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Disturbances of the Perioperative Microbiome Across Multiple Body Sites in Patients Undergoing Pancreaticoduodenectomy

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Abstract

Objective—The goal of this study was to characterize bacterial communities within fecal samples, pancreatic fluid, bile, and jejunal contents from patients undergoing pancreaticoduodenectomy (PDA), and to identify associations between microbiome profiles and clinical variables.

Methods—Fluid was collected from the pancreas, common bile duct, and proximal jejunum from 50 PDA patients. Postoperative fecal samples were also collected. The microbial burden within samples was quantified with ddPCR. Bacterial 16S ribosomal RNA gene sequences were amplified, sequenced, and analyzed. Data from fecal samples were compared to publicly available data obtained from volunteers.

Results—ddPCR confirmed the presence of bacteria in all sample types, including pancreatic fluid. Relative to samples from the American Gut Project, fecal samples from PDA patients were enriched with *Klebsiella* and *Bacteroides*, and were depleted of anaerobic taxa (e.g. *Roseburia* and *Faecalibacterium*). Similar patterns were observed within PDA pancreas, bile, and jejunal samples. Postoperative fecal samples from patients with a pancreatic fistula contained increased abundance of *Klebsiella* and decreased abundance of commensal anaerobes, e.g. *Ruminococcus*.

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Supplemental Digital Content

Supplemental Digital Content 1. PDF

Supplemental Digital Content 2. XLS

Supplemental Digital Content 3. PDF

Conclusions—This study confirms the presence of altered bacterial populations within samples from PDA patients. Future research must validate these findings and may evaluate targeted microbiome modifications to improve outcomes in PDA patients.

Keywords

pancreatoduodenectomy; pancreas; bile; microbiome; microbiota; Klebsiella; pancreatic fistula

Introduction

Pancreaticoduodenectomy (PD), the standard surgical treatment for patients with ductal adenocarcinoma (PDA) of the pancreatic head, is a morbid procedure with complications exceeding 50% even in modern series from high volume centers¹. An unexplored possibility is that the microbiota (either within the gut or in the pancreas) impacts postoperative outcomes. Such a possibility is consistent with compelling recent research demonstrating that the microbiota impacts responsiveness to chemotherapy and risk of anastomotic leak after colon surgery^{2,3}. It is possible that patient survival and pancreatic fistula formation after PDA are both linked to the status of the microbiome.

Historically, the pancreas was viewed as sterile, but recent studies suggest this may not be true⁴. It has been clearly shown that enteric or biliary microbes can colonize the inflamed pancreas in experimental models⁵. Similarly, it has been shown that the pancreatic duct or stents within the duct in the setting of chronic pancreatitis contain a rich biofilm containing bacteria^{6,7}. The bile duct has also been considered to be generally sterile⁶ but a number of studies have demonstrated the presence of bacteria in the biliary system in the setting of bile duct obstruction^{6,8,9}. However, little is known about the presence of microbes within the pancreas or bile duct in the setting of cancer or how these microbes might affect treatment outcomes.

The goal of this study was to characterize the microbiota of the pancreas, the bile duct, the jejunum, and fecal samples in patients undergoing PD. Human fecal samples have been heavily studied, but only rarely has the microbiota been assessed in the perioperative period, when it is likely to be impacted by antibiotics, lack of enteral nutrition, and exposure to nosocomial pathogens. In the case of PD patients, the microbiota may be further impacted by biliary stent placement and/or chemotherapy. If it can be shown that dysbiosis affects patient outcomes for PD patients, then future strategies to improve postoperative outcomes may include simple interventions to modify the microbiota, e.g. dietary modification or fecal transplantation.

Materials and methods

Sample Collection

Fifty patients at the University of Pittsburgh Medical Center were recruited for study participation (January 2014 - December 2014) prior to an electively scheduled pancreaticoduodenectomy. All patients received a magnesium citrate bowel prep regimen and broad-spectrum prophylactic antibiotics (most commonly cefoxitin) prior to surgery and

for 0-2 days postoperatively. During the PD procedure, sterile swabs (BBL CultureSwab EZ, Becton Dickinson, Franklin Lakes, NJ) were used to sample fluid from the common bile duct, the main pancreatic duct, and the proximal jejunum at the site of the pancreatic anastomosis. The first post-operative fecal sample from each subject was also collected in a sterile container. All samples were stored at -80°C until batch processing. The study was conducted with institutional approval from The University of Pittsburgh (IRB #13070219).

