Chapter 2

Intestinal Microbiota, Nonalcoholic Steatohepatitis and Hepatocellular Carcinoma: The Potential Role of Dysbiosis in the Hepatocarcinogenesis

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Abstract

Introduction: Hepatocellular carcinoma (HCC) accounts for the majority of primary liver cancers. Approximately 5–30% of HCC patients lack a readily identifiable risk factor for their cancer, and most of these cases are attributed to nonalcoholic fatty liver disease (NAFLD) and nonalcoholic steatohepatitis (NASH).

Body: Recent lines of evidence have suggested the role of intestinal microbiota, in particular the dysbiosis, in the pathogenesis of chronic liver diseases, such as NAFLD/NASH. Intestinal microbes produce a large array of bioactive molecules from mainly dietary compounds, establishing an intense microbiota-host transgenomic metabolism with a great impact on physiological and pathological conditions. A derangement of intestinal microbiota may lead to microbial translocation of bacteria or their products in the liver, where endotoxins trigger inflammation, and hepatocellular damage, which in turn plays a key role in the development of HCC. The following liver injury and hepatocellular necrosis can promote the activation of a secondary proliferative pathway involving the hepatic progenitor cells (HPCs), a bipotential cell compartment that seems to contribute to hepatocarcinogenesis.

Conclusion: The aim of this chapter is to summarize current knowledge on the potential role of intestinal microbiota in the pathogenesis of NAFLD and the subsequent development of HCC.

Keywords: dysbiosis, nonalcoholic steatohepatitis, hepatic progenitor cells, hepatocellular carcinoma

1. Introduction

Nonalcoholic fatty liver disease (NAFLD) is a common form of a chronic liver disorder worldwide, with an estimated global prevalence of 25% among adults and ~10% among children [1, 2]. NAFLD is traditionally regarded as hepatic manifestations of metabolic syndrome and encompasses the pathological spectrum ranging from simple hepatic steatosis (so-called "nonalcoholic fatty liver or NAFL") to the more aggressive form nonalcoholic steatohepatitis (NASH), which can progress to cirrhosis and its associated complications, including liver failure and hepatocellular carcinoma (HCC) [3, 4].

HCC accounts for the majority of primary cancers of the liver, representing the fifth most common cancer and the third leading cause of cancer death [5]. Many risk factors, including hepatitis B (HBV), hepatitis C (HCV), and alcohol, are well established, but 5–30% of HCC cases lack a readily identifiable risk factor. The majority of these cases of HCC is attributed to NAFLD, in particular in Western countries, and coincides with the growing epidemic of metabolic disorders. Diabetes mellitus and obesity are known to play a pivotal role in the development and progression of NAFLD [6–8]. An increase in the body-mass index and emergence of diabetes mellitus have been associated with progression to cirrhosis, whereas a reduction in body weight, and improved glycemic control promote resolution of liver fibrosis. The risk of progression to end-stage liver disease is influenced by the severity of the underlying liver histopathology. Although most patients with NAFLD remain asymptomatic, 20% of them progress to chronic hepatic inflammation, which in turn can lead to cirrhosis, portal hypertension, and HCC [9, 10].

Recent evidence points to a new factor involved in the development and progression of NAFLD: the intestinal microbiota [11, 12]. Many authors show that patients with NAFLD are characterized by dysbiosis, defined as any change in the composition of the microbiota that deviates from the composition commonly found in healthy people [13]. Intestinal microbes produce a large array of bioactive molecules mainly from dietary compounds, thus establishing intense microbiota-host transgenomic metabolism with a strong influence on physiological and pathological conditions [14]. In this regard, it is important to know the role of the various phyla, genera, or species of bacteria in maintaining the proper (healthy) metabolism or in inducing pathological changes predisposing to metabolic syndrome (or obesity, diabetes, or NASH).

Dysbiosis of the intestinal microbiota increases the ability of bacteria to harvest energy from the host diet and intestinal permeability and may lead to translocation of bacterial endotoxins into the liver [15]. These endogenous mediators can initiate hepatic inflammation and exacerbate hepatocyte damage through production of proinflammatory cytokines. The final result is lipid accumulation in (and death of) hepatocytes, causing steatosis, inflammation, and stimulation of hepatic stellate cells (HSCs) to produce collagen, resulting in fibrosis and cirrhosis [16, 17].

