

Liver tissue microbiota in nonalcoholic liver disease: a change in the paradigm of host-bacterial interactions

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Abstract: Nonalcoholic fatty liver disease (NAFLD) pathogenesis is explained by the complex relationship among diet and lifestyle-predisposing factors, the genetic variance of the nuclear and mitochondrial genome, associated phenotypic traits, and the yet not fully explored interactions with epigenetic and other environmental factors, including the microbiome. Despite the wealth of knowledge gained from molecular and genome-wide investigations in patients with NAFLD, the precise mechanisms that explain the variability of the histological phenotypes are not fully understood. Earlier studies of the gut microbiota in patients with NAFLD and nonalcoholic steatohepatitis (NASH) provided clues on the role of the fecal microbiome in the disease pathogenesis. Nevertheless, the composition of the gut microbiota does not fully explain tissue-specific mechanisms associated with the degree of disease severity, including liver inflammation, ballooning of hepatocytes, and fibrosis. The liver acts as a key filtration system of the whole body by receiving blood from the hepatic artery and the portal vein. Therefore, not only microbes would become entrapped in the complex liver anatomy but, more importantly, bacterial derived products that are likely to be potentially powerful stimuli for initiating the inflammatory response. Hence, the study of liver tissue microbiota offers the opportunity of changing the paradigm of host-NAFLD-microbial interactions from a “gut-centric” to a “liver-centric” approach. Here, we highlight the evidence on the role of liver tissue bacterial DNA in the biology of NAFLD and NASH. Besides, we provide evidence of metagenomic findings that can serve as the seed of further hypothesis-raising studies as well as can be leveraged to discover novel therapeutic targets.

Keywords: Nonalcoholic fatty liver disease (NAFLD); nonalcoholic steatohepatitis (NASH); HSD17B13; microbiota; microbiome; proteobacteria; lipopolysaccharide (LPS); drug prediction

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Nonalcoholic fatty liver disease (NAFLD) pathogenesis is explained by the complex relationship among diet and lifestyle-predisposing factors, genetic variance of the nuclear and mitochondrial genome, special characteristics of the phenome, including obesity, and the yet not fully explored interactions with epigenetic and other environmental factors, including the microbiome (1).

Despite the wealth of knowledge gained from molecular and genome-wide investigations in patients with NAFLD, the precise mechanisms that explain the variability of the histological phenotypes are not fully understood (2,3). Earlier studies on the gut microbiota in patients with NAFLD and nonalcoholic steatohepatitis (NASH) provided clues on the feces bacterial profile/s associated with the disease (4-19) (Figure 1). Nevertheless, the composition of the gut microbiota does not fully explain tissue-specific mechanisms associated with the degree of disease severity, including liver inflammation, ballooning of hepatocytes, and fibrosis. Here, we highlight the evidence on the role of liver tissue bacterial DNA in the biology of NAFLD and NASH. Besides, we provide evidence of metagenomic findings that can serve as the seed of further hypothesis-raising studies as well as can be leveraged to discover novel therapeutic targets.

Non-intestinal microbiotas and liver diseases

With the explosion of knowledge in the field of metagenomics, novel concepts on the pathogenesis of complex diseases emerged, including the putative role of tissue microbiotas.

A remarkable seminal study that surveyed bacteria profiles from up to 27 different body sites from healthy adults over time found that bacterial composition is primarily determined by the body habitat (20). This study demonstrated that the human microbiota varies systematically across body habitats, and it has thus established the basis onto which to explore tissue microbiotas in different disease states (20).

Explorations from studies in the past decade, particularly in the field of cardiovascular disease, suggested that the microbial composition of non-intestinal tissues may contribute to the pathogenesis of non-transmissible complex diseases. For example, Koren *et al.* used pyrosequencing of 16S rRNA genes to survey the bacterial diversity of atherosclerotic plaques and identified *Chryseomonas* in all atherosclerotic plaque samples, and *Veillonella* and *Streptococcus* in the majority (21). More recently, explorations of the microbiome in the circulating compartment showed

that an increase in the proportion of *Proteobacteria* phylum would predict long-term cardiovascular prognosis (22). The putative role of the blood microbiome in the pathogenesis of other diseases, particularly inflammatory diseases, is beyond the scope of this review and it has been thoroughly reviewed before (23).

Notably, emerging studies in the field of liver diseases demonstrated the importance of the non-intestinal microbiotas in the development and/or the prognosis of different disease states, from metabolic, inflammatory, and autoimmune diseases to cancer.

