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MICROBIOTA AND LIVER DISEASE: YEAR IN REVIEW

R. Hassouneh[1], C. Kim[2], J. Behary[2], A. Zekry[2], J.S. Bajaj[3]

[1]Department of Internal Medicine, Virginia Commonwealth University, Richmond, VA, USA
[2]Division of Gastroenterology and Hepatology, University of New South Wales, Sydney, Australia
[3]Division of Gastroenterology, Hepatology and Nutrition, Virginia Commonwealth University and VA Hunter Holmes McGuire, Richmond, VA, USA

Corresponding Author: Ramzi Hassouneh, DO; Email: ramzi.hassouneh@vcuhealth.org

Abstract – Over the past few years, the gut microbiota has been recognized to play a role in maintaining health and disruption in its composition can lead to pathology. In the normal healthy state, the gut microbiome serves as a barrier to invasive species by regulating intestinal permeability as well as preferentially utilizing resources and outcompeting deleterious organisms. Due to their anatomical proximity and shared vascular supply, dysfunction of the gastrointestinal tract can affect the liver and vice-versa. As such, the gut microbiota has been studied in various liver disorders including non-alcoholic liver disease (NAFLD), cirrhosis, and hepatocellular carcinoma (HCC). Data shows that alteration of the gut microbiota, also known as dysbiosis, occurs in all these disorders and may be responsible for disease progression and poorer outcomes. Studies have attempted to reverse the dysbiosis state through alteration of diet, antibiotic therapy targeting harmful microbes, promoting beneficial organisms via prebiotics and probiotics, as well as faecal microbiota transplantation to completely overhaul the gut microbiome. This review will summarize the latest findings regarding the gut microbiota in obesity, NAFLD, cirrhosis, and HCC, as well as clinical trials targeting the microbiome in attempts to correct the pathologic state.

Keywords: Microbiota, Liver disease, Cirrhosis, Non-alcoholic fatty liver disease, Obesity, Hepatocellular carcinoma, Faecal microbiota transplantation, Hepatic encephalopathy, Gut-liver axis.

INTRODUCTION

In recent years, the gut microbiota has emerged as a significant mediator of health and disease in the human body. Incredibly the number of micro-organisms in the gut and their genetic material far outnumber human cells and the human genome[1]. The gut microbiome is involved in the digestion and extraction of vital nutrients and minerals as well as maintenance of the immune system[2]. The gut microbiome functions locally in the gut's immune system by utilizing available resources and outcompeting deleterious microorganisms. Furthermore, it prevents translocation of invasive microorganisms and harmful metabolites by maintaining the intestinal barrier. The liver is the first organ to encounter microbial products that cross the gut barrier and enter portal circulation. Therefore, the gut microbiota may have a significant role in development of liver disease[3,4]. This review will discuss the latest literature describing the role of the gut microbiota in liver diseases.

OBESITY

In healthy individuals, a diverse gut microbiome has been shown to prevent long term weight gain[5]. Mechanisms by which this occurs are complex, and include maintenance of intestinal

integrity, regulated extraction of calories from diet and fat storage in adipose tissue, and release of hormones responsible for satiety[6,7].

Dysbiosis that occurs in weight gain appears to be characterized by a reduction in alpha-diversity and increased Firmicutes to Bacteroidetes ratio. Thus, in a recent study examining the faecal microbiota of obese Italian adults, a reduced abundance of several Bacteroidetes taxa, and enrichment of several Firmicutes taxa along with taxa belonging to *Enterobacteriaceae* with endotoxic activity compared to the normal weight group was observed[8]. In addition, Bacteroidetes abundance seemed to be associated with a favourable weight phenotype as it negatively correlated with body fat and waist circumference, whereas Firmicutes abundance had the opposite relationship to body fat[8]. Similarly, in adults from the UK, the bacterial family *Lachnospiraceae*, genus *Bifidobacterium* and species *Faecalibacterium prausnitzii* demonstrated causal association with trunk fat mass[9].

In obesity, the presence or absence of metabolic risk factors, influence gut microbiota composition. In a recent study[10], a significant difference in microbial composition was observed between obese individuals with at least one metabolic risk factor compared to those without. In this setting, those with at least one metabolic risk factor had reduced phylogenetic and non-phylogenetic alpha diversity[10]. The family *Coriobacteriaceae* and genus *Oscillospira* were associated with a protective effect, with absence of metabolic risk factors[10]. Whereas for obese subjects with diabetes, gut microbiota exhibited an increased abundance of Actinobacteria and reduced abundance of *Akkermansia muciniphila* compared to the control group of obese subjects without diabetes[11].

