*Carcinogenesis*, 2017, Vol. 38, No. 3, 231–240

**doi:10.1093/carcin/bgx007**
Advance Access publication January 25, 2017
Review

REVIEW

# Microbiota, NASH, HCC and the potential role of probiotics

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## Abstract

Hepatocellular carcinoma (HCC) accounts for the majority of primary liver cancers. Clearly identifiable risk factors are lacking in up to 30% of HCC patients and most of these cases are attributed to non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH). Beyond the known risk factors for NAFLD, the intestinal microbiota, in particular dysbiosis (defined as any change in the composition of the microbiota commonly found in healthy conditions) is emerging as a new factor promoting the development of chronic liver diseases and HCC. Intestinal microbes produce a large array of bioactive molecules from mainly dietary compounds, establishing an intense microbiota–host transgenomic metabolism with a major impact on physiological and pathological conditions. A better knowledge of these 'new' pathways could help unravel the pathogenesis of HCC in NAFLD to devise new prevention strategies. Currently unsettled issues include the relative role of a 'negative microbiota' (in addition to the other known risk factors for NASH) and the putative prevention of NAFLD through modulation of the gut microbiota.

## Introduction

Hepatocellular carcinoma (HCC) is the third cause of cancer death worldwide (1). More than 80% of HCC cases occur in less developed countries, particularly East Asia and Sub-Saharan Africa, and are typically associated with chronic hepatitis B (HBV) and C (HCV) infection, although the incidence in these countries is decreasing (2,3). The incidence of HCC in Western countries is increasing (4,5) and coincides with the growing epidemics of obesity and type 2 diabetes (6). These two conditions are clearly associated with the development of non-alcoholic fatty liver disease (NAFLD), considered the most common form of chronic liver disease in the Western world (7,8).

NAFLD is characterized by lipid deposition in the hepatocytes and is considered the hepatic manifestation of metabolic syndrome. NAFLD includes different clinicopathologic conditions ranging from simple hepatic steatosis to non-alcoholic steatohepatitis (NASH) (7,9). While most patients with NAFLD remain asymptomatic, 20% develop chronic hepatic inflammation, which in turn can lead to cirrhosis, portal hypertension, HCC and increased mortality (10). In these patients, the risk of HCC ranges from 2.4% over 7 years to 12.8% over 3 years of follow-up (11,12). The future will see a shift among the main causes of HCC. The incidence of viral forms is decreasing thanks to distribution of the HBV vaccine and the new therapies for

**Received:** August 5, 2016; **Revised:** December 2, 2016; **Accepted:** January 15, 2017

**Abbreviations**

| | |
|---|---|
| DCA | deoxycholic acid |
| FMT | fecal microbial transplantation |
| FXR | farnesoid X receptor |
| HCC | hepatocellular carcinoma |
| HPC | hepatic progenitor cell |
| LPS | lipopolysaccharide |
| NAFLD | non-alcoholic fatty liver disease |
| NASH | non-alcoholic steatohepatitis |
| SASP | senescence-associated secretory phenotype |
| TLR | toll-like receptors |

HCV, whereas the non-viral forms of HCC are on the rise, in particular HCC related to NAFLD/NASH. In the coming years, these forms will become the predominant causes of HCC.

During the last two decades the association between NAFLD, NASH and HCC and the progression from NAFLD/NASH to liver cancer has been a growing area of study (13). A multiple-hit process was recently proposed with successive liver insults leading from fatty accumulation to inflammation and fibrosis (14,15). In particular, the relationship between liver and gut may also play a crucial role in this complex network of multiple interactions (16).

The aim of this review is to summarize current knowledge on the potential role of the intestinal microbiota in the pathogenesis and development of chronic liver diseases, in particular NAFLD, and the subsequent development of HCC.

## Intestinal microbiota: a new 'organ'

### General assessment

In recent times, the neglected and amorphous mass of intestinal bacteria has been promoted to the dignity of an actual organ interacting with other organs of the body both in physiology and pathology.

The human colon harbors bacteria that reside within and colonize the gastrointestinal tract (i.e. autochthonous bacteria), or pass transiently through it (i.e. allochthonous bacteria, probiotics). Autochthonous bacteria can be classified as dominant or subdominant depending on their concentration (17). Only a restricted number of bacterial phyla colonize the gut. A member of the intestinal microbiota has to fulfill several requirements: a metabolic apparatus fit for available nutrients, the ability to escape the host immune response and to replicate quickly enough to avoid expulsion through the anal canal. The dominant microflora belongs to at least five bacterial phyla: *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, *Proteobacteria* and *Verrucomicrobia*. The core ecosystem is dominated by six genera of strict anaerobes: *Bacteroides*, *Eubacteria*, *Bifidobacteria*, *Clostridia*, *Peptostreptococci* and *Ruminococci*, while the most represented aerobic bacteria belong to the genera *Escherichia*, *Enterococcus*, *Streptococcus* and *Klebsiella* remains largely subdominant. Broadly, *Firmicutes* account for about 60–80% of the microbiota of an adult, while the remaining 20–40% are *Bacteroidetes* (18). More than 1000 different bacterial species have been isolated in the human intestine, and more than 50 species are common to >90% of subjects (19).