These studies involved the profiling of the microbiome of a wide range of non-intestinal biological sources, from samples of the peripheral blood to bile acid and bile duct tissue, saliva, and, more recently, the liver tissue (Figure 2). These investigations lead to important discoveries that generated new hypotheses on host-bacterial interactions. For instance, Puri and coworkers investigated the circulating microbiome signature in patients with alcoholic hepatitis and found that the relative abundance of the phylum *Bacteroidetes* was significantly decreased in alcohol-consuming patients (24). In addition, the circulating microbiome of subjects consuming alcohol was significantly enriched with the gram-negative bacilli phylum *Fusobacteria* and its subclasses *Fusobacteriales* and *Leptotrichiaceae* (24).

Cho *et al.* explored the blood microbiome of patients with cirrhosis and hepatocellular carcinoma (HCC) and found that *Staphylococcus*, *Acinetobacter*, *Klebsiella*, and *Trabulsiella* were significantly enriched in HCC-associated microbiomes (25).

Lelouvier *et al.* investigated the relationship between the blood microbiota and liver fibrosis in European cohorts of patients with severe obesity and observed that changes in several taxa correlated with the fibrosis status, including the genera *Sphingomonas*, *Bosea*, and *Variovorax* (26).

Kajihara and coworkers examined the peripheral blood microbiota of patients with liver cirrhosis by 16S rRNA gene sequencing and found that while the abundance of *Enterobacteriaceae* was significantly increased, the abundance of *Akkermansia*, *Rikenellaceae* and *Erysipelotrichales* was decreased in cirrhotic patients (27).

Zhang *et al.* evaluated the blood microbiome in patients with hepatitis B-related acute-on-chronic liver failure and found that that the relative abundance of *Prevotellaceae* independently predicted 28-day mortality (28).

On the other hand, there were interesting studies focused on the examination of the microbiome profile of bile acid and/or bile duct tissue samples. For example,

Human studies of gut microbiota in NAFLD



Figure 1 Summary of human studies of gut microbiota in NAFLD and NASH (4-19). NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; CHD, coronary heart disease; HCC, hepatocellular carcinoma.

Chng and coworkers found that several bacterial families with a significant increase in *Stenotrophomonas* species allowed for distinguishing tumors *vs.* paired non-tumors samples in patients with cholangiocarcinoma (29). Pereira *et al.* collected bile samples from the common bile duct in patients at different stages of primary sclerosing cholangitis and found that *Streptococcus* abundance was positively correlated with an increase in the disease severity (30).

Based on the profiling of salivary microbiota, Bajaj and coworkers (31) reported that salivary dysbiosis was greater in patients with cirrhosis who developed 90-day hospitalizations because of hepatic encephalopathy.

Finally, we have explored the liver tissue bacterial DNA profile in patients with NAFLD and different phenotypic and histological characteristics and found for the first time that the liver tissue of NAFLD patients contains a diverse repertoire of bacterial DNA (up to 2.5×10^4 read counts) (32). More importantly, we observed that the liver

metataxonomic signature may explain differences in the NAFLD pathogenic mechanisms as well as physiological functions of the host (32).

Biogeography of the liver tissue bacterial DNA of patients with NAFLD

The liver acts as a key filtration system of the whole body by receiving blood from the hepatic artery and the portal vein. Therefore, not only microbes would become entrapped in the complex liver anatomy but, more importantly, bacterial-derived products that are likely to be potentially powerful stimuli for initiating the inflammatory response.

Translocation of bacterial products from the gastrointestinal tract to the liver is largely acknowledged as implicated in the complications of liver cirrhosis (33). More recently, the existence of crosstalk between microbes and host liver cells that control metabolism has been proposed

