GUT MICROBIOME AND HEPATIC STEATOSIS (STEATOTIC LIVER DISEASE)

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Abstract (approximately 150 to 250 words)

The gut–liver axis represents complex pathways of communication between the gut, the gut microbiota and the liver that influence hepatic and systemic health. Hepatic steatosis, characterized by an accumulation of lipids in liver tissue, is one of the leading causes of chronic liver disease worldwide, with the potential to progress to more serious and irreversible forms of hepatic disease. It is becoming apparent that interactions between the host and its gut microbiota contribute to hepatic steatosis, but determining whether these interactions initiate hepatic steatosis and/or drive disease progression is difficult. What is clear is that metabolites produced by the gut microbiota influence aspects of hepatic steatosis, and they may prove invaluable in non-invasive diagnostic tools to allow earlier detection of fatty liver disease, reducing the global burden of disease and improving patient outcomes. This chapter summarizes our current knowledge on the role of the gut–liver axis in hepatic steatosis and provides information on microbiota-targeted interventions that may be able ameliorate or treat hepatic steatosis in the future.

Keywords (please provide keywords required to facilitate search of chapter on web; maximum 10 keywords)

Microbiota, metagenome, microbial metabolites, NAFLD, non-alcoholic steatohepatitis, NASH, gut–liver axis

Introduction

Defining and diagnosing hepatic steatosis

Hepatic steatosis (HS), characterized as an excess of fat (triglycerides) in liver tissue, contributes to a spectrum of liver diseases affecting approximately 25 % of the global population (Del Barrio et al. 2023). Because of the complexity of HS-associated conditions, recent advances in research, a need to remove exclusionary confounder terms from clinical use and the desire to remove potentially stigmatizing language from the medical lexicon, the way in which different disorders linked with HS are defined has changed (Rinella et al. 2023). Steatotic liver disease (SLD) is now used as an overarching term to encompass the various aetiologies of HS (Rinella et al. 2023). There has also been recognition that similar, or even the same, biological processes contribute to both non-alcoholic fatty liver disease (NAFLD) and alcohol-related liver disease (ALD) (Rinella et al. 2023). As such, along with the introduction of the term SLD to describe HS, changes have been made to the ways in which variants of NAFLD and non-alcoholic steatohepatitis (NASH) are described (Rinella et al. 2023).

NAFLD, also known as non-alcoholic HS, represents the hepatic component of metabolic syndrome. This condition is now known as Metabolic dysfunction-Associated Steatotic Liver Disease (MASLD). It is the most prevalent chronic liver disease globally, and increases the risk of cardiovascular disease, stroke, hepatocellular carcinoma (40 % of patients present without cirrhosis associated with ALD), cholangiocarcinoma, gallstone diseases, diabetes and chronic kidney disease in those affected (Xiao et al. 2023). MASLD encompasses patients who have HS, as determined by biopsy or imaging, and at least one of five cardiometabolic risk factors known to

be associated with insulin resistance (**Table 1**) (Rinella et al. 2023). However, it is important to note that making a diagnosis of MASLD does not mean that other causes of SLD (e.g. parenteral nutrition, Wilson disease, α-1 antitrypsin deficiency, steatogenic medication) should not be considered, especially in children (Kaufmann et al. 2023; Rinella et al. 2023). Those with no known cause of SLD and with no cardiometabolic risk factors are considered to have cryptogenic SLD. MetALD describes those with MASLD who consume greater amounts of alcohol per week (**Figure 1**) than MASLD patients, and within this group "*exists a continuum across which the contribution of MASLD and ALD will vary*" (Rinella et al. 2023). NASH is now called Metabolic dysfunction-Associated SteatoHepatitis (MASH). It is predicted that by 2030 a large proportion of populations in industrialized countries will be affected by some form of HS, increasing the economic and healthcare burdens associated with SLD (**Figure 2**). This trend is expected to be reflected worldwide, in line with increasing rates of obesity.

Table 1. Cardiometabolic risk factors that, together with the presence of SLD, can be used to give a paediatric or adult diagnosis of MASLD (adapted from (Rinella et al. 2023))

Cardiometabolic risk factor	Paediatric criteria	Adult criteria
Obesity/metabolic syndrome	Body mass index (BMI) ≥85th percentile for age/sex OR Waist circumference >95th percentile OR Ethnicity adjusted	BMI ≥23 kg/m^2 (Asia) or ≥25 kg/m^2 (rest of world) OR Waist circumference >80 cm (male), 94 cm (female) OR Ethnicity adjusted
Insulin resistance	Fasting serum glucose ≥5.6 mmol/L (100 mg/dL) OR Serum glucose ≥11.1 mmol/L (≥200 mg/dL) OR 2-hour post-load glucose levels ≥7.8 mmol/L (140 mg/dL) OR HbA1c ≥5.7 % (39 mmol/L) OR Type II diabetes mellitus OR Treatment for type II diabetes mellitus	Fasting serum glucose ≥5.6 mmol/L (100 mg/dL) OR 2-hour post-load glucose levels ≥7.8 mmol/L (140 mg/dL) OR HbA1c ≥5.7 % (39 mmol/L) OR Type II diabetes mellitus OR Treatment (e.g. metformin) for type II diabetes mellitus
Hypertension	Blood pressure ≥95th percentile for <13 years old or ≥130/80 mmHg (whichever is lower); blood pressure 130/85 mmHg for ≥13 years old OR Specific anti-hypertensive drug treatment	Blood pressure ≥130/85 mmHg OR Specific anti-hypertensive drug treatment (e.g. diuretics)
Dyslipidaemia	Plasma triglcyerides ≥1.15 mmol/L (≥100 mg/dL) for <10 years old; ≥1.70 mmol/L (≥150 mg/dL) for ≥10 years old	Plasma triglycerides ≥ 1.70 mmol/L (150 mg/dL) OR

	OR Lipid-lowering treatment	Lipid-lowering treatment (e.g. fibrates)
Dyslipidaemia	Plasma HDL cholesterol ≤ 1.0 mmol/L (40 mg/dL) OR Lipid-lowering treatment	Plasma HDL cholesterol ≤ 1.0 mmol/L (40 mg/dL) (male), ≤ 1.3. mmol/L (female) OR Lipid-lowering treatment (e.g. statins)

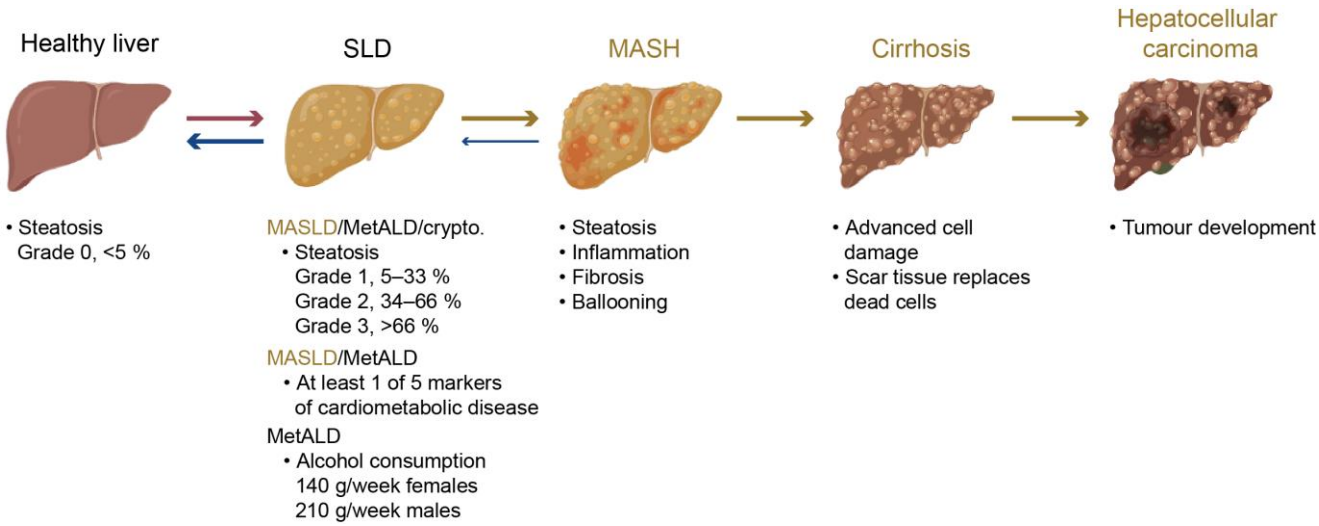

Figure 1. The ways in which different forms of SLD are differentiated, and their potential for disease progression. The system of grading the lipid load of liver tissue (steatosis grade) is based on that of (Kleiner et al. 2005). Shown with yellow text and arrows, the potential for progression of MASLD to MASH, cirrhosis or hepatocellular carcinoma is well documented in the context of the existing literature on NAFLD (Kim et al. 2021). Crypto., cryptogenic.