Under physiological conditions, autochthonous intestinal bacteria are involved in the catabolism of several elements derived from diet or from endogenous secretions: they can modulate the expression of host genes participating in several pathological functions and also interfere with the immune system. The intestinal microbiota is also involved in inflammation mechanisms, redox stress damage, motility, angiogenesis, proliferation, differentiation, fat storage regulation, carcinogenesis, cancer-response to chemotherapy and even cognitive function (20,21,22).

A significant inter-individual variability of intestinal microbiota exists and the host genotype is probably a key factor in this variability (23). Using a metagenomic approach, Arumugam *et al.* (24) showed that notwithstanding its inter-individual variability, the microbiota is not built in a random fashion, but is stratified along three main clusters (so-called enterotypes) based on corresponding *Bacteroides*, *Prevotella* and *Ruminococcus* genera. These three enterotypes utilize different routes to extract energy from fermentable colonic substrates. Besides, microbial communities of the gastrointestinal sites are largely different along the length of the digestive tract (25).

The microbiota of human adult is quite stable over prolonged periods of time (26), whereas in old age it is less stable over a limited time, with an imbalance of the main phyla caused by a decrease of *Faecalibacterium prausnitzii*, which show anti-inflammatory properties (27,28). Due to this intra-individual stability of microbiota as opposed to its extraordinary inter-individual variability, every subject has a unique and distinct microbial pattern.

Interestingly, some temporal variability in microbial composition has been demonstrated in inbred mice in relation to diet changes (29). Both luminal and mucosal adherent microbiota of human flora-associated mice are quite different when animals are fed with a low-fat or a high-fat/sugar 'Western' diet, with a relative increase in bacteria belonging to *Firmicutes* phyla and a reduction of *Bacteroidetes* (30). Switching from a low-fat to a 'Western' diet shifted the structure of microbiota and changed its gene expression and metabolic pathways in a few hours (29). Population studies conducted with metagenomic approaches showed that diet-induced microbiota changes exist, although there is a stable metabolic core among individuals (31,32). More recently, changes in the composition of the microbiota may also occur in humans, only one day after changes in diet (33).

### The role of dysbiosis

Recent studies have shown the emerging role of *dysbiosis* (defined as any change in the composition of the microbiota than that commonly found in healthy conditions) in the pathogenesis of several diseases, including inflammatory bowel disease, allergies and metabolic disorders (34,35,36). The increase in some specific bacteria facilitates the metabolism of absorbed calories, with a progressive development of obesity in the host. The *ob-ob* mice (homozygous for the obese mutation) have an imbalance of the intestinal microbiota compared to the respective wild-type, with an increase in *Firmicutes* and a decrease of *Bacteroides* (so called 'obese microbiota'), hence the increased capacity to harvest energy from diet (37). In particular, the *Bifidobacterium spp*. and *Eubacteruim rectale/Clostridium coccoides* group were significantly reduced in high-fat diet-fed mice compared with a control group (38). A similar difference was found in human obesity with a rise in the microbiota ratio to *Firmicurtes/Bacteroidetes* and re-equilibrium to the benefit of Bacteroidetes in case of a fat restriction diet (39).

Several lines of evidence have also demonstrated the role of dysbiosis in the development of NAFLD, although very little is known about the real composition of intestinal microbiota in these patients. Zhu *et al.* showed that *Proteobacteria*, *Enterobacteriaceae* and *Escherichia* (at phylum, family and genus levels, respectively) were significantly elevated in NASH children, compared with healthy or obese subjects (40). Recently,

another study revealed a different microbiota composition among healthy, simple steatosis and NASH patients: the authors showed a reduction in *Bacteroidetes* in NASH patients compared with the other groups. A lower percentage of this phyla may facilitate the growth of other bacteria increasing the energy intake from dietary fat (41). Qin and colleagues characterized the gut microbiome in liver cirrhosis, comparing 98 patients and 83 healthy individuals. At phylum level, patients with liver cirrhosis had fewer *Bacteroidetes* but higher levels of *Proteobacteria* and *Fusobacteria* than controls (42), without distinguishing patients with NASH or virus-related cirrhosis. The true role of the microbiota in these two different forms remains unclear, but changes in the microbiota are considered a marker of cirrhosis.

## Transgenomic metabolism of dietary compounds

In this light, it is important to know the role of the various phyla/genera/species of bacteria in maintaining a proper and healthy metabolism or inducing pathological changes predisposing to the metabolic syndrome (obesity, diabetes, NASH).

