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Gut microbiota and human NAFLD: disentangling microbial signatures from metabolic disorders

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Abstract

Gut microbiota dysbiosis has been repeatedly observed in obesity and type 2 diabetes mellitus (T2DM), two metabolic diseases strongly intertwined with nonalcoholic fatty liver disease (NAFLD). Animal studies have demonstrated a potential causal role of gut microbiota in NAFLD. Human studies have started to describe microbiota alterations in NAFLD and have found a few consistent microbiome signatures discriminating healthy individuals from NAFLD, nonalcoholic-steatohepatitis or cirrhosis. However, patients with NAFLD often present with obesity and/or insulin resistance and T2DM, and these metabolic confounding factors for dysbiosis have not always been taken into account. Patients with different NAFLD severity stages often present with heterogeneous lesions and variable demographic characteristics (including age, sex and ethnicity), which are known to affect the gut microbiome and have been overlooked in most studies. Finally, multiple gut microbiome sequencing tools and NAFLD diagnostic methods have been used across studies that could account for discrepant microbiome signatures. This Review provides a broad insight into microbiome signatures for human NAFLD and explores issues with disentangling these signatures from underlying metabolic disorders. More advanced metagenomics studies, as well as multi-omics studies using system biology approaches, are needed to improve microbiome biomarkers.

[H1] Introduction

The gut microbiota (that is the microbial community within the gastrointestal tract) has critical physiological roles in host digestion, immunity and metabolism[1,2]. Initially only studied by culture-based methods, the characterization of the gut microbiota[3] has deepened with the rapid development of high-throughput sequencing technology

(shotgun sequencing or pyrosequencing). Constructed gut microbiota reference gene catalogues[4,5] have further enabled the determination of the composition of the gut microbiota and prediction of microbiome functions[6]. In addition to these technological advances, decreasing costs and reduced analytical turnaround due to bioinformatic pipeline development have enabled increasingly accessible and efficient microbiome studies. Thus, knowledge on microbiome characteristics in common diseases, especially metabolic diseases[7], has substantially increased in the past 15 years.

The need for microbiome characterization in metabolic diseases was initially stimulated by pioneering studies using germ-free mice and gut microbiota transfer, which reported the contribution of gut microbiota to weight gain and metabolic alterations[8,9]. Studies using conventional mice receiving lipopolysaccharide (LPS, a major component of the Gram-negative bacterial outer membrane) infusions also provided evidence of the role of gut microbiota in metabolic injuries and its influence on insulin resistance[10]. Since these initial studies, microbiome signatures linked to obesity[11–13] and type 2 diabetes mellitus (T2DM)[14,15] and associated complications were discovered, raising the concept of human gut microbiota dysbiosis (that is,alteration in microbiota composition and functional capacities with modification of microbiome signatures[16]) in metabolic diseases (reviewed elsewhere[17]). Currently, these findings are being pursued to develop microbiota-based therapeutics such as probiotics[18], prebiotics[19], synbiotics[20], and faecal microbiota transplantation **[G] [Au: Please do not delete these marks, they are to flag the glossary terms to our Production team]** (FMT) [21,22] to improve metabolic health and personalized patient care.

Nonalcoholic fatty liver disease **[G]** (NAFLD), and the more advanced stage nonalcoholic steatohepatitis **[G]** (NASH)[23], are common comorbidities of obesity and

T2DM with an increasing burden for society[24]. NAFLD-related liver failure has become the second leading cause of liver transplantation in the Western world [25]. As liver biopsy is the diagnostic gold standard for NAFLD and NASH, and it is an invasive, inconvenient and impractical tool in a public health setting[26], the complete understanding of the complex pathophysiology of these diseases remains limited. Moreover, although mouse models of NAFLD and NASH are helpful, they are not optimal[27] and can limit the translation of results to clinical research[27]. As obesity, T2DM, and NAFLD–NASH are linked clinically and pathophysiologically, exploring the gut microbiome seems to be a relevant approach to gain a better understanding of NAFLD and NASH. Although this level of characterization of NAFLD and NASH is markedly less than that for obesity and diabetes, there is a rapidly growing body of evidence exploring the contribution of the gut microbiome to NAFLD physiopathogenesis[28–30] using high-throughput sequencing in cohorts of individuals spanning the NAFLD–NASH disease spectrum. As there is a large overlap between NAFLD and metabolic disorders in respect to the disease spectrum and contributing factors, some metagenomic signatures of NAFLD might be shared with those already observed in obesity and T2DM. Thus, deciphering signatures specific to liver alterations would be most useful for future NAFLD diagnostic biomarkers. We herein review the gut microbial and gut microbial-derived metabolite signatures associated with NAFLD development and progression focusing on their relationship with disease progression in human. We specifically focused on which microbial signatures are specific to liver injury versus those common to other metabolic diseases and the putative methodological biases that could explain divergent results across the literature.

[H1] NAFLD and related liver fibrosis

NAFLD is defined as the pathological accumulation of lipid droplets in >5% of hepatocytes[23]. This disease can progress towards NASH, which is diagnosed by liver biopsy and the histological examination of the degree of steatosis **[G]** , inflammation and hepatocyte ballooning[23,31]. NASH can also present with liver fibrosis **[G]** [23,31,32], which is the main prognostic lesion for disease progression[33,34], eventually leading to cirrhosis[35] **[G]** and/or hepatocellular carcinoma[36–38] and other liver-related complications among which include ascites, hepatic encephalopathy and portal hypertension[39]. NAFLD is highly prevalent and has become the most common cause of chronic liver disease in the Western world affecting up to 40% of the general population[40] and reaching sometimes 90%[41,42] in obese populations worldwide[43]. NAFLD is closely associated with overweight or obesity and metabolic disorders such as insulin resistance, hypertension and T2DM, and is even recognized as the hepatic component of metabolic syndrome[44,45] (**Box 1**). NAFLD and metabolic syndrome both increase the risk of cardiovascular diseases and T2DM[46]; therefore, NAFLD and metabolic syndrome probably have similar risk profiles[47].

For research purposes, several scores or algorithms based upon histological evaluation of liver biopsy samples have been developed to enable patient classifications in epidemiological studies. For example, the NALFD Activity Score (NAS) is a scoring system calculated from the semi-quantitative evaluation of steatosis, lobular inflammation and hepatocyte ballooning[48]. Although accurate in low (<3) or high (>5) values to exclude or diagnose NASH, respectively, NAS scoring is often inaccurate within the intermediate values (scores 3–4)[31]. As a consequence, European guidelines recommend to only use NAS for disease severity evaluation once the diagnosis has been made[23]. A newer diagnostic algorithm, the Steatosis

Activity and Fibrosis (SAF) score, which includes the semi-quantitative scoring of these factors, to enable the classification of patients as no NAFLD, NAFLD or NASH, has demonstrated improved performance compared with NAS, in particular within the intermediate values of the NAS (scores 3-4)[31]. Indeed, compared with NAS, the SAF score emphasizes the importance of activity, the main culprit of NASH, and therefore provides more accurate and comprehensive histological description. As such, it is now qualified as a true diagnostic score in the European guidelines[23]. Despite the utility of these scoring approaches, they rely on liver biopsy, which has drawbacks such as sampling error, inter-individual variations in pathologist reading and the risk of complications, of which the most worrisome is internal bleeding.

As performing liver biopsies[26] on all patients with NAFLD[49] is unfeasible for disease screening, diagnosis or examining progression in both routine care and research, noninvasive diagnostic methods using plasma samples[50], ultrasonography[51], MRI [52] or liver elastography (including both transient and magnetic resonance[53–55]) have been developed[56–58], and offer good diagnostic performance for liver fibrosis[53,54]. (**Box 2**). These methods have been widely used for early disease detection (steatosis), disease severity assessment, identification of patients needing a liver biopsy for confirmatory diagnosis (patients with divergent results obtained upon two noninvasive tests[53,59,60]) and for assessment of disease progression (fibrosis). Despite their obvious benefit compared with liver biopsy, these noninvasive tools are also hampered by several limitations (summarized in **Box 2)**[58]. They are, in general, not sensitive enough to evaluate the complete spectrum of NAFLD histological lesions[45] and lack validity to be used for routine diagnosis (reviewed elsewhere[54,57,61]). Transient elastography can be seen as an exception, however, as it was validated against liver biopsy with good area under the receiver operator

characteristic (AUROC) values ranging from 0.70 to 0.89 for both steatosis and fibrosis in a large population composed of 450 patients with the complete spectrum of NAFLD fibrosis stages[62].

Despite these noninvasive tests, liver biopsy remains the gold standard for NAFLD and NASH diagnosis. Thus, new biology-based, inexpensive, easily accessible, highly sensitive and specific prognostic and diagnostic biomarkers are urgently needed. As the gut microbiota might have a pathophysiological role in NAFLD development, the use of noninvasive microbiota-related biomarkers from stool (microbiota signatures) and/or blood sampling (metabolic or microbiota-derived signatures) could be an interesting alternative to currently developed noninvasive tests or could be considered as a complementary approach.

