

## The role of the gut microbiota in NAFLD

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**Abstract** | NAFLD is now the most common cause of liver disease in Western countries. This Review explores the links between NAFLD, the metabolic syndrome, dysbiosis, poor diet and gut health. Animal studies in which the gut microbiota are manipulated, and observational studies in patients with NAFLD, have provided considerable evidence that dysbiosis contributes to the pathogenesis of NAFLD. Dysbiosis increases gut permeability to bacterial products and increases hepatic exposure to injurious substances that increase hepatic inflammation and fibrosis. Dysbiosis, combined with poor diet, also changes luminal metabolism of food substrates, such as increased production of certain short-chain fatty acids and alcohol, and depletion of choline. Changes to the microbiome can also cause dysmotility, gut inflammation and other immunological changes in the gut that might contribute to liver injury. Evidence also suggests that certain food components and lifestyle factors, which are known to influence the severity of NAFLD, do so at least in part by changing the gut microbiota. Improved methods of analysis of the gut microbiome, and greater understanding of interactions between dysbiosis, diet, environmental factors and their effects on the gut–liver axis should improve the treatment of this common liver disease and its associated disorders.

NAFLD has become the most common liver disease worldwide<sup>1</sup>. It constitutes a spectrum ranging from simple steatosis through to nonalcoholic steatohepatitis (NASH) and cirrhosis of the liver<sup>2</sup>. In Western countries, it is also the most rapidly increasing cause of hepatocellular cancer<sup>3</sup>. NAFLD largely occurs in overweight individuals and is strongly associated with the presence of metabolic syndrome and the development of diabetes. Much current research centres upon elucidating the factors, sometimes described as ‘multiple parallel hits’, that drive progression from simple steatosis to more complicated NAFLD<sup>4,5</sup>. These factors include intestinal dysbiosis, dietary factors<sup>6–8</sup>, changes to intestinal permeability, genetic factors, as well as endoplasmic reticulum stress and activation of other signalling pathways<sup>9</sup>.

In embryological terms, the gut and the liver are intrinsically linked, with the liver budding directly from the foregut during development. Evidence is increasing that the gut and liver have multiple levels of associated interdependence, and disturbance of the gut–liver axis has been implicated in a number of conditions linked to obesity, including NAFLD<sup>10</sup>. This evidence includes the observations that intestinal permeability is increased in patients with NAFLD compared with those without the disease<sup>11</sup>, an association of liver disease with changes in bacterial flora<sup>9</sup> and the effects of manipulation of the flora on liver injury<sup>12</sup>.

This Review covers the mechanisms via which changes in the gut can influence the development and progression of NAFLD and possible therapeutic implications. The role of bile acids and farnesoid X receptor (FXR) agonists are discussed only briefly as this topic has been well covered recently elsewhere<sup>13,14</sup>. Ultimately, understanding of such mechanisms is hoped to pave the way for new treatments for what has become the most common form of liver disease.

### Gut microbiota

The human gut microbiome includes 10–100 trillion microorganisms, mainly bacteria in the gut, that vastly outnumber our own human cells (BOX 1)<sup>15</sup>. The diversity of such a microbiome can be classified into  $\alpha$ -diversity (within samples) and  $\beta$ -diversity (comparisons between samples from a given population)<sup>16</sup>. Most study to date has focused on the bacterial component of the microbiome. The most common of the bacterial groups are Bacteroidetes and Firmicutes, whereas the predominant Archaea is Euryarchaeota<sup>17</sup>. However, it is likely that non-bacterial organisms such as resident archaeal, fungal and viral populations might also be important, especially in their interactions with the rest of the microbiome (BOX 2).

The development of the microbiome from birth might have important long-term clinical implications

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## Key points

- The incidence of fatty liver disease, and its complications of inflammation, fibrosis and liver cancer, is increasing
- Gut dysbiosis (an unhealthy gut microbiota) contributes to the pathogenesis of obesity-related disorders including the metabolic syndrome and NAFLD
- Considerable differences exist between individuals' microbiota, influenced by the perinatal environment, diet, antibiotic exposure and lifestyle factors; changes in these factors might lead to the development of dysbiosis
- The gut that is compromised by dysbiosis is a portal for increased exposure of the liver to bacteria, bacterial products and injurious components of foods that contribute to NAFLD pathogenesis
- Improved methods of analysis to define healthy and unhealthy microbiotas, and better understanding of dietary and other factors that influence the gut–liver axis will facilitate preventive strategies and treatments for this disease

in NAFLD (BOX 3). Important contributors to the development of the microbiome, including breast milk microbiota, are influenced by the mother's environment and lifestyle, perhaps to prepare the infant for the conditions that they have been born into. Weaning to solid food coincides with a dramatic change in the metabolic capacity of the small intestine<sup>18</sup>. Early life is a critical period for host–microorganism metabolic interactions. This idea is supported by mouse studies showing that transient early microbiota perturbation can lead to long-term durable deranged metabolic phenotypes (including obesity and diabetes) despite microbial community recovery<sup>19</sup>. These perturbations might occur through birthing mode, method of feeding, or even exposure to antibiotics used in livestock<sup>20</sup>.

### Dysbiosis and liver disease

The term dysbiosis refers to disruption of the normal gut microbiota. It can result from a wide range of environmental, immunological or host factors as well as alterations in bile flow, gastric pH or intestinal dysmotility. Evidence linking dysbiosis to the pathogenesis of human liver disease has accumulated rapidly, with a primary focus on its role in NAFLD and related

metabolic disorders. However, it is clear that dysbiosis might have a key role in other diseases; for example, crosstalk between the liver and gut is highly likely to explain the link between ulcerative colitis and primary sclerosing cholangitis<sup>21</sup>.

In fatty liver disease, early evidence linking gut dysbiosis with liver injury came from descriptive human studies showing an association between NASH and small intestinal bacterial overgrowth as assessed by combined <sup>14</sup>C D-xylose and lactulose breath testing<sup>22</sup>. Evidence also indicates that microbial populations are altered in patients with NAFLD (see later for more detail). However, it should be noted that there is considerable overlap with findings in healthy controls and some conflict between the microbiological findings of different studies.

Animal experiments in which the microbiome has been manipulated provide perhaps the strongest evidence supporting the role of dysbiosis in obesity and NAFLD. In a seminal early study it was shown that the microbiome from obese mice is linked to increased intestinal energy harvest from the diet. This trait was transmissible to lean adult germ-free mice when they were co-housed with obese mice<sup>23</sup>. In another study, weight loss achieved by two different dietary measures in mice induced pronounced, division-wide changes in microbial ecology away from those associated with obesity and insulin resistance<sup>24</sup>. Furthermore, insulin resistance, a key feature of NAFLD, can be improved with administration of antibiotics<sup>25</sup>. However, commensal bacteria are still important, with another study showing that the severity of experimental liver fibrosis is increased in germ-free mice<sup>26</sup>. Furthermore, exposure to antibiotics in infancy might have long-term effects on the composition of the commensal gut microbiota, predisposing to obesity and adiposity. For example, administration of subtherapeutic doses of antibiotics to young mice produced persistent changes in the microbiome, which increased colonic short-chain fatty acid (SCFA) production and altered hepatic metabolism of lipids and cholesterol<sup>27</sup>.

Very few similar experiments have been performed to date in humans. In one study, obese men with metabolic syndrome underwent allogeneic (from lean male donors with BMI <23 kg/m<sup>2</sup>) or autologous gut microbiota infusion. 6 weeks after infusion of microbiota from lean donors, the insulin sensitivity of recipients and levels of butyrate-producing intestinal microbiota increased statistically significantly. These findings suggest alteration of intestinal microbiota might be used to increase insulin sensitivity in humans and by implication, could also be of benefit in treating fatty liver disease<sup>28</sup>.

In support of these findings, the metabolic products and effects of the microbiome (that is, the microbial metabolome) seem to be different according to the metabolic phenotype of the host<sup>23,29</sup>. It is also potentially important that dysbiosis might directly affect adipose tissue, influencing levels of adipokines, pro-inflammatory and anti-inflammatory cytokines and fatty oxidation, which could have important downstream effects in the liver<sup>30</sup>.

