Gut microbiota and non-alcoholic fatty liver disease: new insights

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Abstract

Non-alcoholic fatty liver disease (NAFLD) is a severe liver disease that is increasing in prevalence with the worldwide epidemic of obesity and its related insulin-resistance state. A ‘two-hit’ mechanism has been proposed; however, the complete physiopathogenesis remains incompletely understood. Evidence for the role of the gut microbiota in energy storage and the subsequent development of obesity and some of its related diseases is now well established. More recently, a new role of gut microbiota has emerged in NAFLD. The gut microbiota is involved in gut permeability, low-grade inflammation and immune balance, it modulates dietary choline metabolism, regulates bile acid metabolism and produces endogenous ethanol. All of these factors are molecular mechanisms by which the microbiota can induce NAFLD or its progression toward overt non-alcoholic steatohepatitis.

Keywords: hepatic steatosis, microbiota, non alcoholic fatty liver disease, obesity, steatohepatitis

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Introduction

The epidemic of obesity [1,2] has led to the dramatic increase of its related metabolic diseases, namely insulin resistance, type 2 diabetes and non-alcoholic fatty liver disease (NAFLD) [3]. The mechanisms involved in weight gain and the development of obesity are numerous and complex, and research continues to uncover new factors. In the past few years, a potential role of the gut microbiota has emerged in weight regulation [4–8]. The gut microbiota is now considered as a major metabolic internal organ, composed of >10^{14} microorganisms and containing a second genome (named the metagenome), which is up to 100–400 times that of humans [9]. Culture-independent, large-scale tools [10] and associated projects such as the Human Microbiome Project [11] or the MetaHit consortium [9] have enabled major breakthroughs in the understanding of gut microbiota composition and functions in different pathological conditions. Data suggest an important impact of the gut microbiota on health [12] and in the pathogenesis of certain inflammatory and metabolic [13] diseases such as type 2 diabetes [14] and obesity. Recent literature also points to a potential role in the development of NAFLD.

Non-alcoholic fatty liver disease is a very prevalent and severe disease that can lead to cirrhosis, liver carcinoma [15] and death related to liver morbidity. In addition, data show that NAFLD correlates with increased cardiovascular risk assessment scores and most of the clinical surrogates of cardiovascular diseases. A few smaller studies have suggested that NAFLD induces not only increased risk of patent cardiovascular disease, independently of usual risk factors [16,17] and of other components of metabolic syndrome, but also increased risk of related mortality [18,19]. However, no treatment has yet proven effective to improve non-alcoholic steatohepatitis.
Non-alcoholic fatty liver disease is a frequent disease, occurring in 16–30% of the general population, depending on the assessment method [22,23]. Its prevalence rises [24] in parallel with the worldwide epidemic of obesity and metabolic diseases [25], reaching 50–90% of obese individuals, among whom more than one-third present with overt NASH [26]. The diagnosis of NAFLD is made on histological findings, first described by Kleiner and Brunt and their colleagues [27,28]. It includes a wide spectrum of lesions, starting with steatosis, characterized by the accumulation of triglycerides within the hepatocytes [29], in the absence of other liver disease or significant alcohol consumption [21]. Some patients will also develop hepatocyte injuries, such as ballooning and inflammatory infiltrates, both responsible for NASH, with or without concomitant collagen deposition (fibrosis) that potentially leads to end-stage cirrhosis [30].