With obesity, changes in gut microbial composition translate to altered metabolic profile. For instance, levels of faecal short chain fatty acids (SCFAs), known to influence the metabolism of energy, lipids, glucose, and cholesterol, are increased in obese individuals compared to lean counterparts[12]. To this effect, studies[13] have shown levels of SCFAs, especially propionate, are increased two-folds in obese subjects compared to healthy weight controls. Added to this, recent studies have demonstrated that in obese individuals with metabolic risk factors, altered microbiota composition is associated with dysregulated bile acid metabolism. Thus, overweight, and obese individuals with metabolic disease had reduced levels of non-12-OH bile acids including ursodeoxycholate, chenodeoxycholate and lithocholate[14].

Mechanisms by which the microbiota and its metabolites influence obesity are being elucidated and in the future are likely to provide new diagnostic and therapeutic strategies for weight control.

NON-ALCOHOLIC FATTY LIVER DISEASE

Non-alcoholic fatty liver disease (NAFLD) is considered to be the hepatic manifestation of 'metabolic syndrome', a cluster of conditions, including insulin resistance, dyslipidaemia and obesity[15]. NAFLD encompasses a spectrum of liver disease from simple steatosis to non-alcoholic steatohepatitis (NASH), liver fibrosis and cirrhosis. Studies provide evidence for dysbiosis along this spectrum of liver disease, and contribute to disease pathogenesis through promotion of obesity, altered metabolite production, and aberrant immune responses.

A reduction in faecal microbiota diversity is consistently seen in subjects with hepatic steatosis. In this regard, several members of the class Clostridia, especially in orders Lachnospirales and Oscillospirales that are known endogenous ethanol producers have been recently identified to predict the presence of hepatic steatosis in a large population cohort[16]. Moreover, the development of hepatic steatosis in patients post liver transplant was observed with reduced abundance of *Akkermansia muciniphilia* and an increase in *Fusobacterium* compared to those who did not have NAFLD recurrence[17]. Interestingly, patients with a high NAFLD activity score (NAS ≥5) had a higher proportion of Bacteroidetes and a lower proportion of Firmicutes compared to those with a low NAS score[17]. Bacteroidetes was positively correlated with higher hepatic steatosis content, whereas Bifidobacterium showed negative correlation[17].

Dysbiosis has also been associated with histologically confirmed NASH. To this effect, in a study of patients with biopsy proven NASH, a 12-fold increase in the relative abun-

dance of the genus *Collinsella* was observed. *Collinsella* abundance was positively correlated with serum triglyceride, and negatively with serum HDL-C levels, thus suggesting an important role of this genus in influencing host lipid metabolism important in NASH pathogenesis[18].

Added to this, Loomba et al[19], through shotgun metagenome sequencing of faecal samples and serum metabolomic profiling has proposed a model for detection of advanced fibrosis in liver biopsy proven NASH. They demonstrated that at the species level, *Eubacterium rectale* and *Bacteroides vulgatus* were most abundant in mild/moderate fibrosis, *Bacteroides vulgatus* and *Escherichia coli* were most abundant in advanced fibrosis[19]. Consistent with this, Canivet et al[20] also demonstrated increased abundance of the *Escherichia* genus in NASH with advanced fibrosis, including *Escherichia coli* and *Escherichia* unclassified species, along with *Bacteroides plebeius, Enterococcus durans, Megamonas rupellensis,* and *Sutterella wadsworthensis.*

Serum metabolomic analysis identified pathways associated with carbon metabolism and detoxification in patients with advanced fibrosis, while those with mild/moderate fibrosis have increased abundance associated with nucleotide and steroid degradation[20]. Importantly, Oh et al[21], through integration of shotgun metagenomic and untargeted metabolomic profiles using machine learning algorithms was able to accurately detect cirrhosis in patients with NAFLD. In that study, notable composition shifts in patients with NAFLD related cirrhosis (NAFLD-cirrhosis), including enrichment in *Veillonella parvula*, *Veillonella atypica*, *Ruminococcus gnavus*, *Clostridium bolteae*, *Acidaminococcus sp D21*, and decreases in *Eubacterium eligens*, *Eubaterium rectale* and *Faeclibacterium prausnitzii* were observed[21]. Correlations were seen between key microbial species and clinical metadata associated with NAFLD-cirrhosis, for instance, levels of *Veillonella parvula* were inversely correlated with albumin and platelet counts, parameters decreased in cirrhosis. Further, faecal metabolites with the greatest predictive power for detection of cirrhosis included those involved in metabolism of amino acids, bile acids and vitamin D[21].

