World Journal of

Gastrointestinal Oncology

World Journal of Gastrointestinal Oncology

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ABOUT COVER

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RESPONSIBLE EDITORS FOR THIS ISSUE

Intratumour microbiome of pancreatic cancer

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Abstract

Pancreatic cancer is a high mortality malignancy with almost equal mortality and morbidity rates. Both normal and tumour tissues of the pancreas were previously considered sterile. In recent years, with the development of technologies for high-throughput sequencing, a variety of studies have revealed that pancreatic cancer tissues contain small amounts of bacteria and fungi. The intratumour microbiome is being revealed as an influential contributor to carcinogenesis. The intratumour microbiome has been identified as a crucial factor for pancreatic cancer progression, diagnosis, and treatment, chemotherapy resistance, and immune response. A better understanding of the biology of the intratumour microbiome of pancreatic cancer contributes to the establishment of better early cancer screening and treatment strategies. This review focuses on the possible origins of the intratumour microbiome in pancreatic cancer, the intratumour localization, the interaction with the tumour microenvironment, and strategies for improving the outcome of pancreatic cancer treatment. Thus, this review offers new perspectives for improving the prognosis of pancreatic cancer.

Key Words: Intratumour microbiome; Pancreatic cancer; Tumour microenvironment; Chemoresistance; Diagnosis; Prognosis

Core Tip: Recently, with the development of high-throughput sequencing, tumour tissues, which were previously believed to be sterile, have been shown to harbor a low microbiome biomass. The intratumour microbiome is crucial for pancreatic ductal adenocarcinoma (PDAC) diagnosis, treatment, chemotherapy resistance, and immune response. Establishing an awareness of the biology of the tumour microbiome in PDAC supports the establishment of better strategies for PDAC. This review focuses on the possible origins of the microbiome, the localization, the interaction with the tumour microenvironment and the strategies for improving the outcomes of treatment. This review offers new perspectives for improving the prognosis of PDAC.

INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is a malignant tumour that originates from pancreatic ductal cells. Although medical technology has improved the mortality of PDAC patients, the five-year survival remains less than 10%[1]. Since early PDAC patients lack specific clinical manifestations, the detection of PDAC usually occurs in the middle or late stages. Furthermore, advanced-stage PDAC usually cannot be eradicated by surgery, and these patients fail to respond to immunotherapy, chemotherapy or radiotherapy[2]. Therefore, it remains a huge challenge to improve the outlook for individuals with PDAC.

The traditional approaches for PDAC research are centred on the factors of PDAC only while ignoring the role of the microbiome in the tumour microenvironment (TME). Investigation revealed that the host microbiome, particularly the gut microbiome, interacts to influence cellular biological activity and regulate inflammation, immunity and cancer progression[3-6]. Of the 1012 different microbial species known today, only 11 are labeled human carcinogens by the International Association for Cancer Registries[7,8]. A broader range of microbiomes may contribute to carcinogenesis as an important class of 'coconspirators' but is not enough to cause cancer[9-11]. Recently, with increasing research on PDAC, tumour tissues, previously believed to be sterile, have been found to harbour a low microbiome biomass. The tumour microbiome was first proposed in the 19th century, but little progress in this field was made for a considerable period of time[12]. With advances in sequencing technology and a better understanding of the TME, it has been revealed that the intratumour microbiome plays an influential role in tumour progression[8]. However, the abundance of the intratumour microbiome is substantially lower than that of tumour cells. The bacterial portion of the tumour tissue was calculated to be approximately 0.68%. In the case of a three-dimensional or flat tumour environment, this equates to approximately 10^5 to 10^6 bacteria per 1 cm^3 or approximately 34 bacteria per 1 mm^2[8,13]. However, research has revealed that the intratumour microbiome exerts influential impacts on the progression, diagnosis, treatment, chemotherapy resistance, and modulation of immune tone in PDAC[14-17].

Despite the progress made in the study of the intratumour microbiome, there are still many unanswered questions in this emerging field. Furthermore, the understanding of the PDAC intratumour microbiome is far from complete, partly due to the limitations of research techniques[18]. Establishing an awareness of the biology of the tumour microbiome in PDAC supports the establishment of better strategies for early cancer screening and treatment. This review focuses on the possible origins of the intratumour microbiome in PDAC, the intratumour localization, the interaction with the TME, and the roles or strategies in improving the outcomes of PDAC treatment, offering new perspectives for improving the prognosis of PDAC.