Recently, a new algorithm also based upon histological determination has been proposed and validated to better define early stages of NASH, most particularly in morbidly obese patients [31]. Although liver biopsy is the gold standard to confirm the diagnosis, it is an invasive and costly procedure, potentially responsible for secondary effects [32,33]. Furthermore, considering the ever-increasing prevalence of both diabetes [34] and obesity [35], and the subsequent number of patients at risk for liver alterations, it cannot be considered a practical, efficient and large-scale tool to identify those at risk of NASH or advanced fibrosis. In that context, a clinical scoring system including type 2 diabetes, hypertension, sleep apnea and ethnicity has been suggested to propose liver biopsy for those at high risk of NASH [36]. Other imaging techniques include fast magnetic resonance imaging [37], computed tomography, ultrasound [38] and emerging proton magnetic resonance spectroscopy, which is a reliable non-invasive tool to quantify hepatic triglyceride content [39]. The last of these correlates closely with histopathological grade [40] and allows larger sampling than liver biopsy (8–27 cm³). Recently, non-invasive tests, initially used in viral hepatitis B [41] and C [42], have been developed, that are currently validated to screen NAFLD in at-risk populations. For example, Fibromax®, which is an algorithm including an association of gender, age, weight, height and numerous serum biomarkers, has shown good reliability in the prediction of liver abnormalities including steatosis [43], NASH [44] and fibrosis, in both overweight patients and in those at different stages of obesity [45–47].

Regarding the pathogenesis of NAFLD, for a long time, a ‘two-hit’ mechanism was proposed to explain the progression in liver alteration stages, where the first hit consists in hepatocyte lipid accumulation mainly due to obesity and insulin resistance [48]. Then a second hit, occurring in some patients only, plays a role in the shift from steatosis to NASH [49,50]. Several factors have been incriminated in the pathophysiology of NAFLD such as oxidative stress, systemic inflammatory mediators and chronic intermittent hypoxia [51–53]. Furthermore, adipose tissue inflammatory tone [54] also seems to play an important role, because patients with more severe stages of NASH display increased macrophage accumulation in visceral adipose tissue [55]. Moreover, their deep subcutaneous adipose tissue not only displays increased macrophage infiltration but also increased inflammatory gene expression compared with superficial adipose tissue [56]. These associations between macrophage accumulation in adipose tissue and NASH stages were found independently of the diabetes status in morbid obesity.

More recently, an alternative theory favours the determinant effect of free fatty acid lipotoxicity in liver injury, which leads to NASH and occurs in parallel with triglyceride droplet accumulation (i.e. steatosis) [57]. Hepatic steatosis is known to develop in the context of an imbalance of triglycerides afflux in the liver, which is dramatically increased during obesity and even more so when associated with insulin resistance. Indeed in that context, there is a concomitant increased supply (coming from diet, de novo lipogenesis [58] and adipose tissue) and decreased degradation (via impaired β-oxidation) [57]. A special focus should be placed on fructose consumption, which is dramatically increased in the western diet [59]. Indeed, its metabolism induces both de novo hepatic lipogenesis and reactive oxygen species (ROS) production and therefore participates in the two ‘hits’ of NAFLD pathophysiology [60]. Yet to date, the complete pathogenesis of NAFLD is still unknown and many more factors remain still to be uncovered, in particular the precise role of gut microbiota.

**Microbiota Composition in Obesity**

Studies in mice indicated a relation between gut microbiota and weight regulation. Indeed, germ-free mice displayed reduced adiposity despite increased food consumption when...
compared with conventional animals. Most importantly, colo-
ization with caecal content from conventional mice induced a
rapid fat mass gain, even though those mice ingested less food
[4,61,62]. The physiopathogenesis by which microbiota plays a
role in obesity development was finally confirmed after
manipulation in mice and implies multiple mechanisms. The
gut microbiota is able to process otherwise indigestible dietary
polysaccharides [62–64] into short-chain fatty acids [65] that
can subsequently be absorbed by the intestine. Moreover, the
microbiota directly impacts gene regulation to favour
increased storage into adipose tissue [4]. Finally, experiments
comparing the feces of obese and lean individuals demon-
strated that the level of short-chain fatty acids was higher in
the obese whereas residual calories from food were concomi-
tantly reduced. These results suggested that the microbiota
from obese animals displayed increased capacity to extract and
subsequently store energy compared with that of lean animals
[65,66]. Since then, studies in mice using 16S rRNA sequencing
methods have demonstrated that microbiota composition
differs in obese and lean individuals, with increased Firmicutes
and decreased Bacteroidetes levels in the obese although they
ingested the same amount of food [67]. The same result was
also found in a small study in humans, where obese individuals
also displayed increased Firmicutes and decreased Bacteroi-
detes compared with lean ones [68], data that were later
confirmed in larger cohorts [69,70]. However, this result
remains under debate because conflicting data have also
emerged [66,71,72]. Indeed, using two techniques, Duncan
et al. observed no differences in the numbers of Bacteroidetes
in obese or lean patients [71,72]. Likewise, Jumperetz et al.
found the same level of the three dominant phyla (namely
Bacteroidetes, Firmicutes and Actinobacteria) in groups with
different body mass index [73].

