Mediators of Inflammation

The Microbiota and Immune System Crosstalk in Health and Disease

Lead Guest Editor: Rossella Cianci
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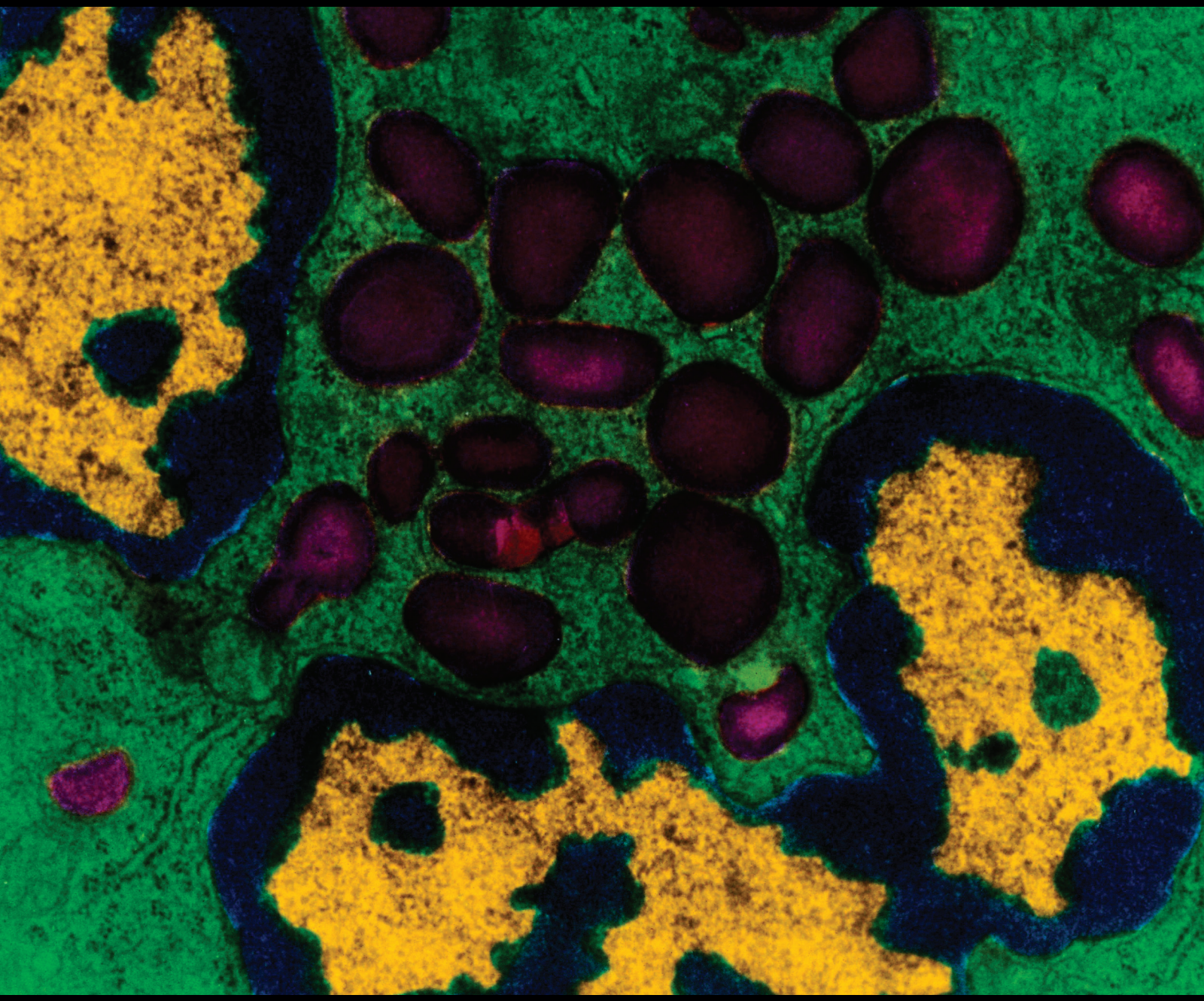

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Editorial Board

Contents

Hindawi
Mediators of Inflammation
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Editorial

The Microbiota and Immune System Crosstalk in Health and Disease

Rossella Cianci,[1] **Danilo Pagliari**,[1] **Ciriaco A. Piccirillo**,[2] **Jörg H. Fritz**,[3] **and Giovanni Gambassi**[1]

[1]*Institute of Internal Medicine, "A. Gemelli Hospital", Catholic University of the Sacred Heart, Rome, Italy*
[2]*Department of Microbiology and Immunology and Program in Infectious Disease and Immunity in Global Health and Centre of Excellence in Translational Immunology (CETI), Research Institute of the McGill University Health Centre, McGill University, Montréal, QC, Canada*
[3]*Department of Microbiology and Immunology and Department of Physiology and McGill University Research Center on Complex Traits (MRCCT) and Centre of Excellence in Translational Immunology (CETI), Research Institute of the McGill University Health Centre, McGill University, Montréal, QC, Canada*

Correspondence should be addressed to Rossella Cianci; rossellacianci@gmail.com

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As gut microbiota, the whole intestinal microbial collection is indicated; it includes trillions of microorganisms and at least 1000 different bacterial species [1]. The microbiome, instead, is the collection of the whole genome sequences of those microorganisms, and it consists of more than 5,000,000 genes. In physiological conditions, a perfect *equilibrium* among commensals and pathogens maintains intestinal homeostasis. Any imbalance precipitates a pathological state known as "gut dysbiosis" [2].

Gut microbiota modulates host physiology and metabolism through different mechanisms. A close relationship exists with sex-based hormones. On one hand, gut microbiota can induce hormonal changes leading to inflammation, and, on the other hand, hormone levels themselves shape microbiota composition. Variations in gut microbiota composition may be responsible for modifications of the host hormonal axis but also in modulation of the immune system function. Recently, a germ-free NOD mice model demonstrated that the colonization by certain commensal bacteria induced an elevation in testosterone levels in males versus females [3]. Other evidences have shown that antibiotics may be responsible for alterations in sterol metabolism-associated gene expression and also in T cell differentiation. This hormone-dependent modulation of gut microbiome explains the different susceptibilities to disease between men and women [4].

Gut microbiota is also strictly linked to the chronological age of each individual. In fact, each stage of human life is characterized by a specific intestinal microbial composition. An epochal turn in human life is set up at birth when sterility of the whole body is challenged by bacterial colonization in the birth canal. During adulthood, gut microbiota becomes stable and in *equilibrium* with the host, then it undergoes significant changes at older ages that correlate with residual physical function and life settings [5].

Diet and food supplements exert a great impact on gut microbial composition and its variability through time. In Western countries, a high-fat diet is a risk factor for disorders, such as obesity, metabolic syndrome, and diabetes all of which are associated with profound modifications of gut microbiota composition [6]. Interaction between food and gut microbiota is also finely tuned by our circadian clock. The disruption of the physiological circadian rhythm increases the likelihood of a gut dysbiosis, possibly contributing to the pathogenesis of several metabolic and inflammatory diseases, including diabetes, inflammatory bowel diseases, and even cancer [7].

In more recent years, the concept of a "gut-brain axis" has been introduced. The endocrine system may be modulated at the intestinal level in a sort of neuro-entero-endocrine system. This system interacts with the immune system at the mucosal level in order to maintain a homeostasis but also to enhance defense against microbial invasion in pathological states. As a result, modifications of microbiota composition may be associated to several disorders of the nervous system, including neuropsychiatric, neurodegenerative, and neuroinflammatory disorders [8].

It is well established that the gut microbiota is in close interaction with the intestinal mucosal immune system. Indeed, the intestinal mucosa may be considered as an immunological niche as it hosts a complex immune-functional organ comprised by T cell subpopulations and their related anti- and proinflammatory cytokines, as well as several other mediators of inflammation, in addition to the microbiota. Both innate and adaptive immune systems are involved in the gut immunological niche [9]. Several barriers protect the immunological niche from the invasion of pathogens. First, there is a mechanical barrier provided by the mucus layer with antimicrobial peptides, such as alpha-defensins, and secretory IgA and by enterocytes that possess tight intercellular junctions. Neutrophils and innate lymphoid cells (ILCs) represent another cellular defensive line. Finally, macrophage-dendritic cell- (DC-) and lymphocyte-associated pattern recognition receptors, such as Toll-like receptors (TLRs) and NOD-like receptors (NLRs), provide a further barrier bridging the activation of both innate and adaptive immune cells. Activated by bacteria, all of these mechanisms determine a proinflammatory adaptive immune response that involves several T cell subpopulations, such T helper 1 and 17 (Th1 and Th17 cells), and their related cytokines, such as IL-1, IL-2, IL-15, IL-17, IL-22, and IL-23 [10].

Any modification in the balance between microbiota and gut immunological niche components may trigger infectious, inflammatory, and endocrine diseases [11], such as metabolic liver disorders, inflammatory bowel diseases, pancreatic disorders, autoimmune diseases, and aging [12, 13]. This holds true also for cancer. There is evidence that gut dysbiosis favors the formation of gastrointestinal tumors—colorectal, gastric, liver, and pancreatic, and it might also be responsible for the modulation of antitumor response during chemotherapy and immunotherapy [14].

In this special issue, a series of manuscripts highlighting the relevance of gut microbiota and immune system crosstalk in several disease states are collected.

S. Bibbò et al. report that leaks in the intestinal mucosal barrier lead to the translocation of bacterial products into portal circulation stimulating innate immunity via TLRs. This translates into a release of proinflammatory cytokines causing chronic liver inflammation, heralding diseases like nonalcoholic fatty liver disease (NAFLD) and nonalcoholic steatohepatitis (NASH).

A similar mechanism seems involved in the pathogenesis of various pancreatic disorders. In their article, D. Pagliari et al. summarize the evidence linking a disruption of the intestinal mucosal barrier to several benign and malignant disorders, such as pancreatitis, diabetes, and pancreatic cancer. A specific microbial profile for each pancreatic disease seems established such that novel tools for prevention and early diagnosis are now underway.

A microbiota is present also in systems other than the gastroenteric tube, and these microorganisms exert additional physiological functions. The skin is not only a mechanical barrier to the external world but it also plays a crucial role in maintaining homeostasis between pathogen-related immune responses and the external environment. F. Abdallah et al. examine the role of skin microbiota in the pathogenesis of immune-mediated disorders, such as atopic dermatitis and *acne vulgaris*.

Similarly, gut microbiota may be implicated in the induction and in the maintenance of local and systemic inflammation in autoimmune diseases. F. Biscetti et al. review the role of the microbiota-related activation of the conserved DNA-binding protein high-mobility group box-1 (HMGB1) in synovial inflammation of patients with rheumatoid arthritis. Since HMGB1 can be activated by the Gram-negative lipopolysaccharide (LPS) via the TLR-4 pathway, it may turn out to be a possible target for novel therapeutic approaches.

In several instances, xenobiotics have been suggested to interfere with inflammatory pathways in the intestinal mucosa eliciting an anti-inflammatory and immune-modulating action. D. Pagliari et al. review the interaction of rifaximin with several components of the gut immunological niche that explain its ability to reduce intestinal dysbiosis and to inhibit the release of proinflammatory mediators.

Interestingly, rifaximin has been successfully employed in patients with colchicine resistance familial Mediterranean fever (FMF). In their article, E. Verrecchia et al. demonstrate that in patients with innate immunity hypersensitivity, such as FMF, gut dysbiosis worsens the clinical manifestations of the disease. Conversely, treatment with rifaximin increases the efficacy of colchicine and ameliorates FMF.

Indeed, other xenobiotics can be involved in the modulation of inflammation. J. Lu et al. explore the anti-inflammatory properties of columbianetin, derived from the root of a Chinese herb *Radix Angelicae Pubescentis*. In LPS-stimulated human peripheral blood mononuclear cells, the molecule suppresses the expression of several NF-κB-mediated proinflammatory cytokines.

New approaches to the treatment of human metabolic and behavioral disorders are being tested. C. Colica et al. have conducted a clinical study on obese and nonobese patients where they administered a probiotic formulation containing a cocktail of *Lactobacillus* spp., *Bifidobacterium* spp., and *Streptococcus* spp. This formulation has produced positive effects on obesity but also on psychological distress.

Finally, changes in gut microbiota have been linked to the chronic, low-grade inflammation associated with aging, named "inflamm-aging." In their article, A. Picca et al. examine how such microbial changes modulate the inflammatory pathways involved in the development of sarcopenia and cachexia. These signaling pathways may provide novel targets for future therapeutical interventions.

The overall aim of this special issue is to collect and summarize current knowledge about the complex interplay among gut microbiota, immune system, mediators of inflammation, and xenobiotics in health and during diseases. We trust that there is abundant food for thoughts.

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Rossella Cianci
Danilo Pagliari
Ciriaco A. Piccirillo
Jörg H. Fritz
Giovanni Gambassi

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Research Article

Anti-Inflammatory Effect of Columbianetin on Lipopolysaccharide-Stimulated Human Peripheral Blood Mononuclear Cells

Junying Lu,[1,2] Keyong Fang,[1] Shiji Wang,[2] Lingxin Xiong,[1,3] Chao Zhang,[1] Zhongmin Liu,[2] Xuewa Guan,[1] Ruipeng Zheng,[1,4] Guoqiang Wang,[1] Jingtong Zheng,[1] and Fang Wang[1]

[1]*Department of Pathogeny Biology, College of Basic Medical Sciences, Jilin University, Changchun 130021, China*
[2]*Department of Intensive Care Unit, First Hospital of Jilin University, Changchun 130021, China*
[3]*School of Pharmaceutical Sciences, Jilin University, Changchun 130021, China*
[4]*Department of Interventional Therapy, First Hospital of Jilin University, Changchun 130021, China*

Correspondence should be addressed to Fang Wang; wf@jlu.edu.cn

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Dysregulated inflammation is increasingly considered as the main cause of many diseases on which NOD1/NF-κB pathway plays an important role. Columbianetin (CBT) is derived from the root of the Chinese herb *Radix Angelicae Pubescentis* for treating inflammatory diseases. Although the anti-inflammatory effect of CBT has been reported, its anti-inflammatory mechanism was poorly studied. In this study, we explored the anti-inflammatory pathway of CBT in lipopolysaccharide- (LPS-) stimulated human peripheral blood mononuclear cell (PBMC) model. Inflammatory cytokine production in culture supernatant was assessed using ELISA assay, and the possible anti-inflammatory pathway of CBT was screened using qPCR array and enrichment analysis with DAVID6.8. To further confirm the targeted pathway of CBT, we pretreated PBMC with the selective NOD1 inhibitor ML130 and then measured the protein levels of the pathway by Western blotting. The result showed that CBT effectively suppressed the expressions of TNF-α, IL-6, MCP-1, and IL-1β in a dose-dependent manner and significantly downregulated 19 out of 32 differentially expressed genes, most of which were involved in the NOD1/NF-κB pathway, and also showed that CBT remarkably inhibited LPS-induced NOD1, RIP2, and NF-κB activation. Furthermore, the inhibitory effects of CBT on NOD1/NF-κB pathways were blocked by ML130. These findings indicated that CBT inhibits the production of inflammatory cytokines induced by LPS involved in the downregulation of NOD1/NF-κB pathways.

1. Introduction

Inflammation is a protective response against infection, tissue stress, and injury in any tissue, and defends and restores physiological functions, but dysregulated inflammatory process could result in chronic inflammation, which is increasingly seen as a major driver of numerous diseases such as obesity, asthma, atherosclerosis, and type 2 diabetes [1, 2]. Nonsteroidal anti-inflammatory drugs (NSAIDs) and corticosteroids are most widely used for the treatment of inflammation; however, the adverse effects of long-term use were unavoidable [3, 4]. Thus, treatment of inflammatory diseases is a big problem. Although a large number of medicinal herbs have been identified as effective anti-inflammatory drugs in the past, but the mechanism of anti-inflammatory herbal compounds has not been elucidated comprehensively [5].

Lipopolysaccharide (LPS), an endotoxin mainly present in the outer membrane of gram-negative bacteria, is the major cause of inflammation. LPS is recognized by specific host pattern-recognition receptors (PRRs) mainly including toll-like receptors (TLRs) and nucleotide-binding and oligomerization domain- (NOD-) like receptors (NLRs) and then induces the activation of NF-κB and mitogen-activated protein kinase (MAPK) signaling pathway, which is involved in the release of various inflammatory cytokines [6, 7].

NOD1, a member of NLRs family, initiates inflammation by recognizing ligands of bacterial peptidoglycan containing γ-D-glutamyl-meso-diaminopimelic acid (iE-DAP), which leads to the activation of NF-κB and subsequently the release of proinflammatory mediators and chemokines via a series of cascade responses [8–10]. Dysregulation of NOD1 function has been described in a variety of chronic inflammatory disorders [11]. Therefore, it has been suggested that inhibitors of NOD1 may be useful as anti-inflammatory agents [12].

Columbianetin (CBT) is one of the main bioactive constituents isolated from the root of *Radix Angelicae Pubescentis* (RAP), which has been widely used in China for a long history as an important component in various prescriptions for treating diseases such as arthritis and asthma [13]. As a member of furocoumarin, numerous investigations have shown that CBT has multiple bioactivities, for example, antioxidative [14], antiproliferation [15], anti-inflammatory [16, 17], and anti-nitric oxide production activities [18]. Although the bioeffects of CBT have been reported, the action mechanism has not been well studied.

In the present study, we aimed to explore the anti-inflammatory pathway of CBT. We used the inflammation model of LPS-stimulated human peripheral blood mononuclear cells (PBMCs) and investigated whether CBT could significantly inhibit LPS-induced cytokine production. We then screened CBT targeted pathway by quantitative real-time polymerase chain reaction (qPCR) array and enrichment analysis, which was further confirmed using NOD1 selective inhibitor ML130.

2. Methods

2.1. Chemicals and Reagents. Lipopolysaccharide (LPS) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA); RPMI medium modified without calcium nitrate was purchased from HyClone (Logan, UT, USA); fetal bovine serum (FBS) was obtained from Gibco (Australia); protein assay kit was purchased from BioTime Biotechnology (Shanghai, China); ELISA kits of human TNF-α, IL-1β, IL-6, and MCP-1 were purchased from RayBiotech (Atlanta, USA); RNeasy Mini Kit, RNase-Free DNase Set, RT^2 First Strand Kit, RT^2 SYBR® Green ROX qPCR Master Mix, and Human Inflammasomes PCR Array were obtained from QIAGEN (Valencia, CA, USA). Minute total protein extraction kit was purchased from Invent Biotechnologies (Eden Prairie, USA). NOD1 antibody and RIP2 antibody were purchased from Cell Signaling Technology (Massachusetts, USA). Anti-IκB-alpha antibody and anti-NF-κB antibody were purchased from Abcam (Cambridge, United Kingdom); secondary antibodies were obtained from Sigma-Aldrich (St. Louis, MO, USA). CBT was purchased from Shanghai Yuanye Bio-Technology Company (Shanghai, China; purity ≥ 98%) and ML130 (Abcam, Cambridge, United Kingdom) was prepared in DMSO and diluted with 10% RPMI-1640. Ten percent RPMI-1640 with 0.1% DMSO was used as a vehicle in control group.

2.2. Isolation of PBMC. PBMC was isolated from heparinized venous blood via Ficoll density gradient centrifugation method. Heparinized venous blood obtained from the normal donors was diluted 1 : 1 with sterile phosphate-buffered saline (PBS), layered over Ficoll-Hypaque, and centrifuged at 1500 rpm for 15 min at room temperature. PBMC was collected from the interphase layer and washed with PBS twice. The cell viability of isolated PBMC was measured by trypan blue exclusion assay. Then PBMC was resuspended at 1×10^6 cells/ml in RPMI-1640 medium containing 10% charcoal-filtered fatal bovine serum and was kept at 37°C with 5% CO_2.

2.3. Inflammation Model of LPS-Stimulated PBMC and Treatment. Isolated PBMC was divided into 7 groups, including blank control group (Control), LPS-stimulated group (LPS), columbianetin-treated group (LPS + 10, 20, 40 μg/ml CBT), ML130-pretreated group (LPS + ML130), and ML130 pretreatment combined with the treatment of 40 μg/ml columbianetin (LPS + ML130 + CBT). The inflammation model and validation of LPS-stimulated PBMC were established according to a previously described protocol [19]. PBMC was stimulated with 1000 ng/ml LPS for 1 h, then received administrations of different concentrations of CBT (0, 10 μg/ml, 20 μg/ml, 40 μg/ml, resp.) for an additional 24 h, while the other two groups were incubated with 30 μmol/l NOD1 inhibitor (ML130) for 4 h prior to the stimulation of 1000 ng/ml LPS and then followed by the treatment with or without 40 μg/ml CBT. Culture supernatants were collected and stored at −20°C until ELISA assays. Intracellular proteins were collected and stored at −20°C until Western blotting analysis.

2.4. ELISA Assay of TNF-α, IL-1β, IL-6, and MCP-1. The concentrations of TNF-α, IL-1β, IL-6, and MCP-1 in culture supernatants were quantified using enzyme-linked immunosorbent assay (ELISA) kits (RayBiotech, Atlanta, USA) according to the manufacturer's instructions.

2.5. Inflammation-Related Gene Expression Measurement Using qPCR Array. The expression of 84 inflammation-related genes was evaluated using quantitative real-time PCR array (SABiosciences, Valencia, CA) according to the instructions. Total RNA was isolated using the RNeasy Mini Kit and then was quantified and qualitied by measuring the absorbance at 260 and 280 nm. Total RNA was purified with DNase. cDNA synthesis was performed by reverse transcription of 20 ng total RNA as described for RT-PCR and then combined with the SYBR Green Master Mix in 96-well plates following the manufacturer's recommendations. Thermal cycling was performed using an ABI Prism SDS 7300 system (Applied Biosystems, Madrid, Spain).

2.6. Enrichment Analysis and the Pathway of Differentially Expressed Genes. Gene expression was analysed using the ΔΔCT method. Screening of differentially expressed genes was based on the standard fold change (FC) ≥ 2 or fold change ≤ 0.5. To investigate the pathway of CBT-targeted inflammatory genes in LPS-stimulated PBMC, enrichment analysis of KEGG pathways was conducted in DAVID6.8

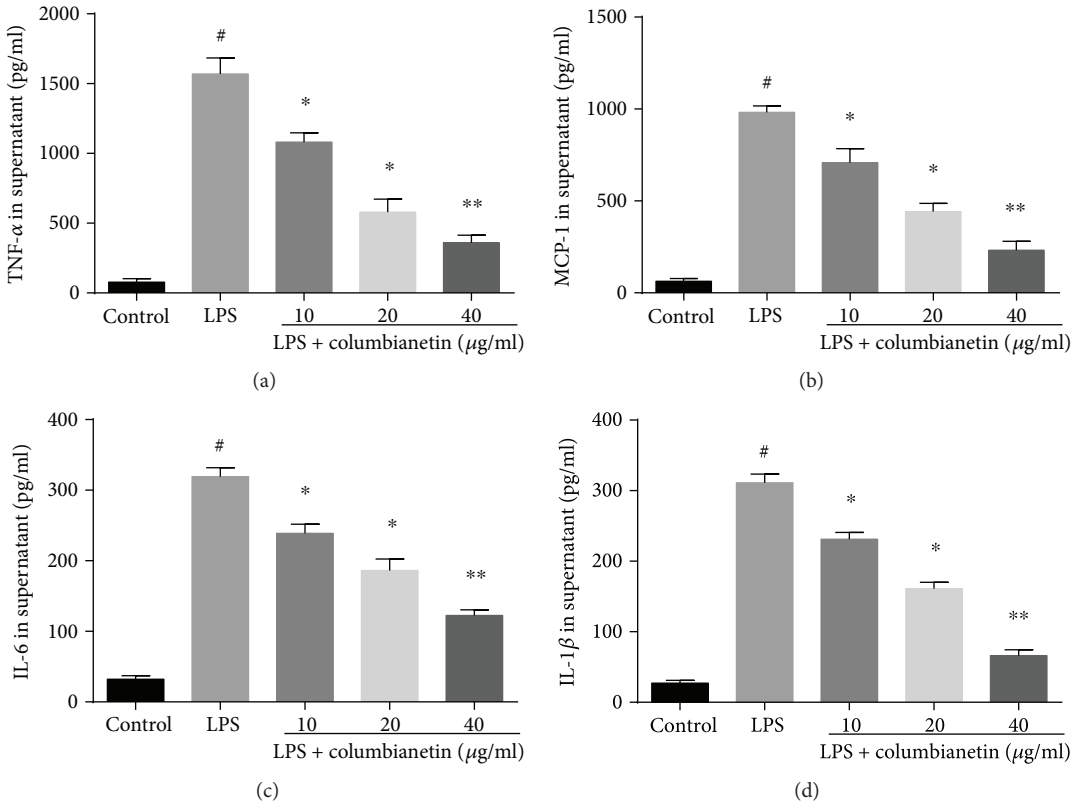

Figure 1: Effects of CBT on TNF-α, MCP-1, IL-6, and IL-1β production in the supernatant of LPS-stimulated PBMC. The values of three independent experiments are presented as mean ± standard deviation (SD) ($n = 3$ in each group). $^{\#}P < 0.01$ versus control group, $^{*}P < 0.05$ versus LPS group, $^{**}P < 0.01$ versus LPS group.

[20]. The significance of the enrichment was determined by P value, and meanwhile, the significance of the P value was evaluated with false discovery rate (FDR). The pathways were screened in the differentially expressed genes using the enrichment scores (−Log10 (P value)). And the significant pathways were screened in accordance with $P < 0.05$ and FDR < 0.05.

2.7. Western Blotting Analysis. After treatment, the cells were collected and lysed in RIPA buffer. The same amounts of proteins (25 μg protein per lane) were loaded onto SDS-PAGE gels separated by 12% SDS-PAGE and then were transferred onto PVDF membranes. The membranes were then blocked with 5% fat-free dry milk and probed with the primary antibody at 4°C for 24 h. After washing the membranes thrice, the membranes were incubated with appropriate secondary antibodies for 2 h. Finally, a coloration solution mixture (Beyotime, Jiangsu, China) was added, and the immunoreactive bands were visualized with ECL chemiluminescent detection.

2.8. Statistical Analysis. The experimental data were expressed as the means ± standard deviation. Kruskal-Wallis ANOVA or a Mann–Whitney U test to compare differences in cytokines and the expression of different proteins among different groups was used in the current study, and a Dunn-Bonferroni test for post hoc comparisons was also used. $P < 0.05$ was considered as statistically significant.

3. Results

3.1. Effects of CBT on Cytokine Production in LPS-Stimulated PBMC. To assess the anti-inflammatory effects of CBT, cytokine expression of LPS-stimulated PBMC was measured by ELISA. As shown in Figure 1, LPS induced a significant release of TNF-α, IL-6, MCP-1, and IL-1β. However, treatment with different concentrations of CBT significantly inhibited LPS-induced inflammatory cytokines (TNF-α, IL-6, MCP-1, and IL-1β) in a dose-dependent manner.

3.2. Effects of CBT on the Regulation of Inflammation-Related Genes. In our present study, we found that CBT could inhibit TNF-α, MCP-1, IL-6, and IL-1β production. To further investigate the possible anti-inflammatory molecular mechanism of CBT, the expression of 84 inflammatory-related genes in PBMC was measured using quantitative real-time polymerase chain reaction (qPCR) array, and then CBT-targeted inflammatory genes were analysed using MATLAB analysis technique. As shown in Supplemental Table 1, LPS caused the differential expression of 44 genes, in which 32 (approximately 72.73%) were upregulated. However, the treatment with CBT significantly downregulated 19 genes which were upregulated by LPS (fold change > 1.5) (Figure 2 and Table 1).

3.3. CBT Targeted Pathways of Differentially Expressed Genes. To elucidate the candidates for CBT targeted pathways,

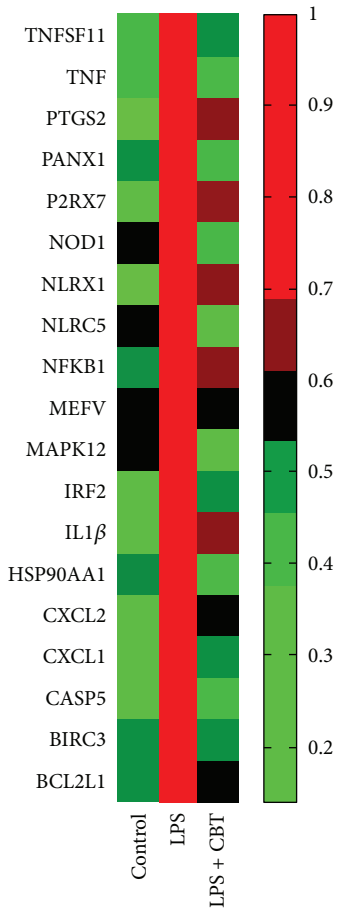

Figure 2: Effects of CBT (40 μg/ml) on the regulation of differential inflammatory-related genes.

we performed enrichment analysis for the differentially expressed genes. As shown in Figure 3, the differentially expressed genes in LPS-stimulated PBMC pretreated with CBT were enriched in 7 pathways. According to Figure 3, the differentially expressed genes were mainly involved in the NOD-like receptor signaling pathway and demonstrated the highest enrichment score.

3.4. Effects of CBT on NOD1/RIP2/NF-κB Pathway. We found that CBT could downregulate the genes involved in NOD-like receptor signaling pathway. The effects of CBT on NOD-like receptor signaling pathway were measured using Western blot. As shown in Figure 4, after LPS stimulation, the expression of NOD1, RIP2, and NF-κB was dramatically higher compared with the control group, while the expression of IκBα was lower. However, after the pretreatment with different concentrations of CBT, the expression of NOD1, RIP2, and NF-κB was evidently reduced and in contrast the expression of IκBα was remarkably increased.

3.5. Effects of CBT and ML130 on NOD1 of LPS-Stimulated PBMC. As shown in Figure 5, the expression of NF-κB and RIP2 was significantly reduced by CBT and ML130 alone to a similar extent ($P > 0.05$). However, the combination of CBT and ML130 did not yield significantly better effects ($P > 0.05$, compared to CBT or ML130 alone, resp.). Our results showed that CBT did not produce any further effect after NOD1 was blocked by ML130. Similarly, ML130 did not further extend the effect of CBT, indicating that these two drugs target the same pathway and exert their anti-inflammatory effects by blocking NOD1 activation.

4. Discussion

Inflammatory response is an important pathophysiological process in which NOD1/NF-κB pathway plays a vital role, and dysregulation of inflammation has been identified as a major cause of numerous common diseases [1, 8]. In the present study, we investigated the protective effects and the related pathway of CBT. Our results showed that its anti-inflammation property was exhibited mainly by downregulating NOD1/NF-κB pathway in LPS-stimulated PBMC. Moreover, the selective NOD1 inhibitor ML130 blocked the effect of CBT on NOD1/NF-κB pathway, suggesting the NOD1-dependent mechanism. Our findings indicated that CBT may exert its protective effect mainly through down-regulation of NOD1/NF-κB pathway for the first time.

It is well known that LPS can induce inflammatory response that is characterized by elevated proinflammatory cytokines such as TNF-α, IL-6, and IL-1β [21]. Besides, *in vitro* model of LPS-induced PBMC, TNF-α, IL-6, MCP-1, and IL-1β was increased notably [19]. A previous study showed that inhibition of these proinflammatory cytokines could attenuate the response of inflammation [22]. Another study also showed that CBT exerted anti-inflammatory effect in LPS-activated RAW 264.7 cells via the inhibition of nitric oxide production [18]. Our results were consistent with previous reports on the anti-inflammatory effects of CBT against LPS-induced inflammation. In the present study, we found that CBT significantly reduced inflammatory cytokine production, which confirmed that CBT had anti-inflammatory effects on LPS-stimulated PBMC.

LPS leads to the release of excessive inflammatory cytokines mainly through the activation of MAPK and NF-κB pathways [6, 7, 23], while the cytokines through the activation of inflammatory and immune cells can regulate the inflammatory response [24]. The MAPK family is comprised by a large group of protein kinases which regulates three major pathways: the extracellular signal-regulated protein kinase 1/2 (ERK), the p38 MAP kinases (p38), and the c-Jun amino-terminal kinase (JNK). And the activation of these reactions is mainly through myeloid differentiation factor 88- (MyD88-) dependent and nondependent TLR4 and NLRS [25]. Moreover, numerous studies show that NLRS and TLR signaling pathways interact at multiple levels in cellular systems [6, 11, 26]. Inflammation induced by LPS is a complex response. As a highly sensitive and reliable method of gene expression profiling, qPCR array combines qRT-PCR and gene chips which can screen differentially expressed genes based on mRNA. qPCR array is a useful tool for studies on signaling pathway [27, 28]. In agreement with these findings, in the current study, the results demonstrated that 44 genes (including MyD88-dependent and nondependent TLR4, NLRS, MARK, and NF-κB) were upregulated

Table 1: Nineteen significantly downregulated genes in the CBT-treated groups (fold change > 1.5) by CBT.

Number	Gene name	M/N	D/M	Description
1	BCL2L1	2.10	1.74	BCL2-like 1
2	BIRC3	2.03	1.96	Baculoviral IAP repeat containing 3
3	CASP5	2.8	2.54	Caspase 5, apoptosis-related cysteine peptidase
4	CXCL1	3.28	2.02	Chemokine (C-X-C motif) ligand 1
5	CXCL2	2.7	1.88	Chemokine (C-X-C motif) ligand 2
6	HSP90AA1	2.08	2.26	Heat shock protein 90 kDa alpha, class A member 1
7	IL-1β	2.82	1.51	Interleukin 1, beta
8	IRF2	1.97	2.07	Interferon regulatory factor 2
9	MAPK12	1.68	3.03	Mitogen-activated protein kinase 12
10	MEFV	1.65	1.85	Mediterranean fever
11	NFKB1	2.1	1.74	Nuclear factor of kappa light polypeptide gene enhancer in B-cell 1
13	NLRC5	1.74	2.96	NLR family, CARD domain containing 5
13	NLRX1	3.51	1.57	NLR family, CARD domain containing 5
14	NOD1	1.76	2.29	Nucleotide-binding oligomerization domain containing 1
15	P2RX7	3.16	1.61	Purinergic receptor P2X, ligand-gated ion channel, 7
16	PANX1	2.20	1.74	Pannexin 1
17	PTGS2	7.1	1.56	Prostaglandin-endoperoxide synthase 2
18	TNF	2.47	2.44	Tumor necrosis factor
19	TNFSF11	2.51	2.01	Tumor necrosis factor (ligand) superfamily, member 11

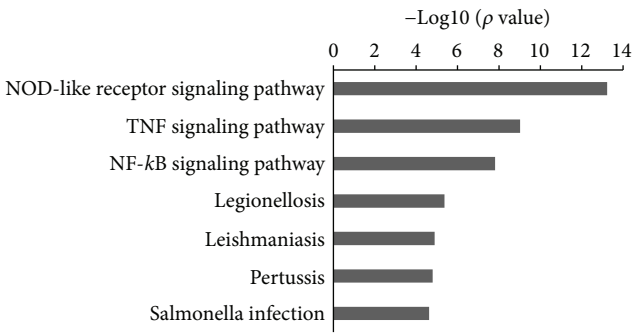

Figure 3: Effects of CBT on the related pathway. The differentially expressed genes of LPS-stimulated human PBMC treated with 40 μg/ml CBT were enriched with DAVID6.8. The black bars showed the pathways in which the differentially expressed gene was found to be involved in the treatment of CBT. The bar plot "−Log10 (P value)" represents the enrichment score of the significant enrichment pathways. And the P value denotes the significance of the correlation between the pathway and the treatment of CBT.

after LPS stimulation. To explore the potential mechanism by which CBT exerts its anti-inflammatory effects, the effects of CBT on LPS-stimulated inflammation-associated genes and proteins were assessed. According to the results of qPCR array, CBT downregulated the expression of 19 genes. We repeated the genes which play an important role in the inflammatory response by qPCR, and the results showed that CBT not only downregulated the levels of NOD1/NF-κB, but also downregulated MAPK12 (Supplemental Figure 1); however, the effect on TLR4 was insignificant (Supplemental Figure 2). Moreover, the differentially expressed genes were mainly involved in the NOD1/NF-κB signaling pathway which demonstrated the highest enrichment score. Thus, NOD1/NF-κB was the potential target of CBT in the current evidence; nevertheless, the effects of the drug on MAPK pathway as well as the other molecular pathways need further investigation. We also found that CBT remarkably inhibited the expressions of NOD1, RIP2, and NF-κB. These results indicated that CBT may exhibited its anti-inflammatory effects mainly by inhibiting NOD1/RIP2/NF-κB signaling pathway.

NOD1 has been reported to play an important role in inflammation [8]. Previous studies showed that blocking of NOD1 can alleviate inflammation via suppressing the activation of NF-κB [29] and the release of inflammatory cytokines [9]. Our findings were similar with these results, in which the pretreatment of ML130 significantly suppressed the activation of NF-κB. We have demonstrated that CBT can inhibit the NOD1/NF-κB signaling pathway, and finally, to further confirm that CBT exerts a protective effect mainly through the inhibition of NOD1/NF-κB pathway, we blocked the activation of NOD1/NF-κB pathway using the selective NOD1 inhibitor ML130 and then tested whether CBT continues to influence NOD1/NF-κB signaling pathway. The results showed that CBT did not produce any further effect after NOD1 was blocked. To further confirm whether CBT is a specific NOD1 inhibitor, it is necessary to study the effect of CBT in NOD1 ligand-induced PBMC.

In summary, the results of this study demonstrated that CBT effectively attenuates LPS-induced inflammation at least partially by inhibiting NOD1/NF-κB activities. CBT might be useful as a potential therapeutic medication for LPS-induced inflammation.

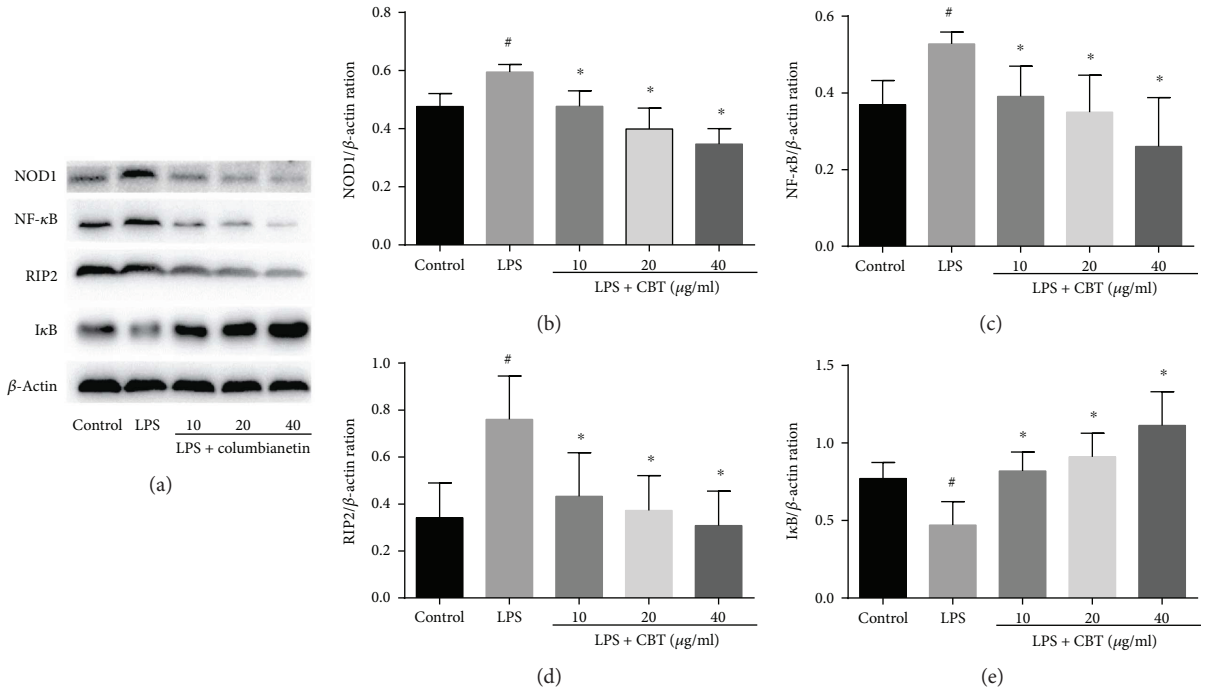

Figure 4: Columbianetin-inhibited LPS-stimulated NOD1/RIP2/NF-κB pathway. The values of three independent experiments are presented as mean ± SD. $^{\#}P < 0.05$ versus control group, $^{*}P < 0.05$ versus LPS group.