There is a broad consensus regarding the association between dysbiosis and colorectal cancer [18–21]. In contrast, the associations of microbiota with NAFLD and cancers other than colorectal are less proven [22, 23]. As suggested by the strong relation between the liver and gut, the microbiota seems to be also involved in the pathogenesis and development of HCC, although the exact molecular mechanisms integrating these events remain unclear (**Figure 1**) [24, 25].

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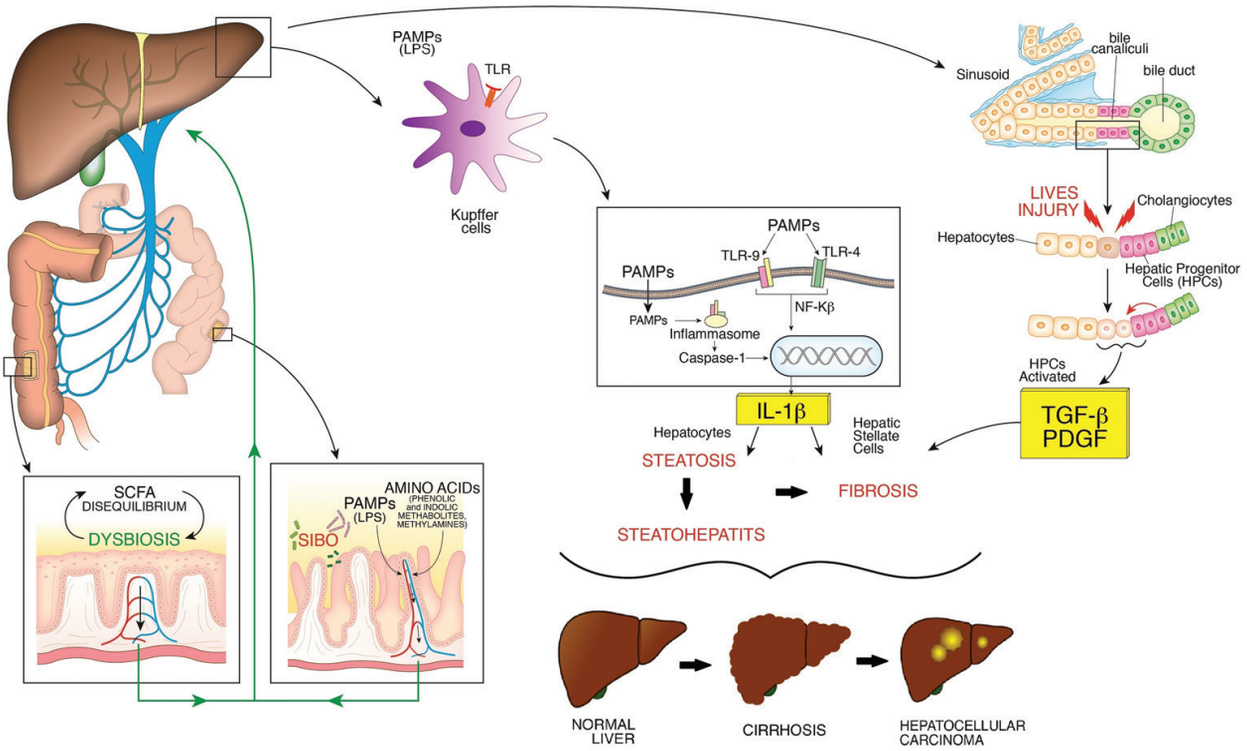

Figure 1. Role of intestinal microbiota and hepatic progenitor cells in the progression of liver injury from steatosis to steatohepatitis and cirrhosis.

Activation of hepatic progenitor cells (HPCs) is one of the factors likely promoting inflammation and hepatocarcinogenesis in NAFLD [26]. Chronic inflammation and DNA-damaging agents such as reactive oxygen species (ROS) induce replicative senescence of mature hepatocytes, and this inhibition can activate a secondary proliferative pathway involving HPCs [26, 27]. Activation of HPCs also leads to the production of several profibrogenic factors, such as transforming growth factor β (TGF-β) and platelet-derived growth factor (PDGF), which activate HSCs and boost the production of collagen [28].

The aim of this chapter is to summarize current knowledge on the potential role of the intestinal microbiota in the pathogenesis of NAFLD and in subsequent development of HCC.

