Role of Intestinal Microbiota in Pathogenesis of NAFLD

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The term “gut microbiota” refers to the microorganism community residing in the intestinal lumen, while the term “gut microbiome” refers to the entire ecological habitat, including the microorganisms as well as their genomes and the surrounding environmental conditions. There has been a growing body of evidence linking the presence of intestinal dysbiosis to the pathogenesis of human liver disease, with a primary focus on metabolic diseases, including non-alcoholic fatty liver disease (NAFLD).

Keywords: NAFLD ; NASH ; microbiome ; probiotic

1. Definition of Intestinal Microbiota

The term “gut microbiota” refers to the microorganism community residing in the intestinal lumen, while the term “gut microbiome” refers to the entire ecological habitat, including the microorganisms as well as their genomes and the surrounding environmental conditions [1]. The adult gut microbiota includes an average 10^{13} bacterial cells, resulting from more than 250 different species of bacteria, fungi, viruses, and archaea [2]. The human intestinal microbiota is mainly composed of bacteria from the *Firmicutes* (60 to 80%), the *Bacteroidetes* (20 to 40%), the *Proteobacteria,* and the *Acinetobacteria* phyla, with high variability among individuals [3][4]. Overall, a wide range of factors may influence the composition and functionality of the gut microbiome, including environmental, immunological, or host factors, as well as alteration in bile flow, gastric pH, or intestinal dysmotility. However, although gut microbiota composition can be modulated by such factors, this is relatively stable in the long term [5]. Of note, the relationship between the host and the gut microbiota is symbiotic and plays a crucial role in modulating the health status. The term ‘dysbiosis’ has sometimes been used to refer to a perturbation of the gut microbiota compared to the ‘normal’ or ‘healthy’ state, although this is an imperfect term since defining the normal, healthy microbiota is itself an area of ongoing debate [4]. Nevertheless, a range of dysbiotic microbiome ‘signatures’ have been associated with a variety of disease states.

2. The Role of the Intestinal Microbiota in the Pathogenesis of NAFLD

2.1. Microbiome Composition

Over the last decade, there has been a growing body of evidence linking the presence of intestinal dysbiosis to the pathogenesis of human liver disease, with a primary focus on metabolic diseases, including non-alcoholic fatty liver disease (NAFLD). Preliminary studies associated non-alcoholic steatohepatitis (NASH) with small intestinal bacterial overgrowth in human subjects [6]. Further animal experiments involving the manipulation of the gut microbiome offered then the strongest evidence, supporting the role of dysbiosis in NAFLD. A specific microbiome composition was associated with increased intestinal energy harvest from the diet in obese mice. Interestingly, this trait was shown to be transmissible to lean, germ-free mice via microbiome transfer [7]. Furthermore, insulin resistance (IR) per se could be ameliorated after the administration of antibiotics [8]. In human studies, when obese men with metabolic syndrome received FMT from lean donors, they showed a significant improvement in IR and an enrichment in their stool of butyrate-producing intestinal microbiota [9].

Over the last years, there has been an explosion of studies exploring modifications in the microbiome and their association with liver disease in NAFLD. A summary of the main changes described in NAFLD is summarised in **Table 1**. Overall, at the phylum level, an increased abundance of *Proteobacteria* and *Firmicutes*—as well as a reduction in *Bacteroidetes* and *Prevotellaceae*—has been noted in the gut microbiome of patients with NAFLD compared to healthy controls [10][11][12][13]. Notably, the majority of the studies have focused on comparing healthy controls vs. patients with NASH or with simple steatosis, as well as comparing different grades of steatosis. It should also be noted that studies comparing the bacterial taxonomic composition of patients with NAFLD vs. those with NASH produced variable and even contradictory findings, as a result of differences in the cohorts analysed and in the methods used to assess liver disease [14]. Unfortunately, there is only small evidence exploring specific changes in gut microbiota with regards to fibrosis stage

in NAFLD, despite this being the main predictor factor in these patients. Furthermore, disentangling the microbial signatures from another co-existing metabolic disease from NAFLD may be challenging [11].

Table 1. Summary of the main alterations of the intestinal microbiota previously described in patients with NAFLD and NASH. The table summarises the main finding from recent studies exploring the association between changes in the microbiome in patients with NAFLD [10][11][12][13].