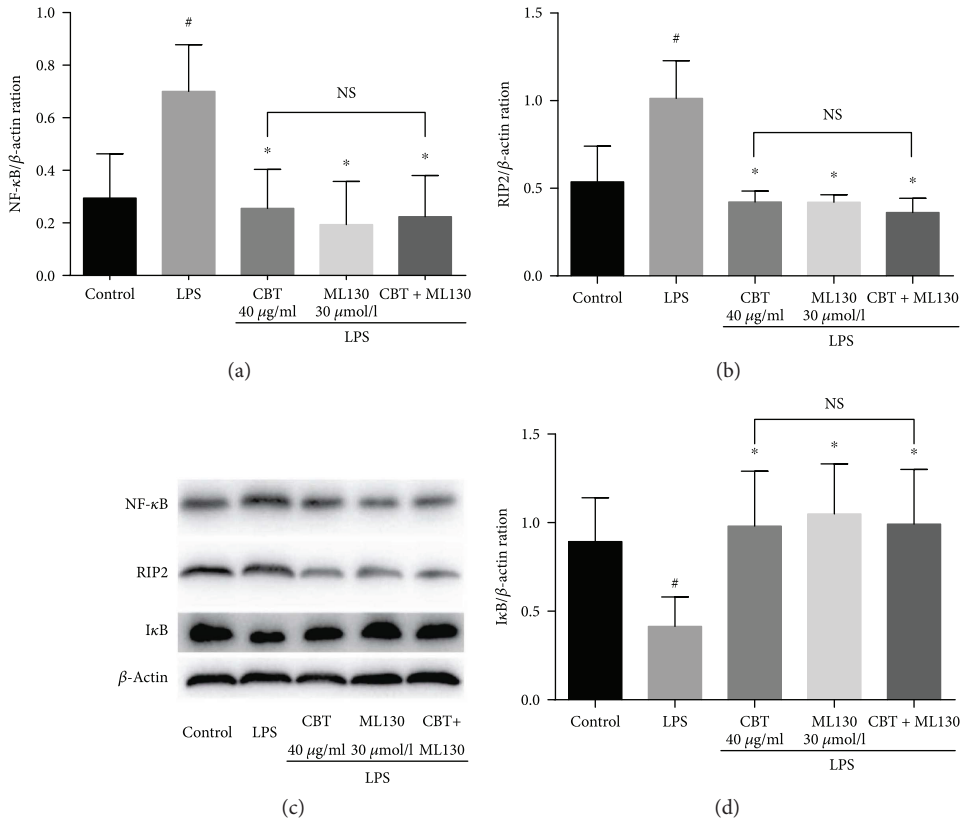

Figure 5: CBT inhibited the expression of NOD1/RIP2/NF-κB pathway by blocking NOD1 activation. The values are presented as mean ± SD of three independent experiments. $^{\#}P < 0.05$ versus control group, $^{*}P < 0.05$ versus LPS group. NS indicates no significance among the three groups ($P > 0.05$). CBT: columbianetin.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Supplementary Materials

Supplemental Table 1: 44 differentially expressed genes in LPS-stimulated PBMC (fold change > 1.5). Supplemental Figure 1: columbianetin inhibited the levels of NOD1/RIP2/NF-κB/MAPK12. The values of three independent experiments are presented as mean ± SD. $^{\#}P < 0.01$ versus control group, $^{*}P < 0.05$ versus LPS group, $^{**}P < 0.01$ versus LPS group. Supplemental Figure 2: CBT (40 μg/ml) has no effect on the expression of MYD88 and TIRAP. The values of three independent experiments are presented as mean ± SD. $^{\#}P < 0.01$ versus control group. There is no significance between the groups LPS and LPS + CBT 40 μg/ml. *(Supplementary Materials)*

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Review Article

Gut Microbiota-Immune System Crosstalk and Pancreatic Disorders

D. Pagliari,[1,2] **A. Saviano**,[1] **E. E. Newton**,[3] **M. L. Serricchio**,[1] **A. A. Dal Lago**,[1] **A. Gasbarrini**,[1] **and R. Cianci**[2]

[1]*Department of Gastroenterology, Pancreatic Unit, Catholic University of the Sacred Heart School of Medicine, Rome, Italy*
[2]*Department of Internal Medicine, Catholic University of the Sacred Heart School of Medicine, Rome, Italy*
[3]*CytoCure LLC, Beverly, MA, USA*

Correspondence should be addressed to D. Pagliari; danilo@nonsololatina.it

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Gut microbiota is key to the development and modulation of the mucosal immune system. It plays a central role in several physiological functions, in the modulation of inflammatory signaling and in the protection against infections. In healthy states, there is a perfect balance between commensal and pathogens, and microbiota and the immune system interact to maintain gut homeostasis. The alteration of such balance, called dysbiosis, determines an intestinal bacterial overgrowth which leads to the disruption of the intestinal barrier with systemic translocation of pathogens. The pancreas does not possess its own microbiota, and it is believed that inflammatory and neoplastic processes affecting the gland may be linked to intestinal dysbiosis. Increasing research evidence testifies a correlation between intestinal dysbiosis and various pancreatic disorders, but it remains unclear whether dysbiosis is the cause or an effect. The analysis of specific alterations in the microbiome profile may permit to develop novel tools for the early detection of several pancreatic disorders, utilizing samples, such as blood, saliva, and stools. Future studies will have to elucidate the mechanisms by which gut microbiota is modulated and how it tunes the immune system, in order to be able to develop innovative treatment strategies for pancreatic disorders.

1. Introduction

The human gastrointestinal tract hosts more than 10^{14} microorganisms, a number 10 to 20 times greater than the total number of cells of the human body, and includes at least 1000 different microbial species, including bacteria, fungi, yeast, viruses, and archaea [1–3]. The ensemble of these populations constitutes the so-called gut microbiota. Instead, the collection of the whole genome sequence of gut microbiota species is called microbiome and consists of more than 5,000,000 genes [4–7].

Gut microbiota is central to the development and modulation of the mucosal innate and adaptive immune system and exerts an important role in the protection against pathogenic microbes by maintaining gut integrity and regulating intestinal barrier permeability. It weighs about 900–1200 g and participates in several physiological functions. Indeed, gut microbiota is constantly involved in facilitating digestion, storing nutrients, secreting vitamins, activating metabolic functions, and shaping intestinal architecture [8]. It is composed of various microbial populations, the most prevalent being the Firmicutes and Bacteroidetes phyla which together represent about 80–90% of the whole gut microbiota [9]. These microbial populations are separated from intestinal epithelial cells by a physical-chemical barrier composed of mucus, mucin glycoproteins, and multiple antibacterial molecules, including alpha-defensins, C-type lectins, lysozyme, phospholipase A2, and secretory IgA [10]. In healthy conditions, all gut microbial species are in a mutualistic or commensal symbiotic state contributing to a perfect and constant homeostasis [11]. In such state, the interaction between gut microbiota, intestinal epithelial cells, and the mucosal immune system creates an environment which controls overgrowth of the host pathogenic flora [12] and limits the colonization of the intestinal tract by foreign pathogens [13–16].

The breakdown of this balance between gut microbiota, the immune system, and the intestinal epithelial barrier results in a pathological condition called dysbiosis [17]. In recent years, several diseases and dysfunctions have been linked to intestinal dysbiosis, including celiac disease, inflammatory bowel disease (IBD), and irritable bowel syndrome (IBS), as well as other conditions [18–24]. In a similar way, given that pancreas is known not to have its own microbial collection, gut microbiota may be involved in the pathogenesis of pancreatic disorders [25]. In this article, we will review the currently available data linking gut microbiota-immune system crosstalk and several pancreatic disorders, such as pancreatitis, diabetes, and pancreatic cancer.

2. Inflammatory Pancreatic Diseases

Acute pancreatitis is an inflammatory disease frequently associated with gallstones or alcohol consumption with a high risk of mortality.

Chronic pancreatitis, instead, is a long-standing, inflammatory disease leading to severe alterations in pancreatic structure and function. The typical clinical manifestations are recurrent episodes of acute pancreatitis in a previously compromised pancreatic gland or a pancreatic exocrine insufficiency due to persistent chronic damage [26].

In either acute or chronic pancreatitis, several alterations in gut microbiota composition have been reported [27].

2.1. Acute Pancreatitis. Hallmark of an acute pancreatitis is an inflammatory state [28, 29] due to an imbalance between pro- and anti-inflammatory cytokines. Recently, Chen et al., in a necrotizing pancreatitis mouse model, demonstrated an overexpression of several proinflammatory cytokines and chemokines, such as TNF-alpha, IL-1beta, IL-6, IL-17A, CXCL1, and IL-18, and a parallel decrease in the Paneth cell-related antimicrobial peptides, such as alpha-defensins and lysozyme [30, 31].

Indeed, pancreatic acinar and Paneth cell-related antimicrobial peptides are essential for gut homeostasis, intestinal immunity integrity, and even for the control of microbiome composition [32]. Recently, in a mouse model, Ahuja et al. have demonstrated that deletion of the Ca^{2+} channel Orai1 in pancreatic acinar cells ($Orai1^{-/-}$ mice) induces several signs of gut inflammation and bacterial overgrowth, leading to bacterial translocation, systemic infection, and death [33]. These experimental findings further confirm the critical role played by antimicrobial pancreatic secretion in modulating gut/pancreatic homeostasis and gut immune system integrity.

As response to inflammation-mediated tissue damage, acinar pancreatic cells produce several molecules that may have the function of damage-associated molecular patterns (DAMPs) [34], such as high-mobility group box protein 1 (HMGB1), heat shock protein 70 (Hsp70), cytosolic protease-caspase 1, nucleotide-binding domain (NLRP3), adenosine triphosphate (ATP), and DNA [35–37]. DAMPs promote activation of the Toll-like-receptors (TLRs) germline-encoded type I transmembrane receptors present on epithelial cells, immune cells, macrophages, and other cells. TLRs act as pathogen recognition receptors (PRRs) and are able to identify pathogen-associated molecular patterns (PAMPs) [38]. To date, in humans, a total of at least 10 different TLRs have been recognized [39]. The TLRs most frequently implicated in the interactions with intestinal bacteria are TLR2 and TLR4, but several other TLRs may be implicated in the pathogenesis of acute pancreatitis [38, 40]. Nishio et al. demonstrated that in mice genetically deficient in the anti-inflammatory cytokine IL-10, the repeated administration of TLR4 and TLR9 ligands was able to induce pancreatic injury [41]. Matas-Cobos et al. comparing 269 acute pancreatitis patients to 269 healthy controls demonstrated that polymorphisms in TLR3 and TLR6 genes were associated with increased severity of pancreatitis [42].

Each TLR responds to distinct DAMPs, leading to the activation of specific intracellular signaling pathways, and to the production of inflammatory cytokines and chemokines [43]. Notably, in the blood of severe acute pancreatitis patients, an increase of TNF-alpha, IL-1, IL-6, and IL-10 has been documented [28, 29]. However, TLR activation is also linked to the transcription of several genes related to some nuclear factors, such as nuclear factor kappa-B (NF-kB), MAP kinase p38, JNK, and IRF-3, crucial in the control of infection and inflammation [11]. Thus, TLRs may be initially responsible for the inflammatory state, but subsequently, they protect the host, repair damaged tissue, and promote a mucosal immune response [38].

Recently, Watanabe et al. proposed that pancreatitis should be thought as a unique form of immune-mediated inflammation [44]. In this model, a pivotal role is played by TLRs (activated by pathogens related DAMPs), in inducing NF-kB-related adaptive immune system cytokines. In this proinflammatory context, damaged acinar cells begin to produce the proinflammatory cytokine IL-33 that, in turn, determines the activation and recruitment of T-cell subpopulations which participate in pancreatic inflammation.

In the context of acute pancreatitis, the inflammation produces intestinal damage by several concomitant pathogenic mechanisms, such as alterations in microcirculation, vasoconstriction in the splanchnic district, and ischemia-reperfusion damage [45, 46]. This, in turn, alters intestinal permeability and leads to a condition known as leaky gut (Figure 1). When there is bacterial overgrowth, leaky gut facilitates the translocation of bacteria and toxins to the pancreas. This worsens pancreatic inflammation resulting in further damage leading to fibrosis or even, in severe cases, necrosis. The bacterial translocation may also be responsible for secondary infections that are associated with a high mortality risk [47].

Moreover, several studies have investigated the relation between inflammatory patterns and microbiota composition during acute pancreatitis. In general, during acute pancreatitis, there is an increase of pathogenic bacteria of the Enterobacteriaceae and Firmicutes families and a decrease of beneficial Bacteroidetes and Lactobacillales [28]. Gerritsen et al. in a mouse model documented that the normal intestinal flora is replaced by an "acute pancreatitis-associated microbiota" [30]. In 2015, Tan et al. published the results of a multicentre prospective clinical study involving 108 acute

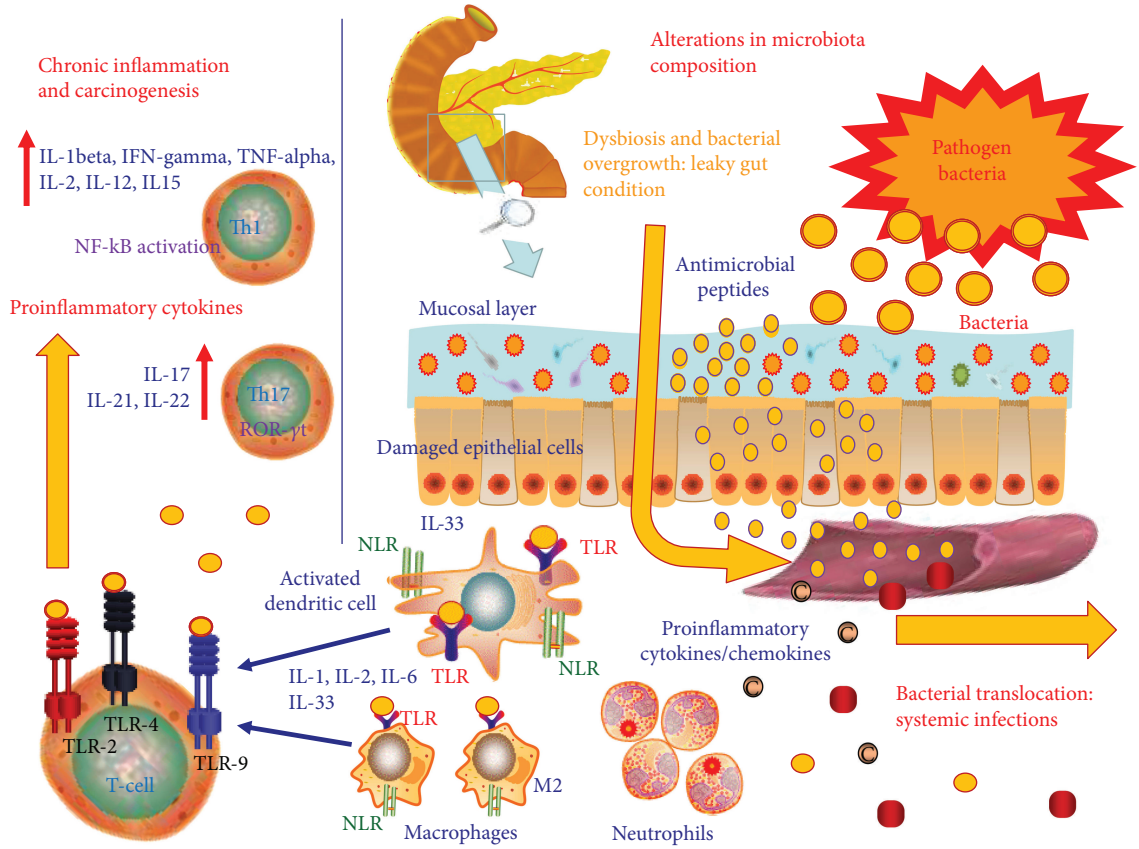

Figure 1: Role of leaky gut in pancreatic inflammation and carcinogenesis. The breakdown of the relationship among physiologic and pathogenic bacteria, the immune system, and intestinal epithelial barrier leads to dysbiosis. The pancreas does not possess its own microbiota, and thus, inflammatory and neoplastic processes affecting the gland may be linked to intestinal dysbiosis. In this way, during bacterial overgrowth, leaky gut is responsible for the translocation of bacteria and toxins to the pancreas. Bacterial translocation is involved in pancreatic inflammation due to toxin diffusion and complications like fibrosis, digestive and absorption disorders, diabetes, or cancer. TLR: Toll-like receptors; NLRs: NOD-like receptors; IL: interleukin; IFN: interferon; TNF: tumor necrosis factor; ROR-γt: RAR-related orphan receptor-gamma t; NF-kB: nuclear factor kappa-B.

pancreatitis patients compared to healthy controls [28]. The authors analyzed the 10 predominant bacteria and measured several serum markers of systemic inflammation, such as plasma endotoxin, TNF-alpha, IL-1, IL-6, and IL-10. The findings have shown that the pathogenetic *Enterococcus*, of the phylum Firmicutes (order Lactobacillales), is increased while *Bifidobacterium*, of the phylum Actinobacteria (order Bifidobacteriales), is decreased. Additionally, IL-6 serum levels correlated directly with Enterobacteriaceae and *Enterococcus* number and inversely with the *Bifidobacterium* and *Clostridium* cluster XI number. The study by Tan et al. was also able to demonstrate that the extent of gut microbiota modifications predicts pancreatitis severity and the occurrence of systemic complications.

It is notable that in the context of acute pancreatitis several commensal bacteria populations have also been identified. These are associated with reduced levels of inflammatory cytokines, such as IL-1beta, TNF-alpha, CXCL1, and IL-18, and are inversely correlated with pancreatitis severity and systemic infectious complications. Thus, it can be hypothesized that the restoration of a physiological gut microbiota composition may be a useful strategy to treat acute pancreatitis [48]. Indeed, the use of probiotics in this clinical setting has been tested, but results are controversial [49]. Qin et al. in 76 acute pancreatitis patients demonstrated that the restoration of a physiological commensal/pathogens *ratio* is able to limit the systemic infectious complications [50]. On the other hand, in several other studies, oral administration of probiotics showed no significant impact on disease outcome or on the prevention of complications [48, 51, 52].

2.2. Chronic Pancreatitis. Chronic pancreatitis results from a long-standing inflammation leading to a chronic damage and severe functional impairment of the gland [53, 54].

It has been reported that about one-third of chronic pancreatitis patients are affected by intestinal bacterial overgrowth but the specific alterations in microbiota composition are not yet fully known [55–59]. Some authors have observed an increase in Firmicutes and a relative decrease in Bacteroidetes [27]. Recently, Jandhyala et al. published a study analyzing three groups of patients: chronic pancreatitis with and without diabetes and healthy controls. Regardless of diabetes, in pancreatitis patients, it was documented a

progressive, duration-dependent reduction of the commensal bacteria *Faecalibacterium prausnitzii* [27]. Notably, *Faecalibacterium prausnitzii* promotes the homeostasis of intestinal epithelium favoring mucin production and tight-junction protein synthesis [60], induces the anti-inflammatory cytokine IL-10 [61], and regulates gut T-cell responses. Thus, the progressive reduction in *Faecalibacterium prausnitzii* observed in chronic pancreatitis patients testifies to a duration-dependent disruption of gut mucosal integrity [27]. Furthermore, *Faecalibacterium prausnitzii* levels negatively correlated with plasma endotoxin ones and an increase of endotoxin levels was associated with an impairment of glucose metabolism. Thus, the reduction in *Faecalibacterium prausnitzii* observed in chronic pancreatitis patients is an additional factor favoring the onset of diabetes or worsening its course. Then, Jandhyala et al. reported a reduction of *Ruminococcus bromii* in chronic pancreatitis patients [27]. *Ruminococcus bromii* has an important physiologic role in the degradation of starch in human colon [62]. Its reduction is related to the disruption of the gut mucosal barrier and is responsible of an alteration of the glucose metabolism.

In other studies, a reduction of *Bacteroidetes*, a Gram-negative bacteria source of lipopolysaccharide (LPS), has consistently been reported. LPS is considered a potent mediator of inflammation. In fact, in binding TLR4, LPS may activate NF-kB-related proinflammatory cytokine production [63]. Chronic pancreatitis patients have higher LPS and endotoxin levels than healthy controls, and these correlate with disease duration. LPS may induce an impairment of pancreatic beta-cells further worsening glucose metabolism [64]. The inflammatory process targets pancreatic islets, and also, T-cell recruitment occurs. In this way, literature data testifies that during chronic pancreatitis there is an increase in both Th1 and Th17 cells [65] and their related proinflammatory cytokines, such as IFN-gamma in pancreatic islets [66].

2.3. Autoimmune Pancreatitis. Pancreatic inflammation may elicit an immune response in the exocrine tissue, leading to either acute or chronic damage. Autoimmune pancreatitis (AIP) accounts for about 5% of all pancreatitis, and it is usually associated with other autoimmune diseases [67]. An increase in serum immunoglobulin G4 (IgG4) is a diagnostic criterion [68]. While genetic factors have been hypothesized [69], the pathogenesis of AIP remains unknown [70].

Interestingly, the gastric *Helicobacter pylori* infection has been shown to be associated with AIP [71, 72]. This bacterium is known to trigger immune responses against host tissues via several molecular mimicry pathways [73]. Guarneri et al. reported a homology between the human carbonic anhydrase II (CA-II) and alpha-carbonic anhydrase of *Helicobacter pylori* (HpCA). CA-II is an enzyme of the pancreatic epithelium whose specific serum antibodies are characteristics of AIP, and the bacterial homolog segments contain the binding motif of the high-risk HLA-DR alleles. These data demonstrated that *Helicobacter pylori* may trigger AIP in genetically predisposed subjects [74].

Other suggestions link bacterial infections with the development of AIP. In a mouse model, *Escherichia coli* induces a severe pancreatic inflammation and fibrosis similar to the human AIP [75]. Numerous studies have reported that specific microbial antigens may trigger the development of AIP activating immune responses. Gram-negative bacteria-associated LPS is able to activate immune response via-TLRs [41]. Several TLRs (TLR2, TLR3, TLR4, TLR5, and TLR7) have been linked with the development of AIP [76–78]. Among these, TLR3 typically recognizes microbial dsRNA activating the Fas/FasL-mediated cytotoxicity, responsible for chronic inflammation [79]. Finally, TLR7 is able to recognize viral ssRNA, thus activating proinflammatory signaling cascades [80].

3. Diabetes

3.1. Type 1 Diabetes. Type 1 diabetes (T1D) is characterized by a loss of insulin secretion due to damage to pancreatic beta-cells caused by an autoimmune process triggered by microbial infections.

Several alterations in gut microbiota composition have been related to the development of T1D. In a recent study on 76 children at high genetic risk, it has been demonstrated that early changes in gut microbiome composition predict T1D onset [81]. In particular, in the microbiome of these T1D predisposed children, *Bacteroides dorei* and *Bacteroides vulgatus* are increased. Instead, in people with late-onset T1D, there is not only a similar increase in *Bacteroides* species but also a reduction of *Clostridium leptum* [38, 82].

Furthermore, several bacterial or viral antigens recognized in children and teenagers have been associated later to the development of T1D [83], including antigens from *Coxsackievirus* A and B, *Echovirus*, *Enterovirus*, and so forth.

During the course of T1D, profound alterations in gut microbiota composition and related metabolites take place [84, 85]. Of importance, changes in the *ratio* of butyrate-producing *Bacteroidetes* and *Firmicutes* bacteria occur [86–88]. Other butyrate-producing and mucin-degrading bacteria, such as *Prevotella* and *Akkermansia muciniphila*, are decreased [89] while short-chain fatty acid- (SCFAs-) producing bacteria such as *Klebsiella* are increased.

Recently, Semenkovich et al. demonstrated bidirectional relationships between gut microbiota alterations and T1D-related inflammation. In fact, in a NOD mouse model, gut microbiota was able to instruct hormonal changes in the testosterone axis (in males) which led to T1D susceptibility, and the hormonal levels, in turn, were able to alter the microbial niches in the gut. This phenomenon may be a possible explanation for the different susceptibility between sexes [84, 90].

In a murine T1D model associated with a reduction in *Lactobacillus* and *Bifidobacterium* species [91], a coexisting high-grade lymphopenia [92] and an upregulation of Th17 cells have been shown [93]. These findings lend support to the hypothesis that alterations in gut microbiota composition are associated with abnormalities of the mucosal immune system and that both mechanisms participate in T1D pathogenesis [94]. In addition, a leaky gut exacerbates T1D either indirectly via beta-cell damage, due to bacterial translocation

and related antigen presentation [95], or directly via beta-cell function impairment mediated by microbial toxins, such as streptozotocin [94].

Diet modification and pharmacological treatment have been similarly studied. Recently, a nonobese diabetic mouse study found that exposure to acidified water is able to increase the presence of mucosal and spleen T-regulatory cells (Tregs) and to decrease Th17 cells, thus decreasing the onset of T1D [96]. A mouse model revealed that insulin treatment is able to somewhat restore microbial populations, positively modulating the microbiota composition towards the normal, healthy state [97]. Xenobiotics have also been implicated in the pathogenesis of T1D. In a recently published study, the neonatal oral administration of vancomycin in a nonobese diabetic mouse reduced the presence of several major *genera* of Gram-positive and Gram-negative bacteria, with one single species *(Akkermansia muciniphila)* becoming dominant [98].

Furthermore, in T1D pathogenesis, a special role is played by mucosal innate and adaptive immunity. To elucidate the role of innate immunity in the susceptibility to T1D, the nucleotide-binding oligomerization domain-containing protein 2 (Nod2) has been identified as a key factor [99]. Nod2, mainly expressed in neutrophils and monocytes/macrophages, recognizes bacterial molecules which possess the muramyl dipeptide (MDP) moiety and stimulates an immune reaction, inducing $CD4^+$ Th1 and $CD4^+$ Th17 cells in pancreatic tissue, contributing to autoantibody production and tissue damage [100, 101].

Recently, Li et al. have generated $Nod2^{-/-}$ nonobese diabetic (NOD) mice with a different gut microbiota composition compared to $Nod2^{+/+}$ NOD mice. $Nod2^{-/-}$ NOD mice appear to be significantly protected from diabetes and present a significant reduction in the proinflammatory cytokine-secreting immune cells and an increase in Tregs [99]. Interestingly, when $Nod2^{-/-}$ NOD mice were housed with $Nod2^{+/+}$ NOD mice, they lost the protection from diabetes, and this evidence confirmed that T1D susceptibility in $Nod2^{-/-}$ NOD mice is dependent on the alteration of gut microbiota, which modulated the frequency and function of IgA-secreting beta-cells and IL-10 promoting T-regulatory cells. Thus, this study has confirmed the close relationship between gut microbiota and T1D susceptibility and the strong interaction between gut microbiota and the immune system.

Several studies have specifically investigated the role of adaptive immune cells in the pathogenesis of T1D. There is evidence that pancreatic islets infiltrating lymphocytes induce beta-cell damage via $CD8^+$ cytotoxic T-cells. This abnormal activation is believed to be the consequence of mechanisms of molecular mimicry and of microbial infections triggering an immune response. Recent studies have focused on the possible role of TLRs. Pancreatic beta-cells express TLR4 which make them sensitive to LPS, promoting and activating transcription of NF-kB-related proinflammatory genes that mediate an immune response against microbial invasion. Thus, the upregulation of TLR4 is a further mechanism to understand the pathogenesis of T1D [71].

3.2. Metabolic Syndrome and Type 2 Diabetes. Metabolic syndrome is defined by a complex cluster of various elements, including visceral obesity, abnormal glucose metabolism, dyslipidaemia, and arterial hypertension. Metabolic syndrome is associated with an increased risk of type 2 diabetes (T2D) and cardiovascular diseases [102]. The disease is characterized by an increased cytokine production (mainly TNF-alpha and IL-1beta) [103], with a persistent low-grade inflammation [104]. This, in turn, generates a continuous recruitment of immune cells in metabolically active tissues, such as adipose tissue, the pancreatic gland, thyroid, liver, and muscle [105, 106]. T2D is a multifactorial disease, and several factors are involved in its pathogenesis, including diet, obesity, and gut dysbiosis [107].

Gut microbiota has conclusively been linked to the pathogenesis of both metabolic syndrome and T2D. Recently, Guo et al. developed a mouse model with high-fat feeding and demonstrated that the diet was able to alter gut microbial communities, the Paneth cell-related antimicrobial peptide production, and even to increase circulating proinflammatory cytokines, such as TNF-alpha, IL-6, and IL-1beta [108]. Thus, it is the intestinal dysbiosis related to diet, rather than adipose tissue per se, that has a pivotal role in developing intestinal inflammation.

Hence, gut microbiota by affecting the production and storage of energy could influence body weight and obesity [8], tissue proinflammatory activity, peripheral insulin resistance, pancreatic intestinal hormone production, and finally bile acid metabolism [109]. Consequently, in metabolic syndrome, the increase in the Firmicutes/Bacteroidetes *ratio* corresponds to body weight and promotes the hydrolysis of nondigestible polysaccharides in the gut, which in turn favors an increase in calories extracted from food [110, 111]. Several metagenomic studies performed on metabolic syndrome and T2D patient stools compared to healthy subjects revealed an increase in the order Lactobacillales with a decrease in *Roseburia intestinalis*, *Faecalibacterium prausnitzii*, *Bacteroides*, *Prevotella genera*, *Bifidobacterium animalis*, and *Methanobrevibacter smithii*. On the other hand, *Staphylococcus aureus*, *Escherichia coli*, and *Lactobacillus reuteri* have been found to be elevated and to predict the development of obesity [107].

Certain types of bacteria, such as *Tannerella* spp., are associated with oral infections and periodontal disease. These are typically characterized by an increase of several proinflammatory cytokines like TNF-alpha, IL-1beta, and IL-6 [112]. Gram-negative bacteria-induced LPS is able to trigger an immune response via LPS-binding protein (LBP), which in turn binds the macrophage receptor CD14. The complex formed by LPS-LBP and CD14 may activate NF-kB and AP-1 proinflammatory genes via TLR4 [113]. LPS may also activate the macrophage and dendritic cell NOD-like receptors (NLRs) that induce NF-kB in association with TLR4 [114]. In this way, a mouse model demonstrated that the lack of TLR4 protects against insulin resistance [115].

Finally, recent evidences demonstrated that intestinal dysbiosis may also mediate alterations in the Th17 cells/Tregs balance. So, the breakdown in the physiological equilibrium between pro- and anti-inflammatory T-cell subpopulations may be responsible for the development and progression of several inflammatory diseases, both in the

gastrointestinal tract and in the systemic ones, including obesity-associated metabolic syndrome and T2D [104]. Thus, intestinal dysbiosis is intimately linked to significant alterations in Th17/Tregs balance contributing to obesity, metabolic syndrome, and T2D. Understanding the complex mechanisms responsible for this alteration will allow to develop novel translational therapeutic strategies to potentially treat these widespread diseases.

4. Pancreatic Cancer

Pancreatic cancer is extremely aggressive, with a very poor prognosis. Only 25% of pancreatic cancer can be surgically removed at the time of diagnosis. About 95% of them are adenocarcinomas that originate from gland, ductal, or acinar cells of the exocrine pancreas [116].

A link among dysbiosis, chronic inflammation, and pancreatic cancer has been well established [117–120]. Importantly, dysbiosis is considered not to have a direct mutagenic action disrupting cell cycle control, activating oncogenic signaling pathways, and producing tumor-promoting metabolites [121–124]. However, intestinal dysbiosis can activate the immune system through several pathways involving tumor-infiltrating lymphocytes (TILs) and their related cytokines, innate immune cells, TLRs, and others. In this way, TILs produce proinflammatory mediators inducing STAT3 and NF-kB pathways that act as tumorigenic factors increasing cellular proliferation and suppressing apoptosis [125–127].

Several germ-free mouse models have allowed to understand the significant impact of gut microbiome in carcinogenesis. In fact, germ-free animals have a significant reduction in cancer development, probably due to decreased gut dysbiosis and related chronic inflammation [1, 128]. In the same way, a reduction in cancer development has been observed in mice after antibiotic treatment that may be responsible for the reduction of the pathogen load in the gut mucosa [117]. Other experimental evidence has highlighted the close relationship among diet, xenobiotics, gut microbiota, and cancer [129]. In one study, mice genetically predisposed to colorectal cancer displayed increased tumor progression in a context characterized by a specific microbiota composition. This tumor-predisposing phenotype could be transferred to healthy mice after microbiota transplant using fecal samples. Interestingly, in these mice, antibiotics were able to limit tumor development, probably blocking the tumor-inducing gut microbiota [129]. However, antibiotics could also have a detrimental role. In a recent case-control study conducted on a very large cancer population, Boursi et al. proved that repeated antibiotic exposure is able to promote cancer formation, probably due to a change in microbiota [130]. This study revealed that especially the use of penicillin was associated with an elevated risk of developing colorectal, esophageal, gastric, and pancreatic cancers [130].

In chronic pancreatitis people who harbor a KRAS mutation, there is an increased risk of cancer [131, 132]. In these individuals, gut dysbiosis is able to accelerate pancreatic carcinogenesis due to the mutated KRAS hyperstimulation by the LPS-driven inflammation and by the TLR-mediated NF-kB proinflammatory gene transcription [133, 134]. The role of Gram-negative LPS-TLR4 interaction in inducing chronic inflammation and cancer has been well recognized [135]. In a recent study, Ochi et al. specifically demonstrated their impact in the pathogenesis of pancreatic cancer [136]. In a mouse model, the administration of LPS was able to significantly accelerate carcinogenic progression. On the other hand, the inhibition of TLR4 limited cancer progression, while the inhibition of the TLR adapter protein myeloid differentiation primary response gene 88 (MyD88) unpredictably worsened pancreatic inflammation and cancer development. The procancerogenetic and inflammatory actions of MyD88 inhibition are mediated by dendritic cells (DCs), which were able to induce pancreatic antigen-restricted Th2 cells and promote the transition from pancreatitis to pancreatic cancer [136].

Pathogens are able to act as carcinogenetic agents after infecting the pancreatic gland through intestinal translocation. Among these, a special role is played by *Helicobacter pylori* [72]. In fact, it has been well established that it may promote the carcinogenesis of the stomach, liver, and pancreas, by inducing the activation of the nuclear factor NF-kB and its proinflammatory cytokines, such as IL-1beta [137]. *Fusobacterium* species have also been linked to the development of pancreatic cancer, and they are associated with worse prognosis [138].

Recently, Ren et al. studied the microbiota profile of 85 pancreatic cancer patients compared to 57 healthy people [139]. This study revealed that gut microbial diversity is significantly reduced in pancreatic cancer and this tumor is characterized by a unique microbial profile. In particular, the microbial alterations in pancreatic cancer regarded an increase in several pathogens, such as *Veillonella*, *Klebsiella*, and *Selenomonas*, and LPS-producing bacteria including *Prevotella*, *Hallella*, and *Enterobacter*, and a related decrease in several commensals, such as *Bifidobacterium*, and some butyrate-producing bacteria, such as *Coprococcus*, *Clostridium* IV, *Blautia*, *Flavonifractor*, and *Anaerostipes* [139]. The evidence of the increase in the LPS-producing bacteria confirms the role of dysbiosis in mediating chronic inflammation and oxidative damage activating the NF-kB pathway and its related proinflammatory cytokine production. In this way, long-standing chronic inflammation and oxidative damage participate in the development of cancer.

Likewise, it has been shown that pancreatic cancer is associated with an alteration of the physiological oral microbiota composition [140]. Oral microbiota is composed of more than 700 bacteria species which contribute to health and physiology of the mouth, teeth, and oral cavity [117]. Alterations in the taxa dominance and diversity among oral microbial communities, particularly regarding those related to the periodontal disease, may be associated with an increased pancreatic cancer risk [140]. Farrell et al. performed a study analyzing salivary microbiota of several pancreatic cancer and chronic pancreatitis patients compared to healthy subjects [141]. These authors demonstrated that pancreatic cancer is related to a specific alteration in salivary microbiota composition. In particular, it was shown

Table 1: Gut microbiota alterations in pancreatic pathologies.

Bacterium (phylum)	Acute pancreatitis [28]	Chronic pancreatitis [27]	Autoimmune pancreatitis (AIP)	Type 1 diabetes (T1D)	Metabolic syndrome and type 2 diabetes (T2D)	Pancreatic cancer
Increase	*Enterococcus* spp. (Firmicutes) Enterobacteriaceae	Firmicutes	*Helicobacter pylori* (**Molecular mimicry** mechanism) [72–75] *Escherichia coli* (**Trigger** mechanism) [75]	*Bacteroides dorei* and *vulgates* (Bacteroidetes) [38, 82] *Klebsiella* spp. (Enterobacteriaceae) [89] *Coxsackievirus* A and B, *Echovirus*, *Enterovirus* [83]	Lactobacillales, *Staphylococcus aureus* (Firmicutes) [107] *Escherichia coli* (Proteobacteria) [107] *Tannerella* spp. (Bacteroidetes) [112]	*Helicobacter pylori* (Proteobacteria) [72] *Fusobacterium* [138] *Leptotrichia* [142] (Fusobacteria) *Veillonella* spp. *Selenomonas* spp. (Firmicutes) [139] *Klebsiella* spp. *Enterobacter* spp. (Enterobacteriaceae) [139] *Prevotella* spp. *Hallella* spp. (Bacteroidetes) [139] **Salivary microbiota:** [140, 141] *Granulicatella adiacens* (Firmicutes) *Porphyromonas gingivalis* (Bacteroidetes)
Decrease	Bacteroidetes *Bifidobacterium* spp. (Actinobacteria) *Lactobacillus* spp. *Clostridium cluster XI* (Firmicutes)	Bacteroidetes *Faecalibacterium prausnitzii* (Firmicutes) *Ruminococcus bromii* (Firmicutes)	—	*Lactobacillus* spp. *Clostridium leptum* (Firmicutes) [38, 82] *Bifidobacterium* spp. (Actinobacteria) [91] *Prevotella* spp. (Bacteroidetes) [89] *Akkermansia muciniphila* (Verrucomicrobia) [89]	*Bacteroides* *Prevotella* (Bacteroidetes) [107] *Roseburia intestinalis* *Faecalibacterium prausnitzii* (Firmicutes) [107] *Bifidobacterium animalis* (Actinobacteria) [107] *Methanobrevibacter smithii* (Methanobacteria) [107]	*Bifidobacterium* spp. (Actinobacteria) [139] *Coprococcus* spp. *Clostridium cluster IV* *Blautia* spp. *Flavinofractor* spp. (Firmicutes) [139] **Salivary microbiota:** [140, 141] *Neisseria elongata* (Proteobacteria) *Streptococcus mitis* (Firmicutes) *Corynebacterium* spp. (Actinobacteria)

that *Neisseria elongata*, *Corynebacterium* spp., and *Streptococcus mitis* decreased, while *Granulicatella adiacens* and *Porphyromonas gingivalis* increased [140, 141]. Recently, Torres et al. conducted a cross-sectional study showing an increase in *Leptotrichia* spp. and a reduction in *Porphyromonas* spp. in pancreatic cancer patient saliva; thus, a higher *Leptotrichia* : *Porphyromonas* (L : P) ratio may become an important pancreatic cancer diagnostic biomarker [142]. Otherwise, Michaud et al. demonstrated that high antibody titer against gut commensal bacteria was associated with a reduction of 45% in the risk of pancreatic cancer compared to those with a lower antibody titer [143]. In the same way, these authors revealed that the highest concentration of serum antibodies to the pathogenetic bacteria *Porphyromonas gingivalis* (associated with periodontal disease) was linked to a 2-fold increased risk of pancreatic cancer [143].

Altogether, these evidences highlight the potential to develop future novel diagnostic tools to detect early pancreatic cancer, utilizing samples easy to collect, such as blood, saliva, and stools. However, at the present time, it is not possible to discriminate whether these gut microbial alterations exert a causal role in the developing of pancreatic cancer or, instead, are a result of cancer formation.

Importantly, it should be noted that chronic inflammation-related pancreatic cancer development may occur even without the presence of bacteria. This type of sterile inflammation may be triggered by distant intestinal dysbiosis or translocation of bacteria components, such as LPS, and it is guided by the activation of the immune system through TLRs. In this way, TLR2, TLR4, and TLR9 have been recently shown to be associated with pancreatic cancer development [144, 145].

Finally, recent evidences have shown that gut microbiota and antibiotics may alter tumor response to chemotherapy by modulating tumor microenvironment [146, 147]. Hence, gut microbiota may modify the efficacy of traditional cancer chemotherapies, the novel immune-target drugs, such as anti-CTLA4 and anti-CD274 therapies, but also the tumor recurrence after pancreatic surgery [121].

In conclusion, pancreatic cancer is considered a very insidious and aggressive disease characterized by late diagnosis and no effective screening methods. In this way, in the one hand, it may be too early to hope in the routine use of gut microbiome modulation for therapeutic purposes, and on the other hand, gut microbiome profiling may have important diagnostic tools in the prediction of pancreatic cancer development, thus improving the survival rates associated with this disease.

5. Conclusions

Gut microbiota is central to the development and modulation of the intestinal homeostasis and mucosal immune system integrity and exerts an important role in the protection against pathogenic microbes by maintaining gut integrity and regulating intestinal barrier permeability.

The pancreas does not possess its own microbiota, and the available evidence demonstrates that alteration of gut microbiota determining dysbiosis and bacterial translocation (Table 1) is correlated with the duration and prognosis of several pancreatic disorders, including pancreatitis, diabetes, and cancer. However, whether gut dysbiosis is the cause or an effect of such pathological conditions remains unclear.

In principle, the pharmacological modulation of gut microbiota may be beneficial in the treatment of pancreatic conditions and related complications. However, the use of prebiotics, probiotics, antibiotics, and anti-inflammatory drugs or the fecal microbiota transplantation either as a preventative or as a therapeutic strategy remains controversial. These procedures have not yet been a subject to the rigorous efficacy and safety testing necessary to recommend their routine use.