### Box 1 | The gut microbiota

Humans can be considered as 'superorganisms' with a karyome (all of our genes in chromosomes), a chondriome (our genes within the mitochondrial system) and a microbiome (all of our microorganisms' genes)<sup>105</sup>.

Traditional culture methods are grossly inadequate to characterize these densely populated heterogeneous microbial communities<sup>184</sup>. These methods are therefore being replaced by new technologies such as 16S ribosomal RNA pyrosequencing (for taxonomic content and as stable phylogenetic markers to define lineages), next-generation metagenomics and metatranscriptomic sequencing (for functional predictions based on gene content), metabolomics and proteomics<sup>55</sup>.

A further issue is that the mucosa-associated flora can differ substantially from that recovered in the faeces<sup>185</sup>. Even though analysis of stool samples offers the easiest method of studying human microbiota, mucosa-associated bacteria might be more important, in which case mucosal biopsy samples are required<sup>186,187</sup>.

However, perhaps the major challenge in assessing the role of the microbiome in disease is that even among normal individuals, proportions of common groups such as *Bacteroides* vary markedly. In fact, every individual's microbiota are unique at a species level, even between twins and within families<sup>29</sup>.

Despite these intriguing findings, it is currently unclear whether an increase in the overall amount and distribution of bacteria in the gut (that is, bacterial overgrowth), the relative abundance of different taxa, the presence of specific harmful microorganisms, the metabolic function of the microbiome, host genetics or combinations of these factors are most important in the pathogenesis of NAFLD. Furthermore, less prevalent components of the microbiome that have received little attention to date, such as fungi, might have a modulating role. For example, *Candida* is able to degrade starches, liberating sugars to be fermented by bacteria such as *Prevotella* (phylum Bacteroidetes) and *Ruminococcus* species (phylum Firmicutes), thus increasing energy production from food in the gut and reducing the energy available for absorption and its utilisation as an energy source in the liver<sup>31</sup>. The main putative mechanisms through which dysbiosis contributes to disturbance of the gut–liver axis and might drive fatty liver disease progression are outlined in FIG. 1 and discussed in detail below.

### Mechanisms linking dysbiosis to NAFLD

#### Effects of bile acids

The role of bile acids in the pathogenesis and potential treatment of fatty liver disease is complex and has been discussed in several reviews<sup>13,14</sup>. Primary, secondary and conjugated bile acids are all implicated in NAFLD pathogenesis. FXR is thought to be the master regulator of bile acid metabolism as it is involved in all phases of the biosynthetic pathway<sup>14</sup>. Initial studies showed FXR-deficient mice fed a 1% cholesterol diet had increased hepatic cholesterol and triglyceride content<sup>32</sup>. It is believed that in NAFLD, gut-derived lipopolysaccharides (LPS) stimulate nuclear factor  $\kappa$ B (NF- $\kappa$ B), which recruits inflammatory cells by increasing levels of TNF and IL-1 $\beta$ , thus leading to fibrosis<sup>14</sup>. FXR stimulation seems to suppress NF- $\kappa$ B and in doing so decreases hepatic inflammation<sup>33</sup>. Another mechanism by which FXR agonism seems to work is by regulating carbohydrate metabolism. This mechanism was shown in animal studies, suggesting that FXR regulates gluconeogenesis via phosphoenolpyruvate carboxykinase, which ameliorates lipid and glucose metabolism and prevents inflammation<sup>14</sup>.

A clinical trial of the FXR ligand obeticholic acid in patients with noncirrhotic NASH (FLINT trial) showed that obeticholic acid 25 mg daily for 72 weeks improved histological NASH<sup>34</sup>, but the major effect was seen in those patients with diabetes<sup>35</sup>. Interestingly, a wide variety of relative benefit was observed between the sites in this multicentre trial, with the odds of a patient achieving histological improvement ranging from 0.6 (in favour of placebo) to 12.4 (REF. 36).

Of note, another study in mice has shown that FXR antagonism might also be beneficial for NASH. In this study, manipulation of the gut microbiota changed intestinal bile acid composition leading to intestinal FXR antagonism. This FXR antagonism reduced ceramide synthesis and *de novo* lipogenesis in the liver<sup>37</sup>. Thus, FXR seems to trigger a broad range of effects and

### Box 2 | Common organisms in the human gut

#### Bacteria

##### Gram-positive

- Firmicutes: largest phylum, several *Lactobacillus* strains used as probiotics. Genera *Eubacterium*, *Faecalibacterium*, *Ruminococcus* and *Roseburia* are butyrate producers
- Actinobacteria: includes genera *Collinsella* and *Bifidobacterium* as well as probiotic strains

##### Gram-negative

- Bacteroidetes: genera *Bacteroides*, *Prevotella* and *Xylanibacter* degrade variety of complex glycans
- Proteobacteria: genus *Escherichia* can produce ethanol; genus *Desulfovibrio* is sulfate-reducing
- Verrucomicrobia: genus *Akkermansia* is involved in mucus degradation but might also have beneficial effects via increasing intestinal levels of endocannabinoids that improve inflammation and gut barrier function

#### Archaea

- Euryarchaeota predominant phylum<sup>17</sup>
- *Methanobrevibacter* predominant genus and involved syntrophically in intestinal methanogenesis; interacts with *Candida*, *Prevotella* (phylum Bacteroidetes) and *Ruminococcus* (phylum Firmicutes) to produce positive energy balance

#### Fungi

- The fungal mycobiome contains over 200 species
- *Candida*, for example, has syntrophic effects

#### Viruses

- Viromes seem to be unique to individuals regardless of their degree of genetic relatedness. Despite remarkable interpersonal variations in viromes and their encoded functions, intrapersonal diversity is very low, with >95% of virotypes retained over time. Moreover, viromes are dominated by a few temperate phages that exhibit remarkable genetic stability<sup>188</sup>
- Bacteriophages are the most common of the enteric viruses
- They can outnumber their hosts by 10-fold and hence provide a constant evolutionary pressure<sup>189</sup>
- They can be predatory and pro-pathological
- They infect prokaryotes and thus might affect microbial community responses to various disturbances.

##### Two main types:

- Tailed, double-stranded DNA viruses of the order Caudovirales (families Podoviridae, Siphoviridae and Myoviridae)
- Non-tailed, cubic or filamentous viruses largely composed of single-stranded DNA viruses (family Microviridae)

#### Important relationships

- Abundance of one major Archaea species, *Methanobrevibacter*, and common fungal species such as *Candida* is increased by carbohydrate ingestion leading to increased methanogenesis
- Syntrophism between *Ruminococcus* and methanogens, in which methanogens such as Archaea consume hydrogen, enabling *Ruminococcus* to extract more energy from the same amount of substrate, thus reducing the energy content of nutrients subsequently absorbed from the gut<sup>31</sup>

## Box 3 | Early development of the microbiome

The human gastrointestinal tract is normally sterile at birth but post-partum is rapidly colonized by microorganisms<sup>164</sup>. Babies born via vaginal delivery are rapidly colonized with maternal faecal organisms<sup>18</sup>. By contrast, babies born via caesarian section are colonized by microorganisms from the mother's non-perineal flora, the air, other infants and nursing staff<sup>18</sup>.

Early colonisation following vaginal delivery is thought to allow immediate mucosal activation of Toll-like receptor 4 (TLR4) and the innate immune system. This activation facilitates tolerance to intestinal microorganisms and helps establish the symbiosis that has evolved between animals and microbiota over millions of years. Important beneficial roles include pathogen displacement, immune system development, metabolism of foodstuffs and the supply of nutrients.

Colostrum and breast milk are a rich source of living bacteria; the bacterial composition of breast milk seems to be influenced by several factors including country of birth, rural or urban location and lactation time<sup>190–193</sup>.

With regards to the possible effect of breast milk feeding on the progression of NAFLD, an observational study of young white children, with follow-up from 3 to 18 years, found that breast milk feeding prevented the development of biopsy-proven steatohepatitis [OR 0.04, 95% CI 0.01–0.10] and fibrosis [OR 0.32, 95% CI 0.16–0.65]<sup>194</sup>.

