Familial Mediterranean Fever (FMF)

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Abstract: The gastrointestinal tract hosts the natural reservoir of microbiota since birth. The microbiota includes various bacteria which establish a progressively mutual relationship with the host. Of note, the composition of gut microbiota is rather individual-specific and, normally, depends on both the host genotype and environmental factors. The study of the bacterial profile in the gut demonstrates that dominant and major phyla are present in the gastrointestinal tract with bacterial density gradually increasing from proximal to distal segments of the gut. At this latter site, the estimated density reaches 10^{11} to 10^{12} bacteria per gram of colonic content. The cross-talk between host gut and microbiota has a major physiological role in metabolic, protective, and structural functions. Dysbiosis can develop in several conditions due to aging, diseases, inflammatory status, and antibiotic therapy. Growing evidences show that the microbiota might also play a role in FMF, by qualitative and quantitative changes of bacterial population. To which extent such perturbations of the microbiota are relevant in driving the phenotypic manifestations of FMF with respect to genetic background, remains to be further investigated.

Keywords: amyloidosis, colchicine, inflammasome, interleukin-1b, *MEFV*

1. Introduction

The gut develops as a natural ecosystem hosting a complex polymicrobial community, referred to as microbiota. The microbiota can undergo major changes during healthy status and diseases[1,2]. The resident symbiotic microorganisms have progressively adapted to a number of factors, i.e., local environment, the host immune responses, antibiotic therapies, and several other conditions [3-5]. In the human gut, there are thousands of different microbial species [6], which influence many facets of human health and disease. Examples include inflammatory bowel disease, irritable bowel syndrome (IBS), periodontal disease, atherosclerosis, rheumatoid arthritis, multiorgan failure, obesity, diabetes mellitus, allergy, and colon cancer. The exact role of intestinal microbiota in other conditions characterized by recurrent genetically-driven auto-inflammatory diseases is largely unknown. Familial Mediterranean Fever (FMF) is an example of monogenic autoinflammatory disease due to

MEFV gene pathogenic variants which lead to a dysfunctional active state of the pyrin protein eliciting proinflammatory cytokine release and pyroptosis (cell death).

Studies focusing on human twins [7], murine quantitative trait loci [8], and genome-wide associations [9], suggest that genes can drive the composition of gut microbiome. This step becomes mainly host genotype-dependent [10,11]. The knowledge of mechanisms linking the genome with the abundance and the composition of gut microbiome, however, deserves further studies. A main interfering factor is the role played by gene-environment interactions [12]. This topic is of great interest, since gut microbiota plays a crucial role in human health and in the maintenance of systemic homeostasis, and several pathological conditions have been linked to variations in the amount and/or composition of the microbiome. On the other hand, the therapeutic manipulation of gut microbiota is a possibly relevant and innovative tool, in particular during diseases characterized by a chronic inflammatory status. Based on genetic (i.e. monogenic disease) and phenotypic characteristics, patients with Familial Mediterranean Fever (FMF) can represent an interesting model to assess the role of genome and environmental variables, in shaping the composition of gut microbiota. A comprehensive view of these dynamics should be useful, in particular, in the management and prevention of acute attacks and complications in FMF patients.

Here, we review critical aspects of the development, distribution, and function of microbiota in the human gastrointestinal tract, with emphasis on the emerging relationship between qualitative and quantitative changes in FMF.

2. Familial Mediterranean Fever

FMF is a rare monogenic autoinflammatory disease which belongs to the group of "periodic fever syndromes". The estimated number of patients with FMF is 150,000 and this feature makes FMF the most common autoinflammatory disease worldwide [13]. FMF occurs more often in individuals of Turkish, Armenian, North African, Jewish, and Arab descent, and in the Mediterranean basin. Cases of FMF also occur in different populations such as Greeks, Italian, and even Japanese [14,15]. Many patients with FMF describe their first attack in early childhood, i.e., before the ages of 10 (65%) and 20 years (90%). Depending on genetic penetrance and phenotypic characteristics, however, the initial attack can occur in subjects older than 50 years of age [14] with extremely delayed onset represented by one case diagnosed at the age of 86 [16].

The diagnosis of FMF relies mainly on the clinical ground together with ethnic origin, family history, and genetic assessment [13,17-20]. Genetic testing can help but it is challenging, since there are 377 mutations in *MEFV* so far (Infevers: an online database for autoinflammatory mutations. Available at https://infevers.umai-montpellier.fr/ accessed 28.07.2020) [21-24]. Whereas some *MEFV* variants appear as clearly pathogenic, many variants are common in the general population and some others have still unknown features in causing the disease [25]. In line with such difficulties in genetic classification of *MEFV* mutations, authors have recently pointed to novel classification tools looking at pathogenicity of variants with recent improvement in *MEFV* variants classification [21,26,27]. On chromosome 16 (16p13.3), the *MEFV* gene (made of 10 exons) encodes for a 781-amino-acid ~95kDa protein named pyrin (also referred to as "marenostrin", TRIM20), a pattern recognition receptor [28-32]. Pyrin is part of the complex molecular platforms involved in the response of the innate immune

system and related cells, originally designed as first-line, fast response to components of pathogenic bacteria. Cells involved in the innate immune system response are macrophages, monocytes, dendritic cells, and neutrophils (myeloid lineage), which express a variety of pattern recognition receptors (PRRs). PRRs, in turn, detect pathogen-associated molecular patterns (PAMPs). The family of Toll-like receptors (TLRs) are membrane-bound PRRs sensing PAMPs in the extracellular milieu and in different types of intracellular endosomes [33]. TLR activation is associated to the expression of proinflammatory factors which induce cytokine release, i.e., as NF-κB. Cytosolic pathogen recognition sensors are the family of nucleotide-binding domain leucine-rich repeat (NLR) proteins, namely NLRP1, NLRP3, NLRP7, and NLRC4, the protein absent in melanoma 2 (AIM2), and pyrin [34]. These cytosolic sensors detect pathogens and endogenous danger-associated molecular patterns (DAMPs) which trigger the intracellular formation of multiprotein complexes, i.e., inflammasomes [35].