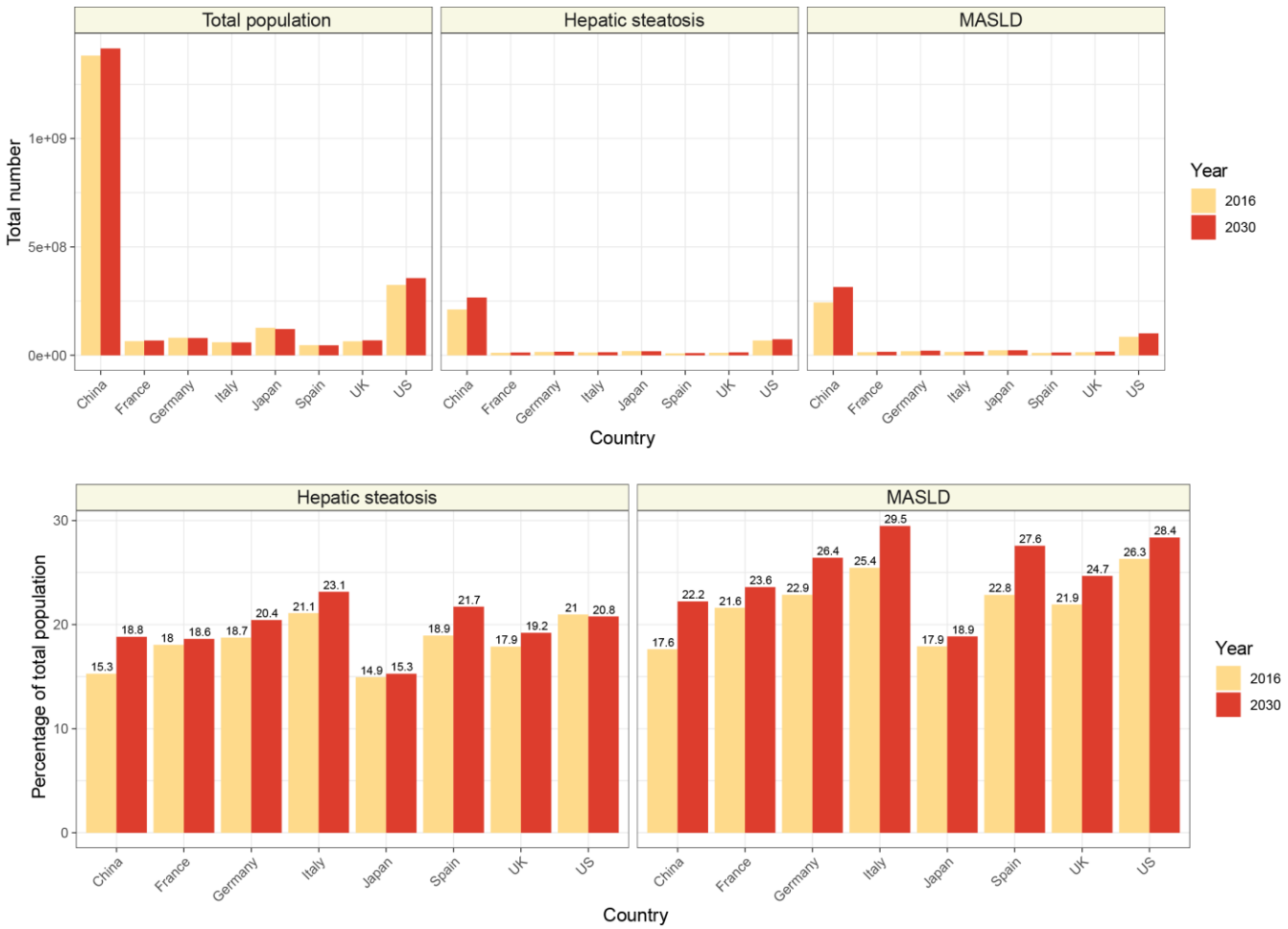

Figure 2. Summary of model forecast data for prevalence of hepatic steatosis (HS) and MASLD in selected industrialized countries for the years 2016 and 2030. Graphs produced from data presented in (Estes et al. 2018). The graphs on the top row represent the data in terms of integers, with the graphs on the bottom row showing the data in terms of proportion of each country's total population affected by HS and MASLD. In the original publication HS was defined as simple steatosis with no progression to NASH, and MASLD was defined as NAFLD.

In the early stages of HS, and because patients often remain asymptomatic, the condition is under-diagnosed. It is only when the patients present with symptoms (e.g. fatigue, pain in upper right quadrant of abdomen) or disease progresses that the condition is detected via abnormal liver blood tests (raised bilirubin, high aspartate aminotransferase to alanine aminotransferase ratio; low albumin in advanced liver fibrosis), ultrasound, computed tomography and/or magnetic resonance imaging. Consequently, the European Association for the Study of the Liver (EASL) has recommended that patients with features of metabolic syndrome (and by extension cardiometabolic risk factors associated with insulin resistance; **Table 1**) should be screened for HS as well as for the presence of fibrosis using non-invasive markers (Forlano et al. 2022). In patients with a high hepatic lipid load, or those who are clinically obese, ultrasound-based tests may not be reliable for diagnosing SLD. Liver fibrosis and cirrhosis can be diagnosed by measuring liver stiffness (transient elastography or magnetic resonance elastography) or imaging, and fibrosis assessed by ELF test, NAFLD fibrosis score or Fibrosis 4 (FIB-4) index. Histological assessment of steatosis, fibrosis, inflammation and ballooning in liver biopsy samples (**Figure 1**) remains the gold standard for diagnosing SLD. Because of its invasive nature, risks associated with infection and expense, alternatives to diagnosis of SLD by biopsy are urgently required. In addition, it is impractical to use biopsy as a diagnostic tool given the increasing global incidence of SLD (**Figure 2**).

This chapter will focus on how the human gut microbiome contributes to SLD, and approaches that may be used to modulate the microbiota/microbiome to improve patient outcomes. Wherever possible, the distinction will be made between MASLD, MetALD and cryptogenic SLD, but this may not always be possible due to lack of information in the literature for patient cohorts, and the need for adoption of the Delphi recommendations around nomenclature for SLD (Rinella et al. 2023) by the wider scientific community.

The contribution of the gut microbiome to steatotic liver disease

The gut microbiota and microbiome

Although the terms are used interchangeably in the literature, from a systems biology perspective it is important to acknowledge the distinction between the microbiota and microbiome. A microbiota represents a collection of viruses, bacteria, archaea and lower eukaryotes (fungi, helminths, protists) in a specific environment (**Figure 3**). Much of what we know about the human gut microbiota comes from the study of bacteria recovered from faecal samples. Up to 1000 species of bacteria can be found in faecal material, representing members of the phyla Bacteroidota (formerly Bacteroidetes), Bacillota (Firmicutes), Actinomycetota (Actinobacteria), Verrucomicrobiota (Verrucomicrobia) and Pseudomonadota (Proteobacteria), with each person harbouring at least 160 different species of bacteria in their faecal microbiota (Hoyles and Swann 2019). Few species of archaea are found in the human gut, but when present they are dominated by *Methanobrevibacter smithii* (Hoyles and Swann 2019). Viruses associated with the gut are predominated by bacteriophages (phages), viruses that can integrate into the genomes of bacteria and archaea or kill these microbes (Shkoporov and Hill 2019).