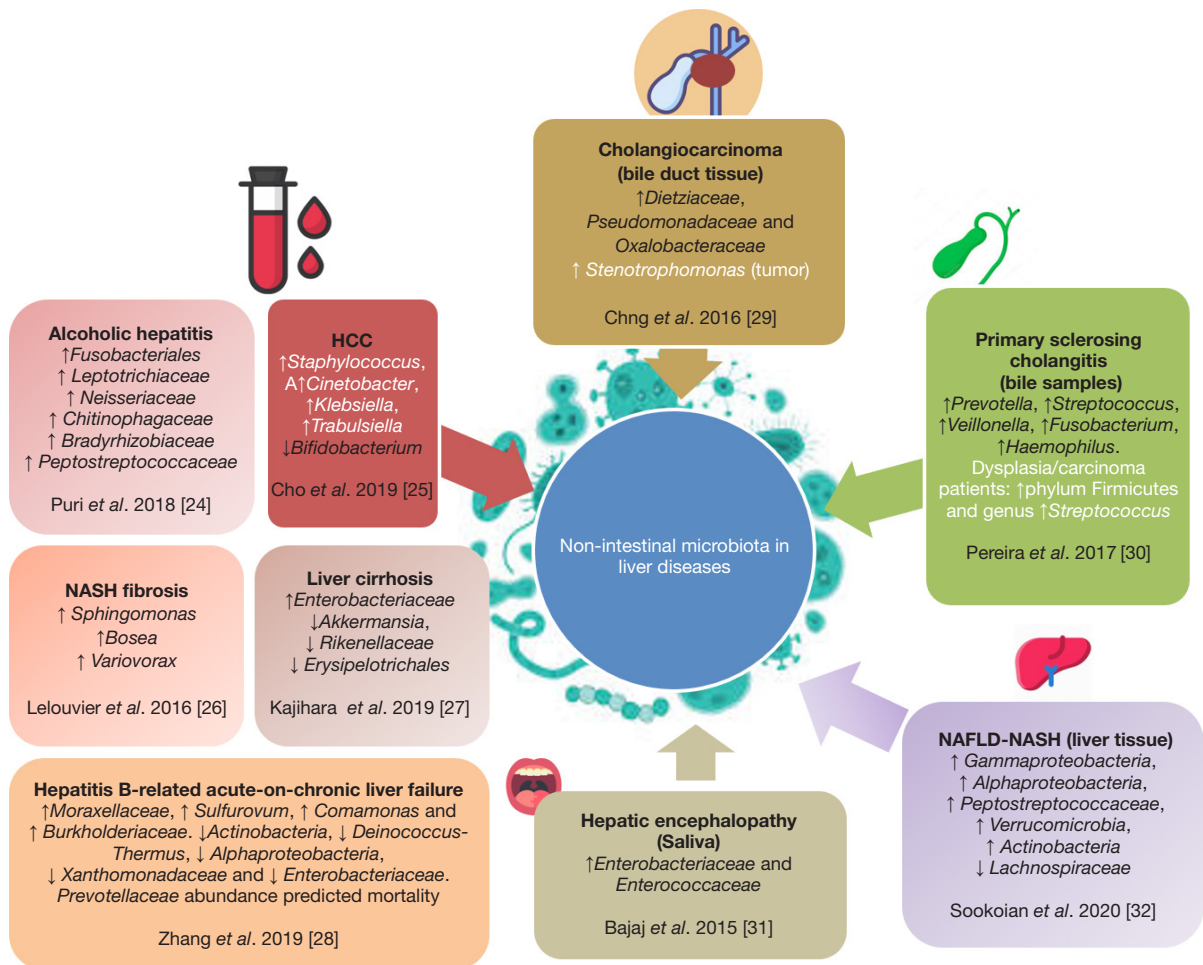


Figure 2 Summary of studies on non-intestinal microbiotas in diverse liver diseases (24–31). HCC, hepatocellular carcinoma.

to explain not only *de novo* hepatic lipogenesis that occurs in NAFLD but also associated liver damage as elegantly reviewed by Tilg et al. (34).

We have profiled the liver tissue microbial DNA in patients with NAFLD and different histological stages (32). Regarding the biogeography of the bacterial DNA found in the liver tissue specimens, we found that the hepatic microbiome profile reflects bacterial DNA communities that can be found not only across the entire gastrointestinal tract—from the oral cavity to the distal colon—but also bacterial DNA potentially derived of other body sites (Figure 3). For example, while we found that many bacterial DNA in the liver tissue samples of patients with NAFLD have been previously reported as microorganisms that inhabit the human intestinal tract, for example, *Bifidobacterium*, *Peptostreptococcus*, *Stenotrophomonas*, *Xanthomonadaceae*, *Lachnospiraceae*, and *Ruminococcaceae*—

among many other taxa (35), we observed bacterial DNA potentially derived from other body sites as well. For example, we found that *Deinococcus Thermus*, which is recognized as one of the most extremophilic phyla of Bacteria (36), was distinctively overabundant in the liver of non-morbidly obese patients with NAFLD (32). *Deinococcus Thermus* species represent a fraction of the bacterial communities residing in the human stomach (37), which is known for its acidic and inhospitable environment. These results might suggest the potential source of liver bacterial DNA that could migrate from the stomach to the liver. Also, this result might suggest the possibility of different stress-related tissue environments in different cohorts of patients, for example oxidative and/or metabolic stress (38–40), which would, in turn modulate bacterial communities' adaptation and survival.