A common feature of inflammasomes is their capability to mediate the activation of caspase-1 and, in turn, to promote the release of the proinflammatory cytokines IL-1β and IL-18. Another step is the induction of inflammatory cell death via pyroptosis, with cellular swelling and lysis, at variance with apoptosis. Pyroptosis requires the caspase-1-mediated cleavage of gasdermin D (GSDMD), translocation of the fragment N-terminal pore-forming domain to the cellular membrane, and release of pro-inflammatory cytokines [36,37]. Notably, the direct binding of lipopolysaccharide (LPS) to caspase-4 and 5 in human cells (caspase-11 in mouse cells) will also result in caspase oligomerization, cleavage of GSDMD, and pyroptosis. In general, pyroptosis appears to amplify the protective immune responses during an infection [36].

The "wild-type" pyrin senses the inactivation of the small RhoA guanosine triphosphatase (RhoA GTPase), originally performed by bacterial toxin, and this step leads to a variety of signal transduction pathways resulting in the formation of a multi-protein complex (inflammasome). Pyrin binds to several effector proteins, such as the serine/threonine-protein kinases PKN1 and PKN2 and actin-binding proteins. RhoA activation is associated to PKN-mediated phosphorylation-dependent pyrin inhibition. The inflammasome also contains the bridging molecule ASC (apoptosis-associated speck-like protein containing a caspase recruitment domain), and the protease caspase-1 [29,35,38,39]. (**Figure 1**).

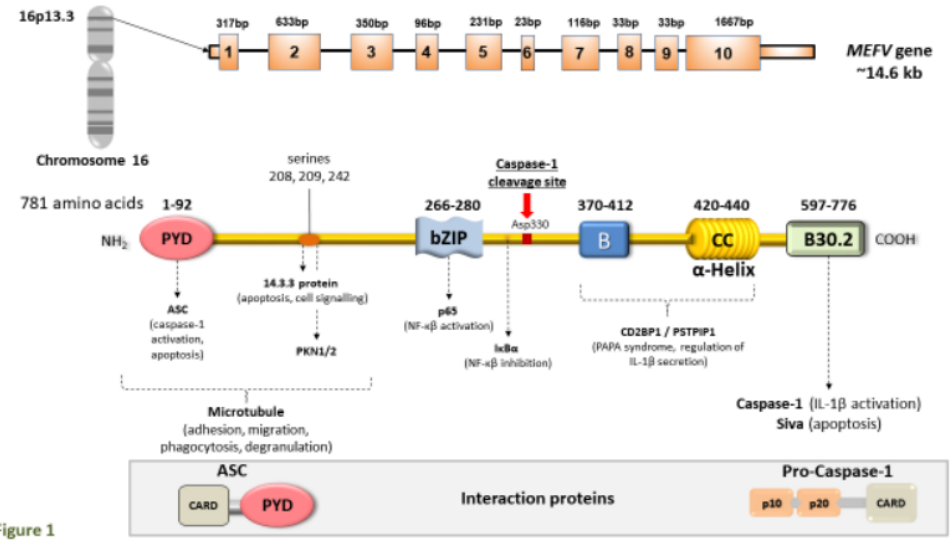

Figure 1

Figure 1. Schematic structure of *MEFV* gene and encoded pyrin (marenostrin) protein

Pyrin protein is encoded by the *MEFV* gene, and consists of 781 amino acids. The most common mutations in FMF are in exon 10 encoding the B30.2 domain (via autosomal-recessive fashion, rather than autosomal-dominant fashion, as for exon 2, 3 and 5). Most important interaction partners appear below the pyrin structure. ASC and Pro-Caspase 1 are also drawn. Pyrin structure includes five different domains, each one responsible for protein-protein interaction, and each domain has a role in the regulation of innate response. From left to right, PYRIN (PYD) domain (residues 1-92) interacts with ASC (apoptosis-associated speck-like protein containing a CARD -caspase-recruitment domain). bZIP transcription factor basic domain (residues 266-280) interacts with the p65 subunit (transcription factor p65) of NF-κB, and IκB-α. B-box zinc finger domain (residues 375-412) and α-helical (CC, coiled-coil) domain (residues 420-440) likely play a role in the oligomerization of pyrin, and interact with the PAPA protein (also named PSTPIP1, proline serine threonine phosphatase-interacting protein, also known as CD2BP1 involved in the organization of the cytoskeleton) and regulation of IL-1β secretion. B30.2 domain (PRYSPRY) (residues 597-776) is the most important, and interacts with caspase-1 and the proapoptotic protein Siva. Further pyrin interactions include binding to microtubule (starting from the N-terminal to bZIP), interaction with 14.3.3 (14-3-3 protein), and with the PKN1/2 (serine-threonine kinases PKN1 and PKN2) at the three serine residues 208, 209, 242 between PYD and bZIP. The position of Asp330 between bZIP and the B-box indicates the caspase-1 cleavage site. Mutations in the B30.2 domain tend to be transmitted in an autosomal-recessive fashion. Mutations in exons 2, 3 and 5 generally exhibit autosomal-dominant pattern of inheritance [15,25-27,32,34,39-49].

The activated inflammasome will then govern the step of pyroptosis, i.e., a pro-inflammatory cell death mode which relies on innate immune response by myeloid cell lineage with release of pro-inflammatory cytokines IL-β1 and IL-18 [36,38,50]. Mutations in the *MEFV* gene are associated with impaired function of pyrin, which becomes insensitive to the microtubule control [32]. This, in turn, leads to increased serum levels of IL-1β, and increased inflammation (**Figure 2 A, B**).