DNA Extraction

Microbial DNA was extracted from all swabs and fecal samples using the MO BIO PowerSoil DNA Isolation kit (MO BIO Laboratories, Inc., Carlsbad, CA). For fecal samples the samples were added directly into bead tubes and incubated at 65°C for 10 minutes followed by 95°C for 10 minutes. After addition of 60 μL of Solution C1 the bead tubes were then shaken horizontally on a lab mixer for 10 min at maximum speed using a MO BIO vortex adaptor. All remaining steps followed the manufacturer's protocol. For swab samples the swab head was cut off directly into bead tubes containing 60 μL of Solution C1 and then incubated at 65°C for 10 minutes. Tubes were then shaken horizontally on a lab mixer for 3 min at maximum speed using a MO BIO vortex adaptor. All remaining steps followed the manufacturer's protocol. To quantify bacterial load, droplet digital PCR was performed as previously described¹⁰.

16S rRNA Amplicon PCR and Sequencing

PCR amplification of the small subunit ribosomal RNA gene (16S rRNA) was performed in triplicate 25 μL reactions. Amplicons were produced utilizing primers adapted for the Illumina MiSeq. Amplicons target the V4 region and primers utilized either the Illumina adaptor, primer pad and linker (forward primer) or Illumina adaptor, Golay barcode, primer pad and linker (reverse primer) followed by a sequence targeting a conserved region of the bacterial 16S rRNA gene as described by Caporaso et al¹¹. The only deviation from the protocol was that PCR was run for 30 cycles. Individual PCR amplicons were purified, quantified, pooled in equimolar ratios, and the library pool was gel purified prior to submission for sequencing on the Illumina MiSeq at the University of Illinois' Roy J. Carver Biotechnology Center High-Throughput Sequencing and Genotyping Unit.

Sequence Analysis

Sequencing reads were demultiplexed and quality filtered using QIIME (v.1.9), then UPARSE was used to cluster reads into operational taxonomic units (OTUs) using an identity cut-off of 0.97, remove chimaeric sequences, and generate a table of OTUs. QIIME was used to assign taxonomic classifications to OTUs, using the RDP classifier trained on the most recent Greengenes database (v. gg_13_8). Unassigned OTUs were treated as potential human contaminants, and OTUs classified as Streptophytes were assumed to result from plant pollen were removed as well. Finally, samples with read counts of less than 400 were excluded from all downstream analysis with the exception of comparison to controls. A second OTU table was generated including samples from the American Gut Project (<http://americangut.org>) using the same parameters, but with shorter read lengths. This dataset was used for alpha and beta diversity comparisons, and for taxonomic comparisons between sites. The American Gut samples were filtered according to the following parameters: age

(18+); antibiotics (none in prior 6 months); quinolone (no); country (USA or Canada); pregnancy (no); BMI (18-30); skin condition (none); nitromidazole (no); smoking frequency (nonsmoker); alcohol frequency (nondaily). Following filtering for read depth, 831 adult fecal samples from the AG project remained in our dataset.

Alpha diversity was calculated using the chao1 metric, on a dataset rarefied to a depth of 2,000 reads per sample. Comparisons of diversity between sites were performed using the QIIME compare_alpha_diversity.py python script on 10 replicates of rarefied datasets, and using the Monto-Carlo permutations. Beta-diversity distances were estimated using QIIME using the weighted UniFrac method, and principle coordinate analysis (PCoA) plots were generated using QIIME. The R vegan library was used to perform PERMANOVA comparisons between PD fecal samples and American Gut (AG) fecal samples on a distance matrix limited to fecal samples. Taxonomic summary tables were generated at all ranks using QIIME, and LEfSe was used to predict taxonomic biomarkers both between sites and between clinical conditions using correction for multiple independent comparisons. Biomarker taxa were further limited to those present at levels higher than 0.01 on average within the group of samples under consideration.

To evaluate whether laboratory contamination contributed to our sequencing results, extraction controls without clinical samples and “no template” PCR controls were analyzed with each set of samples. As expected, environmental contaminants were commonly observed. *Pseudomonas* was the most abundant contaminant. Among the contaminants were also species of *Enterobacteriaceae*, though at much lower levels than present in clinical samples. A heatmap and Hclust clustering of all samples by taxonomic composition placed nearly all controls in a single cluster (see Supplemental Digital Content 1, Figure S1) distinct from clusters containing clinical samples. Further, the abundance of *Pseudomonas* or *Enterobacteriaceae* did not correlate with the abundance of these taxa within corresponding negative controls.