[H1] NAFLD and gut microbiota

Mouse studies and faecal transplant experiments have provided evidence of a causal role of gut microbiota in NAFLD development. First, cohousing experiments with mice prone to developing NASH due to genetic modifications in the inflammasome pathway and healthy wild-type mice demonstrate that microbiota sharing through coprophagia leads to wild-type mice developing liver steatosis and inflammation[63]. Also, direct FMT (from weight-matched obese mice with or without steatosis to germ-free recipients) replicates some NAFLD alterations[64]. These liver alterations include increased hepatic triglyceride content and augmented expression of hepatic genes involved in lipid uptake, lipogenesis, fatty acid catabolism and very low-density lipoprotein export[64]. These phenotypes were traced to gut microbiota composition differences between weight-matched mice with or without steatosis with steatotic mice displaying an increase in two bacterial species (Lachnospiraceae

bacterium 609 and a relative of *Barnesiella intestinihominis*)[64]. Although mouse models might seem to be a solution to explore the microbiota and liver disease, mice experiments present many limitations to extrapolating information to humans. As reviewed in length[65], mouse models do not develop the complete spectrum of histological lesions observed in human NAFLD (that is, hepatocyte ballooning or cirrhosis), nor is it always associated with overweight and/or insulin resistance as in human NAFLD. Whereas some mouse models (choline-deficient mice) can ultimately develop the same final histological alterations as those observed in humans, the pathophysiology completely differs between mice and humans, since the former usually lose weight [65]. Additionally, the microbiota of mice and humans differs substantially[66]: in terms of composition (the vast majority of genera found in mice are absent in humans) and of dominant genera as well as specific genus and species abundance. Finally, mice and humans display major digestive tract architecture differences, which also influences gut microbiota composition[66]. These limitations make the evaluation of the role of gut microbiota within NAFLD in mouse models a challenge. One solution to circumvent this hurdle is using FMT from diseased patients to germ-free mice in an attempt to reproduce the patients' hepatic phenotype. Indeed, FMT from humans with NASH to germ-free mice leads to the transmission of some NASH features among which hepatic steatosis and inflammation, which are exacerbated during high-fat diet (HFD) feeding[67]. However, germ-free mice have an immature immune system[68] and immunity and/or inflammation balance is extremely important in metabolic disease development[10]. As conventional animals have a developed immune system and also allow engraftment of donor microbiota, use of conventional mouse models for FMT studies might be an alternative solution[68] for studying the role of the microbiota in rodent models. Notably,

faecal transfer from obese women with hepatic steatosis to conventional mice fed a chow diet, induces increased hepatic triglyceride content within 14 days[69]. Despite some of these limitations, evidence from rodent studies collectively strengthen the idea that the gut microbiota contributes to NAFLD development.

Several hypotheses have provided mechanistic insights into the pathways of how the gut microbiota might contribute to NAFLD development and progression to NASH, reviewed in detail elsewhere[28,70]. In brief, they include increased intestinal permeability that leads to LPS release to the host, which can trigger tissue and systemic inflammation, and the action of microbially-produced metabolites (including trimethylamine *N*-oxide (TMAO), choline or ethanol) and bile acid signaling, which can also affect immunity[28,70,71]. On the basis of these hypotheses, human studies have compared the gut microbiota composition between patients with NAFLD, NASH, NAFLD-cirrhosis, and healthy liver as controls to discover gut microbiota or microbiota-related metabolite signatures to be used as noninvasive diagnostic tools. We will hereafter focus on microbiota signatures observed in steatosis, NASH or NAFLD-cirrhosis in humans (mainly adults but also review some literature concerning paediatrics)[70,72]. Notably, gut dysbiosis occurs in obesity and T2DM[17]. We discuss signatures that are also seen during those metabolic diseases.

[H1] NAFLD gut microbiome signatures

Owing to heterogeneity in the literature, we have focused on concordant results across studies and describe bacterial signatures associated with the different stages of liver disease severity in humans. We summarize results according to taxonomic levels (bacterial phylum, family, genus and species) that are associated with different NAFLD progression stages (steatosis, NASH), NAFLD-fibrosis, and

cirrhosis and show that some bacterial signatures overlap with those described in obesity or T2DM (**Figure1 & 2**).

[H3] Simple steatosis to NASH signatures. Comparing patients with NAFLD to healthy individuals as controls[69,73], a consistent altered signature is observed at the level of phylum (increased Proteobacteria[69,74–76]), family (increased Enterobacteriaceae[74,77] and decreased Rikenellaceae[77,78] and Ruminoccaceae [73–75]), and genera (increased *Escherichia*[69,77]*, Dorea*[75,78]*, Peptoniphilus*[77,78] and decreased *Anaerosporobacter*[55,73], *Coprococcus*[69,73,77], *Eubacterium*[69,77]*, Faecalibacterium*[55,77] and *Prevotella*[69,79]). Although these initial results suggest a measurable dichotomy in microbial signatures between individuals with hepatic steatosis and controls, there are, however, large discrepancies found across studies with divergent results for phylum, family, genus and species[69,73–81], as described in detail in **Table 1 and 2**.

Similarly to NAFLD, when comparing patients with NASH to healthy individuals as controls, using either liver biopsy[42,69,74,76–81] or noninvasive biomarkers[73,75,78,81], some concordant microbial signatures are observed, which also overlap with NAFLD signatures: phylum (increased Proteobacteria[74–77]), family (increased Enterobacteriaceae[74,77] and decreased Ruminococcaceae[73–75,77,82] and Rikenellaceae[77,78]) and genera (increased *Dorea*[75,78] and decreased *Faecalibacterium*[77,82,83], *Coprococcus*[73,77,82], *Anaerosporobacter*[73,83]). However, similar to results observed for steatosis, the abundance of some bacteria[69,72–81,83] , display opposite trends across the literature as shown in **Table 1 and 2.**

[H3] From NAFLD-fibrosis to NASH-cirrhosis. Few studies have focused specifically on microbiome signatures in NAFLD- fibrosis and even fewer examined

microbial composition as a function of fibrosis progression. Nevertheless, concordant signatures are observed and detailed in **Table 1 and 2**. When compared with patients with advanced fibrosis, individuals with less severe liver alterations or healthy individuals as controls display decreased abundance of Gram-negative bacteria, decreased Fusobacteria phylum, increased Enterobacteriaceae family, (*Bacteroides, Ruminococcus, and Shigella* genera[74,79] and by contrast increased[76] Gram-positive bacteria, Firmicutes phylum, Prevotellaceae family and *Prevotella* genus.

One caution in interpreting the findings is that these studies used various experimental designs each comparing different stages of fibrosis severity (that is, comparison of patients with mild to moderate fibrosis (F0-F2) versus advanced fibrosis (F3-F4), or cirrhosis)[76], whereas others compared patients with no to little fibrosis (F0–F1) to patients with moderate to advanced fibrosis (F≥2)[74]. Finally, another study compared patients with moderate to advanced fibrosis (F≥2) with patients having mild fibrosis (F0–F1) with or without NASH[79]. These differences in experimental design could explain discrepancies found in the proposed microbial signatures (**Table 1 and 2).** For example, *Bacteroides vulgatus* and *Escherichia coli* are the most abundant species in advanced fibrosis (F3-F4)[76]. *B. vulgatus* abundance is also increased from mild–moderate fibrosis to advanced fibrosis[76]. Interestingly, the *B. vulgatus* signature is a common observation associated with metabolic alterations as it is also increased with increasing BMI, specifically in severe obesity, which is characterized by decreased microbial gene richness[84]. Furthermore, *B. vulgatus* abundance increases with increasing haemoglobin A1c (Hba1c) levels [84]. *B. vulgatus* is also associated with insulin resistance[85] and is decreased in obese women receiving prebiotics (inulin-type fructans), a treatment

that improves insulin sensitivity[19]. Likewise, *E. coli* has been shown to be increased in patients with T2DM[86]. These examples illustrate overlapping observations between bacterial signatures linked to NAFLD-fibrosis or NAFLD and those linked with metabolic disorders as obesity and diabetes (**Figures 1 and 2**). Although models have been proposed to use the microbiome as a reservoir for diagnostic signatures of NAFLD-fibrosis[76], further confirmation in independent cohorts and across geographical regions are necessary to assess their clinical relevance.

In patients with cirrhosis (some of whom had 'pure' NASH-related cirrhosis, whereas others were of different aetiology such as viral hepatitis, as detailed later), metagenomic signatures are relatively consistent across studies[76,87–90], confirming the importance of oral microbes invading the intestine in this disease. Taxa including *Prevotella*[88,90]*, Veillonella*[73,90] and *Streptococcus*[87,90,91]*,* being part of the oral cavity bacterial ecosystem, seem to discriminate between patients with cirrhosis and healthy individuals. Likewise, whereas some signatures appear consistent when comparing patients with cirrhosis to healthy individuals as controls (decreased abundance of Lachnospiraceae[87,89], *Veillonella*[4,88], *Prevotella*[4,88] and increased abundance of Enterobacteriaceae[74,87,89,92]), others display contradictory trends across studies[76,87,88]. In general, microbial signatures of cirrhosis are related to a drastic shift in taxa composition leading to an increase of pathogenic taxa and a decrease in taxa proposed to be metabolically beneficial[89] (**Table 1 and 2**). A functional consequence of this taxa shift might be increased endotoxaemia. Indeed, it was demonstrated in mice that during HFD-induced NAFLD, there was a concomitant increase in LPS levels and changes in gut microbiota composition[93,94]. Furthermore, exacerbation of NAFLD into NASH was also associated with increased LPS levels[94]., and bacterial production of antibacterial peptides has been proposed to help maintain

intestinal barrier integrity[89]. Thus, combined effects of increased endotoxaemia, reduced butyrate production and reduced bile acid production (discussed further later) could worsen cirrhosis progression[89]. Another feature also associated with other common diseases is the reduction of levels of *Faecalibacterium prausnitzii*[95] in cirrhosis[90,91]. Indeed, *F. prausnitzii*, known to have anti-inflammatory properties and to be abundant in healthy conditions[11,12], is reduced in abundance in a number of diseases, including intestinal disorders (inflammatory bowel disease[96] or irritable bowel syndrome[97]), obesity[11,12] and diabetes[15,98].