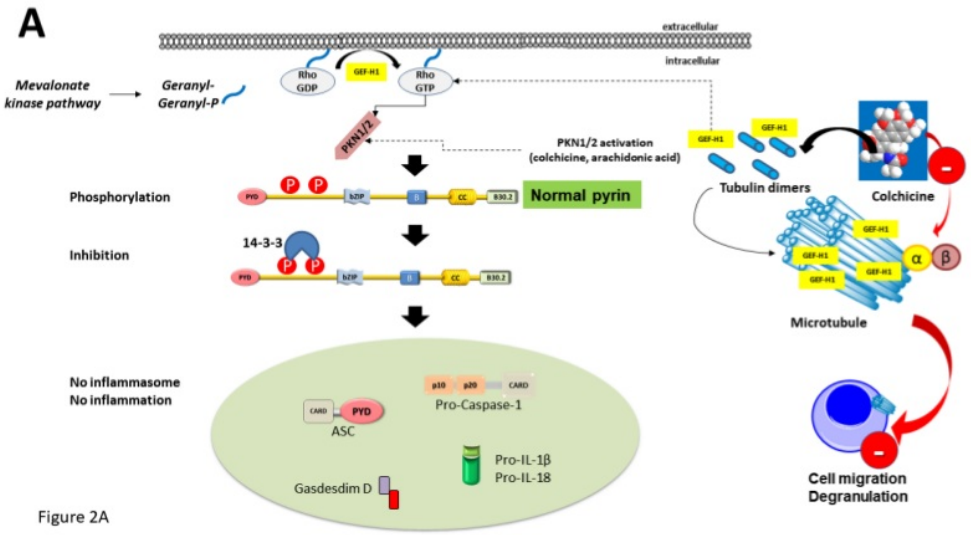

Figure 2A

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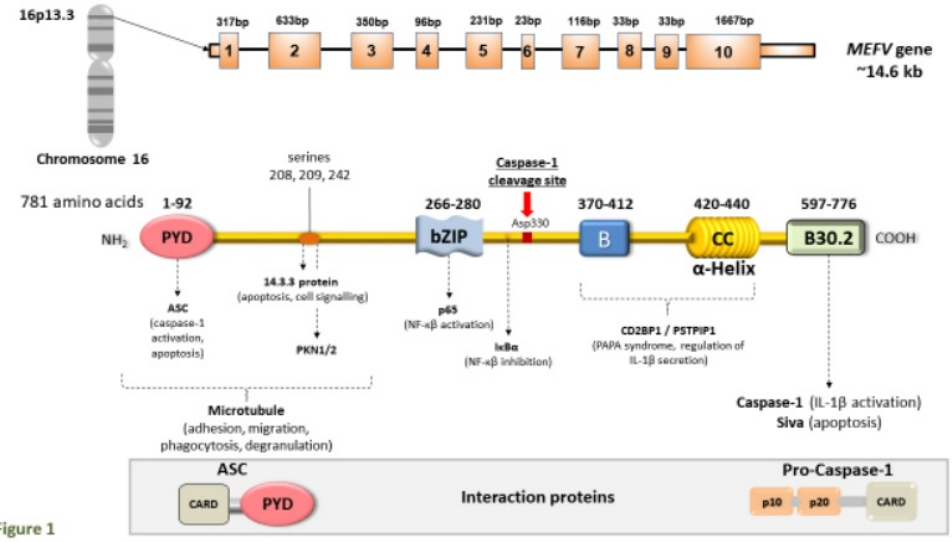

Figure 1

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148 **Figure 2. Mechanisms underlying the assembly of the pyrin inflammasome.**

149 A) In the normal condition, with the functioning pyrin, the mevalonate kinase pathway provides geranyl-
150 geranyl phosphate and, together with release of GEF-H1 (increased by colchicine acting as the inhibitor of the
151 microtubule polymerization), activates RhoA. PKN1 and PKN2 are effector kinases of RhoA mediating the
152 phosphorylation of pyrin and binding to the inhibitory proteins 14-3-3. Inhibition of pyrin can increase with
153 agents activating PKN1/2 or following the release of GEF-H1 (i.e. colchicine).
154 B) If pyrin phosphorylation decreases, i.e., mutated pyrin (i.e., FMF lacking the control by pyrin/marenostrin,
155 due to the mutated *MEFV* gene), low GEF-H1 or defective MVK-pathway function, the activation of PKN1/2 also
156 decreases. This step results in pyrin inflammasome activation and release of mature IL-1β and IL-18. The plasma

membrane pore-forming N-terminal fragment of gasdermin D facilitates IL-1β and IL-18 release. Appropriate stimuli also lead to the assembly of the inflammasome. The first step is the PYD-PYD homotypic interaction of ASC resulting in oligomerization into ASC speck. Pro-caspase-1 is recruited because of CARD-CARD interaction with ASC. This step anticipates the auto-cleavage of pro-caspase-1 into active caspase-1 tetramers (p10/p20) governing the transformation of pro-IL-1/18 into mature IL-1/18. The pyroptosis mediated by Gasdesmin D also contributes to cytoplasmic enrichment with IL-1/18, and further reinforces the inflammatory pathway. Colchicine inhibits the polymerization of intracellular β-tubulin by forming colchicine-tubulin complexes via contact with A and C rings to the C domain of β-subunit of tubulin. This effect blocks the induction of inflammasome in neutrophils and monocytes. This step prevents the dockage of tubulin into the (+) ends of microtubules (cytoskeleton). This colchicine-dependent inhibition of tubulin effectively interferes with white blood cells migration and degranulation [34,41].

On the clinical ground, FMF consists of periodic recurrent febrile attacks, serositis at one or more sites manifesting with abdominal pain, chest pain, joint pain, erysipelas-like dermatitis (meaning limited erythematous skin rash), myalgia, arthralgia, and acute pericarditis. Depending on *MEFV* variants involved, symptoms may appear in childhood and the episodes of fever with abdominal/chest pain usually resolve within 48–72 h (**Figure 3**).