THE ORIGIN OF THE PDAC INTRATUMOUR MICROBIOME

The mainstream view holds that the microbiome in PDAC may originate from the gut and the oral cavity, but this remains controversial (Figure 1). The pancreatic duct and common bile duct open together at the duodenal papilla. The innate anatomy allows microorganisms from the gut to enter the pancreatic tissue retrogradely through the pancreatic duct. Additionally, Okuda *et al*[19] showed that representative bacteria in pancreatic juice strongly colocalized in PDAC tissue[19]. Bacterial signals were detected in pancreatic tissue of wild-type (WT) mice by gavaging fluorescently labelled *Entero-coccus faecalis* and GFP-labelled *Escherichia coli* (*E. coli*)[20]. Similarly, when PDAC mice were gavaged with fungi, the presence of fungi in the tumour tissue was confirmed by staining or fluorescence[17,21]. However, in another design, the presence of bacteria in normal pancreatic tissue was not detected in
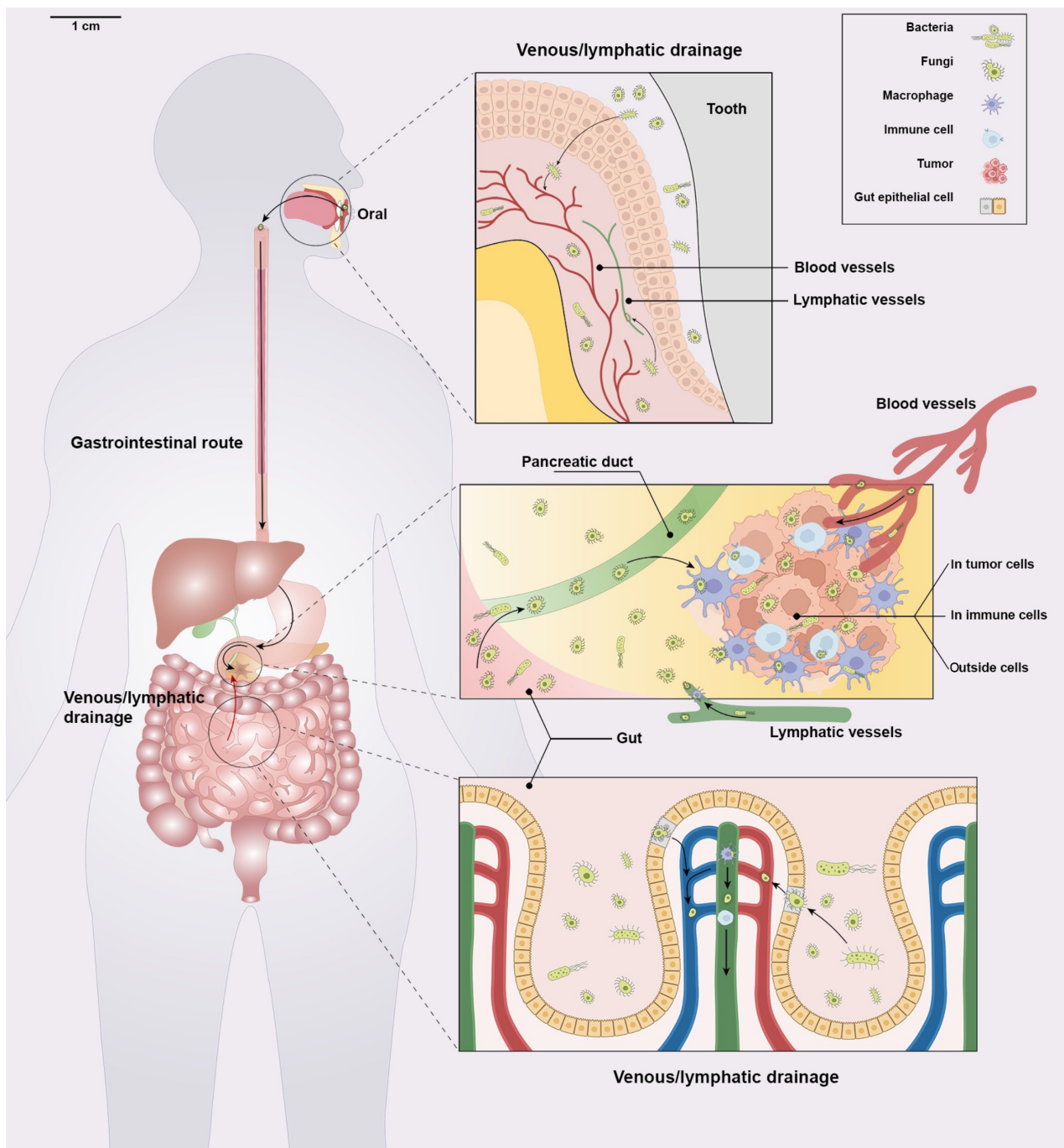
Figure 1 The origin and localization of the intratumour microbiome in pancreatic cancer. The microbiome in pancreatic ductal adenocarcinoma (PDAC) may originate from the gut and the oral cavity. The microbiome located in the oral cavity and gut can reach the pancreas *via* the pancreatic duct. But also exists the possibility of drainage *via* blood and lymph. The microbiome located in the gut migrates through the damaged intestinal epithelial barrier into the pancreas *via* venous blood, especially in the inferior gastrointestinal tract. In the case of oral microbiome, it can also enter the pancreas *via* the venous or lymphatic drainage. And the PDAC intratumour microbiome locates in tumour cells, immune cells and outside cells.

germ-free GF 129SvEv mice after gavage with relatively low doses and frequencies of specific pathogen-free bacteria[22]. This reflects the problem that although animal experiments have indicated that gastrointestinal flora can enter the pancreatic tissue *via* the gastrointestinal route, gavage by specific microbiota at high concentrations and frequencies does not seem to be proportionate to the normal human physiological situation[23]. In 16S rRNA sequencing of intratumour bacteria from PDAC in humans and mice, the bacterial compositions of PDAC and duodenal tissue were quite similar. The highest abundance of bacteria in human PDAC at the phylum level was *Proteobacteria*, which was the same as the highest abundance of bacteria in the duodenum, and patients who received invasive endoscopic procedures (IEP) had a higher abundance of intratumour bacteria than those who did not [15,24-26]. Significantly more abundant 16S rDNA copies were also observed in the pancreatic cyst fluid of intraductal papillary mucinous neoplasm (IPMN) and PDAC in patients with a preoperative history

of IEP[27]. When comparing the microbiomes of PDAC tissue, duodenal fluid, and duodenal tissue from postoperative pancreatic patients, extensive similarities between duodenal and PDAC microbiomes were shown, but some of the microbiome of PDAC was not present in the duodenum[28,29]. Riquelme *et al*[14] also demonstrated that approximately 50% of the intratumour bacteria of PDAC could not be explained by gastrointestinal or adjacent tissue[14]. Even though human-derived bacteria were detected in PDAC in mice that had been gavaged with faeces from PDAC patients, more than half of the intratumour bacteria in mouse PDAC remained unexplained[14]. This suggests that the microbiome in PDAC may have other sources.

Part of the oral microbiome of PDAC patients is also present in PDAC. Normally, the oral microbiome continues to spread to the distal gastrointestinal tract through oral intake alone and exceeds the expected abundance[30]. Coabundance of oral pathogens was found in the pancreatic cyst fluid of IPMN and PDAC[27]. At the phylum level, the intratumour and oral microbiomes of PDAC patients are dominated by *Firmicutes*, *Protebacteria* and *Bacteroidota*. However, *Protebacteria*, highly abundant in PDAC tissues, are not highly enriched in the oral cavity[25]. *Porphyromonas gingivalis* (*P. gingivalis*), an oral disease bacterium strongly associated with periodontitis and other oral diseases, has been most studied in relation to PDAC. Multiple findings indicate that *P. gingivalis* is also available in the PDAC microenvironment[25,29]. By gavaging calcein AM-labelled *P. gingivalis* for 2 weeks in C57BL/6 mice, the presence of *P. gingivalis* in the pancreas and faeces was confirmed by flow cytometry and fluorescence in situ hybridization (FISH)[25]. Thus, it is also possible for the microbiome from the oral cavity to reach the pancreas *via* the gastrointestinal route passing through the pancreatic duct.

Existing studies have indicated that the intratumour microbiome in PDAC has the potential to enter the pancreas through the gastrointestinal anatomy, but the possibility that the microbiome from the oral cavity or gastrointestinal cavity could enter the pancreas through blood and lymphatic drainage is not excluded[23]. *Fusobacterium nucleatum* (*F. nucleatum*), an oral colonizing anaerobic bacterium found in the same PDAC microenvironment as *P. gingivalis*, was injected into the tail vein of mice with rectal cancer and showed an enrichment of *F. nucleatum* in rectal cancer tumour tissue by plate culture or quantitative real-time polymerase chain reaction (qPCR)[31,32]. Tumours of nondigestive tract origin, such as breast cancer, are more likely to have an intratumour microbiome originating *via* blood or lymphatic drainage than tumours of digestive tract origin[33,34]. Although there is no experimental evidence at present that the microbiome can reach PDAC from microbial-rich sites such as the oral cavity or gastrointestinal tract by blood or lymph, much indirect evidence has shown the feasibility of such a transport route. Under healthy conditions, portal blood may contain small amounts of potential pathogens[35]. In cats, *E. coli* enter from the transmural wall of the colon and spread through the bloodstream to the pancreas, especially in cats with acute pancreatitis[36]. Bacterial translocation was detectable in blood from patients with acute pancreatitis by 16S rDNA sequencing[37]. However, this blood drainage seems to be difficult to achieve in disease-free conditions. In germ-free Il10-/- mice with no pancreatic lesions, a mouse with defects in intestinal permeability, oral infection with *Campylobacter jejuni* to accelerate such permeability defects caused them to develop severe colitis, but there appears to be no evidence of bacterial presence in the corresponding mouse pancreas by qPCR or culture[22].

