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MICROBIOME IN LIVER DISEASES: UPDATE FROM A CLINICAL PERSPECTIVE

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Abstract: There is a constant progress in our understanding of gut-microbiota-liver axis. In this review, we provide the readers with a summary of the recent knowledge related to the field of microbiome and liver disease. For this purpose, we reviewed and selected some of the most distinct studies published between April 2019 and March 2020 with the focus on clinical and translational work related to human data. This overview would allow the readers to obtain the current comprehensive insights in the liver-microbiota field. The work includes the summary of associational studies to obesity, non-alcoholic fatty liver disease, liver cirrhosis, autoimmune hepatitis, primary sclerosing cholangitis and finally hepatocellular carcinoma. The work summarizes also recent interventional studies related to liver disease in particular for the treatment of non-alcoholic fatty liver disease and steatohepatitis.

Keywords: Liver, Microbiota, Microbiome, FMT, NAFLD, Liver cirrhosis, Clinical trials.

PRIMARY SCLEROSING CHOLANGITIS

Primary sclerosing cholangitis (PSC) remains one of the least understood microbiome-associated liver diseases. Liwinski et al[1] studied microbiome from 43 patients with PSC with 22 controls without sclerosing cholangitis including. They obtained total 260 specimens, including saliva, duodenal fluid, duodenal mucosa and bile for 16s-rRNA sequencing and performed liquid chromatography mass spectrometry (LCMS) for composition analysis of the bile acids. First, bile microbiome profile was distinct from other regions. Overall, upper GI-tract microbiome differed between patients and controls, while the largest differences were observed in bile. Among the most pronounced changes between PSC patients and controls was the reduced biodiversity based on Shannon entropy, and pronounced presence of *Enterococcus faecalis* in the bile fluid. Enterococcus abundance correlated with the noxious secondary bile acid taurolithocholic acid, which may be potentially carcinogeneic and may be linked to proinflammatory state. Table 1 provides the key information to the studies included in this update review to provide the short summary and overview.

Vieira-Silva et al[2] re-analyzed the microbiome data from 106 patients with PSC and or inflammatory bowel disease (IBD) and evaluated quantitative microbiome alterations taking to account the faecal water content. For this purpose, they adjusted the microbiome alterations on the moisture variation in feces, which may largely have affected results. The authors observed an increased prevalence of *Bacteroides* enterotype across diseases studied with the microbial load inversely correlating with intestinal and inflammation severity. The authors linked cholangitis and/or biliary obstruction (PSC with and without ulcerative colitis) with *Enterococcus* taxa, while *Fusobacterium* was strongly associated with intestinal inflammation and restricted to PSC-Crohn's disease.

TABLE 1. OVERVIEW OF THE INCLUDED STUDIES.