A microbiome constitutes the interaction between the biotic and abiotic factors of a given environment (Marchesi and Ravel 2015). In the context of the gut microbiome, this interaction involves the biotic microbiota and the entirety of human cells lining the host's gastrointestinal tract, and intrinsic and extrinsic abiotic factors that influence the interactions of the biotic components of the microbiome. Some limit the definition of the microbiome to the collection of genes and genomes from the microbiota, but these actually relate to the metagenome (Marchesi and Ravel 2015).

There are approximately 10 trillion bacteria found in the gastrointestinal tract of a healthy human adult. Every individual's gut microbiota is unique to them, complicating clinical studies focused on understanding how the gut microbiome contributes to disease. Different regions of the gastrointestinal tract have their own microbiotas, with the composition of each dictated by several intrinsic and extrinsic factors (Simrén et al. 2013). Intrinsic factors influencing the composition and function of the microbiota include gastrointestinal motility (peristalsis), transit time, immunoglobulin-containing mucus secretions, an individual's age, sex and weight, immune and health status, antimicrobial peptides produced by the host and the microbiota, oxygen and nutrient availability, bacterial co-operation and antagonism, and pH conditions. Extrinsic factors include diet (including functional foods such as probiotics and prebiotics), medications (including proton pump inhibitors, antacids, non-steroidal anti-inflammatory and prokinetics drugs, opioids, laxatives, statins, metformin and antibiotics), physical activity and circadian rhythm (Hoyles and Swann 2019).

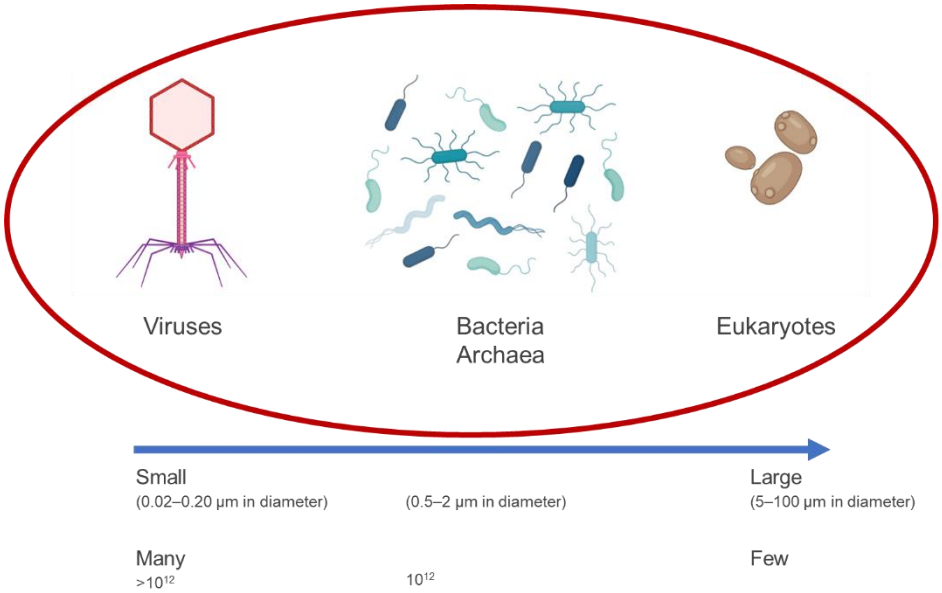

Figure 3. The gut microbiota comprises a community of microbes from different domains of life that co-exist in the human gastrointestinal tract. This environment is predominated by viruses that infect bacteria (phages) and archaea. There are thought to be two to three times more bacteria present in this environment than archaea. No reliable estimates are available for the numbers of lower eukaryotes found in the gastrointestinal tract, though fungi predominate in humans in Western countries (Rowan-Nash et al. 2019; Sender et al. 2016; Shkoporov and Hill 2019). Our knowledge on the human gut microbiota comes mostly from studying faecal samples, with up 10^{10}–10^{11} microbial cells found per wet-weight gram of faeces; this number does not include viruses (Walker and Hoyles 2023).

Host–microbiota co-metabolism, and the gut–liver axis

Co-metabolism

At a genetic level, humans are more microbe than human: >99 % of the genes in the gastrointestinal tract are of microbial not human origin (>3,000,000 vs ~22,000) (Qin et al. 2010). This increased genetic diversity within the gastrointestinal tract allows the microbiota to break down ingested foodstuffs and medications that cannot be acted upon by human digestive functions and enzymes (Clarke et al. 2019). The by-products of microbial metabolism in the gastrointestinal tract are associated with a range of biological functions in mammals through a “network” of microbial–host co-metabolism such that these by-products can influence host intestinal and systemic health (Hoyles and Swann 2019; Zheng et al. 2011) (**Figure 4**).

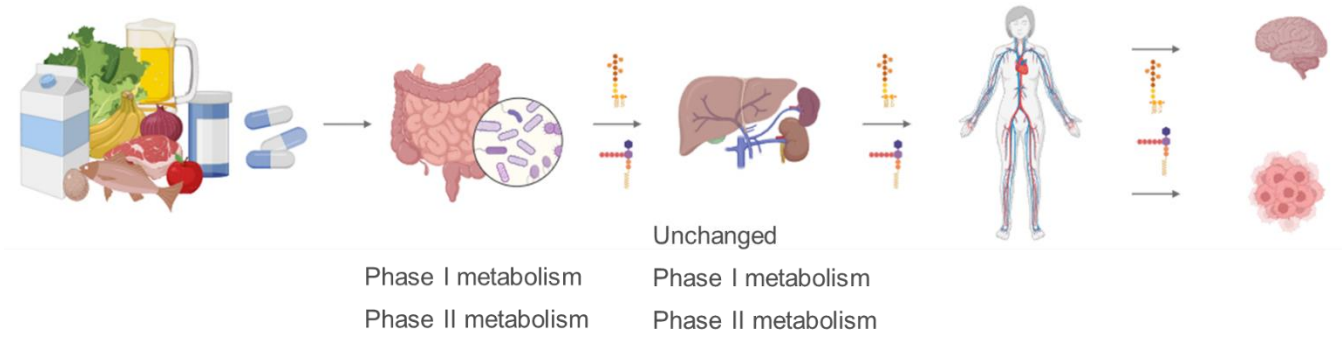

Figure 4. Host–microbiota co-metabolism. Dietary substrates and/or medications are ingested. The gut microbiota breaks down components of these substrates. The contribution of intestinal phase I metabolism to modification of microbial metabolites is unknown, but intestinal phase II metabolism does contribute to modification of some metabolites (e.g. *p*-cresol → *p*-cresol sulfate). Over 75 % of the blood supply to the liver is delivered by the hepatic portal vein (draining venous blood from the small and large intestines), transporting potentially hundreds (Han et al. 2021) of microbial metabolites to the liver. These metabolites can pass through the liver and enter systemic circulation unchanged (e.g. short-chain fatty acids). Other metabolites are subject to phase I (e.g. trimethylamine → trimethylamine *N*-oxide) or phase II (e.g. benzoate + glycine → hippurate; *p*-cresol + glucuronide → *p*-cresol glucuronide) metabolism in the liver before entering the circulation. Whatever biotransformation(s) they undergo in the intestine and/or liver and their route to the systemic circulation, microbiota-associated metabolites can interact with organs (including the brain) and cells (including cancer cells) throughout the body, influencing human biological processes. In healthy individuals, microbiota-associated metabolites are efficiently cleared from the body in urine and/or faeces. Plasma-bound microbiota-associated metabolites such trimethylamine *N*-oxide, indoxyl sulfate and *p*-cresol sulfate accumulate in the blood of those with chronic kidney disease, due to the characteristic impaired renal function of these patients.