In the foreseeable future, the analysis of specific alterations in the microbiome profile may permit to develop novel tools for the early detection of several pancreatic disorders, utilizing samples easy to collect, such as blood, saliva, and stools.

In conclusion, the ways in which gut microbiota is modulated and interacts with the immune system need to be further elucidated to enter a new era of treatment modalities.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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Review Article

Gut Microbiota as a Driver of Inflammation in Nonalcoholic Fatty Liver Disease

Stefano Bibbò,[1] **Gianluca Ianiro**,[2] **Maria Pina Dore**,[1] **Claudia Simonelli**,[2] **Estelle E. Newton**,[3] **and Giovanni Cammarota**[2]

[1]*Department of Clinical and Experimental Medicine, University of Sassari, Sassari, Italy*
[2]*Department of Gastroenterology, Catholic University, School of Medicine and Surgery, A. Gemelli Hospital, Rome, Italy*
[3]*CytoCure LLC, Beverly, MA, USA*

Correspondence should be addressed to Giovanni Cammarota; Giovanni.Cammarota@unicatt.it

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The prevalence of nonalcoholic fatty liver disease and the consequent burden of metabolic syndrome have increased in recent years. Although the pathogenesis of nonalcoholic fatty liver disease is not completely understood, it is thought to be the hepatic manifestation of the dysregulation of insulin-dependent pathways leading to insulin resistance and adipose tissue accumulation in the liver. Recently, the gut-liver axis has been proposed as a key player in the pathogenesis of NAFLD, as the passage of bacteria-derived products into the portal circulation could lead to a trigger of innate immunity, which in turn leads to liver inflammation. Additionally, higher prevalence of intestinal dysbiosis, larger production of endogenous ethanol, and higher prevalence of increased intestinal permeability and bacterial translocation were found in patients with liver injury. In this review, we describe the role of intestinal dysbiosis in the activation of the inflammatory cascade in NAFLD.

1. Introduction

Nonalcoholic fatty liver disease (NAFLD) is a multifactorial condition resulting from a complex interaction of genetic and environmental factors. The prevalence of the disorder has increased in recent decades, as is the burden of metabolic syndrome [1]. NAFLD was defined as a spectrum of liver conditions whose dominant feature is abnormal hepatic triglyceride accumulation. In the absence of inflammation and hepatocellular damage, this condition is simply defined as steatosis or nonalcoholic fatty liver. In a liver with chronic NAFLD, lobular inflammation and signs of hepatocellular damage may occur. This latter condition is called nonalcoholic steatohepatitis (NASH). The natural history of NASH is not completely understood, but one can assume that NASH predisposes to several complications such as liver fibrosis, cirrhosis, and hepatocellular carcinoma [2, 3]. Moreover, the pathogenesis of this disorder remains largely unknown. The so-called two-hit hypothesis suggests that accumulation of triglyceride in hepatic cells may expose the liver to secondary insults, primarily oxidative stress, resulting in chronic injury. This model focuses on liver autonomous dysfunction leading to NASH [4]. In recent years, NAFLD was proposed as the hepatic feature of metabolic disorders, as insulin resistance and metabolic syndrome are strongly linked to the progression of liver disease. However, other organs including adipose tissue, muscle, and gut may play an important role in the progression of NAFLD [1, 3]. The liver and intestine are tightly linked through the portal circulation; consequently, gut-derived products, mainly microbial components, arrive primarily to the liver with obvious pathogenic implications [5]. The intestine is colonized by an enormous array of microorganisms, defined as the gut microbiota or microbiome, which can be considered a functional organ [6]. The gut microbiota plays a key role in the maintenance of human health, being involved in the development and growth of the immune system and regulation of several metabolic pathways [7–9]. Quantitative and/or qualitative alterations of gut microbiota, in other way defined as dysbiosis, are known to lead to disruption of this

homeostasis and, consequently, development of pathology. Disorders associated with the impairment of gut microbiota can include gastrointestinal diseases [10–13], liver diseases [14], and also metabolic disorders such as metabolic syndrome [15] and diabetes [16, 17].

2. The Role of Intestinal Dysbiosis

Our understanding of the relationship between gut microbiota and the development of liver disease has been highlighted in both animal and human studies (see Table 1). Small intestinal bacterial overgrowth (SIBO), increased intestinal permeability, and a number of bacterial endotoxins were reported as putative factors for NASH development [18]. In the first observation, the authors hypothesized an important role for SIBO in the occurrence of NASH, but in recent years, the development of metagenomic sequencing technologies has allowed the description of detailed alterations of the gut microbiota, focusing on qualitative dysbiosis rather than quantitative modifications [19–22].

The prevalence of SIBO in patients with NASH has been widely studied. Wigg et al. reported that patients with NASH have a higher prevalence of SIBO compared to controls (50% versus 22%). In addition, higher levels of TNF-alpha compared to control subjects were observed, although intestinal permeability and serum endotoxin levels were similar in the two groups [23]. These findings were only partially confirmed by further studies. Miele et al. found that subjects with NAFLD had significantly increased gut permeability and a higher prevalence of SIBO, compared with healthy subjects. Both gut permeability and the prevalence of SIBO correlated with the severity of steatosis but not with the presence of NASH [24]. These findings were confirmed in a further study by Shanab et al.; additionally, authors found an enhanced expression of Toll-like receptor 4 (TLR4) and release of interleukin 8 (IL-8) [25]. Moreover, increased intestinal permeability and higher levels of blood lipopolysaccharide (LPS) were found in children with NASH compared to those with NAFLD [26]. These data confirmed findings based on rodent models, showing that higher intestinal mucosa permeability promotes the increase of LPS levels in portal blood and in turn liver inflammatory damage [27–29].

The gut microbiota has been suggested to be responsible for the increase of endogenous ethanol production in patients with NAFLD. A rodent experimental model demonstrated an increased breath ethanol content [30] that was abolished by treatment with neomycin. This observation was also confirmed in humans. Patients with NASH harbored an increased number of alcohol-producing bacteria (in particular *Escherichia coli*) in their microbiome associated with elevated blood-ethanol concentration [31]. An additional study confirmed the results [32]. Patients with NAFLD had a prevalence of SIBO of 37.5%, and *Escherichia coli* was the predominant bacteria in duodenal fluid aspirate. Moreover, patients with SIBO had higher endotoxin levels and expression of Toll-like receptor 4 (TLR4) compared to those without [32]. However, the presence of SIBO appears not to represent an ubiquitous marker of NAFLD. A study performed on 20 patients with NAFLD showed intestinal permeability, and alcohol and endotoxin levels in the plasma were significantly higher compared to controls, but the prevalence of SIBO was similar between patients and controls [33].

The development of modern sequencing techniques (metagenomic approach) has allowed a deeper analysis of the microbiota composition [34]. The first metagenomic characterization of gut microbiota in patients with NASH was reported by Mouzaki et al. [35]. The percentage of *Bacteroidetes* and *C. coccoides* was lower in patients with NASH compared to patients with NAFLD and healthy controls. The percentage of *Bacteroidetes* in patients with NASH remained significantly lower even after adjusting for anthropometric variables (body mass index) and fat intake [35]. Boursier et al. were able to partially confirm the data. In their study, patients with NASH harbored a higher quantity of *Bacteroides* and a lower quantity of *Prevotella*, compared to individuals without NASH [36]. The multivariate analysis adjusted for metabolic factors showed that *Bacteroides* abundance was independently associated with NASH. Differences in taxonomic composition of intestinal microbiota at the phylum level according to NAFLD severity were not detected. On the contrary, dramatic differences were observed at the family level according to severity of hepatic injury. More specifically, *Bacteroidaceae* family increased along with severity of liver lesions, whereas the family of *Prevotellaceae* and *Erysipelotrichaceae* decreased. Authors also evaluated the correlation with the grade of liver fibrosis. Patients with a grade of liver fibrosis of F0/F1 had higher abundances of *Bacteroides* and *Ruminococcus* and lower abundance of *Prevotella* compared to those with F2 liver fibrosis [36]. Analysis of the fecal microbiome and volatile organic compound (for instance ethanol) in patients with NASH revealed a significant increase in fecal volatile compounds in NAFLD patients compared to healthy controls. In the microbiome of NAFLD patients, *Lactobacillus* species and selected members of phylum Firmicutes, in particular *Lachnospiraceae* (*Dorea*, *Robinsoniella*, and *Roseburia)*, were overrepresented, while other members (Ruminococcaceae; genus, *Oscillibacter*) were significantly underrepresented [37]. Further data show that patients with NASH have a higher abundance of *Parabacteroides* and *Allisonella* and lower representation of *Faecalibacterium* and *Firmicutes* families [38].

The intestinal dysbiosis is able to modify the profile of bile acids in patients with NAFLD. In a population of patients with NASH, levels of unconjugated cholic acid and chenodeoxycholic acid were, respectively, increased. The analysis of intestinal microbiota revealed that patients with NASH harbored a lower relative abundance of *Bacteroidetes* and *Clostridium leptum*, independently from other metabolic factors [39]. For instance, *Clostridium leptum* is able to modify bile acids, converting them from primary to secondary bile acids [40]. The correlation of bile acid levels and intestinal dysbiosis with markers of hepatic injury suggests a possible role for bile acids in the progression of NAFLD to NASH [39].

In pediatric patients, NAFLD-specific alterations in gut microbiota composition, different from those found in adults, were also described. Children with NASH had an

Table 1: Gut microbiota alteration in human studies (NASH = nonalcoholic steatohepatitis; SS = simple steatosis; HC = healthy controls).

Study	Subjects	Gut microbiota alterations
Mouzaki et al. [35]	NAFLD (SS or NASH) and HC	↑ *Clostridium coccoides* in NASH versus SS ↓ *Bacteroidetes* in NASH versus SS and HC
Boursier et al. [36]	NAFLD (SS, NASH, and fibrosis)	↑ *Bacteroides* and ↓ *Prevotella* in NASH ↑ *Bacteroidaceae*; ↓ *Prevotellaceae* and *Erysipelotrichaceae* according to the severity of NASH ↑ *Bacteroides* and *Ruminococcus* and ↓ *Prevotella* in patients with F2 fibrosis versus F0/F1
Raman et al. [37]	NAFLD and HC	↑ *Lactobacillus* and selected members of *Firmicutes* (*Dorea*, *Robinsoniella*, and *Roseburia*); ↓ one member of *Firmicutes* (*Oscillibacter*) in NAFLD
Wong et al. [38]	NASH and HC	↑ *Parabacteroides* and *Allisonella*; ↓ *Firmicutes* and *Faecalibacterium* in NASH
Mouzaki et al. [39]	NAFLD (SS and NASH) and HC	↓ *Bacteroidetes* and *Clostridium leptum* in NASH versus HC
Zhu et al. [31]	Children—NASH, obese, and HC	↑ *Bacteriodetes* and *Proteobacteria* and ↓ *Firmicutes* and *Actinobacteria* in NASH versus HC
Del Chierico et al. [41]	Children—NAFLD (SS and NASH), obese, and HC	↑ *Bradyrhizobium*, *Anaerococcus*, *Peptoniphilus*, *Propionibacterium acnes*, *Dorea*, and *Ruminococcus* and ↓ *Oscillospira* and *Rikenellaceae* in NAFLD

increased number of *Bacteriodetes* and *Proteobacteria* and a decreased number of *Firmicutes* and *Actinobacteria* compared to healthy children [31]. In a more recent research, a similar dysbiosis pattern was observed in pediatric patients characterized by a decrease in *Oscillospira* and *Rikenellaceae* and an increase in *Bradyrhizobium*, *Anaerococcus*, *Peptoniphilus*, *Propionibacterium acnes*, *Dorea*, and *Ruminococcus* [41].

3. The Role of Immunity

As discussed before, dysbiosis plays a main role in increasing intestinal permeability, with consequent passage into the portal circulation of bacteria-derived products. Among these, the lypopolisaccharide (LPS), a cell component of Gram-negative bacteria, is the best investigated. LPS is able to activate Toll-like receptors (TLRs) resulting in the production of proinflammatory cytokines and chemokines. Several experimental models of NASH reported high levels of LPS leading to hepatic injury through the recruitment of inflammatory cells [5, 42]. In this pathway, a key role is played by Kupffer cells. They contribute to endotoxin clearance [43] and to inflammatory response, through several TLRs located in their surface that, after being activated by LPS, are able to trigger a cascade of events, leading to the production of inflammatory cytokines, such as IL-1β and TNF-α [44].

Up to 13 different TLRs have been identified in mammals; however, among these, only TLR2, TLR4, TLR5, TLR6, TLR7, and TLR9 are known to be involved in the pathogenesis of NAFLD [45]. TLR2 mainly binds peptidoglycan and lipoteichoic acid that are components of Gram-positive bacterial cells. In a murine experiment, Miura et al. [45] demonstrated that TLR2-deficient mice are resistant to diet-induced steatohepatitis, showing a lower expression of proinflammatory cytokines (TNFα and IL-1β) [46]. In contrast, in other experiments on TLR2-deficient mice, a similar and even more severe susceptibility to steatohepatitis was observed [47, 48]. Studies looking at the interaction between TLR2 and TLR6 found that deregulation of TLR6 expression potentiated the TLR2-mediated liver inflammation. Indeed, the TLR2/TLR6 stimulation promoted the production of proinflammatory cytokines that was higher in patients with lobular inflammation [49]. TLR4 is a receptor for LPS. The importance of this axis has been clarified through TLR4 mutant mice resistant to the development of NAFLD [50]; furthermore, a direct link between TLR4 and Kupffer cells was described in the pathogenesis of steatohepatitis, as the experimental destruction of Kupffer cells was shown to prevent the increased expression of TLR4 [51]. The relevance of this interplay was confirmed in a murine model, where occurrence of NAFLD required endotoxin-dependent activation of hepatic Kupffer cells, associated with SIBO and enhanced intestinal permeability [52]. TLR5 is a receptor for bacterial flagellin. Although few data are reported about its role in the development of metabolic disorders, a murine model suggests that TLR5 deficiency is able to promote obesity, steatosis, and in turn metabolic syndrome [53]. More recent evidence shows that hepatocyte TLR5 protects against diet-induced liver disease [54]. Similarly, a protective role in preventing NAFLD was also reported for TLR7 [55]. TLR9 is a receptor for bacterial DNA, in particular for the unmethylated CpG motif, which is increased in NASH models; the activation of TLR9 signaling on Kupffer cells induces the production of proinflammatory cytokine, such as IL-1β leading to steatosis and inflammation. Moreover, the activation of TLR9 in hepatic stellate cells suggests a role in promoting fibrogenesis [56]. In animal models, the blockage of Il-1 signaling leads to a reduction of TLR9-mediated liver damage, in particular the endogenous IL-1 receptor antagonist, and regulates the extent of TLR9-induced liver injury [57].

The myeloid differentiation primary response gene 88 (MyD88) is the most investigated signaling adaptor for TLRs. The activation of this adaptor by TLRs, mainly TLR4 and TLR9, results in the upregulation of the transcriptional factor

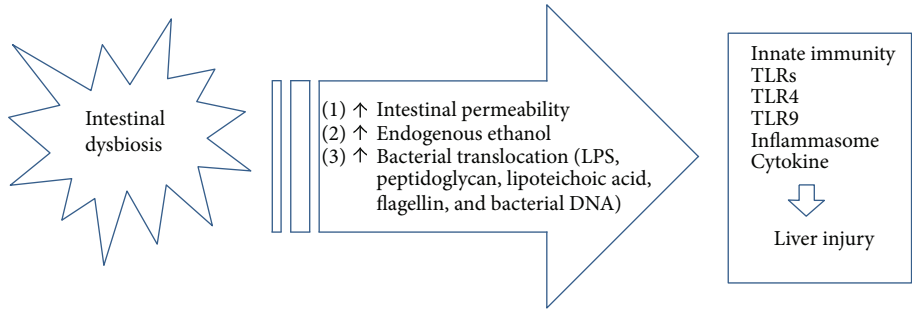

Figure 1: Interactions between gut microbiota and innate immunity in the pathogenesis of steatohepatitis.

nuclear factor kappa beta (NF-κB) and c-Jun N-terminal kinase (JNK) pathway [58]. However, data about the role of Myd88 in the pathophysiology of NAFLD are conflicting. For example, Duparc et al. recently reported that, in a rodent model, the hepatocyte specific deletion of Myd88 predisposes to inflammation, hepatic steatosis, and insulin resistance [59]; other reports suggested that the deletion of Myd88 increases the risk of developing features of metabolic syndrome such as diabetes and hepatic steatosis [60, 61]; conversely, deletion of MyD88 in intestinal epithelial cell-specific murine model partially protected against diet-induced obesity, diabetes, and metabolic inflammation [62].

Finally, the inflammasome, a group of sensors for endogenous and/or exogenous pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) [63], seems to be involved in development of liver steatosis and inflammation. The inflammasome is a multimeric signaling platform that leads to the production of IL-18 and IL-1 through NRLP3 (NOD-like receptors, pyrin domain containing 3) and NRLP6 (and 6). Interestingly, in inflammasome-deficient mice, an increase of *Bacteroidetes* and a reduction of *Firmicutes* were reported, resulting in a higher activation of TLR4 and TLR9 and subsequent inflammatory pathway [64].

4. Concluding Remarks

Gut microbiota alterations and increased intestinal permeability appear to play a major role in promoting inflammation and progression of NAFLD to NASH. The disruption of "normal" microbiota can occur in several conditions including environmental exposures, medications, or diet [65, 66]. It was hypothesized that intestinal dysbiosis may lead to the progression of NAFLD through several pathways. The presence of SIBO is related to endogenous production of alcohol and furthermore to increased intestinal permeability, favoring the passage of bacterial-derived products in the portal circulation. These products (LPS, peptidoglycan, lipoteichoic acid, flagellin, and bacterial DNA) are ligands for TLRs and stimulate the innate immune system in the liver (Figure 1).

Several TLRs, identified in the liver, have a mandatory role in hepatic injury mechanisms, as reported in some animal studies described specifically in the previous section. It has been described that different bacterial products have a selectivity for TLRs, which have different roles in the progression of tissue inflammation. For example TLR4 and TLR9, which bind LPS and bacterial DNA, respectively, promote inflammation and liver fibrogenesis through the activation of Kuppfer cells and hepatic stellate cells. Conversely, certain receptors for bacterial-derived products may have a protective role in the progression of inflammation; indeed, it has been observed that the specific deletion of TLR5 and TLR7 promotes the inflammatory pathways.

Consequently, the altered balance of these receptors can trigger a cascade of events, in particular the secretion of proinflammatory cytokines that drive the inflammation in NAFLD. This condition, previously described as "metabolic endotoxiemia," is a common feature of several metabolic disorders [28, 67].

The reported evidences about the inflammatory pathways mainly derive from animal models. These findings are sometimes conflicting; furthermore, they are not always confirmed by "human" studies. For example, the hepatic deletion of MyD88 seems to promote a proinflammatory "milieu," while the specific deletion in intestinal epithelial cells may have a protective role. There is still much to be understood about the role of the intestine in the inflammatory mechanisms of NAFLD.

In addition, qualitative alterations of gut microbiota are able to interfere with the intestinal absorption of bile acid. Based on this observation, a new role for gut microbiota was proposed. More specifically, intestinal dysbiosis resulting in higher levels of unconjugated bile acid, able to inhibit farnesoid X receptor (FXR) signaling, was observed in animal models [68]. FXR inhibition results in increased production of ceramides that cause lipid toxicity and increased fatty acid synthesis.

In conclusion, to date, evidence for a role of gut microbiota in the progression of NAFLD is still weak, although the reported observations are very intriguing [69]. Research fields that need to be explored are many, from the identification of specific alterations of the gut microbiota, to a more detailed understanding of the mechanisms of innate immunity. The comprehension of the pathogenic pathways of NAFLD in lean patients is a very interesting issue, and several evidences suggest a main role for gut microbiota [70].

These observations allow us to consider a new role for the intestine, suggesting it as one of the main actors in NAFLD/NASH progression. Indeed, the altered production of volatile metabolites by gut microbiota, such as endogenous alcohol, and the uncontrolled passage of bacterial-derived products

in the bloodstream would be able to trigger the inflammation and cellular damage in the liver, even in subjects without overt metabolic syndrome. Assuming these data, the gut can be placed side by side with muscle and adipose tissue as a "director" in the progression of liver disease; it would be very important to understand whether intestinal dysbiosis is a factor necessary for the development of NASH, or is only a precipitating factor.

Finally, further studies are needed, and maybe in a future not too far, they will provide new therapeutic chances for this disorder of growing worldwide interest.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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Review Article

Gut Dysbiosis and Muscle Aging: Searching for Novel Targets against Sarcopenia

Anna Picca,[1] Francesca Fanelli,[2] Riccardo Calvani,[1] Giuseppina Mulè,[2] Vito Pesce,[3] Alex Sisto,[1] Cecilia Pantanelli,[1] Roberto Bernabei,[1] Francesco Landi,[1] and Emanuele Marzetti[1]

[1]*Department of Geriatrics, Neurosciences and Orthopedics, Catholic University of the Sacred Heart, Rome, Italy*
[2]*Institute of Sciences of Food Production, National Research Council, Bari, Italy*
[3]*Department of Biosciences, Biotechnology and Biopharmaceutics, University of Bari, Bari, Italy*

Correspondence should be addressed to Riccardo Calvani; riccardo.calvani@gmail.com

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Advanced age is characterized by several changes, one of which is the impairment of the homeostasis of intestinal microbiota. These alterations critically influence host health and have been associated with morbidity and mortality in older adults. "Inflammaging," an age-related chronic inflammatory process, is a common trait of several conditions, including sarcopenia. Interestingly, imbalanced intestinal microbial community has been suggested to contribute to inflammaging. Changes in gut microbiota accompanying sarcopenia may be attenuated by supplementation with pre- and probiotics. Although muscle aging has been increasingly recognized as a biomarker of aging, the pathophysiology of sarcopenia is to date only partially appreciated. Due to its development in the context of the age-related inflammatory milieu, several studies favor the hypothesis of a tight connection between sarcopenia and inflammaging. However, conclusive evidence describing the signaling pathways involved has not yet been produced. Here, we review the current knowledge of the changes in intestinal microbiota that occur in advanced age with a special emphasis on findings supporting the idea of a modulation of muscle physiology through alterations in gut microbial composition and activity.

1. Introduction

Advances in medicine have led to worldwide population aging with an ever-growing proportion of elderly individuals. In such a scenario, strategies able to extend healthy lifespan and to foster active aging are a top public health priority. Indeed, advanced age is associated with an extraordinarily high prevalence of chronic disease conditions (e.g., cardiovascular disease, diabetes, cancer, and neurodegeneration), which in turn contribute to a number of negative health-related events (e.g., poor quality of life, morbidity, loss of independence, institutionalization, and mortality) [1].

The progressive loss of skeletal muscle mass and strength/function, referred to as sarcopenia, is increasingly recognized as a relevant determinant of negative health outcomes in late life [2]. As such, sarcopenia is endorsed as a meaningful biomarker allowing for the discrimination, at a clinical level, of biological from chronological age [3]. Despite growing interest surrounding the sarcopenia phenomenon, several limitations exist that impede its full appreciation in the clinical arena. Indeed, the lack of a univocal operational definition of sarcopenia and unbiased methods for assessing muscle mass and function represent major limitations in the field [3]. In addition to this, the incomplete knowledge of the pathophysiology of sarcopenia hampers the identification of targets that could be exploited for drug development [4].

A growing body of evidence suggests that the innumerable microorganisms that populate the mammalian gastrointestinal tract (gut microbiota) are tightly linked to the aging process of their host [5, 6]. Indeed, this microbial community, mostly composed of bacteria, participates in crucial

activities of the gut barrier including the generation of metabolites essential for several host functions [7] and the mediation of exogenous chemical effects on their host [8].

Age-related changes in the bacterial composition of the microbiota are well known, and alterations of gut microbiota driven by the diet may affect the health of elderly people [9, 10]. However, the complexity of mammalian gut microbiota and the technical challenges in isolating specific "prolongevity" microbial variants limit the knowledge of the microbiota to taxonomic and metagenomic profiling. The functions of individual microbial genes and the molecular mechanisms through which they intervene in host aging are yet to be elucidated. Even less is known about the implications of microbiota-immune system crosstalk on muscle aging.

Here, we overview the current evidence supporting the involvement of gut microbiota in muscle aging. Special focus is placed on the analytical tools that may help capture the complexity of human microbiota and its crosstalk with several body systems in advanced age.

2. Microbiota in Health and Aging

The human gut microbiota is a complex ecosystem existing in a symbiotic and commensal relationship with 10–100 trillion microbial cells, mostly bacteria but also yeast, virus/phages, fungi, archaea, microeukaryotes, protozoa, helminths, and parasites [11].

Being frequently confused with the microbiota, the term microbiome indicates the gene catalogues these microbes harbor [12], sometimes referred to as our second genome [13].

The gut represents the largest contributor to the human microbiota. Although rich in variety throughout its segments, the human gastrointestinal tract harbors about 10^{14} bacterial cells, which is ten times the number of human cells in the body [14]. Such a bulk of biomass, with 3.3 million nonredundant genes, is almost 150 times the 23 thousand genes present in the cells of the human body and plays a central role in health [15, 16]. Despite a high degree of interindividual variability in gut microbiota composition [17, 18], there is a remarkable similarity in the basal gene metabolic activities across individuals [17].

The human gut controls luminal gastrointestinal content at the interface with the external environment and is involved in several host functions. The microbial ecosystem can impact nutrient absorption through bioconversion of food compounds and is also responsible for the nutritional status of the organism [19]. Indeed, among its activities, gut microbiota is involved in the production of micronutrients, such as essential vitamins and cofactors; regulation of the immune system; transformation of xenobiotics; breakdown of complex lipids, proteins, and polysaccharides into metabolite intermediates [e.g., short-chain fatty acids (SCFA)]; and waste product detoxification and finally represents a barrier against the spread of pathogens [20, 21].

In addition, the gut microbiota participates in host metabolism by contributing to bile acid metabolism and recirculation; absorption of calcium, magnesium, and iron; regulation of fat storage; and activation of bioactive compounds [22, 23].

The gut also serves as an endocrine, immune, and neuronal organ. As the largest endocrine organ, it releases hormones by means of enteroendocrine cells [24], but its role goes well beyond. Besides its barrier-like role that protects the host from pathogen colonization [25], the intestinal microbiota also participates in the development and homeostasis of the host immune system [26, 27]. Indeed, 70% of the body immune cells reside in the gut-associated lymphoid tissue. Immune cells can sense changes in the microbiota through specific gastrointestinal cells and receptors and, in turn, trigger lymphocyte accumulation and differentiation in the gastrointestinal tract [28]. The interaction between gastrointestinal cells and commensal bacteria fosters immunological tolerance or inflammatory responses to pathogens by regulating immune homeostasis in the gut [29]. This crosstalk between microbiota and gut mucosal cells (enterocytes, dendritic cells, lymphocytes, macrophages, and M cells) modulates the production of various cytokines and chemokines. These can be proinflammatory, such as interleukin (IL) 1 and 8, or anti-inflammatory, such as IL10 and transforming growth factor [30].

A bidirectional gut-brain communication involving the microbiota has also been recognized and comprises neural [e.g., enteric nervous system (ENS), vagus, and sympathetic and spinal nerves] and humoral pathways (e.g., cytokines, hormones, and neuropeptides as signaling molecules) [31]. Such a communication network is referred to as the "microbiome-gut-brain axis" [32] and signals gastrointestinal perception to the brain which in turn elaborates a gastrointestinal response.

Through this gut-brain homeostasis axis, the microbiota is able to influence numerous aspects of host health, including organ morphogenesis, immune system and gastrointestinal tract development and maturation, intestinal vascularization, tissue regeneration, carcinogenesis, metabolism [33], bone homeostasis [34], memory formation, emotional arousal, affective behavior [35], intuitive decision-making, and a range of neurological disease [32, 36].

The composition of the gut microbiota drastically changes during the first 2-3 years of life [37]. Primarily dominated by *Bifidobacteria* [38, 39], its development and intraindividual variation in healthy individuals is highly influenced by several factors including mode of delivery (vaginal or cesarean), diet, use of antibiotics, geography, and environmental exposure [40–42]. In adults (<65 years), the interindividual microbial diversity of the gut microbiota reaches its maximum, but a plateau effect is observed afterwards as a consequence of the aging process [37, 43, 44]. An adult-associated core microbiome comprising 66 dominant operational taxonomic units (OTUs) [45] that differs from the core and diversity levels of younger counterparts has been identified [30].

Among the age-associated changes in the microbial population, a reduced abundance of several butyrate producers (*Clostridium* clusters XIVa and IV) has been reported by both 16S targeted Sanger sequencing and next-generation pyrosequencing [46, 47].

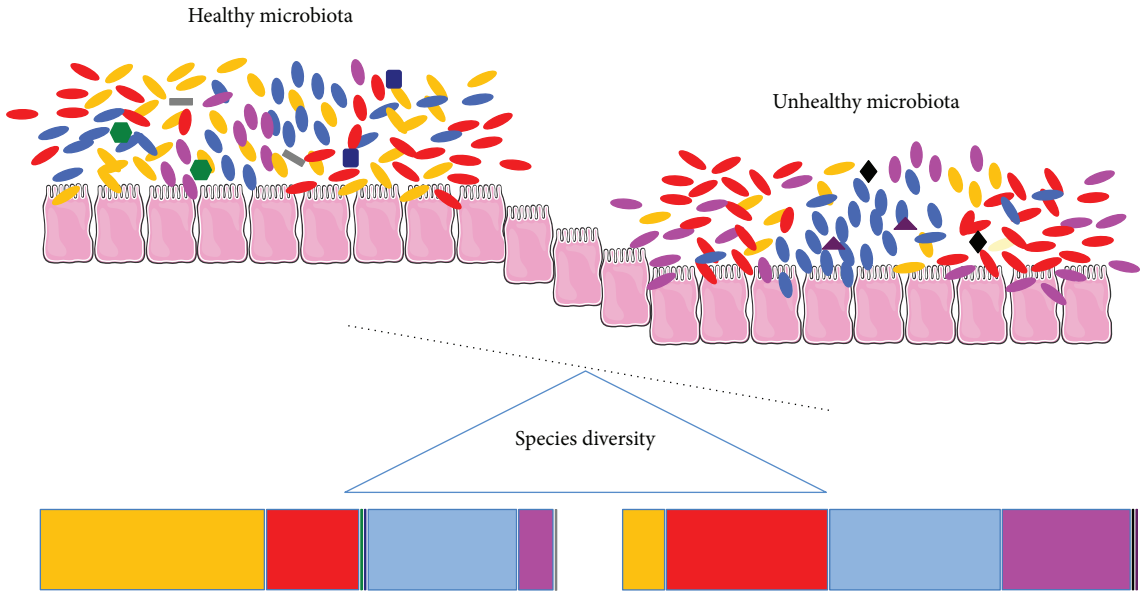

Figure 1: Healthy microbiota is a balanced community of symbiont, commensal, and pathobiont microorganisms. Each microbial class confers distinct characteristics to the host. Either imbalance in alpha-diversity or variations of relative abundance of single microbial taxa results in microbiota imbalance. As such, a sterile inflammation occurs and may predispose the host to opportunistic infections, ultimately leading to acute inflammation.

In addition, analysis of the microbial composition of 161 Irish people aged 65 years and older compared to nine younger controls showed that, even if possessing a unique individual microbiota profile, microbiota of older people was represented predominantly by Bacteroidetes population [48], as inferred by pyrosequencing of 16S rRNA. Using the same sequencing approach, a reduction of *Ruminococcus* and *Blautia* species and an increase in the abundance of *Escherichia* were also found [9]. However, the more evident age-associated trait within the microbial population was the lower Firmicutes/Bacteroides ratio (F/B ratio) reported by Mariat et al. [49] via qPCR analysis and confirmed by Claesson et al. [9] by pyrosequencing of 16S rRNA. A schematic representation of the microbial changes associated with unhealthy microbiota occurring during aging and leading to host inflammation is depicted in Figure 1.

Most gut microbial changes observed during aging are attributable to diet composition. Both environmental and behavioral factors, including loss of sensation, tooth loss, chewing difficulties, changes in lifestyle, increased consumption of high sugar-fat foods and reduction in plant-based foods, and location of residence (community, long-term care, etc.), have been suggested to influence age-associated diet variations. Furthermore, reduced intestinal motility has been indicated to unfavorably affect gut fermentative processes in advanced age. Results from the ELDERMET project, aimed at investigating the association between diet, gut bacteria, and health status in a large cohort of elderly by pyrosequencing of 16S rRNA, showed that the setting of long-term care living represents a major factor affecting diet composition [9]. The authors identified a relationship between diet, microbiota, and health status. In particular, microbial population composition was mainly affected by the consumption of vegetables, fruits, and meat. Furthermore, in elderly people living in long-term care facilities, a higher proportion of Bacteroidetes was found compared with a higher Firmicutes population in community-dwelling persons within the same ethnogeographic region [9].

Taken as a whole, these results support a new hypothetical link between aging and microbiota alterations relying on a proinflammatory loop. In this context, the age-related decline in masticatory function together with a reduction of appetite and gastrointestinal motility induces dietary changes (reduction in fruits and vegetables) that is reflected in microbiota rearrangement (dysbiosis). This alteration, in turn, can activate a proinflammatory loop fueled by the immunosenescence of gut-associated lymphoid tissue releasing proinflammatory mediators which further favors microbiota rearrangements [50].

Regardless of diet, microbiota may also vary in older age in relation to several physiological and immunological statuses, such as antibiotic exposure [51, 52], decreased responsiveness of the immune system, and the existence of a chronic low-grade inflammatory status [53], as well as lifestyle and geographical location [54]. Indeed, bacterial 16S ribosomal RNA genes analyzed by next-generation sequencing in stool samples of Korean women aged 65+ with similar genetic background showed different gut microbial composition according to the location they are living, in island or inland areas [54]. Interestingly, the same approach on fecal samples of Italian elderly inpatients revealed an association between changes in microbial composition and polypharmacy, but not multimorbidity and frailty [55]. Notably, these changes were reported to impact mortality, rehospitalizations, and incident sepsis [55].

Besides the association with aging, dysbiosis has also been related to several undesirable conditions including obesity [56], inflammatory bowel disease [57], type 1 [58] and

type 2 diabetes [59, 60], and nonalcoholic steatohepatitis [61] but has also recently been proposed to be involved in nonmetabolic syndromes such as age-related frailty [9], autism [62, 63], Alzheimer's disease [64], and depression [65].

3. The Importance of Dietary Supplementation on Microbiota

The analysis of microbial community composing human fecal samples of healthy individuals indicated that it is possible to distinguish the human gut microbiota into three main *enterotypes* based on the abundance of specific bacterial genera (i.e., *Bacteroides*, *Prevotella*, or members of the order Clostridiales) [66]. However, recent studies revisited this categorization and proposed the concept of bacterial communities being distributed as a continuum of abundance gradients between microbial genera [67].

Regardless of the exact microbial distribution, distortion of normal microbial balance has been implicated in several chronic conditions, including obesity and metabolic syndrome. Interestingly, antiaging strategies involving dietary manipulations addressing either variation in calorie intake or diet composition have been reported to affect the composition of gut microbiota. Changes in intestinal microbiota composition have been observed after weight loss following calorie restriction (CR), the only life-extending strategy available to date. In particular, an increase in the F/B ratio in obesity and a reduction of the same index with weight loss-producing CR-based interventions were found [68]. Obese people undergoing surgical (laparoscopic sleeve gastrectomy) or diet-based weight loss were also analyzed for changes in gut microbiota composition associated with weight loss interventions. Interestingly, in this case, differences in energy-reabsorbing potential were found to be associated with variation in F/B ratio [69]. A profile of weight gain-associated bacteria has been identified as related to the promotion of the expression of genes linked to carbohydrate and lipid metabolism thereby influencing dietary energy harvest [70]. Structural variations of gut microbiota have also been reported in animal models undergoing CR. For instance, a life-long low-fat diet significantly reshaped the overall structure of the intestinal microbiota in C57BL/6J mice. In particular, enrichment in phylotypes (genus *Lactobacillus*) positively correlated with longevity and a reduction in phylotypes negatively associated with lifespan was found in CR-treated mice [71].

Apart from strategies acting on calorie intake, diet composition (protein-rich versus fiber-rich dietary supplementation) represents a significant modulator of the microbial population of the gut [9, 72]. As such, diet is indicated as the main culprit responsible for metabolic diseases linked to gut dysbiosis. Even for short-term changes in consumption (4 days), animal-based and plant-based diets alter microbial community structure in a specific manner [72]. This change in food intake reflects the exchange between carbohydrate and protein fermentation existing between carnivore and herbivore mammals [72, 73].

Interestingly, Wu et al. [74], although reporting changes in microbiome composition within 24 h of high-fat/low-fiber or high-fiber/low-fat diet, showed that enterotype identity remained stable over 10 days of nutritional intervention. This suggests that food ingredients (e.g., dietary fibers) that are not digested by host enzymes but fermented by gut bacteria could modulate the gut microbiome composition in a relatively short period of time, independent of the effect of changes in transit time [74, 75]. This ability to resist disturbances and restore changes occurring in its composition (e.g., after short-term variations in dietary habits) is referred to as *resilience* [74, 75].

Beyond their primary role as dietary supplements ensuring the minimum nutritional requirements for maintenance and growth, some food components exert several beneficial effects on the host. This is achieved through the interaction with and modification of the gut microbiota. Among these, nutraceutical polyphenols, pre- and probiotics, vitamins, and polyunsaturated fatty acid (PUFA) supplementation have been recently investigated.

The administration of pre- and probiotics has been recommended as a dietary supplement to mitigate some of the age-related alterations in the intestinal microbiota associated with several gastrointestinal and respiratory diseases [76].

Probiotics defined as "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host" [77] exert their beneficial effects on the host by improving gut barrier function, immunomodulation, and production of neurotransmitters as well as by modulating cellular components of the gut-brain axis [78]. On the other hand, prebiotics are "selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microflora that confers benefits upon host well-being and health" [79].

The impact of probiotics, prebiotics, PUFAs, and phytochemicals, including flavonoids and phenolic compounds, on the gut microbiota is well characterized [78, 80–82].

Probiotics, particularly those containing *Bifidobacterium* and *Lactobacillus*, are among the most actively investigated microbiota-targeted interventions aimed at improving health status in advanced age [83, 84]. Results from a clinical trial showed that administration of *Lactobacillus rhamnosus* GG ATCC 53103 in healthy individuals, aged 65 to 80 years, was able to modulate the transcriptional response of the microbiota [85]. Oral supplementation of probiotics containing *Bifidobacterium brevis* B-3 and *Lactobacillus plantarum* HY7714, instead, has been shown to prevent skin photoaging induced by chronic ultraviolet irradiation in both mice and humans [86–88]. Similarly, oral administration of *Lactobacillus brevis* OW38 to aged mice ameliorated both age-associated colitis and memory impairments through the inhibition of lipopolysaccharide (LPS) production by the gut microbiota, p16 expression, and NF-κB activation [89].

It is worth nothing that, when analyzing probiotic-mediated effects, host benefits are mediated through the promotion of microbiota homeostasis, rather than through changes in its composition [90].

Targeting gut microbiota has been indicated as a tool to modulate lean tissue mass. Bindels et al. showed that leukemic mouse model were cachectic mice with gut dysbiosis characterized by selective modulation of *Lactobacillus* spp.

[91]. Following the administration of oral probiotic containing *Lactobacillus reuteri* and *L. gasseri*, an inverse association among serum levels of inflammatory cytokines [IL6 and monocyte chemoattractant protein-1 (MCP-1); the expression of protein associated with muscle atrophy, muscle RING-finger protein-1 (MuRF1); and atrogin-1] and muscle mass was found in these animals. Increased muscle mass and function (grip strength and swim time) have also been found in healthy young mice supplemented with *L. plantarum* [92]. The existence of a relationship between *Lactobacillus* species and skeletal muscle size found in this preclinical model needs to be confirmed in human studies.

The downside of probiotic usage including the potential risk of inducing gastrointestinal side effects, an unfavorable metabolic profile, excessive immune stimulation, and systemic infections in susceptible individuals, as well as horizontal gene transfer, needs also to be considered [93]. Therefore, a more comprehensive evaluation of the incidence and severity of adverse outcomes linked to probiotic consumption needs to be assessed.

Fermented nondigestible compounds, referred to as prebiotics, favor the proliferation of health-promoting bacteria [94] that may positively affect muscle health. Cani et al. [95] reported decreased levels of circulating LPS and inflammation and increased muscle mass in obese mice supplemented with prebiotics (i.e., fiber oligofructose) [95]. As a confirmation of the beneficial effect of prebiotic administration on gut microbiota, a shift in B/F ratio in addition to increased levels of *Lactobacillus* and *Bifidobacterium* spp. were found in follow-up analysis [96]. Further evidence supporting a link between prebiotic administration and effects on muscle mass is that association of proliferation of *Lactobacillus* and *Bifidobacterium* in leukemic mice with restoration of intestinal homeostasis (e.g., increase tight junction proteins) and reduced muscle wasting following administration of symbiotic inulin-type fructans and *Lactobacillus reuteri* [97]. These findings suggest that *Lactobacillus* and *Bifidobacterium* may influence gut-muscle communication and regulate muscle size. Interestingly, *Bifidobacterium* decrease with age [98] and are associated with lower circulating LPS levels [99]. Thus, an age-related decrease in gut *Bifidobacterium* content may underlie increases in circulating endotoxin that are shown to induce muscle atrophy [100]. While no conclusive data show increased muscle mass as an effect of *Bifidobacterium* supplementation especially in humans, there is evidence linking butyrate (associated with *Bifidobacterium* [101]) treatment as a protective strategy to counteract age-related muscle atrophy [102].

