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Review

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MAFLD under the lens: the role of gut microbiota

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Abstract

Obesity, the metabolic syndrome, and metabolic dysfunction-associated fatty liver disease (MAFLD) can be portrayed as transmissible diseases. Indeed, they can be induced, in animal models, by cohabitation or by transplantation of fecal microbiota from other animals or humans with those diseases. As such, to get a 10,000-foot view, we need to see under the lens the microbes that populate our gut. Gut microbiota participates in the harvesting of energy from nutrients, it allows the digestion of otherwise indigestible nutrients such as fibers, and it also produces short chain fatty acids and some vitamins while emitting different compounds that can regulate whole-body metabolism and elicit proinflammatory responses. The metabolic syndrome and MAFLD share physiopathology and also patterns of gut dysbiota. Moreover, MAFLD also correlates with dysbiota patterns that are associated with direct steatogenic or fibrogenic effects. In the last decade, a tremendous effort has allowed a fair understanding of the dysbiota patterns associated with MAFLD. More recently, research is moving towards the delineation of microbiota-targeted therapies to manage metabolic dysfunction and MAFLD. This review provides in-depth insight into the state-of-the-art of gut dysbiosis in MAFLD, targeting clinical hepatologists.

Keywords: MAFLD, gut microbiota, obesity, diabetes mellitus

INTRODUCTION

The steady increase in the prevalence of metabolic-associated fatty liver disease (MAFLD) since its description in the 1980s has turned it into a major health threat[1]. Indeed, MAFLD was the fastest growing

indication for liver transplantation over the last 20 years and is already the leading cause for liver transplantation among women and patients over 54 years in the US[2].

MAFLD is the hepatic expression of metabolic dysfunction and goes hand in hand with obesity and insulin resistance (IR)/type 2 diabetes mellitus (T2DM)[3]. Its pathogenesis is complex and dependent on additional individual susceptibility, whether genetic, behavioral, or acquired[4]. The gut microbiota is one such factor that helps explain individual susceptibility to MAFLD and liver disease progression[5]. In the last decade, we witnessed a research boom on the role of gut dysbiota in MAFLD, regarding its pathogenesis, diagnostic ability, and role as a therapeutic target.

The way the gut microbiota is assessed has been changing since the first studies before the 1990s, when only culture-based methods were available[6]. Even though culture-based methods are cheap, less than 30% of the gut microbiota has been cultured. With the development of culture-independent techniques, there has been an exponential growth in the knowledge of gut microbiota. These techniques have shown the diversity of the gut microbiota, allowing quantitative and/or qualitative information about bacterial species and in-depth comparison of the microbiota from healthy and MAFLD patients[7].

Most studies used culture-independent, biomarker-based profiling techniques that involve sequencing a ubiquitous bacterial gene - small subunit ribosomal RNA (16S rRNA) - which is highly conserved but divergent enough to allow resolution at a genus level and, in some cases, at the level of species or even strains[8]. This provides a relatively accurate fingerprint of microbial community composition but cannot accurately identify bacteria on a strain level, nor does it reflect the microbiota functional properties, even though it is possible, through algorithms, to infer *in silico* metagenomic analyses from 16S RNA data[9].

With recent advances in computational biology and high-throughput sequencing technology (shotgun sequencing or pyrosequencing), research is moving to untargeted whole genome sequencing and "omics" approaches[6]. They include metagenomics (determination of the functional genes encoded), metatranscriptomics (determination of the functional genes expressed), metaproteomics (identification of proteins), and metabolomics (identification of bioactive small molecules)[8,10,11]. Shotgun sequencing metagenomic data allow the characterization of the DNA library from a microbial community with an accurate portrayal of the potential microbial functional properties. However, it does not inform the activity or directionality of a certain present pathway. Those are better inferred by metatranscriptomics and metabolomics[12].

The interpretation and extrapolation of the studies of gut microbiota are challenging. First, the microbiota is as specific to an individual as a fingerprint[13]; that is, each person has a unique collection of bacterial strains and species, although there is the controversial concept of the existence of a core functional microbiome[14,15]. There is also appreciable intraindividual variability with age, medication, and changes of lifestyle[8]. At a population level, the gut microbiota is modulated by several factors, such as geographic localization, different dietary patterns, host genetics, and environmental factors. Most studies did not account for confounding factors that are known to influence the microbiota composition, which may influence the conclusions and the extrapolation of the results to all patients with the same condition. Besides bacteria, gut microbiota also has other communities, such as viral and fungal, but their role in the crosstalk between microbes and host is even less known[8,12]. Sampling may represent another weakness. To start, the choice of the sample will render different results, since the microbiota from stool (representing luminal content) will be different from those from mucosal biopsy samples. The way stool is collected, stored, and processed can have a major impact on the quality of the samples, since it may allow contamination and a

variable degree of proliferation or death of different bacteria that will change the relative proportions of bacterial species[7]. Taking everything into consideration, it is understandable why there are striking differences between studies, which hamper the generalization of the results.

We present an in-depth review of the state-of-the-art of gut microbiota in MAFLD with basilar concepts directed to clinical hepatologists.

OVERVIEW OF THE GUT MICROBIOTA

The term gut microbiota refers to all microorganisms found in the digestive tract. It is considered an essential invisible organ[16], an example of a dynamic and complex symbiotic relationship between host and microbiota that mutually influence each other[17]. The gut microbiota is composed of bacteria, archaea, fungi, and viruses. The most represented bacteria phyla are Bacteroidetes, Firmicutes, Actinobacteria, and Proteobacteria. Three enterotypes are described according to the bacterial genus predominance: *Bacteroides* in type 1, *Prevotella* in type 2, and *Ruminococcus* in type 3[15].

