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The potential of gut microbiome as a non-invasive predictive biomarker for early detection of pancreatic cancer and hepatocellular carcinoma

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Abstract. – OBJECTIVE: The aim of this paper was to discuss the potency of gut microbiome as a non-invasive predictive biomarker for early detection of pancreatic cancer and hepatocellular carcinoma.

MATERIALS AND METHODS: We analysed the available up-to-date literature (PubMed, Embase, Google Scholar databases) regarding the link between gut microbiome and early detection of pancreatic cancer, as well as hepatocellular carcinoma. The following search linked to gut microbiome and aforementioned cancers was used: 'gut microbiome', 'gut microbiota', 'pancreatic cancer', 'pancreatic ductal adenocarcinoma', hepatocellular carcinoma', 'microbial biomarkers', 'fungal microbiota', 'mycobiota'. The search was conducted in English.

RESULTS: The association between gut microbiota imbalance and development of pancreatic cancer and hepatocellular carcinoma has been recognized during last several years. The most common type of pancreatic cancer is pancreatic ductal adenocarcinoma, whose carcinogenesis is strongly related to oral microbial dysbiosis, H. pylori infection, bactibilia, hepatotropic viruses, and intrapancreatic microbiota. It is known that gut-liver axis exists and may affect hepatocarcinogenesis. Currently, the treatment strategies of these cancers are strongly limited and there are not well-recognized screening tools to early diagnose them. The growing attention towards the use of gut microbiome as a predictive non-invasive biomarker to detect pancreatic cancer and hepatocellular carcinoma in early stage has been observed.

CONCLUSIONS: To conclude, the field regarding the link between gut microbiome as a non-invasive biomarkers and early detection of pancreatic cancer and hepatocellular carcinoma exists, however, it is not well-investigated. Additionally, many of the studies were conducted with small sample sizes, whereas biomarkers are ethnicity-dependent and should be validated in wide range of populations. Nevertheless, these aspects are promising and open up new diagnostic options.

Key Words:
Gut microbiota, Microbial biomarkers, Pancreatic ductal adenocarcinoma, Pancreatic cancer, Hepatocellular carcinoma.

Introduction

Gut microbiota plays a pivotal role in human body providing multiple benefits. On the contrary, it may be involved in carcinogenesis[1,2]. The link between gut microbiota imbalance and cancer development, including pancreatic cancer and hepatocellular carcinoma (HCC), has been increasingly studied during last several years. Notably, specific bacteria may participate in the pathogenesis of cancer. Both, pancreatic ductal adenocarcinoma (PDAC) and HCC are one of the most lethal cancer globally. It is estimated that 94% of patients with PDAC die within 5 years of diagnosis and only 26% of patients survive 1-year after diagnosis[3-5]. Additionally, over 75% of pancreatic cancers cases are diagnosed at advanced stages (III/IV)[6]. HCC constitutes around 80% of primary liver cancer cases, which were the third most common cause of deaths related to cancer globally in 2020[7,8]. Currently, the treatment of PDAC is