Complementing several gaps in our metabolic pathways (19), intestinal microbes produce a vast array of bioactive molecules from any dietary compound reaching the colon, establishing an intense microbiota-host transgenomic metabolism with a tremendous impact on our physiology and nutritional state (Figure 1) (43,44). In particular, intestinal microbiota fermentation of indigestible plant polysaccharides involves a remarkable level of syntrophism and metabolic cross-feeding, where primary and secondary fermenters act in concert (45). Plant cell wall polysaccharides—such as hemicellulose, pectins and xylans—can reach the colon solubilized or trapped in the plant cellulose matrix. While the cellulose matrix is degraded by specialized cellulolytic *Ruminococci*, producing acetate and propionate from cellulose, the soluble cell wall polysaccharides are readily metabolized by butyrate producers of the *Clostridium* clusters IV and XIVa (*Faecalibacterium prausnitzii*, *Butyrrivibrio*, *Roseburia* and *Eubacterium rectale*). Conversely, resistant starches are preferentially fermented to propionate, acetate and succinate by *Bacteroidetes* (46,47). These microorganisms are also able to ferment host mucus polysaccharides and plant cell wall polysaccharides, shifting from different carbon sources depending on carbon bioavailability (48,49).

Besides the short chain fatty acids (SCFA) acetate, propionate and butyrate, primary polysaccharide fermenters of the intestinal microbiota produce $H_2$. Molecular hydrogen is the principal energy resource for secondary fermenters of the gut microbial community (50). Acetogens like *Blautia hydrogenotrophica*, sulfate-reducing bacteria like *Bilophila wadswortia* and the methanogen *Methanobrevibacter smithii* can all metabolize $H_2$ producing different endpoint molecules, such as acetate, $H_2S$ and $CH_4$, respectively. Finally, acetate produced by primary and secondary fermenters can be metabolized to butyrate by members of the *Clostridium* clusters IV and XIVa, establishing a balanced syntrophy among members of the intestinal microbial communities (47). Differently from plant polysaccharides, which support a highly sintrophyc metabolic network of several interconnected bacterial groups leading to few endpoint metabolites, essentially SCFA, the amino acids are metabolized by few selected intestinal bacteria in a linear metabolism, resulting in a vast range of possible outputs: SCFA and branched-chain fatty acids (BCFA) but also phenolic and indolic metabolites and metilammines. In particular, the metabolism of dietary amino acids by intestinal microbiota involves proteolytic clostridia, such as members of the *Clostridium* clusters I and XI (51,52), and *Bacteroidetes* (33). Members of enterococci and enterobacteria, a sub-dominant bacteria species, can also efficiently metabolize amino acids. The microbiota metabolism of amino acids involves the production of a vast range of bacterial metabolites, depending on the type of amino acid fermented (52). For instance, the fermentation of simple aliphatic amino acids results in the production of methylamines and a relatively small amount of SCFA, while branched-chain amino acids result in the production of BCFA. Conversely, microbiota metabolism of aromatic amino acids generates a variety of phenolic and indolic metabolites (53).

The microbial metabolites derived from the metabolism of dietary compounds modulate several traits of the host physiology (44,54,55). In particular, acetate, propionate and butyrate

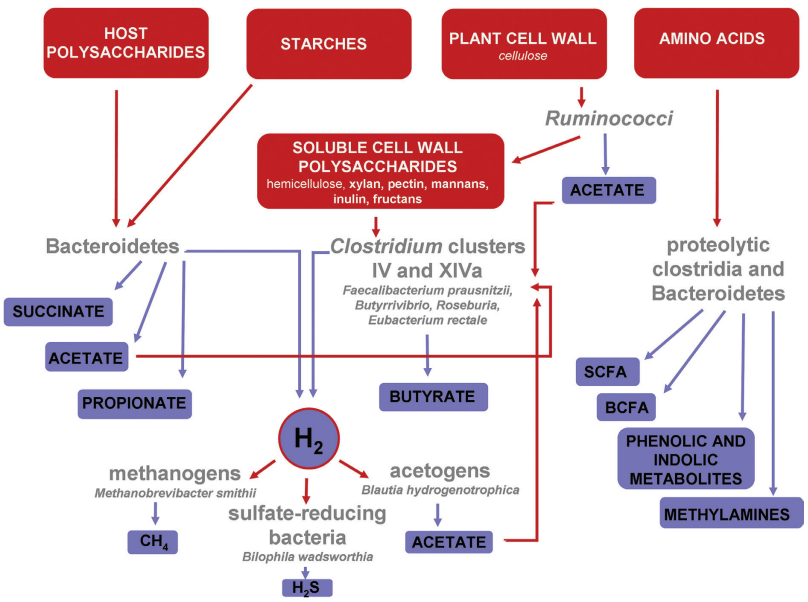


**Figure 1.** The metabolism of different substrates by microbiota. Figure depicts the metabolism of different substrates by the human intestinal microbial community. Fermented substrates are represented in red and corresponding products in blue. The bacterial groups principally involved in the processes are shown in gray.