The term microbiome refers to the combination of genomes and genes from the members of a microbiota[18]. The microbiome acts as a massive functional booster of the host genome, harboring more than 100-fold the number of human genes[19]. Indeed, these extra genes encode enzymes that are not encoded by the host, adding functions that would otherwise be absent in humans, such as the breakdown of complex polysaccharides and the synthesis of polyphenols, essential amino acids, and vitamins[19-21]. There is great functional redundancy across taxa, and changes in microbiota composition may not result in functional consequences[22].

Dysbiosis refers to the permanent disruption of the symbiotic relationship between host and microbiota, with changes in the composition and function of the microbiota. Dysbiosis has a profound impact on host physiology and may promote the development of diseases such as obesity, T2DM, and MAFLD[23-25]. A ubiquitous feature of dysbiosis is the loss of species diversity, which is counterbalanced by the overgrowth of proinflammatory species, promoting intestinal inflammation[23]. Alpha-diversity refers to the bacterial richness in one sample and beta-diversity to the variation in a group of samples[26].

The gut microbiota has several functions with potential benefit for the host homeostasis: maintenance of mucosal barrier integrity; defense against pathogenic microbes; and nutrients, bile acids, and drug metabolism[25].

The interaction between enterocytes and gut microbes regulates epithelial permeability[27]. For example, the commensal *Akkermansia muciniphila* enhances tight junctions' function[28,29]. Consequently, dysbiosis is frequently associated with a leaky gut[23]. Gut microbiota further contributes to defense by constraining pathogens colonization through the competition for attachment sites and the production of bacteriocins that inhibit their competitor's growth[30]. Consequently, in dysbiosis, there is a shift in this balance allowing pathogenic strains to overgrow. Importantly, the gut microbiota is also a key regulator of innate and adaptive immunity[31], acting on gut-associated lymphoid tissues, regulatory T cells, IgA producing plasma cells, innate lymphoid cells, resident macrophages, and dendritic cells in the lamina propria[32].

Regarding nutrient metabolism, the gut microbiota acts on the metabolism of carbohydrates, vitamins, and amino acids. Colonic microbes ferment complex carbohydrates producing volatile organic compounds and short-chain fatty acids (SCFA), mostly propionate, butyrate, and acetate[33]. The critical physiological functions of SCFA are as follows: maintenance of intestinal mucosa integrity, a source of energy for the

host, modulation of glucose and lipid metabolism, control of energy expenditure, and immune regulation[34-36]. Butyrate is the most crucial SCFA for human health, being the major source of energy for colonocytes, presenting anti-colon cancer activity, and potentially decreasing gut permeability[37,38]. Propionate may abrogate hepatic gluconeogenesis and promote satiety, with the potential to reduce adiposity. Acetate is the most abundant SCFA and modulates gut microbiota, for example, being an essential metabolite for *Faecalibacterium prausnitzii* growth[39].

The gut microbiota is responsible for the synthesis of vitamins K and B (including biotin, cobalamin, folates, nicotinic acid, panthotenic acid, pyridoxine, riboflavin, and thiamine)[39,40]. It can also extensively degrade proteins from diet, host enzymes, mucin, and sloughed-off intestinal cells, producing amino acids and their derivatives, including IR-linked branched-chain amino acids (BCAA)[41,42].

The metabolism of bile acids results from the interactive crosstalk between the host and the gut microbiome. Primary bile acids (cholic acid (CA) and chenodeoxycholic acid (CDCA)) are cholesterol-derived metabolites, synthesized and conjugated with glycine or taurine in hepatocytes[43]. They are mostly absorbed by active transport in the terminal ileum, entering the enterohepatic circulation. The gut microbiota metabolizes (deconjugation, 7-dehydroxylation, and 7-dehydrogenation) them into secondary bile acids [deoxycholic acid (DCA) and lithocholic acid (LCA)], changing their structure and function[44]. There is a bidirectional relationship between gut microbiota and bile acids. Bile acids have selective antimicrobial characteristics that modulate the gut microbiota composition[43]. Besides the role of bile acids in fat absorption, they act through different receptors [e.g., farnesoid-X receptor (FXR) and Takeda-G-protein receptor (TGR5)] as well as receptor-independent mechanisms such as modulating membrane dynamics[45]. Bile acids modulate glucose metabolism (e.g., promote hepatic glycogen synthesis and insulin sensitivity and increase pancreatic insulin secretion) and energy homeostasis (facilitate energy expenditure, favor thermogenesis, and mediate satiety in the brain)[46], but they can also induce hepatic lipogenesis, cell injury, and proinflammatory and profibrogenic responses[22].

The microbiota composition varies along the digestive tract, with different strains in the mouth, small intestine, and different colorectal localizations, as well as transversal variations (gut lumen, mucous layer, and intestine villous/cripta)[47].

Several factors regulate the microbiota composition, such as environmental factors (delivery mode, diet, antibiotics, and prebiotics/probiotics), host factors (genetic), environmental factors, and inter-microbial interaction (competition between strains).

Acute perturbations of the gut microbiota, such as acute diarrhea, short-term diet manipulations, or short-term antibiotic use, induce only transient modifications that tend to return to baseline over time, a characteristic known as resilience. However, if the perturbation perpetuates in time (e.g., long-term dietary manipulations), it can overcome microbiota resilience and lead to sustained changes in the gut microbiota[10,48,49].