Those discrepancies have led to further studies, using large-
scale metagenomic tools that enabled interesting discoveries:

First, recent data in humans have focused on the importance of
bacterial diversity rather than the composition in different
phyla [74]. Interestingly, in obese populations with a high
prevalence of obesity-related disease, the overall number of
gut microbiota was reduced. This is in line with previous
results in mouse models [61] and humans [69], where greater
corpulence was associated with reduced diversity. Likewise,
children from rural Africa displayed increased microbiota
richness and biodiversity than same-age healthy children from
Western Europe [75].

Second, although gut microbiota display large inter-individual
variability, a ‘core’ microbiota was pointed out since 40% of
the genes from each individual were shared with at least half of
the individuals in the cohort [76]. Most importantly, based on
studies of the microbiome in different large groups of patients,
three Enterotypes, which can be compared with blood types,
have been identified, that are not driven by corpulence. These
Enterotypes differ according to the abundance of the three
dominant genera, each of them able to process certain types of
nutrients [77]. Indeed, Enterotype 1, enriched in Bacteroides
spp., has been associated with long-term consumption of
animal proteins and saturated fat whereas Enterotype 2
(dominated by Prevotella spp.) is associated with a carbohy-
drate-based diet [78]. Gut microbiota composition seems to
have a strong link with long-term food habits in adults [78].
Moreover, comparison between the microbiota from children
in Western Europe and in rural Africa revealed that the latter
had low levels of Firmicutes and increased levels of Bacter-
oiides in a link with major food consumption differences,
namely high amounts of plant polysaccharides [75]. Other
aspects of the diet have also been related to the microbiota.
Indeed, the overall increase in calorie content induced a
concomitant rapid increase in numbers of Firmicutes and a
decrease in Bacteroidetes in lean and obese humans [73].
Likewise, when submitted to a high-fat [79,80] or western
[61,81] diet, both mouse and humanized mouse models
displayed the same changes. Finally, Ravussin et al. suggested
that the increase in fat content rather than weight modific-
tions drove the increase in numbers of Firmicutes [82]. If those
studies demonstrate the unquestionable effect of acute
changes in the diet on the rapid changes in microbiota
composition, it does not translate in to a switch from one
Enterotype to the other [78].

NAFLD and Microbiota

Several lines of evidence suggest a strong interaction between
gut flora and liver. Deriving from its anatomical position, the
liver receives 70% of its blood supply from the intestine through
the portal vein, so it represents the first line of defence against
gut-derived antigens, and one of the most exposed organs to
gut-derived toxic factors, such as bacteria and bacterial by-
products [83]. The link between gut microbiota and the
development of NAFLD has been shown in mice and in humans.
In mice, Bäckhed et al. observed that the transplantation of
normal caecal microbiota to germ-free mice induced, 15 days
later, a 60% increase in body fat along with a more than two-fold
increase in hepatic triglyceride content [4]. In the early 1980s, a
parallel development of NASH and small intestinal bacterial
overgrowth was reported in humans after intestinal bypass.
Interestingly, hepatic steatosis regressed after antibiotic treat-
ment (namely metronidazole) [84], suggesting a possible
causative role for microbiota in NAFLD.
Gut microflora may stimulate hepatic fat deposition and promote NASH through several mechanisms (Fig. 1):

1. It promotes obesity by improving energy yield from food (see Microbiota and Obesity)
2. It regulates gut permeability, low-grade inflammation and immune balance
3. It modulates dietary choline metabolism
4. It regulates bile acid metabolism
5. It increases endogenous ethanol production by bacteria