However, breast feeding might be a two-edged sword, as mouse studies have shown that obese dams deliver a NAFLD phenotype to their offspring, possibly via increased leptin content in breast milk that results in increased levels of insulin, aspartate transaminase, IL-6, TNF- $\alpha$ , liver triglycerides, steatosis and hepatic fibrogenesis<sup>195</sup>.

could be altered by ileal bile acid changes mediated via the microbiota<sup>38</sup>. However, human and animal bile acid metabolism is different, which might explain some of the conflicting results in the literature<sup>38</sup>. Moreover, the important end points of fibrosis and tumorigenesis need to be more fully addressed when examining the overall effects of FXR modulation in the treatment of fatty liver disease<sup>38</sup>.

#### Effects of short-chain fatty acids

SCFAs, such as acetic, propionic and butyric acid, are the major products of carbohydrate fermentation by gut microorganisms, with the normal gut microbiome producing 50–100 mmol/l per day of these compounds<sup>39</sup>. These SCFAs have effects on energy metabolism, immunity and adipose tissue expansion<sup>40</sup>. Many of these effects are mediated via binding to G-protein coupled receptors expressed in the immune system and on endocrine cells of the gut and adipocytes. The types and amounts of SCFAs synthesized in the gut are changed by the amount of carbohydrate consumed and by dysbiosis, and there are multiple mechanisms through which they might contribute to NAFLD progression.

In rats, a diet containing the SCFAs acetic, propionic and butyric acid, which simulates the caecal fermentation products of sugar-beet fibre (the SCFA group), reduced hepatic cholesterol synthesis and fat content compared with a diet containing whole sugar-beet fibre (the SBF group) and a fibre-free control diet. An explanation for this finding is that intestinal mucosal cholesterol synthesis in the proximal small intestine was lower in the SCFA group than in the fibre-free and the SBF group. These experiments show that SCFAs from the fermentation of fibre, rather than the fibre itself, are responsible for these beneficial effects<sup>41</sup>. Another study in mice fed a high-fat diet (45% palm oil fat) showed

that SCFAs (acetate, propionate and butyrate in the diet at 5% by weight) lowered hepatic fatty acid synthase (FAS) activity and hepatic lipid synthesis. There was also a twofold increase in hepatic lipid oxidation in the SCFA-fed mice, shifting hepatic lipid metabolism towards a more oxidative state. This shift was associated with increased phosphorylation and activation of adenosine monophosphate activated protein kinase (AMPK) and its downstream target acetyl CoA carboxylase (ACC)<sup>42</sup>. Human studies replicating these results are lacking.

**SCFA synthesis and energy harvest.** SCFAs derived from the gut, such as acetate and propionate, provide an energy source to the liver, where they have important roles in hepatic lipogenesis and gluconeogenesis, respectively<sup>43,44</sup>. Acetate, in particular, can potentially be used as a cholesterol or fatty acid precursor<sup>44</sup>. In this way, SCFAs account for ~30% of hepatic energy supply<sup>45</sup>. Thus, changes in microbiota that favour SCFA production can increase energy delivery to the liver and reduce faecal energy loss. For example, in *ob/ob* mice with fatty liver disease, microbial carbohydrate metabolizing genes are enriched, resulting in increased concentration of SCFAs in the caecum and less energy content in the stool<sup>23</sup>.

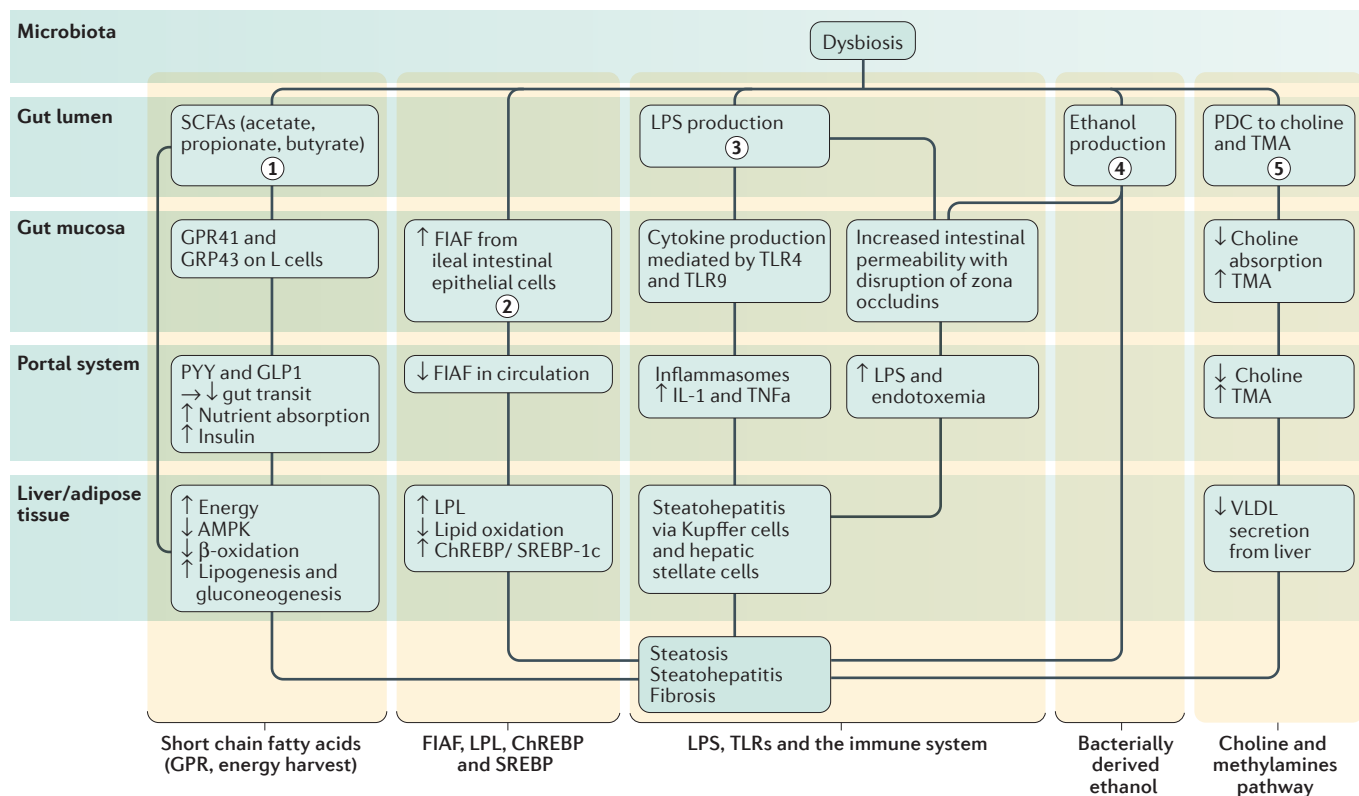
In human studies the role of bacteria in altering energy harvest is less clear. Whereas an early human study showed lower faecal energy excretion in those with obesity compared with lean individuals<sup>46</sup>, a more recent study of 12 lean and 9 obese individuals found no statistically significant difference in energy excretion in stools between the two groups, who consumed a 2,400 kcal per day diet or 3,400 kcal per day diet, respectively<sup>47</sup>. However, a large inter-individual range for the percentage of calories lost in stools was observed. In contrast to other studies, no differences in bacterial abundance between lean individuals and those with obesity were observed.

A study in adults with NAFLD showed a statistically significant association between the presence of steatohepatitis and an increased percentage of *Clostridium coccoides* (phylum Firmicutes) and a reduced percentage of Bacteroidetes, after adjusting for BMI and dietary fat intake<sup>48</sup>. The importance of Bacteroidetes might lie in their major contribution to SCFA production and the metabolic potential of the microbiome. For example, a 20% decrease in faecal Bacteroidetes and a corresponding increase in Firmicutes is associated with a 150 kcal increase in energy harvest from the diet<sup>23,47</sup>. Such a change can occur with just 3 days of overeating, suggesting a very dynamic response in microbiota composition with caloric intake. Although SCFAs produced in the gut supply energy to the liver, they might also have potentially beneficial effects in fatty liver disease by increasing the Bacteroidetes (*Prevotella*)/Firmicutes ratio and thus reducing energy harvest<sup>23</sup>. Indeed, high-fibre diets, a source of SCFAs, promote the Bacteroidetes phylum, *Prevotella*, whereas high-fat diets reduce diversity and promote Firmicutes growth<sup>49</sup>.