In terms of lymphatic drainage, there is evidence of transfer of the gastrointestinal microbiome to mesenteric lymph nodes and transport *via* immune cells[38-40]. Commensal bacteria modulate intestinal immune surveillance by transporting CX3CR1[hi] mononuclear phagocytes to mesenteric lymph nodes along with bacteria captured in the intestinal lumen[38]. During this process, bacteria are screened and transported from the intestine to the mesenteric lymph nodes, which may provide an opportunity for bacteria to enter the pancreas *via* anatomical lymphatic drainage. Although the mechanism is unclear, microbial staining of a variety of tumours in different ways revealed that the intracellular microbiome was found in macrophages[16,24]. Consequently, it is possible that the microbiome within the tumour is transferred to the pancreas by lymphatic drainage through such a mechanism of macrophage transport. Unfortunately, Nejman *et al*[24] did not perform lipopolysaccharide (LPS) staining of PDAC. Furthermore, the immunohistochemistry (IHC) staining of LPS within macrophages may also be due to phagocytosis of local microbiota by macrophages. Macrophages exhibiting positive IHC in LPS rarely exhibit positive 16S rRNA FISH. Thus, the possibility is not excluded that the bacterial LPS staining present in macrophages originates from bacterial components that are not fully processed[16,41]. Bacteria present in the oral cavity, such as *P. gingivalis*, may be captured by lymphatic vasculature during the flow from the oral cavity to the bloodstream and then enter the systemic bloodstream[42]. Sakamoto *et al*[43] analysed microbiota in 153 lymph nodes collected from oral cancer patients and found viable bacteria in 45% of the lymph nodes from 83% of the patients[43]. Overall, the origin of the PDAC intratumour microbiome is still not entirely clear, but the possibility of multiple sources exists. Probing the origin of such a microbiome will facilitate the utilization of diverse approaches to target the intratumour microbiome for the treatment of PDAC patients in the future.

THE LOCATION IN PDAC

The intratumoural microbiome is a novel member of the PDAC tumour ecosystem, and its localization,

especially its subcellular localization, remains unclear (Figure 1). Nejman *et al*[24] performed IHC for LPS and lipoteichoic acid of bacteria in five cancers, including breast, bone, lung, glioblastoma and ovarian cancers, and found that bacteria were predominantly present in tumour cells and immune cells and localized in the cytoplasm and nucleus of cancer cells. FISH was performed on bacterial 16S rRNA; however, bacterial 16S rRNA was mainly localized in the cytoplasm[24]. In multimethod staining of pancreatic, melanoma, ovarian, breast, and lung cancers, Narunsky-Haziza *et al*[16] reported that the fungi were predominantly present in cancer cells of pancreatic, breast, and ovarian cancers, as well as in macrophages of melanoma and lung cancers, and that very few fungi were extracellularly localized[16]. Although Nejman *et al*[24] did not report the dominant localization of bacteria within pancreatic cancer tumours, the results would be expected to be similar. An *in vitro* experiment revealed that *P. gingivalis* could exert tumour-promoting effects in PANC1 cells after *P. gingivalis* infection[44]. Another *in vitro* experiment reported that after coculture of bacteria from IPMN cyst fluid with pancreatic normal cells or pancreatic cancer cell lines for 2 h, most bacterial isolates were discovered to enter and survive in human pancreatic cells[45]. Another line of indirect evidence of the intracellular localization of bacteria was the discovery of bacteria in PDAC tissue-derived extracellular vesicles[46]. However, it is possible that some of these vesicles may also originate in the blood or lymph of the circulation.

Insights into microbial localization inside and outside cells suggest that the microbiome inside cancer cells can alter the transcriptional state, proteome, and metabolic reserve of cancer cells and that the microbiome outside cancer cells can cause metabolic alterations, immune editing, clonal expansion and metastasis, and mutagenesis in cancer cells[13]. Intracellular and extracellular microbial localization studies may also have clinical implications for the selection of antibiotics with different bactericidal mechanisms. In breast cancer, intracellular bacteria can survive cell-impermeable antibiotic treatment (ampicillin and gentamicin) but not cell-penetrating doxycycline treatment[34]. However, it seems to be crucial to elucidate the subcellular localization of the microbiome in tumours. For example, the biological characteristics of microbiomes with different subcellular localizations may differ. Bacteria present in the cytosol can obtain nutrients directly from the interior of the host cell, while the source of nutrients for bacteria present in intracellular vesicles requires input through the membrane[47]. In addition, bacteria in the cytosol spread directly between cells by forming membrane protrusions that eventually enter adjacent cells, thus avoiding the harm of humoural immunity, while vacuolar bacteria can remain free from cytosolic sensors and autophagy[47]. However, the intensity of bacterial effects on target cells depends on the cell type and bacterial strain. The microbiome in PDAC cells is not fully characterized by studies involving normal cells or specific microbiomes[48]. In conclusion, the localization of the intratumoural microbiome within PDAC tumours requires further revelation, and such revelation is of great importance.

PDAC-SPECIFIC INTRATUMOUR MICROBIOME

The "genomics era" has accelerated various fields of biological research, and the impact is particularly noticeable with respect to the human microbiome. As a 'second genome' for cancer, each tumour type was detected to have its own specific intratumour microbiota in approximately 7.2% of sequenced reads in The Cancer Genome Atlas (TCGA) that were not attributed to human origin[16,49-52]. Despite the unavoidable contamination of TCGA-sourced data, the in silico decontamination method and a machine learning (ML) approach to build diagnostic models could effectively distinguish the cancers of TCGA, regardless of the stringency of decontamination[16,49]. Nejman *et al*[24] sequenced 1010 tumour samples with a critical decontamination process and similarly concluded that different tumour types have different microbial compositions[24]. Analogous to the specific microbial community character-istics of ecological differences in nature, in the tumour ecosystem, the specificity of the PDAC intratumour microbiome is reflected not only in the pancancer aspect but also between PDAC patients and normal individuals and between PDAC and the gut.

The intratumour bacterial 16S rDNA of PDAC is abundant, with great differences with respect to glioblastoma and bone cancer, while the Shannon diversity of intratumour bacteria is moderate, between that of ovarian cancer and melanoma[24]. Similar to bacteria, PDAC fungi have higher contents intermediate between breast and ovarian cancers[16]. At the phylum level, *Proteobacteria*, *Bacteroidetes* and *Firmicutes* are prevalent in the bacterial composition of PDAC[15,20,28,29,53]. Moreover, the abundance of Proteobacteria is also higher in PDAC than in breast cancer, glioblastoma, lung cancer, colorectal cancer and melanoma[24]. A similar specificity was also observed in PDAC intratumour fungi, with *Ascomycota* and *Basidiomycota* dominating the panintratumour fungal community at the phylum level. *Ascomycota* is slightly more abundant than *Basidiomycota* in PDAC, and *Yarrowia bubula*, a type of fungus belonging to *Ascomycota*, is the most differentiated fungus between PDAC and other tumours[16]. There was no considerable difference in the abundance of *Malassezia* across cancers, but important differences were shown in the human or mouse intestine *vs* PDAC[17,21].