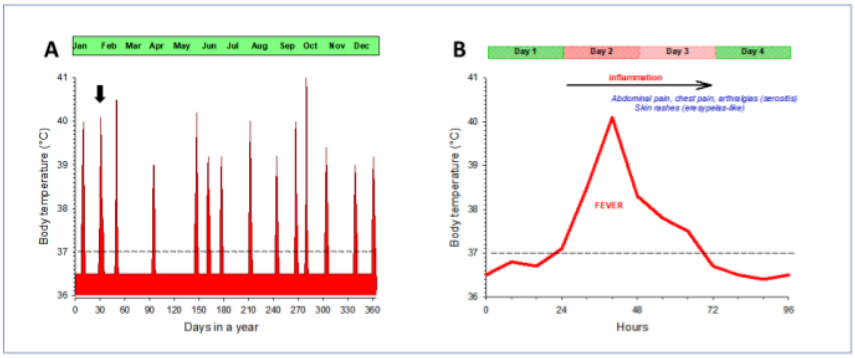

Figure 3

Figure 3. Schematic appearance of time-dependent changes of body temperature in Familial Mediterranean Fever (FMF) before starting the treatment with colchicine.

The profile refers to a typical case belonging to a cluster of families identified in the region of Apulia, Italy [15]. A) Frequency of febrile attacks in a year. The dotted horizontal line represents the cut-off value of 37°C. In between attacks, the temperature has been conventionally set at 36.5°C. This patient reported a total of 14 attacks in a year. The black arrow indicates the febrile attack described in panel B. B) The single febrile attack lasts about 48 hours and is associated with a major auto-inflammatory status and symptoms. Adapted from Portincasa *et al*.

Familial Mediterranean fever: a fascinating model of inherited auto inflammatory disorder. Eur J Clin Invest 43, 1314-1327 (2013) with permission from John Wiley & Sons Ltd [15].

Acute attacks of FMF produce elevation of serum markers of systemic inflammation, including leukocytosis (especially neutrophils), increased erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), fibrinogen, and serum amyloid A (SAA) protein. Depending on *MEFV* variants and intensity of attacks, long-term complications may include secondary (AA) amyloidosis leading to asymptomatic proteinuria, nephrotic syndrome and end-stage kidney disease, small bowel obstruction due to recurrent attacks of peritonitis and adhesions, and even infertility, especially in female patients due to fallopian tube obstruction [15,25,51]. The M694V variant, located in exon 10 of the *MEFV* gene, causes the most severe disease, both in patients homozygous and compound heterozygous for M694V [52,53]. The same is true for variants M694I and M680I, while R761H (or M680Q/R761H) has less penetrance and causes milder symptoms [15,26]. Therapy of FMF should prevent acute attacks, and minimize subclinical inflammation in between attacks. In the most clinically-evident cases, the appropriate therapy will also prevent the development and progression of amyloidosis. All guidelines suggest colchicine as the initial treatment of FMF, with treatment started as soon as a diagnosis of FMF is established and continued indefinitely. In addition, colchicine is effective as a prophylactic treatment for FMF attacks, and for this purpose, all patients should start with colchicine, regardless of the frequency and intensity of attacks [15,19,54,55] (**Figure 2A** and **Figure 4**). A subgroup of patients defined as resistant or intolerant to colchicine needs treatment with alternative biological agents targeting IL-1 inhibition. The human immunoglobulin (IgG) antibody canakinumab targets IL-1β and is effective in FMF [40,42,56-59] **(Figure 4** and **Figure 5**).

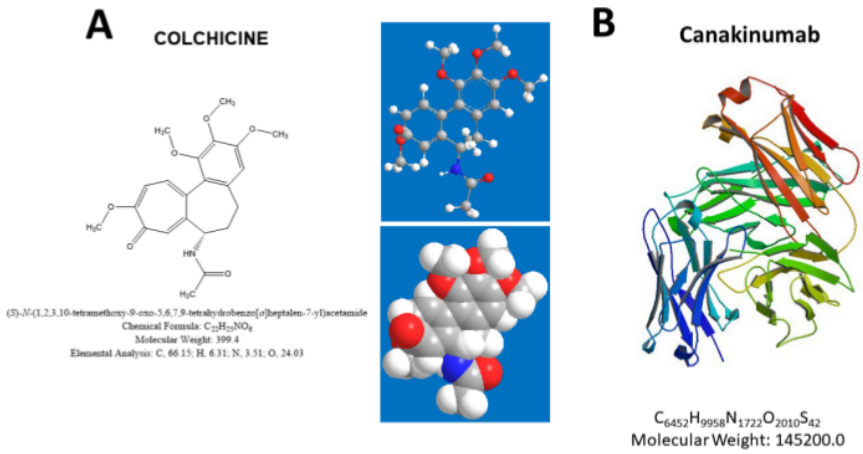

Figure 4

Figure 4. Therapeutic agents effective in FMF.
A) Colchicine: chemical structure, IUPAC names, chemical formula, molecular weight and 3D structures. B) Canakinumab: 3D structure, chemical formula, and molecular weight.

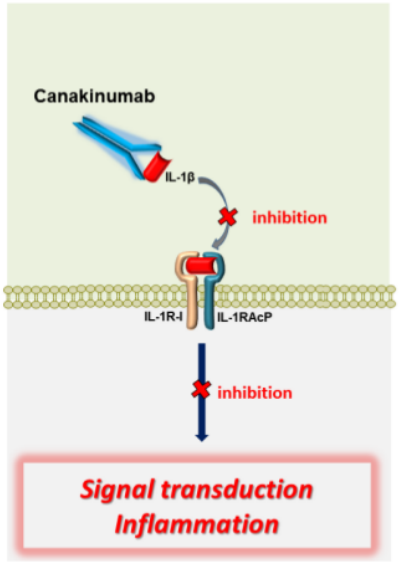

Figure 5

Figure 5. Mechanism of action of biologic agent canakinumab in FMF.