Although a number of studies show an association between increased Firmicutes, decreased Bacteroidetes and NAFLD progression, interestingly, studies in paediatric populations show a reverse association, with an increase in Bacteroidetes content in those with NAFLD<sup>50,51</sup>. This discrepancy might be due to the use of different quantification techniques to those used in adults, different population characteristics and lack of liver histology<sup>50</sup>. However, variation between findings is quite common in major studies of microbiota in NASH, particularly when comparisons of broad phyla are made; findings seem to be more consistent when changes at the family level are examined. For example, a common finding across a number of studies in NAFLD is a decrease in the Ruminococcaceae family of the phylum Firmicutes<sup>48,52–55</sup>.

**Activation of G-protein coupled receptors.** SCFAs act on the G-protein coupled receptors (GPCRs) GPR41 and GPR43 of gut enteroendocrine L cells to produce several effects that might contribute to NAFLD. Activation of

these receptors stimulates PYY release, which slows gastric emptying and intestinal transit and hence enhances nutrient absorption<sup>56</sup>. These L cells also release glucagon-like peptide 1 (GLP-1), which increases glucose-dependent insulin secretion, among other effects<sup>57</sup>. In addition, activation of GPR43 and GPR41 in adipocytes inhibits lipolysis and encourages adipocyte differentiation<sup>40</sup>. GPR43 is also present on intestinal neutrophils and might therefore contribute to NASH pathogenesis by increasing intestinal inflammation and permeability (discussed later)<sup>58</sup>. However, SCFAs, especially butyrate, might also suppress inflammation via effects on T regulatory cells in the mucosa<sup>59,60</sup>. Thus, the balance of different SCFAs produced in the gut might determine their net effect on intestinal inflammation and permeability. Indeed, a study in humans showed that the favourable metabolic effects of faecal transplantation from lean donors into patients with obesity was linked to a marked increase in the proportion of the butyrate producer *Roseburia intestinalis*<sup>28</sup>.



**Figure 1 | Key mechanistic pathways involved in the gut–liver axis in NAFLD progression.** (1) Short-chain fatty acids (SCFAs) have effects on G-protein coupled receptors GPR41 and GPR43, causing release of peptide YY (PYY) and glucagon-like peptide 1 (GLP-1), respectively, from neuroendocrine L cells. Increased energy delivery in the form of SCFAs also inhibits adenosine monophosphate activated protein kinase (AMPK) in the liver, which increases hepatic free fatty acid (FFA) accumulation via decreased  $\beta$ -oxidation. (2) Dysbiosis inhibits secretion of fasting induced adipose factor (FIAF, also known as angiopoietin-related protein 4), which in turn inhibits endothelial lipoprotein lipase (LPL), which is responsible for releasing triglycerides from circulating chylomicrons and VLDL. Decreased circulating FIAF levels result in transactivation of hepatic lipogenic enzymes by carbohydrate-responsive element-binding protein (ChREBP) and sterol

regulatory element-binding protein 1c (SREBP-1c). The net effect is increased triglyceride storage in adipocytes and liver. (3) Lipopolysaccharide (LPS) stimulates Toll-like receptor 4 (TLR4) on endothelial cells and TLR9 on dendritic cells. This activation induces inflammasomes and pro-inflammatory cytokines, which induce NAFLD progression. LPS also has direct effects on Kupffer cells and hepatic stellate cells to drive steatohepatitis to fibrosis. (4) Dysbiosis can also result in increased endogenous alcohol production, which increases intestinal permeability with disruption of tight junctions (zona occludins), allowing endotoxins and ethanol to have direct effects on the liver. (5) The intestinal microbiota converts dietary phosphatidylcholine (PDC) to choline and to hepatotoxic trimethylamine (TMA). Reduced availability of dietary choline inhibits VLDL excretion from the liver inducing steatosis.

**Intestintrophic effect of SCFAs and intestinal gluconeogenesis.** Enterocytes use SCFAs as substrates for glucose synthesis (intestinal gluconeogenesis). This glucose is probably sensed by glucose cotransporter 3 (also known as sodium/glucose cotransporter 3, SGLT3) expressed in the portal system, inducing a signal to the brain that influences food intake and induces satiety<sup>61</sup>. Studies in mice lacking glucose transporter 2 (GLUT-2, also known as SLC2A2) suggest that this signal is mediated by GLUT2 and have demonstrated that common hepatic branch vagotomy does not abolish this anorectic effect of portal glucose<sup>61</sup>. Via this mechanism, production of SCFAs by the microbiota might decrease food intake, reduce fat mass and thus be of benefit in NAFLD<sup>62</sup>.

#### **Effects of Fiaf, ChREBP and SREBP-1**

Another postulated mechanism linking the microbiome to NAFLD is its effects on the intestinal production and secretion of FIAF (fasting-induced adipocyte factor, also known as angiopoietin-related protein 4). A secreted protein that inhibits lipoprotein lipase (LPL), FIAF is produced by L cells of the intestine and at a number of other sites including brown fat, white fat and hepatocytes. In mouse studies, when young are weaned and their diet switches from lipid rich milk to polysaccharide rich chow, FIAF secreted from the ileal epithelial cells is suppressed by developing intestinal microbiota<sup>63</sup>. This suppression of FIAF leads to LPL activation in adipose tissue and the liver, increasing triglyceride storage, which produces a twofold increase in hepatic triglyceride content<sup>63</sup>.

In an experimental model of the metabolic syndrome, dysbiotic microbiota were shown to inhibit FIAF secretion from intestinal cells, leading to activation of LPL and subsequent triglyceride accumulation in both adipose tissue and the liver<sup>63</sup>. One proposed mechanism for this finding is that SCFAs, primarily butyrate, are required to activate secretion of FIAF and that dysbiosis reduces butyrate production in favour of other SCFAs<sup>64</sup>. Interestingly, human and donkey milk given to rats increased concentrations of faecal butyrate compared with cow's milk, leading to metabolically beneficial hypolipidaemic effects<sup>65</sup>.

By increasing hepatic lipids stores, FIAF inhibition also leads to activation of the hepatic proteins carbohydrate-responsive element-binding protein (ChREBP) and sterol regulatory element-binding protein 1 (SREBP-1). Activation of these proteins further stimulates lipogenic enzymes and increases fat accumulation<sup>66,67</sup>. In addition, the metabolism of non-digestible polysaccharides into absorbable monosaccharides by dysbiotic intestinal flora can lead to direct activation of hepatic ChREBP and SREBP-1 and the activation of hepatic lipogenic enzymes<sup>63</sup>.

SREBP-1 is also regulated by AMPK. AMPK normally acts as an energy 'master switch' that regulates lipid metabolism in adipose tissue and the liver. Increased levels of cellular AMP, a marker of reduced cellular energy stores, activate AMPK, which then stimulates ATP-producing catabolic pathways such as fatty acid

oxidation<sup>68</sup>. In NAFLD, this master switch does not activate, and hence there is decreased  $\beta$ -oxidation and increased hepatic steatosis<sup>69</sup>. Hypoadiponectinaemia is thought to contribute to unchanged AMPK expression in NAFLD<sup>68</sup>. AMPK might also be modulated by the microbiota. For example, the *Lactobacillus rhamnosus* GG strain activates AMPK and attenuates alcohol-induced fat accumulation in the liver<sup>70</sup>.

#### **Increased intestinal permeability**

The liver has both an arterial and venous blood supply, with the majority of hepatic blood flow coming from the gut via the portal vein. Therefore, it is exposed to potentially harmful substances derived from the gut, including translocated bacteria, LPS and endotoxins as well as secreted cytokines. One of the key roles of the liver is to rapidly clear these substances from the circulation<sup>71</sup>.

