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Tumor microbiome: Pancreatic cancer and duodenal fluids contain multitudes, …but do they contradict themselves?

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In "Song of Myself," Walt Whitman celebrates the self, praising the joy and wonder of experiencing nature. One of the most famous verses of this poem ("*Do I contradict myself? Very well then I contradict myself, I am large, I contain multitudes*") has been quoted by the award-winning science writer Ed Yong in the title of his compelling book on how ubiquitous and vital microbes are (Yong, 2016). This book, as well as many recent seminal articles, includes chapters on the interaction of human microbiome with the immune system and diseases (Young, 2017; Fung et al., 2017). Notably, growing evidence supports a specific role for the microbiome in cancer initiation, progression, and response to therapy, as recently reviewed also by Gori and collaborators (Zitvogel et al., 2018; Picardo et al., 2019; Gori et al., 2019). Especially the differences in the composition of the microbiome in tumor tissue compared to normal tissue and the modulation of the tumor microbiome influencing therapy efficacy are prompting new clinical approaches (Gopalakrishnan et al., 2018; Routy et al., 2018; Pushalkar et al., 2018).

In 2017, a breakthrough study described several bacterial species within pancreatic ductal adenocarcinoma (PDAC) tissues, showing significantly higher abundance of various gut derived microbes in PDAC patients compared to healthy individuals (Geller et al., 2017). Moreover, some of these bacteria metabolized and thereby inactivated gemcitabine, while co-treatment with antibiotics restored gemcitabine efficacy in murine models. Though gemcitabine is frequently used in the treatment of PDAC and treating patients with antibiotics might seem a straightforward approach, a number of commentaries, including an editorial from the same authors, raised several questions on experimental models, demographic factors, diet, as well as different microbial function/influence on cancer hallmarks, and concluded that the clinical impact of these findings has yet to be determined (Geller and Straussman, 2018; Jobin, 2017; Thomas, 2017; Choy et al., 2018).

Most recently, Riquelme and collaborators provided additional insights on the contribution of the microbiome for the outcome of PDAC patients, comparing long-term (LTS) and short-term (STS) survivors (Riquelme et al., 2019). Through 16S rRNA gene sequencing of frozen tumor tissues of these patients, they demonstrated a relationship between intra-tumoral bacterial diversity and overall survival. The tumor microbial diversity was significantly higher in LTS, and the microbiome of LTS showed significantly more Proteobacteria (*Pseudoxanthomonas*) and Actinobacteria (*Saccharopolyspora* and *Streptomyces*). The hypothesis that gut microbiome can influence tumor microbiota was tested by human-into-mice fecal microbiome transplantation, showing that gut and tumor bacteria from LTS modulated tumor infiltrate and reduced tumor growth in mouse models. These findings provide strong evidence about the key role of the microbiome on tumor progression and prognosis, suggesting the use of microbiome diversity to predict the survival and guide new therapeutic strategies. An early-stage clinical trial is indeed being planned to test fecal transplants in PDAC patients.

However, experimental settings to detect the microbiome are extremely delicate, as contamination with bacteria or bacterial DNA is easily encountered, resulting in potentially biased results. Previous studies suggested that translocation of bacteria into pancreatic tissue occurs presumably by retrograde bacterial migration from the duodenum into the pancreas via the pancreatic duct (Ye et al., 2016). Various critical clinically relevant factors could thus influence the microbiome composition, including usage of proton pump inhibitors (PPIs) and the occurrence of biliary obstruction, which is often palliated preoperatively by endoscopic preoperative biliary drainage (PBD) (Imhann et al., 2016). To get more insight in these unaddressed issues we performed an exploratory study in which we hypothesized that PBD with stent placement in the common bile duct (CBD) could influence the duodenal microbiome, and, consequently, also the microbiome of the pancreas. Duodenal fluid was collected from patients with PBD with stent ($N = 6$) or without stent placement ($N = 5$). Three non-stented patients used proton pump inhibitors (PPIs).

Bacterial DNA was detected in all stented samples, while almost no bacterial DNA was found in non-stented samples (Fig. 1). Duodenal fluids of patients with stent demonstrated a wide microbiome composition, including Proteobacteria and Firmicutes. Strikingly, though they were not contaminated during handling blank samples demonstrated the presence of Bacteroidetes, which were also found in samples without stent placement and samples of STS cases in Riquelme's study. Moreover, *Streptococcus* was most abundant in non-stented patients with PPIs, as reported previously (Imhann et al., 2016). Besides low bacterial DNA in non-stented patients, intraoperatively acquired bile cultures of all these patients were negative. These data demonstrate the