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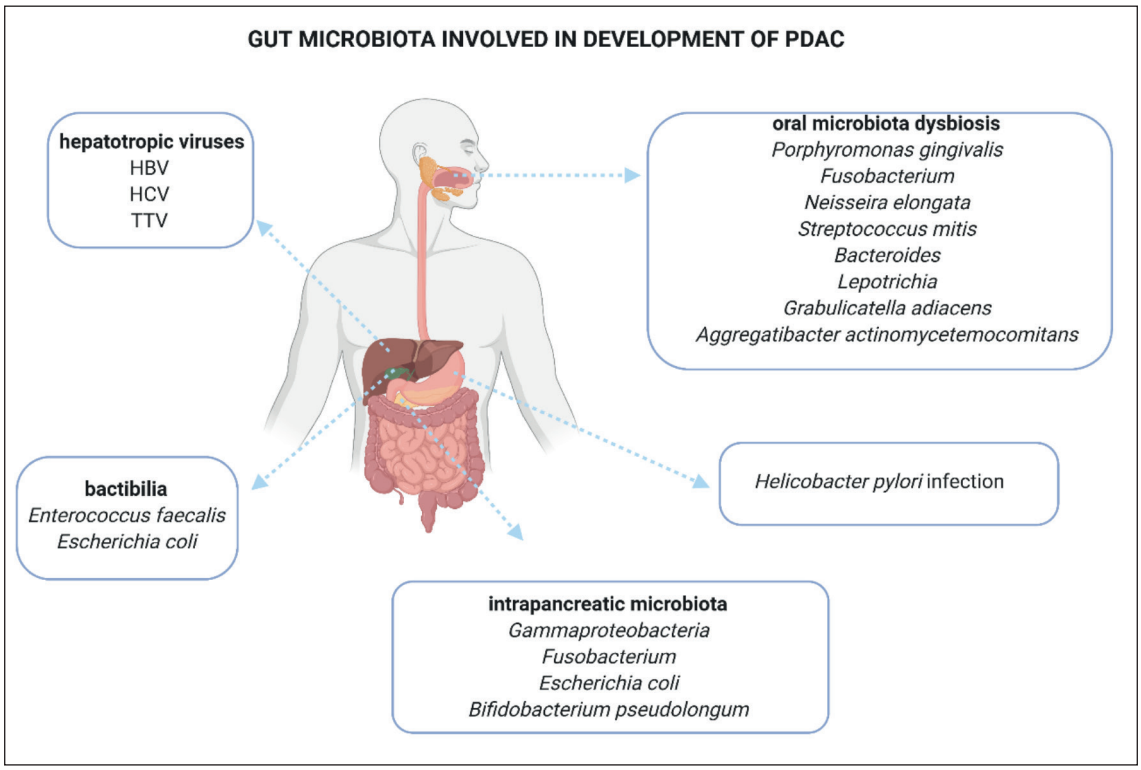

Figure 1. The association between gut microbiota dysbiosis and PDAC development. Own elaboration based on literature[9]. TTV – transfusion-transmitted virus; HCV – hepatitis C virus; HBV – hepatitis B virus; PDAC – pancreatic ductal adenocarcinoma.

strongly limited and depends on the possibility of performing a radical surgery. Therefore, the early detection of PDAC may improve survival rate and patients' quality of life[5]. There is no effective method to screen for PDAC or to detect PDAC; however, gut microbial profile has a potential as a predictive biomarker[6,9]. Recently, its potency has been also recognized in case of HCC.

In this review, we briefly present the impact of gut microbiota dysbiosis on development of PDAC and HCC. Then, we discuss the potency of gut microbiome as a non-invasive predictive biomarker for early detection of these carcinomas based on up-to-date studies.

Pancreatic Cancer

According to GLOBOCAN 2018, there were 458,918 new cases of pancreatic cancer and 432,232 deaths caused by it in 2018[10]. The many types of pancreatic cancer can be histologically divided into two general groups[11]. Notably, pancreatic cancers can be distinguished into: PDAC (85% of cases) and pancreatic neuroendocrine tumour (PanNet, less than 5%). PDAC arises in exocrine glands of pancreas, whereas PanNet occurs in the endocrine tissue of pancreas[12]. The etiopathogenesis of pancreatic cancer is multifactorial, including chronic bacterial infections, environmental, and genetic factors. Among others, chronic pancreatitis, diabetes, tobacco smoking, alcohol abuse, obesity, thus also lifestyle including diet with high amount of saturated fatty acids, are the main risk factors for development of pancreatic cancer[3,5].

Pancreatic Carcinogenesis and Gut Microbiota

The role of gut microbiota in pancreatic carcinogenesis has been recognized during last several years[6]. The association between gut microbial dysbiosis and PDAC development is presented in Figure 1.