can regulate different aspects of our nutritional and immunological state. While butyrate represents an important energy source for host colonocytes (54,56), acetate and propionate regulate lipid synthesis in the liver (55) and intestinal gluconeogenesis (57). Further, supporting insulin secretion, butyrate is also involved in the regulation of host energy storage, and by enhancing the production of leptine and peptide YY (PYY) it regulates appetite control (44). SCFA are strategic modulators of the immune function. Butyrate acts both locally, throughout the regulation of pro-inflammatory cytokine production in the gut (58) and systemically, by modulating extrathymic Treg generation (59). Propionate governs *de novo* peripheral Treg generation and, together with acetate, drives Treg homing in the colon. Propionate has also been reported to enhance the hematopoiesis of dendritic cells with an impaired Th2 activation (59). Conversely, phenolic and indolic metabolites generated by the bacteria metabolism of aromatic amino acids in the gut have been linked to immune activation and diabetes because of the increased expression of pro-inflammatory cytokines (e.g. interleukine-8, IL-8) (53).

Analogously, the production of methylamines from aliphatic amino acids has been associated with diabetes, obesity and NAFLD/NASH through the production of formaldehyde and $H_2O_2$ that can induce inflammation and chronic oxidative stress (60). Finally, the outcome metabolites produced by microbiota secondary fermenters are extremely relevant to host health. While acetate produced by acetogens supports butyrate producers in a feedback process, sulfate reducers are detrimental to host health. Indeed, by producing $H_2S$ from $H_2$, sulfate-reducing microorganisms weaken the gut epithelium, supporting metabolic endotoxemia and inflammation (61).

Via a 'diet-microbiota-host axis', different dietary substrates can modulate intestinal microbiota composition and the correspondent metabolome, with an impact on a vast range of host physiological traits. Favoring fibrolytic microbiota components, complex polysaccharides mainly results in the production of beneficial SCFA. Differently, a high protein intake would support a putrefactive metabolism, resulting in the production of a vast array of harmful metabolites. Finally, resulting in an increased bile acid secretion, saturated fats stimulate the bile-resistant sulfate-reducing gut bacteria *B.wadsworthia* forcing an inflammatory boost due to an increased $H_2S$ production (62).

As well as diet, stressors like antibiotics can affect the intestinal microbiota whose balance is changed after antibiotic treatment (63). It is extremely important to note that reaching a new balance cannot be predicted 'a priori'. Irrespective of diet, stress-induced changes in the gut microbiota caused by antibiotics or chemotherapy might alter the metabolic pattern from NASH-protective to NASH-prone (or vice versa). However, Reijnders *et al.* (64) recently failed to detect any metabolic or inflammatory changes resulting from the antibiotic-dependent gut microbiota shrinkage in obese subjects.

## Intestinal microbiota and liver diseases

A general agreement on the role of intestinal microbiota in colorectal cancer genesis has been reached (65,66), but only sporadic data exist on the linkage with other tumors. These data derive mainly from pre-clinical studies. Animal models have great importance in elucidating the role of microbiota in the pathogenesis of bowel diseases, but some concerns exist in translating these data to humans (65,67).

Some studies showed a strong relationship between liver and gut: the portal system receives blood from the gut, and intestinal blood content can be involved in the induction and progression of liver damage in several chronic liver diseases (68,69). Alterations in intestinal microbiota seem to play an important role in the development of NAFLD and multiple molecular pathways have been postulated to explain the relationship between NAFLD and dysbiosis (Figure 2) (70).

As stated above (71,72), several changes in the composition of intestinal microbiota occur in the course of obesity, including an increase in the *Firmicutes/Bacteroidetes* ratio and a reduction of gut bacterial richness. This 'obese microbiota' increases the capacity to harvest energy from the host diet (37) and promotes the *de novo* hepatic lipogenesis suppressing the expression of fasting-induced adipose factor (Fiaf), a selected lipoprotein lipase (LPL) inhibitor (73,74). Comparing germ-free knockout (Fiaf–/–) and wild-type mice fed with Western diet, Fiaf-deficient mice gained significantly more weight and higher epididymal fat-pad LPL activity than controls (73). These findings demonstrated that adipocyte LPL activation leads to increased lipogenesis and fat storage. The result is a vicious cycle that feeds on itself and favors the development of other metabolic disorders (e.g. insulin resistance) (75,76). Recent studies revealed an abundance of alcohol-producing bacteria (in particular *Escherichia ssp*.) in NASH microbiontes: the consequently increased blood alcohol concentration could promote hepatic oxidative stress and liver inflammation (40,77).