Indeed, 50+-year-old persons supplemented with galactooligosaccharides (GOS, 2×4 g/d for 3 weeks) in a randomized, double-blinded, placebo-controlled trial showed attenuation in age-associated *Bifidobacteria* reduction. In particular, an increase in the number of *Bifidobacteria*, together with higher *Lactobacilli* and butyrate levels, was obtained following GOS treatment. Moreover, SCFA concentration was increased whereas branched chain fatty acid concentrations were decreased by the same treatment. Thus, a more saccharolytic environment was achieved [103]. This and other studies based on GOS supplementation lead one to hypothesize that the administration of the GOS mixture in advanced age might positively affect the microbiota and age-associated markers of immune function [104].

The administration of symbiotic, comprising the probiotic *Bifidobacterium longum* and an inulin-based prebiotic component, has also been demonstrated to have an effect on the age-related changes in the intestinal microbiota. Indeed, an elevation in the number of *Bifidobacteria* as well as increasing members of the phyla Actinobacteria and Firmicutes together with a reduction of Proteobacteria was observed. Furthermore, treatment with this symbiotic caused an enhancement in butyrate production and a reduction in proinflammatory responses [105]. These findings might explain, at least in part, why probiotics have been successfully implemented as strategies to treat respiratory and gastrointestinal infections and enhance responses to vaccinations in older people [106].

Taken as a whole, these findings support the idea that pre- and/or probiotic supplementation may prevent age-related muscle loss by increasing the abundance of *Bifidobacterium* and butyrate producers in old individuals [85, 107].

Although the supply and conversion of nutrients are highly dependent on the composition of gut microbiota, bidirectional interactions between the microbiome, nutrient availability, and gastrointestinal function have also been proposed [108]. The metabolic activity of gut microbiota can modify the exposure, absorption, and potential health-promoting effects exerted by bioactive compounds, functional foods, or nutraceuticals.

Nutraceuticals are recognized among nutrients to beneficially modulate the growth, composition, and functions of the microbial host community in several animal models and recently also in humans [81, 109–112]. However, causality between bioactive compound assumptions and their benefits on host gut microbiota [113] is yet to be established and is challenging especially due to the complexity of endogenous and environmental factors affecting its equilibrium. Nevertheless, functional diets are proposed to prevent or attenuate metabolic diseases in view of their ability to elicit anti-inflammatory responses [114, 115].

As a whole, these findings support the hypothesis of a link between diet, microbiota, metabolism, and inflammation in several conditions and especially in advanced age [116]. Changes in microbiota have recently become the subject of intensive research because of their possible involvement in several conditions associated with inflammation, such as aging. However, a gap exists in the knowledge of how this could influence the variation of muscle mass and strength that accompanies aging. Here, we track some of the molecular pathways shared by age-related microbial alterations, metabolic changes, and sarcopenia in order to identify possible candidates and provide arguments in support of their exploitation in the management of muscle wasting.

4. Sarcopenia: A "Bacterial" Perspective

Muscle wasting is a key feature of several age-related conditions (e.g., sarcopenia, cachexia, and diabetes) leading to

functional impairment and disability. Animal studies suggest a relationship between muscle wasting and alterations in the gut microbiome. Interestingly, muscle wasting induced in a mice model of acute leukemia was attenuated by oral supplementation with specific *Lactobacillus* species [91]. The authors suggested an influence of gut microbiota on muscle physiology through the regulation of amino acid availability.

Muscle protein metabolism is a multifactorial process resulting from the dynamic balance of protein synthesis and breakdown. Muscle protein synthesis is regulated by several anabolic stimuli (e.g., physical activity, food ingestion). Both amino acid (AA) composition of dietary proteins (e.g., prevalence of essential amino acids such as leucine) and their absorption kinetics (i.e., protein digestion speed and AA absorption) influence muscle protein synthesis [117]. Changes in the gut microbiota induced by clinically relevant interventions impact the bioavailability of dietary AAs [118]. Along the gastrointestinal tract, dietary and endogenous proteins are hydrolyzed into peptides and AAs by host- and bacteria-derived proteases and peptidases [119, 120]. The resulting peptides are subsequently released and support the growth and survival of bacteria in the gastrointestinal tract [121], but also regulate energy and protein homeostasis of the whole organism [122, 123]. AAs can also serve as precursors for the synthesis of SCFA by bacteria, thus suggesting an interplay between microbial activity and host AA and SCFA homeostasis [124]. The most abundant SCFA are propionate, butyrate, and acetate. The latter is mainly utilized by muscle cells to generate energy [125]. In addition to this, modulation of proinflammatory responses associated with microbial changes can be triggered by pathogens, various diseases, and malnutrition [126].

The presence of chronic, low-grade systemic inflammation, called "inflammaging," also represents the substrate of aging and a highly significant risk factor for both morbidity and mortality in elderly people [127]. The inflammaging process is characterized by the persistent activation of innate immunity mediated by the NF-κB transcription factor [128] and loss of $CD4^+$ T cells.

Gut microbiota plays a crucial role in maintaining the balance of pro- and anti-inflammatory responses [129]. Aged gut microbiota may elicit an inflammatory response and display lower capability of counteracting adverse microbes or removing their metabolites [30]. The entrance of pathogens into the intestinal mucosa is also facilitated by the secretion of mucins by intestinal epithelial cells [130], which is triggered by a reduction in SCFA levels (especially acetate, n-butyrate, and n-propionate) in the intestines [30]. SCFA serves within the gut not only as an energy source for colonic epithelial cells but also as strong anti-inflammatory molecules regulating host metabolism and immunity [131]. In particular, butyrate modulates intestinal homeostasis through several actions, including the differentiation of $CD4^+$ T cells into regulatory T cells, the induction of tumor growth factor-(TGF-) β secretion by epithelial cells, and the triggering of IL10 and retinoic acid production by dendritic cells and macrophages [131]. These actions allow for resolving local intestinal inflammation and avoiding its dissemination through leakage of bacteria and bacteria-derived inflammatory compounds into the blood [131].

Increased intestinal permeability to LPS is another element in support of a mechanistic link between microbial dysbiosis and systemic inflammation. Indeed, in young mice, high-fat feeding, which is known to compromise epithelial tight junctions and increase intestinal permeability [132], has been associated with decreased glucose tolerance and increased inflammation markers through LPS leakage from the intestine into the circulation [133].

In such a context, chronic inflammation may represent the *trait d'union* of microbial alterations and the development of muscle-wasting conditions in advanced age through a gut microbiota-muscle crosstalk. The molecular players involved in this process are not yet fully understood. Bäckhed et al. [134] showed that germ-free mice are protected from diet-induced obesity through increased fatty acid metabolism. This pathway involves AMP-activated protein kinase (AMPK), which monitors cellular energy status; increased muscular activity of carnitine:palmitoyltransferase-1 (CPT-1), which promotes the entry of long-chain fatty acylCoA into the mitochondria; and higher levels of the fasting-induced adipocyte factors linked to the peroxisome proliferator-activated receptor, gamma coactivator 1-alpha (PGC-1α), the regulator of mitochondrial content and oxidative metabolism. These increased activities counteract the impact of denervation and fasting on muscle atrophy.

The possible involvement of mitochondria in this crosstalk is not surprising if one considers that the maintenance of mitochondrial function is crucial to myocyte viability. Mitochondrial impairment and systemic inflammation play a central role in both cachexia and sarcopenia. Indeed, a role for proinflammatory cytokines [e.g., TNF-α, IL1β, IL6, TNF-like weak inducer of apoptosis (TWEAK)] in the induction of muscle catabolism has been previously reported [135].

Only one study has focused on the interface between chronic inflammation and mitochondrial clearance in skeletal muscle in the context of aging and physical frailty [136]. This investigation made use of IL10-null mice (IL-10tm/tm), a rodent model of chronic inflammation and frailty, and reported severe mitochondrial damage with disrupted organelle ultrastructure and abnormal autophagosomes in skeletal muscle [136]. Although these findings support the existence of a connection among mitochondrial dysfunction, cellular quality control failure, and inflammation, the signaling pathways responsible for such a link have yet to be fully elucidated. Circulating mtDNA is a prominent candidate for such a role, being an important damage-associated molecular pattern (DAMP) associated with inflammation and arising directly from mitochondrial damage [137].

Recent findings by our group also support the idea that mitochondrial impairments in muscle occur in both sarcopenia and cachexia [138, 139]. Trigger candidates of inflammation in sarcopenia and cachexia could be represented by oxidized cell free-mtDNA or nucleoids extruded from damaged mitochondria (Figure 2). These DAMPs would activate the innate immune system and induce the subsequent production of inflammatory mediators. The release of the latter

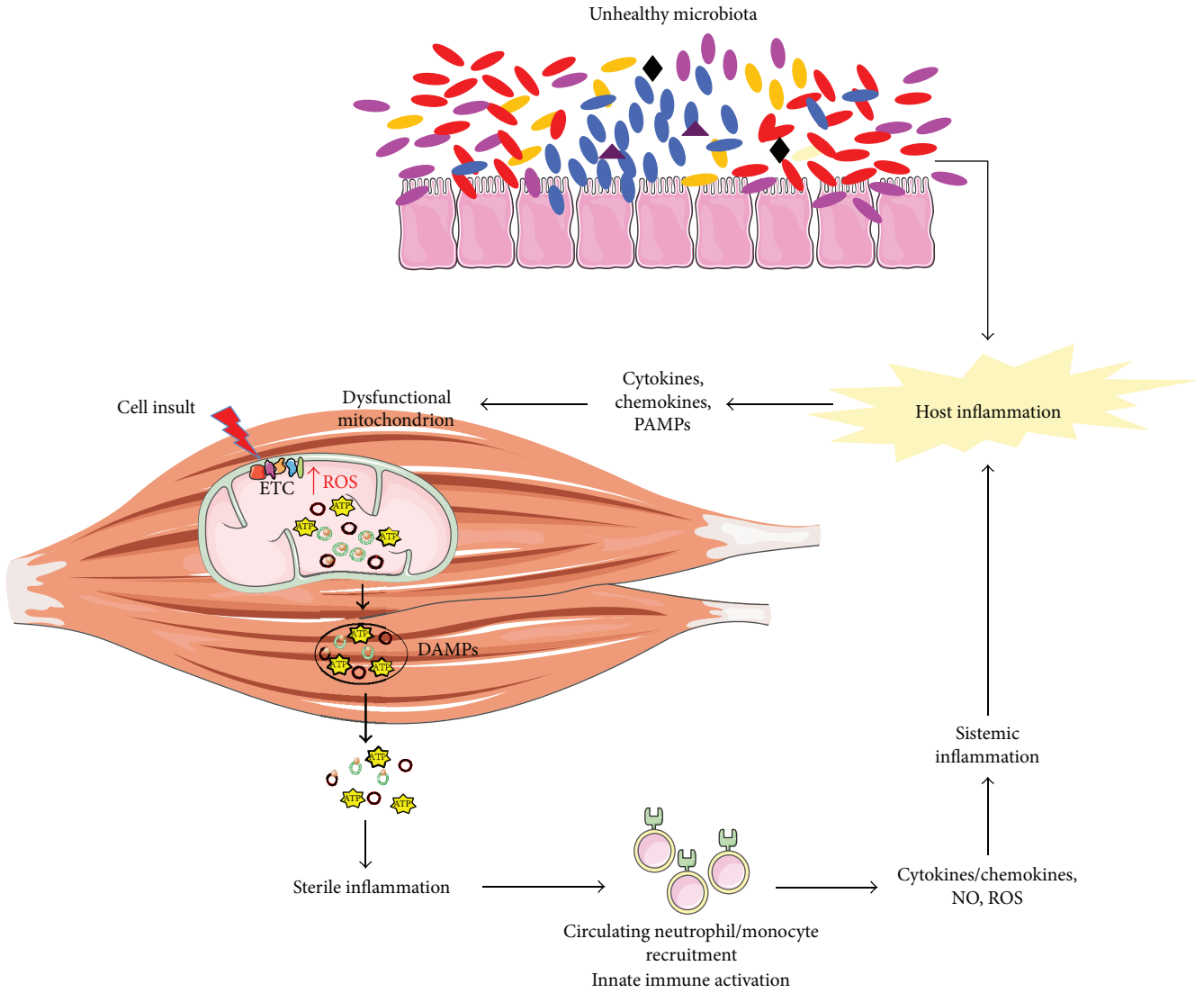

Figure 2: Proposed crosstalk between mitochondrial dysfunction and inflammation in muscle wasting. Imbalanced gut microbiota contributes to host inflammation and fuels the age-associated impairment of mitochondrial quality control in myocytes. This may lead to the release of mitochondrial damage-associated molecular patterns (DAMPs), such as mtDNA and ATP. The subsequent recruitment of local macrophages may maintain a persistent inflammatory milieu by alerting circulating immune cell and mounting a systemic response through the activation of mtDNA-induced inflammatory pathways. Cytokines, chemokines, nitric oxide (NO), and reactive oxygen species (ROS), released in the circulation by inflammatory cells, can induce further mitochondrial damage, thereby establishing a vicious circle and eventually contributing to muscle wasting. ETC: electron transport chain; mtDNA: mitochondrial DNA; TFAM: mitochondrial transcription factor A; PAMPs: pathogen-associated molecular patterns.

could sustain a vicious circle in myocytes through impaired quality control signaling, resulting in further mitochondrial impairment, increased reactive oxygen species generation, and the release of mitochondrial vesicles enriched with DAMPs. This series of events would fuel sterile inflammation, ultimately contributing to muscle wasting [140].

Due to its crucial role in host physiology and health status, age-related differences in the gut microbiota composition have been suggested to relate to the progression of diseases and frailty in old age. The first study correlating gut microbiota composition with frailty severity was conducted by van Tongeren et al. [141]. The authors demonstrated a significant reduction in the proportion of *Lactobacilli*, *Bacteroides*/*Prevotella*, and *Faecalibacterium prausnitzii* and an increase in the proportion of *Ruminococcus*, *Atopobium*, and Enterobacteriacae in older persons with high frailty scores [141].

The finding of dysbiotic shifts of gut microbiota towards a greater abundance of butyrate-producing bacteria such as *Faecalibacterium prausnitzii* in higher functioning persons suggests a positive role for these microbes in muscle function. Indeed, butyrate, by enhancing intestinal barrier function through the reinforcement of tight junction assembly [142], should prevent endotoxin translocation and reduce circulating inflammation [143].

Evidence from metagenomic analysis in a large sample of older adults ($n = 178$), the ELDERMET study, clearly linked

butyrate-generating bacteria with functional capacity by showing that community-dwelling elderly have more butyrate-producing microbes than those in long-stay residence [9]. This finding, together with a greater abundance of Enterobacteriaceae and *Escherichia*/*Shigella* and reduced gut microbial diversity among institutionalized elderly, highlights the need of nutritional strategies aimed at preventing the loss of "healthy" microbes (e.g., butyrate-producing bacteria) for those individuals entering long-term care facilities. Notably, prebiotic supplementation (inulin plus fructooligosaccharides) has been shown to increase muscle strength and endurance in frail older adults [144], thereby highlighting the potential of prebiotic supplementation as a treatment for age-associated deficits in muscle function.

Such findings, although indicating gut microbial changes among the factors affecting muscle mass and quality during aging, are not yet conclusive. Further research aimed at deciphering the pathways involved in microbiota-immune system crosstalk and its implication in muscle aging is warranted.

5. Catching the Microbiota Complexity: Opportunities from Next-Generation Sequencing Approaches

The advent of sequencing technologies has revolutionized the analysis of complex microbiomes and their functions and has allowed for upgrading fundamental theories of evolution [145].

The next-generation sequencing (NGS) revolution has enabled the genomic and functional characterization of novel microbial species, especially pathogens, revealing the diverse composition of microbial communities in several environments and the association of microbial groups with specific activities [146]. Technological advances in sequencing platforms have ensured increasingly long-read lengths that have helped cut down the cost of sequencing, one of the major limitations of the technology [147]. This has led to a dramatic increase in the amount of sequencing data generated. Such a burst in big data production and the parallel exponential increase in computational power have introduced new challenges and bottlenecks related to handling the complexity of the information generated and storing it [148, 149], especially in medicine [150].

Metagenomic studies, among other methods, have taken advantage of increasing computational power to address more complex questions compared with traditional genomic approaches.

Since its inception in 1998 [151], metagenomics has allowed for culture-independent analysis of several complex microbial populations, thus capturing the variability of microbial ecosystems that could not be identified under standard laboratory conditions [152]. This approach has revealed structural diversity, functionality, microbial interactions with the environment, other microbes and the host, and evolutionary processes [13, 153–155].

Targeted metagenomics, known as metagenetic [156], is based on marker gene amplification and the sequencing of 16S ribosomal RNA gene (16S libraries), the domain of which is restricted to *Bacteria* and *Archaea* [157]. Whole-metagenome shotgun analyses, instead, are accomplished by unrestricted sequencing of the collective microbial genomes present in the sample (shotgun libraries). While 16S sequencing approach aims at reconstructing the taxonomic content of the microbial population, the shotgun approach can address the question of how the collective microbial genomes interact in the sample. This allows for a functional microbial characterization by retrieving the complete sequences of protein-coding genes in the sequenced genomes [158]. The choice between these two methods depends on the nature of the study. The 16S approach is generally used with large sample sizes and in longitudinal studies, while the shotgun approach is preferred when a functional characterization within the samples is required [159].

Although being more expensive, the shotgun approach generates more informative libraries when performed with appropriate sequencing depth [154, 160]. Li et al. [161] published a nonredundant reference catalogue of 9,879,896 genes by combining 249 newly sequenced samples in the Metagenomics of the Human Intestinal Tract (MetaHit) project with 1018 previously sequenced samples (data available at http://meta.genomics.cn). Likewise, a shotgun approach was recently used by Xie et al. [162] to construct a comprehensive gut microbial reference gene catalogue from a metagenomic analysis of fecal samples of 250 adult twins from the UK. In this study, the authors demonstrated the heritability of many microbial taxa and functional modules in the gut microbiome, including disease-associated ones. However, the application of shotgun metagenomics to overcome the limited taxonomic resolution and functional inference of metagenetic approaches and to reveal the functional association of gut microbiota in disease conditions is still limited [163].

Especially in relation to human health, the study of 16S is of critical importance, since several disease conditions have been associated with decreased microbiome diversity or with the abundance of specific microbial species. The binning process, which is defined as the assignment of sequences to the corresponding taxonomic group, referred to as operational taxonomic unit (OTU), is pivotal in defining the diversity of the sample and its taxonomic composition. In addition, it facilitates genome assembly and the evaluation of gene association with different taxonomic groups and derived metatranscriptomic or metabolomic analyses [164, 165]. The binning process, the accuracy of which depends mainly on the clustering algorithm and on the preprocessing of the reads [166], is usually carried out with taxonomy-dependent and independent methods: the first performs a standard homology inference against a reference database to classify DNA fragments [167–171], while the second is a reference-free method which applies clustering techniques on features extracted from the sequences [172–176].

Though, when assessing gut microbiota composition by 16S analysis, many sources of bias have been recognized. These include adequacy of the experimental design and data analysis. In particular, the choice of the extraction kit [177],

primer selection and hence the regions to be amplified [178], library preparation methodology [179], sequencing errors [180], and sequencing throughput as well as the choice of pipelines and reference databases for data analysis [181] strongly impact the results.

For all these reasons, the need for a standardized method is required in order to compare datasets generated by different platforms, especially for clinical and diagnostic purposes.

Despite these criticalities, large-scale projects [The NIH Human Microbiome Project, the Metagenomes of the Human Intestinal Tract (MetaHIT), and the ELDERMET project] succeeded in paving the way to a comprehensive determination of the microbial composition of the gut microbiota and its relationship with health and diseases [9, 16, 161, 182–184]. These findings are further supported by advanced computational tools and dedicated pipelines for the analysis of microbial community data [181, 185, 186] including mothur [187], w.A.T.E.R.S [188], the RDP classifier [189], mOTU [190], and QIIME [191], defined as the "gold standard" for 16S metagenomic datasets [192].

Several software programs have been developed to infer metabolic capacity and functionality from 16S libraries. PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) is the first and most used software that associates representative sequences from OTUs to nodes of a reference phylogenetic tree [193]. In addition, it can predict gene content even in sequenced genomes not available by using ancestral state reconstruction algorithms. Other examples are Tax4Fun [194], which relies on the KEGG database, the SILVA SSU Ref NR database, and Piphillin [195], which has implemented an inference tool that works with any current genome database and has improved correlation and accuracy for clinical samples compared with PICRUSt and Tax4Fun [166].

Metagenomics is one of the most powerful tools available to unravel the complexity of gut microbiota. The integration of metagenomic data and other "omic" techniques (e.g., proteomics, metabolomics), within a multidimensional approach, will be crucial to define the determinants of several clinical conditions and thus identify complementary biomarkers [196, 197] and new therapeutic targets [198] based on nutritional and transplantation interventions.

6. Conclusion and Future Perspectives

The identification of specific biomarkers that may aid in the development of noninvasive tools for the assessment and monitoring of the relationship between inflammation and muscle wasting conditions has been sought for a long time. Current research efforts on specific "danger molecules" that stimulate sterile inflammation and link this process with muscular mitochondrial dysfunction could enhance our understanding of muscle wasting pathophysiology. Results from several studies indicate the relevant contribution of microbial changes and activity in the gut to the repertoire of inflammatory molecules involved in the *milieu* characterizing muscle aging. This represents an important matter to be addressed by future investigations to unravel the signaling pathways that may serve as targets for interventions.

Conflicts of Interest

Emanuele Marzetti, Francesco Landi, and Riccardo Calvani are partners of the SPRINTT consortium, which is partly funded by the European Federation of Pharmaceutical Industries and Associations (EFPIA). Emanuele Marzetti served as a consultant for Huron Consulting Group, Genactis, Novartis, and Nutricia. Riccardo Calvani served as a consultant from Novartis and Nutricia. All other authors have no competing financial interests to declare.

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Research Article

Small Intestinal Bacterial Overgrowth Affects the Responsiveness to Colchicine in Familial Mediterranean Fever

E. Verrecchia, L. L. Sicignano, M. La Regina, G. Nucera, I. Patisso, L. Cerrito, M. Montalto, A. Gasbarrini, and R. Manna

Periodic Fever Research Centre, Department of Internal Medicine, Policlinico Gemelli Foundation, Catholic University of Sacred Heart, Rome, Italy

Correspondence should be addressed to R. Manna; raffaele.manna@unicatt.it

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Objective. Familial Mediterranean fever (FMF) is an autosomal recessive disease due to a MEFV gene mutation. Since *Helicobacter pylori* infection has been described to increase the severity and frequency of FMF attacks, we evaluate if overgrowth of small intestinal bacterial (SIBO), associated with a release of bacterial products, can affect the response to colchicine in FMF patients poorly responsive to colchicine. *Methods*. We revised our Periodic Fever Centre database to detect FMF patients who were poorly responsive to colchicine, without a well-defined cause of drug resistance. They were evaluated for SIBO presence, then treated with decontamination therapy. *Results*. Among 223 FMF patients, 49 subjects show colchicine resistance, and no other known causes of colchicine unresponsiveness has been found in 25 patients. All 25 patients underwent glucose breath test; 20 (80%) of them were positive, thus affected by SIBO. After a successful decontamination treatment, 11 patients (55%) did not show FMF attacks during the following three months ($p < 0.01$), while 9 of them revealed a significant reduction of the number of attacks compared to three months before ($p < 0.01$). *Conclusion*. The SIBO eradication improves laboratory and clinical features of FMF patients. Thus, patients with unresponsiveness to colchicine treatment should be investigated for SIBO.

1. Introduction

Familial Mediterranean fever (FMF) is a rare disease due to mutation of the MEFV gene that encodes for *pyrin*, a protein involved in innate immune response regulation through interactions with the inflammasome, a macromolecular complex responsible for IL-β1 production and release. MEFV mutations result in an unbalanced control of flogistic response. Although considered a genetic hereditary disease, the diagnosis of familial Mediterranean fever is exclusively based on the Tel-Hashomer criteria, which are based on major criteria as the presence of high fever and serious pain, presence of amyloidosis, and effectiveness of colchicine and minor criteria as recurrent febrile attacks, erysipelas-like erythema, and a relative affected by FMF. Genetic tests can support but are not mandatory for diagnosis, for which is required the presence of 2 major criteria or 1 major and 2 minor criteria [1].

Colchicine is a fat-soluble alkaloid binding to β-tubulin, hindering its polarization with consequent inhibition of neutrophil chemotaxis and reducing expression of adhesion molecules. It prevents febrile attacks and is an FMF-controlling inflammation. Nevertheless, 5–10% of FMF patients are colchicine nonresponders. This condition may be due to concomitant diseases (vasculitis, inflammatory bowel disease (IBD)) [2, 3] or occult infections acting as trigger factors to reduce drug effectiveness [4, 5]. Chae et al. described that lack of pyrin induces hyperactivity of innate immune response against bacterial antigens such as lipopolysaccharides (LPS) [6]. MEFV-mutated pyrin is less effective than wild-type pyrin in binding to caspase 1 and therefore modulates immune tolerance against bacterial infections. In FMF patients, an increased reactivity to inflammatory conditions such as bacterial infections was observed. Indeed, some authors described cases of FMF patients with concomitant *Helicobacter pylori* (H.p.) infection showing more severe

and frequent febrile attacks. Besides, a reduction of fever attacks and cytokine levels has been demonstrated after H.p. eradication [7, 8].

Small intestinal bacterial overgrowth (SIBO) is a condition characterized by the increase of microorganisms in the small bowel exceeding 10^5 CFU/mL [9, 10]. It might be associated to peculiar anatomic and functional conditions leading to a defective host bacterial removal mechanism. SIBO may reveal through variable symptoms, from a complete malabsorption syndrome, with abdominal distension, dyspepsia, and diarrhea with or without pain, which is a colic type and modified by meals and evacuations of stools, to a total asymptomatic clinical presentation. It is important to emphasize that in spite of a possible overlapping of clinical frameworks, FMF and SIBO are different entities; FMF is indeed characterized by recurrent episodes of high fever associated with arthralgias and thoracic and abdominal pain, which is serous type, stabbing, and continuous. This pain is so important that it is easier to be confused with an appendicitis rather than colic pain.

Due to malabsorption and alteration of the intestinal flora, SIBO might facilitate blood diffusion of bacterial metabolic products, acting as PAMPS [11, 12] and interfere with many of the drugs' bioavailability [13].

Therefore, we hypothesized that SIBO may affect responsiveness to colchicine in FMF.

2. Aim of the Study

We assessed, through a longitudinal retrospective study, a SIBO prevalence in our colchicine-unresponsive FMF patients together with the effect of decontamination therapy on drug responsiveness.

3. Materials and Method

We evaluated our Periodic Fever Centre database between 1997 and 2014 to identify patients with FMF, diagnosed according to the Tel-Hashomer criteria [1, 14], who turned out not to be responsive to colchicine while taking an appropriate drug dosage (up to 0.03 mg/kg oral administration).

We excluded all patients with well-defined colchicine resistance [3], due to certain causes such as vasculitis and other autoinflammatory syndromes. Among the remaining patients, we enrolled only patients who have been evaluated for SIBO and then treated with decontamination therapy. As per conventional clinical practice, a month after the treatment, patients repeated glucose breath test to confirm the success of decontamination and the achievement of SIBO eradication. All patients were still taking colchicine at the same dosage used before decontamination.

In our center, all FMF patients starting a colchicine therapy usually undergo follow-up screening every three months for the first year of treatment; then, in case of responsiveness, it occurs every 6 or 12 months. Responsiveness to therapy is evaluated by physical and blood examinations, as reported below. In FMF patients who were responsive to colchicine, it was not considered necessary to screen for the presence of SIBO because they were asymptomatic.

3.1. Definition of Unresponsiveness to Colchicine. Unresponsiveness to colchicine is defined by FMF attack recrudescence, with usual features occurring more than once during three months since the beginning of the colchicine treatment, at maximum dosage of 0.03 mg/kg/day, according to the patient's characteristics [3].

3.2. Glucose Breath Test. To search the presence of SIBO, an H2 glucose breath test (GBT) was performed in the 20 patients left [15]. We verified that the GBT was performed under standard conditions:

(i) Patients should not have received antibiotics and/or laxatives in the month preceding the test.

(ii) Subjects had a carbohydrate-restricted dinner on the day before the test and to be fast for the next 12 hours before the test, in order to minimize and to give stable values of basal H2 excretion.

(iii) On the day of GBT, patients had rinse their mouths out with chlorhexidine 20 mL at 0.05%.

(iv) Smoking and physical exercise were not allowed for 12 hours before and during the test; end alveolar breath samples were collected with a two-bag system immediately before and every 15 min for 2 hours, after having ingested 200 mL water isoosmotic solution with 50 grams of glucose [16].

According to the literature, GBT was considered indicative of SIBO presence when an increase of H2 levels over the baseline value was >12 p.p.m. with respect to the basal value [17].

3.3. Disease Activity Evaluation. Since the current literature shows no validate scales for disease activity under treatment, our PFC in daily medical practice refers to a questionnaire regarding some of the main features already used by Pras et al. [18] and Mor et al. [19], in their respective severity scores, in order to certify the responsiveness degree to colchicine three months after the beginning of the treatment. Particularly, we use Pras et al.'s score to evaluate the number of attacks in a one-month observation, and Mor et al.'s score for the presence of abdominal and/or thoracic pain, joint pain, attack severity, and limitations in daily life activities. Since some of the topics in our questionnaire make references to subjective parameters, a visual analogic scale (VAS) is used to determine the severity of abdominal, thoracic, and joint pain and daily activity limitation degree.

3.4. Blood Examinations. All patients followed up in PFC undergo blood examinations every three months, as objective parameters to evaluate the disease activity state. Particularly, acute phase reactants, like erythrocyte sedimentation rate (ESR), serum amyloid protein (SAA), and C reactive protein (CRP), are usually checked in outpatient regimen and then analyzed in Gemelli Polyclinic laboratories according to an internationally recognized standard methodology.

3.5. Decontamination Therapy. SIBO positive patients were treated with rifaximin 400 mg three times a day for seven days, according to scientific literature [20]. It was considered unuseful to treat SIBO negative patients to avoid unnecessary treatment.

3.6. Statistical Analysis. A paired sample *t*-test and NPAR tests and Wilcoxon signed-rank test were performed to analyze any changes in the clinical and laboratory features of FMF attack.

3.7. Ethical Aspects. No informed consent was necessary because anonymous retrospective data were collected during conventional clinical practices and analyzed according to the principles of the Helsinki Declaration.

4. Results

Among 223 FMF patients followed up in PFC, 49 subjects (M/F: 28/21; mean age 31.25 ± 9.35SD years; range 11–52) resulted poorly responsive to colchicine. Twenty-four of them were excluded for known colchicine resistance or for other concomitant diseases. In particular, 14 patients had H.P. gastric infection, 2 patients showed respiratory tract infection, 1 patient suffered from intestinal mycosis, 2 patients had IBD, 2 patients had vasculitis (Behçet's disease), 1 patient had TRAPS, 1 patient had marginal lymphoma, and 1 patient had bladder neoplasm.

Genotype of the selected 25 patients documented p.M694V homozygous for 2 patients, p.M680I homozygous for 1 patient, and p.V726A for 1 patient; 11 were heterozygotes (p.M680I, p.M694I, 2 p.V726A, and 2 p.M694V) among whom 5 complex heterozygotes (p.M680I-p.V726A, p.M694I-p.R761H, p.M694V-p.I692del, p.M694V-p.E148Q, and p.K695R-p.R202Q), 5 patients resulted in becoming carriers of polymorphism, and 5 had no MEFV mutations at all.

4.1. SIBO Prevalence in Colchicine Nonresponders. Among the 25 patients left, no other known causes for colchicine unresponsiveness were found. All patients underwent glucose breath test; 20 (80%) of them resulted positive, thus affected by SIBO, and 5 (20%) resulted negative.

4.2. Decontamination Treatment Response. SIBO positive patients underwent decontamination therapy with rifaximin at dosage of 400 mg three times a day. After one month, glucose breath test resulted negative in all those patients, meaning a complete decontamination.

4.3. Evaluation Disease Activity and Colchicine Responsiveness

4.3.1. Clinical Features. Among the 20 patients resulting negative to glucose breath test control after the treatment, 11 patients (55%) did not show any FMF attacks during the following three months ($p < 0.01$), while 9 of them showed a significant reduction in the number of attacks when compared to three months before ($p < 0.01$) (Figure 1).

Each symptom analysis, evaluated by VAS, certified a significant decrease in abdominal, thoracic, and joint pains and daily life activity limitations after decontamination treatment ($p < 0.01$ for all voices) (Figure 2).

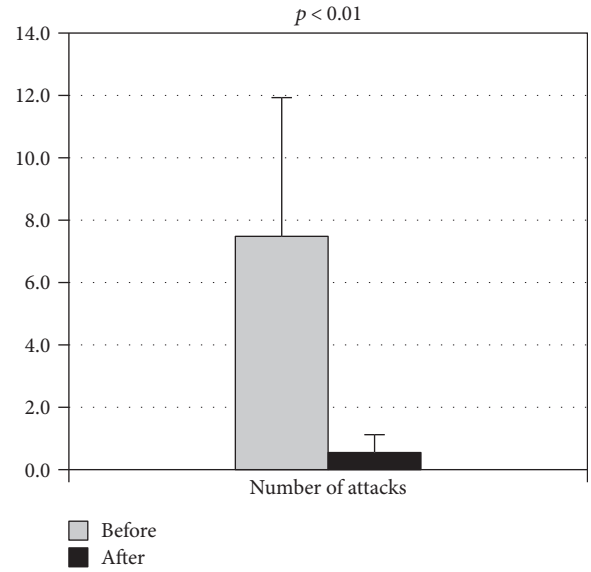

Figure 1: On the *y*-axis, the value of the number of attacks over three months before and after decontamination treatment.

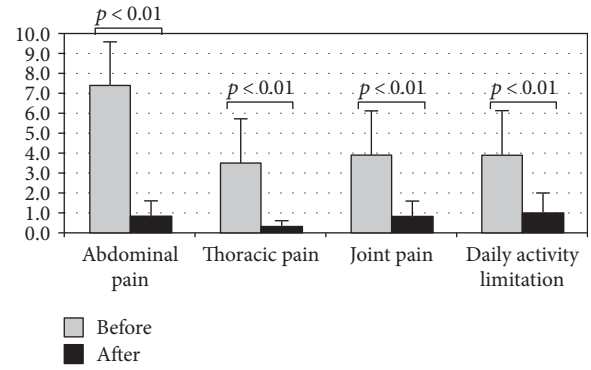

Figure 2: On the *y*-axis, the score obtained by VAS.

Severity disease reduction was reported in all patients showing a decrease of daily life activity limitation (Figure 2).

4.3.2. Laboratory Features. Mean values of acute phase reactant were compared both before decontamination treatment (ESR 34.1 ± 20.8, CRP 21.8 ± 42.2) and after three months, showing a significant decrease (ESR 8.0 ± 3.2, CRP 2.1 ± 0.3) ($p < 0.01$) (Figures 3(a) and 3(b)). Reduction of inflammation parameters has been attributed only to the best control of basic autoinflammatory disease and not to the eradication of SIBO, since it does not induce elevations of systemic inflammatory markers but can cause elevation of local flogistic marker such as fecal calprotectin concentration [21].

5. Discussion

Considering FMF rarity but also the increased interest in natural immunity against bacterial products, we decided to verify retrospectively SIBO influence on colchicine responsiveness and on the clinical severity of the disease.

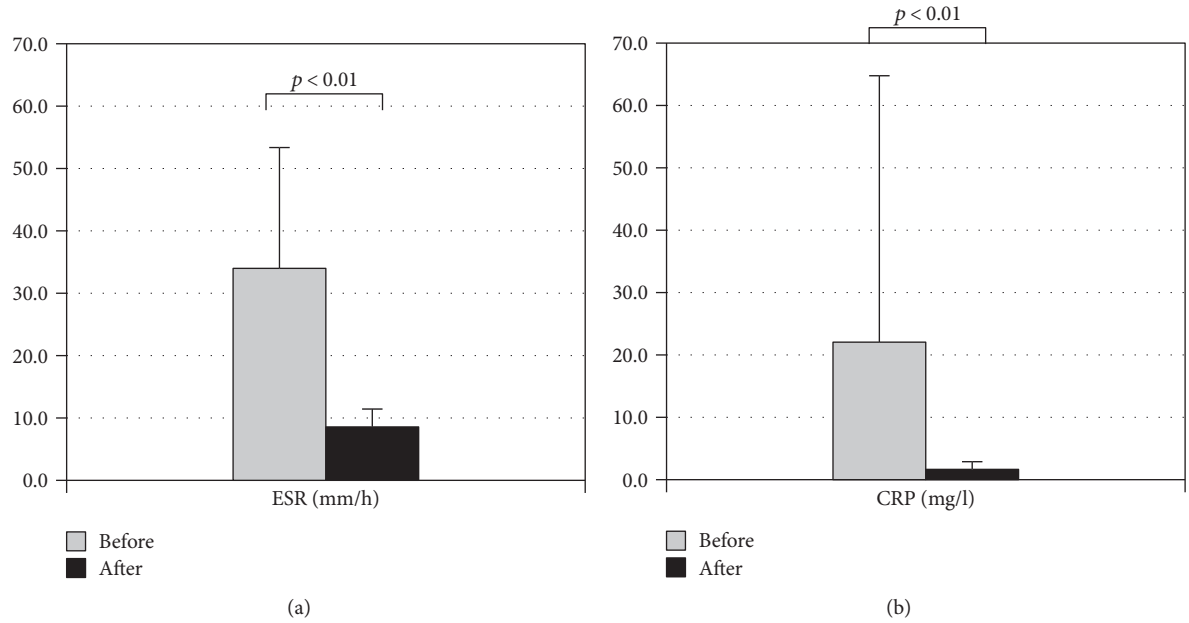

Figure 3: (a) Mean values of ESR before and after decontamination treatment. (b) Mean values of CRP before and after decontamination treatment.

In our cases, we found a higher percentage of colchicine unresponsiveness than the one described in literature: 21.9% versus 5–10%. Nevertheless, there is still no consensus about its definition and no evidence is available regarding the management of this condition. The same definition of colchicine unresponsiveness shows some gaps, because it does not concern partial responsiveness. Indeed, there is no disease evaluation scale concerning treatment responsiveness. In order to find a proper solution, Ben-Chetrit et al. suggested to adopt the method of ACR 20, 50, and 70, so as to establish colchicine effectiveness evaluated on the reduction of FMF attacks per percentage each year, before and after drug administration. The author also made a clear distinction between "true" and "false" nonresponders based on the presence of some factors leading to such condition or the improvement of colchicine tolerance [22].

In fact, there might be various possible explanations at the basis of colchicine unresponsiveness. Lidar et al. in 2004 found a significant reduction of colchicine concentration in mononuclear cells (MNC) of nonresponders compared to responders. This difference was reported to less colchicine treatment compliance in the first group. For this reason, colchicine treatment failure in FMF patients was associated with an inadequate colchicine MNC concentration. Since this condition has been observed also in patients fully adhering to the treatment, it may probably result from a further genetic defect unrelated to the underlying FMF that may alter the drug concentration in MNC [23].

Inadequate therapeutic range of colchicine might also derive from an impaired drug absorption, as observed in SIBO in other diseases [24]. Indeed, the bacterial overgrowth could interfere with the normal adsorption of many substances such as carbohydrates, proteins, lipids, and vitamins; this condition may be due to bacterial fermentation of many sugars but also due to enterocyte injury. According to these considerations, the possibility of colchicine level dosage into MNC could be useful for a better understanding of drug unresponsiveness mechanisms. Unfortunately, in the clinical practice, the colchicine concentration assay is not currently available in MNCs in patients to know if they became responsive to therapy after decontamination treatment or because of better drug absorption.

On the other side, inadequate colchicine bioavailability in MNC might not be the only reason for drug unresponsiveness. Chae et al. [25] reported that *pyrin* gene mutations increase the flogistic response endotoxins, because MEFV mutated-pyrin is less effective than wild-type pyrin in binding to caspase 1 and inhibited it, leading to major activity of caspase 1 after Toll-like receptor activations and therefore to IL-1β production with the systemic inflammatory response to simple stimuli. Therefore, bacterial antigen production or release derived from SIBO may act as trigger factors, enhancing inflammatory cytokine production as IL-1β and sustaining a persistent or occult inflammation, producing an FMF phenotype apparently unresponsive to colchicine [11, 26]. We also considered the possibility of a possible anti-inflammatory activity of rifaximin, but we can exclude this hypothesis in the light of the scientific literature, which appears to be consistent in arguing that it is not absorbed systematically but exposes its function only at the local level.