Comparing PDAC with normal pancreas, the results seem to vary depending on the definition of "normal". When the pancreatic tissue in normal individuals is considered "normal", there are differences in the composition of the intratumour microbiome and a high alpha diversity of the microbiome in

PDAC[17,20,21,54]. The increase in intratumour bacteria in PDAC is 1000-fold compared to the normal pancreas, while the expansion of intratumour fungi is even more remarkable, with a 3000-fold increase compared to the normal pancreas[20,21]. Interestingly, the amount of bacteria in PDAC is considerably higher than the amount of fungi[16]. The gut microbiome from *Pdx1^{Cre}*; *LSL-Kras^{G12D}*; *Trp53^{R172H}* (KPC) mice, which have a higher degree of PDAC malignancy, has a higher capacity to translocate to the pancreas than that of WT mice[20]. Furthermore, the ability of the intratumour microbiome to enter the pancreas seems to be correlated with the level of pathological alterations of the pancreas. The percentage of bacterial DNA positivity in pancreatic cysts increases from 33.0% in non-IPMN to 59.6% in IPMN and 81.5% in cancer[27]. The mechanism of microbial enrichment within the tumour may be attributed to: (1) A hypoxic TME favouring the growth of anaerobic and parthenogenetic bacteria; (2) chemotactic effects of bacterial nutrients present in the necrotic region of the tumour and chemoat-tractive compounds present in the necrotic region of the resting cancer cells; (3) entry of circulating bacteria into the tumour tissue through an abnormally proliferating leaky tumour vascular system; (4) an immunosuppressive TME providing a refuge for microbial immune evasion; and (5) the impaired pancreatic barrier function, which facilitates microbial colonization[23,54,55]. Broad similarity in microbiome composition exists between PDAC and NAT when "normal" pancreas is defined as NAT, but differences also exist[19,25,26,28]. Interestingly, when comparing the microbial compositions of ductal adenocarcinomas in different parts of the pancreas, no differences in the composition or diversity of the microbial community were shown[25,45,56]. Upon comparing different subtypes of PDAC, the 'basal-like' subtype had higher microbial abundance than the 'classical' or 'hybrid' subtypes but was dominated by a few very-high-abundance species[57].

As a possible source of the PDAC intratumour microbiome, several reports have demonstrated significantly higher gut fungal and bacterial alpha diversity than tumours[21,29]. For bacteria, *Proteobacteria*, which account for only 8% of the gut bacteria in PDAC patients, account for nearly 50% in PDAC[20]. Regarding fungi, at the genus level, *Malassezia* was more prevalent in PDAC than in the gut [17,21]. The faeces seem to be incapable of referring to the microbial composition of the gut in different locations. However, comparing bacterial differences between duodenal and PDAC using endoscopic ultrasound-guided fine-needle aspiration (EUS-FNA) also confirmed the higher abundance of *Proteobacteria* in PDAC than in the duodenum[53]. In other words, the enrichment of the gut microbiome in PDAC may be specific as well.

THE INTRATUMOUR MICROBIOME AND THE DIAGNOSIS AND PROGNOSIS OF PDAC

Due to the paucity and nonspecificity of symptoms in patients with early PDAC, early detection of PDAC in clinical practice involves many challenges[58]. Poore *et al*[49], Nejman *et al*[24] and Narunsky-Haziza *et al*[16] provide the most comprehensive analysis of the blood microbiome and solid tumour diagnosis thus far[16,24,49]. Liquid biopsy in cancer allows the detection of miniature amounts of analytes (*e.g.*, DNA, RNA, proteins) shed from the tumour, which enables diagnostic and prognostic analysis of cancer[59] and earlier and more sensitive detection of PDAC by liquid biopsy compared to traditional PDAC examination techniques[60,61]. Traditional liquid biopsy-based diagnostic models have failed to address the presence of the intratumour microbiome. Poore *et al*[49] and Narunsky-Haziza *et al*[16] analysed blood-derived microorganisms from the TCGA and Hopkins cohorts and concluded that ML models based on the blood-derived microbiome can widely distinguish between multiple cancer types[16,49]. In the Hopkins cohort, the ML classification of untreated PDAC in phase I *vs* healthy controls revealed that decontaminated fungal species provided significant performance. This provides a new landscape for cell-free microbial DNA (cf.-mb DNA) models based on multispecies (*e.g.*, tumour, bacterial, fungal) sources in the early clinical diagnosis of PDAC. Despite rigorous computerized decontamination, further examination of decontaminated samples in a rigorous laboratory is required. It is also questionable whether the origin of cf.-mb DNA remains uncertain, although possible sources include oral, gut, and intratumour microbiomes[8].

TCGA microbiome data of solid tumours allow excellent differentiation of tumours by ML[16,49]. Regardless of the low abundance of the intratumour microbiome when compared to the tumour genome, species presence, whether involved in tumour pathogenesis or as opportunistic occupants, potentially contributes to the diagnosis of PDAC. EUS-FNA is a safe histological procedure for the diagnosis of patients with suspected PDAC[62]. Fast frozen EUS-FNA biopsy significantly enhances the diagnostic accuracy of current standard procedures by providing comprehensive genomic and transcriptomic analysis of PDAC patients at all stages[63]. Likewise, this technique is valuable for the evaluation of the intratumour microbiome of PDAC[53,56,64]. The strength of EUS-FNA is the ability to capture microbiome information in inoperable patients with no significant differences in alpha diversity, beta diversity, or taxonomic characteristics between EUS-FNA and surgically resected samples[64]. Since the early phase of intratumour microbiome research, there has been no more application of EUS-FNA for PDAC intratumour microbiology-related diagnosis. The diagnostic idea may be similar to genomic and transcriptional analysis: (1) By using the PDAC-specific microbiome for differentiation; and (2) building a strongly robust diagnostic model of multiple microbiomes, Narunsky-

Haziza *et al*[16] concluded that the TME may be a noncompetitive space for multidomain microbial colonization based on the strong positive correlation observed between fungal and bacterial diversity, abundance and cooccurrence in multiple cancer types[16]. Consequently, utilizing multifeature-based ML seems to be a better choice for diagnosis.