Reference	Author	Disease	Number of patients	Microbiome summary	Intervention
1	Liwinski et al	PSC	43	*Enterococcus faecalis*↑	
2	Vieira-Silva et al	PSC/IBD	106	*Bacteroides*↑; *Enterococcus*↑; *Fusobacterium*↑	
3	Allegretti et al	PSC	10	Improvement in diversity	FMT
4	Wei et al	AIH	91	*Veillonella*↑	
5	Iino et al	NAFLD	205	*Ruminococcaceae*↓; *Faecalibacterium*↓	
6	Kim et al	NAFLD	766	*Fusobacteria*↑; *Ruminococcus*↓	
7	Lang et al	NAFLD	61	*Gemmiger*↑; *Bacteroides*↓	
8	Zhao et al	NAFLD		*Enterococcus faecalis*↑; *Pseudomonas aeruginosa*↑	
9	Schwimmer et al	NAFLD	87	*Prevotella copri*↑	
10	Zhao et al	NAFLD	25	*Faecalibacterium prausnitzii*↑	
11	Mameli et al	Obesity	34	Minor microbial differences	
12	Johnson et al	Adults	34	Microbiome stability	Diet
13	Wan et al	Adults	217	*Alistipes*↑; *Bacteroides*↑ high-fat diet	Low/moderate/
14	Pelligrini et al	Cancer survivors	34		Mediterranean diet with and without probiotics
15	Depommier et al	Obesety	32		*Akkermansia muciniphila*
16	Duseja et al	NAFLD	39		Life-style and probiotics
17	Chen et al	NAFLD	100		Yoghurt
18	Huber et al	Intervention	41	*Bacteroidetes*↑; *Euryarchaeota*↑	Exercise
19	Scorletti et al	NAFLD	104	*Bifidobacterium*↑; *Faecalibacterium*↑; *Scillibacter*↓; *Alistipes*↓	Symbiotic (fructoo-ligosaccharides and Bifidobacterium)
20	Bomhof et al	NASH	14		Oligofructose
21	Ghetti et al	Obesity	40		Diet
22	Chen et al	HBV	97	*Fusobacteria*↑; *Veillonella*↑; *Haemophilus*↑	
23	Zhang et al	HBV-ACLF	50	Microbioal circulation	
24	Bajaj et al	LC	294	*Prevotellaceae*↓	
25	Bajaj et al	LC	15		FMT
26	Bajaj et al	LC	20		FMT
27	Ren et al	HCC	419	*Actinobacteria*↑	
28	Pinero et al	HCC	25	*Erysipelothrichaceae*↑; *Leuconostocaceae*↓; *Fusobacterium*↓	
29	Sydor et al	NASH-HCC	33	*Proteobacteria*↑; *Fusobacteria*↑	
30	Ni et al	HCC	68	Pro-inflammatory bacteria	
31	Iida et al	HCC	107	Blautia	
32	Cho et al	HCC	79	Circulating microbial profile	
33	Jia et al	CCC	84	*Lactobacillus*↑; *Actinomyces*↑; *Peptosptreptococcaceae*↑; *Alloscardovia*↑	

Abbreviations: PSC: primary sclerosing cholangitis; AIH: autoimmune hepatitis; NAFLD: non-alcoholic fatty liver disease; HBV: hepatitis B virus; ACLF: acute on chronic liver failure; LC: liver cirrhosis; HCC: hepatocellular carcinoma; NASH: non-alcoholic steatohepatitis; CCC: cholangiocellular carcinoma; FMT: fecal microbiota transplantation.

With regard to the potential therapeutic possibilities, Allegretti et al[3] performed a pilot open-label clinical fecal microbial transplantation (FMT) study. In total 10 patients with PSC with concurrent IBD (9 patients with ucerative colitis and 1 with Crohn's colitis) and alkaline phosphatase >1.5-fold the upper limit of normal underwent single FMT *via* colonoscopy from a helathy donors. Overall, by the week 24 post-FMT the authors observerd no safety issues or no adverse events. The microbial diversity increased in all patients at 1-weeek post-FMT. In 30% of patients, the authors observed at least 50% decrease in alkaline phosphatase level which interestingly correlated with abundance of engrafter operational taxonomic units.

AUTOIMMUNE HEPATITIS

High-quality characterization of gut microbiome profile was performed by Wei et al[4]. The authors performed 16S-rRNA gene sequencing of 91 patients with autoimmune hepatitis (AIH) and compared to matched healthy controls (n=98) and the results were validated in an additional cohort of 62 independent samples. According to the data, AIH was associated with lower alpha-diversity and distinct overall microbial composition compared to healthy subjects. In particular, depletion of obligate anaerobes and expansion of potential pathobionts, such as *Veillonella*. *Veillonella* genus correlated with aspartate aminotransferase (ASAT) as a surrogate for liver inflammation. Liver histology, such as inflammation grade or fibrosis stage did not correlated with bacterial diversity, however, *Veillonella* significantly differed bewteeen pateitns with mild/moderate and severa inflammation grade. The combination of four AIH-associated genera could differentiate AIH-related gut microbiome from healthy subjects, but the translation value of this observation remains unanswered, as no patients with other diseases were included.