In our series, acute-phase reactants were higher before decontamination therapy and decreased after rifaximin treatment. Indeed, all patients showed clear reduction of clinical, laboratory, and other disease activity parameters, restoring colchicine responsiveness. The result of this retrospective study based on the analysis of PFC registers, even if a control group is lacking, encourages to establish a

multicentre prospective study in order to establish the SIBO prevalence in the general population and in the FMF population and also to confirm the role of SIBO eradication in FMF poorly response to colchicine.

6. Conclusion

We can conclude that SIBO affects the responsiveness to colchicine and the clinical severity in patients affected by FMF. We can assume that impaired intestinal bacterial products of intestinal microbiota may act in patients with innate immunity hypersensitivity as FMF or Crohn's disease, accentuating the clinical manifestations of autoinflammatory diseases. Second, we cannot exclude that SIBO may reduce the absorption of colchicine and cause a lack of its effectiveness.

On the basis of this study, we conclude that patients with FMF should be investigated for a suspected SIBO if presented with a reduced or absent responsiveness to the treatment with colchicine. In our study, bacterial decontamination restored the responsiveness to drug therapy and improved the clinical course of the disease.

Besides, this study suggests that intestinal microbiota modulate the clinical expression of the FMF and colchicine effectiveness. Moreover, these results have a major impact in terms of health economics, because improving the effectiveness of colchicine in patients with autoinflammatory diseases can reduce the use of more expensive drugs as biological agents.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Review Article

The Role of High-Mobility Group Box-1 and Its Crosstalk with Microbiome in Rheumatoid Arthritis

Federico Biscetti,[1] Andrea Flex,[2] Stefano Alivernini,[1] Barbara Tolusso,[1] Elisa Gremese,[1] and Gianfranco Ferraccioli[1]

[1]*Division of Rheumatology, Fondazione Policlinico Universitario "A. Gemelli", Institute of Rheumatology, Catholic University School of Medicine, Rome, Italy*
[2]*Division of Internal Medicine, Fondazione Policlinico Universitario "A. Gemelli", Institute of Internal Medicine, Catholic University School of Medicine, Rome, Italy*

Correspondence should be addressed to Gianfranco Ferraccioli; gianfranco.ferraccioli@unicatt.it

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Rheumatoid arthritis (RA) is a chronic, definitely disabling, and potentially severe autoimmune disease. Although an increasing number of patients are affected, a key treatment for all patients has not been discovered. High-mobility group box-1 (HMGB1) is a nuclear protein passively and actively released by almost all cell types after several *stimuli*. HMGB1 is involved in RA pathogenesis, but a convincing explanation about its role and possible modulation in RA is still lacking. Microbiome and its homeostasis are altered in patients with RA, and the microbiota restoration has been proposed to patients with RA. The purpose of the present review is to analyze the available evidences regarding HMGB1 and microbiome roles in RA and the possible implications of the crosstalk between the nuclear protein and microbiome in understanding and possibly treating patients affected by this harmful condition.

1. Introduction

Among the autoimmune diseases, rheumatoid arthritis (RA) represents one of the most relevant [1, 2]. In fact, patients affected by RA have a poor quality of life, due to articular pain and functional impairment [3–7]. In addition, RA causes an increased risk of other pathological conditions, including cardiovascular diseases [8–16]. Furthermore, immunosuppressant for RA can often determine dangerous and potentially lethal side effects, among which are infections, organ failure, and even death [7, 17–24]. Although RA has been studied over the last decades and several researchers have been focused on identifying new potential drugs, a definite treatment is not available and the disease can progress to severe disability [4, 7, 17, 19, 25–30]. One of the reasons of the delayed defeat of the disease is the lack of a full understanding of the causes responsible for the RA onset. Indeed, while several pathways and mechanisms have been clarified, such as lymphocyte, interleukin, and tumor necrosis factor (TNF) roles, the very initial trigger has not been discovered [1, 23, 27, 28, 31–41]. As in other autoimmune conditions, an infectious event has been proposed to explain the altered immune response and the RA initiation [42]. In this scenario, microbiome obviously represents an attractive candidate. In fact, the altered crosstalk between microbiome and the immune system could underlie the disease onset [43–54]. Among the well-known pathways, the high-mobility group box-1 (HMGB1) plays a role in RA. In fact, this nuclear protein is involved in synovial inflammation observed in RA and could represent a new therapeutic target [55–68]. Recent data demonstrated that the HMGB1 pathway is important in a model of bowel inflammation [69]. The aims of the present review are to evaluate the available data about the role of HMGB1 in the crosstalk between gut microbiome and RA-altered immune response, to try to better understand the mechanisms

underlying this disease, and to see whether it could represent a therapeutic target and, eventually, whether it would be more cost-effective to inhibit or stimulate the activity of HMGB1 in these conditions.

2. Rheumatoid Arthritis

RA is an autoimmune disease, characterized by chronic inflammation of joints and several other tissues, including those of the lungs, vessels, blood, eye, skin, and heart [70, 71]. RA is not a rare disease; in fact, out of every 100,000 people, about 40 are diagnosed with RA every year [72]. In general, at the onset, RA affects the small joints of hands. However, also hips, shoulders, and knees may be involved, and RA can potentially hit every joint [25, 33, 73]. Quality of life of patients affected by RA is worsened by pain, swelling, stiffness, and loss of function in the joints [4, 5, 74]. Furthermore, patients with RA have a reduced life expectancy due to an increased mortality for cardiovascular events, infections, and drug side effects [9–11]. In fact, it has been definitely demonstrated that patients with RA have an increased risk of myocardial infarction and of stroke [75]. The principal reason is that the typical chronic inflammation observed in the RA scenario plays a pivotal role in atherosclerotic plaque formation and destabilization [10, 11, 76, 77]. In addition, the immune dysregulation of T and B cell network can affect other cardiovascular risk factors, such as hypertension and lipid metabolism [9, 78, 79]. Furthermore, sedentary lifestyle and weight gain due to joint impairment could be additional factors. Other morbidity causes are certainly infections. In fact, immunosuppressant therapy and RA itself increase the risk of infectious complication, and about a quarter of deaths are caused by infections [42, 80–86]. Finally, several of the most effective treatments commonly used in patients with RA can have many side effects, including organ failure, cancer and, sometimes, death [18–20, 22].

Although the relevance and the impact of RA are clearly important, an effective treatment has not been yet discovered. The reason of this delay may reside in the relatively unknown initial pathological event. Indeed, several mechanisms have been clarified to explain the fundamental injury: the synovitis and the joint destruction [3]. First, a genetic susceptibility is known. In fact, an association between RA onset and major histocompatibility complex (MHC) class II antigens, specifically the shared epitope found on HLA-DRB1, has been demonstrated [3, 70, 71, 87–89]. However, RA does not seem to be a genetically transmitted disease, and DNA in the strict sense plays a minor role. Regarding the genetic heritage and regulation, novel mechanisms have been elucidated in the last decade, in particular the epigenetic regulatory systems, including the microRNA (miRNA) pathways [41, 90, 91]. Moreover, miRNAs can regulate gene expression and protein function of several cytokines, growth factors, and receptors involved in RA [41]. Alongside the genetic susceptibility, a trigger is required to initiate RA; in fact, studies performed on twins have demonstrated that identical genetics are not sufficient to develop similar disease [92]. Several potential environmental triggers have been implicated, among which are cigarette smoking and infections [93–95]. Taking into account infectious event, the relationship between RA and infective disease is dependent on the immune and inflammatory activation caused by pathogens [42, 96–98]. The T and B cell activation and the beginning of the autoimmune response are the mechanisms involved in the RA onset [32, 79, 99–103]. Another important event is represented by the protein citrullination, a normal post-translational modification required in several physiological processes [104–107]. In RA, there is an autoimmune activity against citrullinated peptides detected as anti-citrullinated peptide antibodies (ACPA), a prototypical biomarker of the disease. After T and B cell activation and autoantibody production, additional cell types come into play to propagate and amplify inflammation, among which are macrophages that produce interleukin- (IL-) 1, IL-6, IL-8, and tumor necrosis factor- (TNF-) α [108–113]. All these phenomena translate into the main event of the disease: joint damage.

3. High-Mobility Group Box-1

The high-mobility group box-1 (HMGB1) is a highly conserved DNA-binding protein, present in the nucleus, that acts as a damage-associated molecular pattern (DAMP) molecule [114]. HMGB1 belongs to the family of the high-mobility group (HMG) chromosomal proteins, distinguished on the basis of their rapid mobility on electrophoresis gels [115]. These nuclear proteins were discovered more than 40 years ago and are subdivided into three superfamilies: the HMGB, HMGN, and HMGA superfamilies [116]. Of the HMGB family that includes HMGB1, HMGB2, HMGB3, and SP100HMG, HMGB1 is the most abundant nonhistone DNA-binding protein [114]. HMGB1 is the typical DAMP molecule, and it is involved in the setting of both sepsis and sterile inflammation [114]. This nuclear protein belongs to the "alarmin" family, a group of signaling effectors that acts as an injury-induced response in mammals [117]. DAMPs interact with several ancestral receptors and pathways and share a significant number of signaling systems with the pathogen-associated molecular patterns (PAMPs) [118]. DAMPs and PAMPs can activate the immune system by using the same ways, starting from completely different pathological triggers. In this scenario, HMGB1 represents the prototypical molecule that can stimulate a lot of immune responses against external injury. In this sense, HMGB1 could be considered exclusively a defensive protein. However, this protein plays also a dangerous and harmful role in numerous conditions by activating detrimental pathways so that many authors suggest the blockade of its function [119–121]. The role of HMGB1 in normal and in disease conditions was originally attributed to the passive release in the extracellular space after the cell damage [122]. Subsequently, a more complex mechanism of action was identified for HMGB1: it is also actively secreted by almost all types of cells, in response to several stimuli, and it can activate different pathways, depending on the tissue where the signaling is triggered and on the kind of receptor involved [118, 123]. The most recent findings have highlighted that the effect of HMGB1 is also closely dependent on the redox status of the milieu where the protein is released [124].

The first information about HMGB1 activity has been collected in models of sepsis and systemic infections [125]; the idea that this alarmin is involved in the sterile inflammation and fibrosis rapidly increased [55, 114, 117, 126] and fibrosis [127]. During the last decade, additional data were collected regarding more variegated effects of this nuclear protein in terms of tissue remodeling and angiogenesis, not necessarily related to septic conditions [115, 128–131].

4. High-Mobility Group Box-1 and Rheumatoid Arthritis

There are several data supporting the role of HMGB1 in RA, particularly suggesting that it plays a role in initiating the synovium inflammation and in maintaining the joint damage mediated by proinflammatory cytokines. Since the first studies by Andersson and coworkers, it has been clarified that HMGB1 can stimulate the release of IL-1, IL-6, and TNF-α [122] and it determines the beginning and the development of inflammation in different experimental models of arthritis. Furthermore, HMGB1 is increased in synovium and synovial fluid of patients with RA, compared with patients with osteoarthritis [132, 133]. Moreover, HMGB1 blockade reduces arthritis induction in experimental models [55, 56, 59, 63, 67, 134, 135]. Finally, HMGB1 administration induces synovial angiogenesis through a vascular endothelial growth factor- (VEGF-) dependent mechanism [55]. Although multiple mechanisms involved in RA pathogenesis have been discovered, there is no fully comprehensive explanation about the HMGB1 pathway in this scenario. In particular, HMGB1 function depends on two principal factors: the oxidation/reduction status and the extracellular milieu where different receptor systems can be found. While the second point is enough studied and we know now that the TLRs, the receptor for advanced glycation end-products (RAGE) and the IL-1 receptor, represent the most important extracellular pathways [61], we less know about the factors that modify the oxidation/reduction status of HMGB1. In fact, depending on oxidation/reduction status, HMGB1 can be in three different conformations: sulfonic, disulfide, or all-thiol form [58, 136, 137]. According to the redox status and following different structures, HMGB1 explicates various functions. For instance, the sulfonic form acts as an immune tolerance inducer, while the disulfide one is a major player in inflammation. In this sense, the HMGB1 pathway is notably plastic and dynamic and depends on the redox status of the extracellular setting, not only on the receptor quality and content [61]. However, it is not yet clear how the environment can modify the redox state and what cell types are involved in this process.

5. Microbiome

The term microbiome refers to the genetic characterization of the entire microbiota in a specific tissue [138]. We know several microbiomes, depending on localization, such as skin, lung, and oral microbiomes [139]. Certainly, the gut microbiome is one of the most important because, together with activities shared with other microbiomes, it plays a fundamental role in digestion and transformation of food [43, 45, 51, 140, 141]. However, the principal function of microbiome is the crosstalk with the immune system to modulate and regulate the immune response against the host. Gut is colonized by billions of bacteria immediately after birth, and the mucosal interface of the intestinal tract is characterized by several types of immune cells and systems, organized in aggregates and organs [140]. The location of these systems is strategically at the border with the outside world, and they require a multipotent and versatile network of signals and receptors. In fact, there we have the pattern recognition receptors (PRRs), an ancestral part of the immune system that can recognize several pathogens with the same pathway [142]. Among PPRs, toll-like receptors (TLRs) are the prototypical receptors that bind elemental fragments of bacteria, such as lipopolysaccharides (LPSs), and also of microbiota [142, 143]. However, given the number and the different types of species of gut microbiome, it seems unlikely that these bacteria activate the immune response normally. Most likely, the interaction between microbiome and intestinal immune system determines a continuous modulation of the two players [43, 144].

6. Microbiome and Rheumatoid Arthritis

The connection between gut and joints was hypothesized several decades ago, when researchers studied different models of inflammatory arthritides, in particular spondyloarthropathies related to inflammatory bowel diseases and secondary to intestinal resections [49]. The interaction between genetic profile and environmental triggers is important in the pathogenesis and development of RA. Oral chronic colonization or infection sustained by *Porphyromonas gingivalis* was linked to RA development [145, 146], and traces of bacteria were found in synovial fluid of patients with RA. Furthermore, prolonged antibiotic therapy against certain bacterial infections is effective in RA disease control [147]. Breaking tolerance in RA could occur in reaction to these pathogens. However, *Porphyromonas gingivalis* is not the only implicated in RA. In fact, data regarding other bacteria are available, and a single infection seems to be not likely as the sole cause. Moreover, the analysis of microbiome from mice prone to arthritis development revealed that microbiome can influence the arthritis susceptibility [148]. Several reports demonstrated that a subpopulation of patients with early RA harbored intestinal microbiota dominated by *Prevotella copri* and that SKG mice harboring the same microbiota had an increased number of intestinal Th17 cells and developed severe arthritis due to autoreactive T cells [149]. Interestingly, a taxon-level analysis-based study revealed an expansion of rare taxa with a decrease in abundant taxa in microbiome of patients with RA, compared with controls; this finding was related to the production of proinflammatory cytokines, such as IL17 [150]. Microbiome alterations do not only affect the expression level of TLRs of cells that exhibit antigens but also contribute to the Treg/Th17 deregulation. Epigenetic modifications triggered by external factors are important pathways leading to an altered gene expression. Crosstalk between microbiome and the mucosal

immune system has been demonstrated being a crucial activator of epigenetic pathways in mammalian, including humans [43]. The most compelling evidence that gut infection-inflammation is a key moment in the occurrence of arthritis comes from the K/BxN and IL1RA−/− mice that do not develop arthritis in a germ-free setting [151]. On the other hand, the evidence that a normal gut microbiota is fundamental in maintaining the homeostasis is shown in the streptococcal cell wall arthritis, in which the normal flora protects against the occurrence of arthritis [152].

RA is a chronic multifactorial autoimmune disease where the immune event represented by the ACPA production can start even 15 years before symptoms, thus suggesting that the initial pathogen phenomenon is not necessarily present in the joints. In this scenario, microbiome represents the ideal theater [44]. Starting from animal models, about forty years ago, several researchers found that the administration of specific bacteria fragments, such as LPS, can induce arthritis and that the presence of gut microbiota is protective against the injury. Furthermore, additional evidence suggested that the balance of the intestinal germ population is fundamental in maintaining homeostasis and protection against environmental pathogens [153]. Recent data demonstrated that alteration of the gut microbiome can influence the balance of pro- and anti-inflammatory immune cells, such as T reservoir, and promote the development of RA [154]. Moreover, it has been found that TLRs play a crucial role in influencing the Th17 differentiation and the Treg inhibition caused by gut microbiome in animal and human models [142]. However, although a lot of possible mechanisms have been elucidated to demonstrate the role of microbiome in RA, a definitive, omnicomprehensive, and convincing explanation has not been yet found.

7. High-Mobility Group Box-1 and Microbiome

Since LPS is one of the most important experimental activators of the HMGB1 pathway, it seems fair to assume that intestinal bacterial *flora* is involved in HMGB1 modulation. However, there is a lack of evidence about the crosstalk between HMGB1 and microbiome due, at least in part, to the difficulty of measuring tissue and fluid protein concentrations in its extracellular form. In fact, once released after cellular injury or activation, HMGB1 can be found in at least three conformations, depending on the oxidation/reduction status, and the commonly used experimental kits are not capable to detect all the conformations [58, 136, 137]. Furthermore, the complexity of the gut and the difficulty of obtaining reproducible data about the redox state of microbiota make it even more difficult task. However, HMGB1 surely plays a role in oral and intestinal homeostasis [155], and recent data demonstrated that this nuclear protein is involved in the inflammatory response of the gut and that the HMGB1 blockade is able to inhibit the LPS-induced injury by a TLR4-dependent mechanism [69]. In this model, TLR4 is considered a pivotal receptor for inflammation and the interaction between HMGB1 and TLR4 of mucosal tissue is important in inducing the intestinal inflammation. However, the inflammatory milieu is rich in oxidizing agents, and the HMGB1 translocation in this scenario could promote the structural modification of the protein. Furthermore, microbiome represents an important source of redox-based signals that modulate critical microbial and host cell functions [156–158]. Moreover, the microbiome modulates the redox status of the host by modifying the glutathione metabolism [159]. In addition, recent data obtained in both *in vivo* and *in vitro* models demonstrated a novel HMGB1-RAGE-mediated redox signaling pathway involved in intestinal inflammation induced by a liver dysfunction model [160]. As shown in Figure 1, HMGB1 conformational modulation depending on microbiome homeostasis could lead to different redox states and consequent activities. In this respect, the maintenance of a proper homeostasis of the microbiome may be important to prevent damage caused by HMGB1 overexpression.

8. Therapeutic Implications

A definitive treatment for all RA patients has not been discovered [23, 161–164]. A multitarget approach is required to better control the disease, and several pathways must be considered to completely treat RA. However, immunosuppressive drugs are not always sufficient [165–167]. For this reason, new therapeutical strategies are desirable and a better knowledge of HMGB1 interaction with microbiome in RA could provide new elements to achieve it. In this regard, a possible attempt could be the HMGB1 pathway blockade. In fact, several data demonstrated that, together with the commonly used monoclonal antibody-based therapies, monoclonal antibodies directed versus HMGB1 can protect against arthritis in experimental models [168, 169]. In particular, in two notably different models of arthritis, collagen-induced arthritis (CIA) and a genetic model of arthritis, Schierbeck and colleagues demonstrated that anti-HMGB1 monoclonal antibody administration significantly ameliorated the clinical courses in these experimental conditions. However, there is no evidence about the redox status and the possible role of microbiome in these models, and further data are needed to better understand the possible implications of an altered homeostasis of microbiome in HMGB1-dependent arthritis and in anti-HMGB1 therapy efficacy. Moreover, in a model where germ-free piglets were orally colonized with enteric bacterial pathogens, HMGB1 result significantly increased, suggesting that the upset balance of the microbiome can affect the HMGB1 pathway *equilibrium* [170]. Since the protein redox state can significantly modify the HMGB1 activity, a therapy capable of controlling the microbiome-oxidizing capacity could represent a new interesting approach. In this respect, probiotics need to be cited. Probiotic administration restores homeostasis of the gut microbiome and can have several beneficial effects [52]. Among the autoimmune disorders, RA seems to benefit from the probiotic therapy [54, 171]. Results obtained from animal models demonstrated that oral therapy with *Lactobacillus casei* ameliorated CIA by downregulating T helper 1 effector functions [172] and by reducing proinflammatory cytokines [173]. Also, data from humans have been achieved. In particular, in 46 patients with RA, *Lactobacillus casei* was orally

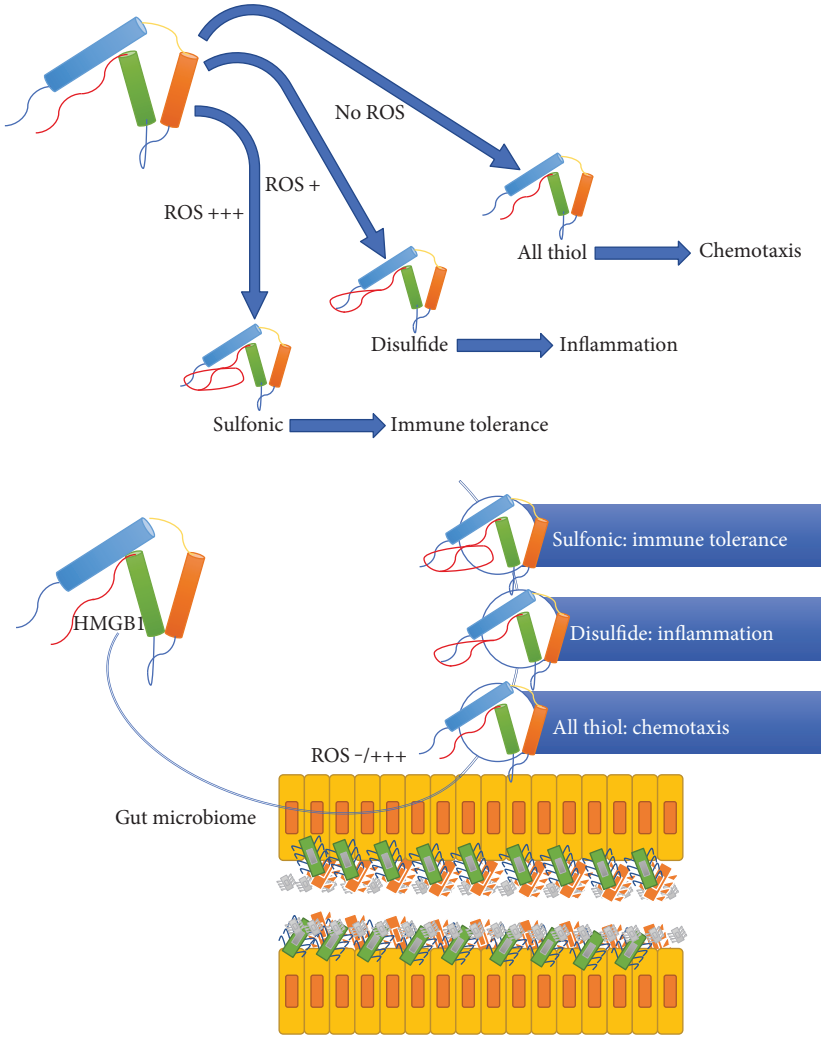

Figure 1: A schematic representation of the interaction and the crosstalk between HMGB1 and gut microbiome in RA pathogenesis. Depending on the oxidation/reduction status after the passage through the gut microbiome, HMGB1 can play several and different roles in RA initiation and maintenance.

administrated for 8 weeks and the disease activity score and serum proinflammatory cytokines were significantly decreased by the intervention [174]. In this setting, it is possible to speculate that homeostasis of microbiome could regulate HMGB1 activities in these patients. However, additional data are required to confirm this hypothesis.

9. Conclusions

RA is a chronic, harmful, and potentially severe disease for which there is no yet a decisive treatment. HMGB1 and microbiome alterations are involved in pathogenesis of RA, and the crosstalk between the protein and the microbiome deserves to be studied more carefully in order to offer a new therapeutic tool for patients with this serious disease.

Conflicts of Interest

The authors declare that they have no conflicts to disclose.

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Review Article

Skin Immune Landscape: Inside and Outside the Organism

Florence Abdallah,[1] Lily Mijouin,[2] and Chantal Pichon[1,3]

[1]*Centre de Biophysique Moléculaire, CNRS UPR4301, Orléans, France*
[2]*Remedials Laboratoire, 91 rue du Faubourg Saint-Honoré, 75008 Paris, France*
[3]*Collégium Sciences et Techniques, Université d'Orléans, Orléans, France*

Correspondence should be addressed to Chantal Pichon; chantal.pichon@cnrs-orleans.fr

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The skin is an essential organ to the human body protecting it from external aggressions and pathogens. Over the years, the skin was proven to have a crucial immunological role, not only being a passive protective barrier but a network of effector cells and molecular mediators that constitute a highly sophisticated compound known as the "skin immune system" (SIS). Studies of skin immune sentinels provided essential insights of a complex and dynamic immunity, which was achieved through interaction between the external and internal cutaneous compartments. In fact, the skin surface is cohabited by microorganisms recognized as skin microbiota that live in complete harmony with the immune sentinels and contribute to the epithelial barrier reinforcement. However, under stress, the symbiotic relationship changes into a dysbiotic one resulting in skin disorders. Hence, the skin microbiota may have either positive or negative influence on the immune system. This review aims at providing basic background information on the cutaneous immune system from major cellular and molecular players and the impact of its microbiota on the well-coordinated immune responses in host defense.

1. Introduction

Remarkable advances have been achieved over the past years to understand and characterize the immunobiology of the skin. As the largest organ of the integumentary system, the skin covers the internal organs of the body to maintain its temperature, to prevent water loss, and to provide a physical barrier against external insults. Far from being a simple mechanical barrier, the skin constitutes a network of effector cells and molecular mediators that constitute a highly sophisticated "skin immune system" (SIS) as described by Bos and Kapsenberg in 1986 [1]. The cutaneous homeostasis maintenance is dependent on the cross-talk between several immune sentinels present in the different compartments of the skin and the interplay between innate and adaptive immune responses. This SIS includes resident cellular players (keratinocytes, Langerhans cells, fibroblasts, mast cells, macrophages, endothelial cells, or recruited leucocytes) and a wide variety of soluble inflammatory mediators (antimicrobial peptides (AMPs), cytokines, and chemokines). This system allows the maintenance of cutaneous homeostasis and is also responsible for the activation and regulation of normal and pathological inflammatory reactions. More recently, the dynamic cutaneous ecosystem was shown to affect profoundly the immune response. Moreover, the skin forms a complex and dynamic ecosystem colonized by about 10^{12} microorganisms including bacteria, fungi, and viruses known as skin microbiota. These organisms play an important role in the protection against invading pathogens and in the development of inflammatory-mediated diseases. Taken together, the total skin environment favors the interaction between the immune cells and the host microbial community. It results in a highly defined and organized defense response that can be divided into three major steps: (1) interplay of the cutaneous ecosystem and pathogen invasion, (2) onset of the immune response, and (3) immunological memory.

2. Step 1: Interplay of the Cutaneous Ecosystem and Pathogen Invasion

The skin is the first and largest barrier of the human body. It covers the human organism and ensures a constant dialogue

with the external environment full of exogenous factors, such as foreign pathogens, ultraviolet (UV) radiation, and allergenic and chemical irritants. Therefore, through evolution, a dynamic cutaneous ecosystem has been developed in order to protect the host from undesirable insults and aggressions. This ecosystem comprises (1) a sophisticated immune system and (2) a normal flora inhabited by many commensal microorganisms such as bacteria, fungi, and viruses that constitute a cutaneous microbiome known as skin microbiota. Both are interconnected and mutually regulated to form a biological and immunological barrier. In this paragraph, we will review briefly the features of the skin microbiota and the mechanism by which commensal microorganisms such as *Staphylococcus epidermidis* and *Staphylococcus aureus* and fungi such as *Candida albicans* and *Malassezia* spp. invade the skin and become pathogenic.

The skin microbiota extends from the skin surface to deeper layers in the dermis and dermal adipose tissue. Almost 25% of skin microorganisms grow in the dermis at the level of the sebum glands and through hair follicles [2]. These microorganisms are classified as resident and transient microorganisms [3, 4]. The resident microorganisms transmitted during birth from the mother or acquired from the contact with the daily life surroundings (animals, plants, persons, chemicals, and climates) are long lasting. On the other hand, the exposition to new settings (e.g., changes in the environment due to travelling) leads to the development of transient microorganisms that is eradicated once back to usual conditions. Therefore, each individual has a unique and specific signature of skin microbiota encountered during infancy and stabilized during adulthood [5, 6]. This skin microbiota lives in symbiosis with the SIS actors forming a strong biological shield against pathogens [4]. Hence, the resident flora has evolved tightly with the host gaining ability to train, to induce, and to modulate local immune reactions when appropriate [7]. For instance, the protection is acquired either directly through bacteriocin production, pathogen adhesion inhibition, and toxin degradation or indirectly through interaction and activation of the host SIS. In the last decades, studies have shown the importance of commensal microbes to promote immune development and to prevent infection without inducing detrimental inflammatory responses. The interconnection of the microbiota and the development of the SIS was pointed out [8]. Previous studies have demonstrated the importance of repopulating the skin with commensal microbes to restore an effective immune response against invading pathogens. For instance, *S. epidermidis* colonization in germ-free mice is sufficient to restore effective T cell immunity to parasites such as *Leishmania major* through modulation of IL-1, IL-17, and IFN-γ inflammatory response [9] (Figure 1). Moreover, lipoteichoic acids (LTA) present in the cell wall of *S. epidermidis* inhibit *Propionibacterium acnes*-induced inflammation via miR-143 induction [10]. *S. epidermidis* is also able to enhance host defense mechanisms by inhibiting the growth of group A *Streptococcus* and *S. aureus* [11, 12] (Figure 1).

S. epidermidis is also able to restrain *S. aureus* pathogenicity [13, 14] (Figure 1). In addition, *Malassezia* spp. is one of the most dominant cutaneous fungi that possess a protective role against bacteria and fungi in the skin via its antimicrobial activity. *Malassezia* spp. growth depends on processing of external lipids by enzyme production to yield short fatty acids such as azelaic acid. The latter is responsible for the antimicrobial activity in normal pH skin [15]. As mentioned earlier, the skin is a complex and dynamic environment. Its complexity derives from the intricate relationship existing between microbiota and immune responses. Commensal microbes colonize different areas of the skin surface that would be otherwise available for pathogens. So far, the cutaneous microbiota is an essential partner in protecting the skin from pathogen invasion [16]. Nevertheless, for many reasons, the protective symbiotic effect can turn into harmful and devastating opponents of the SIS leading to dysbiosis responsible for infection genesis and/or cutaneous disease development. There are several causes of symbiosis to dysbiosis shift. For instance, a modified immune tolerance and disrupted microbial homeostasis such as cutaneous lesions (open wound, catheter, burns, and insect bite), extensive scrubbing, hormonal deregulation, other environmental factors (antibiotics, cosmetics, and cold), or genetic predisposition/alteration may weaken the microbial barrier and modify the composition or the virulence of microbial communities, thereby facilitating the genesis of infection [17–19]. Indeed, in optimal conditions, the alliance between skin immunity and local flora allows simultaneously the protection against the external pathogens and tolerance maintenance towards resident microorganisms. Conversely, altered resident microbial communities or harmful local expansion of some members of skin microbiota can terminate this alliance. Clear evidences have been reported to prove the link between dysbiosis and skin diseases or infections, albeit the mechanisms are not yet fully understood [20]. Studies showed that dysbiosis is a major trigger of acute or chronic inflammatory disorders such as atopic dermatitis, acne, and rosacea [16, 21]. For example, *S. aureus* produces δ toxins triggering local allergic cutaneous responses which may also prevent wound healing and cause epithelial barrier deterioration [3, 22] (Figure 1). The correlation between skin immune disorders and microbiota will be developed in the last part of this review. Any shift among these populations can lead to aberrant skin immune responses. It is a vicious cycle: chronic and/or excessive immune responses can modify the composition of resident microbiota, allowing the attraction of new invasive microorganisms. These reactions can be further amplified by a positive feedback which leads eventually to a loss of skin homeostasis and to numerous pathologies [7]. In the following paragraph, examples of skin infections caused by skin colonizers are described.

Staphylococci are common bacterial colonizers of human skin [23], hence associated with high occurrence of skin infection breaking through the barriers. *S. epidermidis*, in particular, is the most frequent microorganism isolated from human epithelia and is an essential member of skin resident microflora [24]. In normal conditions, *S. epidermidis* has a sane adaptable relationship with its host. Nonetheless, its ability to form biofilms renders it highly resistant in case of infection. Biofilm-associated infections are extremely hard to clear, due to the difficulty to bypass the extracellular

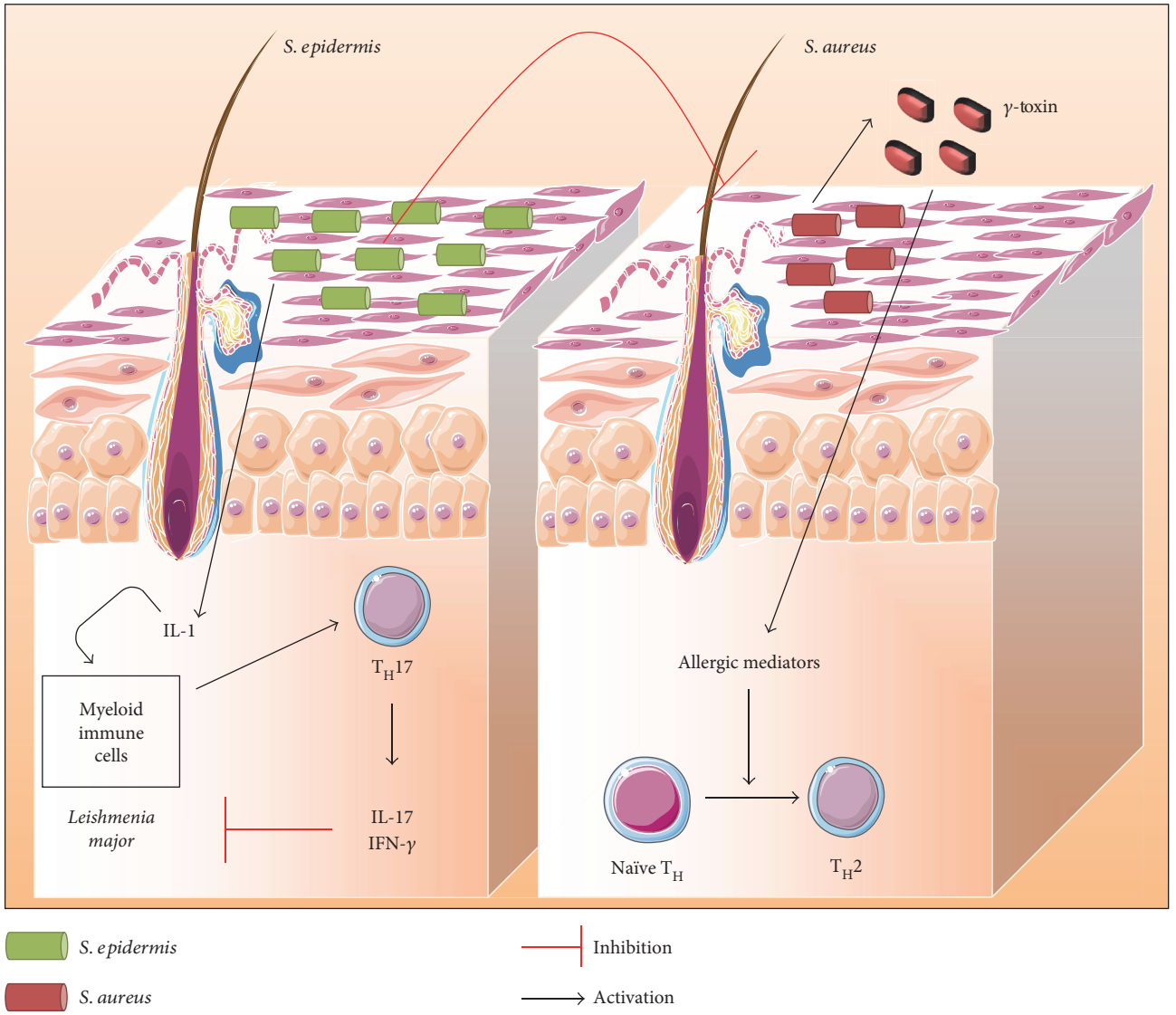

Figure 1: Illustration of skin microbiota interaction with the SIS. The alliance between skin immunity and local flora allows protection against external pathogens and maintenance of tolerance to resident microorganisms, simultaneously. In a healthy state, resident bacteria such as *S. epidermidis* are able to contain *S. aureus* pathogenicity. Moreover, *S. epidermidis* was shown to be required for the production of IL-17 and IFN-γ by T cells inhibiting *L. major* growth. However, altered resident microbial communities or local expansion of some members of skin microbiota with harmful potential alters this alliance. *S. aureus* is able to produce δ toxins that trigger local allergic responses in skin resulting in T_H2 inflammatory immune response. Adapted from [157].

matrix [25]. This matrix acts as (i) a physical barrier restricting many antibiotics and chemical diffusion and (ii) a mechanical barrier restraining immune cell passage. Only a few cells, like neutrophils, are able to bypass this barrier, by using hydric channels, to penetrate the matrix and access bacteria [25]. In general, the host's immunity is not sufficient to clear off biofilm-associated infections thereby endorsing chronic infection. *S. aureus* is part of the human transient microflora but is considered "semipermanent", since 30 to 50% of the human population are believed to be healthy carriers of *S. aureus* [26, 27]. Moreover, the skin of patients suffering from inflammatory chronic diseases initiating epithelial barrier disorders (e.g., atopic dermatitis) is often colonized by *S. aureus* [28]. As mentioned previously, a breach at the cutaneous level grants an easy and rapid access for microorganisms to deep tissues and the bloodstream and risks of the dissemination of the infection. Most common *S. aureus* infections include those in the skin and soft tissues, and the infection risk is enhanced by the use of medical implants, such as prosthetic joints and intravascular catheters [29]. In the late '60s, there was an emergence of methicillin-resistant *S. aureus* (MRSA) [30]. The virulence of this pathogen still poses a significant therapeutic challenge [31]. To date, the majority of research in the *Staphylococcus* field is dedicated to the understanding of *S. aureus* infection occurrence. The interference of *S. aureus* with the host's immune responses has been well described over years but remains quite dependent on the

models [32]. The host's defense against *S. aureus* includes (i) the skin barrier and outcompetition with other strains, for example, *S. epidermidis*, as described previously; (ii) the innate immune responses, mostly driven by antimicrobial peptide (AMP), complement, neutrophil, and macrophage activation; and (iii) the adaptive immune response. *S. aureus* is an excellent model of bacteria being part of a semiresident flora, but able to switch as a pathogen as soon as it is left uncontrolled by other members of resident flora [33–36]. The coevolution of this particular microorganism with the host's SIS and its ability to get specific virulence genes easily and rapidly makes it a quite interesting target to understand how this system is dynamic.

More recently, there is increasing awareness of the importance of fungi and their interactions with the immune system influencing the immune homeostasis and inducing disease. When the chemical composition (pH, pathological sweat secretion) of host epidermis is disrupted, *Malassezia* spp. gains in pathogenicity and releases lipases, phospholipases, and an array of bioactive indoles. These molecules alter the function of the epithelial barrier resulting in immune deregulation and diseases [37, 38]. Another common cause of fungal infections worldwide is *Candida albicans* [39] despite being in most cases harmless commensal fungi. The dryness of the skin renders hard the growth of *C. albicans* and their cutaneous concentration remains low, yet a normal constituent of the resident skin microflora is about 70% of the population [40, 41]. This fungus is unable to cause severe disease when present at low rates, but inappropriate immune response or disruption in the normal floral occupancy can cause uncontrolled proliferation of this germ on the skin, thereby leading to cutaneous invasion and infection. *C. albicans* interacts with the host's defenses in three major ways: (i) innate response, (ii) adaptive response, and (iii) neuronal response [42–44].

3. Step 2: Onset of the Immune Response

3.1. Innate Immune Response. The innate immune system is designed to directly and rapidly respond to foreign pathogens by activating recognition systems and effector mechanisms (Figure 2). The major innate immune cells are macrophages, dendritic cells, and natural killer cells that express a wide variety of pattern recognition receptors (PRR) including two transmembrane proteins, Toll-like receptors (TLRs) and C-type lectin receptors. They also express two cytosolic proteins: retinoic acid-inducible gene-I-like receptors and NOD-like receptors (NLRs) [45, 46]. The most well-characterized PRR is the TLR family composed of 11 and 12 members in humans and in mice, respectively. TLRs detect a broad range of pathogen-associated molecular patterns (PAMPs) and conserved microbial sturctures, including lipopolysaccharide (LPS), peptidoglycan, flagellin, and nucleic acid ligands. TLR signaling is characterized by the activation of the critical transcription factor "nuclear factor kappa B (NF-κB)" and mitogen-activated protein kinase (MAPK) pathways through adaptor proteins including MyD88, TIRAP/Mal, TRIF, and TRAM [47]. Consequently, genes involved in inflammatory responses (a panel of AMP, cytokines, and chemokines) such as IL-6, TNF-α, IL-8, and IL-12 are upregulated [45, 47]. The resulting inflammatory environment stimulates the neighboring cells to produce more inflammatory mediators and attracts innate immune cells to the stressed site. These recruited cells induce reactive oxygen species (ROS) and nitrogen oxide species (NOS) production [45, 47]. They also promote cell lysis and phagocytosis and boost cell autonomous defenses such as apoptosis to eliminate invaders [48, 49].