Finally, interest has turned to intrahepatic microbial signatures of liver disease progression in NASH. In the first study of its kind, Sookoian et al[22], demonstrated that liver DNA profile significantly differs between morbidly obese and non-morbidly obese patients with NAFLD. Morbidly obese subjects with more severe histological features of NASH (including more balloon degeneration and fibrosis) were associated with higher liver tissue levels of *Peptostreptococcaceae*, Verrucomicrobia, Actinobacteria and Gamma Proteobacteria. Mechanisms by which these taxa promote liver injury are the subject of ongoing study.

Gut microbiota and mechanisms of NAFLD

There are multiple recent publications implicating gut dysbiosis, related aberrant bile acid metabolism and an increase in gut permeability in the pathogenesis of NAFLD. Transitional increase in serum and faecal bile acid levels have been observed as patients with NAFLD who progressed from minimal to severe fibrosis[23]. Furthermore, serum levels of bile acids (glycolic acid and deoxycholic acid) correlated with the abundance of *Bacteroidaceae* and *Lachnospiraceae*, which in turn was associated with the development of advanced fibrosis[23].

Highlighting the role of aberrant bile acid metabolism in NASH progression, Takahashi et al[24] showed that administration of the bile acid sequestrant sevelamer to HFD-mice reduced steatosis, inflammation, and fibrosis, with associated restoration in the bile acid and microbiota composition, leading to increased abundance of *Lactobacillus* and decreased abundance of *Desulfovibrio*[24]. Similar findings were observed by Gupta et al[25], where HFD mice displayed loss of intestinal epithelial barrier, leading to severe NASH, with associated increased bile acid concentration, but this was attenuated by *in vivo* delivery of sevelamer. Elobixibat, an ileal bile acid transporter inhibitor, reduced the methionine and choline-deficient diet-induced hepatic inflammation and fibrosis in mice, restored intestinal tight junction protein levels and reversed the reduced abundance of *Lachnospiraceae* and *Ruminococcacae* and increased abundance of *Enterobacteriaceae*[26].

Another mechanism that altered gut microbiota which is suggested to impact NASH progression is through hepatic accumulation and activation of B lymphocytes with enhanced

proinflammatory cytokine secretion and antigen-presenting ability, as seen in NASH mouse livers[27]. FMT from human NAFLD donor to mice led to progression of NASH with increased hepatic B lymphocyte accumulation[27].

Pharmacotherapy for gut microbiota in NAFLD

Human studies of pharmacotherapy to target gut dysbiosis in NASH patients are limited. Fibroblast growth factor 19 (FGF19) functions as a hormone that regulates bile acid synthesis as well as glucose and lipid metabolism[28]. FGF19 is present in reduced levels in individuals with insulin resistance and NAFLD and appears to return to normal values in obese patients who undergo gastric bypass surgery[29]. In a phase 2 trial, administration of aldafermin, an analog of the gut hormone FGF19, in NASH patients was associated with a dose-dependent enrichment of the genus *Veillonella* which was also associated with an improvement in the serum bile acid profile and reduced liver fat content[30]. Furthermore, serum triglyceride concentrations declined over time in patients treated with aldafermin but not placebo[30]. At week 24, 38% of NAFLD patients receiving aldafermin exhibited improvement in fibrosis stage, compared to 18% in the placebo group, reflecting a trend towards fibrosis improvement by aldafermin[30].

Metformin combined with healthy lifestyle recommendations in non-diabetic obese children was associated with a reduced abundance of *Bacillus* at the genus level and a trend towards a reduced abundance of Actinobacteria at the phylum level compared to placebo with the same lifestyle recommendations[31]. A nationwide population study in Korea[32] showed an association between proton pump inhibitor use and increased risk of NAFLD with hazard ratio of 1.50 after adjusting for confounders including age, sex, BMI, smoking, alcohol intake, exercise, income level and comorbidities, possibly through the gut-liver axis related to previously described changes in the gut microbiota.