OBESITY AND NON-ALCOHOLIC FATTY LIVER DISEASE

Due to epidemiologicaly confirmed increase of obesity and metabolic liver disease, including non-alcoholic fatty liver diseases (NAFLD), there is an increasing efferot to characterize microbial alterations in this group of patients and identify sustained therapeutic options. In the past year, a number of associational and interventional studies that provide valuable knowledge on NAFLD has been published.

NAFLD in Adults

One of the associational studies was published by Iino et al[5] where the authors screened 1148 subjects using ultrasonography to identify 205 participants with NAFLD and matched the results with non-NAFLD controls. Using a relative abundance level of more than 1% the authors observed significant decrease in *Ruminococcaceae* family and genus *Faecalibacterium* in matching 153 pairs of NALFD and non-NAFLD groups, which confirm the possible involvement of *Faecalibacterium* in pathogenesis of NAFLD. The influence of gut microbiome on the persistence of NAFLD has been studied by Kim et al[6]. The 16s-rRNA analysis of the 766 subjects revealed that patients with persistent NALFD had lower richness compared to control group. This group was also associated with relative high abundance of *Fusobacteria* and low abundance of genera *Oscillospira* and *Ruminococcus* of the family *Ruminococcaceae* and genus *Coprococcus* of the family *Lachnospiraceae*. Future studies will be certainly needed to address the role of microbiome in prediction of NAFLD disease development.

The potential effect of diet on gut microbiome and NAFLD-activity has been studied by Lang et al[7]. Using detailed food records in a cohort of 61 biopsy-proven NAFLD, the authors identified significantly higher daily intake of proteins in subjects with higher NAFLD-activity score. Protein intake was associated with microbial alterations specifically with higher *Gemmiger* and lower *Bacteroides*. In addition, microbial metabolites gain substantial attention. Zhao et al[8] identified N,N,N-trimethyl-5-aminovaleric acid (TMAVA), a metabolite that is linked to intestinal microbes to be increased in

plasma from subjects with liver steatosis. The authors identified *Enterococcus faecalis* and *Pseudomonas aeruginosa* responsible for the metabolization of trimethyllysine to TMAVA, which was further modulated by antibiotic treatment in mice model.

Obesity and NAFLD in Children

Obesity and NAFLD are increasingly present in children and adolescents. Schwimmer et al[9] performed a prospective observational study of 87 children with biopsy-proven NAFLD and 37 obese children without NAFLD. Lowest α-diversity was observed in children with NAFLD and specifically NASH in comparison to controls. NALFD and its severity were associated with greater abundance of genes encoding inflammatory bacterial products and specifically high abundance of *Prevotella copri* correlated with severe fibrosis. Zhao et al[10] studied gut microbiome in children in China. The authors also observed significant differences in bacterial composition. In comparison to controls, obese NAFLD children showed higher abundance of Phylum *Proteobacteria.* The only species that was different between obese children with and without NAFLD was *Faecalibacterium prausnitzii*. In comparison to the previous discussed study, the authors observed no difference in term of α-diversity among the groups.

Mameli et al[11] questioned if the taste perception and salivary microbiota composition may be associated with obesity. The results of their study revealed that obese children presented with lower ability to correctly identify taste qualities and had lower number of fungiform papillae compared to non-obese subjects. Analysis of the oral microbiome revealed only slight increase α-diversity, while only minor differences in microbial abundance was observed.

Interventional Studies Related to Obesity and NAFLD

Most excitingly from the translational point of view is though the increasing number of studies that evaluated the effect of various interventions on microbiome in obese and/or NAFLD subject published over the last year. Figure 1 provides an overview on the selected interventional studies.

First of all, the diet is one of the most important determinants of the gut microbiome variation and recently very important work has been published that links the dietary diversity and microbial stability, which is crucial for the future understanding of interventional studies. Johnson et al[12] performed a fecal shotgut metagenome analyses of daily fecal specimens of 34 healthy subjects and correlated with 24 h food records. The authors confirm that microbiome composition is crucially dependent on dietary history and was strongly related to food choice and nutrient profile, but the responses to diet were highly personalized. Most interestingly, dietary diversity but not monotonous diet was associated with microbiome stability.