Canakinumab is a fully human selective anti-IL-1β monoclonal antibody and binds human IL-1β. Subsequent binding of IL-1β to the IL-1R is inhibited with prevention of the intracellular signal transduction events and further proinflammatory. Abbreviations: IL, interleukin; IL-1R-I, interleukin-1 receptor, type1; IL-1RAcP, IL-1 receptor accessory protein. Adapted from [40-42].

3. Gut microbiota

Gut microbiota consists in a huge collection of microbes in the human gut. The human microbiota is responsive to lifestyles, age, birth mode, and contributes to maintaining the mucosal barrier [60]. Gut microbiome also contribute to maintain a healthy function of the epithelial intestinal cells and of the immune system [61], producing local peptides with antimicrobial function and immunoglobulins [62-64]. Differences in the gut microbiome composition exist between elderly and younger individuals [65].

3.1 *Development of the gastrointestinal microbiota*

The interaction between microbiota and the human gut begins very early in life.

Growing body of evidence indicates that the intrauterine environment is not sterile since the maternal-fetal transmission of microbiota occurs during pregnancy [66]. The genetic background of the infant may influence gut microbial colonization but also the gut microbiota is affected by many prenatal factors (maternal diet, obesity, smoking status, and use of antibiotic agents during pregnancy). At birth, the neonate also acquires bacteria from the mother and the external environment [67,68]. Facultative aerobes anticipate the increase in strict anaerobes [69]. *Bifidobacteria* are more prevalent during breast- or formula feeding [70-72]. Feeding with solid food in the infant is associated with further microbial changes and diversity and already in the infant the gut microbiota resembles that of the adult [73]. Factors such as environmental and genetic influences, type of delivery, maternal and infant diet, gestational age, and exposure to antibiotics will contribute to changes of type of microbiota in infancy [5,74]. According to this scenario, the gut microbiota

stabilizes after the first 12 months of life.

3.2 Characterization of the gut microbiota

Most of the gut bacterial species cannot be cultivated [70,75-78], while culture-independent molecular approaches provide information on composition and diversity of the gut microbiota [79-83]. Techniques include sequencing and phylogenetic micro-arrays [84,85], studies of metagenomes to compare assembled sequences to reference databases [3]. Metaproteomics, metabolomics and metatranscriptomics require methodologies that are more sophisticated. The Human Microbiome Project (HMP) employed the 16S and metagenomic profiling to investigate the microbial communities from multiple body sites from healthy individuals, the relation between microbiome and diseases, and to identify a standardized and useful dataset [86,87]. The human intestinal microbiome contains about 3.3 million microbial genes, and this amount is about 150 times greater than the human genome [88]. The human body contains a large global number of species-level phylotypes (> 1,000) but phylogenetic individual diversity is low, with only 160 different bacterial species [88]. Few bacterial phylotypes are prevalent among individuals, sharing a phylogenetic core [89].

3.3 Bacterial communities in the human gut

In healthy conditions, the dominant phyla are *Bacteroides* and *Firmicutes* [90,91] while minor phyla are *Proteobacteria, Actinobacteria, Fusobacteria, Spirochaetes, Verrucomicrobia,* and *Lentisphaerae* [10,84]. Microbial density increases along the human gut raising from 10^4–10^8 cells in the jejunum and ileum to 10^{10}–10^{12} cells in the colon and faeces [3,92]. Different microbiota ecosystems change depending on intestinal pH, redox potential, supply of nutrients, intestinal motility, and secretions [3]. (**Figure 5**).

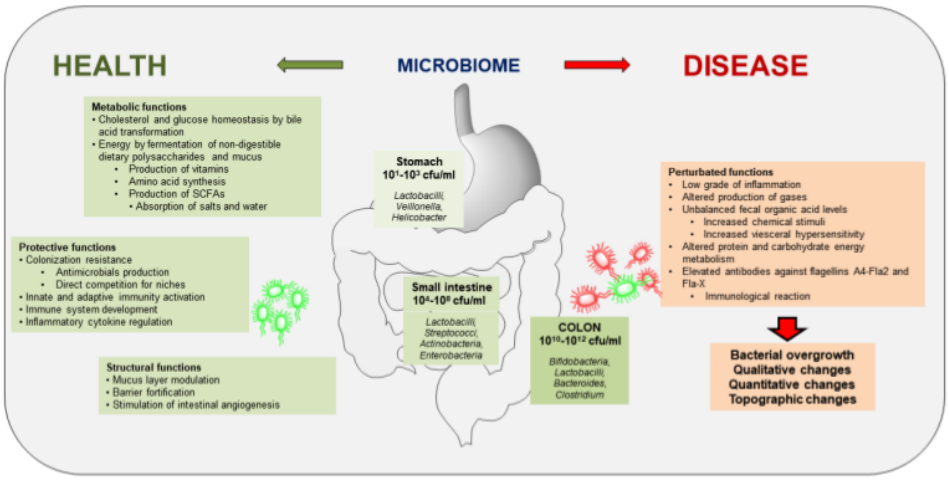

Figure 6

Figure 6. Distribution and function in health and disease of human microbiome in the gut.

The microbiome is the core of bacterial community in the gastrointestinal tract. In healthy subjects, bacteria show variations in microbial density and types across the intestine with density increasing dramatically from the stomach to the colon. Microbial composition also changes. The crosstalk between bacteria and host contribute to maintain physiological metabolic, protective and structural functions. In disease, qualitative and/or quantitative alterations of microbiome occur, including low-grade inflammation, abnormal gases and faecal organic acid levels, impaired protein and carbohydrate energy metabolism and immunological responses [2,93-97].