However, the evaluation of gut microbiota contribution in liver disease progression (from steatosis to NASH, and NASH-cirrhosis) is limited and bacterial markers are frequently identified in one study, yet not confirmed in independent cohorts. As the origin of liver disease is heterogeneous by nature, most studies in cirrhosis have included patients with different aetiologies including hepatitis B[87,90], biliary disease-related cirhosis[88,90], alcohol-related cirrhosis[87,90], NASH or a combination of different diseases. Patients were also included at different stages of disease severity with compensated cirrhosis **[G]** or decompensated cirrhosis **[G]**[89,90]. These differences could collectively explain why associated metagenomic signatures of liver disease progression are not frequently replicated. Nevertheless, a study exploring well-characterized patients with non-NAFLD, NAFLD without advanced fibrosis or NAFLD-cirrhosis provides a potentially promising diagnostic signature, which includes 27 bacterial features and 3 demographic characteristics (BMI, age and gender). This signature is able to robustly identify NAFLD-cirrhosis and was further confirmed in an independent cohort with an AUROC of 0.92[91], which remained accurate after adjustment for T2DM. Although promising, this signature will need to be further confirmed in different cohorts including various ethnicities and

individuals from different geographical regions. Furthermore, to use these microbial signatures in clinical practice, validated assays will have to be developed to enable easy and reproducible diagnosis.

Although the literature provides initial information regarding gut bacterial groups as promising signatures of different stages of liver disease progression, whether the microbiota is a causal factor, which interacts with the complex pathophysiological processes driving disease from mild fibrosis to severe fibrosis[74,76,79] and eventually cirrhosis still needs to be demonstrated.

[H1] Gut-derived metabolites and pathways

Studies have evaluated the metabolomic signatures associated with NAFLD or NAFLD-fibrosis and have been extensively reviewed[99]. Among these signatures are molecules produced by bacterial communities (**Figure 3**) such as LPS[100], short-chain fatty acids such as butyrate, propionate and acetate (the balance of which mediates beneficial or detrimental effects on the liver)[71] and products derived from bile acid metabolism acting on FXR within the liver or the intestine[101–103] . Changes in these metabolites are suspected to have a role in the pathophysiology of liver injuries[99]. Herein, we choose to focus on novel studies exploring substrates of gut microbiota metabolism or circulating gut microbiota-derived metabolites. Importantly, all of these metabolites have also been demonstrated to be involved in obesity and metabolic alterations, including T2DM. For example, evidence from mice during obesity, LPS is increased and promotes the activation of insulin resistance pathways in tissues[93]. Although, SCFAs have beneficial effects on metabolic health, they are also involved in energy harvesting and, therefore, potentially contribute to increased weight gain[104]. Levels of SCFAs have been found to be increased in faecal samples from individuals

who are obese as compared with healthy individuals[105]. Overall, understanding their specific role in NAFLD physiopathology is complex.

[H3] Choline, betaine and circulating methylamines. Mice fed a choline-deficient diet[106] are recognized as a representative model of NAFLD[107,108] and reducing dietary choline leads to both increased liver fat and gut bacteria modifications[108]. Choline is an essential nutrient and a component of phosphatidylcholine, which is a precursor of acetylcholine (neurotransmitter) found in food. Choline is a substrate that can be oxidized to betaine. HFD-induced NAFLD mice fed a diet with standard levels of choline exhibit a decrease in systemic phosphatidylcholine with increasing severity of NAFLD[109]. This observation was translated to humans in which patients with an increasing severity of NAFLD display a decreased ratio of betaine:choline[110]. As demonstrated in mice and humans[111,112], dietary choline is metabolized by the gut microbiota into trimethylamine (TMA), which is further metabolized in the liver by the enzyme FMO3 and results in the production of TMAO[17]. Increased circulating TMAO is proposed as a biomarker of cardiovascular events and kidney dysfunction[17,112,113], and the increase of circulating levels TMAO positively correlates with the increase of *Deferribacteres* and *Tenericutes* in the gut in mice[113]. In humans, elevated levels of TMAO were seen in individuals with prevotella enterotype, and several OTUs were significantly increased in patients with higher concentrations of TMAO **[Au: in humans?]** [113]*.* Mouse studies demonstrate that increased NAFLD severity is associated with increased urinary levels of both TMA and TMAO[109]. These observations can be seen as paradoxical as increased consumption of choline and phosphatidylcholine correlates with increased production of TMA and TMAO[111,112] . The pathophysiological explanation by which TMA and/or TMAO has a role in NAFLD development therefore needs further

examination[114], but the proposed mechanisms include a reduction of host choline bioavailability due to a switch in microbiota metabolism to methylamine production as well as urinary excretion[114]. In human studies, TMAO is independently associated with increasing severity of NAFLD when comparing patients with NAFLD to healthy individuals[110].

[H3] TMAO and bile acids. Another major function of the gut microbiota is the deconjugation of primary bile acids into secondary bile acids. Overall, primary bile acids are involved in cholesterol metabolism, facilitate the absorption of dietary fat and fat-soluble molecules, and have a role in regulatory pathways[115]. Primary and secondary bile acids have endocrine functions and modulate numerous host metabolic pathways through different receptors[116]. Secondary bile acids are notably preferential ligands of the G protein-coupled bile acid receptor-1 (TGR5) a key actor of energy, glucose and lipid metabolism in the host[103]. The gut microbiota not only regulates secondary bile acid metabolism but also inhibits the liver synthesis of lipids by alleviating FXR inhibition[117]. Differences in bile acid pool size and composition has been associated with metabolic diseases[118]. Thus, gut dysbiosis could influence bile acid pool, composition and homeostasis. Evidence from mice and humans suggests that bile acid bioconversion by the gut microbiota (deconjugation, dehydrogenation and dehydroxylation) is related to NAFLD and NASH progression[119], as previously reviewed[101]. Interestingly, a decrease in bile acids could be associated with NAFLD through TMAO production since TMAO induces a decrease in the total bile acid pool by inhibiting two key enzymes involved in bile acid metabolism: CYP7A1 and CYP27A1[110,113,120]. In agreement with this hypothesis, patients with advanced cirrhosis exhibit a decreased conversion of bile acids with concomitant modifications

of their microbiota composition, including higher Enterobacteriaceae but lower Lachonospiraceae, Ruminococcaceae and *Blautia* abundance [121,122].

[H3] 3-(4-hydroxyphenyl) lactate. A study published in 2019 has shown that 3-(4-hydroxyphenyl) lactate is associated with increased severity of NAFLD-fibrosis both in a test and validation cohort, both comprising 156 individuals (one from the Twin and family study, the other from an independent prospective study)[123] (**Figure 3**). Interestingly, 3-(4-hydroxyphenyl) lactate is a gut microbiota-derived product of aromatic amino acid metabolism. These results are in line with another study performed in patients with different stages of steatosis, which showed decreased microbial gene richness and an alteration in aromatic amino acid and branched-chain amino acid metabolism in steatosis[69]. This metabolite could be used as non-invasive biomarker of NAFLD, but needs further confirmation.

[H3] Ethanol. Production of ethanol by the gut microbiota could also play a part in NAFLD physiopathology. In children, the gut microbiota of individuals with NAFLD exhibits increased abundance of ethanol-producing bacteria as compared with those who were obese or healthy children as controls[77]. In the absence of ethanol consumption, adults with NASH display increased breath ethanol concentrations[124], which could be attributed to those with NAFLD producing more gut microbiota-derived ethanol as compared with healthy controls. These results suggest that gut microbiota ethanol production might serve as a liver toxin contributing to the development of NAFLD and its progression towards NASH[125]. A study performed in mice and further validated in humans, indeed displayed that some bacteria (namely *Klebsiella pneumoniae*) were able to produce ethanol from glucose, in the absence of any alcohol consumption[126].

[H3] ***Short-chain fatty acids***. Short-chain fatty acids (SCFAs), a group comprised of butyrate, acetate and propionate, are locally produced in the colon through microbial fermentation of normally non-digestible complex carbohydrates (dietary fiber)[99,127]. Their role and mechanism of action in NAFLD development has been extensively reviewed[127,128]. Microbially produced SCFAs are absorbed primarily through diffusion or co-transport in the colon whereas their intestinal signalling effects are mediated by activation of G-protein-coupled receptors (GPR41 and GPR43)[129]. SCFAs have been proposed as an important substrate to increase liver triglyceride levels and promote energy storage and weight gain[130] as SCFAs are involved in fatty acid synthesis and gluconeogenesis[131]. Human studies comparing NAFLD, NASH and healthy individuals as controls have observed an increased faecal concentration of SCFAs in patients with NAFLD and/or NASH [132] concomitantly with an increase in abundance of bacterial groups involved in their production. Furthermore, this increased faecal SCFA and microbial signature observed during NASH[132] are associated with reduced numbers of resting regulatory T-cells (rTregs) (CD4+CD45RA+CD25+) and higher Th17:rTreg ratio in peripheral blood, which are systemic immunological features previously observed in NASH[133] .