In a randomized controlled trial[33] of patients with non-alcoholic fatty liver disease (NAFLD), administration of synbiotic therapy for 1 year led to increases in faecal *Bifidobacterium* and *Faecalibacterium* species and reductions in faecal *Oscillibacter* and *Alistipes* species compared to baseline[33]. Unfortunately, while increased abundance of traditionally beneficial microbiota was observed, no changes were seen regarding markers of liver fat or fibrosis.

Faecal microbiota transplantation in NAFLD

Faecal microbiota transplantation (FMT) is the next natural step in modulating the gut microbiota in diseased states. This can be achieved by delivering FMT from a healthy donor into an unhealthy individual *via* enema, endoscopically, or by capsule. FMT is already approved for treatment in refractory C. difficile infection and is now being investigating for treatment of liver disease[34].

Several human studies examined the effect of faecal microbiota transplantation (FMT) in obese and NAFLD patients. An RCT in a group of obese, type 2 diabetic patients who underwent FMT, those who received lifestyle advice combined with FMT from lean healthy donors had a higher proportion of patients who achieved more than 20% lean-microbiota engraftment rate, compared to those who received FMT alone and those who received lifestyle advice and sham FMT (100%, 88.2% and 22% respectively)[35]. Repeated FMTs enhanced this effect. Groups that received FMT had increased butyrate-producing bacteria[35]. FMT combined with lifestyle intervention was associated with increased abundances of *Bifidobacterium* and *Lactobacillus*, as well as reduced total and low-density lipoprotein cholesterol and liver stiffness compared to FMT alone[35].

Recently, the effect of endoscopic delivery of FMT from a thin and healthy donor on patients with NAFLD was tested in an RCT[36]. Fifteen patients received allogenic FMT whereas six patients received autologous FMT. FMT was delivered endoscopically directly into the distal duodenum. The rationale for this delivery method is to directly target and treat SIBO which is a common complication of chronic liver disease. Markers of NAFLD activity were assessed, including insulin resistance, hepatic proton density fat fraction, and intestinal permeability. Patients who received allogenic FMT experienced a significant reduction in small intestine permeability 6 weeks after treatment as tested using the lactulose: mannitol urine test.

Autologous FMT from faecal sample attained from individuals with abdominal obesity or dyslipidaemia after six months of green plant-based Mediterranean diet led to a reduced weight regain, waist circumference gain and insulin rebound over the following eight months[37]. This was not observed with the other two dietary interventions studied[37]. Furthermore, only the green-Mediterranean diet was associated with a significant alteration in the microbiome composition during the weight loss phase which was maintained after FMT[37].

Overall, FMT for obesity and NAFLD continues to evolve in the literature with more data needed for better identification of donor and recipient factors that can influence response to this type of gut-based intervention.

CIRRHOSIS

Prolonged liver inflammation results in cirrhosis and end stage liver disease which comes with multiple complications, including spontaneous bacterial peritonitis (SBP), ascites, hepatic encephalopathy (HE), and hepatocellular carcinoma. Compared to healthy individuals, individuals with cirrhosis have slower intestinal transit time, overgrowth of intestinal flora, and alterations in faecal microbiota composition[38]. Multiple studies have shown that individuals with cirrhosis have a significant reduction in beneficial gut organisms such as Bacteroidetes and an increase in harmful organisms such as Proteobacteria and Fusobacteria[39-43].

As previously described, these changes in microbiome profile in a diseased state is known as dysbiosis. Beneficial gut organisms have multiple beneficial roles, some of which are mediated by the production of bile acids and SCFAs[44]. Bile acids are involved in the lysis of pathogens, as well as regulation of immune inflammatory signalling by facilitating differentiation of various T cells[45]. SCFA are essential in maintaining luminal pH, enterocyte structure, as well as regulating the function of gut lymphoid tissue[46]. In cirrhosis, overgrowth of pathogenic organisms results in reduced levels of bile acids and SCFAs. Moreover, poor liver synthetic function further exacerbates reduced levels of bile acids. In turn, pathogenic organisms mainly of the *Enterobacteriaceae* family produce endotoxins and lipopolysaccharides leading to the prototypical pro-inflammatory state of cirrhosis. These changes are particularly harmful in cirrhosis due to underlying portal hypertension which leads to blood bypassing the reticuloendothelial system and subsequent delivery of harmful metabolites to the systemic circulation.