Wan et al[13] performed a 6-month randomized controlled-feeding trial in 217 healthy young adults. In comparison to higher-fat or moderate-fat diet, lower-fat diet (20% energy) led to increased α-diversity (Shannon index) and increased abundance of *Blautia* and *Faecalibacterium*. Higher-fat diet was associated with increased *Alistipes*, *Bacteroides* paralleled by a decrease in short-chain fatty acids, and accumulation of proinflammatory markers. In a 16-week randomized open-label pilot study[14], breast cancer survivors were randomly assigned to a Mediterranean diet with and without probiotics (*Bifidobacterium longum* BB536, *Lactobacillus rhamnosus* HN001). The combination with probiotics positively influenced gut microbiota and was associated with improvement of metabolic and anthropometric parameters compared to diet alone. Depommier et al[15] performed a randomized double-blind placebo controlled pilot study to evaluate the effect of *Akkermansia muciniphila* (*A.muciniphila*) in overweight/obese insulin-resistant volunteers. *A.muciniphila* live and pasteurized form was safe and well tolerated. Compared to placebo, pasteurized bacteria improved insulin sensitivity, reduced insulinemia and total cholesterol values following a 12-week diet.

Various interventions were studied in subjects with NAFLD. Life-style and multistrain probiotics[16] over one year treatment showed significant improvement in liver histology and ALT compared to placebo group. Twenty four week treatment with yogurt in comparison to milk was associated with amelioration of insulin resistance in obese Chinese women with NAFLD[17]. In an observational study by

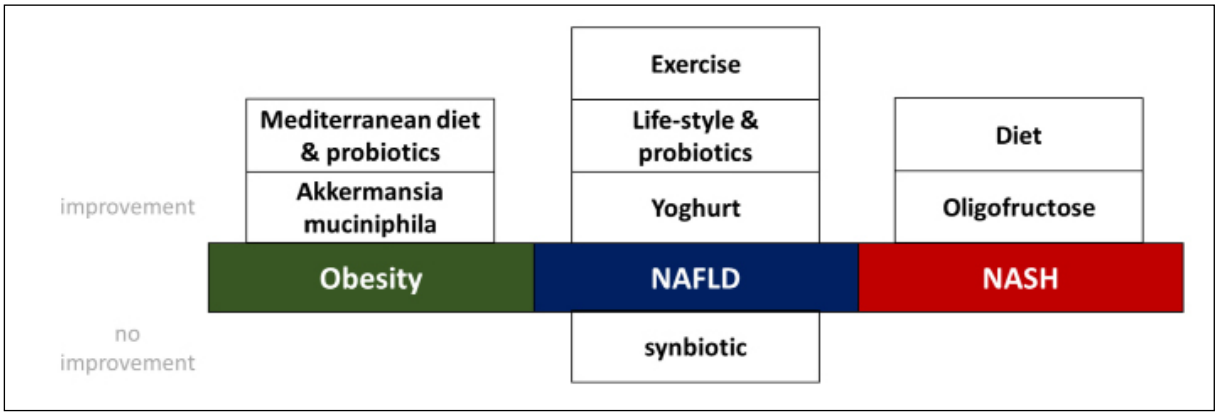

Figure 1. Overview of the interventional studies in obesity, NAFLD and NASH subjects.

Huber et al[18], physical exercise over 8 weeks was associated with improvement of liver biochemistry and microbial improvement, such as metagenomics richness, increase of *Bacteroidetes* and *Euryarchaeota* and decrease of *Actinobacteria phylum*. Scorletti et al[19] performed a double-blind phase 2 trial of 104 patients with NAFLD in UK. In comparison to placebo, symbiotic agents (fructo-oligosaccharides and *Bifidobacterium animalis* subspecies lactis BB-12) led to fecal microbiome alteration but did not reduce liver fat content or markers of liver fibrosis following 1-year treatment. Patients in symbiotic cohort showed higher proportion of *Bifidobacterium* and *Faecalibacterium species* and reduction of *Scillibacter* and *Alistipes*.