In addition, the findings of our study suggested that some

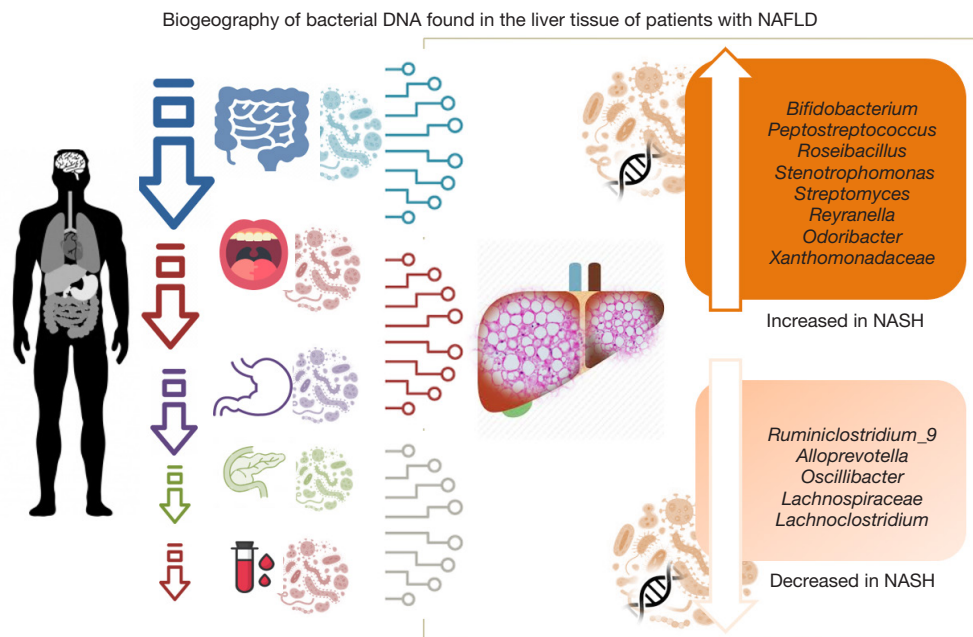


Figure 3 Summary of biogeography of bacterial DNA found in the liver tissue of patients with NAFLD. Summary indicates results of the study of Sookoian et al. (32). NAFLD, nonalcoholic fatty liver disease.

bacterial DNA might be derived from the oral microbiome (41), for example, *Actinobacteria* and *Prevotella*, and some species of *Deltaproteobacteria*.

Other features deserve particular attention. For example, we found the presence of bacterial DNA of *Gemella* genus in patients with higher scores of portal inflammation (32). *Gemella* is abundant in the proximal small intestine, and some species of this genus, for instance, *Gemella* spp can translocate to other organs and thus cause systemic diseases (42).

Likewise, we found that the abundance of bacterial DNA of *Peptostreptococcus* was significantly increased in the liver of morbidly obese patients with NASH (32). The presence in the gut of members of the *Peptostreptococcaceae* family is often associated with compromised health, for instance, elderly hospitalized patients with *Clostridium difficile* infection exposed to previous antibiotic (43), ulcerative colitis (44), and colorectal cancer (45).

Microbiota or bacterial DNA can have negative and positive effects on the host. For instance, we found that depletion of DNA from *Lachnospiraceae* family members was particularly associated with severe histological features (32). Several members of the *Lachnospiraceae* family are butyrate producers; microbial butyrate-producing communities are often reported as associated with the healthy microbiota.

Besides, microbiota-derived butyrate presumably

presents several health benefits. For example, butyrate is able to beneficially affect oxidative stress in the healthy human colon (46), it might have anti-proliferative effects by interfering with prostaglandin E2 synthesis (47), and butyrate may modulate immunological homeostasis in the gut (48). The decrease in butyrate from gut bacteria has been also linked to energy imbalance and disturbances in body weight and glucose control (49).

A recent experimental study showed that *Lachnospiraceae* administration attenuates obesity, inflammation, and dysbiosis (50). It has been also reported diminution of *Lachnospiraceae* microbial populations in patients with inflammatory bowel diseases (51). Remarkably, a human study showed that gut microbial function, inflammation, and cognition were markedly improved in cirrhotic patients by capsular fecal transplant containing *Lachnospiraceae* (52).

Furthermore, we found that overabundance of DNA derived from gram-negative Proteobacteria, particularly *Gamma*proteobacteria, was a distinguishing feature of NASH and NASH-related severe histological outcomes (32). For example, DNA derived from *Stenotrophomonas* was 3.1-fold increased in the liver of morbidly obese patients with NASH. Likewise, DNA derived from *Xanthomonadaceae* family members was consistently increased in all NASH patients regardless of their body mass index status (32). We

also found that DNA from *Alpha* and *Gamma Proteobacteria* was consistently associated with increased lobular and portal inflammation scores (32).

Concurrently, we demonstrated not only that the liver tissue of NASH patients contains DNA from *Gammaproteobacteria* but we found that lipopolysaccharide (LPS)—the major component of the outer membrane in gram-negative bacterial pathogens, predominantly localized in the portal tracts (32).

Bacterial DNA induces inflammatory response

Bacterial DNA is not immunologically inert. Quite the opposite, bacterial DNA is sensed by the immune system as a signal of infectious danger mostly because the structure of bacterial DNA presents high frequency of the dinucleotides CG and lacks of methylation (53).