Spontaneous Bacterial Peritonitis

The mechanism of SBP in cirrhosis involves translocation of bacteria from the intestine into ascitic fluid. This in part can be explained by findings showing that mesenteric lymph nodes in individuals with cirrhosis have higher proportions of pathogenic bacteria as well as endotoxins compared to healthy individuals[47]. The most common organisms in infected ascitic fluid are *Escherichia coli* and *Klebsiella pneumoniae* which belong to the *Enterobacteriaceae* family and are typically found in increased numbers in faecal samples of cirrhotic individuals[48]. Patients with cirrhosis are particularly sensitive to further alterations of their gut microbiome and as such proton pump inhibitors which are thought to cause intestinal overgrowth of *Enterococcus* species also place patients at higher risk for SBP[49,50].

Hepatic Encephalopathy

Many of the alterations in gut microbiota population seen in cirrhosis have been linked to HE[51]. Compared to those without HE, patients with HE have greater abundance of *Veillonellaceae* which is linked to increased levels of pro-inflammatory cytokines including interleukin (IL)-6, tumour necrosis factor alpha, IL-2, and IL-3[52]. Poorer cognition in patients with HE was found to be associated with increased levels of these pro-inflammatory cytokines. Due to the effectiveness of rifaximin in preventing repeat episodes HE, it is not surprising that individuals with HE have a higher population of urease-producing Proteobacteria compared to individuals without HE[53]. Ammonia producing microbes such as *Streptococcus* species were

found to be in much larger quantities in the oral cavities of individuals with HE[54]. Functional neurologic changes associated with poorer cognition, including astrocytic injury have been detected using magnetic resonance imaging in patients with higher levels of ammonia-producing gut organisms[55].

In a healthy individual, the proximal intestine contains few bacteria in comparison to the distal intestine and colon likely due to acid and enzymes secreted from the nearby stomach which have a strong bactericidal effect[56]. Furthermore, continuous downward peristalsis and prevention of retrograde flow by the ileocecal valve play an active role in keeping the upper intestine free of colonic bacteria. However, given the enormous population of colonic bacteria, a small amount of retrograde migration of organisms into the small intestine leads to a substantial increase in bacterial population and to small intestinal bacterial overgrowth (SIBO). SIBO is highly present in individuals with cirrhosis compared to healthy individuals affecting up to 60% of these patients[57]. One proposed mechanism is the slower intestinal transit time seen in cirrhosis in addition to cirrhosis-associated immune dysfunction. SIBO has been linked to increased risk of decompensation of chronic liver disease and development of HE[58]. Individuals with cirrhosis have an over-abundance of gram-negative bacteria in the jejunum including *E. coli* and *K. pneumoniae* even more so compared to non-cirrhotic patients with SIBO[59].

Targeting the Gut Microbiota in Cirrhosis

Modification of altered gut microbiota in cirrhosis is a hallmark of treatment of HE. Non-absorbable disaccharides such as lactulose and lactitol work by decreasing serum ammonia by accelerating intestinal transit whereas antibiotics such as rifaximin decrease abundance of harmful bacteria. As such, most research regarding therapeutics targeting the gut microbiome in cirrhosis have aimed to treat HE.

Dietary changes have been shown to be helpful in increasing populations of beneficial micro-organisms and reducing organisms linked with systemic inflammation. Comparing individuals with cirrhosis in Turkey and the United States, a Turkish diet rich in vegetables and fermented mild products led to higher microbial diversity and was associated with a lower risk of 90-day hospitalizations compared to their U.S.-based cohort[60].

Probiotics and synbiotics (combination of probiotics and prebiotics) have been utilized in multiple studies to modulate altered gut microbiota in cirrhosis. In patients with cirrhosis and covert HE, treatment with probiotics decreased hospitalization rates and ameliorated progression to overt HE compared to placebo. More recently, the effect of the probiotic *Lactobacillus casei* was tested in patients with cirrhosis and compared to placebo[61]. While it did not lead to changes in clinical outcomes including significant infections compared to placebo, there was significant improvement in cytokine profile with reductions in plasma monocyte chemotactic protein-1, plasma interleukin-1B, interleukin-17a, and macrophage inflammatory protein-1B. This suggests that treatment with *L. casei* shifts the cytokine profile towards an anti-inflammatory phenotype. A meta-analysis of synbiotic therapy prior to liver transplantation or liver resection showed reduced rates of peri-operative infection and may improve liver function compared to placebo[62].