**Lipopolysaccharide and Toll-like-receptor 4 signalling**
NAFLD and other insulin-resistant states are associated with activation of the innate immune system. The gut microbiota participate in the development and homeostasis of the overall immunity of the host [85]. Indeed, the cross-talk between host and bacteria at the mucosal interface is responsible for innate and adaptive immune responses that protect the host and maintain intestinal homeostasis. This depends on specific pattern recognition receptors, including Toll-like receptors (TLRs) and NOD-like receptors that recognize highly conserved microbial molecules called ‘pathogen-associated molecular patterns’ (PAMPs), whereas the endogenous products are distinguished in damage-associated molecular patterns (DAMPS) [83,86,87]. The TLRs, acting as immune sensors of PAMPs and DAMPs, initiate an adaptive immune response and a signalling cascade leading to activation of pro-inflammatory genes, such as tumour necrosis factor-α (TNF-α), interleukin-6 (IL-6), IL-8, IL-12 [87]. The most studied PAMP is the lipopolysaccharide (LPS), a component of the gram-negative bacteria cell membrane, the active component of endotoxin. The latter binds to lipopolysaccharide-binding protein (LBP), which subsequently binds to CD14. The LPS–LBP–CD14 complex activates TLR-4, present in Kupffer cells, triggering an essential inflammatory cascade, including stress-activated and mitogen-activated protein kinases, Jun N-terminal kinase, p38, interferon regulatory factor 3 and nuclear factor-κB pathway [88,89]. Translocation of nuclear factor-κB to the nucleus induces the transcription of numerous pro-inflammatory genes such as TNF-α and IL-1β [90]. Other bacterial endotoxins such as peptidoglycan also contribute to stimulate pro-inflammatory cascades though activation of the NOD1 receptor [91]. Many studies, mostly carried out in animal models, enabled LPS–TLR-4 signalling activation to be related to insulin-resistance and NASH. Cani et al., first described how a 4-week high-fat diet led to a moderate increase in plasma LPS concentration in mice, defining this effect as ’metabolic endotoxaemia’, because this increase was still 10–50 times lower than values that could
be reached during sepsicaemia [80,92]. Importantly, 4 weeks of continuous infusion of LPS in mice mimicked the high-fat diet phenotype, namely an increase in insulin resistance, liver triglyceride content and adipose tissue inflammation. Besides, they demonstrated that these effects were CD14-dependent, because the phenotype was significantly reversed in CD14 knockout mice [80]. The role of the gut microbiota was further demonstrated by antibiotic treatment reducing the intensity of the disease in high-fat diet and ob/ob mice [93–95]. Since then, other studies in TLR-4 null mice (−/−) have confirmed that TLR-4 is essential for hepatic fat deposition and NASH development [96–98]. Hepatic stellate cells might also have an important role in generating the liver inflammatory cascade linked with endotoxinemia. Indeed, they have been shown to be the target through which TLR-4 promote fibrogenesis via enhancement of transforming growth factor-β (TGF-β) signalling [99,100].

Another important contributor to microbiota dysbiosis, participating in the activation of lipid peroxidation and ROS production—both involved in the second-hit mechanism in NAFLD/NASH progression—is the inflammasome. Inflammasomes are cytoplasmic multi-protein complexes composed of leucin-rich-repeat containing proteins and nucleotide-binding domain (NLRPs) [101,102], which are sensors of PAMPs and DAMPs. They govern cleavage of pro-inflammatory cytokines such as IL-1β and pro-IL-18. Most DAMPs induce ROS production, which is known to activate the NLRP3 inflammasome [103]. Recently, Henao-Mejia et al., showed that changes in gut microbiota associated with NLRP6 and NLRP3 inflammasome deficiency were linked with exacerbated hepatic steatosis and enhanced TNF-α expression. Furthermore, co-housing inflammasome-deficient mice with wild-type mice transferred the phenotype, providing direct evidence that inflammasome-mediated dysbiosis is implicated in NASH progression [104]. Studies in humans have demonstrated increased endotoxin levels in adults and children with NASH [105–107]. Furthermore, several studies showed that endotoxin levels increased with the severity of the disease [108,109]. Likewise, chronic endotoxinemia, which behaves as a real ‘hepatotoxicin’, may be of particular importance in inducing hepatic inflammation, fibrosis and insulin resistance.