Oral microbial dysbiosis

The composition of oral microbiota differs significantly between healthy individuals and patients with pancreatic cancer[13]. Among oral pathogens mentioned aboved, *P. gingivalis*, *Fusobacterium*, *N. elongata*, and *S. mitis* are known as keystone bacteria contributing to PDAC development. *P. gingivalis* (Gram-negative oral anaerobe)

is a periopathogen involved in chronic periodontitis, which promotes dysbiosis *via* interference with innate immunity[14,15]. In European prospective cohort study, it was noted that increased serum antibodies to the ATTC 53978 strain of *P. gingivalis* could triple the risk of pancreatic cancer[16]. *P. gingivalis* initiates Toll-like receptors (TLRs) signalling pathways; thus, it contributes to pancreatic carcinogenesis. Moreover, p53 gene can be activated by *P. gingivalis* invasion. Notably, high rates of tumor suppression of gene p53 mutations were observed in pancreatic cancer patients[17]. *P. gingivalis* inhibits the apoptosis of epithelial cells[17]. In Fan et al[18] study including 361 patients with PDAC and 371 healthy controls, it was shown that the aboudance of *P. gingivalis* and *A. actinomycetemocomitans* correlated with a higher risk of PDAC development.

H. pylori infection

H. pylori, which is Gram-negative bacterium, is not only known as one of the major factors contributing to development of gastric cancer, but also it is involved in the carcinogenesis of pancreatic cancer[13,19]. Takayama et al[20] have investigated the effects of *H. pylori* infection on human pancreatic cancer cells. It was observed that IL-8 and vascular endothelial growth factor (VEGF) levels were increased by *H. pylori* infection. Moreover, this infection activates proliferation factors like nuclear factor-kappaB (NF-κB), activator protein 1 (AP-1), and serum response element (SRE) of human pancreatic cells. Additionally, cytotoxin-associated gene A protein (CagA) secretion in pancreatic cancer cells was also observed. Overall, these results confirmed that *H. pylori* infection may be strongly associated with the development of pancreatic cancer[20].

KRAS gene mutations are observed in over 90% cases of PDAC[21]. Inflammation within the pancreas may be triggered by lipopolysaccharide (LPS) and mediated by Toll-like receptors (TLRs). Notably, LPS from *H. pylori* stimulates mutations of this gene and initiates the pancreatic carcinogenesis[21]. In Ochi et al[23] rodent study it has been proven that LPS triggers the progression of pancreatic cancer.

Intrapancreatic microbiota

Pancreas is known as a sterile organ; however, gut microbiota can migrate into it and promote the tumorigenic inflammation[13]. Pancreatic microenvironment may have an impact on the efficiency of anti-cancer therapy. Interestingly, Geller et al[24] have confirmed that intra-tumoral microbiota exists and may affect the efficiency of chemotherapy. It was detected that 76% of human specimens of PDAC were positive for bacteria, mainly *Gammaproteobacteria*. Additionally, it was observed that these bacteria modulate the sensitivity to gemcitabine contributing to drug resistance and consequently negatively affect the efficiency of chemotherapy[24]. Additionally, Pushalkar et al[25] have presented that pancreatic cancer microbiome promotes oncogenesis by inducing intra-tumoral immune suppression. It was shown that *Bifidobacterium pseudolongum* was significantly abundant in gut and tumor. Moreover, this bacterium accelerates the oncogenesis in TLR dependent manner[25].