Nevertheless, SCFA action is quite complex, as they also can provide metabolic benefits. GRP43 activation by SCFAs reduces pro-inflammatory production and immune cell (T cells)[134] infiltration, whereas *GRP43*$^{-/-}$ mice or germ-free mice with lower levels of SCFA display increased inflammation both at the level of circulating immune cells as well as in the colon , a feature usually seen in NASH[135]. However, inflammation during NASH development was not assessed in these models and the effect of SCFAs on liver inflammation needs further investigation.

Furthermore, although dietary fibres have been shown to be beneficial for metabolic health[10], some interventional studies using soluble fibres have, in contrast, led to increased liver disease in mice with genetically-induced or high fat diet-induced microbial dysbiosis[136,137]. Thus, although soluble fibres can induce positive metabolic effects, such as those observed on glucose metabolism, the effects of soluble fibre supplementation in NASH mouse models need further exploration before interventional studies in patients. Moreover, due to interindividual variability in the gut microbiota, dietary supplementation of fibre might need to be personalized because of possible different effects in different individuals, which could also be further complicated due to the variations of soluble fibres available. Most importantly, each SCFA exerts specific and somehow different metabolic effects. Thus, assessing their balance both at the fecal and systemic level in patients with NASH and after a dietary intervention would probably decipher more precisely their overall role in NAFLD development, exacerbation or improvement. Finally, it was reported that SCFAs could have a beneficial role in NAFLD through epigenetic modulation via histone deacetylase (HDACs) inhibition. Indeed, it was shown in rats that histone deacetylase (HDACs) inhibition decreased liver gene expression involved in NAFLD mostly lipogenic genes, such as acetyl-CoA carboxylase (Acc), fatty acid synthase (Fasn), and sterol regulatory element binding protein 1c (Srebp1c)[138] . This finding provides important mechanistic insights, reviewed elsewhere[127,128].

New bacterial metabolites and derived factors will be identified as contributors to liver disease in the future. Thus, it will be essential to determine how these factors, together with changes in LPS, biliary acid metabolism and SCFAs, contribute to NAFLD development and progression. Investigations are now needed to determine

the relevance of these molecules as biomarkers and/or predictors for diagnosis of NAFLD and NASH and its progression.

[H1] Issues in NAFLD metagenomics studies

The number of clinical studies investigating gut microbiota signatures associated with NAFLD and/or NASH or fibrosis is increasing rapidly. However, careful interpretation is needed when reviewing the literature due to the heterogeneous cohorts used across studies with differences in sex, ethnicity, liver disease severity stages, BMI, presence of T2DM, patient populations (paediatric or adult), corpulence, and other associated metabolic diseases. The gut microbiota sequencing technologies used vary among studies, and other critical factors influencing the gut microbiota, such as dietary consumption or drug intake are scarcely measured or under-reported.

[H3] Population variability in demographic characteristics. Although there is some consistency in the literature for microbiome signatures, there is a noticeable lack of reproduced findings or confirmation in independent cohorts. This heterogeneity might originate from different methodological approaches and from major inter-individual variability among recruited patients regarding particular demographic characteristics. While one study examined only women[69], most studies examined adults[69,73–76,79,80,83] and children[77,78,81] of both sexes. Yet some studies have controlled for sex[69,73–75,80,110] and most have also controlled for age[69,73–77,79,90,110]. Nevertheless, it is important to note that there are histological specificities for adolescent and adult NAFLD[139,140], as recalled in recent pediatric clinical guidelines [141]. For example, in paediatric NASH, the ballooning degeneration, classic zone 3 fibrosis, and parenchymal inflammation often seen in adult NASH are less

common in children NASH[140,141]. Furthermore, several histological types are found in children: type 1 resembling the adult form, type 2 NASH is mainly characterized as NASH yet with no or minimal ballooning degeneration, and finally a third type with with overlapping features[139]. Studies were also performed in different geographical regions (North America, Canada[75,80] and USA[76,81]), Asia (China[73,83]), Europe (France[79], Italy and Spain[69]) probably with various ethnic backgrounds and cultural and food habits. Importantly, ethnicity seems to strongly influence microbial composition even in individuals living in the same geographical area. For example, gut microbial diversity differed substantially in different ethnic groups all living in the Netherlands and ethnicity accounted for a major part of these differences [142]. Thus, whereas there are different liver disease risks across ethnicities[143], it might translate into differential microbiome-related signatures[142]. Notably, some studies controlled for dietary habits[73,74,77,80,81,110], which could relate, in part, to cultural differences.

[H3] Population variability in corpulence. Obesity, and particularly abdominal obesity, is a well-known risk factor for NAFLD[144], making these diseases strongly inter-dependent. In published reports, although some studies included lean individuals[73,74,83], others examined individuals who were overweight[74,83] or obese (mixing different classes of obesity)[69,75,76,78–81,87]. Several studies even further stratify by the severity of obesity classes (class I: BMI 30-35kg/m² [75,77,79]), one study in particular focused on severe or morbid obesity (BMI> 35 or BMI >40 [69]), and it has been demonstrated that gut microbiota dysbiosis is exacerbated with increasing obesity severity[11,84].

Metagenomic studies clearly demonstrate relationships between corpulence and gut microbiome changes. Microbial gene richness, for example, strongly

decreases with increasing BMI[11,12,84]. Furthermore, although individuals with obesity generally share common bacterial signatures and modified functional properties, some signatures differ across the obesity spectrum[11], with peculiar signatures only found in populations with extreme BMIs (above 40kg/m²)[84]. On the basis of these findings, microbiome-related signatures in studies comparing patients with NASH with different degrees of obesity and lean or overweight patients with NAFLD as controls[80] or patients with different classes of obesity and NASH and healthy lean individual as controls[74] might be overestimated and mostly related to the degree of obesity[145]. The link between closely associated metabolic disorders, such as obesity and NAFLD is likely to be more complex than compositional shifts in bacterial composition alone. Thus, it remains difficult to conclude whether these signatures are solely related to liver alterations, BMI or both. In one study, Shen *et al*.[74] did not find any statistically significant shift in gut microbiota, when comparing patients with NAFLD stratified by BMI. Patient groups were, however, of small size (*n*=47) and the study had limited power to definitively conclude the absence of NAFLD–NASH specific microbial signatures according to corpulence. By contrast, Wang *et al.* focused their analytical comparisons between NAFLD and healthy individuals with normal and comparable BMI[73]. They demonstrate a microbiome signature of patients with NAFLD independently of corpulence differences. Finally, to limit bias owing to differences in corpulence, studies exploring NAFLD microbiome signature have adjusted their statistical analysis on BMI (for example, according to the studies, the preformed partial Spearman's rank-based correlation (pSRC) coefficients adjusted on BMI, or linear regression adjusted for BMI, multivariate models, analysis of covariance (ANCOVA) or finally logistic regression analysis) [69,73,74,76,79,80,82,89,110].

Despite the described interactions between corpulence and liver disorders (from steatosis to cirrhosis considering individuals who are lean or obese), Enterobacteriaceae is consistently increased in both individuals with NAFLD and those who are obese in numerous studies[74,77,87,89,146,147]. Decreased microbial gene richness is found both in individuals with obesity[11,12] and patients with NAFLD in some studies[74,91], but also in lean individuals with NAFLD[73]. More importantly, BMI is a proxy of obesity and future studies should extend phenotyping to other measures related to body fat amount and distribution (for example, abdominal versus gynoid distribution) **[G]** to examine the interplay between fat distribution, different stages of liver alterations and microbiome alterations. Indeed, microbial gene richness is negatively correlated with increasing visceral fat deposition[84].

[H3] Population variability in metabolic diseases and related treatments. Another major confounder lies in obesity-associated metabolic comorbidities, which are also involved in NAFLD physiopathology. Diabetes is a major risk factor involved in NAFLD development [148] and its presence strongly exacerbates NAFLD to overt NASH, including forms associated with mild and advanced fibrosis[149]. Metabolic syndrome[15] or T2DM[14,15,85] *per se* are known to be associated with microbial signatures. Thus, it might be tricky to disentangle microbial signatures from NAFLD and linked metabolic disorders, such as diabetes. Four NAFLD studies[74–76,79] included patients with T2DM and two other studies evaluated the signature and predicted function of the gut microbiome of patients with T2DM without known NAFLD[14,15]. Despite the difference in geographical demography (European women with diabetes[15] and Chinese adults with diabetes[14,15]), these studies showed one consensus finding: a statistically significant reduction in abundance of butyrate-

producing bacteria in T2DM. However, they did not examine whether patients with T2DM had NAFLD; extrapolating from epidemiological evidence, most probably, those patients had both NAFLD and T2DM. Clostridia[73,150] and *Lactobaccillus*[15,75] are two common species signatures found in both T2DM and NAFLD (increased abundance of *Lactobacillus* and decreased abundances of Clostridia in both patients with NAFLD and those with T2DM compared with healthy groups). At a predicted functional level, both patients with T2DM and those with NAFLD display consistent decrease in butyrate-producing bacteria[15,73,77]. *E. coli* is consistently enriched in studies exploring either solely cirrhosis or solely diabetes [14,90]. By contrast, the *Roseburia* genus shows opposite trends across studies[15,73,75,77] (**Table 2**). However, one study included only six individuals with T2DM among their NAFLD cohort[75], potentially explaining these discrepant results since *Roseburia* is also known to be decreased in T2DM[15]. Importantly, some studies looking for a NAFLD microbial signature actually controlled their analysis for the presence of T2DM[69,73,76,79,90] to limit this bias. For example, microorganisms signature remained associated with NAFLD after proper adjustments for T2DM but results were not always replicated in all studies (for example, *Propionibacterium acnes*[69], *Bacteroides fragilis*[69], *Anaerosporobacter*[73], Enterobacteriaceae[79]) In studies including patients without T2DM, only one controlled for insulin resistance[82].