Obesity and NAFLD are also associated with small intestinal bacteria overgrowth of Gram-negative organisms and increased intestinal permeability through the disruption of gut barrier integrity (78). The intestinal barrier is made up of epithelial cells linked together through tight junctions, which play a pivotal role in maintaining this complex structure (79). Several studies suggested that the diet-induced changes in gut microbiota can alter the intestinal tight junction proteins (in particular, zonula occludens-1 and occludin) and promote intestinal inflammation, increasing the leaky gut (80,81]. Therefore, dietary factors and dysbiosis may affect intestinal barrier function and lead to the so-called microbial translocation (MT), defined as the migration of viable microorganisms or bacterial endotoxins, also called pathogen-associated molecular patterns from the intestinal lumen to the mesenteric lymph nodes and other extraintestinal sites (82). Intestinal microbiota is the primary source of bacterial endotoxins [lipopolysaccharide (LPS)] produced by Gram-negative bacteria. Normally, these bacterial molecules cross the mucosa only in trace amounts, enter the portal blood, and are cleared in the liver. Some mechanisms have been identified in this process that relies on a balance between the barrier functions of the gut and the detoxification ability of the liver (15,83,84,85). LPS and other endotoxins may influence intestinal permeability and activate molecular mechanisms of innate immune response, acting as possible inductor of necro-inflammatory lesions and severe fibrosis in NAFLD (84). A link between bacteria overgrowth and NAFLD/NASH was first demonstrated by Wigg et al (86). More recently, Miele *et al.* described an increased intestinal permeability in 35 NAFLD patients, showing that the increased leaky gut was caused by disruption of the intestinal tight junctions, confirming by decreased expression of the zonula occludens-1 protein in these patients compared with healthy subjects. Moreover, they found a higher prevalence of small intestinal bacteria overgrowth in NALFD patients, correlated with the severity of steatosis (87). This is the first evidence in humans of the relationship between gut permeability, tight junction alterations, small intestinal bacteria overgrowth and NAFLD.

The gut–liver axis is the route by which bacteria and their potential hepatotoxic products can easily reach the liver (88).