The ability of the innate immune cells to communicate with epithelial cells leading to an effective immune response is a key feature of the cutaneous immune system. It is of great importance to understand the cellular and structural composition of the skin that dictates the hierarchy of the skin immune response. Therefore, a quick brief overview of the constitution of the skin is essential before developing the steps of the cutaneous immune response.

The skin is made up of various cell types, each characterized with specific functions according to their location. It has three layers: (i) the epidermis, the outermost layer containing predominantly keratinocytes and, to a lesser extent, melanocytes, $CD8^+$ T cells, and Langerhans cells with a simple cell composition; (ii) the dermis, the intermediate layer with greater cell diversity—dendritic cells, macrophages, natural killer cells, $CD4^+$ T cells, innate lymphoid cells, fibroblasts, and so forth—and with lymphatic and blood vessels which allow cell migration traffic [50]; and (iii) the hypodermis, the innermost layer, composed mainly of adipocytes, which ensures thermoregulation. The epidermis is separated from the dermis by the dermoepidermal junction and from the external environment by the stratum corneum (Figure 3). The latter represents a true barrier of protection. It is composed of cells made up mainly of proteins called corneocytes, whose intercellular space is highly constituted of lipids. The dynamic interaction between all these cells coordinates the immune response.

The first sensors of pathogen invasion are *keratinocytes (KCs)* which represent 95% of the epidermal cell type and ensure its structural integrity [51]. The corneal layer made of dead KCs constitutes the skin's mechanical barrier. KCs are the initiators of the immune response [52] and thus could be perceived as immune sentinels. KCs of the granular spinous and basal layer can sense nonspecific external stimuli such as UV rays and chemicals and detect a wide range of microbial ligands via TLRs expressed on their surface. So far, TLR1, 2, 3, 4, 5, 6, and 9 have been shown to be expressed in a constitutive or inducible manner in KCs [53–58]. As a response to stimulation, KCs produce a wide panel of cytokines (IL-1, TNF-α, IL-6, G-CSF, TGF-β, and IL-10), chemokines (CXCL-8, IP-10), growth factor (IL-6, GM-CSF, and TGF-α), and AMPs (β-defensins, cathelicidins, S100 family members, and sebum) resulting in either direct neutralisation of the pathogen or indirect activation of other immune sentinels to launch a specific immune response [59] (Table 1). The nature of the immune response depends on the stimulus. For example, UV rays and chemicals activate the inflammasome-dependent proinflammatory signaling pathway leading to IL-1β secretion [60, 61], whereas a dominant T_H1 immune response accompanied with type 1 interferon (IFN)

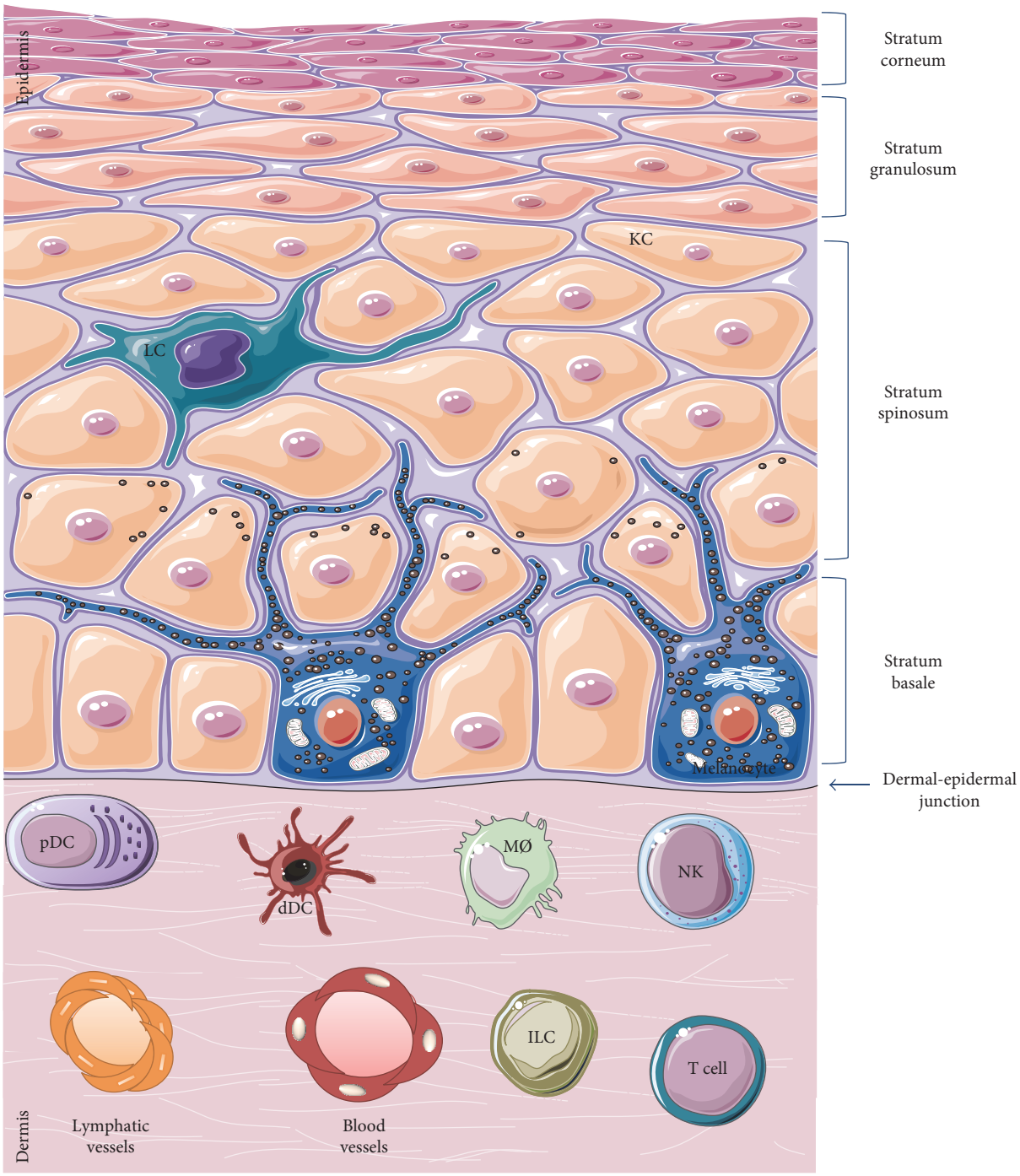

Figure 2: Skin anatomy and cellular constituents. The protection of the body from the external environment is provided by the multilayered structure as well as by the complex cellular composition of the skin. The epidermis is the outermost layer composed of different strata made of keratinocytes (KC) from the most exposed surface to the least differentiated deeper area: stratum corneum, stratum granulosum, stratum spinosum, and stratum basale. Immune cells that ensure immunosurveillance such as Langerhans cells (LC) and specialized cells that produce melanin such as melanocytes are found in the epidermis. The dermis is the intermediate layer composed of several specialised immune cells such as plasmocytoid dendritic cells (pDC), dermal dendritic cells (dDC), macrophages (MØ), natural killer cells (NK), innate lymphoid cells (ILC), and T cells responsible of the immune response. In addition, blood and lymphatic vessels are present throughout the dermis. The hypodermis (not represented) is the innermost layer constituted mainly of adipose tissue.

production is obtained upon PAMP-TLR pathway activation. This elicits the cell-mediated immunity against infection [62]. Furthermore, another immunological function of the KCs has been described in ingraft versus host disease. Nickoloff and Turka demonstrated that MHC class II-expressing KCs act as nonprofessional antigen-presenting cells that are able to activate and maintain T cell tolerance [63]. The costimulatory pathways (BB1 and B7-H1) initiated

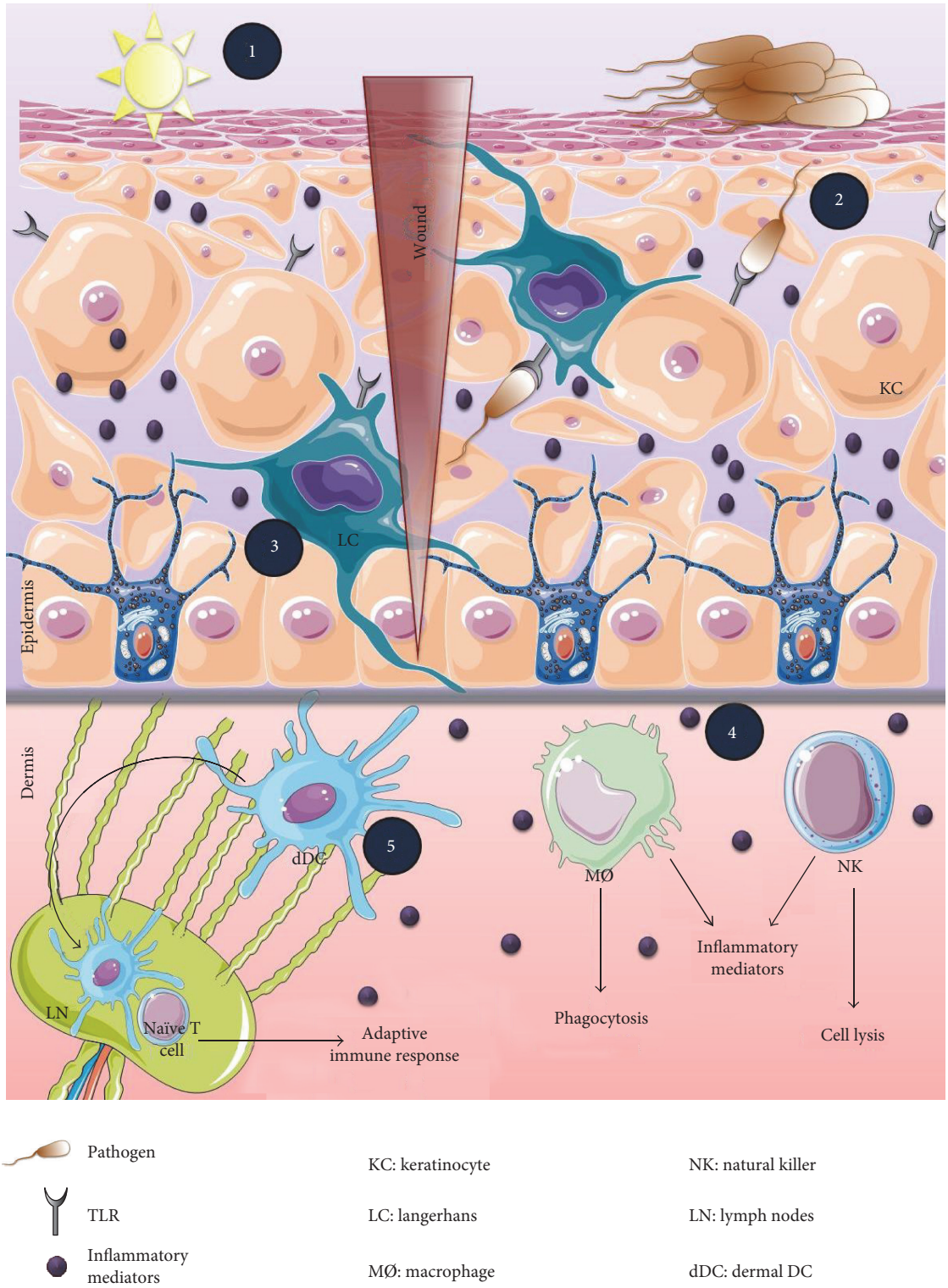

Figure 3: Initiation of a primary cutaneous immune response. The skin is a primary immunological barrier to the external environment. The uppermost layer "corneal layer" is composed of dead keratinocytes that provide a physical barrier. However, the pathogens can access directly to the interior of the host through skin wounds and by outcompeting the normal flora (1). TLR-bearing cells (KCs and LCs) recognize pathogens and establish a highly coordinated immune response: antimicrobial production to neutralize the pathogen (2), inflammatory mediator secretion to alert the immune cells (3), activation of innate immune cells such as natural killer cells (NK) to induce cell lysis and/or phagocytosis such as macrophages to engulf pathogens (4), and maturation of dermal DCs that migrate into draining lymph nodes to prime T cell responses (adaptive immunity) (5).

by KCs differ from those coming from professional cells (B7-1 and B7-2). Taking all described differences into consideration, T cells' interaction with KCs remains crucial in mounting the immune response to local antigens and also in maintaining self-tolerance. Note that KCs can also interfere with the adaptive immune response; they are playmakers in coordinating immune responses due to their ability to cross-talk with other epithelial and immune cells.

Melanocytes are epithelial cells recently described for their potential in modulating the immune response through inflammatory cytokine production. They are mostly located in the epidermal basal layer towards the dermoepidermal junction. They are oval, fusiform, and smaller than KCs. The expression of melanocyte-specific proteins such as tyrosinase (tyr), TYRP1, DCT, Pmel17/gp100, MART-1, and/or MITF allows differentiated melanocyte identification. The main features of these cells are melanin production and melanosome transfer from differentiated melanocyte to KCs. The melanin presence in the skin defines its pigmentation and is involved in the photoprotection against UV rays [64]. The contact between KCs and melanocytes is crucial, and the underlying molecular mechanisms are still a subject of investigations [65]. Besides melanogenesis, the role of melanocytes in the inflammatory response is minimally studied although they have been described to produce various inflammatory cytokines.

Fibroblasts are also implicated in the immune response via their interaction with KCs. They are the main cellular constituents of connective tissues. These cells are major contributors in extracellular matrix (ECM) protein synthesis, through collagen and fibronectin secretion, as well as remodeling, by the production of proteinases. Even though, they secrete a complex mixture of growth factors, cytokines, and chemokines, they still are not considered immune sentinels. Fibroblasts communicate with nearby cells through the paracrine and autocrine system. For instance, the fibroblast-keratinocyte interaction modulates the levels of MMP-2 and MMP-9 and their inhibitors resulting in a better healing quality at a late stage of the wound healing process [66]. Thus, the dialogue between fibroblasts and KCs via cytokines plays a fundamental role in generating skin immunity (Table 1).

In parallel, Langerhans cells *(LCs)* are the first immune cells that come in contact with skin-invading pathogens. LCs are in intimate association with KCs and represent 2 to 4% of the epidermal cell population with a half-life range between 53 and 78 days [67]. LCs have been described for the first time, 150 years ago by Langerhans [68]. They are specialized residents of skin dendritic cells (DCs) of hematopoietic origin derived from bone marrow [69]. LCs within the spinous layer demonstrate a dendritic morphology that extends through tight junctions to the stratum corneum where it can capture antigen without disturbing the epithelial barrier [70]. LCs express C-type lectins on their plasma membrane langerin (CD207) in mice [71] and CD1a in human [72] and Fcγ and Fcε receptors [73]. These surface C-type lectins are PRRs that recognize mannosylated ligands found on the surface of a wide range of pathogens [46, 74] which leads to receptor-mediated endocytosis and trafficking to the Birbeck granule where they may participate in antigen processing [75]. Unlike conventional DCs, *in vitro* studies showed that LCs are weak stimulators of T cell responses and have phagocytic capabilities. However, during culture, they become mature by acquiring immunostimulatory activity with increased MHC-II molecule expression and decreased Birbeck granule number and phagocytic capacity [76]. They play a primary defense role by monitoring the presence of infection and damage within the epidermis. They have been found to be major contributors in inducing IgG to neutralize *S. aureus* during cutaneous infection [77]. Activated LCs capture antigens and migrate into draining lymph nodes where naïve T cells are activated. Yet, the definitive function of LC and its contribution in the adaptive immune response is not fully understood.

Table 1: Major constituents of the innate immunity.

Compartments	Cells	Inflammatory mediators
Epidermis	KC	AMPs, IL-1β, IL-8, IL-10
	LC	IL-1, TNF-α, IL-10, IL-15
Dermis	Fibroblasts	IL-6, TNF-α, IL-8, IL-1, MMP-9, MMP-2
	MØ	ROS, NO, L-1, IL-6, IL-8, IL-12, IL-10, TNF-α, TGF-β
	NKs	IL-4, IL-10

KC: keratinocyte; LC: Langerhans cell; MØ: macrophage; NK: natural killer; AMPs: antimicrobial peptides; IL: interleukin, TGF: tumor growth factor; TNF: tumor necrosis factor; MMP: matrix metalloproteinases; ROS: reactive oxygen species; NO: nitric oxide.

The optimal outcome of the innate immunity is to eliminate pathogens and prevent full-blown infections from happening. For this purpose, *macrophages* (MØ), phagocytic cells, play a key role in inflammation dampening and host defense activation. MØ control the immune response in three phases. During the first phase, MØ recognize the crystallized fragment (Fc) of IgG-covered microbes via FcγRI (CD64$^+$) leading to pathogen destruction via antibody-dependent cell cytotoxicity (ADCC) and phagocytosis. Alternatively, microbes coated with the complement C3b are identified by MØ with the help of the complement receptor C3bR leading to their lysis or phagocytosis. In the second phase, MØ developed another strategy to destroy pathogens. It is based on proinflammatory mediator secretion including the production of ROS and nitric oxide (NO), as well as proinflammatory cytokine secretion such as TNF-α, IL-6, and IL-1β (Table 1). The duration of this proinflammatory phase depends on the balance between the capacity of the microorganism to survive and the capacity of MØ to remove them. Finally, the last phase is meant to suppress inflammation and to improve apoptotic body removal. It involves anti-inflammatory mechanisms triggered by TGF-β and lipid mediator production [78, 79].

When the innate immunity and signaling are insufficient to clear off a pathogen and to resolve pathogen invasion, the adaptive immune system kicks in. The quantity and the quality of an adaptive immune response depend on the strength of the innate immune response. Although innate

and adaptive immune responses are distinct, they are highly interconnected. The coordination between innate and adaptive immunity is assured by dendritic cells (*DCs*), which are professional antigen-presenting cells known as immune system gatekeepers. They were discovered in 1973 by Steinman and Cohn [80]. In 2011, Steinman was awarded by a Nobel Prize for his work that demonstrated that DCs play a crucial role in the immune system by linking the innate and the adaptive immunity. DCs represent a complex heterogeneous network of subsets that differ in ontology and specific functions. The first step in DC generation occurs in the bone marrow where two precursors committed to either conventional myeloid DCs (mDCs) or nonconventional plasmacytoid DCs (pDCs) were derived. The last step of DC differentiation is also dependent on the DC subset. For instance, mDCs undergo differentiation in the periphery whereas pDCs complete their development in the bone marrow. The maturity of DCs is highly dependent on pathogens they encounter. Different features of the DC population can be observed according to local environmental cues. Regarding the status of the tissue, steady or inflammatory, several subsets of resident or recruited DCs with differential phagocytic activity and capacity to produce cytokine could be identified. One common function among the heterogeneity of these cells is their antigen processing, and presenting cells implicated in T cell tolerance [81–83] make them strategic cells able to participate in both innate and adaptive immunity. DCs recognize antigen via a diverse array of TLR1, 2, 4, 5, 6, 11, and 12. *Dermal DCs* are divided into several subsets. In the mouse dermis, two resident DCs were identified in normal skin: $CD103^{+}CD11b^{-}$ ($CD103^{+}$ DCs) and $CD103^{-}CD11b^{+}$ ($CD11b^{+}$ DCs) [84]. They share functional homology with human $CD141^{hi}CD14^{+}$ DCs and $Cd1a^{+}CD1c^{+}$ DCs, respectively. Since these DCs have the power to catch cutaneous antigens, mature, and migrate to draining local lymph nodes, they become migratory skin DCs [85, 86]. The migratory DCs act as antigen-presenting cells (APCs) and are able to interact with antigen-specific lymphocytes such as T cells subsequently activating the adaptive immune response. Plasmacytoid DCs (pDCs) are quite rare in human skin; they are acting during viral infection by the production of large amounts of IFN-α via TLR7 and 9 activation [87, 88].

3.2. Adaptive Immune Response. In contrast to innate immunity, the adaptive immune system provides a more delayed and specific response. A unique feature of the adaptive immunity is its ability to generate and to retain memory providing a more rapid response in the event of subsequent immunologic challenge. The adaptive immune response consists of humoral and cellular immune reactions carried by adaptive B and T cells, respectively. T cells are major contributors in safeguarding the cutaneous barrier. They are located next to papillary venules and beneath the dermoepidermal junction as well as adjacent to cutaneous appendages in the dermis. The activations of adaptive B and T cells through antigen-specific receptors demand antigen encounters either free antigen or bound antigen by APC to become effector cells. In the following paragraph, the mechanisms of B and T cell activation, maturation, and functions will be overviewed (Figure 4).

The activation of naïve T cells requires two signals provided by APC, mainly DCs. In fact, once inside the lymph nodes, DCs migrate to T cell areas, seeking out antigen-specific T cells by furnishing the necessary signals to induce their activation and differentiation into effector cells. The first signal is MHC molecules presented by DCs after microbial antigen capturing and processing. The second signal is the promotion of CD28 expressed on naïve T cells via costimulatory molecules B7-1 (CD80) and B7-2 (CD86) expressed on DCs (Table 2). The induction of CD80 and CD86 is a very crucial step, which is launched by microbial pathogen recognition [48]. Resident and recirculating T lymphocytes in the skin are a major subtype of leukocytes produced in the bone marrow and matured in the thymus. They are famous for their capacity to recognize a wide range of antigens due to their ability to rearrange the DNA encoding for their T cell receptor (TCR) [89]. Two types of T cells exist, $\alpha\beta$T cells and $\gamma\delta$T cells, which differ in the structure of TCR displayed on their membrane. The other variability is the cluster of differentiation expressed on the surface of T cells that result in two different subpopulations of T cells: $CD8^{+}$ and $CD4^{+}$ T cells. $CD8^{+}$ T cells recognize antigenic peptides presented by MHC-I molecules on APC and are qualified as cytotoxic T lymphocytes (CTLs) responsible for cellular lysis through the secretion of enzymes (perforins and granzymes) that alter the cytoplasm of target cells (Table 2). CTLs use also other mechanisms to kill intracellular pathogens by triggering caspase activation leading to apoptosis. Also, CTLs produce TNF-α and IFN-γ, which have antitumor and antiviral microbial effects. $CD4^{+}$ T cells are essential for both the T cell-mediated and antibody-mediated branches of the immune system. They recognize antigenic peptides presented by MHC-II molecules. T_H cells have been described to be differentiated into two subsets of conventional T cells T_H1 and T_H2 during inflammatory diseases. The differentiation depends on the cytokinic environment and the nature of the antigen (parasite, virus, bacteria, fungi, and extracellular or intracellular organisms). MØ and DCs release IFN-α and IL-12 that stimulate T_H1 response resulting in IFN-γ and lymphotoxin secretion, recruiting phagocytic cells as MØ engulfing the intracellular pathogens [90] (Table 2). T_H2 polarization is dependent on IL-4 liberation by naïve $CD4^{+}$ T cells. T_H2 response is important in the defense against large extracellular organisms such as helminths, utilizing cytokines such as IL-4, IL-5, and IL-13, promoting eosinophilia and mastocytosis (Table 2). Severe consequences occur when the balance between T_H1 and T_H2 is disturbed. T_H1 can be associated with autoimmunity and chronic inflammatory disease such as psoriasis whereas T_H2 can lead to allergic diseases such as atopic dermatitis [91–93]. More recently, two populations of $CD4^{+}$ cells were identified: T_H17 and T_H22. APCs release IL-23 that results in T_H17 differentiation. T_H17 produces mainly IL-17 and IL-22 promoting immunity against various fungal and bacterial infections [94, 95] (Table 2). The differentiation of T_H22 was promoted by TNF-α and IL-6 released from DCs. T_H22 are a subset of circulating T cells with skin-homing potential that produce IL-22 but not IL-17 and IFN-γ [96, 97] (Table 2). Numerous skin

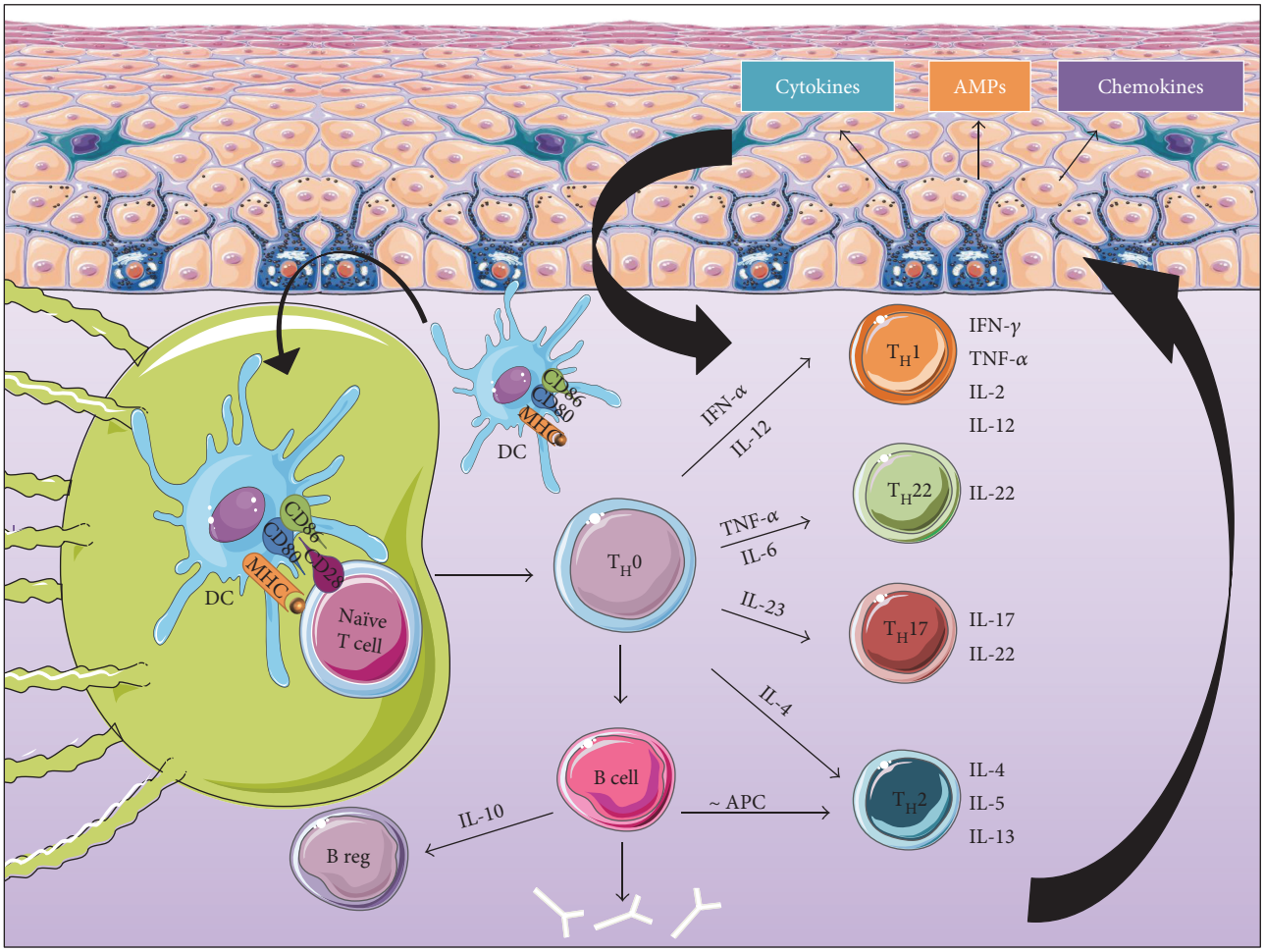

Figure 4: Major actors of the adaptive immune response. The adaptive immune system mounts a stronger, antigen-specific immune response when the innate immune response fails to eliminate pathogens. The first phase consists of the activation of antigen-presenting cells such as DCs allowing their migration into lymph nodes where they prime naïve T cells. Activated T cells migrate back to the site of infection where they induce cell-mediated and humoral immunity causing mediator release by the immune cells present at the site of infection. The resulting cytokinic environment stimulates epidermal cells mainly KCs to release further mediators that activate and maintain the dermal immune response. Hence, a positive feedback loop forms. DCs: dendritic cells; KCs: keratinocytes; TH: T helper; MHC: major histocompatibility class; IFN: interferon; CD: cluster of differentiation; IL: interleukin; TNF: tumor necrosis factor.

Table 2: Major constituents of the adaptive immunity.

Compartments	Cells	Inflammatory mediators
Dermis	DCs	MHC, CD80/CD86, IFN-β
	CTL	Enzymes, caspases
	T_H1	IFN-γ, IL-2, TNF-α, IL-12
	T_H2	IL-4, IL-5, IL-13 IL-4, IL-6, IL-15
	T_H17	IL-17, IL-22
	T_H 22	IL-22
	B cells	IgA, IgE, IgG, IgD, IgM

DCs: dendritic cells; CTL: cytotoxic T lymphocytes; TH: T helper; MHC: major histocompatibility class; IFN: interferon; CD: cluster of differentiation; IL: interleukin; TNF: tumor necrosis factor; Ig: immunoglobulin.

disorders are caused by the deregulation of T_H17 and T_H22 immune responses leading to both psoriasis and atopic dermatitis [94, 98, 99].

The role of cutaneous B cells is poorly documented. In general, once naïve B cells encounter a circulating antigen in the periphery, they complete their maturation process. Mature activated B cells release antibodies into blood and tissue fluid in order to target antigens. When immunoglobulins cover the antigen, a series of different reactions can occur including complement activation and pathogen opsonisation in order to neutralize and evacuate pathogens. Moreover, activated B cells can serve as APC to prime T helper cells into T_H2 mediating a humoral immunity. In 2002, Shlomchik and colleagues have demonstrated that B cells producing IL-10 have suppressive capacity and thus can be qualified as B regulatory cells (Breg) [100, 101] (Table 2). IL-10 is a key cytokine, and besides its suppressive function and anti-inflammatory virtue, it acts as a growth factor promoting B cell maturation into antibody-producing plasma cells [102, 103]. B cells have been found in skin dermis during chronic inflammations caused by cutaneous leishmaniasis, diffuse cutaneous sclerosis, and atopic dermatitis [104–106]. They

play a role in cutaneous inflammation via interactions with both innate immune cells and T cells. More recently, B cells are found to inhibit Treg and T_H17 responses via IL-10 production [107]. These cells were found to improve cutaneous inflammatory responses in murine models of skin inflammation [108]. They were also detected in the lymphocyte population of human skin and described as innate-like B cells that migrate from central reservoirs into the skin [109].

The diverse immune responses are cross-connected. A bridge between innate and adaptive immunity is required for a better infection resolution and an enhanced immunosurveillance. In fact, the inflammatory mediators released from the adaptive immune response stimulate epidermal cells mainly the KCs. They react in turn by secreting further mediators that can stimulate the dermal adaptive immune cells. A positive feedback loop is then formed to amplify the immune response. This coordination between the cells in the different compartments of the skin and those in the lymphatic and blood systems leads to neutralization of the pathogen. After being challenged, the SIS keeps a memory of the antigen nature, in case of a second exposition, to be more reactive and directly effective.

4. Step 3: Immunological Memory

The T cell-mediated immunity is the central element of the adaptive immune system as developed earlier in this review. Recall that adaptive immunity consists of three essential phases: T activation, effector function, and persistence "memory." This paragraph focuses on skin resident T cells that belong to the memory T cell subset. The T cells arrive to the skin after pathogen challenge and are maintained as memory populations. They are sustained by growth factors supplied by KCs and other tissue resident cells [110]. In fact, skin resident T cells in healthy skin accounts for 2×10^{10} cells that correspond to nearly twice the number of T cells in the whole circulation [111]. This huge amount of skin resident T cells is necessary to afford immunosurveillance of the cutaneous barrier exposed continuously to external environment with a high risk of pathogen invasion. In other words, these memory T cells provide long-lasting and rapid responses to pathogen re-encounter. Among these memory T cells, a combination of resident and recirculating memory T cells exist [112]. Recently, Watanabe and colleagues developed a skin xenograft model (nude NGS mice were grafted with human neonatal foreskin) that allowed them to identify four distinct populations of memory T cells: two resident subsets—effector memory (T_{EM}) and resident memory (T_{RM}), and two recirculating subsets—migratory memory (T_{MM}) and central memory (T_{CM}) [112–115]. These subsets can be distinguished by their localization and functional activities. T_{EM} are the first actors during an immune response. They express high levels of CD44 and lack homing addressins (L-selectin and CCR7) [114, 116, 117]; thus, they are not circulating T cells and can be found in nonlymphoid tissues. They exert immediate effector function and secrete cytokines mainly interferon-γ and T_H1, T_H17, and T_H22 proinflammatory cytokines [115]. However, these T_{EM} disappear once the infection is resolved leaving the place for T_{CM}. By contrast to T_{EM} [118], T_{CM} express high levels of homing addressins (L-selectin, CCR7, cutaneous lymphocytes antigen "CLA," and CCR4) [111, 119, 120], which allow their migration in both directions either in lymph nodes (LNs) or in skin. They can also produce IL-2 and T_H2 cytokine (IL-4 and IL-13) [115]. Interestingly, upon rechallenge, persisting T_{CM} are activated in the LN where they extensively proliferate and convert into T_{EM} phenotype to assure an effective appropriate local immune response [114, 121]. Therefore, T_{CM} play a key role in maintaining long-lasting immunologic memory. Recent discoveries described a new powerful subset of resident memory T cells (T_{RM}) that remain in tissues after infection ready to act in case of antigen re-encounter [122]. They have more potent effector function than circulating T cells and have limited proliferation properties. T_{RM} phenotype is quite similar to that of T_{EM}. The emerging studies were able to highlight the importance and the efficacy of T_{RM} in providing an immediate and highly protective rapid local immunity, though the molecular mechanism by which the T_{RM} are regulated is not fully understood. There are two subsets of T_{RM} including $CD103^+$ cells which are enriched in the epidermis with increased cytokine production (IFN-γ, TNF-α, and IL-22) and $CD103^-$ cells present in the dermis with a lower effector function [115]. Recently, Watanabe et al. defined a new subset of recirculating T cells T_{MM}. They are CLA^+, $CCR7^+$, and L-$selectin^-$, recirculating between the skin and LNs. Nonetheless, since they lack L-selectin, these cells are suspected to reside in the skin after infection resolution. They are considered an intermediate in cytokine production between T_{CM} and T_{EM} [115]. Further studies are needed for a better comprehension of these T_{MM}.

5. Association of Skin Disorders with Cutaneous Microbiota Disturbance

In dermatology, antimicrobial agents are used to clinically improve several skin diseases. Therefore, scientists investigate the microbial contribution and association with different skin disorders. However, a direct causative relationship between a microbe and a disease remains partially identified. In fact, the four criteria of Koch's postulates are hard to satisfy in some cases. For instance, isolated microorganisms from sick skins are often considered commensal in steady-state condition. Hence, they fail to cause disease when introduced into a healthy organism. In the following part of this review, we illustrate the different ways in which skin disorders are due to skin immune response deregulation associated with microbiota dysbiosis and vice versa.

5.1. Skin Immune Disorder Correlation to Microbiota. Atopic dermatitis (AD) is a chronic T_H2-type inflammatory skin disease associated with cutaneous hyperreactivity to environmental triggers [123]. It affects at least 15% of children and 3% of adults [124]. Patients suffer from relapsing eczematous lesions with severe pruritus. These lesions are characterized by inflammatory DC, MØ, and eosinophil infiltrations [125, 126]. AD is frequently connected with barrier dysfunction and transepidermal water loss associated with filaggrin gene (FLG) mutation that enhances susceptibility

to microbial colonization such as *S. aureus* infections [127–129]. *S. aureus* itself is capable of penetrating the epidermis in case of increased cathelicidin expression and increased expression of IL-4, IL-13, IL-22, and other cytokines [127]. Flares of the disease are associated with an expansion of *S. aureus* on lesional skin and a substantial loss of biodiversity in skin microbiome [130]. However, the resolution of AD lesions is preceded by a restoration of microbial diversity demonstrating the implication of the cutaneous microbiota and AD development [130]. *S. aureus* release high levels of antimicrobial agents weakening other resident microorganisms and replacing them. *S. aureus* found in atopic skin produce toxins that contribute to inflammation and skin barrier dysfunction via host inflammasome activation. To cross the epithelial barrier, *S. aureus* promote peptidoglycan acetylation, superoxide dismutase, and catalase production to avoid phagocyte-mediated killing [35]. *S. aureus* escape the immune system by a plethora of secreted and surface-associated immune evasive molecules. *S. aureus* redirects host defense by fibrin formation or by disruption of adaptive responses, therefore preventing the establishment of protective immune responses [131–133]. Moreover, *S. aureus* adhesion to KCs stimulates their endogenous protease activity resulting in skin barrier integrity disruption [34]. KCs sense *S. aureus* via NOD-2 signaling activation and initiation of an IL-17 response, concomitant with AMP secretion [34, 134]. The bacterial replication within the skin will then induce the release of exoproducts (peptidoglycan, lipoproteins), which will promote inflammation and thus cytokine production [33] and dermal macrophage activation [135]. Indeed, macrophages will perceive these exoproducts via TLR and will activate neutrophil extravasation and migration to the site of infection [36]. Neutrophil adhesion and phagocytosis are then activated in IL-17-dependent T cell signaling [136]. The overall induced immune response aims at eliminating the bacteria. Until today, studies considering the different compartments/tissue layers populated by skin microbiome in AD have not been investigated in detail. These studies are crucial to develop new potential therapeutic targets.

5.2. Microbiota Correlation to Skin Immune Disorders. Acne vulgaris known as acne is one of the most common skin diseases that usually occur in puberty. Multiple factors can be at the origins of acne development in the sebaceous unit. The latter is colonized by *Propionibacterium* spp., a lipophilic bacterium that hydrolyses triglycerides present in sebum into free fatty acids resulting in skin acidification and emollition [5, 137, 138]. The correlation between *Propionibacterium acnes* and acne vulgaris has been well established since 1975 [139]. During puberty, increased sebum secretion induces proliferation of specific *P. acne*s subtypes, *S. epidermidis*, and *Corynebacterium* [140–144]. However, *P. acnes* relative abundance does not differ between individuals with acne and healthy ones [144, 145]. These findings raise the question how *P. acnes*, a commensal bacterium, functions as a pathogenic factor in acne. Metagenomic analysis demonstrated that certain strains were highly associated with acne and other strains were enriched in healthy skin [145, 146]. Thus, the pathogenicity and virulence of *P. acnes* are strain specific. Acne pathogenesis initiates and propagates due to abnormal keratinization resulting in pilosebaceous inflammation [147]. Other causes are attributed to a complex interplay of increased sebum productions, changes in the endocrine system, and local inflammatory cytokine secretion due to the activation of the innate immunity by *P. acnes* [147–150]. *P. acnes* mainly trigger the inflammatory process via TLR2 and TLR4 activation resulting in IL-1 secretion leading to KC hyperproliferation and further production of IL-1 [151]. As a matter of fact, IL-1 plays a key role in acne formation. It maintains an inflammatory milieu that boosts cellular proliferation and stimulates different cells such as neutrophils, endothelial cells, and follicular cells to generate further inflammatory mediators such as AMPs (β-defensin family, cathelicidin, and granulysin), cytokines (IL-1, IL-6, IL-10, IL-12, and TNF-α), chemokine (CXCL-8), matrix metalloproteinase (MMP-9), and NF-κB [150–155]. In clinic, antibodies against *P. acnes* secretory factors were able to decrease acne inflammation demonstrating the essential role of *P. acnes* in acne-dependent inflammation. One of the novel approaches to treat acne is to supplement skin microbiota with healthful *P. acnes* strains or *S. epidermidis* known to inhibit pathogenic *P. acnes* growth as probiotic application (refer to Interplay of the Cutaneous Ecosystem and Pathogen Invasion) [156].

6. Conclusions and Perspectives

This review aims to give basic concepts of the skin immune system, the mechanisms underlying the immune response activation upon pathogen invasion, and the influence of skin microbiota on health and on disease. It is important to visualize the skin as a complex network of immune (innate and adaptive immunity) and epithelial cells that are in constant communication with the external environment and in effective activation of the internal environment (immune response) in order to maintain skin homeostasis. Although considerable attention was directed at the characterization of the interaction of the skin microbiome, there are much more factors that influence both the skin microbiota and the SIS. For instance, the age, sex, ethnicity, endocrine system, neurological system, and genetic predisposition are all contributors in both skin ecosystem and immune host defense. Despite the tremendous efforts made in this field, we are far from a full understanding of the immune regulation of the skin in health and in disease. More studies are needed to improve our understanding of the peaceful and mutual beneficial exchange between the host SIS and microorganism colonization. We are also far from understanding the global view and the cross-talk between the different axes of the SIS in a steady state and in a disorder state. Moreover, the translation of findings obtained from genetic mouse models into human skin poses a great challenge since there are fundamental differences between the mouse and human cutaneous composition and immune responses. Finally, the ultimate objective of studying the skin and the associated microflora is to find new efficient therapeutic invention

against skin diseases, which constitute a large health and economic issues in the society.

Conflicts of Interest

The authors declare no competing financial interest.