Medication use is another critical feature influencing microbiome signature variability among individuals[151,152]. Studies exploring microbiome signatures of NAFLD[74–76,79,80] included patients with T2DM whereas others excluded them[73,77,78,81,83], but most do not clearly state the list of current medications that study participants are taking. Only three studies evaluating microbial signature during NAFLD have controlled for medication use[69,73,76]. Metformin is the first line of

pharmaceutical therapy prescribed in T2DM and is commonly used in NAFLD since 50–75% of NAFLD patients have T2DM according to population and ethnicities[153,154].The effect of metformin on the gut microbiome is well-documented[86,155,156]. Metformin increases the abundance of *Akkermanisa muciniphila*[155,156], a bacteria associated with improved insulin sensitivity in mice and humans[18,157,158]. Furthermore, treating mice on a HFD with metformin recapitulates the improvement in insulin sensitivity and switches the microbiota composition towards that of mice on chow diet[156]. Patients with T2DM taking metformin display a specific microbiome signature with an increase in *Escherichia* species[86], which could explain, in part, the increased *E. coli* found in patients with NAFLD-fibrosis[69,76,77] . Owing to increased risk of cardiovascular events, statins are frequently prescribed to patients with T2DM and NAFLD–NASH and could strongly influence the gut microbiome[159,160]. A study in mice observed a reduction in microbial diversity upon statin treatment, a modification in bile acid pools and a reduction of SCFA producing bacteria which was also confirmed in humans[160]. Nevertheless, those findings need further confirmation in larger-scaled studies. Proton pump inhibitors, which are frequently given to patients with cirrhosis, have been shown to switch microbiome composition towards an increased abundance of oral bacteria in the gut microbiota [161], thus the 'oral microbiome' signature observed in cirrhosis could well be related to drug intake rather than disease. It remains critical in microbiome studies examining patients with NAFLD or NASH to collect information on diabetes history and drug intake. Although some studies have controlled for medication, no study has yet controlled for each of the above potential biases when looking for a microbial signature of NAFLD.

[H3] Variability in liver injury diagnostic methods. Another source of variability in the reviewed studies is the different grades and stages and heterogeneity of liver disease alterations (steatosis, NASH and fibrosis)[75,79,81]. Even if the use of the SAF score seems to improve inter-observer variability, the inter-individual pathologist variation when examining biopsy samples is acknowledged in NAFLD diagnosis[162,163]. As liver lesions are closely intertwined, deciphering the specific microbial signatures of each histological lesion is challenging. Studies have focused on different stages of disease progression. One study focused on the steatosis state[69], three on NASH[77,81,83], and seven investigated the NAFLD spectrum (from steatosis to NASH)[73–76,78–80]. Two reports focused on different fibrosis stage[74,79] and three on cirrhosis[87,89,90], whereas one study investigated a larger disease spectrum of fibrosis to cirrhosis[76]. However, in the latter study, patients with fibrosis probably displayed concomitant lesions of NASH and/or steatosis. As most studies do not focus on the same stages of disease progression, it is rather challenging to underline concordant microbial signatures.

The diagnostic method used to classify NAFLD lesions is also an important factor to consider when examining these studies. Liver biopsy, the most reliable diagnostic method and currently considered as the gold standard, was used in nine studies[69,74,76,77,79,80,83,88,90]. However, others used less reliable and noninvasive tools such as ultrasonography, MRI or blood tests[73,75,78,81] (such as liver enzymes (alanine aminotransferase, aspartate aminotransferase and γ-glutamyl transferase)[73,75,78,81], and also other metabolism-related biomarkers (levels of fasting serum glucose[73,78], insulin[78], triglyceride[78] or complete lipid profile[73]), or liver-related biomarkers (albumin and platelet count [73]) (Box **2**). These noninvasive tools are designed to specifically characterize one histological aspect, it might well be that these patients displayed

heterogeneous lesions (steatosis and some degree of fibrosis) that were not investigated. Furthermore, the degree of lesions severity is variably expressed. For example, some studies considered simple steatosis[78], whereas other took into account the steatosis score and discriminated steatosis severity from S0 to S3[69]. These aspects are critical and could partly explain disparities found in clinical studies exploring microbiome signatures of NAFLD and liver disease progression. In addition to placing a priority on using liver biopsy when possible, diagnostic tools and the interpretation of their results used to assess liver alterations should be taken into consideration when comparing across the literature.

[H3] Bias due to circadian rhythm. Another overlooked factor in the literature examining gut microbiota and NAFLD is the contribution of circadian rhythm. Circadian rhythm is a well-known and pivotal regulator of liver metabolic pathways, which are altered in NAFLD development[164]. For example, jetlag (seen as a perturbation of the circadian clock) is associated with the worsening of metabolic alterations (that is, the hallmarks of NAFLD), which show further perturbations during obesity[165]. Animal studies and experimental models have shown that feeding time also influences the circadian rhythm and, subsequently, host physiology[166]. Some human population cohorts confirmed those findings[167]. Studies performed in different mouse models (genetic invalidation of clock genes, antibiotic treated or conventional mice with or without modification of the light–dark phases) have shown that the gut microbiota displays rhythmic oscillations in the colon during the day in terms of proliferation, composition, functions and metabolite production[168–170] and depend upon function of the host circadian clock. These oscillations (that is, existence of time-of-day-specific profiles of microbiota functionality) were demonstrated to be

controlled by host feeding intake. Interestingly, modifying feeding times results in a shift of cycling bacteria. Moreover, timed feeding can restore the loss of fluctuations in circadian clock deficient mice[169] . Perturbations of the circadian rhythm are also associated with metabolic impairment and microbiota dysbiosis, both in experimental models as well as in humans[169]. On the other hand, the gut microbiota and its circadian oscillations also influence the rhythmic expression of host intestinal and liver genes that are not known to be involved in the circadian clock[168]. Additionally, these gene expression patterns are modified in the absence of gut microbiota as genes from several metabolic pathways lose their oscillatory patterns, whereas genes from other metabolic pathways gain rhythmicity with the lack of microbiota in animal models[168]. As such, germ-free mice submitted to light–dark cycle display impaired circadian hepatic gene expression[168], demonstrating the role of gut microbiota in the circadian clock effects. Thus, gut microbiota changes could influence the rhythmicity of several host metabolic pathways contributing to NAFLD physiopathology. This aspect could be due to microbiota functions rather than composition as gut microbiota-produced metabolites from the diet can also modulate liver clock gene expression, observed when treating hepatic organoids with different SCFA[170]. Furthermore, in clock gene knockout models, modulating feeding time can rescue abrogated host gene expression oscillation[170]. These observations highlight the combined contribution of both circadian clock and diet acting on gut microbiota and host physiology. Whether these results obtained in mice models are relevant in human needs further investigation. This aspect also raises the question if the methodology of studies examining gut microbiota signatures in NAFLD need to consider patients' circadian clock phenotype and food intake rhythm, which could help explain some discrepancies in gut microbial signatures observed across studies.

[H3] Sequencing methods. Another important point to be considered when comparing different results across the literature is the methods used to sequence and analyze the gut microbiota. Furthermore, sampling (home faeces auto-collection use different devices that have not all been evaluated, one complete bowel movement stool or a sample of that material, mucosal or luminal microbiota in studies including surgery sampling, faeces versus caecal or other parts of the intestine in mouse experiments) itself as well as the specific steps of analysis, including steps before sequencing (i.e. sample concentration, lysis, purification and extraction), also need to be harmonized to enable comparisons between studies as reviewed herein[171]. Studies used a variety of methods such as quantitative PCR[80], 16S ribosomal RNA (rRNA) sequencing [74,78,79], or shotgun sequencing **[G]** [69,76,90]. It has been previously shown that Sanger, Roche 454, or Illumina-based 16S rRNA or shotgun sequencing can lead to different results[172]. The results from 16S rRNA sequencing are less granular and accurate than those from whole shotgun sequencing, because the 16S approach sequences a single region of the bacterial genome whereas shotgun sequences the complete genome[173]. Species prediction is not optimal using 16S rRNA sequencing[171], and the results derived from pyrosequencing frequently lack numerous species because of the choice of tagged-primers[174]. Shotgun sequencing produces extended information regarding read sequences, since it can sequence and amplify the complete genome. Shotgun sequencing, therefore, produces more information as it also includes unknown metagenomic species, which potentially lead to increased discovery potential. Metagenomic sequencing enables the prediction of functional potential based on gene and species annotations.