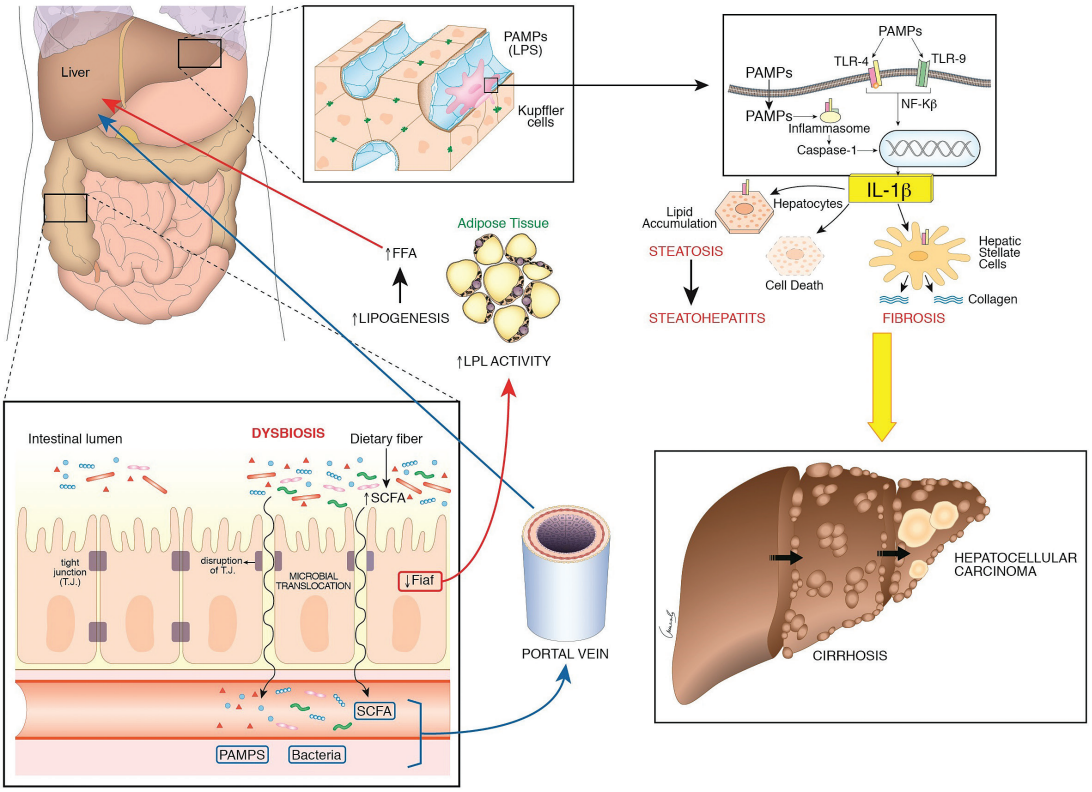


**Figure 2.** The putative role of gut microbiota in the progression of liver injury from steatosis to steatohepatitis and cirrhosis. Dysbiosis of the gut microbiota may lead to microbial translocation, defined as the migration of viable microorganisms or bacterial endotoxin (PAMPs, products of amino acid fermentation, SCFA) from the intestinal lumen to the mesenteric lymph nodes and other extraintestinal sites. The gut-liver axis is the route by which bacteria and their potential hepatotoxic products can easily reach the liver. The final effect is the production of pro-inflammatory cytokines (e.g., IL-1β and IL-8) that play a pivotal role in the induction and progression of non-alcoholic liver disease to NASH and cirrhosis. In particular, IL-1β promotes lipid accumulation and cell death in the hepatocytes, causing steatosis and inflammation and stimulates the hepatic stellate cells (HSCs) to produce fibrogenic mediators, resulting in fibrosis. The combination of oxidative stress and chronic inflammation inhibits hepatocyte proliferation and promotes the activation of human liver progenitor cells (HPCs). Once activated, HPCs produce several pro-fibrogenetic factors (e.i. transforming growth factor-β and platelet-derived growth factor) that stimulate the HSCs to produce collagen and promote the development of fibrosis. Both gut microbiota and HPCs play a key role in the pathogenesis of cirrhosis and HCC.

The final effect is the activation of the signaling cascade triggered by specific immune receptors which results in the expression of pro-inflammatory cytokine genes, including tumor necrosis factor-α (TNF-α), that may exacerbate hepatocyte damage (89,90). In the liver, the bacterial components stimulate toll-like receptors (TLR), a highly conserved family that recognize specific pathogen-associated molecular patterns and are expressed on Kupffer cells, bilary epithelial cells, hepatocytes, endothelial cells and dendritic cells (91). This TLR-endotoxin interaction results in the activation of nuclear transcription factors to release numerous pro-inflammatory mediators, which induce liver injury and fibrosis (92,93).

A breakdown in TLR tolerance also seems to contribute to the progression of NAFLD/NASH, and hepatic TLR expression is increased in these patients (94). Bacterial DNA, LPS and other endogenous mediators may activate the liver's innate immune system through TLR4 and TLR9 signaling, leading to Kupffer cell production of interleukin-1β (IL-1β). In turn, IL-1β promotes lipid accumulation and cell death in the hepatocytes, causing steatosis and inflammation, and stimulates the HSCs to produce collagen, resulting in fibrosis (95,96). Studies on TLR4-mutant mice showed reduced lipid accumulation following a high-fructose diet or methionine-choline deficient diet compared to their TRL4-wild-type controls, suggesting that LPS may contribute to disease progression (95,97). Furthermore, high-fat diets reduce hepatic natural killer T (NKT) cell numbers through hepatic IL-12 production, resulting in increased hepatic production of pro-inflammatory cytokines and the exacerbation of liver inflammation (98).

The inflammasome contributes to the pathogenesis of NAFLD. The inflammasome is a cytoplasmic multi-protein complex that recognizes a diverse set of inflammation-inducing stimuli that directly activate caspase-1. Activated caspase-1 triggers the release of strong pro-inflammatory cytokines such as IL-1β and/or interleukin-18 (IL-18), which are involved in the pathogenesis of most chronic liver diseases such as NAFLD/NASH (99,100). To date, five main inflammasome subtypes have been characterized: NLRP1 (NALP1), NLRP3 (NALP3, cryporin), NLRC4 (IPAF), AIM2 and NLRP6. They have different recognition sites and ligand specificity, but all culminate in caspase-1 activation (101,102). In particular, NLRP3 inflammasome is activated by microbial pathogen-associated molecular patterns and therefore is the principal inflammasome subtype involved in NAFLD progression, promoting insulin resistance and β-cell death (103). Csak et al (104) were the first to describe the role of NLRP3 inflammasome activation in NASH. They found inflammasome upregulation in high-fat diet mice, indicated by increased caspase-1 activity and higher serum levels of IL-1β compared to controls, but a similar increase in inflammasome gene expression in human NASH. In another study (105), ablation of NLRP3 inflammasome (NLRP3–/– mice) reduced the expression of IL-1β and IL-18 in diet-induced obese mice compared to wild

type mice. However, other studies reported the development of hyperphagia, obesity and insulin resistance in both IL-18–/– and ASC–/– (a component of NLRP3 inflammasome) mice fed a high-fat diet (103,106). These discrepancies may reflect the role of multiple inflammasome components in the various metabolic processes and further studies are needed to fully understand this association.

Recent evidence has demonstrated that dysbiosis could promote the development of NAFLD/NASH by modifying the bile acid metabolism. The bile acids synthesized from cholesterol by the liver modulate glucose and lipid metabolism through their binding. In turn, this activates the G protein-coupled receptor TGR5 and the hepatic nuclear farnesoid X receptor (FXR). This ultimately inhibits bile acid synthesis, lipogenesis and gluconeogenesis in the liver, and modulates immune function (107). Swann *et al.* (108) found less diversity and a predominance of taurine-conjugated bile acids (in particular, in the liver) in germ-free mice, compared with conventional animals. These changes in bile acid composition could influence NAFLD pathogenesis, in particular because of the alteration of taurine-conjugated bile acids (a FXR antagonist) (109). FXR-knockout (FXR–/–) mice fed with high-fat diet developed some features of NAFLD, such as hepatic steatosis and necroinflammation (110,111). Using a murine NAFLD model, McMahan *et al.* (112) showed that the administration of TGR5/FXR agonists in these mice improved NAFLD histology and decreased the hepatic inflammation, inhibiting the production of pro-inflammatory cytokines from macrophages. A recent Japanese study treated a pool of NAFLD mice with either antibiotic or tempol (an antioxidant), finding increased levels of taurine-conjugated bile acids that were able to inhibit the FXR pathway in the liver (113). Although these findings stem from animal models while human studies are still in progress, the evidence suggests dysbiosis and bile acids/FXR axis are involved in the development of NAFLD/NASH.