Tight junction proteins, such as zonula occludens, normally seal the junction between intestinal endothelial cells at their apical aspect and thus have a vital role in preventing translocation of harmful substances from the gut into the portal system. Dysbiosis can disrupt these tight junctions, increasing mucosal permeability and exposing both the gut mucosal cells and the liver to potentially pro-inflammatory bacterial products. For example, animal studies have shown that hepatic steatosis induced by a high-fat diet is associated with dysbiosis and increased intestinal permeability, with translocation of bacterial LPS from Gram-negative bacilli<sup>66</sup>. The relationship between gut permeability and NAFLD is highlighted by the finding in a high-fat dietary model of NAFLD that colitis induced by TNBS (2,4,6-trinitrobenzenesulfonic acid) increased circulating LPS levels and worsened steatohepatitis, as measured by the NAFLD Activity Score (NAS) and liver enzyme levels<sup>67</sup>. Notably, LPS has effects beyond the liver and gut; for example, chronic low doses of LPS administered subcutaneously impair fasting glucose and insulin, alter hepatic insulin sensitivity, increase visceral and subcutaneous fat, increase adipose tissue macrophage numbers and raise hepatic triglyceride content<sup>66</sup>.

Translocated microbial products might contribute to the pathogenesis of fatty liver disease by several mechanisms. One pathway is via Toll-like receptors (TLRs) that recognize gut-derived bacterial products, especially LPS. There is evidence that dysbiosis-induced permeability changes increase portal levels of gut-derived TLR ligands, which activate TLR4 on hepatic Kupffer cells and stellate cells to stimulate pro-inflammatory and profibrotic pathways via a range of cytokines, including IL-1, IL-6 and TNF<sup>71-76</sup>.

Intracellular cascades involved in this process include stress-activated and mitogen-activated protein kinases, JNK (c-Jun N-terminal kinase), p38 mitogen-activated kinases and the NF- $\kappa$ B pathway<sup>77</sup>. Specifically, mucosal TLR activation might contribute to hepatic steatosis via intestinal epithelial MYD88, which acts as a sensor to switch host metabolism towards obesity according to nutritional status<sup>78</sup>. This mechanism was shown in mice with an inducible intestinal epithelial cell (IEC)-specific deletion of MYD88. When fed a high-fat diet,

mice with IEC-specific MYD88 knockout had improved oral glucose tolerance and associated hepatic lipid and triglyceride content compared with wild-type mice<sup>78</sup>.

TLR signalling in the mucosa can also lead to the production of inflammasomes. These multiprotein cytoplasmic complexes are assembled in the cytosol via activation of TLR4, TLR9 and other pattern-recognition receptors (PRRs) by exogenous pathogens, pathogen-associated molecular patterns (PAMPs, such as LPS) or internal host damage-associated molecular patterns (DAMPs). Inflammasomes then activate a variety of processes, including cleavage of pro-caspase-1 to form active caspase-1, which results in cell death dependent on caspase-1 and 3 (REF. 79). Another downstream effect of inflammasome production is the cleavage of pro-IL-1 $\beta$  and pro-IL-18 to release IL-1 $\beta$  and IL-18, which have pro-inflammatory and profibrotic effects<sup>80</sup>. Reactive oxygen species (ROS) are also released in this process.

Activation of the NLRP3 (NACHT, LRR and PYD domains-containing protein 3) inflammasome by LPS from gut microbiota via TLR4 and TLR9 appears to be important for fibrosis development in NAFLD<sup>81</sup>. Specifically, NLRP3 gain-of-function in mice leads to earlier onset and increased severity of steatohepatitis. Furthermore, increased NLRP3 inflammasome components, which correlated with hepatic collagen type 1 $\alpha$  expression, were observed in liver samples from patients with NASH<sup>81</sup>. However, it seems that the role of inflammasomes in NAFLD is complex, as NASH severity is increased in inflammasome-deficient mice<sup>82</sup>. Specifically, this study showed that NLRP6 and NLRP3 inflammasomes and the effector protein IL-18 negatively regulate NAFLD progression via modulation of the gut microbiota. In inflammasome-deficient mice, changes in the configuration of the gut microbiota were associated with worse NASH through influx of TLR4 and TLR9 agonists into the portal circulation and consequent increased hepatic TNF expression<sup>82</sup>. Within this same study, co-housing of inflammasome-deficient mice with NASH and wild-type mice resulted in NASH in the wild-type animals via coprophagy (the ingestion of each other's faeces)<sup>82,83</sup>. Interestingly, co-housing of inflammasome-deficient mice with NASH and *db/db* mice (a model of obesity, diabetes and dyslipidaemia) also worsened NASH in the latter group, which was mediated by CCL5-induced intestinal inflammation<sup>82</sup>. These experiments show that altered interactions between the gut microbiota and the host, produced by defective NLRP3 and NLRP6 inflammasome sensing, might drive the progression of NAFLD<sup>82</sup>. Subsequent studies suggest that some degree of inflammasome activity is physiological and that these complexes might act as steady-state sensors and regulators of the colonic microbiota<sup>82</sup>.

In experimental models, permeability can be studied through tissue analysis such as tight junction histology<sup>84</sup>. In humans, a number of noninvasive methods for measuring permeability have been explored. Most current techniques rely on excretion of an orally administered disaccharide (usually lactulose) and a monosaccharide (mannitol or L-rhamnose) that appear

in blood and urine after transitioning the intestinal barrier. The calculated urinary excretion ratio of these two sugars correlates with small intestinal permeability<sup>85</sup>. Multi-sugar tests have also been developed that enable pan-intestinal permeability assessment<sup>86</sup>. Permeability can also be more directly measured via urinary excretion of ingested <sup>51</sup>chromium-radiolabeled ethylenediaminetetraacetic acid (EDTA)<sup>87</sup>. All these tests have the benefit of being noninvasive, but might be affected by other factors such as antibiotic use, current diet, diabetes, obesity and normal heterogeneity. Another approach has been to measure volatile organic compounds (VOCs) that are formed by fermentation of dietary non-starch polysaccharides. Such a 'fermentome' can be present in the gaseous phase in exhaled air, sweat, urine and faeces. The presence of these VOCs at extra-intestinal sites is therefore presumed to be due to altered gut permeability<sup>88</sup>.

In a study from 2009, gut permeability was compared between patients with biopsy-proven NAFLD, healthy volunteers and patients with untreated coeliac disease (as a model of intestinal permeability change). The patients with NAFLD had significantly increased gut permeability (as measured by urinary excretion of <sup>51</sup>chromium-radiolabeled EDTA compared to healthy volunteers), although it was lower than in patients with untreated coeliac disease. Furthermore, in the patients with NAFLD both gut permeability and the prevalence of small intestinal bacterial overgrowth correlated with severity of steatosis but, interestingly, not with steatohepatitis<sup>87</sup>. In another human study, plasma IgG levels against endotoxin were found to be increased in biopsy-proven human NASH and progressively increased with NASH grade. This finding suggests a relationship between chronic endotoxin exposure and human NASH severity in which increased permeability drives endotoxaemia, which in turn triggers inflammatory cytokine responses and insulin resistance<sup>89</sup>.

Another interesting consideration is the gut-spleen axis and the possibility that increased gut permeability exposes the spleen to LPS, resulting in local immune activation<sup>90</sup>. Indeed, colloid scintigraphy, a method of measuring Kupffer cell activity, shifts to the spleen in patients with progressive NASH<sup>91</sup>. As suggested by animal studies in arthritis, this shift might stem from a reduction in the number and function of splenic B regulatory cells<sup>92</sup>.

Some studies show that some patients with NASH are free from endotoxaemia, suggesting that alternate pathways might be involved<sup>93,94</sup>. However, important caveats regarding measurement and interpretation of peripheral endotoxaemia in humans must be considered. Serum LPS antibody levels and plasma LPS binding protein levels have broad ranges that overlap with normal physiological ranges<sup>89,95</sup>. Furthermore, human studies are limited in that peripheral LPS levels might not reflect portal LPS levels and might change longitudinally over time<sup>93</sup>. In other words, increased gut permeability might expose the liver to deleterious levels of LPS without sufficient LPS escaping liver clearance to produce a marked increase in systemic levels.