The newborn microbiota is transferred vertically and is influenced by the delivery route: either colonized by microbes from the maternal vagina or from maternal skin flora with cesarean section birth[50,51]. Microbiota changes with age, increasing its diversity from childhood to adulthood and then decreasing in the elderly[23].

Diet seems to shape gut microbiota in infants (breast milk or formula) and adulthood. For example, preclinical and epidemiological studies showed an association between high-fiber diets rich in fruits and

vegetables and higher gut microbiota diversity and richness[32], as well as decreased Firmicutes/Bacteroidetes ratio, increased levels of *Prevotella* and *Xylanibacter,* and a decrease in proinflammatory pathogens Enterobacteriaceae, *Shigella,* and *Escherichia*. On the contrary, the high-fat, high-protein Western diet is associated with a decrease in diversity and an increase in *Firmicutes*[48,52-54].

Geographic localization is also a strong determiner of gut microbiota composition. Indeed, a study evaluating individuals from four districts within one province of China found that host location showed the strongest association with microbiota variation[55]. Furthermore, in developed countries, there is a trend of a loss of microbiota diversity. Geographic variations might be the result of different modulators of the gut microbiota, such as diet, lifestyle, sanitary conditions, proportion of infants delivered by cesarean sections, use of antibiotics, and genetics[56,57].

Studies with twins showed that host genetics is significantly associated with the abundance of specific gut microbial taxa, dubbed hereditable taxons. For example, there seems to be an association between Bifidobacteriaceae and the LCT locus (higher abundance of *Bifidobacterium* in lactose-intolerant subjects that maintain a regular intake of dairy products)[58-60].

Longitudinal studies showed that the microbial composition of a unique host may change in function of diet, drug intake, lifestyle (smoking, travelling, and physical activity), co-morbidities, and colonic transit time[10,49].

GUT MICROBIOTA AND OBESITY

The pathophysiology of obesity is complex and does not reduce to a simple arithmetic between the energy consumed and expended. Behavioral, genetic, and environmental factors concur.

The first evidence for the role of gut microbiota in obesity and the concept of obesity as a transmissible disease comes from seminal preclinical studies from the beginning of the century[61]. Those studies showed that rodents devoid of microbiota were resistant to diet-induced obesity and would adopt an obese phenotype when submitted to fecal microbiota transplantation (FMT) from genetically or diet-induced obese rodents but not from lean donors[62-64]. Furthermore, the FMT-acquired obese phenotype could be reverted by cohousing with lean rodents as a result of invasion by specific strains of Bacteroidetes, but only in mice fed a low-fat diet, clearly illustrating the interplay between diet and microbiota[65]. The potential obesogenic gut microbiota is also evident in humans, with reports of new-onset obesity in patients with recurrent *Clostridium difficile* after FMT from overweight donors[66].

Gut microbiota has a paramount role in the regulation of whole-body energy homeostasis and may induce obesity through direct host interactions or indirectly via microbial metabolites.

The gut microbiota is able to ferment indigestible carbohydrates into SCFA, which accounts for roughly 10% of harvested energy from diet[37]. Acetate is the main source of energy from SCFA and the most obesogenic one, being able to induce hyperphagia (through induction of ghrelin and glucose-stimulated insulin secretion), hypertriglyceridemia, IR, hepatic and adipocyte lipogenesis, and ectopic lipid deposition in the liver and skeletal muscle[67,68]. Conversely, butyrate and propionate are anti-obesogenic, both increasing the expression of the anorexigenic adipokine leptin[69,70] and gut hormones peptide tyrosine-tyrosine (PYY) and glucagon-like peptide-1 (GLP-1)[71]. Butyrate enhances energy expenditure by promoting mitochondrial activity and upregulating genes for lipolysis and fatty acid oxidation[72]. Propionate directly induces intestinal gluconeogenesis, which abrogates adiposity and weight gain,

independently of food intake, through the activation of gut–brain neural circuits mediated by portal vein glucose sensors[73]. Butyrate is mainly produced by the Firmicutes *Faecalibacterium prausnitzii*, *Eubacterium rectale,* and *Rosuberia intestinalis*. Acetate is also produced by Bacteroidetes such as *Bacteroides* and *Prevotella*, as well as *Lactobacillus*, *Bifidobacterium,* and *Akkermansia*[70,74].

Gut microbiota downregulates the intestinal expression of fasting-induced adipose factor (FIAF). FIAF is a lipoprotein lipase inhibitor that inhibits adipose tissue and hepatic cellular uptake of fatty acids from circulating lipoproteins. As such, FIAF inhibition by the gut microbiota promotes liver steatogenesis and expansion of the adipose tissue[61].

The interplay between dietary tryptophan and gut microbiota may also contribute to obesity. In the gut, the main metabolic pathway of tryptophan is the kynurenine pathway, which results in different bioactive metabolites. Several gut bacteria have the potential to shunt tryptophan into two minor pathways: the aryl hydrocarbon receptor (AhR) pathway directly by metabolizing it into indoles (e.g., *Lactobacillus*) and the serotonin pathway indirectly by SCFA and secondary bile acids regulation of serotonin synthesis (e.g., *Prevotella*)[75,76]. The AhR pathway culminates in the production of GLP-1 and IL-22, the latter being able to decrease mucosal inflammation and permeability by inducing resistance to *Candida albicans* colonization[77,78]. Serotonin also acts on energy homeostasis by acting in brain reward centers and increasing lipolysis in white adipose tissue while decreasing thermogenesis in brown adipose tissue[79,80].