Few species of bacteria, i.e., *Lactobacilli*, *Enterococci*, oral *Streptococci* and other gram-positive aerobic or rare facultative anaerobes from the oropharynx prevail in the jejunum (concentrations of 10^4 CFU/mL of jejunal content). The microbiota at this level is sensitive to and controlled by physiological factors including pH, bile acids, pancreatic secretion, and gastrointestinal motility [92]. The ileum becomes a more favourable environment for microbial growth where *enterobacteria* and other coliforms reach a concentration of 10^9 CFU/mL [53]. Again, peristalsis in the small intestine and appropriate gastric acid secretion prevent qualitative, quantitative and/or topographic changes of the intestinal microbiota, namely the bacterial overgrowth [98]. The colon hosts up to 10^{12} CFU/mL of several bacterial species, mainly anaerobes (*Bifidobacteria*, *Lactobacilli*, *Bacteroides*, and *Clostridium*) [99]. In the faeces, anaerobic bacteria predominate with a great diversity (range 3,000 to 5,000 species) [100]. About 90% of the faecal mass is made of bacteria and optimal temperature and viscosity can facilitate bacterial growth. The human gut hosts three bacterial enterotypes, not country- or continent-specific. These clusters extract energy and produce vitamins differently [101].

3.4 Functions of the intestinal microbiota

The gut microbiota plays a key role for metabolic, protective and structural activities.

- Metabolic functions include extraction of energy from indigestible dietary polysaccharides. Bacteria also release nutrients not produced by the human host (vitamin B complex and vitamin K) [102]. In addition, bacteria produce amino acids, and short-chain fatty acids (SCFAs: acetate, propionate, and butyrate). In the colon, the microbiota has a major role in the biotransformation of primary to secondary bile acids during the ongoing enterohepatic re-circulation of bile acids [96]. In turn, bile acids contribute to the suppression of significant bacterial colonization of the small intestine [103,104]. Shift towards different patterns of bile acids and bile acid pool may affect cholesterol and glucose homeostasis [105], as well as other metabolic pathways [106,107]. Gases and SCFAs [108] derive from the fermentation of undigested dietary carbohydrates by bacteria within the intestinal lumen. SCFAs, in turn, contribute to the protection of the colonic mucosa [108]. SCFAs also influence the lipogenesis in adipose tissue and cholesterol synthesis [109], and have hypocholesterolemic action by inhibiting liver cholesterol synthesis [108,110]. In obese subjects, gut microbiota harvests energy more effectively from otherwise non-digestible carbohydrates. Mechanisms include increased production of SCFA and decreased intestinal expression of *Fiaf* (fasting induced adipose factor), with higher availability of fatty acids to the liver and adipose tissue [90,111-114].

- The protective activity of the intestinal microbiota consists of prevention of pathogenic colonization, development of the immune system, and regulation of inflammatory cytokines. The microbial community prevents the infections by pathogens via production of antimicrobial peptides, but also directly competes for metabolic niches [115]. In addition, the intestinal microbiota influences the development and balance of the immune system, contributing to B cell development [116], regulatory T cells, T helper type 1, 2 and 17 cells [117]. SCFAs *per se* may have an immunomodulatory effect by regulating inflammatory responses [118,119].
- Structural activity. The microbiota interacts with the mucus layer which acts as a barrier to inflammatory molecules [120]. The SCFA butyrate improves the colonic defensive border [121]. Further beneficial effects derive from the strong immunoactivating activities of microbiota and components like LPS, peptidoglycans, superantigens, bacterial DNA, heat shock protein (Hsp).

2.5 *The microbiota gut–brain axis*

The "brain-gut axis" is a bidirectional pathway which involves neural pathways and immune cells [122]. More extensively, the "microbiota-gut-brain axis" is a system where intestinal microbiota and brain communicate through immune-endocrine-neuronal pathways and could modulate mood, behaviour, and perception [123-125]. The central nervous system (CNS), the neuro-endocrine and neuro-immune systems, the autonomic nervous system (ANS), the enteric nervous system (ENS), and the intestinal microbiota are all involved in this complex scenario where GI function can modulate brain signalling [125]. Indeed, gut microbiota plays a role in the stress response, anxiety [126-128], and also memory function [129]. In animal models exposed to infection or inflammation the intestinal microbiota modulates the system via a neural protection [129-131] and can modulates behaviour [132]. Brain development could be modulated via neuronal circuits involved in motor control and anxiety behaviour [127]. Indeed, gut bacteria might interact with the brain derived neurotrophic factor (BDNF) concentration, a neurotrophin involved in neuronal growth, with modulation of cognitive and emotional behaviours [126-128]. In addition, changes in gut microbiota composition or exposure to specific commensal bacteria affect hypothalamic-pituitary-adrenal (HPA) axis. This pathway influences the response to stress with a role in stress-related mood or behavioural disorders [124,126,133,134]. Microbiota-vagus nerve interaction would affect the communication between visceral and immune signals to CNS [131-133,135].

4. Gene-bacteria interplay and composition of gut microbiota in FMF patients

Autoinflammatory genes, such as *MEFV*, drive an exaggerated innate immune response to various signals *in vitro*, including microbial products [46]. In parallel, the *NOD2/CARD15* gene is a major susceptibility gene for Crohn's disease, a type of inflammatory bowel disease (IBD) characterized by recurrent course. Similarly to the *MEFV* gene, the *NOD2/CARD15* gene is localized to chromosome 16 [136]. Both *MEFV* and *NOD2/CARD15* genes encode similar superfamily proteins, acting as intracellular pattern recognition receptors [137], and likely play key roles in the regulation of apoptosis, cytokine processing, and inflammation. Patients with CD are carriers of mutated

teins, which sense bacterial products and activate the innate immune response [138]. *NOD2/CARD15* mutations were not associated to an increased susceptibility to develop FMF. However, in a cohort of 103 FMF children, patients with NOD2/CARD15 mutations had a higher rate of erysipelas-like erythema, acute scrotum attacks, a trend for a higher rate of colchicine resistance, and a more severe disease ascompared with patients without mutations [139].