Microbial metabolism of dietary substrates and medications in the upper gut is poorly studied because samples can only be obtained from this environment in an invasive manner. However, the microbiota of the small intestine contributes to synthesis of essential amino acids, with up to one-fifth of circulating plasma lysine and threonine produced by the ileal microbiota (Hoyles and Swann 2019). Phase I and II metabolism of xenobiotics (including products of microbial metabolism of dietary substrates and medications) occurs in many organs in the body, but predominates in the liver (Clarke et al. 2019; Pant et al. 2022). Phase I metabolism involves modification of xenobiotics (via oxidation, reduction, hydrolysis, deamination, demethylation, dehalogenation, epoxidation, and/or peroxigenation) to make them more water soluble, facilitating their excretion from the body. Phase II metabolism involves conjugation (via glutathionylation, glucuronidation, sulfation, acylation and/or glycation) of xenobiotics or their phase I derivatives to (in general) increase their metabolic weight, reduce their biological activity and make them more hydrophilic for excretion. Dietary substrates such as choline, trimethylamine *N*-oxide (TMAO) and carnitine are broken down to trimethylamine (TMA) by bacteria in the small intestine, with TMA subject to phase I metabolism in the liver (Hoyles et al. 2018b). Benzoate, an end-point product of microbial polyphenol metabolism and likely produced in the small intestine, is subject to phase II conjugation in the liver, producing hippurate, known to be a marker of and contributor to improved metabolic health (Brial et al. 2021). The microbiota in the large intestine gains most of its nutrients from undigested dietary substrates and host-derived products such as mucins, desquamated intestinal epithelial cells, and gastric and pancreatic secretory products. By-products of microbial fermentation of dietary fibre include short-chain fatty acids (acetate, propionate, butyrate, succinate), CO_2, CH_4, H_2S, H, lactate, pyruvate and ethanol (Hoyles and Swann 2019). Butyrate is the main energy source for epithelial cells lining the large intestine, protecting against colorectal cancer, and stimulating the antimicrobial activity of macrophages and regulatory T cells (Hsu and Schnabl 2023). Microbiota-driven proteolysis in the large intestine leads to the formation of potentially

toxic metabolites (**Figure 5**), including ammonia, amines, sulfides, phenols and indoles, though the impact of many of these on host health is still poorly defined (Clarke et al. 2019).

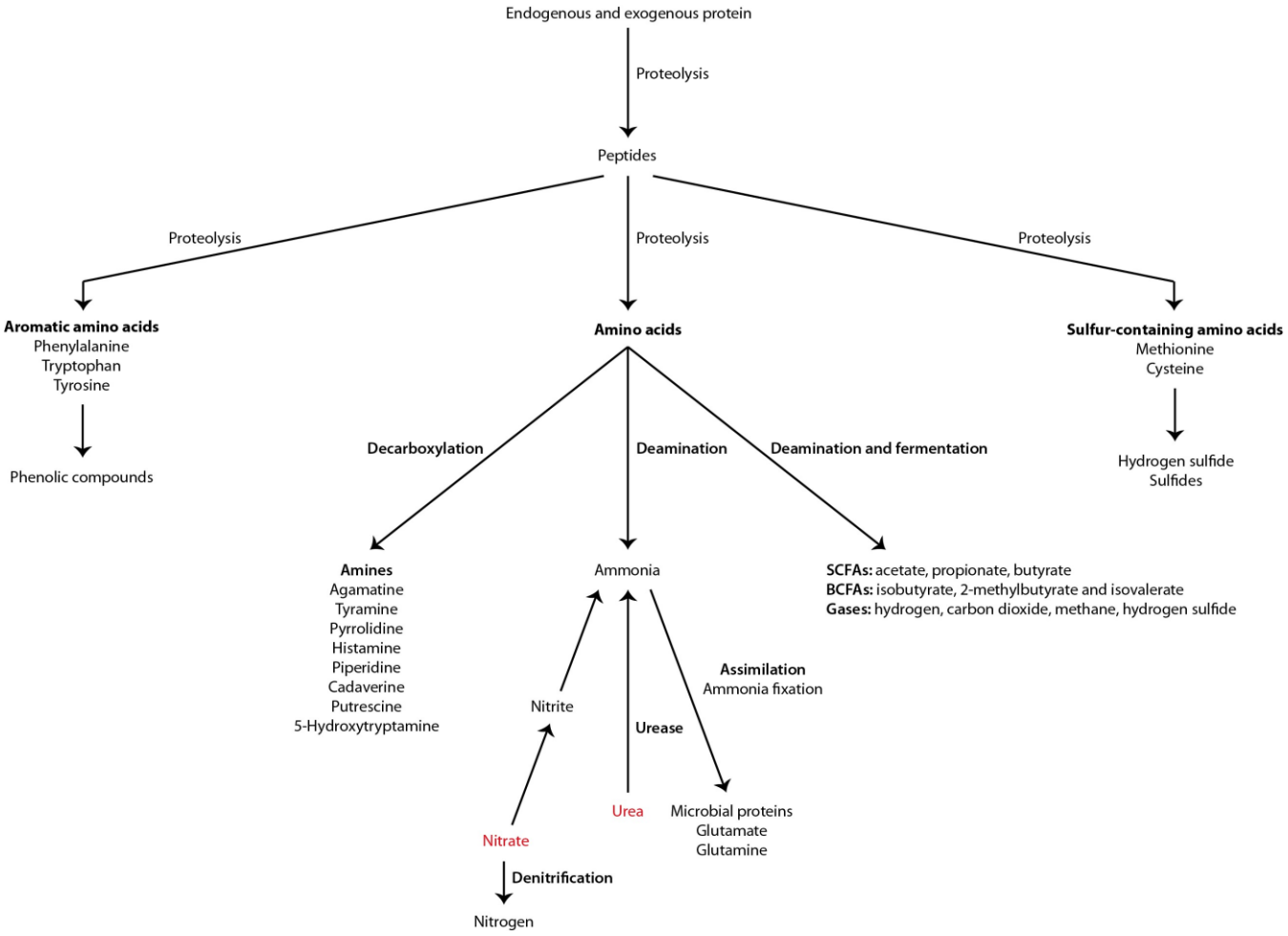

Figure 5. Microbial use of proteins, peptides and amino acids in the large intestine. Amino-acid-utilizing bacteria are more dominant than peptide-utilizers in the human faecal microbiota. Compared with carbohydrate fermentation, protein and amino acid use by the gut microbiota results in a wider range of metabolites. Non-protein sources of ammonia are shown in red. Phenolic compounds produced by the gut microbiota include the methylphenol *p*-cresol, a by-product of microbial metabolism of tyrosine and phenylalanine. Adapted from (Hughes et al. 2000; Mafra et al. 2013).

Metabolic retroconversion

The term “metabolic retroconversion” was originally used to describe the way in which TMAO, derived predominantly from fish-based foodstuffs, was subject to host–microbiota co-metabolism. The process was suggested to involve “*a cycle of reductive followed by oxidative reactions to regenerate TMAO*” (Al-Waiz et al. 1987). Studies using *in vitro* fermentation systems inoculated with mixed microbiotas derived from human faeces demonstrated that TMAO is broken down by *Enterobacteriaceae* to TMA (Hoyles et al. 2018b). Metabolic retroconversion of TMAO was confirmed in a mouse model in the same study. As described above, microbially produced TMA is subject to phase I metabolism in the liver, with hepatic flavin mono-oxygenases converting it to TMAO. If one considers metabolic retroconversion as the breakdown of compound A to compound B by the gut microbiota with conversion back to compound A by the host, urea is also subject to this process (**Figure 6**). There is increasing interest in the role of nitrogen metabolism in MASLD, as increased microbial production of

NH_3 and decreased hepatic removal of this metabolite are characteristics of the disease (Delgado et al. 2022). The exact contribution of the microbiota to host NH_3 levels (i.e. within the gut, accumulation within the liver and/or in hyperammonia) is unknown. Re-establishment of the nitrogenous balance in MASLD may, however, be one approach to tackling the disease (Delgado et al. 2022).