## Microbiota and development of HCC

Several lines of evidence suggested that the gut microbiota is also involved in the development of HCC, in particular by increasing LPS levels and creating a subsequent pro-inflammatory microenvironment in the liver.

Using toxic murine models of HCC, Yu *et al.* (114) demonstrated that a depletion of host microflora after an antibiotic treatment could suppress tumor formation, with a significant reduction of the number and size of HCC nodules in treated mice comparing with untreated mice. In line with these findings, a study by Dapito *et al.* (115) reported that mice growing in germ-free conditions developed fewer and smaller HCC than conventional mice, and a chronic treatment with a low, non-toxic dose of LPS led to a significant increase of the number and size of HCC. Moreover, the same authors showed that TLR4 might be directly involved in the pathogenesis of HCC, without significant differences in tumor incidence (114,115).

Obesity and high-fat diet are increasingly recognized as major risk factors for HCC, but the exact molecular mechanisms integrating these events remain unclear. Anyway, alterations of intestinal microbiota seem to play a key role in this pathogenetic pathway. Yoshimoto *et al.* (116) studied hepatocarcinogenesis in obese mice, showing that the administration of antibiotics and gut sterilization could decrease the development of HCC in treated mice, modulating the dysbiosis and the subsequent secretion of pro-inflammatory and pro-carcinogenetic factors. These data suggest that gut sterilization and antibiotic treatments could prevent the development of HCC, but did not lead to the regression of already established tumors.

The senescence-associated secretory phenotype (SASP), a process occurring in normal cells in response to stress conditions, has a crucial role in promoting obesity-associated HCC development in mice model (116,117). Senescence cells develop a secretory profile composed of inflammatory cytokines, chemokines and proteases, called SASP. Some of these factors block cancer development by arresting cell proliferation, but other SASP factors (e.g. IL-6 and IL-8) foster inflammation and tumorigenesis promotion, indicating that the SASP contributes positively and negatively to cancer development depending on the biological context (118,119). In obesity conditions, alterations of microbiota increase the levels of deoxycholic acid (DCA), a bacterial metabolite causing DNA damage. The enterohepatic circulation of DCA provokes the SASP phenotype in HSCs, which in turn secrete various inflammatory and tumor-promoting factors. These events, together with DCA activation of various cell signals, result in the promotion of HCC development in mice after exposure to chemical carcinogens, demonstrating the pivotal role of the DCA-SASP axis in HSCs in the pathogenesis of HCC.

Other factors seem to be involved in the progression of chronic liver diseases and HCC. Human hepatic progenitor cells (HPCs) have been studied in regeneration after severe hepatocellular necrosis (120,121,122), but recent studies show that this cell compartment is also activated in NAFLD (123,124). Activation of HPCs led to the production of several pro-fibrogenetic factors, such as transforming growth factor-β and platelet-derived growth factor, that activate the HSCs and boost the production of collagen (125). Roskams *et al.* studied murine models of fatty liver disease and human patients with NAFLD. The increased oxidative stress promoted replicative senescence in mature hepatocytes and expansion of progenitor cells in both mice and humans (126). Moreover, the degree of HPC activation seems to be correlated with the severity of chronic liver diseases (127,128,129).

In line with these data, several studies analyzed the precursor lesions of HCC and found that HPCs and intermediate hepatocyte-like cells were present in 50% of small cell dysplastic foci and hepatocellular adenoma (130,131). These findings support the hypothesis that some human HCCs arise from HPCs. Furthermore, HCCs expressing HPC markers (i.e. CK19) have a worse prognosis than HPC marker-negative HCCs (132,133). Although the available data suggest that HPCs are involved in fibrogenesis and NAFLD progression, and their activation in the setting of chronic liver disease may increase the risk for HCC, further studies are needed to better clarify the role of these cells in the liver's response to NAFLD injury, and hepatocarcinogenesis.

## New microbiota modulation strategies

Several pharmacological interventions have been tested for the treatment of NAFLD with varying success, but no drug therapy is currently recommended for this condition. To date, a combination of dietary modifications and increased physical activity remains the mainstay of NAFLD management (134,135).