Xu et al. found that pyrin is a specific immune sensor for bacterial modifications of Rho GTPases, and responds to *Clostridium difficile*, which is a frequent cause of nosocomial diarrhea. Pyrin does not directly recognize the microbial products but detects pathogen virulence activity [29]. This finding has shed some light on FMF pathogenesis. On this way, colchicine is the principle therapy for FMF-patients and the aim of treatment is to prevent acute attacks and the consequences of chronic inflammation [140]. Colchicine is a fat-soluble alkaloid binding to β-tubulin, hindering its polarization with consequent inhibition of neutrophil chemotaxis while reducing expression of adhesion molecules, therefore it prevents febrile attacks and is an FMF-controlling inflammation. Nevertheless, 5-10% of FMF patients are colchicine non-responders [141]. This condition may be due to concomitant diseases (e.g. vasculitis and IBD) [142,143] or occult infections acting as trigger factors to reduce drug effectiveness [144,145].

MEFV variants are mainly represented by missense mutations in the C-terminal half of the pyrin protein [25-27,146]. In homozygous mutant mice expressing a truncated pyrin, the bacterial endotoxin lipopolysaccharide (LPS) induced increased fever and lethality. The mutant pyrin was less effective than wild-type pyrin in binding to ASC and inhibiting caspase 1and IL-1β production. Thus, one possibility is that FMF patients become more responsive to transient bacteremia and bacterial pathogens, LPS release and therefore to systemic inflammatory response [147].

Two studies described that FMF patients with concomitant *Helicobacter pylori* (HP) infection show more severe and frequent febrile attacks. Of note, HP eradication was associated with a reduction of fever attacks and cytokine levels [148,149].

Small intestinal bacterial overgrowth (SIBO), is a condition characterized by the increase of microorganisms in the small bowel exceeding 10^5 CFU/mL [150,151] and increased bacterial fermentation of a non-adsorbable carbohydrate substrate [152]. The occurrence of SIBO could exacerbate the FMF phenotypic expression. SIBO may reveal through variable symptoms, from a complete malabsorption syndrome, with abdominal distension, dyspepsia, and diarrhea with or without colicky pain, eventually modified by meals and evacuation of stools, to a totally asymptomatic clinical presentation. Due to malabsorption and alteration of the intestinal microbiota, SIBO might facilitate blood diffusion of bacterial metabolic products, acting as pathogen associated molecular patterns (PAMPs) [153,154]. This condition might also interfere with a physiological intestinal permeability [155] as well as with the bioavailability of drugs [156]. SIBO could be responsible of unresponsiveness to colchicine, while SIBO decontamination therapy by the non-absorbable antibiotic rifaximin leads to a decrease in FMF attacks [157]. Therefore, bacterial antigen production or release derived also from SIBO, may act as trigger factors, enhancing inflammatory cytokine production such as IL-1β and sustaining a persistent or occult inflammation, producing an FMF phenotype apparently unresponsive to colchicine.

Full characterization of gut microbiota in FMF patients required. Major difficulties derive from phenotypic variations and gene-environment interactions. FMF patients, as compared with healthy

subjects might exhibit a different composition in gut microbiota [158,159]. In principle, the profile of microbial products and metabolites in the human metabolome from FMF patients (in particular the specific profile of long chain fatty acids) might become a marker for the disease [160]. Similarly, increased blood levels of short chain fatty acids appear in the acute phase of the disease, as a consequence of active inflammation [161].

In a series of 19 FMF patients explored during an attack as compared with healthy controls, a poorer microbiota with loss of diversity has been described, and major shifts in bacterial populations within the *Bacteroidetes*, *Firmicutes* and *Proteobacteria* phyla (i.e., as compared with controls, lower proportion of *Prevotellaceae*, *Dialister* and *Prevotella*; increase in *Porphyromonadaceae*, *Phascolarctobacterium*, *Faecalibacterium*, and *Parabacteroides*). In the same subjects, during remission, the amount of *Enterobacteriaceae*, *Acidaminococcaceae*, *Ruminococcus* and *Megasphaera* was higher than in controls. Conversely, *Roseburia* was reduced. Thus, genetic factors may play a key role in the gut-microbiota interaction, with the existence of a microbiota profile specific for FMF, and with the most diverse gut bacterial community observed during remission [159]. Additionally, a combined analysis of mutations in the *MEFV* gene and gut bacterial diversity suggested that the described depletion of total numbers of bacteria, loss of diversity, and major shifts in bacterial populations depended on the allele carrier status of the host [159]

Different results derive from a more recent study on 41 FMF patients. Data from this series also showed specific changes in gut microbiota which were linked with FMF but, more specifically, a decrease in α-diversity and a significantly altered microbiota composition, with several operational taxonomic units belonging to the order *Clostridiales* [158]. Variations with the study of Khachatryan et al [159] might depend on the different statistical method employed for the analysis (multivariate analysis), a lower number of enrolled subjects in the previous study, and the different country of origin of patients [158].

Moreover, Pepoyan et al [162] observed that M694V/V726A pyrin mutations leading to FMF disease may contribute to gender-specific differences in microbial community structure in FMF patients, although this study was performed on a small number of subjects analyzed.