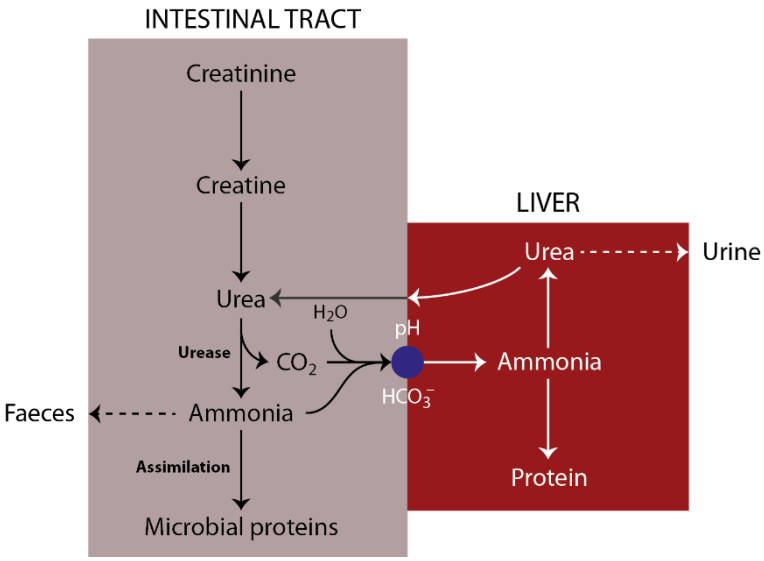

Figure 6. Production of ammonia by the gut microbiota, and its metabolic fates. Breakdown of urea to ammonia (NH_3) by the gut microbiota and its conversion back to urea by the liver is an example of metabolic retroconversion. Numerous members of the gut microbiota (e.g. bacteroides, bifidobacteria, clostridia, enterobacteria) have urease activity, enabling them to convert urea to NH_3 and CO_2. Microbial-mediated NH_3 production occurs in the stomach, small intestine and large intestine (Delgado et al. 2022). Urea is also subject to enterohepatic recirculation, a process in which it and a range of different chemicals (e.g. bile acids, bilirubin, drugs) circulate from the liver to the bile and enter the small intestine, from where they are absorbed by enterocytes and transported back to the liver. HCO_3^-, bicarbonate. Bicarbonate produced during renal ammonia generation is used in the hepatic metabolism of ammonia to urea (Weiner 2017).

The gut–liver axis

The interactions of microbial metabolites with the liver constitute one aspect of the gut–liver axis, involving the complex interplay between the gut, gut microbiota and the liver (**Figure 7**). Some microbiota-associated metabolites contribute directly to liver function: e.g. D-lactic acid produced by microbes in the small intestine promotes phagocytosis of circulating/translocating pathogens by Kupffer cells (Hsu and Schnabl 2023). There is extensive communication between the liver and gastrointestinal tract via the biliary tract, portal vein and other mediators (Tripathi et al. 2018). It was proposed that SLD results from "*multiple hits*" from the gut and/or adipose tissue that together promote liver inflammation (Tilg and Moschen 2010). If one considers the complexity of the gut–liver axis (**Figure 7**) it is not difficult to visualize the "*multiple hit*" concept, and the difficulty of distinguishing cause from effect with respect to the onset and progression of SLD. As such, gut- and adipose-derived modulators are unlikely to be the only contributors to fatty liver disease.

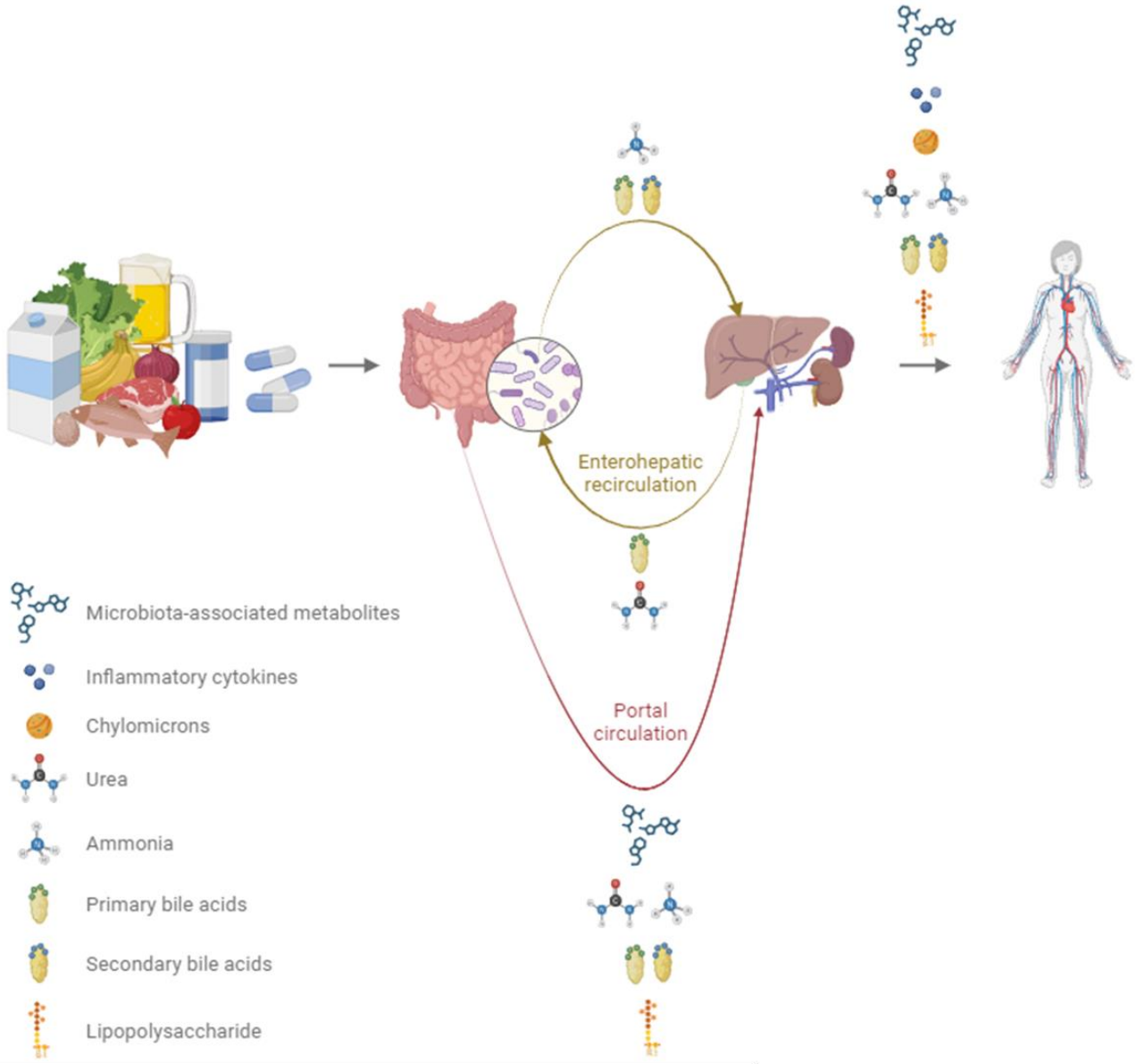

Figure 7. The gut–liver axis and its complexity. Foods and medications we ingest are broken down by gut bacteria, releasing a range of microbiota-associated metabolites into the gastrointestinal tract. Lipopolysaccharide from the cell walls of Gram-negative bacteria (e.g. *Escherichia*, *Klebsiella*, *Citrobacter*, *Bacteroides*) are also present in the gastrointestinal tract. By-products of microbial metabolism and cell-wall components, along with nutrients (carbohydrates, proteins, lipids) from foods, can be transported to the liver via the portal circulation, where they remain unchanged or are subject to hepatic phase I/II metabolism before entering systemic circulation. Enterohepatic recirculation refers to the passage of substances (e.g. bile acids and other steroids, urea, bilirubin, drugs) from the liver to bile, and their transport to the duodenum. These substances are actively absorbed from the small intestine by ileal enterocytes into the hepatic portal circulation, and then transported back to the liver. Incorporates information from (Hsu and Schnabl 2023).