Interactions between bacterial derived products, including bacterial DNA and RNA induce host inflammatory responses by activating the innate immune defense system, including the inflammasome (54). Activation of innate immune system in response of highly conserved microbial structures is known as pathogen-associated molecular patterns (PAMPs) (55). Collectively, these findings suggest that bacterial DNA itself may be a significant microbial mediator of inflammation in affected tissues by stimulating pro-inflammatory cytokine production, including interferon factor alpha (IFN α) (53) and tumor necrosis factor alpha (TNF α) (56), priming of the inflammasome (57,58), and activation of toll like receptors (59).

Advances in genomics and bioinformatics revealed that bacterial DNA might potentially integrate into the human genome through an RNA intermediate and such integrations are detected more frequently in tumor than normal cells (60). This finding not only transforms current knowledge of host-bacterial-interactions but also opens new avenues of research in the field of NASH and the development of HCC, particularly in non-cirrhotic patients.

Bacteria-bacteria interactions are another interesting mechanism to explain pathogen-host effects. We hypothesized that differences in liver bacterial DNA diversity between cohorts of NAFLD patients with different phenotypic features, including severely obese versus moderate obese patients, would also reflect putative differences in systemic ecological diversity. We thus performed network analysis of bacterial DNA communities, and observed that bacterial DNA found in the liver of

morbidly obese patients with NAFLD clustered separately from the bacterial DNA of the liver of NAFLD-non-morbidly obese patients. More importantly, while clusters of bacterial DNA communities presented significant intra-cluster positive correlations, significant negative co-occurrences between clusters were observed, for example between *Gammaproteobacteria* and *Lachnospiraceae*, which were precisely linked to severe and benign histological phenotypes, respectively (*Figure 4*); this pattern was replicated at all taxonomic levels.

Liver tissue metagenomic findings can be leveraged to identify therapeutic targets against NASH

The putative interplay between the liver tissue cellular components and bacteria-derived products has never been explored before in humans. A rodent study that investigated ethanol-associated compositional microbiome changes along the intestinal tract and the liver showed that ethanol increases bacterial richness in the jejunum and changed bacterial communities in the ileum by inducing a shift towards gram-negative bacteria (61).

As mentioned in the preceding paragraphs, we have shown that the presence of bacterial DNA in the liver tissue of patients with NAFLD would be implicated as a contributing factor in the disease pathogenesis (32).

Microbiome and host genetics are often explored as independent contributing factors in complex disorders, albeit there is a clear and biologically plausible interplay between these two factors. It becomes then obvious that understanding the putative synergistic effect/s between the microbiome and/or its byproducts and the genetic background of the host may serve to predict and design novel personalized treatments. The goal would be to identify novel pathways and interactions with microbial components that could provide effective therapies for NASH. Nevertheless, there are gaps in our understanding of how and which of these putative interactions between the tissue microbiota and the host-genetics impact on the disease biology.

Recently, a study by Abul-Husn *et al.* revealed that rs72613567, a loss-of-function variation in HSD17B13 (17-beta-hydroxysteroid dehydrogenase 13) gene, confers protection against chronic liver injury and mitigates the progression of NAFLD and alcoholic liver disease in European Americans (62). We further replicated these findings and demonstrated that the rs72613567 A-INS allele