Bactibilia

Bactibilia is defined as microbial colonization in the bile fluid[9]. In Serra et al[26], it was noted that the carcinoma of the pancreatic head was associated with bactibilia and *Pseudomonas* spp. ($p<0.0001$) as well as *Escherichia coli* ($p<0.0001$) were the most common bacteria. Another study revealed that *Enterobacter* and *Enteroccocus* spp. were the major microbes presented in bile samples[27]. Interestingly, Sydor et al[28] investigated the potential link between liver and gut in non-alcoholic steatohepatitis (NASH) related hepatocarcinogenesis. Participants were divided into 5 groups, i.e., NASH-non-HCC (n=23), NASH-non-HCC-cirrhosis (n=11), NASH-HCC (n=14), NASH-HCC cirrhosis (n=19), and healthy controls (n=20). Controls were younger than patients with a mean BMI of 23.3 kg/m², whereas the majority of NASH-non-HCC and NASH-HCC patients were overweight or obese. It was shown that serum level of total and individual bile acid (BA) was higher in NASH group in comparison to healthy subjects. Moreover, serum fibroblast growth factor 19 (FGF19) levels were significantly higher in NASH-HCC cirrhosis group compared to NASH-HCC and healthy controls. Notably, it was associated with tumor markers and attenuation of the synthesis of BA. *Bacteroides* and *Lactobacilli* were involved in BA metabolism. Additionally, the abundance of *Lactobacilli* was related to liver injury in both, NASH as well as NASH-HCC. Overall, the authors demonstrated that microbiota-related changes in BA may promote the liver injury and hepatocarcinogenesis[28].

Hepatotropic Viruses

Hepatotropic viruses (HBV, HCV) widely spread viral infections affecting respectively

around 400 and 180 millions of people globally[29]. HBV and HCV are known to cause hepatitis as well as HCC; notwithstanding, they can also take part in carcinogenesis of extra-hepatic carcinomas including PDAC[9,30]. These viruses contribute to pancreatic carcinogenesis *via* several mechanisms including induction of inflammation, modification of tissue viscoelasticity, and modulation of PI3K/AKT signalling pathway by HBVX protein (HBX)[9]. The clear association between anti-HCV positivity and significantly increased risk of PDAC development (OR=1.21, 95% Cl: 1.02-1.44) was noted in Fiorino et al[29].

Transfusion transmitted virus (TTV) was isolated in 1997 from patients with acute post-transfusion hepatitis[31]. It is suspected as a causative agent of non-A to non-E hepatitis[32]. Additionally, it is also considered as a risk factor for PDAC development[9]. Tomasiewicz et al[31] presented two cases of patients with TTV infection who developed pancreatic cancer; nevertheless, this result only indicates the possible association between TTV infection and development of pancreatic cancer.

Gut Microbiome as a Non-Invasive Diagnostic Biomarker for Pancreatic Cancer

As mentioned above, oral microbiota dysbiosis is strongly associated with development of pancreatic cancer. Its potency as a non-invasive diagnostic biomarker for detection of this cancer is observed. The link between variation of salivary microbiota and chronic pancreatitis as well as pancreatic cancer was evaluated in Farrell et al[33]. It was shown that in the saliva of patients with pancreatic cancer (n=10), 31 bacterial species/cluster were increased and 25 were decreased compared to healthy controls (n=10). Two bacterial species, i.e., *N. elongata* and *S. mitis* were distinguishing features between patients with pancreatic cancer and healthy controls (96.4% sensitivity and 82.1% specificity). The authors have proven that salivary microbiota may be a potential biomarker for detection of pancreatic cancer[33].