### Changed gut motility

Deficits in mixing and transit of gut contents can lead to bacterial overgrowth and nutrient malabsorption. In the case of SCFAs, malabsorption causes release of PYY from the distal ileum, which slows gastric emptying and small intestinal transit<sup>56</sup>. Mixing and transit are controlled by enteric neurons. We have found that a diet high in fat, cholesterol and fructose resulted in degeneration and loss of 15–30% of enteric neurons and damage to remaining neurons, which is attributed to lipotoxicity<sup>96</sup>. Neuronal loss was associated with hepatic steatohepatitis and fibrosis, even in the absence of diabetes. Other studies in post-infectious IBS found that *Bacteroides* spp. and methanogenic Archaea were over-represented and this dysbiosis was linked to decreased gut transit<sup>97</sup>. Thus, reduced gut motility, in which the nutrients are not mixed and adequately absorbed, could contribute to dysbiosis and the progression of steatohepatitis.

### Bacterially-derived ethanol

NAFLD and alcohol-induced liver injury have very similar histological features and might share many common pathogenic pathways. Several lines of evidence suggest that nondietary ethanol might be involved in the development of NASH. Dysbiosis might increase intestinal ethanol production; for example, 1 g of *Escherichia coli* can produce 0.8 g of ethanol per hour in anaerobic conditions<sup>98</sup>. In addition, Proteobacteria (especially *Escherichia coli* and other Enterobacteriaceae), which produce alcohol, were found to be substantially increased in patients with NASH<sup>55</sup>. The alcohol hypothesis in NASH is supported by the finding that *ob/ob* mice that develop NASH have higher early-morning breath alcohol content compared with their lean littermates<sup>99</sup>. This finding is abrogated by neomycin treatment<sup>99</sup>. In human studies, small increases in breath ethanol were detected in women with obesity<sup>100</sup>. Elevated blood ethanol levels have also been observed in patients with NASH, with corresponding upregulation of hepatic alcohol metabolizing capacity (alcohol and aldehyde dehydrogenases and CYP2E1 metabolism)<sup>101</sup>. In children, blood ethanol levels were also found to be statistically significantly increased in patients with NASH<sup>55</sup>. Ethanol produced in the gut might contribute to liver injury by increasing intestinal permeability and portal LPS levels, triggering TLR and inflammasome activation<sup>102</sup>; once absorbed, ethanol might also have direct toxic effects in the liver (FIG. 1).

### Choline and methylamines

Choline, a component of cell membranes, is found in foods such as red meat and eggs. It can also be endogenously synthesized<sup>103</sup>. In the liver, choline is used in the synthesis of VLDL. Thus, choline deficiency prevents synthesis and excretion of VLDL, leading to hepatic triglyceride accumulation. In human studies, patients with more aggressive NAFLD were shown to have choline depletion linked to increased choline metabolism in the gut and high levels of the taxa Erysipelotrichia (from the phylum Firmicutes)<sup>104</sup>. An explanation for this finding comes from a mouse association study showing that

taxa such as Erysipelotrichia are associated with both choline depletion and increased urinary toxic methylamines (which have been linked to liver injury)<sup>104,105</sup>. Interestingly, the liver metabolizes methylamines to trimethylamine *N*-oxide (TMAO), another toxic metabolite that has adverse effects on glucose homeostasis and is implicated in atherosclerosis — this process might be one mechanism underlying the excess cardiac mortality in patients with NAFLD<sup>103</sup>.

### Factors affecting the gut–liver axis

#### Dietary factors

**Dietary fat and cholesterol.** Dietary fat and cholesterol seem to interact synergistically to induce the metabolic and hepatic features of NASH<sup>106</sup>. In mice, a diet high in saturated fat such as palm oil increases steatosis, decreases microbial diversity and increases the Firmicutes to Bacteroidetes ratio, potentially increasing energy harvest from the gut, as well as inducing upregulation of genes related to lipid metabolism in the distal small bowel<sup>107</sup>. A study found that mice fed lard (animal fat high in saturated fats and cholesterol content) have increased hepatic TLR4 activation and white adipose tissue inflammation with reduced insulin sensitivity compared with mice fed fish oil. Lard-fed animals had significantly decreased phylogenetic diversity and increased levels of *Bacteroides*, *Turicibacter* and *Bilophila* whereas fish-oil-fed mice had increased levels of Actinobacteria (*Bifidobacterium* and *Adlercreutzia*), lactic acid bacteria (*Lactobacillus* and *Streptococcus*), Verrucomicrobia (*Akkermansia muciniphila*), Alphaproteobacteria and Deltaproteobacteria<sup>108</sup>. In another study, a high-fat diet changed the balance of SCFAs produced in the gut to one favouring the development of NASH with decreased formation of butyrate and increased production of acetate<sup>109,110</sup>. On the other hand, rats fed a high-fibre diet had reduced hepatic inflammation<sup>109</sup>.

**Fructose and sucrose.** Dietary fructose has been strongly implicated in NAFLD progression. In a mouse study, fructose-exposed animals had considerably increased intestinal macrophage counts and lower tight junction occludin protein expression, associated with increased endotoxaemia and bacterial translocation and increased TLR1, TLR2, TLR3, TLR4, TLR6 and TLR8 expression in the liver<sup>111</sup>. In this study, sucrose-induced steatohepatitis was abrogated in fructokinase knockout mice, suggesting a role of fructose derived from sucrose in steatohepatitis<sup>112</sup>.

The possible role of fructose is highlighted by a study of patients with NAFLD that found that only 15% of hepatic triglycerides were derived from dietary fat, with 59% derived from serum non-esterified fatty acids and 26% from *de novo* lipogenesis from dietary sugars, especially fructose<sup>113–115</sup>. By comparison, the contribution of *de novo* lipogenesis to the liver triglyceride content can be <5% in the fasted state<sup>116</sup>. Fructose does not require insulin for its metabolism and directly stimulates SREBP1c, hence promoting lipogenesis even in the setting of insulin resistance<sup>117</sup>. Thus, some argue



that *de novo* lipogenesis from fructose is a central abnormality in NAFLD and more important than high dietary fat intake<sup>117</sup>. Association studies in a large cohort demonstrated an increased risk of NAFLD in those with regular sugar-sweetened beverage intake, especially if overweight<sup>118</sup>. However, accurately assessing consumption of sugars is challenging<sup>119</sup>. Moreover, genetic predisposition might have a role as healthy children fed a high-fructose diet are more likely to increase liver fat if their parents have diabetes<sup>120</sup>. Even so, it might be difficult to distinguish early-life and perinatal effects from genetic factors in this type of study.

**Glycotoxins.** Glycotoxins or advanced glycation end-products (AGEs) are formed in food when reducing sugars react non-enzymatically with the amino groups on proteins<sup>121</sup>. Levels of AGEs are particularly high in baked, fried and broiled foods when cooking occurs at high temperatures. The main receptor for AGEs, known as the receptor for AGEs (RAGE), has been found to be involved in chronic gastritis due to *Helicobacter pylori* by favouring its adhesion to epithelial cells<sup>122</sup>. Similarly, in Crohn's disease increased RAGE expression is found in phagocytes infiltrating inflamed areas<sup>123</sup>. RAGE expression is particularly increased in epithelial and lamina propria cells of inflamed bowel and contributes to on-going chronic inflammation in IBD<sup>124</sup>. Hepatic stellate cells also express RAGE, and AGEs have been shown to increase proliferation and expression of collagen in these cells<sup>125</sup>. Furthermore, a high-AGE diet (typical of Western diets) increased steatosis, oxidative stress and fibrosis in a mouse model of fatty liver disease<sup>125</sup>. Further studies are required to determine whether AGE-RAGE signalling in the gut and liver contributes to the pathogenesis of fatty liver disease.