In a histology-confirmed placebo-controlled trial in NASH patients, Bomhof et al[20] demonstrated that prebiotic oligofructose improved liver steatosis and NAS scores. Oligofructose treatment was associated with enrichment of *Bifidobacterium* and reduction of *Clostridium cluster XI* and I. Ghetti et al[21] demonstrated in a randomized trial that nutritional treatment was associated with body-weight reduction, improvement of liver enzymes. Fluorescence *in situ* hybridization (FISH) method revealed certain quantitative alterations in microbial composition; but no microbiome sequencing was performed.

CHRONIC VIRAL HEPATITIS

Chen et al[22] aimed to evaluate an association between microbial composition and chronic hepatitis B (HBV) progression. Microbiome analysis of 97 samples revealed that HBV infection was associated with higher abundance of *Fusobacteria*, *Veillonella*, *Haemophilus*. Indicator analysis identified 57 OTUs that were associated with changes from non-liver cirrhosis (non-LC) to LC disease progression. Specifically, *Dialister succinatiphilus* and *Alistipes onderdonkii* decreased with diseases progression. Zhang et al[23], have further reported on increased circulating microbial burden in HBV-related acute on chronic liver failure (ACLF), but the study importantly was missing non-HBV-ACLF.

LIVER CIRRHOSIS AND COMPLICATIONS

Little is known regarding the diet and microbial composition in LC patients and possible outcome. Bajaj et al[24] evaluated two cohorts of from USA and Mexico and analyzed salivary and fecal microbiome. Patients with decompensated LC had lower diversity in feces and saliva, and lower *Prevotellaceae* particularly in those with lower animal fat and protein consumption. *Lachnospiraceae*, *Ruminococcaceae* and *Prevotellaceae* were associated with lower hospitalization risk independent of MELD score and ascites.

Having shown the feasibility of FMT for hepatic encephalopathy, Bajaj et al[25,26] evaluated the safety of FMT in patients with hepatic encephalopathy using capsules. Twenty subjects on lactulose and rifaximin were randomized either to 15 placebo or to FMT capsules from a single donor

with defined microbial composition enriched in *Lachnospiraceae* and *Ruminococcaceae*. In this population, the FMT *via* capsules was safe and well tolerated and was associated with improved duodenal mucosal diversity increased with higher *Ruminococcaceae* and *Bifidobacteriaceae* and lower *Streptococcaceae* and *Veillonellaceae*. Gut-microbial function was beneficially affected by capsular FMT with improved inflammation and cognition.

HEPATOCELLULAR CARCINOMA

Another hot area of the liver microbiome research is related to understanding of microbiome alterations in patients with hepatocellular carcinoma (HCC). Ren et al[27] characterized the microbiome alteration in HCC and its potential as non-invasive biomarker for HCC. Analysis of the cohort of 419 samples included subjects with early and advanced HCC, liver cirrhosis (LC) and healthy controls. Gut microbial diversity increased from LC to LC with HCC, but showed no differences to controls. In similar fashion Simpson- and Invsimpson-Index showed similar pattern. Overall, Phylum *Actinobacteria* was increased in HCC in comparison to LC subjects, but not to controls. Thirteen genera including *Gemmiger*, *Parabacteroides, Clostridium XIVb, and Paraprevotella* were increased in HCC cohort, while 12 genera such as *Alistipes, Phascolarctobacterium,* and *Ruminococcus* were decreased in HCC in comparison to controls. Using the 30 microbial markers the authors could achieve an area under the curve (AUC) of 80.64%, which could be validated in 3 additional validation cohorts form China, including advanced HCC.

Pinero et al[28] studied gut microbiota in HCC subjects in the cohort of south American population. Following comparison of gut microbiota from 25 matched LC patients with and without HCC, the authors observed 3-fold increase of *Erysipelotrichaceae*, 5-fold decrease in family *Leuconostocaceae* and decrease in genus *Fusobacterium* for subjects with HCC. The authors predicted that the identified microbial pattern might be linked with inflammatory milieu.