Altered choline metabolism
Choline is an important phospholipid component of the cell membrane, and a key partner of fat metabolism in the liver, and of very-low-lipoprotein assembly. Lastly, it promotes lipid transport from the liver [118]. A choline-deficient diet promotes liver steatosis, which is reversible by choline infusion [119]. Enzymes produced by the gut microbiota catalyse the conversion of dietary choline into toxic methylamines (dimethylamine and trimethylamine). The uptake by the liver of those amines and their transformation to trimethylamine-N-oxide can induce liver inflammation [120,121]. Interestingly, Spencer et al. showed that variations in levels of Gammaproteobacteria and Erysipelotrichi in human faecal microbiota were directly associated with changes in liver fat during choline depletion [122]. Hence, microbiota dysbiosis can promote
Modification of bile acid metabolism

Representing the main components of bile, bile acids are secreted into the duodenum and work to emulsify liposoluble dietary nutrients to facilitate their digestion and absorption. They also have a strong antimicrobial activity. Bile acids damage bacterial cell membranes by interacting with membrane phospholipids, which results in bactericidal activity [125]. Recent studies have demonstrated that dietary fats (high in saturated fat), by promoting changes in host bile acid composition, can markedly alter conditions for gut microbial assemblage, resulting in dysbiosis [126,127]. Reciprocally, the gut microbiota is able to modulate bile acid metabolism, through farnesoid X receptor stimulation [89]. Dekaney et al. showed that farnesoid X receptor (FXR) null and germ-free mice failed to induce the expression of bile acid transport genes, following ileo-caecal resection, compared with wild type mice [128]. Indeed, bile acids are ligands for a G-protein-coupled receptor (TGR5/Gpbar-1) and activate nuclear receptors such as FXR (NR1H4). FXR and its downstream targets play a key role in the control of hepatic de novo lipogenesis, very-low-density lipoprotein-triglyceride export and plasma triglyceride turnover [129], whereas TGR5 binds secondary bile acids and promotes glucose homeostasis, by stimulating secretion of glucagon-like peptide 1 [130]. Administration of specific TGR5 agonists lowers serum and liver triglyceride levels, thereby reducing liver steatosis [131]. By modifying bile acid metabolism and FXR/TGR5 signalling, gut flora could therefore contribute indirectly to the development of NAFLD [132].

Hepatotoxic microbial by-products: role of ethanol

Intestinal microflora produces a number of potentially hepatotoxic compounds such as ethanol, phenols, ammonia, which are delivered to the liver by the portal circulation. Those compounds activate Kupffer cells (resident hepatic macrophages) and stimulate their production of nitric oxide and cytokines [88]. Acetaldehyde and acetate are two major metabolites of ethanol. While acetate is a substrate for fatty acid synthesis, acetaldehyde may lead to the production of ROS. This could be involved in liver injury by contributing to the disruption of intestinal barrier function and to the two-hit mechanisms of NASH. Remarkably, Zhu et al. studied gut microbiota of patients with and without NASH in obese individuals and in healthy children. They observed an increase of alcohol-producing bacteria in the gut microbiota of children with NASH, associated with an elevated blood alcohol concentration, without dietary alcohol consumption [133]. Furthermore, they reported that Escherichia (a well-known ethanol producer [134]) stood out as the only abundant genus that differed between NASH and obese patients without NASH. Interestingly, changes in Enterobacteria seem to be a frequent feature in obesity and weight changes. Importantly, this endogenously produced alcohol has a well-established role in the generation of ROS and consequently liver inflammation [135]. Moreover, this could participate in the increase of gut permeability [88,136,137].