The autoinflammatory state *per se* can play a critical role in the determination of microbiota variations observed in FMF patients. Armenian FMF patients showed an elevated systemic reactivity against gut microbiota. Inflammatory alterations were also present in the absence of acute attacks, with increased levels of IgG antibodies against commensal microbiota (i.e. *Bacteroides*, *Parabacteroides*, *Escherichia*, and *Enteroccocus* antigens) [163]. Another study found a specific association between the presence of AA amyloidosis (i.e. subjects with a complicated disease) and two operational taxonomic units belonging to *Clostridiales* [158]. This difference does not appear to be attributable to the use of colchicine, the commonest drug employed in FMF patients, since this drug, in vitro, does not seem to be able to affect the gut microbiota [164]. Additionally, the oral administration of colchicine in subjects with FMF is not able to normalize the altered profile of microbial long chain fatty acids, microbial products circulating in the systemic metabolome [165]. Conversely, as suggested in other diseases linked with chronic inflammation, it is possible that the appearance of amyloidosis can depend on changes in the gut microbiome [158,166-168], independently from genetic factors. Studies exploring gut microbiota in FMF patients report discrepant results [158,159]. Evidences point to a dominant role of environmental factors over host genetics [12]. FMF patients display

further variations in the microbiome linked with the presence of AA amyloidosis [158], i.e., when the most severe form of FMF occurs.

Alimov et al. [169] investigated the role of bile acid analogues (BAA) in activating the pyrin inflammasome. Both BAA473, and less potently BAA485, led to IL-18 release from peripheral blood mononuclear cells (PBMCs). Further, BAA473 induced secretion of IL-18 from a human colonic adenocarcinoma cell line and the basolateral side of a human intestinal organoid. Finally, ASC and pyrin were required for IL-1ß and IL-18 secretion and colchicine blocked BAA473-mediated inflammasome activation confirming the specific role of pyrin in the process. Increasing evidences are accumulating on the role of the gut microbiome on bile acid bioconversion with interindividual variations driving susceptibility to infections, altered metabolism, and immune response [170-173]. Thus, genetic factors can represent one of several variables determining the gut microbiota profile in subjects with FMF.

5. The role of environmental factors and the determination of phenotype

Despite the genetic origin, environmental factors might influence the prevalence of different FMF phenotypes. One factor might be the living country [174,175]. These observations are relevant, in particular, in the determination of individual susceptibility to amyloidosis [174].

An analysis on FMF patients from 14 countries demonstrated that the living country, rather than the *MEFV* genotype, was the major factor determining an increased risk of amyloidosis [174]. Furthermore, a comparison between Turkish children with FMF living in Turkey or in Germany showed a more severe course of the disease in those living in Turkey, pointing to the environment as a strong influence of the FMF phenotype [175]. In this scenario, environmental factors affecting gut microbiota could have a role in determining onset and severity of complications in the context of a monogenic disease of the innate inflammatory pathway. Gut microbiota might also play a role in evolution of AA amyloidosis, the most severe complication of FMF [158].

Finally, it is possible that different levels of basal state activation of pyrin, dependent on the *MEFV* genotype, could subtly influence the intestinal homeostasis in the gut conferring an interindividual diverse risk to develop chronic inflammation. In this light, microbiota metabolites are capable of modulating other inflammasomes [176].

6. Possible therapeutic implications

The link between gut microbiota, FMF acute attacks and FMF complications (i.e. AA amyloidosis), together with evidences pointing to an environmental modulation of gut microbiota, could allow novel therapeutic strategies in FMF patients. One evidence is that FMF patients with high serum C-reactive protein (CRP) levels in remission showed normalization of CRP after a specific probiotic therapy [177,178]. This approach appeared to restore the integrity and functionality of the gut microbiota [179].

In particular, *Lactobacillus acidophilus* INMIA 9602 Er-2 strain 317/402, a probiotic strain isolated from faeces of a healthy newborn infant [180], produces a small anti-microbial peptide (bacteriocin acidocin LCHV), which has a broad spectrum of activity against human pathogens, including methicillin-resistant *Staphylococcus aureus* and *Clostridium difficile* [181]. The strain's clinically proven positive effects have been shown in several studies, including FMF patients [182]. Interestingly, *Lactobacillus acidophilus* INMIA 9602 Er-2 strain 317/402 was able to reduce Enterobacteriaceae, thus

bacterial-related intestinal dysbiosis, but also the relative abundance of *Candida albicans*, which is increased in FMF-patie[illegible] In our setting we recently tested a combination of eight living species totaling 450 billion bacteria (*Streptococcus thermophilus DSM24731®, Bifidobacterium breve DSM24732®, Bifidobacterium longum DSM24736®, Bifidobacterium infantis DSM24737®, Lactobacillus acidophilus DSM24735®, Lactobacillus plantarum DSM24730®, Lactobacillus paracasei DSM24733®, Lactobacillus delbrueckii ssp. bulgaricus DSM24734*). Preliminary results suggest that this combination given during the intercritical period, might improve symptoms in the subgroup of FMF patients with more severe genetic variants, and partially resistant to colchicine.

However, well-designed, large, comprehensive, prospective and definitive studies are missing on the effects of probiotics in FMF patients to prevent the attacks, to reduce symptoms and to prevent complications (i.e. amyloidosis).

7. Conclusions

The microbiota has an essential role in the host gut and is sensitive to genetic and environmental changes in both health and disease. FMF, as a model of rare inherited monogenic autoinflammatory disease, offers a background of periodic inflammatory changes, with a major involvement of the innate immunity. The microbiota is highly sensitive to such inflammatory changes. In addition, it might govern specific autoinflammatory responses in FMF. FMF symptoms might be sensitive as [illegible]l, and this emerging topic, deserves high attention, as a model of environment-genetic interaction. Gut microbiota is emerging as a key factor in determining the FMF phenotype. In FMF patients, the microbiome abundance and its composition could depend on both genetic and environmental factors with the genome, however, playing a minor role. On the other hand, environmental variables could be critical in shaping the disease severity and complications onset (i.e. AA amyloidosis) in the long term. Further studies urge to exploit gene-environment interactions in FMF patients. Moreover, possibly beneficial effects deriving from external manipulation of gut microbiota request additional studies investigating how specific probiotic treatments could improve symptoms and microbiome growth, without reducing the beneficial effects of main therapeutic options in FMF patients.

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