Dysbiota may also modulate the effects of the gut microbiota on bile acids, which may impact metabolism and energy homeostasis[81,82]. For example, an impairment in the production of secondary bile acids leads to the accumulation of primary bile acids with proinflammatory and leaky gut-inducing effects[83].

The gut microbiota is a main regulator of the intestinal mucosal barrier integrity, and gut dysbiota can induce a leaky gut through direct mucosal inflammation or indirectly via microbe metabolites[84]. The increased permeability to microbial products such as lipopolysaccharides (LPS) has been dubbed metabolic endotoxemia, which contributes to the inflammatory state that characterizes obesity, but it also has profound metabolic effects, inducing IR, leptin resistance, adipose tissue expansion, and hepatic steatosis[85-87]. Furthermore, gut inflammation increases its vascularization and enhances nutrient absorption[88].

Recent research is gathering information to characterize obesity-associated gut microbiota. Obesity is characterized by decreased gut microbiota richness and diversity[89], with a corresponding decrease in microbiome gene count[90].

Obesity-associated gut microbiota seems not only quantitatively but also qualitatively different. Most, but not all, studies have described an increased Firmicutes/Bacteroidetes ratio[91,92]. The huge variability between studies suggests that more than a taxonomic core, there is a microbiome core for obesity that is associated with higher efficiency in energy harvesting from diet and proinflammatory states[89,93,94]. However, some taxonomic associations have been fairly consistent, with obesity being associated with decreased relative abundance in the families Rikenellaceae and Christensenellaceae and the genera *Bifidobacterium*, *Oscillospira*, *Faecalibacterium,* and *Akkermansia,* all of which are involved in SCFA metabolism, mucosal integrity, and protection against a leaky gut[70]. Conversely, obesity has been associated with increased relative abundance in the families Prevotellaceae and Coriobacteriacea and the genera *Roseburia* and *Eubacterium*. H2-oxidizing methanogenic Archaea with the potential for higher carbohydrate fermentation efficiency also seem to be increased[70,94,95], as well as pathogenic proinflammatory Gram-negative bacteria,

such as *Escherichia* or *Shigella*[94].

The diet seems to have a major role in the acquisition of an obese-associated gut microbiota[96], with high-fat, high-sucrose Western diets promoting a shifting microbiota composition, depleting "favorable" bacteria (e.g., *Bifidobacterium* and *Faecalibacterium*) while replenishing "unfavorable" bacteria (e.g., *Blautia* and *Acinetobacter*)[97].

GUT MICROBIOTA AND DIABETES MELLITUS

Type 2 diabetes mellitus (T2DM) shares behavioral risk factors with obesity, as well as similar dysbiota profiles.

Human observational studies have shown a lower - but not -diversity in T2DM patients[98,99]. Furthermore, a recent metaanalysis showed that T2DM is associated with an increased abundance of Firmicutes (e.g., Veillonellacea) and decreased Bacterioidetes[98]. At the genus level, the most striking associations were an increased relative abundance of *Ruminococcus*, *Coprococcus*, *Eubacteria*, *Blautia,* and *Prevotella*, whereas potentially protective bacteria were *Dorea*, *Bifidobacterium*, *Bacteroides*, *Faecalibacterium, Akkermansia,* and *Roseburia*[98-100].

Collectively, T2DM patients have a decrease in butyrate-producing bacteria and increased expression of microbiota genes related to inflammation and oxidative stress, leading to a leaky gut[101,102].

Increased intestinal permeability leading to endotoxemia and a continuous state of low-grade inflammation can induce IR[103]. For example, LPS binding to TLR4 induces IR by direct phosphorylation of insulin receptor[85,104,105]. Indeed, *Roseburia*, *Bacteroides,* and *Akkermansia*, which seem to be depleted in T2DM, either induce anti-inflammatory or inhibit proinflammatory cytokines[28,106,107]. *Bacteroides* and *Akkermansia muciniphila* further protect from the leaky gut by directly strengthening epithelial tight junctions[29,108].

Gut dysbiosis may also have a profound effect on glucose metabolism and overall energy homeostasis[100]. For example, the potentially anti-diabetogenic *Bifidobacterium* induces gut secretion of GLP-1 and PYY, promoting insulin sensitivity and beta cell function[109,110]. Microbial metabolites also modulate glucose homeostasis. For example, regarding SCFA, propionate reduces insulin sensitivity, leading to compensatory hyperinsulinemia[111]; acetate increases glucose-stimulated insulin secretion and weight gain[67]; and butyrate has insulin-sensitizing actions by indirectly promoting the gut barrier abrogating metabolic endotoxemia[112] and through the induction of insulin-sensitizing gut hormones GLP-1, PYY, and GLP-2[113].

Prevotella copri is associated with increased risk for T2DM, and the link may be the production of BCAAs[114], which are able to induce mTORC1-dependent IR[115].

Trimethylamine-N-oxide (TMAO) may be another link between gut dysbiota and T2DM[116]. TMAO induces IR through ER stress-induced FoxO1 expression[117,118]. Dietary choline can be used for phosphatidylcholine synthesis, oxidized into betaine or metabolized into trimethylamine (TMA) by gut microbiota, mainly Firmicutes and Proteobacteria such as *Escherichia coli*[119]. TMA is further oxidized in the liver to TMAO by hepatic flavin-containing monooxygenases (FMAO3). TMAO has been consistently associated with T2DM[120] and an increased cardiovascular risk[121,122].

Lastly, T2DM patients have lower levels of secondary biliary acids, decreasing the contribution of the latter to insulin sensitivity via TGR5 induction of GLP-1 secretion[123].