In addition to the sequencing technology, the bioinformatic pipelines used to analyze sequencing data also contributes to the variability of results. Taxonomic analyses in 16S rRNA sequencing is easier due to established standard pipelines such as QIIME[175] and Mothur[176] and functional profiling in 16S rRNA is predominantly done with PICRUST[177]. However, until now, few studies precisely describe the microbial species that could be used as indicators of liver disease status[76,90] and even fewer provide clues for the discovery of new species. Although microbiota catalogues are currently quite exhaustive, the variability of sequencing method and sequencing depth might influence findings. The use of whole metagenome sequencing can provide this information, but requires pipeline harmonization and dedicated bioinformatics expertise[178].

The lack of power in statistical analysis is another issue regarding the reliability of microbial biomarkers that can be used with confidence. Uncertainty about the absence or presence of certain aspects of NAFLD and unbalanced population distributions might affect statistical results. Obtaining a validated healthy and liver-biopsied group is difficult owing to obvious ethical reasons. Indeed, in other fields such as oncology, ethical concerns have already been raised about research biopsy and their potential risk to harm participants as well as the adequacy of voluntary informed consent [179,180].Thus, this leads to a lack of statistical power and phenotypic uncertainty because of limitations in non-invasive testing. In addition to finding microbial abundance differences between groups, *P* values are often used and interpreted as whether the abundance difference is statistically significant or not. However, *P* values alone do not provide reliable results. Some studies perform only *P* value tests without False Discovery Rate (FDR) correction[77,79,80,83,87,89]. By contrast, nine studies used FDR or Bonferroni corrections[69,73–76,78,81,88,90]. For example, in

Mexican children with obesity, *B. plebeius* is negatively correlated with BMI percentile, but this finding is no longer statistically significant after FDR correction[181]. FDR should be mandatory when exploring metagenomics signatures. Importantly though, Falony *et al.* estimated that a sample size of approximately 1,700 individuals would be needed to adequately assess the relationship between obesity, NAFLD and microbiota composition in a cohort study when correcting for age, gender, ethnicity and other variables[151], which questions the feasibility to actually achieve such a trial.

[H3] Multi-omics approaches. As a complement to gut metagenomics, additional 'omics' data and systems biology approaches are a good method to further confirm microbial signature for a specific disease. By using both metagenomics and metabolomics[182] or lipidomics[183,184] , the gut microbiome compositional and functional signatures can be characterized and further linked with concentrations or production of gut-derived metabolites in blood, urine or faeces. An additional approach could also be to combine metagenomics to metatranscriptomics data, bringing some clarity to the gut microbiota genes specifically activated and providing further functional insights. Such approaches have already been used in other metabolic diseases (such as T2DM or obesity[84,185]) and are emerging in the field of NAFLD[69]. The use of approaches combing multi-omics techniques, coupled with computational science, will probably enable better insights regarding microbiota contribution in the pathophysiological pathways involved in these metabolic diseases and help stratify patients based on their multi-omics profiles. However, it does not preclude researchers from designing correct clinical trials in particular choosing carefully the best control groups.

[H1] Conclusions

Clinical studies have revealed gut microbiome signatures in NAFLD, NAFLD-fibrosis and cirrhosis, which could serve as future noninvasive diagnostic biomarkers for liver disease diagnosis or evolution prognosis. Nevertheless, the relevance of identified signatures needs to be further examined in longitudinal studies where physicians can prospectively examine the deterioration of liver status. This strategy was conducted for gut microbiota-derived factors in Crohn's disease[186] and in the cardiovascular field[111]. However, discrepancies are nevertheless observed across studies in NAFLD, which might originate from their large heterogeneity in terms of microbiome sequencing method, bioinformatic pipelines, liver diagnostic method and disease severity spectrum, as well as clinical and demographic characteristics. Obesity and T2DM are associated with strong microbiota dysbiosis and identified liver disease microbiome signatures are often biased by the additional presence of obesity and/or diabetes status, which are not always accounted for. There is, therefore, an urgent need for more investigations with strong study designs (**Box 3**). Studies should account for confounding metabolic disorders, such as T2DM and obesity, population background, medication and dietary intake. An option could also be to additionally examine identified-signatures in well-selected and clinically harmonized cohorts of patients with either T2DM or obesity. However, considering some potential confounding factors, such as circadian rhythm, might prove to be a complicated task for both researchers and patients. Future studies should also consider investigating gut microbiome signatures in a two-step manner; that is discovery and validation cohorts using varied and documented ethnic backgrounds and include patients with a biopsy-proven NAFLD and/or NASH diagnosis. Where possible, repeated exploration with a second longitudinal study incorporating liver biopsy should also be considered

to confirm clinical evolution. Overall, whether optimal study design is feasible remains an open question and in any case should be considered in the context of large-scale research consortia. Moreover, the understandable reluctance from physicians and patients, as well as ethical committees, to repeat liver biopsy is also a limitation. Finally, advanced methods in predicting microbiome signature (such as deep learning combined with multi-omics approach) are needed in this field. Exploring the whole microbial ecosystem in which the interaction of microorganisms could be as important as the abundance of single or multiple family, genus or species is a priority. Similarly, the importance of microbiome-altering factors, such as phages or viruses and fungi is worth examining. Combining microbiome signatures with systemic microbial-derived metabolites could help in the future to diagnose patients with liver alterations in routine care. The potential establishment of reliable biomarkers will determine how future NAFLD–NASH treatments modulate these signatures to develop biomarkers enabling follow-up for therapeutic response.

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J.A.-W. contributed to the research, discussion of content, writing and editing of this manuscript. C.V.; J.W.; P. L.; A.G.H.; J.V. contributed to the research, discussion of content, writing of this manuscript. M.N. and K.C. initiated the project and contributed to the discussion of content, writing and reviewing/editing the manuscript.

Competing interests

M.N. is in the Scientific Advisory Board of Caelus Pharmaceuticals, the Netherlands. K.C. is on the Scientific Advisory Board of LNC therapeutics and CONFO therapeutics and has contract consultancy and contract collaboration with Danone Research. None of these are directly relevant to the current paper. There are no

patents, products in development or marketed products to declare. The other authors declare no competing financial interests.

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Key points:

- Whereas animal studies have demonstrated a potential causal role of gut microbiota in nonalcoholic fatty liver disease (NAFLD), human studies have started to describe microbiome signatures in NAFLD.
- Proteobacteria is consistently enriched in steatosis and nonalcoholic steatohepatitis.
- The invasion of oral bacteria into the distal intestine (such as *Prevotella* or *Veillonella*) is observed in cirrhosis.
- *Faecalibacterium prausnitzii* abundance is reduced in cirrhosis and other diseases including diabetes, obesity and irritable bowel syndrome.
- Bacterial signatures (*Clostridia* and *Lactobaccillus*) overlap between NAFLD and metabolic diseases (type 2 diabetes mellitus)
- Discrepant microbiome signatures across studies could be linked to heterogeneity of geographical regions, ethnicity, population characteristics, microbiome sequencing tools, NAFLD diagnostic tools, disease spectrum, drug consumption and circadian rhythm.

Table 1 | Taxonomic gut microbiota signatures of NAFLD and NAFLD-fibrosis progression

This table indicates the microorganisms found in different studies as microbial signature(s) of NAFLD or NAFLD-fibrosis at different taxonomic levels. The table displays whether the particular microorganism changes its abundance with the disease progression from normal state to nonalcoholic fatty liver disease (NAFLD, and nonalcoholic steatohepatitis) and also from normal to high fibrosis. Cases where previous studies found discordant results are also indicated.

Characteristic	Microorganism	Status in NAFLD	Status in fibrosis
Taxonomic level			
Phylum	Verrucomicrobia	Increased[69]	Not assessed
Phylum	Fusobacteria	Increased[74]	Increased[87]
Phylum	Proteobacteria	Increased[69,74–77]	Discordant results[76,87,88]
Phylum	Firmicutes	Discordant results[69,73,75–78,83]	Discordant results[76,88]
Phylum	Bacteroidetes	Discordant results[73,74,78–80]	Discordant results[79,87]
Phylum	Actinobacteria	Discordant results[69,78,80]	Not assessed
Class	Gammaproteobacteria	Increased[81]	Increased74
Class	Bacteroidia	Increased[73]	Not assessed
Class	Epsilonproteobacteria	Increased[81]	Not assessed
Class	Clostridia	Decreased[73]	Not assessed
Family	Streptococcaceae	Increased[74]	Increased[87]
Family	Enterobacteriaceae	Increased[74,77]	Increased[74,77,89]
Family	Pasteurellaceae	Increased[75]	Increased[87]
Family	Veillonellaceae	Increased[75]	Increased[87]
Family	Erysipelotrichaceae	Increased[74]	Not assessed
Family	Kiloniellaceae	Increased[75]	Not assessed
Family	Succinivibrionaceae	Increased[83]	Not assessed
Family	Peptostreptococcaceae	Decreased[73]	Not assessed
Family	Ruminococcaceae	Decreased[73–75,77]	Decreased[89]
Family	Bifidobacteriaceae	Decreased[77]	Not assessed
Family	Rikenellaceae	Decreased[77,78]	Not assessed
Family	Lachnospiraceae	Discordant results[69,74,75,77]	Decreased[87,89]