Bile is produced in the liver, and comprises cholesterol, secretory IgA, antimicrobial molecules, phospholipids, bicarbonate (**Figure 6**), and the primary lipid-soluble bile acids cholic acid and chenodeoxycholic acid, and their water-soluble taurine- and glycine-conjugated bile salts taurocholic acid, glycocholic acid, taurochenodeoxycholic acid and glycochenodeoxycholic acid (Hsu and Schnabl 2023). Primary bile acids, synthesized in the liver from cholesterol, and their conjugates are stored in the gall bladder prior to their excretion in bile. Bile ducts channel bile into the duodenum, where bile acids play a role in the absorption of lipids and aid in the innate immune system via their direct bacteriostatic effect, where by acting as detergents

they disrupt microbial cell membranes and contribute to modulation of the microbiota (Joyce and Gahan 2016). Secretory IgA and antimicrobial molecules present in bile also contribute to modulation of the microbiota. Bile acids influence this modulatory process indirectly via activation of farnesoid X receptor (FXR), a bile acid receptor, stimulating production of antimicrobial molecules by intestinal epithelial cells and maintaining the gut barrier (Hsu and Schnabl 2023). Bacteria in the large intestine dehydroxylate or deconjugate primary bile acids to a range of secondary bile acids, with up to 66 and 55 bile acids detected in blood and urine, respectively (Sarafian et al. 2015). These bile acids are reabsorbed in the ileum, before being transported back to the liver and converted to primary bile acids before being excreted again (Joyce and Gahan 2016). Bile acids can self-regulate their synthesis: activation of the FXR target gene fibroblast growth factor 19 (FGF19) in the ileum suppresses hepatic bile acid synthesis. Activation of FGF19 also contributes to regulation of insulin sensitivity, and stimulation of hepatic protein and glycogen synthesis (Hsu and Schnabl 2023). The movement of bile acids from the liver to the gut and back to the liver is referred to as enterohepatic recirculation (**Figure 7**). Up to 95 % of bile acids and salts delivered to the duodenum are subject to enterohepatic recirculation.

The role of the metabolic endotoxin lipopolysaccharide (LPS) in the gut–liver axis is complex. It is often assumed that the presence of LPS, derived from the cell walls of Gram-negative bacteria, is never a good thing, contributing to increased permeability of the gut barrier and hepatic inflammation. In homeostasis, the gut barrier prevents translocation of living bacteria across the lamina propria lining the intestinal tract into the portal circulation. LPS can activate toll-like receptor 4 (TLR4) on Kuppfer and stellate cells in the liver, activating NF-κB and NLRP3 (inflammasome) signaling pathways that produce pro-inflammatory and pro-fibrotic mediators that aggravate SLD. LPS also contributes to innate immune suppression in the liver and control of local inflammation, through so-called “LPS tolerance”. That is, continuous exposure of Kuppfer cells to LPS in the portal venous blood leaves these cells immunosuppressive, resulting in downregulation of the TLR4 signaling pathway. LPS tolerance also affects sinusoidal endothelial cells of the liver (Horst et al. 2016). Increased intestinal permeability is observed in less than half of patients with early liver disease (Hsu and Schnabl 2023). This suggests that increased hepatic LPS, such as that seen in individuals affected by SLD, may not be as important in early-stage liver disease as often cited.

SLD and the gut microbiome

Perturbation of the faecal microbiota, with respect to its taxonomic diversity and functional capacity, has long been associated with metabolic diseases such as SLD (Sharpton et al. 2019; Walker and Hoyles 2023). This perturbation, characterized by an increase in the presence of facultative microbes (i.e. those that can grow in the presence or absence of oxygen) at the expense of more-specialist anaerobic bacteria (such as those responsible for the production of short-chain fatty acids, e.g. *Faecalibacterium* and *Roseburia* spp.), is referred to as dysbiosis. Few early studies into NAFLD characterized both the microbiota and its metabolites (Sharpton et al. 2019). In NAFLD, there are inconsistencies in the species or groups of bacteria associated with dysbiosis and HS: the facultative bacteria *Escherichia* spp. tend to increase in abundance, while obligately anaerobic bacteria (i.e. *Coprococcus* spp.) tend to decrease in abundance as disease severity increases. As SLD progresses to NASH, NAFLD-related advanced fibrosis or NAFLD-related hepatocellular carcinoma – all associated with concomitant increases in gut permeability, circulating LPS and systemic inflammation – dysbiosis becomes more pronounced with significant increases seen in *Escherichia coli*, *Enterococcus Bacteroides* spp., and significant decreases seen in *Faecalibacterium* spp. (Sharpton et al. 2019). These trends are seen across a range of diseases (e.g. obesity, type II diabetes, Alzheimer’s disease, coeliac disease, gastrointestinal cancers, inflammatory bowel diseases) associated with inflammation (Walker and Hoyles 2023). Dysbiosis is considered a risk factor for progression of fatty liver disease, but whether it is the cause of disease progression or merely an indicator of the increased systemic inflammation and metabolic dysregulation associated with SLD remains to be determined.

In the limited number of early studies that examined faecal or serum microbiota-associated metabolites and their association with hepatic disease, no single metabolite was consistently associated with SLD (Sharpton et

al. 2019). It is only in recent years and with the advent of multi-omics studies incorporating microbiomic, metabolomic and/or transcriptomic approaches that the roles of microbial metabolites in SLD have started to be appreciated (Koh et al. 2018; Hoyles et al. 2018a; Caussy et al. 2018; Yuan et al. 2019; Zhao et al. 2020).

Histidine can be metabolized directly to imidazole propionate by *Adlercreutzia equolifaciens*, *Shewanella oneidensis* and *Brevibacillus laterosporus*, or via the intermediate urocanate by bacteria encoding urocanate reductase (*Anaerococcus*, *Aerococcus*, *Streptococcus*, *Adlercreutzia*, *Eggerthella*, *Lactobacillus*, *Shewanella*, *Brevibacillus* spp.). Imidazole propionate impairs insulin signalling and glucose tolerance, contributing to the development of type 2 diabetes (Koh et al. 2018). In European patients with type 2 diabetes levels of imidazole propionate were found to be higher when compared to their non-diabetic counterparts, with imidazole propionate associated with inflammatory bowel disease and reduced diversity in the gut microbiota (Molinaro et al. 2020). Although imidazole propionate has not been directly implicated in SLD, it may play a role in impairing insulin signalling in humans, as the metabolite was associated with increased activation of p62 and mTORC1 in liver from subjects with type 2 diabetes (Koh et al. 2018). Dysbiosis and significantly increased levels of imidazole propionate have been observed in the serum of male Göttingen Minipigs fed a choline-deficient amino-acid-defined high-fat diet (Lützhøft et al. 2022). Expression of mTOR and binding of mTORC1 were increased, and expression of insulin substrate receptors 1 and 2 (INSR1, INSR2) and the glucagon receptor were decreased. At the time of writing, the influence of imidazole propionate on human SLD has not been investigated, but the observations of Koh et al. (2018) and Lützhøft et al. (2002) suggest increased circulating levels of this metabolite could influence not only metabolic processes associated with type II diabetes (Koh et al. 2018) but also those involved in the onset/progression of fatty liver disease.