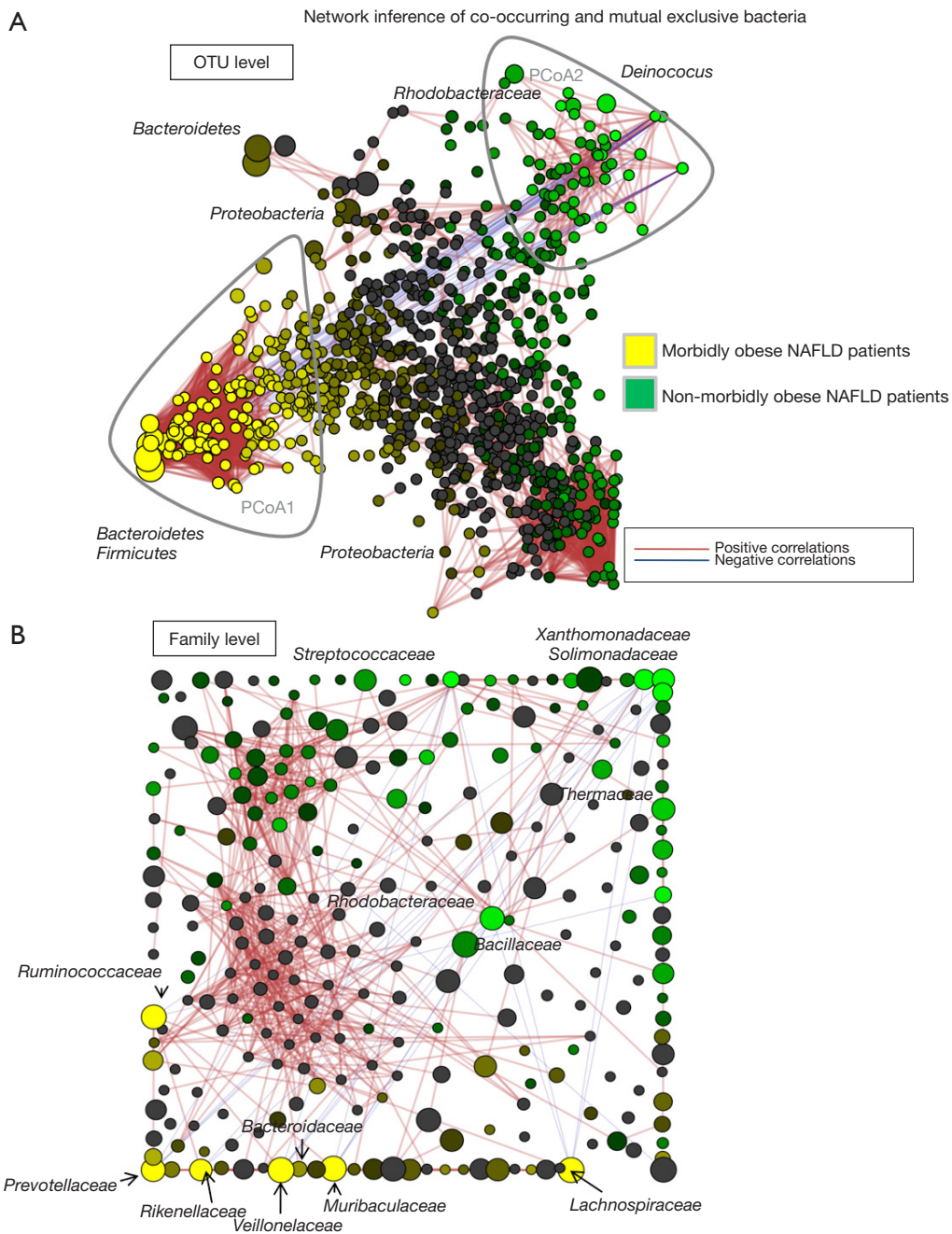


Figure 4 Network analysis of liver bacterial DNA communities and their interactions. Network analysis for identifying co-occurring bacteria, mutually exclusive bacteria, and clusters of co-occurring bacteria was performed by the Calypso online software (<http://cggenome.net/wiki/index.php/Calypso>). Co-occurrences were calculated by Spearman's rho; thus, different taxa, which are represented by nodes, and positive and negative correlations are shown in the network. Network analysis was performed at the OTU level (top 1,000 most abundant taxa) (A) and family level (B) for bacterial DNA communities residing in the liver of morbidly obese (yellow nodes) and non-morbidly obese (green nodes) subjects with NAFLD (32). The network plot shows co-occurring and mutually exclusive bacteria. Each node represents an OUT or family (a and b, respectively), and each edge represents a significant pairwise association between them. Taxon abundance is represented as node size. Nodes are placed by PCoA and are connected via edges based on Spearman's rho correlations, signifying positive (red lines) and negative (blue lines) associations. Correlations (presented as edges) are subsequently sorted for adjusted (FDR) statistical significance ($P < 0.05$) and $R > \pm 0.6$. NAFLD, nonalcoholic fatty liver disease.

protects against NAFLD [odds ratio (OR) per adenine allele =0.667; 95% CI, 0.486–0.916; $P=0.012$] (63). In addition, we found that the A-INS allele protects against NASH (OR =0.612; 95% CI, 0.388–0.964), ballooning degeneration (OR =0.474; 95% CI, 0.267–0.842), lobular inflammation (OR =0.475; 95% CI, 0.275–0.821), and fibrosis (OR =0.590; 95% CI, 0.361–0.965) (63). Subsequent studies from other groups (64–67) found similar results. Importantly, as shown by immunohistochemistry, there was a dose-dependent decreasing effect of the rs72613567 A-INS on the expression levels of the HSD17B13 protein in the liver (63).

Notably, we have performed the liver transcriptome analysis of NAFLD patients carrying the rs72613567 (–/–) homozygous genotype versus the reference group [rs72613567 (A/A) homozygous genotype], and we found that the most (≥ 3.5 -fold) downregulated and upregulated genes in the liver of patients carrying the rs72613567 (–/–) genotype were in biological pathways related to the immune system, including antigen presentation and interferon-related processes, as well as cytokine signaling (63). Accordingly, previous evidence from experimental studies showed that hydroxysteroid (17 beta) dehydrogenase 13 deficiency triggers hepatic steatosis and inflammation in mice (68). Together, these observations raised the possibility that therapeutic inhibition of HSD17B13 could represent a new strategy to treat NASH patients with more severe liver histology (1,63,69).