Family	Prevotellaceae	Discordant results[74,77]	Decreased[79]
Family	Lactobacillaceae	Discordant results[73,75]	Not assessed
Family	Porphyromonadaceae	Discordant results[73,75]	Not assessed
Family	Fusobacteriaeae	Not assessed	Increased[87]
Family	Enterococcaeae	Not assessed	Increased[89]
Family	Staphylococcaceae	Not assessed	Increased[89]
Family	Bacteroidaceae	Not assessed	Decreased[87]
Family	Clostridiales XIV	Not assessed	Decreased[89]
Genus	*Shigella*	Increased[74]	Increased[74]
Genus	*Bacteroides*	Increased[79]	Increased[79]
Genus	*Ruminococcus*	Increased[79]	Increased[79]
Genus	*Acidaminococcus*	Increased[69]	Not assessed
Genus	*Akkermansia*	Increased[69]	Not assessed
Genus	*Eggerthella*	Increased[69]	Not assessed
Genus	*Flavonifractor*	Increased[69]	Not assessed
Genus	*Escherichia*	Increased[69,77]	Not assessed
Genus	*Lachnospiraceae_incertae_sedis*	Increased[74]	Not assessed
Genus	*Robinsoniella*	Increased[75]	Not assessed
Genus	*Dorea*	Increased[75,78]	Not assessed
Genus	*Porphyromonas*	Increased[77]	Not assessed
Genus	*Anaerococcus*	Increased[78]	Not assessed
Genus	*Bradyrhizobium*	Increased[78]	Not assessed
Genus	*Peptoniphilus*	Increased[73,78]	Not assessed
Genus	*Allisonella*	Increased[83]	Not assessed
Genus	*Parabacteroides*	Increased[83]	Not assessed
Genus	*Haemophilus*	Decreased[69]	Decreased75
Genus	*Eubacterium*	Decreased[69,77]	Decreased76
Genus	*Coprobacter*	Decreased[69]	Not assessed
Genus	*Holdemania*	Decreased[69]	Not assessed
Genus	*Subdoligranulum*	Decreased[69]	Not assessed
Genus	*Coprococcus*	Decreased[69,73,77]	Not assessed
Genus	*Moryella*	Decreased[73]	Not assessed
Genus	*Pseudobutyrivibrio*	Decreased[73]	Not assessed
Genus	*Anaerosporobacter*	Decreased60,70	Not assessed
Genus	*Alistipes*	Decreased[77]	Not assessed
Genus	*Faecalibacterium*	Decreased[77,87]	Not assessed
Genus	*Oscillospira*	Decreased[78]	Not assessed
Genus	*Prevotella*	Discordant results[74,77,79,81]	Discordant results[77,87,89]
Genus	*Oscillibacter*	Discordant results[69,75]	Not assessed
Genus	*Bifidobacterium*	Discordant results[69,77]	Not assessed
Genus	*Blautia*	Discordant results[74,77–]	Not assessed

		[79]	
Genus	*Lactobacillus*	Discordant results[73,75]	Not assessed
Genus	*Roseburia*	Discordant results[73,75,77]	Not assessed
Genus	*Bacilli*	Not assessed	Increased[87]
Genus	*Megasphaera*	Not assessed	Increased[87]
Genus	*Atopobium*	Not assessed	Increased[88]
Genus	*Dialister*	Not assessed	Increased[88]
Genus	*Clostridium*	Not assessed	Increased[90]
Genus	*Streptococcus*	Not assessed	Increased[90]
Genus	*Neisseria*	Not assessed	Decreased[87]
Genus	*SR1 genera incertae sedis*	Not assessed	Decreased[88]
Genus	*Alistipes*	Not assessed	Decreased[90]
Species	*Clostridium coccoides*	Increased[77]	Not assessed
Species	*Propionibacterium acnes*	Increased[79]	Not assessed
Species	*Bacteroides fragilis*	Decreased[79]	Increased[76]
Species	*Escherichia coli*	Increased[77]	Increased[76]
Species	*Eubacterium rectale*	Higher in moderate NAFLD[76]	Decrease[76]
Species	*Ruminococcus obeum CAG:39*	Not assessed	Increased[76]
General characteristics			
Gram stain	*Gram positive*	Decreased[73,76]	Decreased[76]
Gram stain	*Gram negative*	Increased[73,76]	Increased[76]

Table 2 | Study cohorts of gut microbiome in NAFLD research

Reference	Sequencing method	T2DM Status	BMI status	NAFLD diagnostic method	Average age (years)	Sample size	Country
Hoyles et al.[69](2018)	Shotgun	No	Obese	Liver biopsy	43	63	Italy
Shen et al.[74] (2017)	16S rRNA	Yes	Overweight	Liver biopsy	48	47	China
Raman et al.[75] (2013)	Pyrosequencing	Yes	Obese	Ultrasonography, blood sample	50	66	Canada
Loomba et al.[76] (2017)	Shotgun	Yes	Obese	Liver biopsy	48	86	USA (white & hispanic)
Zhu et al.[77] (2013)	Pyrosequencing	ND	Obese	Liver biopsy	13	67	USA
Del Chierico et al.[78] (2016)	16S rRNA	No	Obese	liver biopsy, ultrasonography	11,5	115	Italy
Wang et al.[73] (2016)	Pyrosequencing	No	Lean	Ultrasonography	43	126	China
Boursier et al.[79] (2016)	16S rRNA	Yes	Obese	Liver biopsy	66	64	France
Mouzaki et al.[80](2013)	qRT-PCR	Yes	Obese	Liver biopsy	43,7	50	Canada
Michail et al.[81](2015)	16S rRNA & shotgun	No	Obese	47 Ultrasonography, 3 liver biopsy	13,4	50	USA
Wong et al.[83] (2013)	Pyrosequencing	Yes	Overweight	Liver biopsy	49	61	China
Chen et al.[87] (2011)	16S rRNA pyrosequencing	ND	NA	NA	47,5	66	China
Chen et al.[88](2016)	16S rRNA pyrosequencing	ND	NA	Ultrasonography or biopsy	50,5	48	China
Bajaj et al.[89] (2014)	Pyrosequencing	ND	Obese	Blood sample	63	244	USA
Qin et al.[90] (2014)	Shotgun	Yes	Obese	Liver biopsy	45	237	China[a]

[a]Only the Chinese cohorts (discovery and validation) used for liver cirrhosis study is considered here. NA, not applicable; NAFLD, nonalcoholic fatty liver disease; ND, not determined; qRT-PCR, quantitative RT-PCR; rRNA, ribosomal RNA; T2DM, type 2 diabetes mellitus.

Figure 1: Overlapping microbiota species and genera signatures in NAFLD, diabetes and obesity. a) Specific genera signatures are observed during obesity, type 2 diabetes mellitus (T2DM) and nonalcoholic fatty liver disease (NAFLD, including nonalcoholic steatohepatitis (NASH)), and common genera signatures in these diseases are highlighted. b) Specific microbial species signatures are observed during obesity, T2DM and NAFLD, with common genera signatures in these diseases highlighted in the figure. For both panels, microorganisms with an up arrow are found more abundant in diseases than in healthy individuals as controls. Microorganisms noted with a down arrow are found less abundant in diseases than in healthy

individuals. Microorganisms with both up and down black arrows have been reported with contradictory results showing them to be either more or less abundant in diseases depending on the study. Microorganisms in black with both up and down purple arrows are differentially abundant between diabetes, and/or obesity and/or hepatic disease. In this case, if one disease is associated in all publications with an increase of the organism, a colored “+ or -” is added (e.g. opposite differences in abundance of *Bifidobacterium* are associated with obesity and NAFLD progression so there are both up and down purple arrows, and obesity is associated with an increase of Bifidobacterium so a red “+” is added after the organism name, because obesity is associated with red in the figure, if a blue – is indicated it means by contrast that it is decreased in T2DM).

Figure 2: Microbiota species and genera signatures in NASH-related fibrosis, cirrhosis, diabetes, and obesity. A) Specific genera signatures are observed during obesity, type 2 diabetes mellitus (T2DM) and liver fibrosis or cirrhosis, and common genera signatures in two or three of these diseases are highlighted. B) Specific microbial species signatures observed during obesity, T2DM and liver fibrosis or cirrhosis, with common genera signatures in two or three of these diseases highlighted. For both panels, microorganisms with an up arrow are found more abundant in diseases than in healthy individuals as controls. Microorganisms with a down arrow are found less abundant in diseases than in healthy individuals as controls Microorganisms with both up and down black arrows have been reported with contradictory results showing them to be either more or less abundant in diseases depending on the study. Microorganisms in black with both up and down purple arrows are differentially abundant between diabetes, and/or obesity and/or

hepatic disease. In this case, if one disease is associated in all publications with an increase of the organism, a colored “+ or -” is added (e.g. opposite differences in abundance of *Clostridium* are associated with T2D and fibrosis progression so there are both up and down purple arrows, and fibrosis is associated with an increase of *Clostridium* so a green “+” is added after the organism name, because NAFLD/fibrosis is associated with red in the figure, if a blue – is indicated it means by contrast that it is decreased in T2DM).