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Review Article

The Intricate Link among Gut "Immunological Niche," Microbiota, and Xenobiotics in Intestinal Pathology

Danilo Pagliari,[1] Giovanni Gambassi,[1] Ciriaco A. Piccirillo,[2,3,4] and Rossella Cianci[1]

[1]*Department of Internal Medicine and Medical Sciences, "A. Gemelli Hospital", Catholic University of the Sacred Heart, Rome, Italy*
[2]*Department of Microbiology and Immunology, McGill University, Montréal, QC, Canada*
[3]*Program in Infectious Disease and Immunity in Global Health, Research Institute of the McGill University Health Centre, Montréal, QC, Canada*
[4]*Centre of Excellence in Translational Immunology (CETI), Research Institute of the McGill University Health Centre, Montréal, QC, Canada*

Correspondence should be addressed to Rossella Cianci; rossellacianci@gmail.com

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Inflammatory bowel diseases (IBDs) are diseases characterized by various degrees of inflammation involving the gastrointestinal tract. Ulcerative colitis and Crohn's disease are characterized by a dysregulated immune response leading to structural gut alterations in genetically predisposed individuals. Diverticular disease is characterized by abnormal immune response to normal gut microbiota. IBDs are linked to a lack of physiological tolerance of the mucosal immune system to resident gut microbiota and pathogens. The disruption of immune tolerance involves inflammatory pathways characterized by an unbalance between the anti-inflammatory regulatory T cells and the proinflammatory Th1/Th17 cells. The interaction among T cell subpopulations and their related cytokines, mediators of inflammation, gut microbiota, and the intestinal mucosa constitute the gut "immunological niche." Several evidences have shown that xenobiotics, such as rifaximin, can positively modulate the inflammatory pathways at the site of gut immunological niche, acting as anti-inflammatory agents. Xenobiotics may interfere with components of the immunological niche, leading to activation of anti-inflammatory pathways and inhibition of several mediators of inflammation. In summary, xenobiotics may reduce disease-related gut mucosal alterations and clinical symptoms. Studying the complex interplay between gut immunological niche and xenobiotics will certainly open new horizons in the knowledge and therapy of intestinal pathologies.

1. The Role of Mucosal Immunity in the Intestinal Mucosal Barrier

Human bowel has a sophisticated immune system that protects from pathogen's infections, while maintaining a tolerance to food antigens and nonpathogen *bacteria* [1]. The mucus layer over the gut epithelium itself contains antimicrobial products and secretory IgA and it is the first defensive component. However, it is the intestinal *epithelium* with its secretory antibacterial peptides [2] and innate and adaptive immune system cells that regulates gut immunity (Figure 1). Intestinal mucosal immune cells are specifically organized to form a so-called gut-associated lymphoid tissue (GALT), where cells are activated by bacterial antigens. These structural and immunological defense mechanisms of the human gut have been referred to as the "mucosal firewall" [3].

It has been well established that $CD4^+$ T cells can differentiate into several subtypes that may have both pro- and anti-inflammatory properties [4, 5]. Th1, Th2, and Th17 cells generate mucosal inflammation and tissue damage while regulatory T cells (Tregs), instead, have anti-inflammatory properties and limit mucosal inflammation and promote tissue repair. Thus, T cell subsets and their related cytokines contribute to the physiological maintenance and the

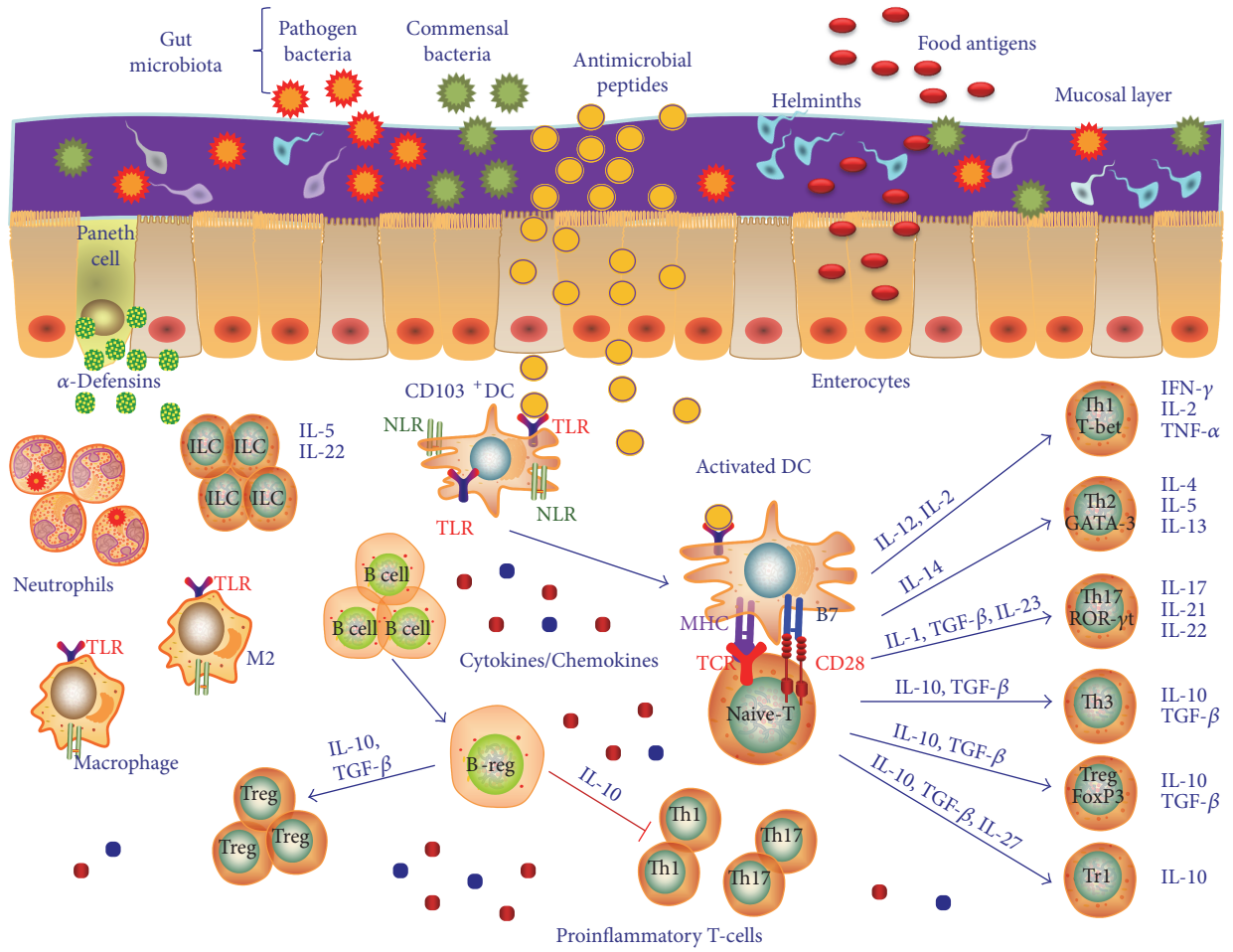

Figure 1: The complex interactions in the gut "*immunological niche*". The human bowel is a sophisticated immune system that protects from pathogen's infections. The intestinal mucosal layer represents a mechanical barrier. The mucus over the gut epithelium contains antimicrobial peptides and it is the first defensive component. However, it is the intestinal *epithelium* with its secretory antibacterial peptides, innate and adaptive immune system cells, and their related pro- and anti-inflammatory cytokines and chemokines that regulate gut immunity. Intestinal mucosal immune cells are specifically organized to form a so-called gut-associated lymphoid tissue (GALT), where cells are activated by bacterial antigens. These structural and immunological defense mechanisms of the human gut have been referred to as the "*immunological niche*." TLR: Toll-like receptors; Treg: regulatory T cells; NLR: NOD-like receptors; TCR: T cell receptor; IL: interleukin; GM-CSF: granulocyte-macrophage colony-stimulating factor; DC: dendritic cell; MHC: major histocompatibility complex; PD1: programmed death 1; PD-L1: programmed death-ligand 1.

pathological transformation of intestinal mucosa by constantly modulating the gut homeostasis and inflammation [6]. These T cell subpopulations present in the gut mucosa are associated with a specific cytokine cocktail [5]. Thus, several cytokines and their receptors with pro- and anti-inflammatory functions resulted involved in inflammatory diseases of the bowel [5, 7], such as IFN-γ and IFN-γR1, TNF-α, IL-1R1, IL-2 and IL-2RA, IL-4, IL-5, IL-9, IL-12β and IL-12R, IL-13, IL-15, IL-17A, IL-18, IL-21, IL-22, IL-23 and IL-23R, TGF-β , IL-10, and IL-27 [8, 9]. The well-known proinflammatory T cell subpopulations are Th1 cells that are characterized by the specific production of IFN-γ and IL-12 and the counter-regulatory Th2 cells which produce humoral immunity-promoting cytokines like IL-4, IL-5, and IL-13. IL-12, a major product of activated DC, stimulates Th1 differentiation and production of effector cytokines, such as IFN-γ and TNF-α. As a classic activator of cell-mediated immunity, IFN-γ activates macrophages, natural killer (NK) cells, and $CD8^+$ T cells. For Th2 cells, IL-4 acts as the major Th2 differentiation factor and promotes IL-4 and IL-13 expression. In particular, IL-13 is a cytokine with proinflammatory functions, as it participates in the disruption of gut epithelial barrier and in the promotion of mucosal fibrosis via TGF-β1 expression [10]. Furthermore, another proinflammatory T cell subset is constituted by Th9 cells characterized by the production of IL-9. IL-9 acts impairing gut mucosal healing, barrier function, and epithelial cell proliferation [11].

Th17 cells are key initiators of proinflammatory responses in gut mucosal surfaces. Th17 cells via their production of IL-17A and IL-17F are generally proinflammatory and play an important role in host defense against infection to extracellular pathogens, by recruiting neutrophils and macrophages to infected tissues. Their development depends

on signals mediated by IL-6 (and downstream activation of STAT3) and TGF-β, IL-21, and IL-23 and by induction of the lineage-specifying transcription factor, retinoic acid-related orphan nuclear receptor (RORγT) [5]. IL-17A is involved in local chronic inflammation inducing proinflammatory cytokine expression leading to mucosal destruction and altering mucosal healing. Among Th17 cell cytokines, a key role is played by IL-23 that orchestrates the survival and maintenance of the Th17 phenotype and in turn the crosstalk between innate and adaptive immunity in the gut [12]. Interestingly, aberrant expression and activity of the IL-17/IL-23 axis is frequently involved in the pathogenesis of several inflammatory bowel pathologies [13, 14]. Consistently, studies report that polymorphisms in the *il23r* gene are associated with inflammatory bowel diseases (IBDs) [15, 16]. Another proinflammatory cytokine implicated in IBD pathogenesis is IL-21 which is secreted by T follicular helper (Tfh) cells, and it is implicated in the differentiation of germinal center B cells into high-affinity antibody-secreting plasma cells and memory B cells that ensure sustained immune protection and rapid recall responses against previously encountered foreign antigens [17]. IL-21 can also play important roles in T cell subset development. Tfh cells differentiate from naïve $CD4^+$ T helper cell precursors after antigen activation in the presence of IL-6 and IL-21 and induction of B cell lymphoma 6 (BcL6) [4, 18]. It must be noted that while Th17 cells are prominent inducers of chronic inflammatory responses in disease states of the gut, this subset can protect the intestinal mucosa from microbiota and pathogens by its ability to resist infection and promote IgA secretion. Moreover, Th17 cells also produce IL-22 which has important functions in host defense at mucosal surfaces as well as in tissue repair [19]. While it is produced by innate lymphoid cells (ILCs) and Th cell subsets, including Th17 cells, IL-22 acts only on nonhematopoietic stromal cells like epithelial cells and keratinocytes. Although IL-22 is beneficial to the host in many infectious and inflammatory disorders, it can be pathologic due to its proinflammatory properties, which are further enhanced by other proinflammatory cytokines like IL-17 [20].

Several innate-like lymphocyte populations are involved in key homeostatic and pathogenic interactions with gut microbiota. Among these populations, a crucial role is played by ILCs, of which exist 3 subpopulations: ILC1, ILC2, and ILC3 [21]. These cells are part of the innate immune system, their actions are strictly related to the presence of commensal microbiota, and they interact between both the innate and adaptive arms of the immune system [22]. As it relates to the regulation of intestinal immune responses, ILC3 participates in the maintenance of mucosal barrier homeostasis by producing the anti-inflammatory cytokine IL-22, and they stimulate neutrophils and macrophage recruitment and proliferation in the gut, producing IL-17 and granulocyte-macrophage colony-stimulating factor (GM-CSF), respectively. In turn, macrophages may stimulate ILC function by producing IL-1β [23]. On the other hand, ILCs can stimulate T cells by favoring antigen presentation by intramucosal antigen-presenting cells (APCs), such as DCs [22].

The abovementioned cytokines and chemokines are principally related to several T cell subtypes of the adaptive immunity. Among these molecules, some of them have a bridge action in participating in both innate and adaptive immune response and include cytokines like IFN-γ, TNF-α, IL-1, IL-2, IL-12, and IL-15 [24]. Among these cytokines, a special role is played by IL-15 which plays key roles in the intestinal mucosal barrier [25]. IL-15 is a member of the IL-2 family of cytokines whose signaling pathway constitutes a bridge between innate and adaptive immune responses. On one hand, IL-15 regulates the differentiation and activation of proinflammatory Th1 and Th17 cells, and on the other hand, it blocks the activation of the immunosuppressive Tregs. In addition, IL-15 may mediate enterocyte apoptosis [26]. Consistent with these actions, several studies have reported an upregulation of IL-15 in inflamed tissues of patients with bowel pathologies, such as IBDs, and diverticular disease [27]. These data suggest that IL-15 may exert a direct pathogenetic role in these conditions. However, there is also evidence that IL-15 may potentiate the immune response against cancer. For these reasons, IL-15 is still considered both a friend and a foe of human physiology and pathology [28].

An essential role of the immune system is to eradicate pathogens while suppressing the potential for immune pathology. Triggering and maintaining immune tolerance within the intestine represent a unique challenge to the mucosal immune system. A variety of immune-regulatory cell subsets within the T cell, B cell, dendritic cells (DCs), and macrophage (M2 phenotype) compartments, each endowed with unique suppressive functions, are critical for ensuring sustained immune tolerance in the intestinal tissue microenvironment through active inhibition of innate and adaptive immune responses [20].

One of the predominant anti-inflammatory cell types are Tregs, of which there are many subtypes [29] including $CD4^+CD25^{high}$ Treg cells. These cells are characterized by the expression of the forkhead box P3 (FoxP3), the master-switch, lineage-specifying transcription factor that orchestrates the transcriptional landscape and drives the development and function of this Treg subset. $FoxP3^+$ Tregs are essential mediators of immune tolerance by modulating innate and adaptive immune responses to self and nonself antigens [30]. Developmental, homeostatic, or functional deficits in these cells can provoke autoimmune disease and all the while augment responses to pathogens, tumors, or allergens [29]. $Foxp3^+$ Tregs also have a positive role in limiting tissue inflammation, maintaining immune tolerance, and promoting mucosal healing in the gut [31]. In fact, studies have shown that their number is inversely correlated with the clinical course and severity of IBDs [6]. Moreover, the development of Tregs is strictly linked to the presence of commensal gut microbiota [32]. In fact, evidence in experimental mouse models showed that, in the absence of gut microbiota, the number of Tregs resulted significantly reduced and it was subsequently restored to normal proportions after gut recolonization with flora [33, 34].

DCs play an important role in activating immune responses but also in the induction of tolerance to microbial

and dietary antigens [35]. DCs are environmental sentinels scanning for various innate and danger signals and poised in tissue to influence the immune activation or suppression decision. To this end, they are present in the mesenteric lymph nodes, in the gut *lamina propria*, and in Peyer's patches and participate in the control of intestinal inflammation [36]. Normally, DCs, particularly the tolerogenic $CD103^+$ DC subset, are recruited in the gut during inflammatory conditions and in turn efficiently act stimulating the differentiation of $Foxp3^+$ Tregs [37]. Overall, $Foxp3^+$ Tregs, in concertation with other immunoregulatory cell types, including T regulatory 1 (Tr1) cells, T helper 3 (Th3) cells, regulatory B cells (Bregs), $CD103^+$ DCs, and M2 cells, are instrumental in establishing a global context of immune tolerance to a spectrum of potentially pathogenic microorganisms in the gut flora [20].

2. The Gut Microbiota and Mucosal Immunity Crosstalk

Gut microbiota is the collection of all microbial populations that reside in the gastrointestinal tract. It can weigh up to a total of 1 kg and contains tens of trillions of microorganisms, a 100 times more genes than the host, and includes at least 1000 different bacterial species. It is increasingly recognized that gut microbiota plays a pivotal role in the gastrointestinal tract homeostasis [38]. A strong reciprocal interaction between gut microbiota and host immunity has been proven as the former has coevolved in a symbiotic relationship with mucosal immunity. These commensal *bacteria* are called "keystone species," and overall, they can be considered a "superorganism" that is an integral part of the human gastrointestinal tract [39].

The human intestine has a large surface area that constitutes an entrance door for the antigens that we introduce with the food. In addition, the human gut is covered with many bacterial communities, some of which may be dangerous for the host. Hence, the principal role of the intestinal immune system is to protect the host from pathogens preventing infections. To this end, in mucosal immune cells, several surface receptors are endowed that mediate the interaction with microbial antigens. Among these pattern recognition receptors (PRRs), the most important are the Toll-like receptors (TLRs) and the NOD-like receptors (NLRs) [40].

In physiological homeostasis, there is a perfect balance between microbial load and the immune response generated against it [41]. The immune system correctly functions to ensure tolerance to food antigens and defense against microbial infections. A homeostatic role is played by TLR signaling. Commensal microbiota also participates to immune tolerance by promoting the differentiation of anti-inflammatory Tregs [42]. Conversely, in a disease state or during an infection, the normal gut homeostasis is lost with an excess of tissue inflammation. In this altered environment, TLRs, activated by pathogens, lose their homeostatic role and promote the activation and development of an inflammatory response, contributing to acute and chronic intestinal inflammatory states [43].

Host and microbial metabolisms are also key modulators of innate and adaptive immune responses in mucosal environments. While both occur simultaneously, the two are profoundly interdependent: while the host depends on the microbiome for a spectrum of digestive and metabolic enzymes, the microbiota, particularly in the gut, produces a wide array of metabolites from endogenous compounds produced by microbes and the host [44], but primarily from the anaerobic fermentation of dietary components in the colon [45]. The epithelial cell layer that constitutes the host microbe mucosal interface permits microbial-derived metabolic products to access and interact with host cells and, in turn, shape downstream inflammatory and immune responses. One salient example of such metabolite is short-chain fatty acids (SCFAs), like propionic acid and butyric acid, which are produced by colonic microbial fermentation of undigested or partially digested dietary fibres. They signal in host cells via G protein-coupled receptors (e.g., GPR41 and GPR43) on the surface of epithelial and immune cells, having a range of effects on host immune functions [46]. The effects of SCFAs include inhibition of histone deacetylase activity and altered gene expression in host cells and augmented epithelial barrier function which promotes gut homeostasis via several mechanisms: (1) enhanced mucus production by intestinal goblet cells, (2) inhibition of NF-κB, (3) activation of inflammasomes and IL-18 production, (4) increased B cell secretion of secretory IgA, (5) diminished maturation of DCs, and (6) increased number and function of colonic $Foxp3^+$ Tregs. SCFAs are not restricted to the gut and can also find their way to other organs, such as the lungs, where they directly or indirectly act on local APCs to modulate inflammatory responses that are associated with airway disease (allergic or infectious) [46–49].

3. Microbiota and Host Immune System Interactions in Inflammatory Bowel Diseases

IBDs, such as Crohn's disease (CD) and ulcerative colitis (UC), are chronic and multifactorial pathologies of the gastrointestinal tract. In these bowel diseases, there is an imbalance between proinflammatory and anti-inflammatory responses, but their full pathogenetic mechanisms are still incompletely understood [50]. It is known that in genetically predisposed individuals, an inappropriate immune response against luminal agents is activated with an abnormal production of cytokines and other mediators of inflammation [9]. The genetic factors involved in the pathogenesis of IBDs include genes encoding proteins of immunity involved in environmental sensing of microbial-derived products and signals.

In particular, nucleotide-binding oligomerization domain-containing protein 2 (NOD2) is an autophagy-related gene that activates an immune reaction against components of the bacteria cell wall, including peptidoglycans, in both Gram-positive and Gram-negative bacteria such as in the case of *Shigella* and *Listeria* [51]. NOD2 is expressed on the cell surface of various epithelial and innate immune cells, such as neutrophils, DCs, stromal cells, macrophages, and others [52]. NOD2 has several homeostatic

functions like intracellular bacterial sensing, inducing the expression of several antibacterial peptides, such as α-defensin, and participating in the immune tolerance mechanisms by the suppression of the TLR axis [53]. Under physiological conditions, when the equilibrium between pro- and anti-inflammatory factors is maintained in the gut, NOD2 expression levels are very low [51]. On the other hand, in pathological situations, when proinflammatory factors are predominant over the anti-inflammatory ones, NOD2 level expressions are increased, particularly due to the overexpression of the proinflammatory cytokine TNF-α [51]. The activation of NOD2 by antimicrobial peptides determines the initiation of a signaling cascade which is responsible for the production of several proinflammatory cytokines and chemokines involving the activation of NF-κB [52]. The breakdown of the physiological equilibrium between immunity and microbiota determines alterations in the NOD2 functions [54]. The bidirectional function of NOD2 is linked to the inflammatory levels present on the gut surface. Interestingly, NOD2 polymorphisms are associated with an increased risk for the development of CD, and aberrant NOD2 activity may alter the functions of both epithelial and innate immunity cells. Primarily, NOD2 in the epithelial compartment is able to selectively reduce the levels of alpha-defensins in Paneth cells in the small bowel [55, 56]. NOD2 activation is also linked to disturbance of hematopoietic cells and DC activity [57]. Moreover, NOD2 polymorphisms are related to a reduction of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) in response to *bacterial* peptidoglycan [58]. Finally, other genetic factors involved in the pathogenesis of IBDs include the signal transducer and activator of transcription 3 (STAT3), and IL-23 receptor (IL-23R) [25].

Both humoral and cell mediated immunity are involved in the pathogenesis of IBDs [59]. IBDs are also characterized by an influence of environmental factors. In fact, IBDs pathogenesis is influenced by the TLR axis activated by both commensal and pathogenetic *bacteria*, leading to the promotion of inflammatory pathways responsible for tissue damage [25]. Likewise, IBDs are characterized by a profound modification of the gut microbiota. Typically, pathogens grow and proliferate to suppress the physiological *flora* and generate a dysbiotic state. The consequent intestinal barrier dysfunction determines the translocation of pathogens in the *lamina propria*. Human GALT is directly affected in at least two distinct ways. First, the outgrowth of opportunistic classes of *bacteria* drives increased inflammation [60]. In this scenario, TLRs mediate the activation of the proinflammatory transcription factor NF-κB with the consequent production of proinflammatory cytokines and chemokines [61]. Secondly, the loss of benign fermenting *bacteria* that produce "keystone" metabolites results in reduced immunoregulation [3]. In particular, studies report that IBDs present specific gut microbiota alterations, characterized by a reduction in bacterial diversity and an increase in bacterial instability [13]. There is an increase of *Bacteroidetes* and *Proteobacteria* in both CD and UC [62, 63]. CD is further characterized by an increase in some pathogens of the *Enterobacteriaceae* family [64], such as *Salmonella*, *Shigella*, *Escherichia*, invasive *Fusobacteria*, and *Actinobacteria* [62]. Likewise, both CD and UC present a reduced number of *Firmicutes*, *Lachnospiraceae*, and *Ruminococcaceae* [65]. Finally, in CD, fewer *Bifidobacteria* have been reported [66].

Considering the immunopathogenesis of IBDs, it is well known that CD is related to a Th1 and Th17 immune profile, while UC is related to a Th2 response [67]. Then, both pathologies are characterized by the unbalance between proinflammatory T cell subsets and anti-inflammatory Tregs [59]. In fact, both in CD and UC, Tregs and their related anti-inflammatory cytokines resulted as decreased. Several literature data have reported a strong interaction among mucosal immunity, TLRs, and gut microbiota. These interactions may regulate gut physiology, immune tolerance to external dietary antigens, protection from external infections, regulation of gut microbial populations, and the commensal/pathogen ratio. A special focus has been placed on TLR2 and TLR4 signaling. In fact, their signals promote Treg proliferation and survival [68]. Then, other evidences supported the fact that such bacteria may directly influence the development of Th17 cell differentiation. In this way, *Bacteroides fragilis* promotes Treg differentiation and IL-10 and TGF-Beta production and inhibits Th17 cell differentiation [69].

4. Pharmacological Modulation of the Immunological Niche

These data suggest that the intestinal mucosa holds a complex immune-functional set that is central in the regulation of physiological homeostasis. For this reason, the gut mucosa may be considered an "immunological niche," that is, a definite immune-functional region, that is, constituted by T cell subpopulations and their related anti- and proinflammatory cytokines, several mediators of inflammation, and gut microbiota [26, 70]. Perturbations and disruption of the immunological niche are critical steps in the pathogenetic pathways contributing to the development of inflammatory bowel pathologies. However, it is likely that we may also use the concept of immunological niche to explain the mechanisms of several other "inflammatory" diseases. An immediate therapeutic strategy would be to use agents that can modulate the immunological niche reducing inflammation and rebalancing gut immunity [71]. While there is no such definitely proven agent, there is a growing interest in the possible role of xenobiotics, especially rifaximin [72].

5. The Role of Xenobiotics in the Interactions with the Immunological Niche: The Case of Rifaximin

Xenobiotics are chemical molecules that are not normally produced by humans. They may interfere with host metabolism and produce effects of modification of the host physiology and pathology. Among xenobiotics, rifaximin seems to be of special interest [73]. Rifaximin is a semisynthetic agent based on rifamycin with a broad-action spectrum of antibiotic activity against both $Gram^+$ and $Gram^-$ *bacteria*. Rifaximin

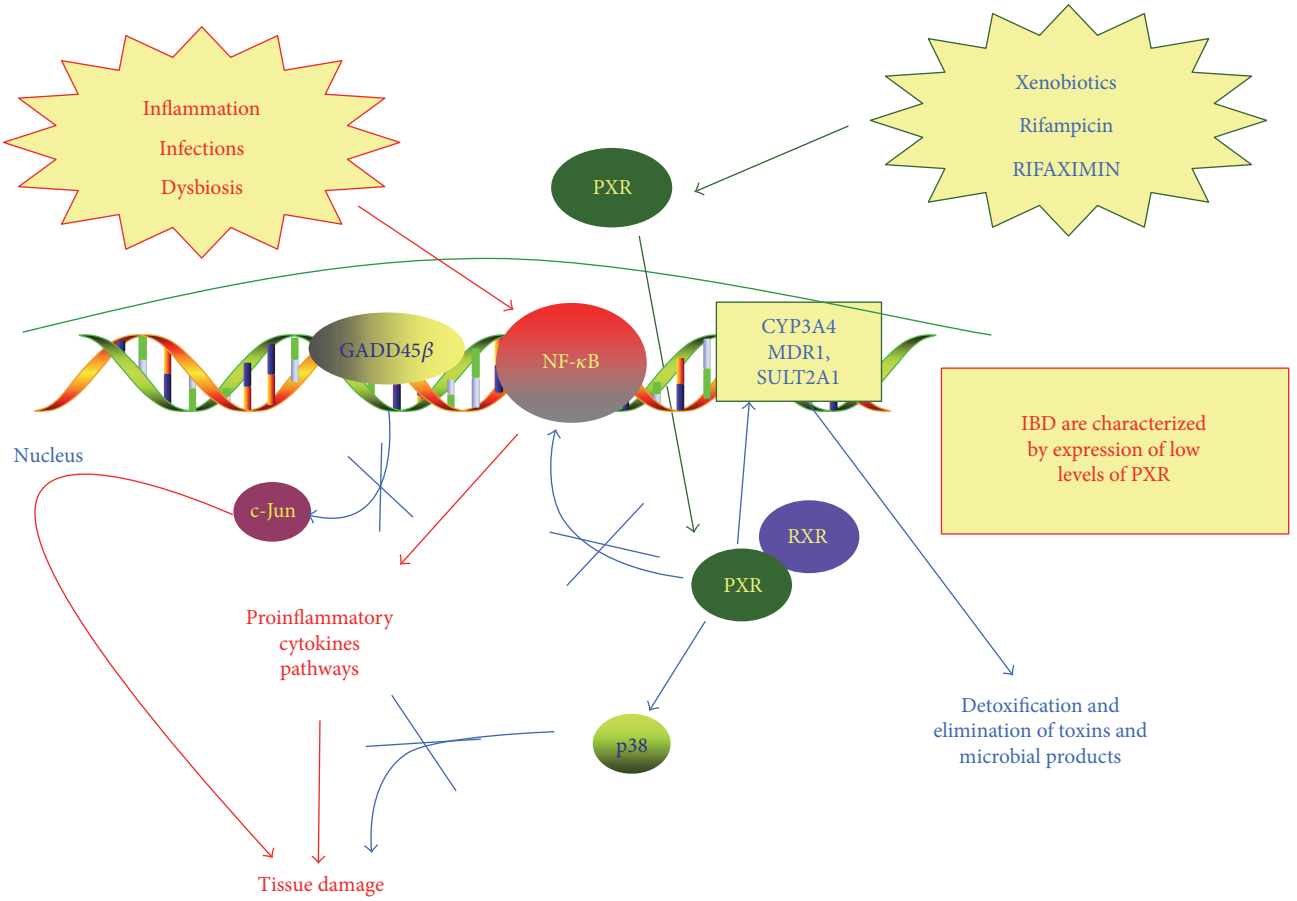

Figure 2: The role of rifaximin in modulating gut inflammation via the PXR/NF-κB pathway. Rifaximin is an effective agonist of the nuclear receptor PXR. PXR, greatly expressed in liver and intestinal mucosa, acts as a driver of detoxification processes and contributes to intestinal cell survival during exposure to several xenobiotics. After being activated by its ligands, PXR translocates into the nucleus where it binds its receptor and then regulates DNA transcription. The anti-inflammatory effects of rifaximin may not only be linked to the reduction of ileal *bacteria* load but it may also have an indirect action on inflammation. In fact, rifaximin, being an effective agonist of PXR, may regulate the inflammatory process. In particular, rifaximin, activating PXR, can inhibit NF-κB activity and the consequent transcription of several proinflammatory cytokines, such as TNF-alpha and IL-1β. The activation of PXR then upregulates p38 MAP kinase signal cascade via GADD45β upregulation. All these mechanisms are linked to the prevention of tissue damage and to favor gut mucosal healing.

has poor oral bioavaibility; thus, it acts locally in the gastrointestinal tract having only few systemic effects [74].

Differently from common antibiotics, rifaximin may modify gut microbiota toward a relative abundance of certain species of protective *bacteria*. Several data have confirmed that rifaximin is able to increase the proliferation and growth of the protective *Lactobacilli* while inhibiting that of several pathobionts, including *Clostridia* and *Firmicutes*, with negative effects [75, 76]. These changes in microbiota composition may contribute to the anti-inflammatory effects of rifaximin on the intestinal mucosa [77]. Indeed, *Lactobacilli* are able to downregulate mucosal inflammation, improving the function of intestinal barrier, and restoring the normal mucosal permeability [78].

The anti-inflammatory effects of rifaximin may not only be linked to the reduction of ileal *bacteria* load but it may also have an indirect action on inflammation. In fact, rifaximin is an effective agonist of the nuclear receptor PXR [79]. PXR, greatly expressed in liver and intestinal mucosa, acts as a driver of detoxification processes and contributes to intestinal cell survival during exposure to several xenobiotics. After being activated by its ligands, PXR translocates into the nucleus where it binds its receptor and then regulates DNA transcription (Figure 2). PXR can inhibit NF-κB activity and the consequent transcription of several proinflammatory cytokines, such as TNF-alpha and IL-1beta [73, 80]. In 2010, Cheng et al. [81] demonstrated that rifaximin reduces the expression of the NF-κB-related proinflammatory genes activating PXR function. In contrast, rifaximin is not able to modify the expression of these genes in PXR-*null* transgenic mice. For this reason, PXR is considered an anti-inflammatory molecular factor. In support of this, it has been proven that IBDs are characterized by the expression of low levels of PXR. Thus, rifaximin activation of PXR function may have positive anti-inflammatory properties. Finally, rifaximin may limit inflammation-mediated damage activating the p38 MAP kinase that is directly able to promote tissue repair [82].

6. The Role of Rifaximin in Inflammatory Bowel Diseases

It is well known that gut microbiota plays an important role in the development of IBDs [83]. Thus, the modulation of the gut microbiota has been put as focus of several clinical and research areas [84]. The manipulation of intestinal *bacteria* can be achieved by several modalities that involved xenobiotics, such as prebiotics, probiotics, and antibiotics, and fecal transplants [85, 86].

Prebiotics are fermentable substances participating in the modulation of gut flora. Prebiotics have several actions on the gut mucosa, such as the improvement of IL-10 DCs and TLR2 and TLR4 cells. Then, they exert positive modulation of gut microbiota populations improving the growth of beneficial resident *bacteria* as a consequence of the manipulation of the luminal substrate composition. Moreover, prebiotics improve the intestinal barrier and regulate the mucosal immune system [87].

On the other hand, probiotics are live microorganisms that, administered in therapeutic doses, confer a health advantage on the host. Probiotics present several positive actions on the gut mucosa. In fact, probiotics restore the microbial balance, protect the host against pathogens, and modify gut-associated lymphoid tissue and the mucosal immune system. A synergic action between prebiotics and probiotics seems to be associated with a reduction of the concentration of pathogenetic metabolites and dangerous microflora [85].

In particular, several antibiotics may modulate the course of IBDs by reducing pathological *bacteria*, such as *Escherichia coli*, *Bacteroides fragilis*, and other gram-negative *Enterobacteriaceae* present in the gut lumen, and by altering the composition of gut microbiota to favor beneficial *bacteria* [83]. Literature data demonstrated that antibiotics may be effectively used in the treatment of IBDs, due to their action in reducing bacterial overgrowth, resolving septic systemic complications and local infections, such as abscesses and fistulas [88]. Importantly, it has been shown that antibiotics may be used even to maintain IBD remission [89]. Among xenobiotics, rifaximin seems to influence remission in both CD and UC [90].

In 2005, Shafran and Johnson performed a clinical study on active CD patients. Rifaximin was administered at a dose of 200 mg twice daily for 16 weeks, and it induced a high clinical remission (59% cases) [91]. Then, in a further study, Shafran and Burgunder performed a retrospective analysis of CD patients receiving adjunctive therapy with rifaximin (mean dose 600 mg daily for 16 weeks) showing clinical remission in a high amount of cases (70%) [92]. In 2012, Prantera et al. performed a clinical trial comparing the twice-daily rifaximin administration of 400 mg, 800 mg, and 1200 mg versus placebo. This trial demonstrated that the administration of rifaximin 800 mg twice daily for 3 months was able to induce clinical remission of moderately active CD [93]. Surprisingly, the administration of rifaximin 1200 mg and 400 mg twice daily for 3 months had no significant higher induction of clinical CD remission versus placebo [93]. The treatment resulted safe and well tolerated by all patients. Furthermore, a similar trial on active, moderate CD reported significantly higher rates of remission after 12 weeks of treatment among patients receiving rifaximin 800 mg twice daily versus placebo. Thus, rifaximin may be used as an adjunct to standard therapy, although the authors did not address the surprisingly high clinical remission rates observed in both the rifaximin and placebo groups [94].

Differently from CD, data on the efficacy of rifaximin in UC are anecdotal [95]. In particular, in 1999, a clinical trial with rifaximin in unresponsive-to-steroids UC patients was conducted. This trial demonstrated that 400 mg rifaximin twice daily for 10 days significantly reduced clinical symptoms and mucosal healing in those patients [96]. In 2006, Guslandi et al. conducted a small clinical trial on 30 UC patients with steroid intolerance. Rifaximin 400 mg twice daily was added for 4 weeks to the mesalamine 2.4 g daily treatment, and clinical remission was obtain in a large amount of cases [97, 98]. In addition, it has been evaluated that the combined treatment with *Saccharomyces boulardii* 500 mg daily *plus* rifaximin 400 mg daily did obtain clinical remission in all mesalamine-resistant UC patients [99].

Recently, a clinical trial has been conducted with a fully humanized anti-IL-17A monoclonal antibody in CD patients [100]. However, this trial failed in this disease but it had satisfactory results in psoriasis [101]. In fact, literature data confirmed that the overexpression in the IL-17/IL-23 axis related to the increased level in Th17 cells constitutes a favorable prognostic factor in the pathogenesis of IBDs [6, 13].

Other innovative therapies for IBDs have been developed using humanized antibodies against the cytokines overexpressed in these diseases. To date, several anti-cytokine antibodies are approved for IBD therapy, including anti-TNFs (infliximab, etanercept, adalimumab, golimumab, certolizumab, etc.), anti-IL-17 (secukinumab, brodalumab), anti-IL-12/23 p40 (ustekinumab), and IL-23 p19 (tildrakizumab) [9].

7. The Role of Rifaximin in Diverticular Disease

A better understanding of the potential role of rifaximin is derived from a series of studies conducted by our group in patients with diverticular disease. In 2009, we demonstrated that in diverticular disease, there are several modifications in T cell subpopulations, both in peripheral blood and in colonic mucosa. In particular, these patients have an increased tissue recruitment of $CD103^+$ lymphocytes [102]. These cells are characteristic of the intestinal homing, because they typically move from the peripheral blood to the gut mucosa [70].

It has been now well established that in patients with diverticular disease, rifaximin is able to ameliorate clinical symptoms reducing bacterial overgrowth and related mucosal chronic inflammation. Indeed, gut $CD103^+$ lymphocytes are reduced after 2 months of rifaximin treatment. This is the first demonstration that rifaximin has the ability to not only modify gut microbiota and inhibit tissue inflammation but may also directly modulate T cell circulation and mucosal immunity. In 2014, we demonstrated that the number of TLR2 and TLR4 lymphocytes both in peripheral blood and in sigmoid mucosa is significantly altered in diverticular

patients relative to controls [103]. An increased number of TLR2 and TLR4 cells in the peripheral blood of diverticular patients indicates an increase in activated circulating T cells. Moreover, after *placebo*, we demonstrated that the number of TLR2 and TLR4 lymphocytes increased. This evidence indicates that TLRs are indirect markers of bacterial overgrowth. These data reveal that rifaximin may act limiting bacterial overgrowth and then reducing the related TLR activation. TLRs mediate the activation of both innate and adaptive immune response and may also activate the proinflammatory transcriptional factor NF-κB [43]. NF-κB has a key role in the development of immune response against pathogenic *bacteria*. In fact, NF-κB is associated with a transcription and secretion of a Th1 proinflammatory cytokine pattern [43]. In this way, these data demonstrate the effective role of rifaximin in modulating local and systemic TLR expression and T cell circulation and further confirm the well-established anti-inflammatory properties of this drug in addition to its antibacterial action [72]. Therefore, considering its multiple activities, rifaximin could be redefined as a "eubiotic" agent acting as a gut microenvironment modulator (Table 1).

Table 1: The beneficial effects of the interaction between gut microbiota and immunological niche: the role of rifaximin as a "eubiotic" agent.

Rifaximin may act on both innate and adaptive immune cells and has a role on both mucosal and systemic immunity. Thus, it may have 3 levels of action	
(1) Gut microbiota	Positively selecting commensal gut microbial communities: (i) Increasing the proliferation and growth of the protective *Lactobacilli* (ii) Inhibiting the proliferation of several pathobionts, including *Clostridia* and *Firmicutes*
(2) Inflammation	Inhibiting the PXR-induced transcription of NF-κB proinflammatory-related genes, such as TNF-alpha and IL-1beta
(3) Mucosal and systemic immunity	(i) Reducing TLR activation (ii) Interfering with T cell circulation and gut homing of $CD103^+$ lymphocytes and inhibiting proinflammatory T cells, such as Th1 and Th17 cells

8. Conclusions

Inflammatory bowel pathologies are a heterogeneous group of diseases characterized by various degrees of inflammation involving the gut mucosa. Several mediators of inflammation are involved in their pathogenesis, such as T cell subpopulations and their related pro- and anti-inflammatory cytokines, TLRs, and the pathogen/commensal ratio. A strong interaction among these factors has been well evaluated. In fact, on the one hand, gut microbiota may strictly modulate mucosal immunity, and on the other hand, mucosal immunity may influence the composition of gut microbiota. This complex interaction between gut microbiota and mucosal immunity is also mediated by other several factors that participate in the inflammatory pathways.

Inflammatory bowel pathologies are shared by the disruption of the physiological homeostasis present in the gut mucosa. First, the alteration in the pathogen/commensal ratio precipitates in the pathological condition of dysbiosis. During dysbiosis, the normal physiology of the gut mucosa is altered and there is bacterial translocation from the lumen to systemic circulation due to the leaky gut condition. In this pathological *scenario*, there is a dysregulation in the cytokine production, with the proliferation of proinflammatory T cell subsets and inhibition of anti-inflammatory ones. All these complex interactions among T cell subpopulations, gut microbiota, and the mediators of inflammation occur in the anatomical subset of the gut mucosa, which we have defined as "immunological niche."