HSD17B13 as a hydroxysteroid dehydrogenase (HSD) belongs to the superfamily of short-chain dehydrogenases/reductases (SDR); these enzymes are involved in the steroid hormone metabolism among others. Disruption in the signaling of members of the SDR family may cause a wide range of inflammatory diseases, from hypertension to cancer (70). Likewise, the metabolic activity of microorganisms co-inhabiting the human body, particularly the gut, may act as a parallel endocrine site by producing hormones that can interfere with human physiology. For example, it has been long speculated that the composition of the gastrointestinal microbiota may be involved in the development of cancer (71). Also, interference with steroid hormone metabolism, e.g., progesterone, estradiol and cortisol, may affect the inflammatory response (72).

Hence, it was speculated about a possible relationship between host-genetics and microbiota that would modulate the inflammatory response through to the activity of steroid metabolism, and this relationship might alternatively affect bile acid signaling as well (70).

Meanwhile, it remains to be explored whether the

rs72613567 variant in HSD17B13 presents any direct or indirect interaction effect with the microbial composition of the body, including the liver or bile acid bacterial composition, which might explain in part the missing heritability of NAFLD and NASH (1). Unfortunately, knowledge of the function and regulation of HSD17B13 is still missing; no crystal structure was deposited in the protein database (PDB) for HSD17B13 either. Therefore, criteria for exploration of HSD17B13 druggability predictions are currently inconclusive. Conversely, HSD17B11 (estradiol 17-beta-dehydrogenase 11)—a protein of the same family that HSD17B13, has been largely studied (*Figure 5A*). HSD17B13 shares ~65% of its amino acid sequence with HSD17B11 (E value: $1.3e^{-131}$) being the closest family member (*Figure 5B*), and similarly to HSD17B13, HSD17B11 is located in the endoplasmic reticulum. As a proof-of-concept, we modeled the putative druggability (the ability of a binding site to bind a drug-like ligand) of HSD17B11 to demonstrate the potentiality of targeting HSD17B13. Together, the analysis of available HSD17B11 protein structure was straightforward suggesting that proteins in the same family, including HSD17B13, are predicted to be druggable (*Figure 5C,D*). Analysis of structural ligandability of HSD17B11 (*Figure 5C,D*) shows a binding site conformation with a putative pocket for binding small drugs.

Variants in many other candidate genes remain unexplored, for example rs59846192 in DMRTB1 (DMRT like family B with proline rich C-terminal 1) that has been associated with relative abundance of *Lachnospira* in earlier studies (73).

Nevertheless, the most relevant message is that microbial associations with host-genome variants are body site-specific (74). Hence, associations of host-genome variants with the fecal microbiome do not necessarily explain the effect of liver tissue microbial and host-genetics interactions.

Areas of future research

An important yet poorly explored aspect of the microbial and host-genetics interactions is the possibility of drug-metabolizing proteins encoded by the microbiome genes might interfere with patients' response to medicinal drugs. There is evidence from recent literature that intestinal metagenomic abundance of bacterial phyla, genera, species, and their gene products are able to interfere with many drugs used to treat human diseases, for instance norethindrone acetate and famciclovir (75). A study