The essential role of the intratumoural microbiome in regulating the immune tone of the tumour TME makes it a favourable predictor of prognosis in PDAC patients. When comparing the alpha diversity of the intratumour microbiome in long-term survival (LTS) PDAC patients [overall survival (OS) > 5 years] *vs* short-term survival (STS) PDAC patients (OS < 5 years), patients with LTS had higher diversity than those with STS[14]. LTS patients showed a predominance of *Alphaproteobacteria*, *Sphingobacteria* and *Flavobacteria* at the class level, while STS patients presented with *Clostridia* and *Bacteroidea.* Similarly, another study on the prognosis-related intratumoural microbiome of Chinese PDAC patients reported higher alpha diversity in LTS patients than in STS patients, although the two studies did not have the same threshold for OS time[65]. It seems, however, that the role of high microbiome diversity in predicting the prognosis of PDAC tumours is not common to all tumours. For instance, high microbial diversity in gastric adenocarcinoma tumours is associated with poor survival[66]. Most likely due to genetic, ethnic, dietary, and geographical variability, the dominant species between LTS and STS obtained from these two cohorts were not identical[65,67]. Similar to the aforementioned diagnostic approach using the intratumour microbiome, microbiome data from TCGA were found to be a better prognostic predictor than clinical covariates alone in adrenocortical carcinoma, cervical squamous cell carcinoma, low-grade glioma and subcutaneous melanoma by the ML approach[68]. The combination of tumour microbiome abundance data and gene expression data allowed for modest improvements in predictive performance. In PDAC, Riquelme *el al*[14] constructed a prognostic signature employing *Pseudoxanthomonas*, *Saccharopolyspora* and *Streptomyces*, together with *Bacillus clausii*, that effectively predicted the prognosis of patients in the MD Anderson Cancer Center cohort (AUC = 97.51) and Johns Hopkins Hospital cohort (AUC = 99.17)[14].

Overall, the PDAC microbiome has shown incipient clinical relevance in diagnosis and prognosis, but the low biomass of the tumour microbiome makes decontamination particularly critical[69]. Laboratory means and computerized decontamination to achieve more reliable and reproducible results make the use of intratumour microbiome information for PDAC cancer diagnosis and prognosis more reliable. However, few studies have applied strict contamination controls to the cancer genome, although the efficiency of the application can be increased by adding samples or performing computerized decontamination[13]. Furthermore, genetic, ethnic, and geographical differences create heterogeneity in the microbiome composition of populations in different regions, which adds limitations in the use of microbial information for diagnostic and prognostic judgements. However, recently, one of the methods using transfer learning to overcome regional effects has yielded better robustness in cross-regional disease diagnosis using gut microbial features[70]. In other words, the microbial information within the tumour seems not to lose its meaning due to the presence of various restrictions. Further investigation of the meaning of the intratumour microbiome of PDAC in diagnosis and prognosis is desirable.

THE INTRATUMOUR MICROBIOME-IMMUNE-PANCREATIC CANCER AXIS

Innate and adaptive immunity comprise the body's powerful immune system, and they serve in the surveillance, recognition and elimination of tumours. The innate immune system reacts rapidly and nonspecifically when the body encounters pathogens, while adaptive immune responses develop more slowly but specifically and lead to classical immune memory[71]. Research over the years has focused on the adaptive immune system; however, studies of the adaptive immune system have led researchers to reassess the role of innate immunity as an essential hub for adaptive immune activation[71-73]. The human gut microbiome, as the largest microbial reservoir in the body, coevolved with the immune system and interacts directly through metabolic crosstalk[74]. The gut microbiome regulates host innate and adaptive immunity and influences disease development through its metabolic and microbial intrinsic components[75]. Similarly, in tumours, microbial mechanisms exist that are known to manipulate components of the intestinal epithelial barrier, regulate the activity of lymphoid organs, and modulate the immune tone of the TME[76]. For PDAC, immune cells, as an important component of the PDAC microenvironment, serve influential roles in regulating the growth, metastasis and treatment of PDAC[77-79]. Current findings demonstrated that the PDAC intratumour microbiome, by regulating immune tone in the TME, impacts PDAC progression and the immunotherapeutic response[14,20-22]. This mechanism of intratumour microbiome regulation of PDAC by influencing TME immune tone can be described as the intratumour microbial-immune-pancreatic cancer axis[8] (Figure 2). The intratumour microbiome similarly influences PDAC by modulating adaptive and innate immunity in the TME.

The complement system is a member of innate immunity and consists of approximately 20 different serine proteases. Similar to the coagulation pathway, complement activation entails several steps that are tightly regulated[80,81]. The complement system is activated in three major ways: The "classical activation pathway", the "bypass activation pathway" and the "lectin activation pathway"[80,82]. The convergence point for all complement activation pathways is the formation of C3 convertase complexes
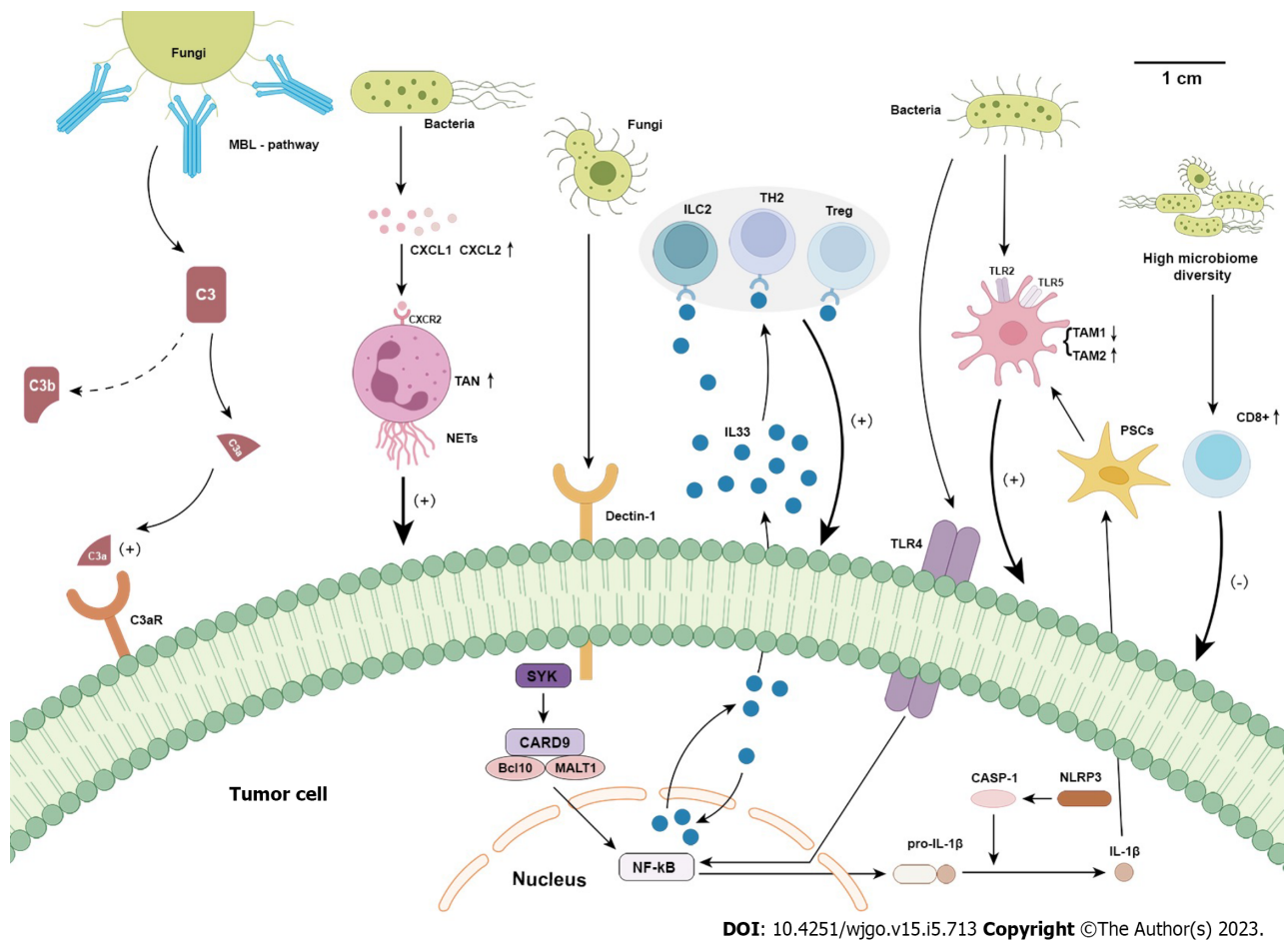
Figure 2 The intratumour microbiome-immune-pancreatic cancer axis. The intratumour fungi can activate the complement 3 (C3) complement cascade through the "lectin activation pathway". And C3a, as a fragment after C3 complement cascade reaction, promotes pancreatic ductal adenocarcinoma (PDAC) cells proliferation by binding to C3a receptors on the surface of cancer cells. Moreover, the intratumour fungi (*Malassezia globosa* or *Alternaria alternata*) and their cell-free extracts facilitate interleukin (IL)-33 secretion through activation of the dectin-1 receptor-mediated Src-Syk-CARD9 pathway. And IL-33 secretion promotes T helper 2 cell, group 2 innate lymphoid cells and Tregs enrichment in tumour microenvironment (TME), thus promoting PDAC progression. The intratumour bacteria promotes the secretion of neutrophil chemokines in the TME of PDAC thereby promoting tumour-associated neutrophils 2 (TAN2) enrichment in the TME. A portion of the effect of TAN2 may be through neutrophil extracellular traps. The PDAC intratumour bacteria also reduces the TAM1 polarization and decreased the antigen-presenting ability of TAM1 though through activation of toll-like receptors (TLR)2 and TLR4 on the surface of cells. TAM1 inhibition is accompanied by an increase in TAM to TAM2 conversion. It also promotes the secretion of IL-1β through TLR4 on the surface of PDAC cells. And IL-1β secretion promotes TAM2 activation through an indirect pathway that activates pancreatic stellate cells. Finally, the high diversity of intratumour microbiome promotes the activation of CD8+ T cells, which inhibits PDAC. C3: Complement 3; PDAC: Pancreatic ductal adenocarcinoma; Th2: T helper 2 cell; ILC2: Group 2 innate lymphoid cells; TME: Tumour microenvironment; TAN2: Tumour-associated neutrophils 2; NETs: Neutrophil extracellular traps; TAM: Tumour-associated macrophages; TLR: Toll-like receptors; PSCs: Pancreatic stellate cells.