**Artificial sweeteners.** Noncaloric artificial sweeteners are widely used by patients with obesity and metabolic disorders. However, evidence suggests that they contribute to the development of glucose intolerance by producing compositional and functional alterations in microbiota<sup>126</sup>. A study showed that intake of saccharin, sucralose or aspartame induced glucose intolerance associated with changes in microbiota, including an increase in *Bacteroides vulgatus* and under-representation of *Akkermansia muciniphila*<sup>126</sup>. These effects were fully transferrable to germ-free mice and could be abrogated by antibiotics<sup>126</sup>. This effect might reflect GLUT2 upregulation in the small intestine<sup>127</sup>. Specifically, apical GLUT2 transporters alter their membrane insertion rate and activity in response to  $\beta$ -adrenergic agonists, gut-derived hormones (for example GLP-1 and GLP-2) and leptin levels<sup>127</sup>. Moreover, fructose entering the portal blood is almost completely extracted at first pass by the hepatic GLUT2 transporter, where it is oxidized and subsequently converted to lactate and glucose. Lactate and glucose are then directed to *de novo* lipogenesis or converted to glycogen for storage<sup>127</sup>.

Artificial sweeteners have also been linked to obesity, diabetes, cardiovascular disease, the metabolic syndrome and learning difficulty<sup>128</sup>. As a result, clinical guidelines

now recommend restriction of their intake<sup>129</sup>. Whether they have any direct link to NAFLD needs further investigation, as a study of mice with free access to solutions containing 30% glucose, fructose, sucrose or artificial sweetener for 8 weeks did not show statistically significant hepatic lipid changes compared with water alone<sup>130</sup>. Another study, however, has shown that aspartame, an artificial sweetener, accumulates in the liver of both healthy and cirrhotic rats and might increase the risk of NAFLD via mitochondrial dysfunction and ATP depletion in the liver<sup>131</sup>. By contrast, other artificial sweeteners such as xylooligosaccharide might actually reduce liver triglyceride levels<sup>132</sup>. Further human studies are therefore required to clarify the relationship between artificial sweeteners and NAFLD<sup>133</sup>.

**Fermented green tea.** Green tea (*Camellia sinensis*) and its processed products (for example, oolong and black tea) were found to restore the Firmicutes/Bacteroidetes and *Bacteroides/Prevotella* ratios in mice with NASH induced by high-fat diets<sup>134</sup>. The proposed mechanism is that the phylum Bacteroidetes is more capable of cleaving glycosidic linkages of polyphenols than Firmicutes. Consequently, mRNA expression levels of lipogenic and inflammatory genes are downregulated in white adipose tissue. Importantly, hepatic triglyceride levels and hepatic lipogenesis-related *Srebp1*, *Acaca* (which encodes ACC1), *Fas* and stearoyl-CoA desaturase (*Scd1*) genes were all downregulated. The confounding factor here, however, is that both green tea and fermented green tea contain caffeine, which could be contributing to these effects.

This issue was addressed in another study in which the major polyphenol in green tea, epigallocatechin gallate (EGCG), was given to mice fed a high-fat, Western-style diet in a dose equivalent to a human drinking 10 cups of green tea per day<sup>135</sup>. EGCG was found to induce lipid clearance via hepatic autophagy both *in vitro* and *in vivo*. The mechanism in this experiment was found to be increased phosphorylation of AMPK and decreased lipid accumulation in cultured primary hepatocytes<sup>135</sup>.

In another experiment, mice on a hyperlipidic diet given additional EGCG had decreased weight gain, reduced retroperitoneal and relative mesenteric adipose tissue, lower insulin resistance as measured by homeostatic model assessment (HOMA-IR), and decreased insulin levels and liver fat accumulation compared with those on a hyperlipidic diet alone<sup>136</sup>.

In human studies, an epidemiological cross-sectional study of 1,024 Japanese male workers did not find an association between green tea consumption (categorized by  $\geq$  three cups of green tea a day) and hepatic steatosis on ultrasonography<sup>137</sup>. However, in a double-blind, placebo-controlled, randomized clinical trial of 80 participants receiving 500 mg of green tea extract (containing 31.4% EGCG but notably also 2.3% caffeine) versus placebo, those receiving the green tea capsules had significantly reduced alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels after 12 weeks ( $P < 0.001$ )<sup>138</sup>.

**Caffeine.** Considerable experimental and epidemiological evidence suggests that intake of coffee inhibits the development of NAFLD<sup>139,140</sup>. One potential mechanism is that coffee and its bioactive components (alkaloids and phenolic compounds) modulate the gut microbiota<sup>141</sup>. In a study of healthy adult volunteers who consumed three cups of coffee a day for 3 weeks, *Bifidobacterium* spp. were reduced. In corresponding mouse studies, caffeine reduced expression of aquaporin-8, a water channel protein expressed in the intestinal mucosa that facilitates water transport, in the proximal colon<sup>142</sup>. Downregulation of aquaporin-8 might contribute to more rapid gut transit and decreased energy harvest. In rats fed a high-fat diet, coffee altered the Firmicutes/Bacteroidetes ratio, which was associated with increased levels of the beneficial SCFA butyrate, and decreased body weight, adiposity and levels of liver triglycerides<sup>143</sup>. However, whether these changes are cause or effect of alterations in the microbiome requires further study.

**Berberine.** Berberine is an alkaloid herbal compound derived from *Rhizoma coptidis*, a traditional Chinese herb. Berberine has been used for the treatment of metabolic syndrome, as well as for NAFLD<sup>144</sup>. In a rodent model fed a high-fat diet, berberine increased SCFA-induced bacterial proliferation (*Blautia* and *Allobaculum*), and reduced adiposity, levels of MCP-1 (also known as CCL2) and leptin and increased insulin sensitivity and adiponectin levels<sup>145</sup>. In another mouse model of steatohepatitis, berberine administration resulted in increased *Bifidobacteria* and *Lactobacillus* counts with concomitant attenuation in NAFLD activity score, ALT and hepatic IL-1, IL-6 and TNF levels<sup>146</sup>.

### Sleep

Adequate sleep and adherence to a circadian rhythm seem to be important for metabolic health<sup>147</sup>. Inadequate sleep in participants with obesity involved in an exercise program decreased fat loss and increased loss of fat-free body mass. These results were accompanied by increased serum levels of acylated ghrelin, reduced energy expenditure and increased hepatic glucose production<sup>148</sup>. In a study published in 2014, weekly phase reversals of the light–dark cycle to disrupt circadian rhythm did not alter the microbiome in mice fed normal chow, but did produce a number of changes in mice fed a high-fat, high-sugar diet. In this latter group, bacterial composition from the phylum Firmicutes was altered, with both increases and decreases in the relative abundance of various taxa (for example, *Desulfosporosinus* and *Desulfotomaculum* decreased and *Ruminococcus* and *Sporosarcina* increased in relative abundance). Furthermore, there was a relative decrease in Bacteroidetes compared to Firmicutes<sup>149</sup>. This particular study did not look at changes in the liver. However, the same group of researchers have found that circadian disruption increases intestinal permeability and promotes alcohol-induced steatohepatitis in mice<sup>150</sup>. Certain bacteria, such as cyanobacteria, are known to harbour a circadian timing mechanism<sup>151</sup>. However, whether host or microbiota circadian systems are responsible for

these changes to microbiota or translate to worsening of NAFLD is unknown.

Obstructive sleep apnoea is common in patients with NAFLD<sup>152</sup>. In one study, patients with NASH were found to have worse oxygen desaturation, lower mean nocturnal O<sub>2</sub> levels, higher apnoea–hypopnoea index and higher respiratory disturbance index than those with simple steatosis<sup>153</sup>. These findings are supported by work in a mouse model of NAFLD, which found that chronic intermittent hypoxia increased hepatic lobular inflammation, fibrosis, lipid peroxidation and pro-inflammatory cytokine expression<sup>154</sup>. Some evidence from mouse models suggests that intermittent hypoxia increases  $\alpha$ -diversity and Firmicutes abundance in relation to Bacteroidetes and Proteobacteria phyla<sup>155</sup>. Thus, one possible explanation for these effects of obstructive sleep apnoea in NAFLD could be that hypoxic conditions within the bowel enrich obligate anaerobes<sup>155</sup>.