Several evidences have shown that xenobiotics may positively modulate the gut immunological niche. Most specifically, xenobiotics may interfere with components of the immunological niche, leading to an activation of anti-inflammatory pathways, and inhibition of several inflammatory mediators. As a result, xenobiotics may reduce disease-related gut mucosal alterations and clinical symptoms.

In summary, while further research is warranted, the complex interplay between gut immunological niche and xenobiotics has the potential to open new horizons in our knowledge of inflammatory bowel pathologies and their treatment.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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Clinical Study

Evidences of a New Psychobiotic Formulation on Body Composition and Anxiety

Carmela Colica,[1] Ennio Avolio,[2] Patrizio Bollero,[3] Renata Costa de Miranda,[4,5] Simona Ferraro,[6] Paola Sinibaldi Salimei,[7] Antonino De Lorenzo,[7] and Laura Di Renzo[7]

[1]*CNR, IBFM UOS of Germaneto, University "Magna Graecia" of Catanzaro, Campus "Salvatore Venuta", Germaneto, 88100 Catanzaro, Italy*
[2]*Comparative Neuroanatomy Laboratory of Ecology Department, University of Calabria, Ponte Pietro Bucci, Arcavacata di Rende, 87030 Cosenza, Italy*
[3]*Department of Systems Medicine, University of Rome Tor Vergata, Via Montpellier 1, 00133 Rome, Italy*
[4]*PhD School of Medical-Surgical Applied Sciences, University of Rome Tor Vergata, Via Montpellier 1, 00133 Rome, Italy*
[5]*CAPES Scholarship (Proc No. BEX 13264/13-3), CAPES Foundation, Ministry of Education of Brazil, 70040-020 Brasília, DF, Brazil*
[6]*Health Center Srl, Biomedical and Nutritional Center, 87100 Cosenza, Italy*
[7]*Section of Clinical Nutrition and Nutrigenomic, Department of Biomedicine and Prevention, University of Rome Tor Vergata, Via Montpellier 1, 00133 Rome, Italy*

Correspondence should be addressed to Laura Di Renzo; laura.di.renzo@uniroma2.it

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Background. Gut microbiota is implied in obesity, because of its ability to harvest energy from diet, and in the regulation of behavior. Given the link between gut microbiota, body composition, obesity, and anxiety, the aim of this study was to evaluate the effects of a new psychobiotic formulation. *Methods*. Eligible patients were randomly divided into three groups: psychobiotics oral suspension group (POSG); dietary treatment group (DTG); combined treatment group (CTG). All subjects underwent body composition and psychological profile evaluation. *Results*. Significant changes in body composition parameters in each group were relieved after all treatments. Hamilton anxiety rating scale (HAM-A) highlighted a significant reduction of the total score for all study population after treatments in POSG ($p = 0.01$) and CTG ($p = 0.04$). A reduction of HAM-A total score in anxious subjects in POSG or CTG and a significant reduction of positive subjects for HAM-A in POSG ($p = 0.03$) and in CDG ($p = 0.01$) were shown. *Discussion*. Three-week intake of selected POS represents a good approach to solve problems related to obesity and behavior disorders. However, new clinical trials need to be performed on a larger population and for a longer period of treatment before definitive conclusions can be made. This trial is registered with NCT01890070.

1. Introduction

Gut microbiota is an ensemble of 100 trillion microorganisms present in the gastrointestinal tract (GI), which belong to more than 1000 species and 700 strains [1], and plays a crucial role in human's physiology, due to its ability to maintain energy homeostasis and stimulate immunity as an endocrine organ, in a symbiotic relationship with the host [2]. Several factors like genetics, diet, infection, drug consumption, age, and sex could influence the nature of gut microbiota, both temporarily and definitely. An external change of its composition could induce a dramatic variation on the hosts' health [3, 4].

A growing body of evidence demonstrated the possible involvement of gut microbiota in fat mass accumulation and cardiometabolic disease onset. The World Health Organization described obesity as a disorder with an excessive body fat accumulation, abandoning the old definition, which restricted this condition to the simple body mass index (BMI) classification, to embrace a broader range of phenotypes,

including subjects within the normal BMI range but with a critical percentage of body fat mass [5–10]. Obesity condition seems to be mostly caused by the obesogenic environment [11], which consists of a series of bad lifestyle habits from the disproportionate intake of calories, especially from simple carbohydrates, and the reduction of physical activity. Moreover, obese subjects could present some behavioral disorders [12], and some genetic profiles seem to be associated with the body weight regulation [13–16].

Gut microbiota seems to be implicated in obesity onset given its ability to harvest energy from the diet, through its influence on gut epithelium and motility [17], and increase triglyceride storage in the host adipocytes, inhibiting the fasting-induced adipose factors [18]. Furthermore, gut microbiota influences several metabolic processes such as lipogenesis, fatty acid oxidation, triglycerides, and cholesterol production [19]. Numerous *in vivo* studies observed that microbiota plays a strong role in adipose tissue accumulation. In fact, germ-free mice, although they ingest more calories than their littermates, result leaner than conventional mice. At the same time, microbiota transplantation from conventional to germ-free mice highlights a dramatic increase in body fat, triglyceride production, and insulin resistance, without changing their food habits [17, 20, 21].

In recent years, researchers focused their attention on the relationship between gut microbiota and brain development and function, discovering a bidirectional communication pathway between them, defined as microbiota-gut-brain axis.

Several studies highlighted the role of microbiota in the regulation of mood and behavior, like stress, anxiety, depression, and autism [22–24], as well as the potential therapeutic effects deriving from its modulation.

Due to the psychotropic effects in animal models and human clinical trials, the term "psychobiotics" was introduced [25]. In fact, it has been demonstrated that treatment with probiotics formulation, containing *Lactobacillus acidophilus*, *Lactobacillus casei*, and *Bifidobacterium bifidum*, showed positive effects on psychological distress [26, 27].

In vivo studies observed a relation between altered gut microbiota composition and anxiety related behavior, with increased exploration of aversive zones and improved serotoninergic function in germ-free mice when compared to specific pathogen-free counterparts [24, 28, 29].

Moreover, the anxiety-like behavior increases during pathogen infection and GI inflammation in animal models [30].

At the same time, anxiety and other psychological disorders seem to be related to body composition and obesity [31]. Several studies observed learning, memory, and function deficits in obese subjects, linking obesity to the exacerbation of depression and anxiety disorders [32–35]. Vice versa, depression disorders have a strong positive association with eating behaviors and fat mass, especially in subjects who do not follow a Mediterranean-like eating pattern [36].

Up today, very few studies demonstrated the beneficial effects of psychobiotics on the health status of obese subjects. An improvement of psychosocial behavior was seen in subjects with a fat mass surplus that underwent weight loss dietary treatments [37].

Given the link among gut microbiota, body composition, obesity, and the risk of developing anxiety, the aim of this study was to evaluate the differences deriving from the combination of 3-week administration of a new psychobiotic formulation, (psychobiotics oral suspension, POS) with or without dietary treatment (DT), consisting of a hypocaloric diet, on general population.

The evaluation was performed based on anthropometric, bioimpedance analysis (BIA), dual X-ray absorptiometry (DXA) measurements, and anxiety assessment with Hamilton anxiety rating scale (HAM-A).

2. Methods

2.1. Study Design and Subjects. This research was conducted using a prospective intervention study design, between January 2017 and April 2017. Forty-five subjects were recruited sequentially within a routine medical checkup program at the Section of Clinical Nutrition and Nutrigenomics, Department of Biomedicine and Prevention of the University of Rome "Tor Vergata." POS was administrated 1 time/day, 2 h before lunch in order to ensure gastrointestinal transit and absorption.

Eligible patients were randomly divided into three groups (1 : 1 : 1 ratio): (1) psychobiotics oral suspension group (POSG), subjects took daily note 1 bag of 3 g of POS, and they did not change their ordinary diet; (2) dietary treatment group (DTG), subjects followed a hypocaloric diet; (3) combined treatment group (CTG), subjects followed the hypocaloric diet and took daily note 1 bag of 3 g of POS. Each group followed the assigned treatment consecutively for 3 weeks. At the beginning and at the end of each treatment, body composition evaluation and psychodiagnostic tests were performed.

Subjects were asked to maintain their usual lifestyle habits and to report any illness or abnormality arisen during the study.

The primary outcome of this study was the evaluation of nutritional status according to body composition changes measured by anthropometry, BIA, and DXA, due to the different treatments. The secondary outcome was the evaluation of anxiety disorder through the HAM-A test, pre- and posttreatment each.

All participants recruited in the study authorized their participation by reading and signing the informed consent, drafted in accordance with the provisions of the Ethics Committee of Medicine, University of Rome Tor Vergata and with the Helsinki Declaration of 1975 as revised in 1983. This trial is registered with NCT01890070, http://www.ClinicalTrials.gov.

2.2. Exclusion Criteria. Exclusion criteria included age < 20 y or >75 y, pregnancy, breastfeeding, type 1 diabetes, presence of intestinal bacterial overgrowth, characterized by high levels of hydrogen and methane production in the small bowel, acute diseases, endocrine disorders, liver, heart or kidney dysfunctions, history of chronic medication, antibiotic therapy up to ten days before enrollment, smoke, drug or alcohol abuse, and participation in another diet trial. No

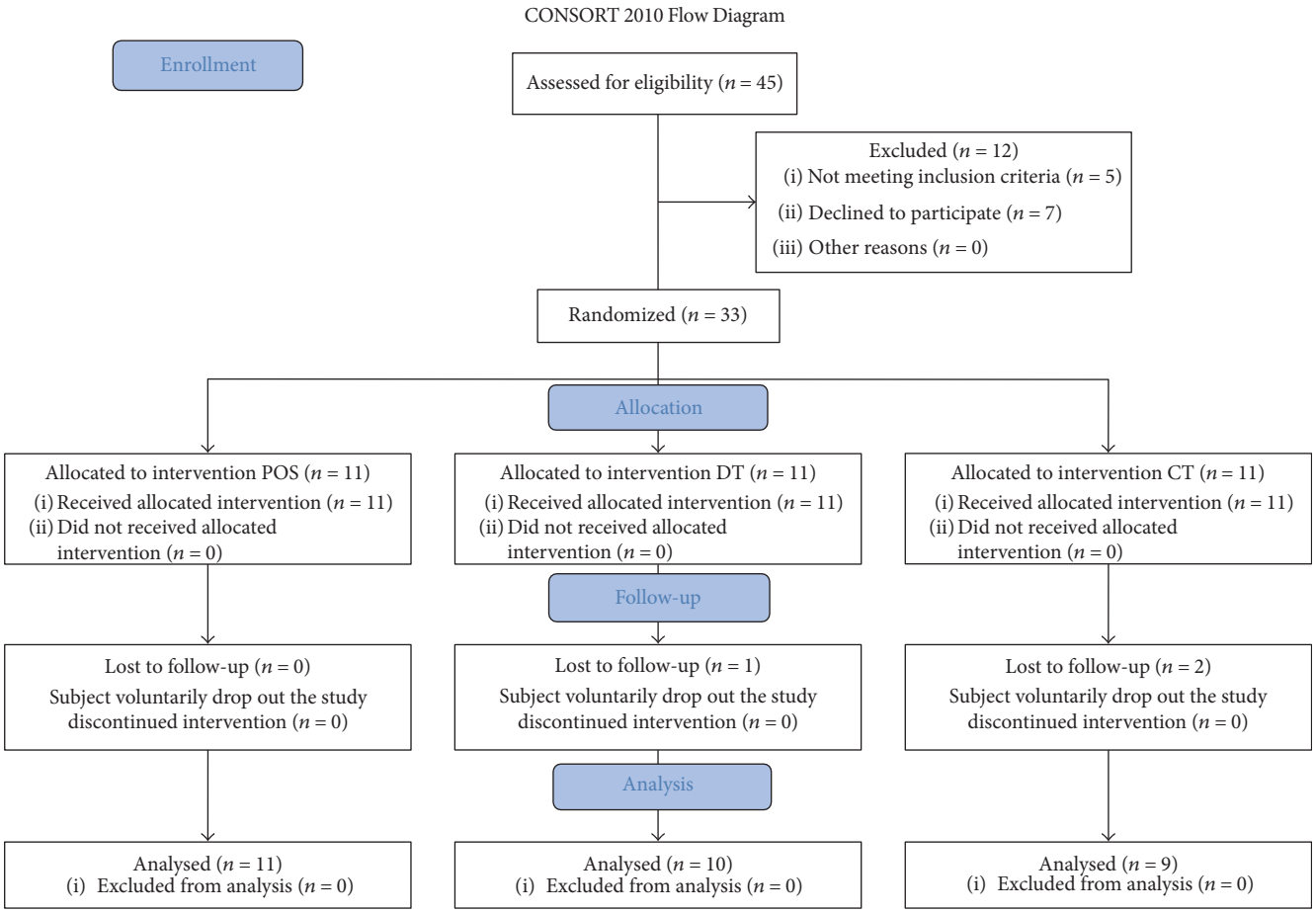

Figure 1: Study flow diagram according to Consort, 2010.

subjects with known alterations of intestinal transit following organic pathologies (abdominal surgery, diabetes mellitus, scleroderma, hypothyroidism, etc.) were included in the study. Subjects were advised not to consume any antibiotics or probiotics for the month prior to study initiation and to avoid using it for all the duration of the study.

2.3. Psychobiotics Oral Suspension Composition. 1 bag of POS of 3 g contained: 1.5×10^{10} colony-forming unit CFU of *Streptococcus thermophilus* (CNCM strain number I-1630), 1.5×10^{10} colony-forming unit CFU of *Lactobacillus bulgaricus* (CNCM strain numbers I-1632 and I-1519); 1.5×10^{10} colony-forming unit CFU of *Lactococcus lactis* subsp. *lactis* (CNCM strain number I-1631); 1.5×10^{10} colony-forming unit CFU of *Lactobacillus acidophilus*; 1.5×10^{10} colony-forming unit CFU of *Streptococcus thermophiles*; 1.5×10^{10} colony-forming unit CFU of *Lactobacillus plantarum*; 1.5×10^{10} colony-forming unit CFU of *Bifidobacterium lactis* (CNCM I-2494); $1.5 \times 1\ 10^{10}$ colony-forming unit CFU of *Lactobacillus reuteri* (DSM 17938), maltodextrin from corn, anticaking agent (silica), casein, lactose, and gluten < 3 ppm LLOQ (lower limit of quantitation), (Biocult strong, HOMEOSYN, Rome, Italy).

2.4. Anthropometric Analysis. According to the International Society for the Advancement of Kinanthropometry protocol and National Institute of Health Guidelines, waist circumference (WC) and hip circumference (HC) were taken using a flexible steel metric tape to the nearest 0.5 cm. Body weight (Kg) was measured to the nearest 0.1 Kg, using a technical balance (Invernizzi, Rome, Italy). Waist/hip ratio (WHR) was also evaluated in relation to clinical risk thresholds, that is, WHR > 1 for men and WHR > 0.9 for women. Height (m) was measured to the nearest 0.1 cm using a stadiometer (Invernizzi, Rome, Italy). BMI was calculated using the formula: BMI = body weight/height^2 (Kg/m^2).

2.5. Bioelectrical Impedance Analysis (BIA). Resistance, reactance, impedance, phase angle, total body water (TBW), intracellular water (ICW), and extracellular water (ECW) were assessed by BIA phase sensitive system (BIA 101S, Akern/RJL Systems, Florence, Italy) [38, 39]. Impedance index (II) was evaluated with the following formula [40]:

$$\mathrm{II} = \frac{\mathrm{height}^2\ (\mathrm{cm}^2)}{\mathrm{resistance}\ (\Omega)}. \quad (1)$$

Measurements were taken according to Di Renzo et al. [39].

2.6. Dual X-Ray Absorptiometry (DXA). Body composition analysis was assessed by DXA (i-DXA, GE Medical Systems,

Milwaukee, WI, USA) according to the previously described procedures [12].

Total body fat (TBFat), total body lean (TBLean), android body fat (ABF), and gynoid body fat (GBF) were expressed as a percentage (%) of the total body mass. TBFat percentage was estimated by the ratio between the TBFat (Kg) and the sum of TBFat (Kg), TBLean (Kg) and bone mineral content (BMC) (Kg) multiplied by 100. The intermuscular adipose tissue (IMAT) was evaluated according to Bauer et al. [41].

2.7. Dietary Intervention. The energy intake for DT was calculated based on basal metabolic rate (BMR) of each subject, with Weir's formula: $\mathrm{BMR} = [(3.94 \times \mathrm{VO2}) + (1.106 \times \mathrm{VCO2})] \times 1.44$ VO2, where VO2 is the volume of oxygen uptake (mL/min), estimated by the following formulas:

$$\begin{aligned} \mathrm{VO_2\ \ woman} &= \mathrm{TBLean\ \ DXA} \times 4.5, \\ \mathrm{VO_2\ \ man} &= \mathrm{TBLean\ \ DXA} \times 5.3, \end{aligned} \tag{2}$$

and VCO2 is the volume of carbon dioxide output (mL/min), evaluated with the following formula:

$$\mathrm{VCO_2} = \mathrm{VO_2} \times 0.85. \tag{3}$$

Protein intake was determined considering 2 g of protein/Kg of TBLean, representing 21–26% of daily caloric intake. Carbohydrate intake was between 44% and 51% of total energy intake, and fat intake was between 27% and 31% of daily caloric intake (<10% of saturated fatty acids, <300 mg/day of cholesterol). The fiber intake was 30 g/day [42].

2.8. Psychodiagnostic Instruments. Hamilton anxiety rating scale (HAM-A) was administered by instructed physicians and was used to measure the severity of anxiety symptoms. The 14 items on the scale define several symptoms and measure psychic and somatic anxiety. Each item has a score from 0 to 4, respectively, from the absence to the severe presence of the related symptom. The total score, which has a range from 0 to 56, describes three different scenarios: <17 indicates mild anxiety severity, 18–24 from mild to moderate anxiety severity, and 25–30 from moderate to high anxiety severity. In this work, we considered anxious subjects those who had a score higher than or equal to 18 [43, 44].

2.9. Statistical Analysis. Nonparametric tests for asymmetrically distributed data were conducted in all analyses and presented as median (minimum and maximum). Kruskal Wallis test was carried out to compare the three groups at baseline. To evaluate differences before and after 21 days of treatment, Wilcoxon test was performed in each group. To describe, quantitatively, variable change after treatments, we used a ratio of the absolute variation to the baseline value (percent variation = Δ%). Categorical variables were compared among groups by Chi-square (χ^2) or Fisher's test. McNemar test was used for the comparison between groups at baseline (T0) and after treatment (T1). Statistical analyses were carried out using IBM SPSS 21.0 for Windows (Armonk, NY: IBM Corp. USA). In all statistical tests performed, the null hypothesis was rejected at the 0.05 level of probability.

Table 1: Overall description of anthropometric, body composition, and anxiety data.

	Overall $n = 30$ Median (min–max)
Age (years)	45.00 (21.00–72.00)
Weight (Kg)	77.75 (50.30–121.00)
Height (cm)	165.00 (150.00–186.00)
BMI (Kg/m^2)	26.87 (20.12–39.93)
WC (cm)	89.25 (69.50–122.50)
HC (cm)	108.50 (85.50–132.00)
WHR	0.82 (0.69–1.09)
Phase angle (°)	7.05 (5.10–930)
II (cm^2/Ω)	53.97 (39.35–94.28)
TBFat (Kg)	28.45 (14.03–44.64)
ABFat (Kg)	2.50 (0.70–4.38)
GBFat (Kg)	5.20 (2.72–7.70)
IMAT	1.21 (0.46–2.04)
TBLean (Kg)	43.23 (35.63–58.13)
HAM-A (points)	11.00 (0.00–30.00)

All results were expressed as median (minimum–maximum). BMI: body mass index; WC: waist circumference; HC: hip circumference; WHR: waist-to-hip ratio; II: impedance index; TBFat: total body fat; ABFat: android body fat; GBFat: gynoid body fat; IMAT: intermuscular adipose tissue; TBLean: total body lean; HAM-A: Hamilton anxiety rating scale (negative test (nonanxious subjects) if total score < 18 and positive test (anxious subjects) if total score ≥ 18).

3. Results

Out of forty-five subjects recruited, twelve were excluded from the trial: five did not meet inclusion criteria, seven declined to participate, and other three subjects voluntarily stopped the treatment (Figure 1).

During the trial, three subjects dropped out of the study and, finally, thirty patients between 21 and 72 years old with a BMI between 18.5 and 39.9 Kg/m^2 and without metabolic complications met the inclusion criteria and completed the trial. No changes to trial outcomes occurred after it commenced. The median age of subjects was 45 years, 83.3% female and 16.7% male (Table 1). At baseline, no statistical significance was observed between groups (Table 2).

Significant changes in body composition parameters in each group were relieved after all treatments. In fact, POSG and CTG showed a significant reduction in II ($p = 0.03$ and $p = 0.01$, resp.), whereas both DTG and CTG highlighted a significant reduction in weight ($p = 0.01$), BMI ($p = 0.01$), waist circumference ($p = 0.01$ and $p = 0.04$, resp.), TBFat (Kg) ($p = 0.03$ and $p = 0.04$, resp.), and IMAT ($p = 0.03$; $p = 0.04$, resp.). Significant reduction in hip circumference ($p = 0.02$) and TBLean (Kg) ($p = 0.02$) were observed only in DTG, whereas waist/hip ratio ($p = 0.04$), PA ($p = 0.02$), ABFat (Kg) ($p = 0.04$), and GBFat (Kg) ($p = 0.04$) parameters were significantly reduced in CTG (Table 3).

The HAM-A test performed on POSG and CTG highlighted a significant reduction in the total score for all study population after both treatments ($p = 0.01$ and

Table 2: Anthropometric, body composition, and anxiety analysis of the 3 groups.

	POSG $n = 11$ Median (min–max)	DTG $n = 10$ Median (min–max)	CTG $n = 9$ Median (min–max)	p
Age (years)	42.00 (26.00–65.00)	36.50 (21.00–72.00)	51.00 (44.00–72.00)	0.12
Weight (Kg)	67.80 (50.30–111.80)	77.00 (62.20–99.00)	79.50 (57.20–121.00)	0.45
Height (cm)	166.00 (155.00–186.00)	166.50 (150.00–173.00)	165.00 (161.00–185.00)	0.81
BMI (Kg/m^2)	25.11 (20.12–39.45)	28.60 (26.10–38.60)	29.20 (22.07–39.93)	0.10
WC (cm)	77.00 (69.50–121.00)	94.25 (85.00–106.50)	90.00 (74.00–122.50)	0.27
HC (cm)	104.50 (85.50–132.00)	108.00 (102.00–120.00)	112.25 (89.00–132.00)	0.27
WHR	0.77 (0.69–1.05)	0.86 (0.80–0.94)	0.82 (0.71–1.09)	0.25
Phase Angle (°)	6.50 (4.60–10.20)	7.05 (5.10–9.30)	6.90Z (4.90–9.00)	0.71
II (cm^2/Ω)	61.54 (39.35–69.05)	57.12 (42.79–82.53)	52.76 (45.40–94.28)	0.91
TBFat (Kg)	23.29 (14.03–24.57)	29.52 (23.00–44.64)	34.39 (15.05–44.19)	0.13
ABFat (Kg)	1.36 (0.70–2.40)	2.60 (1.29–4.23)	2.53 (0.82–4.38)	0.17
GBFat (Kg)	3.90 (2.97–4.95)	5.70 (4.35–7.70)	5.86 (2.72–7.05)	0.25
IMAT	0.89 (0.46–2.00)	1.22 (0.87–1.79)	1.47 (0.50–2.04)	0.25
TBLean (Kg)	35.88 (34.43–51.36)	43.23 (35.63–58.13)	41.58 (39.59–54.96)	0.35
HAM-A (points)	11.00 (3.00–28.00)	13.50 (7.00–30.00)	6.00 (00.00–23.00)	0.06

All parameters were evaluated at baseline among the 3 groups, by Kruskal Wallis test. All results were expressed as median (minimum–maximum). Statistical significance attributed to results with $^*p < 0.05$. POSG: psychobiotics oral suspension group; DTG: dietary treatment group; CTG: combined treatment group; BMI: body mass index; WC: waist circumference; HC: hip circumference; WHR: waist-to-hip ratio; II: impedance index; TBFat: total body fat; ABFat: android body fat; GBFat: gynoid body fat; IMAT: intermuscular adipose tissue; TBLean: total body lean; HAM-A: Hamilton anxiety rating scale (negative test (nonanxious subjects) if total score < 18 and positive test (anxious subjects) if total score ≥ 18).

$p = 0.04$, resp.). No significant difference was seen in the HAM-A of DTG (Table 3). However, according to the total score, the sample was divided into anxious (total score ≥ 18) and nonanxious subjects (total score< 18) within each group. At baseline, no statistical significance was observed between POSG, DTG, and CTG for anxious and nonanxious subjects ($p = 0.06$). A notable reduction in the HAM-A total score in anxious subjects that had undergone the POS or combined treatment ($\Delta = -5$ points and $\Delta = -9.5$ points, resp.) was highlighted, while anxious DTG patients had HAM-A total score increased (Figure 2).

Table 4 shows absolute numbers of anxious and nonanxious subjects in the 3 groups, before and after treatment. Furthermore, a significant reduction in the number of anxious subjects was observed in POSG ($p = 0.03$; $\Delta\% = -39.3\%$), as well as in CTG, where all anxious subjects became nonanxious ($p = 0.01$; $\Delta\% = -100\%$) (Figure 3).

4. Discussion

Neurotransmitters and neuromodulators, secreted by bacteria, are able to modulate the state of the hosts' mood: gamma-aminobutyric acid is produced by certain *Lactobacillus* and *Bifidobacterium* species; norepinephrine is released by *Escherichia*, *Bacillus*, and *Saccharomyces* spp.; 5-hydroxytryptamine is released by *Candida*, *Streptococcus*, *Escherichia*, and *Enterococcus* spp.; and dopamine is produced by *Bacillus* and acetylcholine by *Lactobacillus* [45].

Fat mass increase in humans is related to several environmental factors, especially bad lifestyle habits, like sedentary living and excess of daily caloric, carbohydrate, and fat intake [11]. It is well known that the increase of body fat mass represents a strong risk factor for the development of metabolic and cardiovascular diseases [46–48].

Gut microbiota could play an essential role in fat mass increase and obesity development by invading the intestinal mucosa and causing systemic inflammation. On the other hand, the integrity of the intestinal barrier and a healthy intestinal microflora induce an anti-inflammatory effect that causes a consequent reduction in fat mass body composition [49].

Gut microbiota has been studied for decades in order to evaluate its impact on different aspects of human health and body composition [18–20], and recently, the role of probiotics with or without diet has been evaluated in terms of changing the overall health status [50, 51], body weight, body composition, and obesity [52, 53].

In our study, we enrolled normal weight, preobese, and obese up to the second-degree patients based on BMI, and, at the same time, we performed DXA to evaluate body composition. At baseline, the population resulted homogeneous in the three groups for the studied variables as reported in Table 2. On average, the population chosen had a TBFat percentage over 30%, who are considered obese according to De Lorenzo et al. [54].

The statistical comparison among the three groups exhibited that the subjects who belong to POSG did not report significant differences between time T0 and T1, in weight, waist and hip circumference, and body composition, except for the II ($p = 0.03$, $\Delta\% = -1.92\%$). In accordance with the literature [55], the DTG demonstrated significant reductions in weight ($p = 0.01$; $\Delta\% = -2.27\%$),

Table 3: Comparison between before and after treatment in each group.

	POSG ($n = 11$)			DTG ($n = 10$)			CTG ($n = 9$)		
	T0 Median (min–max)	T1 Median (min–max)	p	T0 Median (min–max)	T1 Median (min–max)	p	T0 Median (min–max)	T1 Median (min–max)	p
Weight (Kg)	67.80 (50.30–111.80)	66.30 (49.70–107.90)	0.39	77.00 (62.20–99.00)	75.25 (60.50–93.30)	0.01*	79.50 (57.20–121.00)	76.50 (56.10–119.50)	0.01*
BMI (Kg/m^2)	25.11 (20.12–39.45)	24.75 (20.12–38.52)	0.39	28.60 (26.10–38.60)	27.57 (23.90–35.80)	0.01*	29.20 (22.07–39.93)	28.10 (21.64–38.58)	0.01*
WC (cm)	77.00 (69.50–121.00)	78.00 (68.00–121.00)	0.07	94.25 (85.00–106.50)	88.50 (80.00–100.00)	0.01*	90.00 (74.00–122.50)	86.50 (71.00–115.00)	0.04*
HC (cm)	104.50 (85.50–132.00)	105.00 (87.00–121.50)	0.40	108.00 (102.00–120.00)	104.50 (96.00–116.00)	0.02*	112.25 (89.00–132.00)	109.50 (88.00–119.00)	0.17
WHR	0.77 (0.69–1.05)	0.76 (0.66–1.05)	0.28	0.86 (0.80–0.94)	0.84 (0.76–0.93)	0.09	0.82 (0.71–1.09)	0.81 (0.74–1.00)	0.04*
Phase angle (°)	6.50 (4.60–10.20)	6.30 (4.90–8.60)	0.15	7.05 (5.10–9.30)	6.50 (4.70–8.60)	0.06	6.90 (4.90–9.00)	5.70 (5.10–6.60)	0.02*
II (cm2/Ω)	51.52 (39.35–69.05)	50.53 (39.35–66.92)	0.03*	57.12 (42.79–82.53)	53.43 (40.99–76.08)	0.06	55.11 (45.40–94.28)	51.93 (43.42–91.02)	0.01*
TBFat (Kg)	23.29 (14.03–24.57)	22.70 (14.43–25.50)	0.59	29.52 (23.00–44.64)	29.13 (22.76–40.33)	0.03*	34.39 (15.05–44.19)	28.18 (13.60–43.05)	0.04*
ABFat (Kg)	1.36 (0.70–2.40)	1.24 (0.67–2.33)	0.11	2.60 (1.29–4.23)	2.65Z (1.23–3.60)	0.07	2.53 (0.82–4.38)	1.81 (0.61–4.23)	0.04*
GBFat (Kg)	3.90 (2.97–4.95)	3.91 (2.90–4.87)	0.28	5.70 (4.35–7.70)	4.90 (4.10–7.77)	0.07	5.86 (2.72–7.05)	5.20 (2.43–6.76)	0.04*
IMAT	0.89 (0.46–2.00)	0.85 (0.47–1.18)	0.59	1.22 (0.87–1.79)	1.20 (0.86–1.69)	0.03*	1.47 (0.50–2.04)	1.15 (0.44–1.72)	0.04*
TBLean (Kg)	35.88 (34.43–51.36)	35.87 (35.39–50.63)	1.00	43.23 (35.63–58.13)	42.61 (35.62–57.21)	0.02*	41.58 (39.59–54.96)	40.42 (39.51–54.11)	0.22
HAM-A	11.00 (3.00–28.00)	7.00 (1.00–28.00)	0.01*	13.50 (7.00–30.00)	10.00 (6.00–31.00)	0.72	6.00 (00.00–23.00)	5.00 (0.00–19.00)	0.04*

All parameters were evaluated before and after treatments by Wilcoxon test. All results were expressed as median (minimum–maximum). Statistical significance attributed to results with $^*p < 0.05$ between T0 and T1. POSG: psychobiotics oral suspension group; DTG: dietary treatment group; CTG: combined treatment group; BMI: body mass index; WC: waist circumference; HC: hip circumference; WHR: waist-to-hip ratio; II: impedance index; TBFat: total body fat; ABFat: android body fat; GBFat: gynoid body fat; IMAT: intermuscular adipose tissue; TBLean: total body lean; Ham-A: Hamilton anxiety rating scale (negative test (nonanxious subjects) if total score < 18 and positive test (anxious subjects) if total score ≥ 18).

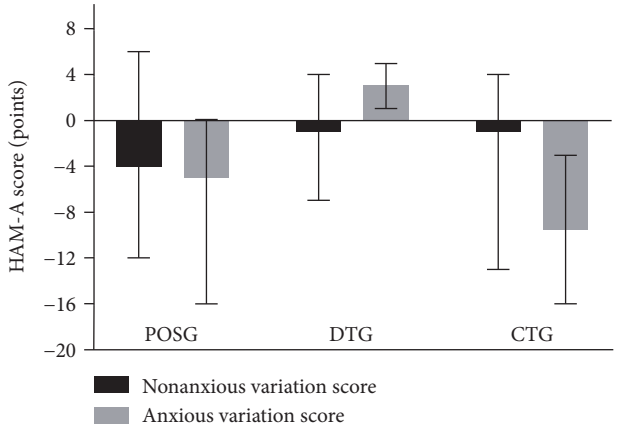

Figure 2: Hamilton anxiety rating scale (HAM-A) score variation before and after treatments in anxious and nonanxious subjects. Nonanxious subjects (negative test) if total score < 18 and anxious subjects (positive test) if total score ≥ 18. Variation score is shown as median, minimum, and maximum. 609 statistical significance attributed to results with $p < 0.05$ by Kruskal Wallis test. Anxious variation score among groups: $p = 0.10$ and nonanxious variation score among groups: $p = 0.67$. POSG: psychobiotics oral suspension group; DTG: dietary treatment group; CTG: combined treatment group.

Table 4: Absolute numbers of anxious and nonanxious subjects in all groups, before and after treatment.

HAM-A score	POSG $n = 11$		DTG $n = 10$		CTG $n = 9$	
	T0	T1	T0	T1	T0	T1
HAM-A < 18	7	9	8	7	8	9
HAM-A ≥ 18	4	2	2	3	1	0

Frequency of anxiety subjects was evaluated before and after treatment in POSG, DGT, and CTG. Negative test (nonanxious subjects) if total score < 18 and positive test (anxious subjects) if total score ≥ 18. HAM-A: Hamilton anxiety rating scale; POSG: psychobiotics oral suspension group; DTG: dietary treatment group; CTG: combined treatment group.

BMI ($p = 0.01$; $\Delta\% = -3.60\%$), waist circumference ($p = 0.01$; $\Delta\% = -6.10\%$), hip circumference ($p = 0.02$, $\Delta\% = -3.24\%$), TBFat (Kg) ($p = 0.03$, $\Delta\% = -.32\%$), IMAT ($p = 0.03$; $\Delta\% = -1.64\%$), and TBLean (Kg) ($p = 0.02$, $\Delta\% = -1.64$).

In the CTG, a higher variation of TBFat loss ($p = 0.04$, $\Delta\% = -18.06\%$) and a statistically significant difference in the reduction of android ($\Delta\% = -28.46\%$) and gynoid fat mass ($\Delta\% = -11.46\%$) ($p = 0.04$) were highlighted. This preliminary data underlines the role of probiotics as a supplement for diet, as described by Kim et al. [56]. The 21-day period treatment is probably not enough to point out the positive effect of probiotic treatment alone on the improvement of weight and body composition, taking into account that most pharmacological treatments for obesity have a duration of at least three months, and in all cases, it is advised that they are coupled with lifestyle changes [57].

IMAT was significantly reduced in subjects treated only with diet ($p = 0.03$, $\Delta\% = -1.64\%$), whereas the group under diet with probiotic intake showed a greater IMAT reduction

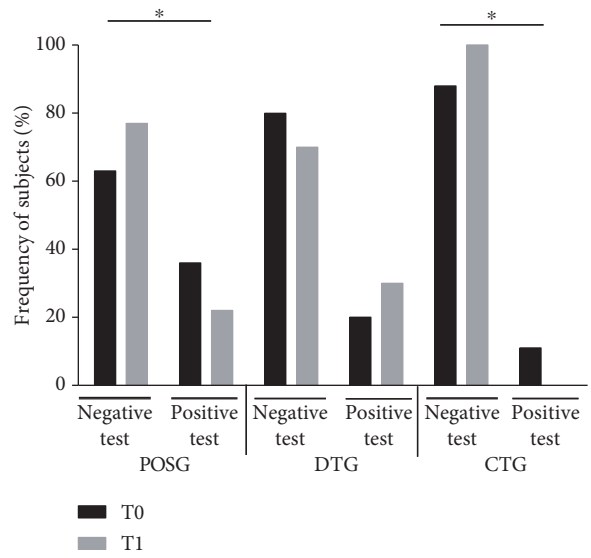

Figure 3: Frequency change of anxious subjects in POSG, DTG, and CTG after treatment. Frequency of anxiety was evaluated before and after treatment in POSG, DTG, and CTG. Negative test (nonanxious 619 subjects) if total score < 18 and positive test (anxious subjects) if total score ≥ 18. Statistical significance attributed to results with $^{*}p < 0.05$ between T0 and T1 by McNemar test. POSG: $p = 0.03^{*}$; DTG: $p = 0.10$; CTG: $p = 0.01^{*}$. POSG: psychobiotics oral suspension group; DTG: dietary treatment group; CTG: combined treatment group.

($p = 0.04$, $\Delta\% = -21.77\%$). This result is the first evidence reported in the literature, and we speculate that it could be attributed to the capacity of probiotics to decrease the intestinal permeability with a consequent reduction of lipopolysaccharide and inflammatory cytokine levels [58].

The improvement of inflammatory state and oxidative status induced by probiotics administration [59, 60] could be able to contribute to the proper fatty acids and glucose metabolism, with the improvement of insulin resistance related to a better IMAT [61]. In CTG, the significant reduction of ABFat and waist/hip ratio, both related to insulin resistance and cardiovascular risk [62], could be due to an improvement of insulin profile.

BIA results show that POS intervention led to a significant reduction in II and, consequently, a resistance increase. The same trend was marked by Valentini Neto et al. [63], even if not significant.

However, a significant decrease of phase angle was observed in CTG, probably due to body water loss which translates into a resistance increase, despite of TBFat loss. Since in POSG we observed a phase angle reduction and a significant reduction of II, we can hypothesize a synergic action between diet and POS.

Multiple studies have demonstrated the existence of a clear link between gut microbiota and brain function. Given this, the gut microbiota appears to be a key regulator of mood and behavior [22]. Moreover, probiotics due to their effects on gut microbiota seem to have a positive impact on the management of psychological disorders, such as anxiety. In our study, POS supplementation led to a significant decrease

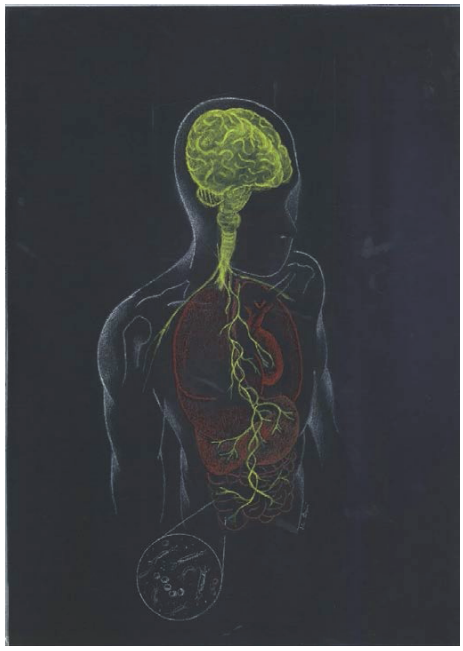

Figure 4: Gut-microbiota axis.

of HAM-A score both in POSG than in CTG. This could be attributed to the effects of POS on molecular pathways in the central nervous system, which could also act on gut microbiota [23, 24]. We did not observe any significant difference in DTG on HAM-A score. This confirms that POS might have played a role in anxiety rather than only diet.

Based on the positive anxious score (total score ≥ 18), we observed that in both groups with POS, there was a significant decrease in the number of subjects that had a positive HAM-A test, with a score improvement of 5 points in POSG and 9.5 points in CTG. These results confirm that POS supplementation is associated with a reduction of anxiety, as shown by Wang et al. [64]. However, a balanced diet, associated with POS, seems to have a greater effect on the improvement of anxiety symptoms (Figure 4).

5. Conclusions

Despite the limitations of our study, related to the study design and the low sample size, our results highlighted that a three-week intake of selected psychobiotics represents a good approach to solve the problems related to obesity and behavior disorders. However, new clinical trials need to be performed on a larger population and for a longer period of treatment before definitive conclusions can be made.

Abbreviations

BMI: Body mass index
POS: Probiotics oral suspension
DT: Dietary treatment
BIA: Bioimpedance analysis
DXA: Dual X-ray absorptiometry
HAM-A: Hamilton anxiety rating scale
POSG: Psychobiotics oral suspension group
DTG: Dietary treatment group
CTG: Combined treatment group
WC: Waist circumference
HC: Hip circumference
WHR: Waist-to-hip ratio
II: Impedance index
TBFat: Total body fat
TBLean: Total body lean
ABF: Android body fat
GBF: Gynoid body fat
BMC: Bone mineral content
IMAT: Intermuscular adipose tissue
BMR: Basal metabolic rate.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

Authors' Contributions

Carmela Colica contributed to the interpretation of the data and drafted the manuscript. Patrizio Bollero, Ennio Avolio, Simona Ferarro, and Paola Sinibaldi Salimei collected the data and performed the experiments. Renata Costa de Miranda analyzed the data. Antonino De Lorenzo had primary responsibility for the final content. Laura Di Renzo conceived, designed the experiments, and drafted the manuscript. All the authors read and approved the final manuscript.

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