Further supporting the role of gut dysbiosis in T2DM pathogenesis, a small study with FMT in obese patients improved insulin sensitivity and glucose tolerance after 12 weeks, independent of weight loss[124], even though this was not consistently replicated by others[125-127].

GUT MICROBIOTA AND MAFLD

MAFLD is the hepatic manifestation of metabolic syndrome with strong associations with obesity and T2DM[4]. Unsurprisingly, it shares common core pathogenesis, which extends to sharing the gut microbiota[5]. However, not all MAFLD patients are obese or overweight[128], and one third of T2DM patients will not present MAFLD[129]. Predictably, specific microbiota features may be associated with MAFLD beyond the associations shared with metabolic dysfunction.

There is strong indirect evidence that MAFLD is associated with gut dysbiosis. Similar to obesity, MAFLD can be a transmissible disease in rodents by cohousing or direct FMT of microbiota-devoid mice from mice with genetically or diet-induced hepatic steatosis or from patients with nonalcoholic steatohepatitis (NASH), even when those mice were fed a healthy isocaloric diet[61,130-134]. Furthermore, antibiotic treatment prevents the development of hepatic steatosis in dietary and genetically induced obesity in animal models[135,136], as well as in obese patients submitted to intestinal bypass bariatric surgery[137]. There is also strong preclinical and clinical evidence of an association among small intestine bacterial overgrowth[138,139], leaky gut[140], and MAFLD. Although only up to 40% of patients with MAFLD present an increase in intestinal permeability, this is six folds higher than the level expected in healthy subjects[141-143].

Studies comparing gut microbiota from healthy subjects, obese patients, and patients with MAFLD found greater differences between obese and non-obese phenotypes and greater similarities between obese and MAFLD phenotypes[144]. Both obesity and MAFLD seem to be associated with decreased bacterial species diversity[145], although the diversity is even lower in patients with MAFLD[146]. In obesity, at the phylum level, an increased Firmicutes/Bacteroidetes ratio has been consistently described. In MAFLD, the relative abundance of Firmicutes and Bacteroidetes was variable[144,147-150]; however, when direct comparisons of the microbiota between obese patients and MAFLD patients were made, their ratio was similar in some studies[144,146] or reversed with decreased Firmicutes and increased Bacteroidetes in MAFLD patients, particularly in patients with steatohepatitis[134,151,152]. At the family level, both obesity and MAFLD are associated with a relative decrease in Lachnospiraceae and Ruminococcaceae[153], and, at the genus level, there was a decrease in *Faecalibacterium* as well as an increase in *Prevotella* in some but not all cohorts[6,143,152,154].

Faecalibacterium, *Eubacterium*, *Rosuberia,* and *Coprococcus* are all butyrate-producing bacteria that consistently were shown to be decreased in MAFLD[147,152]. *Faecalibacterium praunitzii*, in particular, belongs to the Ruminococcaceae family[155], accounts for over 5% of the total gut microbiota in healthy humans[156], and regulates hepatic fat content, upregulating fatty acids oxidation and increasing hepatic sensitivity to adiponectin[157]. It also secretes anti-inflammatory compounds such as salicylic acid[158] and microbial anti-inflammatory molecule (MAM) protein[159], which abrogate adipose tissue inflammation[155].

Akkermansia depletion is associated with disruption of gut epithelial barrier, is characteristic of obesity and T2DM, and was also reported in MAFLD[147].

Similar to T2DM and the metabolic syndrome, TMAO levels increase (with correspondent decreases in betaine) in parallel with the severity of hepatic steatosis[160]. *Prevotella* gut colonization seems to be associated with increased levels of TMAO[161], and several studies described increased *Prevotella* abundance in MAFLD patients[143]. The increase in TMAO in MAFLD may result in metabolic dysfunction-independent steatogenic effects, since TMAO synthesis shunts choline away from the synthesis of betaine (a methyl donor with antioxidant and anti-inflammatory properties[162]) and phosphatidylcholine (necessary for lipoprotein export from the liver[163]). Furthermore, TMAO has direct steatogenic effects in the liver by suppressing bile acid-mediated hepatic FXR signaling, which results in SREPBP-1c-mediated induction of lipogenesis. This was matched with a shift in the composition of the bile acid pool, with a decrease in potent FXR agonists tauroCDCA and glycolithocholic acids and an increase in FXR antagonist taurocholic acid[164]. Different bacteria belonging to Firmicutes and Proteobacteria are able to produce TMA, such as some *Clostridium* species, *Escherichia fergusonii,* and *Proteus penneri*[165]. Some of those bacteria have been associated with MAFLD. For example, *Escherichia fergusonii* was associated with non-obese hepatic steatosis in animal models and humans[166].

Gut dysbiota may have steatogenic effects independent of the ones leading to obesity and IR/T2DM, mainly through bile acid metabolism and microbiota end-products/metabolites. Indeed, when comparing morbid non-diabetic obese women with and without steatosis, a 75% shared variation between the gut microbiome and the molecular phenomics was found (i.e., the sum of hepatic transcriptome and plasma and urine metabolome), which was associated with steatosis, suggesting a causal effect[134].