Fecal Microbiota Transplantation in Cirrhosis

Multiple clinical trials have been completed to demonstrate safety and efficacy of FMT in cirrhosis. In the first clinical trial to date involving patients with recurrent HE, stool was obtained from a healthy volunteer with high populations of *Lachnospiraceae* and *Ruminococcaceae*[63]. Patients underwent pre-treatment antibiotic therapy prior to receiving a one-time FMT enema while continuing their home rifaximin and lactulose. The FMT group experienced less serious adverse events and had no episodes of HE up to 150 days after treatment compared to the non-treatment group. Furthermore, cognitive testing *via* psychometric HE score (PHES) and EncephalApp Stroop (EAS) demonstrated improved cognition in the treatment arm after 20 days compared to their baseline cognition.

Concerns regarding safety of FMT have been raised especially with transmission of harmful microorganisms. Although a rare adverse event, cases of transmission of drug-resistant *E. coli* have been reported leading to bacteraemia and death[64]. This led to a 2019 update in the FDA's protocol regarding screening donors for these drug-resistant organisms. Long term safety of FMT enema was tested using a similar protocol to the previously discussed study except for the addition of proton pump inhibitors to their pre-treatment regimen[65]. No adverse events occurred in the treatment group over a 12-month period suggesting FMT *via* enema was safe for long-term use. Patients who had received FMT enema had fewer hospitalizations and HE episodes compared to no treatment. Cognition was also improved when measured using PHES and EAS. Notably, no statistically significant difference in stool microbiota was detected between the FMT group and placebo group.

As FMT can be administered in multiple ways, safety of capsular FMT was addressed in a phase 1, randomized, placebo-controlled trial[66]. FMT was harvested in the same fashion (from a healthy donor with high abundance of *Lachnospiraceae* and *Ruminococcaceae*) and delivered in capsular form. Patients were followed for 5 months after treatment and only one serious adverse event occurred in the treatment arm while eleven serious adverse events were reported in the control arm. While cognition improved in the treatment group as measured by EAS, it did not change when measured by PHES. Significant changes in microbiome signatures were detected in the duodenum, sigmoid, and stool of patients treated with capsular FMT. Most noteworthy, there was a significant reduction in abundance of the ammonia producing *Streptococcaceae* organism in the duodenum of patients in the treatment group. Additionally, FMT delivery *via* endoscopy was retrospectively analysed in patients with cirrhosis and refractory HE[67]. The treatment group had significant and sustained reductions in arterial ammonia levels, Child-Pugh score, and model for end-stage liver disease score after 20 weeks of treatment.

The effects of FMT enema have been evaluated in patients with alcohol-use-disorder-related cirrhosis in a phase 1, double-blind, randomized clinical trial[68]. Patients received either one placebo or one FMT enema treatment from a donor enriched in *Lachnospiraceae* and *Ruminococcaceae* and followed for 6 months. At day 15, subjective craving for alcohol were reduced when measured by questionnaire and objectively reduced as demonstrated by lower levels of urinary ethyl-glucuronide in the FMT group compared to baseline. Cognition and psychosocial quality of life were improved in the treatment group compared to placebo. Compared to baseline, patients treated with FMT had reduced IL-6 and lipopolysaccharide-binding protein. Furthermore, stool microbiota in treated patients showed increased abundance of SCFA producing taxa such as *Ruminococcaceae* which was not detected in the placebo group. There was a statistically significant decrease in incidence of any serious adverse events and alcohol-use-disorder related serious adverse events in the treatment group compared to placebo.

In addition to changes in stool microbiota signatures, antibiotic resistance is a common complication of cirrhosis leading to poor prognosis. FMT has been postulated to be a potential therapeutic that may reduce the incidence of organisms with multidrug resistance genes. This was addressed in two studies, one with capsule FMT and one with enema FMT in patients with cirrhosis on rifaximin, lactulose, and proton pump inhibitors[69]. Antibiotic resistance gene (ARG) burden was detected in stool samples using metagenomics and mapped against the Comprehensive Antibiotic Resistance Database. After 4 weeks, patients who received capsular FMT had lower levels of beta-lactamase ARG expression compared to baseline and had lower abundance of vancomycin, beta-lactamase, and rifamycin ARGs compared to placebo. Patients who received FMT *via* enema underwent pre-treatment antibiotic therapy and interestingly after 7 days these patients showed an increase in vancomycin and beta-lactamase ARGs compared to baseline. However, after 15 days these changes were reversed and decreased below baseline levels. Yet, quinolone resistance ARG expression increased at day 15 compared to baseline.