Non-alcoholic steatohepatitis (NASH) is increasingly recognized the most leading risk factor for HCC development. Sydor et al[29] studied the link between liver and gut in NASH-related hepatocarcinogenesis and evaluated gut microbiome and bile-acid signaling in well characterized patients with and without LC or HCC and controls. At family level abundance of *Actinobacteria* was lower in LC independently to HCC, while increased *Proteobacteria* and *Fusobacteria* abundance was observed in NASH-HCC with LC. Interestingly, low abundance of *Bacteroidetes* was associated with increased detected of less abundant bacterial strains. The authors observed a progressive increase in *Lactobacillus* abundance from controls to NASH-HCC with LC, which was further associated with serum bile acid metabolites and liver injury.

Ni et al[30] aimed to study the relationship between the degree of microbial alterations and prognosis of HCC at different stages in 68 patients with HCC and 18 controls. They confirmed that gut microbiota of HCC patients is associated with pro-inflammatory bacteria, while no difference was observed between different stages of HCC. Proteobacteria were significantly increased in HCC subjects compared to healthy controls. Although the authors provide the information regarding the different stages of HCC, prognostic relevance has not been shown.

Iida et al[31] analyzed the gut microbiome of HCC subjects (n=107) to investigate an association between use of antibiotics and prognosis following hepatic arterial infusion chemotherapy. Subanalysis of the data from the randomized controlled trial revealed that among 26 types of antibiotics, administration of carbapenem before or during chemotherapy was associated with worse progression-free survival (PFS) and overall survival (OS). In multivariate analysis, overall antianaerobic drugs were an independent predictor of worse prognosis. *Blautia* in fecal microbiota was associated with better PFS and OS in a prospective subcohort of HCC patients.

Another approach was used by Cho et al[32]. The authors studied circulating microbial metagenomics for detection of HCC (n=79), LC (n=83) and in matching healthy controls (n=201). Blood microbial diversity was lower in HCC compared to LC and controls, but no specific bacterial taxa were identified for HCC or LC. To evaluate the diagnostic performance of circulating microbial profile the authors tested several models. Among the proposed models, a 5-genera microbiome signature (*Pseudomonas, Streptococcus, Staphylococcus, Bifidobacterium* and *Trabulsiella*) adjusted on age and sex showed an AUC of 0.908 in testing and 0.875 in validation cohorts.

CHOLANGIOCELLULAR CARCINOMA

Cholangiocellular carcinoma (CCC) associated microbiome is still not well characterized. Jia et al[33] evaluated gut microbiota, bile acid metabolism and cytokines in a cohort of 84 subjects with intrahepatic CCC (iCCC), HCC, liver cirrhosis (LC) patients and controls. Gut microbiome of iCCC patients had the highest α- and β-diversity and highest abundance of 4 genera *Lactobacillus, Actinomyces, Peptosptreptococcaceae* and *Alloscardovia* compared to HCC, LC and controls. *Lactobacillus* and *Alloscardovia* correlated positively with tauroursodeoxycholic acid (TUDCA) plasma-stool ratio and could be used to discriminate iCCC form other conditions. Abundance of *Ruminococcaceae* was associated with iCCC with vascular invasion, which may be predictive for disease prognosis. The role of TUDCA was validated in addition in a xenograft mice model.

CONCLUDING REMARKS

This brief review of the publications on liver and microbiome of the last year highlights scientific gain in this rapidly evolving research field. Increasing understanding of the gut-microbiome-liver axis will have very likely a major impact on the prevention and management of various liver diseases. Similar as in a previous year, substantial knowledge has been obtained for the battle with obesity and NAFLD, but also in liver diseases, such as PSC and AIH. In the long-term view, a major effort is needed to unravel the microbiome-carcinogenesis interplay. However, the highlight of the year 2020 is the SARS-CoV-2 pandemic. Over the last view months, enormous effort has been made to understand the role of SARS-CoV-2 and its impact on gastrointestinal and liver diseases. Respective microbiome studies in COVID-19 and related GI involvement are not yet available though.

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Conflict of Interest

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