In a study of Spanish and Italian female non-diabetic patients with HS, increased circulating levels of the branched-chain amino acids valine, leucine and isoleucine were seen as hepatic lipid load increased (Hoyles et al. 2018a). Valine and leucine can be produced by the gut microbiota and the host (Shoaie et al. 2015). Therefore, it was not possible for Hoyles et al. (2018a) to determine whether the increased levels of these branched-chain amino acids seen in patient sera were from the microbiota or host. It was only through an additional combined analysis of liver transcriptomic and serum metabolomic data that these authors were able to demonstrate the entire host hepatic pathway involved in degradation of branched-chain amino acids was downregulated in HS (Menghini et al. 2022). This exemplifies the complexity of host–microbiota co-metabolism and its contribution to disease, and the need to consider host- and microbiota-associated processes together in microbiome studies.

Hoyles et al. (2018a) noted that it was possible to detect significant changes in the functional potential of the faecal microbiota as hepatic lipid load increased in HS patients when no obvious changes in species representation were apparent. That is, functional changes in the faecal microbiota preceded obvious taxonomic changes in HS, and thus may be a more-suitable focus for future microbiome studies in early liver disease. This observation may also go some way to explain the inconsistent results seen in studies focused solely on taxonomic changes in the NAFLD-associated faecal microbiota (Sharpton et al. 2019). Microbial gene diversity and hepatic *INSR* expression were inversely associated with progression of HS. Along with decreased hepatic *INSR* expression in HS, reduced p-Akt phosphorylation was seen (reducing the response to insulin), concomitant to increased expression of hepatic lipoprotein lipase and increased levels of liver triglycerides. An integrated analysis of microbiomic and metabolomic data suggested a direct role for phenylacetate, a product of aromatic amino acid metabolism, in the development of HS (Hoyles et al. 2018a). Phenylacetate is the main phenolic compound found in faeces and is a product of microbial metabolism of phenylalanine. As hepatic transamination of phenylalanine to phenylacetate is low, except in the case of phenylketonuria, the increased levels of phenylacetate seen in HS are of microbial origin (Delzenne and Bindels 2018). Treatment of primary hepatocytes with phenylacetate triggered accumulation of lipids in hepatic cells. When phenylacetate was

applied to hepatocytes together with palmitic acid, a synergistic effect on lipid deposition was observed, suggesting phenylacetate is not the sole metabolite contributing to HS. Feeding phenylacetate to mice contributed to HS in the mice, confirming phenylacetate does have a role in mammalian HS. 3-(4-Hydroxyphenyl)lactate, a microbiota-associated metabolite sharing structural similarity with phenylacetate, has been proposed to influence HS and fibrosis based on predicted shared gene effects, but this metabolite's role in SLD has yet to be demonstrated (Caussy et al. 2018).

Similar to phenylacetate, the microbiota-associated metabolite *N*,*N*,*N*-trimethyl-5-aminovaleric acid (TMAVA) has been implicated in HS (Zhao et al. 2020). TMAVA is produced by *Enterococcus faecalis* and *Pseudomonas aeruginosa* from trimethyllysine, and initially was found in higher levels in the plasma of 15 patients with HS compared with controls. Analyses in replication cohorts (1157 subjects with or without diabetes, 767 subjects with or without HS) confirmed the association of TMAVA with HS. Inclusion of TMAVA in water in combination with high-fat feeding caused mice to develop HS, confirming the role of TMAVA in HS. In murine models, TMAVA inhibits γ-butyrobetaine hydroxylase, reducing carnitine synthesis and fatty acid oxidation to promote steatosis (Zhao et al. 2020).

Hepatocytes convert ethanol into acetate and triglycerides, contributing to the development of HS. High-alcohol-producing strains of *Klebsiella pneumoniae* were found in moderate to high abundance in over 60 % of NAFLD/NASH patients in a Chinese cohort (Yuan et al. 2019). Transfer of these *K. pneumoniae* strains to mice caused the mice to develop HS. This is an unusual finding, and may represent a cause of SLD in only a small subset of patients, though further studies are required to determine the exact contribution of high-alcohol-producing bacteria to the development of HS (Yuan et al. 2019).

Findings from the above-mentioned studies have led to the suggestion that diagnostic tests based on measuring serum metabolites could be used for non-invasive screening of SLD. A proof-of-concept study, including derivation and validation cohorts, identified 10 serum metabolites (5α-androstan-3β monosulfate, pregnanediol-3-glucuronide, androsterone sulfate, epiandrosterone sulfate, palmitoleate, dehydroisoandrosterone sulfate, 5α-androstan-3β disulfate, glycocholate, taurine, fucose) that had greater diagnostic accuracy than the FIB-4 index or the NAFLD fibrosis score for detecting advanced fibrosis (Caussy et al. 2019). A combination of 31 serum metabolites linked to amino acid, lipid, nucleotide and peptide pathways had excellent accuracy for the detection of NASH in patients with biopsy-proven NAFLD. However, the diagnostic performance of this panel of metabolites for the detection of NASH requires follow-up in independent cohorts (Caussy et al. 2019).

Microbiome-targeted interventions to ameliorate SLD

Current recommendations for the treatment of SLD revolve around dietary and lifestyle interventions, as there are no licensed drug-based therapies for the disease (Del Barrio et al. 2023; Kaufmann et al. 2023). A Mediterranean diet is recommended, along with regular moderate- or vigorous-intensity physical activity, to encourage 10 % weight loss with the intention of reducing levels of SLD biomarkers (e.g. intestinal permeability, circulating aspartate transferase and alanine transferase levels, altered metabolome, inflammation) (Del Barrio et al. 2023; Kaufmann et al. 2023). However, many individuals affected by SLD are unable to reach weight-loss goals and/or fail to make the necessary long-term changes to dietary and exercise habits required to reverse the disease. As such, tractable alternatives that modulate the microbiota/microbiome are being investigated to improve patient outcomes in SLD (Del Barrio et al. 2023).

Probiotics, prebiotics, synbiotics and postbiotics

Probiotics are "*live microorganisms that, when administered in adequate amounts, confer a health benefit on the host*" (Hill et al. 2014). Probiotic products (usually dietary supplements or foods) have been formulated using one or more species of bacteria representing lactobacilli, bifidobacteria, streptococci or bacilli. The probiotic market is largely unregulated, and these functional foods are not considered a medicine. As such, there are no clear guidelines on their prescription or administration for treatment or amelioration of SLD (Kaufmann et al. 2023). Another issue around their clinical use is that not one probiotic has been identified that elicits health benefits in all individuals who ingest it. A small number of studies have shown the potential of probiotics in improving markers of health in rodent models of SLD, but many of these interventions remain untested in patient cohorts (Kaufmann et al. 2023). Although their long-term effectiveness is unknown, short-term (8 week to 12 month, depending on duration of study) benefits of probiotics in NAFLD/NASH patients include decreases in alanine and aspartate transaminase, high-sensitivity C-reaction protein, inflammation, total body fat, intrahepatic fat and/or γ-glutamyl transferase levels (Kaufmann et al. 2023). However, the lack of standardized tests and reporting, and different probiotic strain(s) and doses used across studies make it impossible to compare outcomes. Reports in the literature also focus on small (*n*=28 to 89) cohorts in which disease status is often heterogeneous. Many larger and unified clinical studies are required to determine the true value of probiotics in SLD (Kaufmann et al. 2023). Recent improvements in our understanding of microbial genomics and the biological functions of gut bacteria have led to the development of next-generation probiotics (Khan et al. 2023) that have yet to be tested in SLD patient cohorts, but which offer greater potential for targeted improvements to metabolic health.