2. Microbiota-host transgenomic metabolism of dietary compounds

Intestinal microbes produce a vast array of bioactive molecules from any dietary compounds, thus establishing intense microbiota-host transgenomic metabolism with a tremendous impact on our physiology and nutritional state [29]. In particular, fermentation of indigestible plant polysaccharides by the gut microbiota involves a remarkable interspecies metabolic network, where primary and secondary fermenters act in concert [30]. Plant cell wall polysaccharides—including hemicellulose, pectins, and xylans—reach the colon solubilized or trapped in the plant cellulose matrix. The latter is solubilized by specialized cellulolytic ruminococci,

which produce acetate and propionate from cellulose. Furthermore, soluble cell wall polysaccharides are readily metabolized by butyrate producers of *Clostridium* clusters IV and XIVa (e.g., *Faecalibacterium prausnitzii*, *Butyrivibrio*, *Roseburia*, and *Eubacterium rectale*). On the other hand, soluble starches are preferentially fermented to propionate, acetate, and succinate by Bacteroidetes [31, 32]. These microorganisms are also capable of fermenting host mucus polysaccharides and plant cell wall polysaccharides, shifting from one carbon source to another depending on their bioavailability [33, 34].

Primary fermenters of polysaccharides produce both short-chain fatty acids (SCFAs: acetate, propionate, and butyrate) and molecular hydrogen (H_2). In turn, H_2 is the principal energy resource for secondary fermenters in the gut microbial community, and many of them compete for H_2 in the gut [35]. Indeed, acetogens such as *Blautia hydrogenotrophica*, sulfate-reducing bacteria such as *Bilophila wadsworthia*, and methanogen *Methanobrevibacter smithii* can all metabolize H_2, thereby 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 *Clostridium* clusters IV and XIVa; this phenomenon establishes balanced syntrophy among members of intestinal microbial communities [32].

The metabolism of dietary amino acids by the intestinal microbiota involves proteolytic clostridia, such as members of *Clostridium* clusters I and XI [36, 37], Bacteroidetes, and some enterococci and enterobacteria [38]. The metabolism of amino acids involves production of a variety of bacterial metabolites, also depending on the type of amino acid being fermented [37]. In particular, in addition to SCFAs, fermentation of simple aliphatic amino acids results in the production of methylamines, whereas branched-chain amino acids lead to the production of branched-chain fatty acids. Microbiota-mediated metabolism of aromatic amino acids generates a variety of phenolic and indolic metabolites [39].

The microbial metabolites derived from the metabolism of dietary compounds modulate several traits of the host physiology [29, 40]. In particular, SCFAs perform a key multifactorial function in human physiology and homeostasis [41]. For instance, acetate, propionate, and butyrate modulate several parameters of our nutritional state. Although butyrate represents an important energy source for host colonocytes [40, 42], acetate and propionate regulate lipid synthesis in the liver [41] and intestinal gluconeogenesis [43]. Furthermore, by supporting insulin secretion, butyrate is also involved in the regulation of the host energy storage and is known to regulate appetite by enhancing the production of leptin and peptide YY [29].

SCFAs are also strategic modulators of immune function. Butyrate acts both locally, throughout regulatory mechanisms governing production of proinflammatory cytokines in the gut [44], and systemically, by modulating the extrathymic formation of regulatory T cells [45]. In contrast, propionate governs the de novo formation of peripheral regulatory T cells and, together with acetate, guides their homing in the colon. Moreover, propionate has been implicated in the enhancement of hematopoiesis of dendritic cells with impaired T helper 2 type of activation [45].

Certain microbial metabolites generated by amino acid fermentation in the gut have a detrimental effect on the host [39]. In particular, phenolic and indolic metabolites generated by the bacterial metabolism of aromatic amino acids in the gut have been linked with immune

activation and diabetes [39]. Similarly, production of methylamines from aliphatic amino acids is associated with diabetes, obesity, and NAFLD or NASH [46]. Finally, the endpoint metabolites produced by secondary fermenters in the microbiota are relevant to host health. Although acetate produced by acetogens supports butyrate producers in a feedback process, sulfate reducers are detrimental for host health because they support inflammation [47].

The microbiota-mediated metabolism of complex polysaccharides mainly results in the production of beneficial SCFAs, whereas protein fermentation involves production of a vast array of harmful metabolites. Therefore, we can hypothesize that the gut microbiota-host mutualism evolved in the context of a plant-based diet, with only occasional consumption of meat. In fact, according to the aforementioned observations, a plant-based diet should lead to massive production of SCFAs by a saccharolytic intestinal microbiota, preventing the accumulation of detrimental metabolites as a result of bacterial proteolytic fermentation processes [38]. Finally, recent studies support a direct connection between the intake of saturated fats and proinflammatory dysbioses of the intestinal microbiota [48]. High intake of saturated fats results in an increase of bile acid secretion, stimulating the growth of bile-resistant sulfate-reducing bacteria *B. wadsworthia* in the gut and forcing an inflammatory boost as a result of increased H_2S production.