Some bacteria produce ethanol, which is directly steatogenic[144]. Accordingly, several studies, including on pediatric populations, showed MAFLD/NASH to be associated with increased endogenous ethanol levels[144,167]. Examples of ethanol-producing bacteria are Proteobacteria such as *Escherichia coli*[168]. Interestingly, an increase in Proteobacteria relative abundance is one of the most consistent findings in MAFLD patients[6,151,153,154]. An increase in Proteobacteria, and particularly *Escherichia coli,* is a distinguishing gut microbiota trait between obese and MAFLD/NASH patients[143,144,146,147,169]. Of note, an overgrowth of *Escherichia coli* in the gut microbiota has been linked to subtherapeutic doses of antibiotics that may be a contaminant of Western diets[170]. Additionally, in a Chinese cohort of MAFLD patients, 60% presented an increased relative abundance of another Proteobacteria: high alcohol-producing *Klebsiella pneumoniae*[171]. From that cohort, one patient presented severe NASH and auto-brewing syndrome; that is, he developed ultra-high alcohol blood concentration after an alcohol-free, high-carbohydrate diet[172]. Transplantation of isolates of high alcohol producing *Klebsiella pneumonia* from that patient into a rodent recipient induced hepatic steatosis[171,173]. An increased relative abundance of *Klebsiella* was corroborated by other groups[147].

Proteobacteria are also relevant as a source of LPS[134]. Indeed, the microbiome of patients with MAFLD/steatohepatitis is characteristically enriched in genes for LPS synthesis[151].

T2DM is associated with decreased *Lactobacillus*[174]; however, in MAFLD, different groups described an actual increase in *Lactobacillus* relative abundance[143,151,153]. The increased abundance of *Lactobacillus* in MAFLD is counterintuitive since it is a known probiotic that competitively inhibits pathogens, heightens the epithelial barrier function, and has immunomodulatory properties[175]. However, *Lactobacillus* also produces volatile organ compounds and ethanol, which are known potentiators of steatogenesis and steatohepatitis[176]. Indeed, the *Lactobacillus* genus comprises more than 180 species with different sugar fermentation properties, the end result of some species being predominantly lactic acid (*L. acidophilus* and *L. salivarius*) and others ethanol (*L. casei*, *L. brevis,* and *L. plantarum*)[5].

Gut microbiota can modulate the development of MAFLD through other microbial byproducts. MAFLD-associated microbiota also showed an increased genetic potential for hepatic inflammation and deregulation of AAA (tryptophan, tyrosine, and phenylalanine) and BCAA (valine, leucin, and isoleucine)[134]. BCAAs are associated with obesity and IR[114]. Steatohepatitis has been associated with an increase in the genus *Bacteroides* and *Prevotella copri* which have the ability to produce BCAA[143,154,177]. Another microbiota-associated metabolite that was strongly associated with MAFLD is the AAA-derived phenylacetic acid, which showed direct steatogenic effects on hepatocytes *in vitro*, promoting hepatic lipids uptake (by induction of lipoprotein lipase), *de novo* lipogenesis (by inducing fatty acids synthase), and inhibiting the insulin receptor pathway[134]. 2-butanone seems to decrease in NAFLD[153] but increase in steatohepatitis, which may be the result of increased *Streptococcus pneumonia*[143,146,154]. 2-butanone is a strong inductor of CYP, and even though it is not hepatotoxic *per se*, it potentiates hepatic lesions from toxic-associated steatohepatitis[178,179].

The effects of the gut microbiota on bile acid metabolism also have a crucial role in the development of MAFLD. MAFLD is associated with an increased abundance of bacteria that produce bile salt hydrolases and hence deconjugate bile acids (e.g., *Clostridium*, *Lactobacillus*, *Escherichia,* and *Bacteroides*), as well as bacteria that facilitate the conversion of primary to secondary bile acids (predominantly *Clostridium* species[143,148,180]). Primary bile acids activate, whereas secondary bile acids inhibit FXR[142]. FXR activity is known to be decreased in MAFLD, which is associated with an increased synthesis of hepatic bile acids, metabolic deregulation, steatogenesis, inflammation, and possibly fibrogenesis in the liver[181].

Non-obese MAFLD shares microbiota signatures with obese MAFLD. In fact, some studies even found an obese-associated microbiota in lean patients with MAFLD, such as an increased Firmicutes/Bacterioidetes ratio[182] and a decrease in Lachnospiraceae (e.g., *Coprococcus*) and Ruminococcaceae (e.g., *Ruminococcus*). However, other microbiota associations with lean MAFLD suggest a different microbiota profile that could induce steatogenesis by mechanisms unrelated to the metabolic syndrome[155]. For example, lean *versus* obese NAFLD is associated with decreased *Lactobacillus* abundance[182,183]. Furthermore, non-obese steatohepatitis is associated with a different mycobiome, with a lower fungal richness, increased abundance of *Candida albicans* and *Mucor* spp, and decreased *Saccharomyces cerevisiae*[184]. *C. albicans* is known to induce a Th17 proinflammatory response[185], and its richness is associated more with hepatic inflammation than steatosis[184].

GUT MICROBIOTA AND PROGRESSION OF MAFLD

The presence and severity of liver fibrosis dictate the risk for progression to liver cirrhosis and morbi-mortality[186]. The gut microbiota changes according to the severity of liver disease, from mild to significant fibrosis, compensated cirrhosis, and decompensated cirrhosis. The differences found in the gut microbiota allowed the proposal of gut microbiome-based metagenomic signatures for MAFLD with advanced fibrosis and cirrhosis[169,187,188], with apparent great diagnostic accuracy.

Interestingly, while - and -diversity seem to decrease with mild disease, they paradoxically increase with severe disease, with increased dispersion in cirrhosis[169].