Interestingly, Mendez et al[3] have shown that microbial gut dysbiosis and polyamine metabolism may be used as a predictive marker for early detection of PDAC. The study was conducted with spontaneous pancreatic animal model $KRAS^{G12D}$ $TP53^{R172H}$Pdx-Cre (KPC) at 1 month of age. It was observed that *Proteobacteria* and *Firmicutes* were dominant in the early stage of PDAC development. Moreover, the significantly elevated serum polyamine concentration was detected in both, KPC models and patients with PDAC. Polyamine promotes rapid cells proliferation *via* contributing to purine/pirymide biosynthesis. This study confirmed that this class of metabolites is significantly deregulated in precancerous stage. It is known that members of *Lactobacillus* spp. affect polyamine metabolism; however, their role in pancreatic cancer is still unclear. *Lactobacillus reuteri* was detected in 4-months old animals in KPC model and it was associated with polyamine metabolism. Overall, it was the first study presenting that microbial dysbiosis and its altered metabolic pathways may potentially be exploited to develop non-invasive biomarker panel in the context of PDAC[3]. Additionally, Half et al[34] investigated the role of gut microbiome alterations in pancreatic cancer as potential biomarkers in Israeli cohort. This study included patients with pancreatic cancer (n=30), pre-cancerous lesions (n=6), control subjects (n=13), and patients with non-alcoholic fatty liver disease (n=16). The faecal microbiota was analysed using amplicon sequencing of the bacterial 16S rRNA gene. In patients with pancreatic cancer under-representation of: *Clostridiacea*, *Lachnospiraceae*, and *Ruminococcaceae* as well as over-representation of: *Veillonellaceae*, *Akkermansia*, and *Odoribacter* were noted. Notwithstanding, the authors emphasized that a low incidence and high variability in microbiome signature can be used in clinical practice in combination with other established biomarkers[34]. The potency of gut microbiome as a non-invasive diagnostic biomarker was also assessed in Ren et al[35] prospective study. Eighty-five samples from patients with pancreatic cancer and 57 matched control samples were collected. The characteristic of gut microbiome was analyzed using 16S rRNA Miseq sequencing. Based on 40 genera associated with pancreatic cancer, gut microbial markers achieve high classification power with AUC of 0.842. Overall, gut microbiome profile was unique in pancreatic cancer, suggesting being a potential non-invasive tool to diagnose this cancer[35].

Additionally, tumor diversity and composition may influence pancreatic cancer outcomes, which was proven in Riquelme et al[36] study. The tumor microbiome compositions in PDAC patients with short and long-term survival were analysed using 16S rRNA gene sequencing. The higher alpha-diversity in the tumor microbiome of patients with long-term survival was detected; moreover, intratumoral microbiome signature (*Pseudoxanthomonas-Streptomyces-Saccharopolyspora-Ba-*

cillus clausii) was associated with the prediction of long-term survivorship[36].

To conclude, the possibility to use gut microbiome as a predictive biomarker for the screening of the pancreatic cancer or as a useful tool for survival prognosis exists and may be used in combination with other diagnostic tools. Due to strongly limited data including its potency, before gut microbiome can be introduced as a single biomarker in clinical practice, further trials are necessary.

Hepatocellular Carcinoma

Currently, HCC is the third leading cause of cancer mortality globally[37,38]. The etiopathogenesis of HCC is multifactorial and it is presented in 2[39-41]. It is estimated that around 80-90% of HCCs occur in advanced fibrotic or cirrhotic livers; therefore, the liver cirrhosis consequently leads to the development of HCC[37].

Hepatocarcinogenesis – Gut Microbiota and Gut-Liver Axis

Gut microbiota imbalance may attribute to hepatocarcinogenesis and promote the progression of HCC[44,45]. Grąt et al[46] presented the characteristics of gut microbiota associated with the presence of HCC in patients with liver cirrhosis who underwent liver transplantation. It was shown that the increase of fecal counts of *E. coli* was significantly associated with the presence of HCC suggesting the role of this bacterium in the hepatocarcinogenesis. Additionally, the high level of *E. coli* and other Gram-negative bacteria correlated with increased serum concentration of LPS[46]. In Yu et al[47] study with rodents it was shown that LPS induced development of HCC. The reduction of LPS level using antibiotics in rats or genetic ablation of its Toll-like receptors 4 (TLR4) in mice prevented the excessive tumor growth and multiplicity[47]. Moreover, in another study[48], it was shown that the level of *Lactobacillus* spp., *Bifidobacterium* spp., and *Enteroccocus* spp. is reduced in patients with HCC.