Aside from the diet, there are some stressors that can influence the balance of a microbiota; in particular, antibiotics modify the microbiota, which, after this treatment, is characterized by a different equilibrium [49].

3. Microbiota and liver diseases

In the last two decades, there has been considerable growth in the number of publications evaluating the associations among NAFLD, NASH, and HCC. The progression from NAFLD or NASH to hepatic carcinogenesis represents another growing area of study [50]. A "two-hit" mechanism has been proposed for the NAFLD and NASH pathogenesis. The "first hit," hepatic steatosis, is closely associated with lipotoxicity-induced mitochondrial abnormalities that sensitize the liver to additional proinflammatory insults. The "second hit" includes enhanced lipid peroxidation and increased production of ROS [51]. Recently, some investigators proposed a multiple-hit process with successive liver injuries leading from fat accumulation to inflammation and fibrosis [52]. In particular, there is a report of a relation between the liver-gut correlation and the development of liver diseases [53].

Alteration of a microbiota seems to be involved in the induction and progression of liver damage, in addition to direct injury resulting from various casual agents [54].

Using a metagenomic approach, Turnbaugh et al. compared animals fed a low-fat diet or high-fat high-sugar "Western" diet and demonstrated a relative increase in the number of bacterial cells belonging to the Firmicutes phylum and a reduction in the number of bacterial cells belonging to Bacteroides during the Western diet [55]. The switch from a low-fat to the Western diet shifts composition of the microbiota and increases the ability of the bacteria to

harvest energy from the host diet, with progressive development of obesity [56]. In mouse models, Ley et al. observed a similar difference: a rise of the ratio of Firmicutes/Bacteroides in the microbiota in obese humans and re-equilibrium in favor of Bacteroidetes in case of a fat-restricted diet [57].

Therefore, in obese subjects, there are several changes in composition of the intestinal microbiota, which are characterized by upregulation of Firmicutes and a decline of Bacteroidetes (resulting in the so-called "obese microbiota") and a reduction in gut bacterial richness [58, 59]. Small intestinal bacterial overgrowth (SIBO) by Gram-negative organisms may promote insulin resistance and induce choline deficiency: all of these factors are implicated in NAFLD [60]. The intestinal microbiota is the primary source of bacterial endotoxins (e.g., lipopolysaccharide; LPS) produced by Gram-negative bacteria. LPS normally crosses the mucosa only in trace amounts and enters portal blood to be cleared in the liver. LPS can initiate inflammation and insulin resistance associated with obesity [16, 61].

Quantitative and qualitative alterations of the gut microbiota may lead to increased intestinal permeability via several mechanisms, including regulation of tight junctions, and may favor microbial translocation defined as migration of bacteria or their products—also termed pathogen-associated molecular patterns (PAMPs)—from the gut to mesenteric lymph nodes or to other organs [62–64].

A link between bacterial overgrowth and NAFLD or NASH was first demonstrated by Wigg et al. [13]. In another study, Miele et al. [65] compared intestinal permeability in the three groups of human subjects (NAFLD, celiac disease, and healthy controls) and observed higher prevalence of SIBO and of leaky gut in the NAFLD group, thereby demonstrating the role of this increased permeability in the pathogenesis of hepatic fat deposition.

The gut-liver axis is the way bacteria and their possible hepatotoxic products (e.g., LPS, DNA, or RNA) can easily reach the liver. The final effect is activation of the signaling cascade triggered by a specific immune receptor resulting in the expression of proinflammatory cytokine genes, which may exacerbate the hepatocyte damage and contribute to the subsequent development of HCC [66, 67].

Bacterial components stimulate a toll-like receptor (TLR), which represents a highly conserved family of receptors that recognize specific PAMPs and are expressed on Kupffer cells, biliary epithelial cells, hepatocytes, HSCs, endothelial cells, and dendritic cells [68]. An interaction of a TLR with an endotoxin results in activation of nuclear transcription factors, leading to the release of numerous proinflammatory mediators, such as tumor necrosis factor α (TNF-α), which can induce liver injury, fibrosis, and insulin resistance [69, 70].