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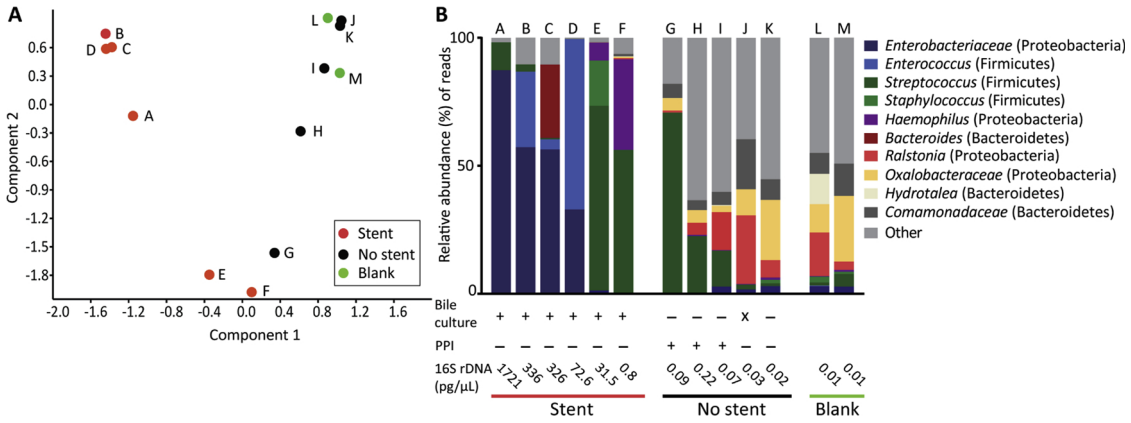

Fig. 1. The duodenal microbiome of PDAC patients with and without stent in the CBD.

A. PCA plot of duodenal microbiome of PDAC patients with (red) and without stent (black) in the CBD in comparison to blank quality controls (green; Component 1 = 38.4% variance and Component 2 = 13.4% variance). The plot is based on the 16S rDNA amplicon sequences, clustered in OTUs. Samples with stent are separated from the samples without stent. Most samples from the patients without stent clustered with the negative controls.

B. Relative abundance of major bacterial taxa at genus or higher taxonomic level in duodenal fluid of patients with (left) and without stent (middle), as well as negative (blank) control samples (right). Viability of microbiological bile culture (positive growth = +, negative growth = –, missing data = x), PPI usage (yes = + and no = –) and 16S rDNA amount (pg/µL, determined by qPCR) per individual sample are shown below.

Methods. Endoscopic PBD was performed in patients with obstructive jaundice by plastic stent placement. Duodenal fluid of patients with histopathologically confirmed PDAC was collected directly after resection of the primary tumor by sterile aspiration in the duodenum and stored at −80 °C. Clinical data was collected prospectively and all patients provided written informed consent. Bile cultures were collected perioperatively to investigate bacterial growth in bile. To isolate bacteria, duodenal fluid was disrupted by zirconium beads (BioSpec) and genomic DNA was isolated with the Mag Mini DNA Isolation Kit (LGC). Sequencing of the V4 region of 16S rDNA was performed on the Illumina MiSeq Platform (2 × 251 nt) and sequences were clustered into operational units (OTUs) and assigned taxonomy (but allowing max. 25 mismatches in the read overlap). Principal Component Analysis (PCA) of OTU-data was performed after Log2 transformation to visualize clustering of the microbial profiles in PAST (v.3.11). The relative abundance of bacterial taxa at genus or higher taxonomic level was calculated for each sample and analyzed with GraphPad Prism (v.7).

importance of evaluation of possible factors influencing the microbiome composition and inclusion of adequate control samples. The presence of stent placement and PPI usage however were not reported in the article by Riquelme and collaborators, while biliary obstruction was reported in ~50% of patients.

Since future studies have to validate the potential of using the bacterial signature as a biomarker of expected survival and treatment success, we urge a thorough inclusion of clinically relevant factors, such as the above-described presence of stent replacement and use of PPIs, in order to translate these results to practical implications.

The knowledge of the exact composition of microbiota in both cancer tissues and accessible gut specimens such as duodenal fluids should indeed be fully reviewed using metagenomic sequencing on all the bacterial genes (or, as better defined by Marchesi and Ravel, “metataxonomics” (Marchesi and Ravel, 2015)), but parallel extensive studies should also improve the understanding on the variation in the microbiome between individuals, as well as the influence of clinical and demographic factors and other medical-procedures which can have important consequences on microbiome composition and function.

In conclusion, due to microbiome, all human beings have an inner life, in every sense, and we are looking forward to exploiting this diversity, and seeking for unraveling the contradictions reported so far, in order to benefit cancer patients.

Declaration of Competing Interest

The authors declare no conflicts of interest.

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