Advanced fibrosis, as compared to absence or mild fibrosis, has been associated with increased anaerobic bacteria (e.g., *Bacteroides vulgatus*, *Holdemanella,* and *Prevotella copri*, even though an increase in the latter was not consensual[22,177]) and Gram-negative bacteria (e.g., the Proteobacteria *Escherichia coli* and *Shigella*)[151,187,189-191]. Interestingly, *Prevotella* abundance is associated with alcohol consumption, even when moderate[190], which may explain an additive effect in metabolic dysfunction and alcohol-associated liver

disease. *Prevotella* has antioxidant properties, such as the production of superoxide dismutase. However, that can have an adverse effect by creating a more permissive milieu to other bacteria less resistant to oxidative stress, hence promoting intestinal inflammation[192]. Similarly, *Holdemanella biformes* is another immunogenic commensal that has been associated with gastrointestinal inflammatory diseases and host lipid metabolism[193]. Lastly, *B. vulgatus* has been linked to metabolic dysfunction, with relative abundance variation correlating with body mass index, poor glucose control, and IR[114,194].

Several reports showed an association between advanced fibrosis and decreased *Ruminococcus obeum*[22,155,177] and *Eubacterium rectale*[187]. Both species are fiber-fermenting bacteria with the ability to produce SCFA and have also been negatively associated with the development of primary sclerosing cholangitis in patients with inflammatory bowel disease[195]. Indeed, SCFAs inhibit hepatic inflammation (a major driver of fibrogenesis) via promoting intestinal barrier integrity and directly with anti-inflammatory actions on Kupffer cells[196].

The metagenome associated with advanced fibrosis is correlated with bacterial changes. For example, advanced fibrosis was associated with the anaerobic bacteria derived 3-phenylpropanoate[187]. Increased abundance of the anaerobic bacteria *Prevotella* and *Holdemanella* is also associated with oxidative stress and a proinflammatory environment, as a consequence of changes in metabolic pathways such as urea cycle and vitamin B biosynthesis[190,197].

Besides bacteria, the virome in patients with advanced fibrosis is different, with a decreased variability and a decreased relative abundance of bacteriophages, which may have a direct impact on modulating the taxonomic composition of the bacterial microbiota[198]. In addition, regarding mycobiome, *Candida albicans* relative abundance and immunogenicity seem to increase in patients with advanced fibrosis[184].

Cirrhosis seems to be associated with invasion of the gut by oral commensals, such as *Prevotella*, *Veillonella,* and *Streptococcus*, possibly through altered bile acid metabolism[199,200]. Conversely, there is a further decrease in beneficial commensals such as *F. prausnitzii* and *Coprococcus*[188,199].

Decompensated cirrhosis is associated with a further dramatic shift in gut microbiota, with an increased abundance of pathogenic Enterococcaceae and Enterobacteriaceae and decreased Bacteroidaceae and the Lachnospiracea Clostridiales IV[201,202], which is correlated with endotoxemia[203]. Again, when cirrhosis decompensates, there is a decrease in the synthesis of bile acids and their fecal concentrations, which abrogates bile acids' negative effect on Gram-negative bacteria, increasing the ratio of Gram-negative to Gram-positive bacteria (e.g., Lachnospiraceae)[149].

MAFLD-associated hepatocellular carcinoma is associated with intestinal inflammation, as evidenced by increased fecal calprotectin and increased cytokines IL-8, IL-13, and CCL-3/4/5, which is correlated with a decrease in *Akkermansia* and *Bifidobacterium*[200]. Furthermore, there is also an increase in pathogenic Enterobactereacea compared to MAFLD cirrhosis without hepatocellular carcinoma. In contrast to the increased intestinal inflammation, MAFLD-HCC microbiota induces a peripheral T cell immunosuppressive phenotype, expansion of regulatory T cells, and attenuation of effector CD8+ T cells, which may be responsible for a decreased tumoral immunosurveillance[204].

MAFLD-associated hepatocellular carcinoma is also associated with increased total bile acids and serum FGF-19, which is correlated with an increase in *Lactobacilli*[205]. A gut dysbiota-dependent increase in DCA has been described in MAFLD cirrhosis and may have pro-tumoral effects. Indeed, DCA induces a senescence-associated secretory phenotype in hepatic stellate cells, which elicits the secretion of

proinflammatory and tumor-promoting factors[206]. Furthermore, DCA further inhibits tumoral immunosurveillance by inhibiting the expression of CXCL16 in hepatic sinusoidal cells, hence recruiting fewer anti-tumoral CRCR6+ NKT cells[207] [Figure 1].

INTERVENTION IN GUT MICROBIOTA FOR THE TREATMENT OF MAFLD

As of today, no treatment has been approved for MAFLD, and gut dysbiosis is a highly appealing treatment target. Microbiome-targeted therapies include different strategies, such as probiotics and prebiotics, antibiotics, FMT, and bacteriophages. Furthermore, treatment strategies applied today, for example, diet and exercise, bariatric surgery, and some anti-diabetic agents such as metformin[208] and liraglutide[209], act through modulation of the microbiota. Metformin is able to alter the gut microbiota composition in a way that leads to an improvement in glucose intolerance[208]. Indeed, metformin has a dose-dependent antimicrobial effect with a decrease of specific bacteria[210] such as *Bacterioides fragilis*, and depletion of *Bacterioides fragilis* is associated with inhibited FXR signaling mediated by glycoursodeoxycholic acid[211]. Metformin also promotes the growth of specific bacteria such as *Akkermansia muciniphila*, which is known to have beneficial metabolic and anti-inflammatory effects[212]. There is also scarce preclinical evidence of treatment of Chinese herbs such as Jiangzi granules and *Ixeris chinensis* showing beneficial effects on MAFLD through the modulation of the gut microbiota[213-215]. Furthermore, alcohol consumption promotes *Prevotella* growth, high protein intake decreases *Bacteroides* abundance[216], and exercise increases SCFA-producing bacteria and anti-inflammatory *F. prausnitzii* and *A. muciniphila*[217].