Disease Severity	Bacterial Microbiota Changes	
NAFLD vs. healthy controls	Phylum	↑ ***Proteobacteria***
	Family	↑ ***Enterobacteriaceae*** ↓ ***Rikenellaceae, Rhuminococcaceae***
	Genera	↑ ***Escherichia coli***, ***Dorea, Peptoniphilus*** ↓ ***Anaerosporobacter, Coprococcus*** ***Eubacterium***, ***Faecalibacterium***, ***Prevotella***
Severe steatosis or NASH vs. controls or mild steatosis	Phylum	↑ ***Fusobacteria***
	Family	↑ ***Enterobacteriaceae*** ↓ ***Prevotellaceae, Clostridiaceae***
	Genera	↑ ***Bacteroides, Ruminococcus***, ***Shigella***, ***Escherichia coli*** ↓ ***Clostridium***

Abbreviations: NAFLD: non-alcoholic fatty liver disease, NASH: non-alcoholic steatohepatitis.

2.2. Short-Chain Fatty Acids

Short-chain fatty acids (SCFA)—such as acetic, propionic, and butyric acid—play a crucial role in modulating the interaction between the host and the gut microbiota. Specifically, SCFA are major products of fermentation of undigested carbohydrates (and amino acids) by gut microorganisms up to a daily production of 50–100 mmol/l [15]. The SCFA influence the energetic metabolism, the immune response, and the expansion of the adipose tissue [16]. Many of the effects of the SCFA are mediated via G-protein coupled receptors (GPCRs), which are mainly expressed in the immune system cells, the adipocytes, and the intestinal endocrine cells. Within the intestine, SCFA act on GPCRs, slowing gastric emptying, intestinal transit, and nutrient absorption [17]. Moreover, specific SCFA, such as butyrate, might also suppress inflammation directly as a result of their interaction with T regulatory cells in the intestinal mucosa [18][19].

Interestingly, SCFA were able to reduce the amount of hepatic steatosis, via modulating fatty acid synthetase activity and hepatic lipid synthesis in mice fed with a high-fat diet. In the same model, there was also a two-fold increase in hepatic lipid oxidation in the SCFA-fed mice, mainly due to an enhanced lipid oxidative state [20]. Despite clear results arising from animal models, the role of SCFA in altering the energy harvest has been less elucidated in humans. An early study reported a lower faecal energy excretion in those with obesity when compared with lean ones [21]. Among others, Bacteroidetes are the main contributors to the production of SCFA, with changes in their abundance impacting the level of SCFA. Specifically, it has been demonstrated that a 20% decrease in faecal Bacteroidetes and a correspondent increase in Firmicutes translates into a 150 kcal increase in energy harvest from the diet [7][22]. Of note, such functional change in the microbiota composition can occur after a few days of overeating, hinting at the presence of a dynamic response with caloric intake. On a similar note, another study including adults with NAFLD showed an association between the presence of NASH and an increased percentage of *Firmicutes* vs. a reduced percentage of *Bacteroides*, after adjusting for BMI and dietary fat intake [23]. Not only microbiota but also diet may influence the production of SCFA. Specifically, it is well known that dietary fibres represent an important source of SCFA. Moreover, high-fibre diets may promote the Bacteroidetes phylum, *Prevotella*, whereas high-fat diets reduce diversity and promote Firmicute growth [24].

2.3. Bile Acids

Bile acids (BAs) are potent “digestive surfactants” that promote the absorption of lipids, including fat-soluble vitamins. Moreover, BAs are involved in the primary pathway for the metabolism of cholesterol and account for ~50% of its daily turnover [25]. BAs are mainly synthetised in the liver, resulting from the conversion of cholesterol into more water-soluble compounds [26]; BAs are then secreted into the hepatic canaliculi and stored in the gallbladder. After excretion and the digestive process, about 95% of the BAs are re-absorbed from the terminal ileum, while only 5% reach the colon. In the colon, the remaining fraction of BAs is passively reabsorbed after modifications, i.e., deconjugation and oxidation. The intestinal microbiota is actively involved in modulating the pool of circulating and excreted BAs, which in turn participate

actively in hydrolysis and dehydrogenation reactions [27]. Overall, BAs display several functions as they are involved not only in the digestion and absorption of lipids, but they also act as signalling molecules modulating the metabolism of glucose and lipids through the farnesoid X receptor (FXR) and the C protein-coupled bile acid receptor TGR5 [28]. In the liver, FXR activation results in the downregulation of free fatty acid (FFA) synthesis and de novo lipogenesis [29]. FXR is also involved in carbohydrate metabolism, as this regulates hepatic gluconeogenesis, and prevents hepatic inflammation [30].