on the surface of target cells, and upon formation of C3 convertase, the complement system is able to perform its duties. C3 is primarily synthesized by hepatocytes, but increasing evidence suggests that C3 is also locally secreted by a variety of cell types, including monocytes/macrophages, fibroblasts, endothelial cells, epithelial cells, and cancer cells, including PDAC[83-85]. The positive role of the complement system in fighting heterologous pathogens has been extensively studied, but it appears to serve as a promoter of tumour growth in a variety of tumours[82,83]. On the one hand, tumour-associated macrophages (TAMs) in the PDAC microenvironment can protect pancreatic cancer cells from complement-dependent cytotoxicity by regulating CD59, and on the other hand, intratumour fungi can promote fungal-tumour cohabitations using complement cascade reactions[21,86]. In preclinical experiments, the intratumour fungi of KPC mice, especially *Malassezia*, can activate the C3 complement cascade through the "lectin activation pathway"[21]. C3a, as a fragment produced after the C3 complement cascade reaction, promotes PDAC cell proliferation by binding to C3a receptors on the surface of cancer cells[21]. However, the role of the MBL-C3 mechanism of intratumoural fungi may be more significant than that[87]. An *in vitro* experiment showed that C3a-C3a receptor binding could promote the epithelial-to-mesenchymal transition (EMT) by activating the ERK pathway in PDAC cells [88]. Additionally, C3a receptors are expressed not only in tumour cells, but also on myeloid cells and CD4+ T lymphocytes[89-93]. This suggests that the intratumour fungal MBL-C3 mechanism in PDAC may have a broader role in the TME and necessitates further investigation.

Neutrophils, as the predominant specialized phagocytes in the body, play an important role in the body's resistance to pathogens such as bacteria, fungi, viruses and parasites[87,94-96]. Neutrophils function primarily through three major strategies: Phagocytosis, degranulation and the release of neutrophil extracellular traps (NETs)[97]. NETs are reticular structures composed of nuclear or mitochondrial DNA fibres decorated with antimicrobial enzymes and histones that are released to trap and kill pathogens[87,98]. NETs in tumours induce tumour recurrence, enhance tumour migration and invasiveness, and promote tumour cell proliferation[99]. The interaction between neutrophils and the microbiome is also reflected in PDAC. It was recently reported that *P. gingivalis* promotes the secretion of neutrophil chemokines (CXCL1 and CXCL2) in the TME of PDAC, thereby promoting tumour-associated neutrophil 2 (TAN2) enrichment in the TME[25]. In addition, the enrichment of TAN2 and the progression of PDAC can be blocked by CXCR2 inhibitors. In addition, neutrophil elastase (NE) in the TME was observed to be coexpressed with myeloperoxidase, a component of NETs[25]. However, the mechanism of increased neutrophil-associated chemokines and NE in the PDAC microenvironment caused by intratumour *P. gingivalis* is unclear. However, the toxicity factors of *P. gingivalis*, such as gingipains, serine proteases, lipid phosphatases or fimbriae, have been reported to manipulate the immune response of neutrophils in periodontitis[100].

Group 2 innate lymphoid cells (ILC2s), initially identified as important cells that protect the host from worm infection, also appear to play roles in asthma, inflammation and cancer. It was revealed that ILC2s seem to serve as "bipartisan politicians" in different tumours, and this feature was also likely exhibited in different immunogenic pancreatic cancers[17,101,102]. Kras-mutated PDAC promotes the infiltration of Th2 cells, ILC2 cells and Tregs in the TME through interleukin (IL)-33 secretion mediated by the Kras-MEK-ERK pathway. Meanwhile, intratumour fungi (*Malassezia globosa* or *Alternaria alternata*) and their cell-free extracts facilitate IL-33 secretion through activation of the dectin-1 receptor-mediated Src-Syk-CARD9 pathway[17]. PDAC infiltrates into tumour-promoting immune cells, including Th2 and ILC2 cells, to contribute to the protumourigenic program through their cytokine networks, leading to PDAC progression[103-105]. However, ILC2 cells in PDAC may have opposite effects. From another study, ILC2 was reported to inhibit PDAC tumour progression through the ILC2-CD103$^+$DC-CD8$^+$T axis[102]. High/Low TME immunogenicity apparently leads to distinct effects of ILC2 cells in PDAC. In other words, although there are no reports on the inhibition of PDAC by intratumour fungi, the bifacial impact of ILC2 cells in PDAC provides a clue to the antitumour effects of intratumour fungi in PDAC with respect to the pro/inhibitory effects of the intratumour microbiome under different immunogenicities.