Gut microbiota modulation as a new therapeutic strategy

Probiotics are defined as live commensal microorganisms, that when consumed in adequate quantities, confer a health benefit to the host (FAO/WHO 2001) [138]. Probiotics have been suggested as a treatment to prevent chronic liver damage, because they prevent bacterial translocation and epithelial invasion, inhibit mucosal adherence by bacteria, produce antimicrobial peptides, decrease inflammation and stimulate host immunity [139,140]. The main probiotics on the market are lactobacilli, streptococci and bifidobacteria [141]. Although the literature on the subject is large, it is difficult to draw conclusions about the true effect of probiotics on NAFLD. Indeed the animal models and bacterial strains tested across the studies are different. Likewise, it must be noted that gut microbiota will always outnumber the probiotics that can be administered. Moreover, probiotic processes will always be confounded by the variability of the human microbiome, diet and genetics [142,143]. Several animal models have provided evidence that probiotics may reduce NASH progression [144–148]. The most studied probiotic is VSL#3 mixture, which was first given for 4 weeks in ob/ob mice fed with a high-fat diet [144]. Treatment with VSL#3, improved liver histology, reduced hepatic total fatty acid content, and decreased serum alanine aminotransferase levels, a surrogate marker of NAFLD. Ma et al. provided further evidence that VSL#3 ameliorates insulin resistance and hepatic steatosis by preventing the expected natural killer T-cell depletion induced by the high-fat diet [145]. Several strains of lactobacillus have demonstrated a protective effect on NAFLD [149–151], whereas a recent meta-analysis pointed out the association of certain species (L. fermentum and L. ingluviei) with weight gain [152]. Probiotics have been shown to have several anti-inflammatory effects, which could contribute to clinical benefit in NAFLD, reviewed elsewhere [88]. Regarding human studies, preliminary data showed that both VSL#3 and a symbiotic (association of pro/prebiotic) given to NAFLD patients for 2–3 months improved liver enzymes, TNF-α and oxidative stress markers [153]. More recently, two randomized double-blind placebo-controlled studies, showed a significant decrease in liver aminotransferases with probiotics in children [154] and in adults [155]. These promising results are strongly indicative of a great

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potential benefit to use probiotics in the treatment of NAFLD. Nonetheless, stated in a Cochrane meta-analysis, larger randomized studies are still needed [156].

Prebiotics
Prebiotics are indigestible carbohydrates that stimulate the growth and activity of beneficial bacteria, particularly lactobacilli and bifidobacteria [141]. Lactulose is a commonly used prebiotic, which has proven its efficiency in reducing symptoms in liver patients by increasing bifidobacteria [157]. Other prebiotics include inulin-type fructans that can be differentiated by their degree of polymerization such as long-chain and short-chain, i.e. oligofructose and fructo-oligosaccharides [158]. Several beneficial effects of prebiotics on gut microbiota, serum lipids and liver physiology have been shown [159]. Besides, in several animal models of NAFLD, lactulose and oligo-fructose have been shown to reduce hepatic steatosis development [160,161]. The potential mechanisms include reduced de novo fatty acid synthesis, reduced body weight through stimulation of gut peptides (glucagon-like peptide 1 and peptide YY), reduced inflammation and pro-inflammatory cytokines (IL6, TNF-α), improved glycemic control, modulation of gut microbiota and alterations in volatile organic compounds [83,159]. Yet, studies assessing the effects of prebiotics on NAFLD in humans are lacking. Nevertheless, a pilot study already gives encouraging results, since Daibiouli et al. showed a reduction in liver enzymes and fasting insulinemia in seven patients with NASH who were treated with oligo-fructose for 8 weeks [162].

Conclusion
Gut–liver axis plays a central role in the pathogenesis of NAFLD, mainly through the crosstalk of the intestinal microbiota with the host immune system modulating inflammation, insulin resistance and intestinal permeability. Large-scale randomized trials using antibiotics, probiotics and prebiotics with imaging and histological endpoints are needed.

Transparency Declaration
The authors declare no competing interests.

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