Ongoing trials are taking place to further elucidate the effectiveness of different routes of FMT administration. The PROspective, randomized placebo-controlled feasibility trial of Faecal microbiota Transplantation (PROFIT) is a single-centre trial assessing the feasibility and effectiveness of FMT delivered endoscopically into the small bowel of patients with cirrhosis[70]. While the trial is ongoing, early data has shown that patients with cirrhosis who received FMT

had significantly reduced plasma ammonia concentrations at day 30 compared to baseline[71]. Stool analysis showed that ammonia level was increased after 30 days in the placebo group and not in the treatment group. The PROFIT trial is increasing their study cohort to recruit 300 more patients over a two-year period to compare capsular FMT to their previously studied endoscopic FMT.

A criticism of most of the FMT data is small sample size. While this is being addressed by the PROFIT trial as described above, we are also aiming to address this in a 100-participant trial to further analyse modes of delivery and dosages. This study has four groups: 1) simultaneous capsular and enema FMT, 2) capsular FMT and enema placebo, 3) capsular placebo and enema FMT, and 4) capsular placebo and enema placebo. Patients are to be followed for 6 months. The primary outcome is serious adverse events while secondary outcomes are changes in microbial diversity of stool, blood, and saliva as well as changes in intestinal permeability, and cognitive ability assessed by EAS and PHES.

HEPATOCELLULAR CARCINOMA

Hepatocellular carcinoma (HCC) is a complication of chronic liver disease that is associated with poor prognosis, now representing the fourth leading cause of cancer-related mortality worldwide[72]. Development of HCC in patients with cirrhosis has been associated with alterations in gut microbiota, with reduced bacterial richness and production of aberrant metabolites observed as hallmark changes as liver disease progressed from cirrhosis to HCC[73,74].

Studies attempting to identify a core microbiome characterising HCC have so far yielded variable results, namely due to the methodology used (16S rRNA versus metagenomics), differing aetiology and extent of the underlying liver disease (advanced versus early).

In this regard, studies in patients with NAFLD or hepatitis C-related HCC, an increased abundance of *Clostridium* and *CF231* genus of *Paraprevotella* and reduced abundance of Alphaproteobacteria was noted compared to those with cirrhosis but without HCC[73,74]. Furthermore, metagenomic analyses of stool microbiota identified a consortium of species that were NAFLD-HCC specific including *Enterobacteriaceae* at the family level and *Bacteroides caecimuris* and *Veillonella parvula* at the species level[74]. These consortia of microbiota were SCFAs-producing, and this was confirmed in subsequent metabolomic analysis in stool and serum samples[74]. The data contrasted with studies mainly focusing on studying HBV related liver cancer, whereby dysbiosis characterised HCC patients with cirrhosis but not HCC patients without cirrhosis[75]. There was reduction in SCFAs producing genera such as *Bifidobacterium* and *Lactobacillus* and increased lipopolysaccharide-producing genera such as *Enterococcus*[74]. The contrasting data related to abundance of SCFAs producing microbiota in NAFLD-related HCC versus HBV- related HCC is likely attributable to the underlying liver disease and the fact that, unlike the HBV cohort, the NAFLD group has several metabolic risk factors, likely to influence bacterial metabolites, including SCFAs. Notably, a study including a small number of patients and using 16S rRNA analyses reported that changes in gut microbiota could distinguish HCC, regardless of the presence or absence of metabolic risk factors[76]. The study, however, was not well powered, and clearly, larger studies are needed with adequate control groups in addition to in-depth analyses to dissect out metabolic versus HCC-specific microbial signatures[76].

In terms of the relationship between the microbiome and the immune milieu in HCC, Behary et al[74] have demonstrated an *ex-vivo* culture model that bacterial extracts from patients with NAFLD related HCC promotes an immunosuppressive milieu with increased expansion of T regs and other anti-inflammatory cytokines. Furthermore, work from Huang et al[77] examining the association between gut microbiota and tumorigenesis transcriptome demonstrated a correlation between several microbial species and down regulation of key genes involved in regulating anti-tumour NKT cell responses[77].