Miura and colleagues [71] showed that TLR9 ligands induce the production of IL-1β by Kupffer cells in a mouse model of NASH. IL-1β then promotes lipid accumulation in (and death of) hepatocytes, causing steatosis and inflammation and stimulates HSCs to produce fibrogenic mediators, such as collagen, resulting in fibrosis. In particular, TLR9-deficient mice ($TLR9^{-/-}$) show a significant reduction in hepatic lipid accumulation when compared with their wild-type counterparts [71]. In addition, TLR4 contributes to the development of inflammation and

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fibrosis by inducing production of proinflammatory cytokines (TNF-α, IL-1β) and cooperates with TLR9 to induce active IL-1β in Kupffer cells [72, 73].

The inflammasome is a cytoplasmic multiprotein complex that recognizes a diverse set of inflammation-inducing stimuli and directly activates caspase 1. Activated caspase 1 causes a release of strong proinflammatory cytokines, such as IL-1β and/or IL-18, which are involved in the pathogenesis of the majority of chronic liver diseases, such as NAFLD and NASH [74, 75]. In particular, the NLRP3 inflammasome is activated by microbial PAMPs (via a two-step process involving a TLR), and therefore, it is the principal inflammasome subtype involved in the NAFLD progression and promoting insulin resistance and β-cell death [11]. Csak et al. [76] described for the first time the role of NLRP3 inflammasome activation in NASH. In mice on a high-fat diet, those authors observed upregulation of the inflammasome, according to increased caspase 1 activity and higher serum levels of IL-1β, in comparison with controls. Another study confirmed these data, pointing to a contribution of the inflammasome to the pathogenesis of NAFLD or NASH [77].

Recent evidence revealed that dysbiosis can promote the development of NAFLD or NASH by modifying the bile acid metabolism. Bile acids can modulate glucose and lipid metabolism via their binding to and activation of G protein-coupled receptor TGR5 and farnesoid X receptor (FXR): nuclear hormone receptors expressed by hepatic Kupffer, stellate, and endothelial cells. In FXR-deficient mice, researchers have demonstrated glucose intolerance, insulin resistance, and elevated circulating levels of free fatty acids, which lead to the development of severe hepatic steatosis [78–80].

FXR regulates hepatic inflammation and fibrosis and is important for hepatocarcinogenesis. Fickert et al. [81] studied FXR knockout mice ($FXR^{-/-}$) and showed that the FXR loss alleviates fibrosis of the hepatic biliary tree. $FXR^{-/-}$ mice develop spontaneous HCC at age >12 months [82, 83]. Selective reactivation of intestinal FXR can restore bile acid enterohepatic circulation and protect $FXR^{-/-}$ mice from spontaneous development of HCC [84].

4. NAFLD and hepatic progenitor cells

Several lines of evidence suggest that another factor is implicated in the development and progression of chronic liver diseases. Namely, HPCs are a bipotent cell population that can differentiate into hepatocytes or into biliary epithelium cells and reside in the terminal biliary ductules and in the so-called "canals of Hering" [85, 86]. They represent a heterogeneous cell population expressing phenotypic markers of both immature hepatocytes (such as α-fetoprotein) and bile duct cells (such as bile duct-type cytokeratins) [26, 87].

HPCs have been studied regarding regeneration after severe hepatocellular necrosis [88], but recent studies revealed that this cellular compartment is also activated in chronic viral hepatitis, alcoholic liver disease, and NAFLD [89]: the most important hepatocarcinogenic conditions in the Western world. Activation of progenitor cells in these diseases suggests that they are a possible target cell population for hepatocarcinogens [67, 90].

In the healthy liver, replacement of necrotic and apoptotic hepatocytes involves proliferation of adjacent hepatocytes within the lobules [26]. Nonetheless, this primary pathway is often impaired by a variety of insults, including experimental toxins, viral infection, steatosis, oxidative stress, and alcohol. Chronic inflammation, the presence of growth factors, and DNA-damaging agents like ROS and reactive nitrogen species induce replicative senescence of hepatocytes, and this inhibition activates a secondary proliferative pathway involving HPCs [91–93].