Probiotics are live non-pathogenic microorganisms that induce health benefits in humans when in appropriate quantities. The most used probiotics are *Lactobacilli* and *Bifidobacteria*[218]. Prebiotics are fermentable dietary fibers (such as inulin and fructo-oligosaccharides) that selectively promote the growth of probiotics. The combination of probiotics and prebiotics is dubbed synbiotics[8]. Small short-term clinical trials have evaluated the effect of probiotics on MAFLD. Different metaanalyses showed probiotic therapy to result in biochemical improvement of aminotransferases and lipid profile, as well as of steatosis and fibrosis by non-invasive techniques, while there are no data on liver histology[218-220]. Comparing different studies with probiotics is hampered by the fact that each study uses different formulations of probiotics. Furthermore, even though we can find some patterns of dysbiosis in MAFLD, the gut microbiota is unique for each patient, which anticipates a highly variable effect of probiotics. The effect of probiotics is also expected to be only transient, since there is no evidence of persistent probiotic engraftment with a persistent shift in the microbiota composition[221]. The best approach would be personalized probiotics therapy, with the selection of specific commensal bacteria that would already be present in the individual baseline microbiota to allow better engraftment[222].

The evidence for prebiotics in the treatment of MAFLD is even scarcer, with very small trials in adults suggesting a mild effect on body mass index and aminotransferases levels[223].

Less is known regarding the effect of antibiotics on MAFLD. Small studies administered rifaximin, a gut-specific antimicrobial agent, to patients with NASH and showed a mild reduction in body mass index, endotoxemia, and liver enzymes at doses of 1200 mg per day[224,225], while there was no effect for lower doses[226].

FMT consists of collecting stools from a healthy donor and transferring it to a patient. The transference can be accomplished by colonoscopy, nasogastric tube, enema, or gastric-resistant pills[42]. Small studies already tested the effect of FMT on the metabolic syndrome and showed benefits in glucose and lipid metabolism, albeit with no effect on body weight[126,127,227,228]. Regarding MAFLD, a small pilot study performed FMT in 21

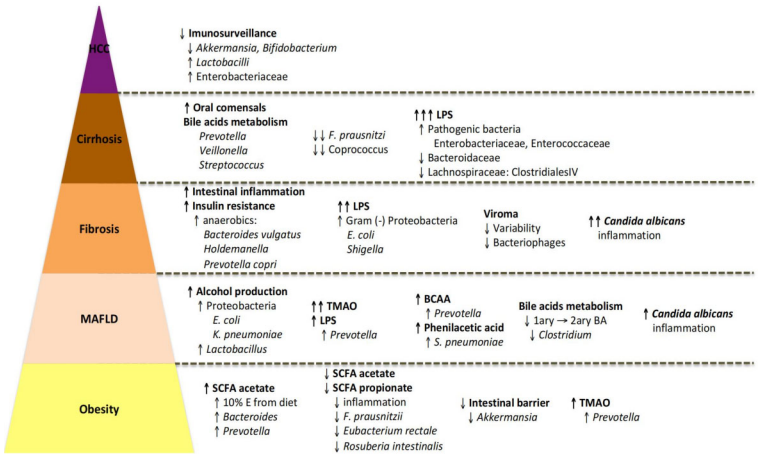

Figure 1. Step-by-step acquisition of unhealthy patterns of gut dysbiosis with the severity of liver disease in patients with MAFLD. TMAO: Trimethylamine-N-oxide; MAFLD: metabolic-associated fatty liver disease; HCC: hepatocellular carcinoma; LPS: lipopolysaccharides; SCFA: short-chain fatty acids.

patients and did not achieve improvements in IR or liver fat content, but a reduction in small intestine permeability was achieved, suggesting a beneficial effect on the intestinal epithelial barrier[229].

An innovative way to modulate microbiota is through bacteriophages therapy. Bacteriophages are ubiquitous viruses that can infect and destroy bacteria, often with species-level specificity. This approach has not been studied in MAFLD[230]. However, preclinical data on alcoholic hepatitis approached with a bacteriophage that specifically targets cytolytic *Enterococcus faecalis* show promising results[231].

CONCLUSIONS

Dramatizing, we are only 50% humans, since roughly as many bacteria as the human cells that compose our body colonize us. Half of the microbes have profound effects on our health and disease pathogenesis. This is the case for the development of the metabolic syndrome and its liver associated disease, MAFLD. Furthermore, gut dysbiosis has the ability to promote MAFLD by direct steatogenic metabolic syndrome-independent effects.

The study of gut microbiota has benefited from the tremendous recent advances in molecular testing, and today we can fairly accurately characterize an individual's microbiota and microbiome. However, generalizations for a population and even more so for different populations are hampered by inter- and intra-individual differences, as well as geographic differences. As such, even though microbiota-targeted therapies for MAFLD are quite appealing, they are still far from clinical practice application. The field is moving towards a personalized microbiome targeted therapy, in which first we would characterize the individual gut microbiota, and afterwards, we would modulate the microbiota in a species-specific fashion in order to modulate the individual gut microbiota into a healthier, metabolically beneficial, and anti-steatogenic flora.

DECLARATIONS

Authors' contributions

Conceptualized the manuscript: Machado MV
Wrote the manuscript: Machado MV, Sousa P

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All authors declared that there are no conflicts of interest.

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