The microbiome composition has also been shown to predict outcomes from HCC. Thus, a high tumour burden in HBV related HCC has been shown to associate enrichment of *Bacteroides, Lachnospiracea incertae sedis*, and *Clostridium XIVa*[77]. In contrast higher abundance of *Clostridium sensu stricto* and *Anaerotruncus* has been proposed to be associated with protection against HCC development regardless of underlying liver disease aetiology[76].

Recent studies examining liver samples from HCC and adjacent tissue have demonstrated the existence of an intratumor microbiome in the liver[77,78]. To this effect, Chakladar et al[78] recently reported a significant microbiome dysregulation landscape within HCC liver samples obtained from subjects with HBV and alcohol related HCC. The combination of alcohol and HBV was associated with distinctive microbial composition, with increased abundance of certain species correlating with worse prognosis[78]. Several of the abundant microbial species correlated with the expression of key immune associated cytokines such as CCL28, CCL26, CSF3, and SOCS3[78]. The authors concluded that the intratumor microbiome was likely to influence cytokine expression and immune system regulation in HCC[78].

Gut Based Interventional Studies in HCC

According to recent studies, gut-based modulation strategies has been shown to ameliorate the complication of chronic liver disease[79,80]. To date, interventional and longitudinal studies to examine the role of the microbiome in HCC have been limited to animal models. Progressive gut dysbiosis was observed in high-fat/high-cholesterol diet-fed mice that progressed from normal to hepatic steatosis to HCC, with progressive increase in *Mucispirillum*, *Desulfovibrio*, *Anaerotruncus* and *Desulfovibrionaceae* and decrease in *Bifidobacterium* and *Bacteroides*[81]. There were also alterations in gut microbe metabolites with increased taurocholic acid and decreased 3-indolepropionic acid[81]. Zhang et al[81] also observed that atorvastatin reversed the high dietary cholesterol-induced dysbiosis and prevented NAFLD-HCC development. Vancomycin-induced selective depletion of *Lachnospiraceae* and *Ruminococcaceae* belonging to the phylum Firmicutes, *Bifidobacteria* of the phylum Actinobacteria, which ferment fibres and *Clostridium cluster XIVa* which produce secondary bile acids prevented HCC development in inulin-fed, Toll-like receptor 5 deficient, dysbiosis-susceptible mice[82].

Of note, combined administration of SSL6 and sorafenib demonstrated anti-tumour effect in mice[83]. Li et al[84] found an increased gut microbial diversity in advanced HCC patients treated with immune-checkpoint inhibitors (ICI). They also observed different microbial diversity and composition in responders and non-responders to immunotherapy[84]. Among responders, an increased abundance of *Faecalibacterium* genus was associated with a greater progression-free survival (PFS)[84]. Among non-responders, an increased abundance of the Bacteroidales order was associated with a reduced PFS[84]. Antibiotic-induced dysbiosis increased the efficacy of the γδT cell anti-tumour response in mouse models, with increased levels of released cytotoxic cytokines[85]. Furthermore, increased levels of the microbial metabolite 3-indopropionic acid (IPA) following antibiotic therapy was associated with an increased cytotoxic ability of γδT cell both *in vitro* and *in vivo*[85]. However, Spahn et al[86] showed gut decontamination with antibiotic therapy for 30 days post initiation of ICI in HCC patients was associated with a reduced median overall survival. Therefore clearly, the field for how and when to manipulate the gut microbiome to improve therapeutic responses in HCC is still under investigation.

CONCLUSIONS

It has become clear that the gut microbiota plays a crucial role in our health and particularly liver diseases, including NAFLD, cirrhosis, and HCC. While our understanding of the underlying mechanism by which it performs its functions is still incomplete, restoring populations of beneficial organisms and correcting dysbiosis appears to improve outcomes in liver disorders. Studies have shown that this can be performed through dietary changes, antibiotics, probiotics, and synbiotics. The most exciting therapy targeting gut microbiota is FMT and early studies have demonstrated safety and efficacy. However, further evaluation of the gut microbiota is needed as well as development of standardized treatment protocols for each disease before these therapies can be implemented in clinical practice.

Conflict of Interest

The authors declare no conflict of interest.

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