The combination of oxidative liver damage and inhibited hepatocyte proliferation, as observed in NAFLD and NASH, seems to provide a strong stimulus for activation of HPCs and plays a key role in the pathogenesis of HCC. Roskams et al. [91] studied three murine models of fatty liver disease (genetically obese ob/ob mice and normal mice with fatty livers induced either by ethanol or methionine choline-deficient diets) and patients with nonalcoholic fatty liver disease or alcoholic liver disease. Mice with fatty liver show greater numbers of progenitor cells than controls do, and mitochondrial ROS production is significantly increased in all three groups. This increased oxidative stress promotes replicative senescence in mature hepatocytes and expansion of progenitor cells, in both mice and humans [91].

The magnitude of progenitor cell activation seems to correlate with the severity of liver disease [89, 91]. In a recent work, Richardson et al. showed that NASH with portal or linking fibrosis (disease stages 2–4) is associated with more frequent replicative arrest of hepatocytes and with expansion of HPC numbers as compared to steatosis alone [94].

Literature data are suggestive of the involvement of the inflammatory infiltrate in the activation of progenitor cells, through the secretion of inflammatory cytokines, in particular TNF-α [95, 96]. Expression of these cytokines is upregulated during hepatic injury and performs an important function in HPC activation [97, 98]. The result is production of some profibrogenic factors that activate HSCs and boost the production of collagen [28].

Other signaling pathways participate in the complex mechanism controlling the behavior of HPCs. *Must1*, *Must2*, and *Yap* genes are important for proliferative control and tumorigenesis in the liver. Defects in this signaling pathway lead to sustained liver overgrowth and eventual development of either HCC or cholangiocarcinoma in mice [99]. Studies in humans confirmed that a loss of regulation of Mst1 or Mst2 is a common aberration in HCC and may account for Yap activation in these tumors. In fact, approximately 30% of HCCs show reduced Yap phosphorylation and aberrant overexpression of Yap [100, 101].

Approximately, a half of human HCCs (28–50%) express one or more markers of progenitor cells that are not present in normal mature hepatocytes [102, 103]. When analyzing the precursor lesions of HCC, many authors detected HPCs and intermediate hepatocyte like cells in 50% of small cell dysplastic foci and in hepatocellular adenoma [90, 104]. These findings support the hypothesis that some human HCCs arise from HPCs. Moreover, HCCs expressing HPC markers have a worse prognosis than HPC marker-negative HCCs. Wu et al. observed significantly shorter survival of patients with HCCs expressing CK19 [105]. Similar findings were made by Uenishi et al. [106]. In a recent study, Durnez reported that CK19-positive HCC shows a higher rate of tumor recurrence after a liver transplant as compared with CK19-negative HCC [103].

The available data suggest that HPCs are involved in fibrogenesis and progression of NAFLD and that their activation during chronic liver disease may increase the risk of HCC. Nonetheless, further studies are necessary to better clarify the function of these cells in hepatocarcinogenesis and in the liver's response to NAFLD injury.

5. Conclusion

Recent pieces of evidence are indicative of the role of the intestinal microbiota—in particular its dysbiosis and activation of HPCs—in the clinical course of NAFLD and in the subsequent development of HCC. Intestinal microbes produce a large array of bioactive molecules mainly from dietary compounds, thus establishing intense microbiota-host transgenomic metabolism with a strong impact on pathological conditions. Derangement of the intestinal microbiota may lead to translocation of bacteria or their products to the liver, where endotoxins trigger inflammation and hepatocellular damage, which in turn is crucial for the development of HCC.

The subsequent liver injury and hepatocellular necrosis can activate a secondary proliferative pathway involving HPCs: a bipotential cell compartment that seems to contribute to hepatocarcinogenesis.

Better knowledge of these factors is necessary for understanding the HCC pathogenesis in NAFLD and for discovery of new therapies, but further research is necessary to identify the carcinogenesis process.

Abbreviations

Nonalcoholic fatty liver disease	NAFLD
Nonalcoholic fatty liver	NAFL
Nonalcoholic steatohepatitis	NASH
Hepatocellular carcinoma	HCC
Hepatitis B	HBV
Hepatitis C	HCV
Hepatic stellate cells	HSCs
Hepatic progenitor cells	HPCs
Reactive oxygen species	ROS
Transforming growth factor β	TGF-β
Platelet-derived growth factor	PDGF
Short-chain fatty acids	SCFAs
Molecular hydrogen	H_2

Small intestinal bacterial overgrowth	SIBO
lipopolysaccharide	LPS
Pathogen-associated molecular patterns	PAMPs
Toll-like receptor	TLR
Tumor necrosis factor	α (TNF-α)
Farnesoid X receptor	FXR

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