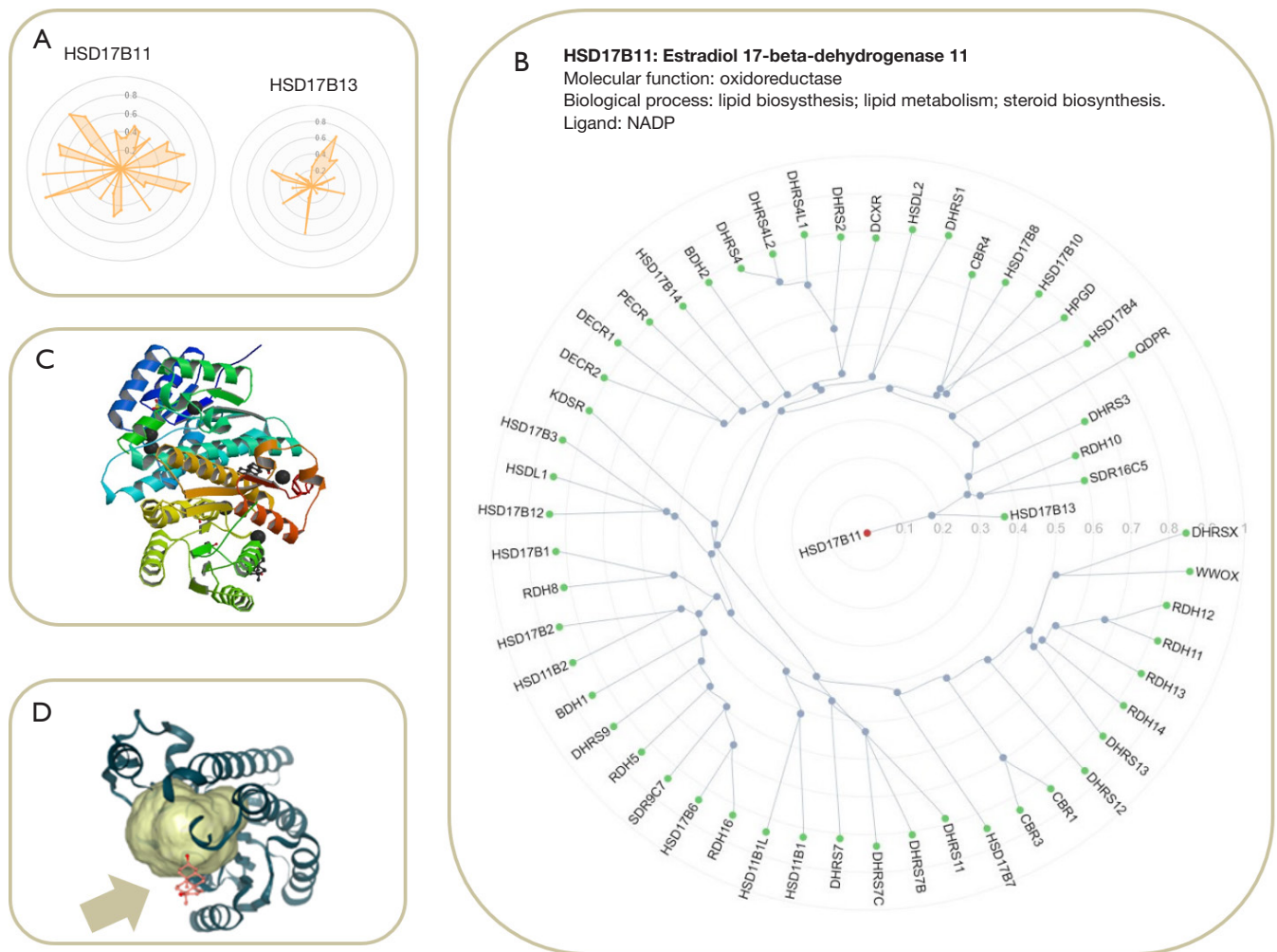


Figure 5 Homolog-based druggability prediction of HSD17B13. (A) Radar plot (illumination graph) depicting the variety of knowledge on HSD17B11 and HSD17B13 obtained by Pharos (Pharos NIH Druggable Genome Knowledgebase available at <https://pharos.nih.gov/>). The radar chart is a visualization of gene-attribute associations; this radial plot summarizes the level of accumulated knowledge about both genes/proteins above mentioned. The further the point is away from the center of the radial plot, the more knowledge exists about the target. Knowledge value: 0 to 1 scale. HSD17B11: most knowledge about: PubMed ID: 0.86, small molecule perturbation: 0.83, transcription factor binding site profil: 0.8, gene perturbation: 0.74, tissue sample: 0.71. HSD17B13: most knowledge about cell type or tissue: 0.68, microRNA: 0.58, tissue sample: 0.53, cell line: 0.45, chemical: 0.44; (B) family cladogram based on network proteins of the short-chain dehydrogenases/reductases (SDR) family. The graph shows human HSD17B11 and HSD17B13 as the closest linked members. (<https://cansarblack.icr.ac.uk/>); (C) structural ligandability of HSD17B11 by X-ray diffraction (<https://cansarblack.icr.ac.uk/>); (D) “bindability” of HSD17B11 protein: predicted binding site pocket suggests high chances of binding of small molecules with high affinity. (<https://cansarblack.icr.ac.uk/>).

that combined genomics of human gut commensals and gnotobiotic animal experiments to quantify microbiota and host contributions to drug metabolism provided remarkable evidence on the microbiome drug-metabolizing activity (76). The model consisted of eight compartments, including three sections of the small intestine, three sections of the

large intestine, gallbladder, and a central compartment, representing processes occurring in serum, liver and other tissues (76). This investigation demonstrated that while bacterial drug conversion influences both intestinal and systemic drug metabolite levels, the main factors affecting systemic drug metabolite levels were systemic

metabolite elimination and biliary secretion rates of the drug (76). Surprisingly, drug metabolites produced by the gut microbiota substantially contributed to systemic exposure (76). It remains to be explored whether tissular metataxonomic profiles represent microbiotas. Tissular microbiotas, in particular the liver, might affect drug pharmacokinetics, possessing potential implications in novel discoveries for the treatment of NASH. Deciphering the interrelationship between liver tissular microbiota and anti-NASH drugs pharmacokinetics and pharmacodynamics would be a breakthrough in the understanding of interpersonal variability in drug response.

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Footnote

Conflicts of Interest: Both authors have completed the ICMJE uniform disclosure form (available at <http://dx.doi.org/10.21037/hbsn-20-270>). SS and CJP serve as unpaid editorial board members of *Hepatobiliary Surgery and Nutrition*.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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