Gut dysbiosis and the leaky gut promote hepatocarcinogenesis *via* several mechanisms[37]. Firstly, it is observed the release of cancer promoting and senescence promoting metabolites (deoxycholic acid) from gut microbiota. Secondly, it is noted that increased exposure of liver to gut-derived microbe-associated molecular patterns

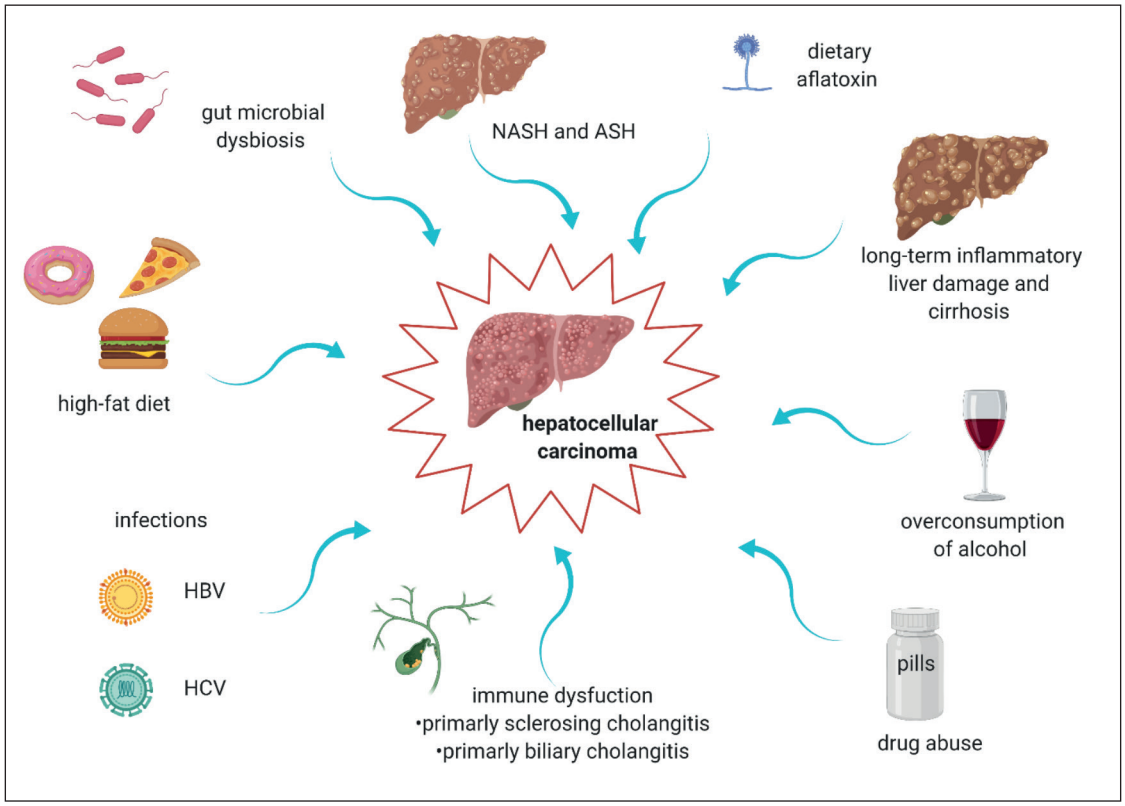

Figure 2. The major risk factors for the development of HCC. Own elaboration based on literature. HBV – hepatitis B virus; HCV – hepatitis C virus; NASH – non-alcoholic steatohepatitis; ASH – alcoholic steatohepatitis[39-43].

(MAMPs) promotes hepatic inflammation, fibrosis, proliferation, and the activation of anti-apoptotic signals[37]. The increase of intestinal permeability, bacterial translocation, and accumulation of LPS leads to overgrowth of intestinal bacteria, thus alters the composition of gut microbiota[43].