A prebiotic is "*a substrate that is selectively utilized by host microorganisms conferring a health benefit*" (Gibson et al. 2017). Traditionally considered carbohydrate-based products (e.g. inulin) that could be selectively broken down by bifidobacteria or lactobacilli present in the large intestine, the subtle change made to the definition in 2017 suggests that plant-derived polyphenols or polyunsaturated fatty acids converted to respective conjugated fatty acids could be considered as prebiotics in future if sufficient evidence of their efficacy in humans could be demonstrated. Gastrointestinal effects of prebiotics (i.e inulin, the oligosaccharides FOS, GOS, XOS, MOS) include inhibition of pathogens and immune stimulation, while cardiometabolic benefits include, among other things, increased insulin sensitivity and a reduction in blood lipid levels (Gibson et al. 2017). Fewer prebiotic than probiotic studies have been conducted in humans with SLD (i.e. NAFLD/NASH). These studies suffer the same issues as described for probiotics with respect to low numbers of participants (*n*=14 to 89), short duration (12 weeks to 9 months), lack of uniformity of intervention/design and reporting, though similar changes in biomarkers of disease have been seen as for probiotic interventions (Kaufmann et al. 2023). In theory, because of their ability to selectively modulate the microbiota to generate by-products of microbial metabolism known to be beneficial to metabolic health (e.g. short-chain fatty acids) (Canfora et al. 2019; Canfora et al. 2015), prebiotics should have a more profound effect on SLD than probiotics, but this has not been demonstrated clinically.

A synbiotic is "*a mixture comprising live microorganisms and substrate(s) selectively utilized by host microorganisms that confers a health benefit on the host*" (Swanson et al. 2020). Host microorganisms here includes microbes usually found in the gastrointestinal tract or introduced microbes (e.g. probiotics), as they can both be targets for the substrate component of the synbiotic. Synergistic synbiotics comprise co-administered substrates designed to be selectively used by the co-administered microbe(s), and the selective use of the substrate included in these synbiotics must be demonstrated in the same study demonstrating a health benefit. Complementary synbiotics include a probiotic combined with a prebiotic. As with probiotics and prebiotics, the few studies to date incorporating synbiotics have shown variable effects on SLD, include low numbers of patients and non-unified approaches in study design (Kaufmann et al. 2023). It should also be noted that inclusion of a *Bifidobacterium* or *Lactobacillus* spp. in a synbiotic intervention does not mean the bacterium

in question has been demonstrated to be a probiotic (i.e. not all bifidobacteria or lactobacilli have probiotic traits), so it is often not possible to determine what whether a synergistic or complementary synbiotic has been used in a specific trial.

Postbiotics are preparations "*of inanimate microorganisms and/or their components that confers a health benefit on the host*" (Salminen et al. 2021). These constitute "*deliberately inactivated microbial cells with or without metabolites or cell components that contribute to demonstrated health benefits*", and the microbial cells do not have to come from recognized probiotics (Salminen et al. 2021). Pure microbial metabolites are not considered postbiotics, even though several studies have demonstrated the benefits of microbiota-associated metabolites to host health in rodent and human studies (Canfora et al. 2019; Canfora et al. 2015; Clarke et al. 2019; Hoyles and Swann 2019). To date, no postbiotic studies have been conducted in SLD cohorts.

Faecal microbiota transplant

Faecal microbiota transplantation (FMT) involves taking a faecal sample from a healthy donor and using processed faecal material to transfer the donor's microbiota to the gastrointestinal tract of a patient affected with a disease, the implication being that restoration to a "healthy" microbiota could treat or ameliorate the patient's condition (Forlano et al. 2022). To date, FMT has only been used successfully in a clinical setting for the treatment of recurrent *Clostridioides difficile* infections. FMTs from humans to mice have been used to demonstrate the role of the gut microbiota in HS (Hoyles et al. 2018a). Similarly, FMT of a microbiota from a NASH patient harbouring high-ethanol-producing *Klebsiella pneumoniae* induced NAFLD in mice (Yuan et al. 2019). Mice with high-fat-diet-induced HS had reductions in liver lipids and pro-inflammatory cytokines, an increase in intestinal butyrate and improved intestinal-barrier function after an 8-week FMT intervention (Forlano et al. 2022). On the back of such studies, it has been proposed that FMT could be used to modulate the whole microbiota of SLD patients to reduce their intestinal permeability and hepatic liver load (Del Barrio et al. 2023; Forlano et al. 2022). As of September 2023 there were 11 clinical trials registered with ClinicalTrials.gov (https://clinicaltrials.gov/search?cond=NAFLD&intr=FMT) involving FMT and NAFLD/NASH/steatosis. None of these trials has returned their findings to date. A pilot study in NAFLD patients delivered FMT by endoscope to the distal duodenum of 15 patients who received faecal material from one of three healthy, lean faecal donors (allogenic FMT) and six patients who received their own faecal material (autologous FMT) (Craven et al. 2020). By chance, patients randomized to the autologous group were healthier (i.e. less severe NAFLD) than those in the allogenic group. FMT recipients were monitored up to 6 months post-FMT. It was concluded that duodenoscopy administered FMT did not improve insulin sensitivity or the hepatic proton density fat fraction in NAFLD patients but did contribute to repair of intestinal permeability, but this may have been an artefact of the higher baseline permeability observed in the allogenic group. A more recent study aimed to determine to determine the clinical efficacy and safety of colonoscopy-delivered (daily for 3 days, from healthy donors) FMT for NAFLD patients (Xue et al. 2022). The study was non-randomized, as patients were allowed to select whether they joined the FMT or non-FMT group. No significant differences were seen in baseline information between the groups of patients, nor were any significant differences seen in blood lipid and liver function tests before and after treatment in the FMT group. There was, however, a significant ($p < 0.05$) reduction in hepatic fat (measured by attenuation) after FMT.

As our knowledge develops around the components of FMTs that elicit effects in patients, it might become possible to design defined synthetic consortia of microbes (and/or metabolites) that could be used to treat patients with SLD rather than use FMT (Del Barrio et al. 2023). This would remove the need to conduct expensive screening exercises to identify "healthy" faecal donors, in which between only 3 and 25 % of all individuals make it through screening (Del Barrio et al. 2023), and would reduce the risk of side-effects (e.g.

diarrhoea, abdominal cramps, abdominal distension, bloating, abdominal pain, fever, flatulence or constipation; (Del Barrio et al. 2023)) to patients.

Phage therapy

The association of high-ethanol-producing strains of *K. pneumoniae* with NAFLD (Yuan et al. 2019) and cytotoxin-excreting *Enterococcus faecalis* with hepatocyte death and injury (Duan et al. 2019) has led to attempts to use phage therapy as a means to treat liver disease (Duan et al. 2019; Gan et al. 2023). While lytic phages can be isolated against a range of pathogenic and commensal bacteria with relative ease, these entities often have narrow host ranges, sometimes infecting only one to a few different strains of a single species of bacteria. Each individual's gut microbiota is unique, so no two individuals will harbour exactly the same strains of, for example, *K. pneumoniae* or *E. faecalis*, limiting the clinical efficacy of phage therapies designed based on studies involving only one to a few different strains of bacteria. With rapid advances being made in the field of synthetic biology, it is likely that broad-host-range phages will be engineered in future to target a range of bacteria implicated in SLD.

Conclusion

Microbiota characterization based on taxonomic profiling is of limited use in studies concerning early-stage liver disease, and more focus should be put on functional characterization of the gut microbiota in SLD. The contribution of individual members of the microbiota to SLD is difficult to assess, with only high-alcohol-producing strains of *K. pneumoniae* having been linked to development of HS in some individuals. It is clear a range of microbiota-associated metabolites contribute to the phenotype of HS/SLD, but the roles of these metabolites in the initiation and/or progression of hepatic disease remain largely unproven. More mechanistic studies are required to determine the numerous ways in which microbiota-associated metabolites influence hepatic biological processes and metabolic pathways, so that targeted interventions can be designed to reduce the global burden of SLD. Interventions intended to modulate the microbiota for improved metabolic and hepatic health have potential, but have yet to be tested in large-scale, well-designed clinical studies involving well-characterized patient cohorts.

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