The spectrum of macrophage activation states in tumour tissues is complicated, and TAMs are typically classified into two categories: M1 classically activated macrophages (TAM1) or M2 alternatively activated macrophages (TAM2)[106]. TAM1 promotes tumour remission and the Th1 response by secreting tumour necrosis factor-α and IL-12, while TAM2 exhibits an immunosuppressive phenotype and releases cytokines such as IL-4, IL-13, and IL-10 to promote the Th2 response[107]. A complex mechanism exists for the interaction between the intratumour microbiome and TAMs. Ablation of the microbiota with antibiotics leads to a decrease in TAM2 in KPC mice in situ and a concomitant increase in TAM1. Moreover, cell-free extracts from *Bifidobacterium pseudolongum*, a member of the PDAC intratumour microbiota, reduced TAM1 polarization and decreased the antigen-presenting ability of TAM1[20]. TAM2 tumour-promoting efficacy weakens when Toll-like receptor (TLR) signalling is eliminated *in vivo*. However, the TLR signalling-mediated effects do not seem to be limited to TAMs. According to a separate study, the pro-oncogenic effect of the intratumour microbiome in PDAC probably results partly from TLR4-mediated IL-1β production in PDAC cells[108]. Tumour-derived IL-1β partially contributes to the upregulation of TAM2 by regulating the activation and secretory phenotype of pancreatic stellate cells. In addition, the concentration of IL-1β seems to be positively correlated with the number of bacterial 16S rDNA copies in PDAC and IPMN cyst fluid[27].

In regard to adaptive immunity, CD8$^+$ T cells are an essential component. CD8+ T cells are recruited to infiltrate the TME and specifically kill target cells through recognition of antigens presented by MHC class I molecules[109]. Antibiotic ablation of the microbiota significantly increased the proportion of intratumour T cells in KPC mice. In parallel, the decrease in the microbiome increased the CD8$^+$:CD4$^+$ T-cell ratio and the number of cytotoxic phenotypic CD8$^+$ T cells[20]. A significant reduction in CD8$^+$ T cells in the TME was observed in PDAC mice gavaged with *Alternaria alternata* and *P. gingivalis*[17,25]. However, the effects of the intratumoural microbiome on CD8$^+$ T cells in the TME may be associated with the heterogeneity of the PDAC microbial community. The LTS patients had higher alpha diversity and a higher density of CD3+ and CD8+ T cells than STS patients. Furthermore, the enrichment of *Saccharopolyspora*, *Pseudoxanthomonas* and *Streptomyces* in LTS patients was positively correlated with CD8$^+$ T-cell density[14]. This may imply that the intratumour microbiome is not just tumour-promoting. The recruitment mechanism of the PDAC intratumour microbiome is still unclear, but it seems that the PDAC ecosystem allows "co-occurrence" of microbiomes that are beneficial to PDAC[16]. The evidence derived from pancreatic cancer mice with Col1 gene knockout showed reduced *Bacteroidales*, increased *Campylobacterales* and high infiltration of CD8$^+$ T cells compared to the tumour ecosystem of control mice[54]. Knockdown of the Col1 gene resulted in reduced malignancy of PDAC and altered intratumour microbial composition. Meanwhile, the ablation of the microbiome at this time resulted in shorter survival time and reduced infiltration of CD8+ T cells in the TME of the knockout Col1 mice.

In conclusion, complex interactions exist in the intratumour microbiome-immune-pancreatic cancer axis, which may more predominantly act as contributors to PDAC progression by modulating immune tone and thus influencing PDAC progression. However, the impact of the PDAC intratumour microbiome exceeds that of promoting the production of an immunosuppressive TME; depending on the PDAC ecosystem, it may also help to form an immune-promoting TME. At present, problems remain in the study of the PDAC intratumour microbiome: For example, the gavage of the microbiome or the oral administration of antibiotics cannot rule out the effects of the gut microbiome in mice[22]. Therefore, the mechanisms related to bacteria and fungi in PDAC still need to be further investigated.

THE INTRATUMOUR MICROBIOME AND CANCER THERAPY

Chemotherapy resistance

Gemcitabine, the classic chemotherapy regimen for PDAC, is also used in other solid tumours, such as ovarian cancer, bladder cancer and non-small cell lung cancer[110-113]. Gemcitabine is a cytidine analogue for which clearance is mainly due to the rapid and extensive inactivation of its main metabolite, 2',2'-difluorodeoxyuridine, by cytidine deaminase (CDD)[110]. CDD was recently identified by Geller *et al*[15] as a potential contributor to microbial-induced chemoresistance[15]. They classified bacteria according to the length of the bacterial CDD gene into 880-nucleotide-long CDD (CDD_L), 400-nucleotide-long short form CDD (CDD_S) and CDD-deficient bacteria. All species expressing CDD_L were resistant to gemcitabine, whereas only a minority of CDD_S and CDD-deficient bacteria mediated this effect. *In vivo* experiments confirmed that CDD_L-expressing *E. coli* increased tumour resistance to gemcitabine. Instead, the combination of gemcitabine and the antibiotic ciprofloxacin impeded resistance to this anticancer drug. Finally, by culturing bacteria from 15 fresh human PDAC tumours, they observed that bacteria from 14 PDAC samples enabled human colon cancer cell lines to become fully resistant to gemcitabine. This bacterial-driven gemcitabine deamination could be restored by exogenous delivery of the CDD inhibitor tetrahydrouridine[114]. Geller *et al*[15] also reported that bacterial suspensions also reduced the efficacy of oxaliplatin; however, this effect was not mediated by CDD. Although the enzyme or bacterial product that mediates oxaliplatin catabolism remains elusive, it is clear that bacteria can confer oxaliplatin resistance to cancer cells in a similar manner[15]. Nevertheless, *Clostridium nucleatum* was also revealed to induce resistance to oxaliplatin in colorectal cancer indirectly through the TLR4/MYD88 pathway[115]. It seems that the application of chemotherapeutic agents also altered the intratumour microbial composition of PDAC patients and affected the efficacy of chemotherapeutic agents. Significantly higher relative abundance of *Enterobacteriaceae* was observed in samples from patients treated with the combination of gemcitabine and paclitaxel compared to those treated with gemcitabine only and those not receiving neoadjuvant chemotherapy at all[26]. These *Enterobacteriaceae* are believed to be associated with chemotherapy resistance. Consequently, there may be various mechanisms involved in the chemotherapy resistance of PDAC with respect to the intratumour microbiome, showing the potential to improve tumour treatment outcomes by influencing the microbiome.