Nowadays, it is known that gut-liver axis exists (which is observed in close anatomical, functional, and bidirectional interaction of the gastrointestinal tract and liver) and affects the pathogenesis of liver disease including HCC[40]. Notably, the complex network of interaction including metabolic, immune, and neuroendocrine crosstalk regulate the gut-liver axis. Tight junctions in gut epithelium are a natural barrier to both, bacteria and bacterial products. Antigens from food or pathogenic microorganisms pass by this connection and they are recognized by dendritic cells. Antigens can also activate the adaptative immune system *via* modulating T cell response. Pathogen-associated molecular patterns (PAMPs) minimal concentration (LPS, flagellin, and peptidoglycans) activate nuclear factor kappa-B (NF-κB) *via* TRLs and nod-like receptors (NRL). It leads to pro-inflammatory cytokines production. Moreover, stellate cells, which are involved in promotion and progression of fibrosis can be also activated by PAMPs[43].

Bile acids (BAs) microbiota may also be associated with hepatocarcinogenesis. G protein-coupled bile acid receptor (TGR5) and nuclear hormone receptor farnesoid X receptor (FXR) are bile acid-sensing receptors[49]. Among others, TGR5 modulates inflammation and proliferation as well as secretion of bile. Overexpression of TGR5 was observed in intra- and extrahepatic cholangiocarcinoma and cystic cholangiocytes[50]. BAs are metabolized by enzymes which are derived from intestinal bacteria. Gut microbiota can be modulated by BAs. In turn, gut microbiota regulates the composition of the BA pool[49,51]. Notably, the disruption of BAs-microbiota crosstalk promotes inflammation and consequently contributes to development of HCC[49].

Hepatocarcinogenesis and Mycobiota

There are relatively few studies showing that fungal gut microbiota (mycobiota), when disturbed, can lead to carcinogenesis[4]. The fungal microbiota profile is strongly related to the disease stage and may promote carcinogenesis, e.g., by the activation of the inflammatory process[52]. Aykut et al[53] noted that certain fungi species may migrate from the gut lumen to the pancreas. This migration can increase the risk of the PDAC. In both mice and humans, PDAC tumors harbored a ~3000-fold increase in fungi compared to normal pancreas. That is significantly higher than the number of mycobiota in normal pancreatic tissue. *Malassezia* (*Malassezia globosa*) was linked to oncogenesis (observations on mice models), while oral administration of antifungal amphotericin B slowed down the growth of the tumor in cases of slowly progressive and invasive models of the PDA[52].

Van Asbeck et al[52] showed that the activation of the complement cascade through the activation of mannose-binding lectin (MBL), increases the risk of the PDA. Induced expression of MBL in humans was associated with higher mortality among patients with the PDA[52].

There are no studies confirming that fungi may be involved in the pathogenesis of HCC. In a paper published in 1994, it was indicated that hepatobiliary and pancreatic diseases (included HCC) were accompanied by a fungal infection[52]. Several species were related to hepatic dysfunction. *Candida* spp. pointed out to the highest range (56%); *Aspergillus* spp. was linked to 36%, whereas *Cryptococcus* spp. was reported in 8% of cases[54]. Kew showed that the aflatoxine B1, known as a metabolite of *A. flavus* and *A. parasiticus*, may be a serious hazard linked to the development of HCC[55].

Gut Microbiome as a Diagnostic Biomarker for HCC

Currently, alpha-fetoprotein is the most common biomarker used to detect liver cancer; nevertheless, it is not presented in around 30% cases mainly in early stages[56]. Therefore, there is a strong need to discover novel biomarkers. Recently, Ren et al[57] have evaluated the potential of microbiome as a non-invasive biomarker for HCC. Total number of 486 faecal samples from East, Central, and Northwest China were collected. The analysis was conducted with 16S rRNA Miseq sequencing. There were 3 groups, i.e., early HCC, cirrhosis, and healthy controls. *Actinobacteria* was increased in early HCC versus cirrhosis. 13 genera *Gemmiger* and *Parabacteroides* were increased in HCC compared to cirrhosis[57]. Additionally, butyrate-producing genera was decreased, and lipopolysaccharide-producing was increased in HCC in comparison to healthy controls. Interestingly, 30 microbial markers were identified through a fivefold cross-validation on a random forest model, an area under the curve of

80.64% between 75 early HCC and 105 non-HCC samples. This was the first study characterizing the gut microbiome in early HCC and presenting the potency of gut microbiome as a non-invasive tool to diagnosed early stage of HCC[57].