Based on these gut–microbiota interactions, a novel therapeutic approach is to interfere with microbiota. Manipulation of the human intestinal microbiota is essentially based on the use of probiotics and prebiotics. Besides the traditional probiotic genera *Bifidobacterium* and *Lactobacillus*, a new group of probiotic bacteria, the so-called 'next generation probiotics' is currently emerging (136,137). These microorganisms represent a new

potential for therapeutic microbiota modulation and mainly belong to butyrate-producing members of *Clostridium* clusters IV and XIVa (eg. *Faecalibacterium prausnitzii*) or to the health-promoting mucin degraders *Akkermansia muciniphila* (138). On the other hand, probiotic strains can be tailored to different ages. For instance, probiotics specifically targeted to infants can be isolated from infant stools or human milk (139) or, analogously, adult and elderly tailored probiotic strains need to be isolated from healthy people at the corresponding ages. Beneficial modulation of the intestinal microbiota can also be obtained by community-based approaches such as administering synthetic microbial communities (140,141). Alternatively, the whole microbiota can be reconstructed by fecal microbial transplantation (FMT) (142,143), representing the process of transplanting the fecal bacteria community from a healthy individual to a recipient whose microbiota has been altered. With extraordinary potential in the treatment of *C.difficile* infection and colitis, the key factor in determining FMT success is the composition of the donor microbiota. Finally, specific dietary approaches can be designed to preserve ecosystem diversity and an helath-promoting saccharolytic metabolism (144).

Several animal studies demonstrated the profound effect of probiotics on NASH, reducing de novo fatty acid synthesis, metabolic endoxiemia and inflammation (145,146,147,148). Despite these findings, the potential effectiveness of the probiotics in human NAFLD patients is still unclear, because of the few numbers of trials achieved. Loguercio et al provided the first evidence that probiotic treatment with VSL≠3 could reduce the serum level of tramsaminases and improve some parameters of liver function in a group of patients with different type of chronic liver diseases (including 22 biopsy-proven NALFD) (149). Other studies demonstrated an improvement of hepatic histology and biochemical parameters in after probiotic treatments (in particular, *B.longum* or *Lactobacillus ssp*) (150,151), but few randomized controlled trials support the therapeutic use of probiotics in human. More recently, a randomized double-blind placebo-controlled trial studied 52 patients with NAFLD treated with symbiotic or placebo. A symbiotic supplementation in addition to lifestyle modification is superior to lifestyle modification alone for the treatment of NAFLD, at least partially through attenuation of inflammatory markers in the body (152).

To date, there are two trials still underway that evaluated the effect of symbiotic treatment of NAFLD, but the results are not yet available (NCT01680640 and NCT0258351).

## Conclusion

In Western countries, NAFLD/NASH will become the most common cause of liver cirrhosis and HCC due to the growing epidemic of obesity and metabolic syndrome. A better understanding of the pathogenic mechanisms underlying the development of NASH and NASH-related malignancies is therefore of paramount importance.

Intestinal microbiota and activation of HPCs seem to play a pivotal role in the induction and progression of liver damage in NAFLD/NASH. Specifically, dysbiotic microbiota may increase the capacity to harvest energy from the host diet, promote *de novo* hepatic lipogenesis, increase intestinal permeability and lead to translocation of both bacteria and endotoxins from the intestinal lumen to extra-intestinal sites. Moreover, intestinal bacteria produce a large array of bioactive molecules from mainly dietary compounds, establishing an intense microbiota-host transgenomic metabolism with a major impact on physiological and pathological conditions. The final effect is the production of pro-inflammatory cytokines (e.g., IL-1β and IL-8) playing a pivotal role in the induction and progression to NASH and cirrhosis.

The microbiota appears to be further involved in the pathogenesis of HCC, but the exact molecular mechanisms integrating these events remain unclear. The transition from a healthy to a dysbiotic NASH-prone microbiota may occur during childhood or in adulthood following antibiotic therapy, chemotherapy or other stresses. Establishing a NASH-prone microbiota may represent an additional risk factor in the development of both NASH and NASH-related malignancies in patients with metabolic syndrome. Even more interestingly, the role of the gut microbiota must be considered in patients who developed NASH in the absence of any known risk factors (i.e. lean patients without metabolic syndrome). These patients could develop illnesses as serious as liver cirrhosis and HCC mainly due to a dysbiotic microbiota.

In conclusion, a better knowledge of the factors involved in promoting inflammation and hepatocarcinogenesis in NAFLD is necessary to understand HCC pathogenesis in NAFLD and devise new therapies and/or preventive strategies. In particular, three main issues await definition: (1) the relative role of the microbiota vis-à-vis other well-known risk factors for NAFLD and HCC development; (2) the prospective identification (through metagenomic approaches) of bacterial biomarkers able to reveal patients at high risk for severe liver disease and/or HCC; (3) the potential role of the manipulating the NASH-prone microbiota to block or even reverse the carcinogenic process.

## Acknowledgements

G.B., S.D.L., M.C. and G.S. were responsible for drafting the manuscript and critical revision for content. M.A.P., S.B., F.T. and G.B. critically revised the manuscript for content.
*Conflict of Interest Statement:* None declared.

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