Figure 3: Gut-derived metabolites and factors that could drive progression of NAFLD. This figure illustrates how main gut-derived metabolites are involved in the development and progression of nonalcoholic fatty liver disease (NAFLD), fibrosis and cirrhosis.

Lactate, ethanol, TMAO can propel NAFLD progression (TMAO induces a decrease in the total bile acid pool size, which in turn can affect FXR signaling and NAFLD), as can LPS. On the other hand, short-chain fatty acids (SCFAs) can have anti-inflammatory properties, which could prevent progression of NAFLD.

Box 1: Definition of metabolic syndrome

According to formal clinical guidelines[187], metabolic syndrome is defined as:

Android obesity with a waist circumference above the cut-off of 94cm and 80cm, respectively, for white men and women, plus any two of the following factors:

- Fasting plasma glucose ≥100mg/dl (5.55mmol/l) or with T2DM
- Systolic blood pressure level ≥130 mmHg or diastolic blood pressure level ≥85mmHg or patients taking any hypertension-lowering drugs
- Serum triglyceride levels ≥150mg/dl (1.69 mmol/l) or patients taking any kind of lipid-lowering treatments
- HDL cholesterol levels <40 mg/dl (1.04 mmol/l) in men or <50mg/dl (1.29 mmol/l) in women, or patients taking any kind of lipid-lowering treatments.

Box 2: Summary of usefulness of NAFLD diagnostic tools.

Diagnostic tool	Cost	Detection abilities				Key features
		Steatosis	NASH	Fibrosis	Cirrhosis	
Liver biopsy[56,57]	$$	Y	Y	Y	Y	Gold standard for diagnosis; poor patient tolerance as painful, inconvenient and potential for complications; possibility of false-negative results (liver injuries are not homogeneous, biopsy is only 1/50,000 of the liver mass). Scores or algorithms based on histology are available including the NAS score (score of 3 and 4 accurately diagnoses NAFLD or NASH) and SAF algorithm, which has improved diagnostic accuracy compared with NAS.
Serological markers[56a]						
AAR = AST/ALT[57]	$	N	N	Y	Y	AUROC 0.83 for stage 3-4 ; this inexpensive tool has good negative predictive value, but positive predictive ability is limited; good accuracy.
FIB-4[57]	$	N	N	Y	N	AUROC 0.8 for stage 3-4) ; one of the best noninvasive tests in diagnosing advanced fibrosis in NAFLD; this tool is inexpensive; limitations for patients that have no advanced fibrosis.
NAFLD fibrosis score[57]	$	N	N	Y	N	AUROC 0.88 for stage 3-4; this inexpensive tool identifies advanced fibrosis well.
BARD score[×57]	$	N	N	Y	N	AUROC 0.81 for stage 3-4); good prediction for patients with no fibrosis (95-97%), but does not predict fibrosis well in patients with mild NAFLD (specifically in patients with obesity or T2DM), which limits its clinical use.
Procollagen III (PIINP)[57,58]	**$**	N	Y	Y	N	AUROC 0.77-0.82); could be a useful test to identify the highest risk patients before in depth-analysis, but it is less predictive than hyaluronic acid; needs further validation.
Cytokeratin-18[57]	**$**	N	Y	N	N	AUROC 0.83; needs to be validated.
Fibrotest[×57]	**$**	N	N	Y	N	AUROC 0.75–0.8 for stage 2–4; AUROC 0.81–0.92 for stage 3–4; not available in the UK.
Fibromax[188]	**$**	Y	N	Y	Y	High power for prediction with high predictive positive value (0.9); low negative predictive value (48.3%); difficulty to predict absence of steatosis.
Morphological phenotype						
Ultrasonography∞[56,57]	**$**	Y	N	N	N	AUROC 0.97l good predictive tool for steatosis, but does not provide information regarding fibrosis.
Computed tomography[56]	**$$**	Y	N	N	N	high accuracy; cannot distinguish NASH from steatosis; exposure to radiation.
Transient elastography[56]	**$**	Y	Y	Y	N	AUROC >0.8); less reliable those who are overweight or obese.
Acoustic radiation force impulse imaging[×56,57]	**$**	N	N	Y	N	AUROC 0.97l increasingly available on ultrasonography machines, but not available on every machine; still needs validation.
Real-time shear wave elastography[×]	**$**	N	N	Y	N	AUROC 0.85–0.88; results might be invalid in patients aged >52 years or with severe obesity or patients with T2DM; results might be different from liver biopsy; accurate if >30% of hepatocytes are steatotic.

Magnetic resonance imaging[56]	**$$**	Y	N	N	N	This accurate tool for steatosis diagnosis is less reliable for grading steatosis in patients with advanced fibrosis or cirrhosis;. It cannot be performed in patients with claustrophobia and the measurements are affected by hepatic iron deposition
Magnetic resonance spectroscopy[56]	**$$**	Y	N	N	N	Medium accuracy; results of this tool might be affected by respiration movements, claustrophobia and implanted devices;.it is not systematically available.
Magnetic resonance elastography[56]	**$$**	N	N	Y	N	AUROC >0.9; access to this tool is limited

[a]mostly useful to diagnose one liver alteration (NAFLD or fibrosis) but not always accurate; some markers are expensive. Several serological markers are available. **$ Low cost, $$ Moderate cost, $$$ Expensive.** *NAS, Non-alcoholic fatty liver disease score; NAFLD, Non-alcoholic fatty liver disease; NASH: Non-alcoholic steato-hepatitis; AUROC area under the receiving operator characteristic;* ***AAR = AST/ALT ,*** *aspartate transaminase/alanine transaminase ratio;* ***FIB-4, fibrosis-4;*** *T2DM, type 2 diabetes mellitus;* ***PIINP,*** *amino terminal peptide of type III procollagen.*

Box 3: Proposal for new study investigations

Study population:
Inclusions of study groups with similar ethnic origin, including several groups of individuals (i.e. obese with NAFLD, obese with T2D and NAFLD, with T2D individuals all treated at least with metformin). The diagnosis of NAFLD/NASH should rely on liver biopsy.

Metagenomic data:
Future studies should choose shotgun metagenomics sequencing data and put aside 16S rRNA. This may allow to predict taxonomic composition but also functional composition. With this kind of data, MGS, genes and Gut microbial Kegg Modules (GMMs) associated with NAFLD could be systematically predicted in each dataset.

Statistical analysis:
Adjustments on diabetes, obesity, sex and age should be processed if they are variations on these parameters in the dataset (e.g. ANCOVA, logistic regression, p trends by contrasts, linear regression adjusted). This would allow to determine if found signatures are originating from NAFLD per se or to the underlying metabolic diseases. Sex disparity is also seen (with more prevalent obese women and more prevalent NAFLD in men), thus, results should be adjusted on sex, and eventually ethnicity if they are different ethnic groups in studies. More globally, taking into account clinical data from the patient in the gut microbiome analyses should be a good systematic practice in gut microbial analyses.
Also, treatments (e.g. for type 2 diabetes and lipid lowering drugs such as statin) should be taken into account. Indeed, treatment impacts both clinical data (i.e. normalizing systemic metabolic parameters) as well as the gut microbiome profiles).
Moreover, while it represents yet an additional constraint, food intakes should be added in those studies due to the major impact of the diet on the gut microbiota.
While a majority of studies tries to find species or genes that are disease specific to be used as biomarker signatures, studying the whole microbial ecosystem might also be interesting, using co-occurrence and co-abundance networks. The structure of the community might be the signature of the disease. This could also enhance knowledge into disease pathophysiology and further lead to therapeutic development.

Glossary terms

Faecal microbiota transplantation: transfer of faeces from a donor to a receiver to obtain a beneficial clinical outcome by modifying the recipient's gut microbiota.

Nonalcoholic fatty liver disease: a liver disease characterized by pathological hepatic fat accumulation from simple steatosis to nonalcoholic steatohepatitis.

Nonalcoholic steatohepatitis: a severe stage of NAFLD characterized by steatosis, hepatocyte ballooning (that is, cell injury) and inflammation, which can be associated and/or evolve to fibrosis, cirrhosis and hepatocellular carcinoma

Steatosis: corresponds to intrahepatic fat of at least 5% of liver weight, which can be reversible upon lifestyle modifications.

Liver fibrosis: the excessive accumulation of extracellular matrix proteins including collagen that occurs in most chronic liver etiologies.

Cirrhosis: corresponds to the histological development of regenerative nodules surrounded by fibrous bands in response to chronic liver injury.

Compensated cirrhosis: the liver at the stage of severe fibrosis yet can still performs its basic functions; thus, compensated cirrhosis is not associated with specific clinical symptoms.

Decompensated cirrhosis: the liver at the stage of severe fibrosis and liver dysfunction leading to clinical symptoms such as internal bleeding, ascites, hepatic encephalopathy.

Gynoid distribution: refers to the body fat that is preferentially placed around the hip.

Shotgun sequencing: involves randomly breaking up DNA sequences into lots of small segments, which are further sequenced to obtain reads. Computational programs then reassemble the sequence by looking for regions of overlap

Table of contents blurb

The gut microbiota has been linked to nonalcoholic fatty liver disease (NAFLD), but metabolic confounding factors (such as obesity and diabetes) complicate analysis. This Review provides a broad insight into microbiome signatures for human NAFLD and explores issues with disentangling them from underlying metabolic disorders.