The strategy of applying antibiotics

In preclinical studies, the administration of antibiotics to PDAC mice to ablate the gut and intratumour microbiota of mice achieved inhibition or promotion of tumour progression[20,21]. In clinical trials, antibiotic monotherapy seems to improve the prognosis of patients with PDAC. In a retrospective clinical study enrolling 580 patients, patients with metastatic PDAC with a history of antibiotic use beyond 48 h had longer OS and progression-free survival (PFS) than patients with metastatic PDAC who did not use antibiotics but were not dependent on the use of preoperative antibiotics[116]. However, such an effect may be limited to specific patients. Another study noted that postoperative quinolones improved postoperative survival for patients with positive *Klebsiella pneumoniae* cultures in the bile but failed to show statistically significant improvement in postoperative survival for patients with negative *Klebsiella pneumoniae* cultures[117].

The combination of antibiotics with gemcitabine to reduce microbial-induced chemoresistance seems to be an effective strategy for the treatment of PDAC patients. The combination of antibiotics with gemcitabine improved OS and PFS in patients with metastatic PDAC, and improvement in PFS was observed in patients using FOLFIRINOX in combination with antibiotics[116]. Similarly, a retrospective study of 430 patients with PDAC reported that patient treatment with the combination of gemcitabine and antibiotics was more effective than monotherapy with gemcitabine[118]. In other words, the combination of antimicrobials with gemcitabine may increase the efficacy of gemcitabine while probably also increasing gastrointestinal and haematological adverse effects. Furthermore, the combination of quinolones with gemcitabine increased the incidence of haematological, gastrointestinal, obesity, and transaminase elevations, while the combination of β-lactam antibiotics with gemcitabine increased the incidence of haematological adverse events[118]. However, the combination of quinolones with gemcitabine improved PFS in patients with negative *Klebsiella pneumoniae* bile cultures[117]. Apart from aggravating the adverse effects of chemotherapeutic drugs, the combination of antibiotics and

chemotherapeutic drugs may lead to the development of drug-resistant bacteria and disrupt the commensal relationships of microbiota in the long-term use of antibiotics[119]. Research has shown that 7 d of continuous antibiotic use in healthy individuals will perturb gut microbes and require at least 1 year to return to normal[120]. Therefore, it is imperative to investigate antibiotic strategies that target microbiota to improve the prognosis of PDAC patients and preserve the beneficial microbiota.

Despite the limited efficacy of immunotherapy in PDAC, the significance of the intratumour microbiota in altering the immune tone of PDAC offers new therapeutic options[121,122]. The complex relationship between the microbiota and immune regulation within PDAC tumours makes the outcome of antibiotic combination immunotherapy unclear. In a meta-analysis enrolling 2740 cancer patients, antibiotic use was associated with significantly lower OS and PFS in patients treated with immune checkpoint inhibitors[123]. Moreover, the modulatory mechanism of the intratumour microbiome on the TME in PDAC may not be dependent on the surviving microbiome. The application of cell-free extracts of microbiota in preclinical studies also achieved the modulation of TME immune tone[17,65]. Therefore, the strategy of applying antibiotics in combination with immune checkpoint inhibitors in PDAC also faces some urgent challenges to be solved.

The application of probiotics

In view of the access of the PDAC microbiome to the pancreas *via* the pancreatic duct, oral administration of probiotics offers a potentially effective strategy. While this strategy needs to be confirmed by clinical trials, the role of the gut/pancreatic microbiome and its metabolites in PDAC has been demonstrated in many preclinical studies. For example, *Lactobacillus* can decrease the number and grade of pancreatic precancerous lesions, retard the growth of pancreatic cancer cells in Kras mutant mice, and inhibit the EMT process in cancer cells[124]. Heptelidic acid, a metabolite of the probiotic *Aspergillus oryzae*, activates the p38 MAPK signalling pathway and induces apoptosis in pancreatic cancer cells [125]. *Megasphaera* and the short-chain fatty acids derived from its metabolism enriched in LTS PDAC patients stimulated macrophage activation *in vitro* and improved the efficacy of programmed cell death protein 1 inhibitors *in vivo*[65]. However, the use of probiotics in PDAC is also problematic. Probiotics may not only destroy the ecosystem of innately colonized microbiota but could also hinder the re-establishment of the microbial ecosystem after antibiotic treatment[126]. It is also noted that probiotics may induce infections in patients, especially those who suffer from immune deficiency[122,127]. Incorporating the once-unappreciated intratumour microbiome into research would provide a good direction to improve the prognosis of pancreatic cancer patients.

CONCLUSION

In recent years, the field of the intratumour microbiome has provided new insights into the field of oncology. PDAC is a highly malignant tumour, and some of the biological processes in PDAC are tied to the intratumour microbiome. Investigating the causal relationship and molecular interactions between PDAC and the commensal microbes in the TME is expected to provide new ideas for mankind in the conquest of PDAC. In this review, we reveal that the microorganisms within PDAC tissues may originate from the gut and oral cavity *via* circulation, the lymphatic system, and the gastrointestinal system. The microbial enrichment within PDAC tissues is specific. The PDAC intratumour microbiome is capable of regulating immune tone through immune cells, such as TAMs, TANs and lymphocytes, and the complement system. In addition, targeting the microbiota associated with PDAC has potential clinical applications in the diagnosis and treatment of tumours. Overall, the study of the intratumour microbiome is still at an early stage, and many issues remain to be addressed. For example, the origin and pathways of the intratumour microbiome in PDAC have not been fully explained. The details about the specific intratissue localization of the microbiome and its subcellular localization are unclear. The complex interactions between specific microbiomes and the TME have not been fully revealed. Alternatively, the majority of microbiota appears not to be culturable from tumours in a straightforward manner, limiting the ability to directly utilize intratumour microbiota for studies[128]. Furthermore, fundamental and clinical research on the association between the intratumour microbiome and genomic mutations in PDAC is still inadequate. However, based on the previously described mechanism by which intratumoural fungi enhance KRAS mutations mediating IL-33 secretion by PDAC, the existence of interactions between genomic alterations in PDAC and intratumoural microbes has been shown[17]. In addition, current research mainly focuses on intratumoural bacteria and fungi, and there is less research on the interactions between viruses and tumours. An association between hepatitis B virus (HBV) and hepatitis C virus and PDAC risk has been shown, and HBV expression can be found in PDAC tissue[129,130].

In the future, it is essential to investigate the causal relationship between PDAC and intratumoural microbial interactions and to use more advanced technologies, such as single-cell sequencing, for related research. Furthermore, KRAS mutations are a major burden in the conversion of pancreatic precancerous lesions to PDAC; thus, the relevance of KRAS mutations in the intratumour microbiome of PDAC needs further investigation[131]. Additionally, the mechanisms of virus-host interactions are still

not available, and it is essential to investigate the mechanisms associated with viruses in tumours to improve the theoretical system of tumour microbiology. Finally, a more precise and personalized application of antibiotics or probiotics to improve chemoresistance and immunotherapy in PDAC patients is a huge challenge. Thus, more sophisticated and effective clinical trials are required in the future to identify such potentially beneficial patients and improve their prognosis.

FOOTNOTES

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