An increased level of BAs has been widely demonstrated in liver tissue [31], plasma [31][32], and faeces [32] in patients with NASH. There is unanimous consensus that higher levels of serum BAs in patients with NASH and NAFL are mainly driven by increased levels of conjugated BAs, while evidence on secondary BAs is still conflicting [33]. A large body of work has demonstrated a dysregulation of BAs metabolism in patients with NASH, including elevated primary conjugated BAs, decreased levels of specific secondary BAs, and alteration of excreted BAs [31][32][34]. Moreover, the expression of BAs transporters also seems to be impacted in patients with NASH or simple steatosis [35][36]. Specifically, the concentrations of cholic, chenodeoxycholic, and deoxycholic acids were significantly increased in the liver of patients with NASH compared to controls [32]. Moreover, cholic acid has been strongly associated with inflammatory markers, with deoxycholic acid showing an opposite trend [32]. Interestingly, a recent study suggested that there might be a specific trend in taurine-conjugated vs. glycine-conjugated BAs, with the first being elevated and the latter suppressed in patients with NASH [37]. Nevertheless, it should be noted that many studies have not accounted for confounding factors such as obesity and insulin resistance, which exert an independent influence on BAs metabolism.

2.4. Other Gut-Derived Metabolites

Another postulated mechanism linking the microbiome to NAFLD is its effects on the stimulation of adipose tissue. Specifically, disturbances of the microbiota can result in changes in the production of the intestinal form of fasting-induced adipocyte factor (FIAF). FIAF is a secreted protein which inhibits lipoprotein lipase (LPL) in several extra-intestinal sites, such as white adipose tissue, brown adipose tissue, muscles, and hepatocytes [38]. Inhibiting intestinal FIAF has been linked to increased lipolysis in the adipose tissue and to reduced fatty acid oxidation in the muscles [38]. In the liver, FIAF inhibition results in the activation of lipogenic enzymes and increased fat accumulation [39][40].

Finally, several studies have also demonstrated that the gut microbiota may influence host metabolism in NASH, following an augmented production of dietary ethanol. An early study linked dysbiosis with increased production of ethanol from the intestine; for example, 1 gr of Escherichia coli was able to produce 0.8 gr of ethanol per hour in anaerobic conditions [41]. Additionally, Proteobacteria—a phylum which includes alcohol-producing bacteria—were found to be substantially increased in the gut of patients with NASH [42]. Interestingly, ethanol per se may contribute to liver injury by increasing intestinal permeability and lipopolysaccharide (LPS) levels in the portal circulation, ultimately triggering inflammation [43]. Furthermore, the gut microbiome may elicit the inflammatory response in the hepatocytes and macrophages directly via increased flux of tryptophan metabolites through the portal system [44]. Some other molecules, i.e., ethanol and choline-related metabolites, have also been described as contributors to the development of NAFLD [45].

Given the recent exploration of the role of the microbiota in NAFLD, there has been an increasing interest in evaluating the manipulation of the intestinal microbiome as a potential treatment option for patients with NAFLD. In this sense, different strategies have been investigated, including the use of nondigestible prebiotics, probiotics, and symbiotics [46].

2.5. Effect of Microbiome on Insulin Resistance and Adipose Tissue

Several studies have suggested that the gut microbiome may modulate insulin resistance and adipose tissue. Specifically, metabolic inflammation represents the most important player in the way intestinal bacteria have to modulate energy homeostasis. A 40% reduction in microbiome diversity was associated with low-grade systemic inflammation and adipokines production, mainly mediated by LPS production [47]. Similarly, other bacterial products may elicit inflammation and insulin resistance, activating the toll-like receptor (TLR) pathway [48]. The SCFA production also has a direct effect on insulin resistance, as butyrate and propionate may modulate gluconeogenesis and de novo lipogenesis [49]. Moreover, results from the animal model have shown how intestinal bacteria may influence adipose tissue differentiation, with some species favouring brown over white adipose tissue [50][51].

Interestingly, by modifying a dietary pattern, it is possible to change the effect that the microbiome has on insulin resistance. It has been demonstrated that calorie restriction increases *Lactobacillaceae* and reduces *Firmicutes* and *Bacteroidaceae*. Interestingly, a regime of every-other-day fasting led to similar changes [52]. Among others, *Akkermansia muciniphila* appears to be one of the species mediating the beneficial effects of calorie restriction, as it reduces adiposity and increases insulin resistance [53].

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