### Exercise

No overall consensus exists as to which diet or lifestyle approach is right for patients with NAFLD<sup>156</sup>. However, given that cardiovascular complications are the leading cause of death in patients with NAFLD<sup>157</sup>, regular and moderate exercise is independently associated with a 25–35% decrease in cardiovascular risk over a 20 year period<sup>158</sup>. Moreover, diet and exercise probably interact, and some interesting evidence suggests that changes in microbiota contribute to the beneficial effects of exercise. In obese mice, exercise induces a change in the microbiome with a decrease in the percentage of Lactobacillaceae and increased numbers of Turicibacteraceae<sup>159</sup>. Exercise-induced changes in the microbiome also seem to increase butyrate concentration in the rat caecum with downstream beneficial effects on NF- $\kappa$ B-dependent pathways<sup>160</sup>. In another study in mice, exercise was associated with a relative increase in butyrate-producing Bacteroidetes and Firmicutes bacteria as well as the diversity of the gut microbiota<sup>159</sup>. In elite athletic human males, protein consumption also appeared to positively correlate with microbial diversity. Moreover, athletes and controls with low BMI had increased populations of the genus *Akkermansia* compared with a high BMI control group<sup>161</sup>. *Akkermansia muciniphila*'s interactions with L cells and manipulation of host immunity can have favourable metabolic effects<sup>162</sup>. Specifically, it has been found that live *Akkermansia* administration increased intestinal levels of endocannabinoids, which control gut inflammation and improve gut barrier function<sup>162</sup>.

Interestingly, catechins, such as ECGC in green tea, improved running endurance and energy metabolism in mice as measured by treadmill running time to exhaustion<sup>163</sup>. The mechanism, at least partly, was thought to be via increased metabolic capacity and utilization of fatty acid as a source of energy in skeletal muscle during exercise<sup>163</sup>.

### Probiotics, prebiotics and symbiotics

Strategies aimed at favourably changing the intestinal microbiota through ‘bacteriotherapy’ include the use of

nondigestible prebiotics (which encourage the growth of certain species), probiotics (which are living bacteria or fungi), and symbiotics (which are products that contain both probiotics and prebiotics)<sup>164</sup>.

Probiotics have a number of potentially important effects that could be beneficial in NAFLD. These effects include antimicrobial properties, enhancement of mucosal barrier integrity and immune modulation<sup>164</sup>. Although probiotics seem to be able to change the microbiota, there are important limitations in most studies of their effects in NAFLD, including variable dosing, small numbers of participants, lack of liver biopsy samples and the use of additional constituents such as oligo-elements, vitamins and prebiotics<sup>165</sup>.

Administration of VSL#3 (VSL Pharmaceuticals Inc., Maryland, USA), a mixture of eight probiotic strains, for 4 months in children with obesity improved NAFLD as defined by decreased ultrasonographic steatosis and BMI; one possible mechanism is that this preparation increased circulating levels of GLP-1 (REF. 166); GLP-1 has many functions, including stimulation of glucose-dependent insulin secretion, inhibition of postprandial glucagon release, delay in gastric emptying, increased satiety and induction of pancreatic beta-cell proliferation<sup>167,168</sup>.

In adult patients with NAFLD, treatment with *Bifidobacterium longum* with fructooligosaccharides, in addition to lifestyle modification, markedly reduced levels of TNF, C-reactive protein, serum AST, HOMA-IR, serum endotoxin, steatosis and NASH activity index after 24 weeks compared with lifestyle modification alone<sup>169</sup>. In another study, 20 adult patients with histology-proven NASH were randomly allocated to receive a probiotic formula containing *Lactobacillus plantarum*, *Lactobacillus delbrueckii*, *Lactobacillus acidophilus*, *Lactobacillus rhamnosus* and *Bifidobacterium bifidum*. Patients receiving this formula had reduced intrahepatic triglyceride content (as measured by proton-magnetic resonance spectroscopy) and improved ALT levels<sup>170</sup>. In a further analysis by the same group, these findings were associated with a decrease in abundance of Firmicutes and an increase in Bacteroidetes with no differences in biodiversity<sup>52</sup>.

Prebiotics have been found to reduce plasma cholesterol, triglycerides and increase HDL concentrations in diabetes trials<sup>171</sup>. Symbiotic supplementation has also been shown to reduce plasma fasting insulin and triglyceride levels in patients with diabetes<sup>172</sup>. However, high-quality clinical studies to support their use in obesity related NAFLD are lacking at this time<sup>173</sup>. More extensive reviews on this important and emerging topic have been published elsewhere<sup>164,173–176</sup>.

### Conclusions

Evidence supporting the central role of the microbiome in human diseases such as obesity and its related disorders, including NAFLD and the metabolic syndrome, is increasing. Much of this evidence initially came from elegant experiments in mice showing that phenotypes could be altered by transfer of gut microbiota from obese animals to lean littermates and included the finding that disease could be reversed with antibiotics. Studies

examining how dysbiosis might drive NAFLD have identified a number of plausible mechanisms, including changes in SCFA metabolism, increased intestinal permeability and LPS activation of TLR and inflammasomes, endogenous ethanol production, decreased choline availability and TMA (trimethylamine) production. Several of these ‘multiple hits’ are probably involved, but their relative importance might vary between individuals.

Most evidence in this field comes from animal experiments and further human study is needed. A major difficulty is the variation in the normal functional human microbiome, and that different techniques used to assess dysbiosis in humans might produce different results. Hopefully these difficulties might be overcome with the development of more sophisticated techniques such as next-generation sequencing. It is also becoming increasingly clear that the metabolic effects of dysbiosis are of central importance, and that study of the gut metabolome in disease might provide further insights into mechanisms and offer new therapeutic strategies.

Dietary factors such as glycotoxins, lipid composition, fructose, sucrose, artificial sweeteners, soy, tea and caffeine intake also affect the microbiome and the liver. Additional evidence that lifestyle factors such as quality of sleep, exercise, as well as exposure to probiotics, prebiotics and symbiotics can have specific gut and liver effects that influence progression of NAFLD might offer opportunities for therapy.

The multiple mechanisms and interactions detailed in this Review, including the heterogenous nature of the dysbiosis that can occur in NASH, might mean that the most relevant pathway in an individual will depend on host and microbiome characteristics<sup>29</sup>. Moreover, fatty liver injury can be a common response to a wide range of insults with a broad range of initiating factors. With the burgeoning increase in the breadth and depth of genomic, metabolomic, lipidomic and proteomic techniques, such complexities and their mechanisms can hopefully be unravelled, enabling a personalized medicine approach based on knowledge of pathogenic pathways, as well as clinical and prognostic biomarkers<sup>55,177</sup>. However, positive results in phase II and III human clinical trials in NASH with agents including intestinal FXR agonists (obeticholic acid)<sup>34,178</sup>, bile acid pathway modulators (Aramchol (Galmed Pharmaceuticals, Israel))<sup>179</sup>, CCR2 and CCR5 dual inhibitors<sup>180</sup>, anti-lysyl oxidase-like 2 monoclonal antibodies (simtuzumab)<sup>181</sup>, GLP-1 agonists<sup>182</sup> and dual peroxisome proliferator-activated receptor alpha/delta agonists (Elafibranor, also known as GFT505)<sup>183</sup> suggest common pathways exist that can be targeted in the majority of patients with NAFLD<sup>38</sup>.

Future studies should therefore focus on a more complete description of the microbiome, its metabolic functions and interactions with the diet via longitudinal studies in large cohorts. Hopefully, this approach will elucidate how the microbiome and the development of dysbiosis influences the metabolic and disease phenotype, allowing therapies to be targeted to individual microbiological, metabolic and lifestyle factors.

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### Author contributions

All authors contributed to researching data, discussing, writing and reviewing/editing the manuscript.

### Competing interests statement

The authors declare no competing interests.