In 2020, Zheng et al[58] presented the characteristics of gut microbiota of liver cirrhosis-induced HCC (LC-HCC) as well as non-liver cirrhosis-induced HCC (NL-HCC) and investigated the impact of gut microbial dysbiosis on pathogenesis of HCC. The fecal samples were taken from 24 patients with hepatitis, 24 patients with liver cirrhosis, and 75 patients suffering from HCC (n=52 LC-HCC and n=23 NL-HCC) in Northeast China. The samples were sequenced on Illumina Hiseq platform and the bioinformatic analysis of gut microbiota was conducted with QIIME and Microbiome Analyst. It was observed that the fecal microbial diversity was significantly increased in HCC compared to liver cirrhosis. Moreover, the abundance of butyrate-producing bacteria was decreased whereas LPS-producing increased in LC-HCC patients. It was also noted that gut microbial dysbiosis in HCC patients was related to liver cirrhosis whereas not to viruses (HCV, HBV) and alcoholic liver disease. Additionally, three microbial biomarkers (i.e. *Enterococcus*, *Limnobacter*, and *Phyllobacterium*) were detected, which may be used to diagnose HCC[58].

Piñero et al[59] have compared the gut microbiome in cirrhotic patients with those with HCC and those without HCC. This study included patients with cirrhosis who were followed-up in Liver Unit at Austral University Hospital, School of Medicine, in collaboration with HERITAS (Rosario), CONICET and the National Academy of Medicine from Argentina. The analysis of gut microbiome was conducted with 16S rRNA sequencing (Illumina MISeq Platform). The differences of composition of gut microbiome between patients with HCC and without were observed. A 3-fold increase of *Erysipelotrichaceae* and a 5-fold decrease in *Leuconostocaceae* family, a 5-fold decrease of *Fusobacterium* genus as well as an increased *Bacteroides*/*Prevotella* ratio was shown in patients with HCC in comparison to those without HCC. Additionally, three operational taxonomic units (OUT's) were identified as potential biomarkers of HCC including abundance of genera i.e., *Odoribacter* and *Butyricimonas* with a decrease abundance of genus *Dorea*[59].

The oral microbiome dysbiosis in HCC patients may also be used as a non-invasive prognostic biomarker to detect this carcinoma, which was shown in Lu et al[60] study including 35 cirrhotic patients with HCC in early stage and 25 matched healthy subjects. The analysis of oral microbiome was conducted using 16S rRNA gene sequencing. It was observed that *Oribacterium* and *Fusobacterium* could distinguish patients with HCC from healthy controls[60].

Overall, the potency of gut microbiome as a prognostic biomarker to detect HCC is based on strongly limited data. The studies above mentioned were published most recently (in 2019 and 2020) showing that this scientific field is new and requires further investigations.

Conclusions

The association between gut microbial dysbiosis and carcinogenesis has been intensively analyzed for last several years. The development of PDAC is related to imbalance of oral microbiota, *H. pylori* infection, bactibilia, hepatotropic viruses, and intrapancreatic microbiota. Recent studies revealed that gut-liver axis exists and gut microbial dysbiosis promotes hepatocarcinogenesis.

Nowadays, growing attention towards using gut microbiome profile as a predictive or diagnostic biomarker of pancreatic cancer and HCC is observed. However, many of above-mentioned studies were conducted on small samples sizes. Notably, biomarkers are strongly ethnicity-dependent and should be validated in wide range of populations. Moreover, these data were focused only on bacterial part of gut microbiota in this context. The further studies should also assess the potency of mycobiota (fungal microbiota) as prognostic biomarkers.

Conflicts of interest

All authors report no relevant conflicts of interest, financial or otherwise.

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