Results

Patient cohort

Clinical information for all study subjects is shown in Table S1 (Supplemental Digital Content 2). The median age of the study population was 66 years. Of the 50 patients, 11 underwent an open PD procedure, and 39 underwent robotic-assisted procedures. Surgical pathology confirmed a cancer diagnosis in all but five study subjects. Eight patients (16%) developed a postoperative pancreatic fistula, and eight patients (16%) developed surgical site infections requiring treatment. Survival at 1 year was 84%.

We collected 40 postoperative fecal samples and 149 total swabs (pancreatic fluid, bile, or jejunal contents) from the 50 study subjects. Many samples could not be analyzed due to insufficient DNA yield or inadequate number of sequencing reads after preparation of 16S rRNA amplicons. The final number of samples included in this analysis (see Table S2 in Supplemental Digital Content 2) is 36 fecal samples and 22, 26, and 17 pancreatic duct, bile duct, and jejunal samples respectively.

Quantitative PCR demonstrates presence of bacterial DNA in intraoperative pancreatic and biliary samples

We performed droplet digital PCR with a universal bacterial primer to detect bacterial DNA in representative sets of samples from six patients. These patients were selected because samples from each body site passed quality filtering and thus it was possible to make comparisons across body sites. As expected, fecal samples contained far more bacterial DNA than other environments and there was a statistical significance across sample types (Kruskal Wallis test $p = 1.328\text{e-}09$) (Figure 1). Post-hoc Mann-Whitney tests with Bonferroni correction showed all sample types including pancreatic duct fluid contained more bacterial DNA than negative controls used for reference ($p < 0.05$). The microbial burden within swabs of bile and jejunal contents varied significantly among patients. Interestingly, the density of bacterial DNA per swab was not significantly different among pancreas, bile, and jejunal samples. These results document that pancreatic duct fluid in PD patients contains microbial populations similar in density to that seen in the jejunum and biliary tree.

Microbial diversity across body sites

Alpha diversity is a measure of how many taxonomic groups are present in a sample and how evenly the abundance of these groups is distributed¹². Using data from the American Gut (AG) Project (<http://americangut.org>), we found that alpha diversity within PD fecal samples (Figure 2) was significantly lower than samples from participants in the AG project (chao1 metric, non parametric test, t-stat -11.35, $p=0.01$). Similarly, alpha diversity was significantly lower in pancreas, biliary, and jejunal samples in comparison to PD and AG fecal samples ($p\text{-value}\leq 0.01$). No significant difference in diversity was observed between the intestinal, bile and pancreatic groups ($p\text{-values} > 0.05$).

To compare the composition of microbial communities, we calculated weighted UniFrac distances, an accepted measure of beta diversity¹³. Community composition differed significantly between fecal samples from AG participants and PD patients, though with a very small effect size (PERMANOVA, $p < 0.05$, pseudo- $F=16.9$, $R^2=0.02$). PCoA analysis produced a cluster of PD fecal samples that grouped together apart from the pancreas, bile and intestinal samples (Figure 3A). Community membership within pancreas, bile, and jejunum samples overlapped significantly (Figure 3B).

Taxonomic features of bacterial communities in fecal, pancreas, bile, and jejunal samples

As seen in other studies, fecal samples from PD patients and healthy volunteers were generally dominated by the phyla Bacteroidetes and Firmicutes (Figure 4 and Table S3 in Supplemental Digital Content 2). However, the PD fecal samples were markedly enriched with sequences from the genera *Klebsiella*, *Bacteroides*, and *Parabacteroides*. PD samples were significantly depleted of sequences from many commensal anaerobic taxa important for intestinal health. At the taxonomic level of genus, we found that PD fecal samples were depleted of *Roseburia*, *Blautia*, and *Faecalibacterium*, which are anaerobic taxa associated with production of short chain fatty acids via fermentation. Samples from healthy volunteers contained a higher abundance of *Escherichia* and *Pseudomonas* than PD patients.

The observed changes within PD fecal samples could be a manifestation of perioperative antibiotic exposure and bowel rest. These samples were collected after a median of 5 days following surgery (Table S2 in Supplemental Digital Content 2), and the abundance of *Klebsiella* in the postoperative fecal samples generally correlated with postoperative antibiotic exposure (see Figure S2 in Supplemental Digital Content 3). However, we also found that intraoperative pancreas, bile, and jejunal samples were enriched with the same taxa, notably *Klebsiella*, suggesting that these taxa were present at the time of surgery. Taken together, these findings support the conclusion that pathogenic taxa such as *Klebsiella* were present at the time of surgery, and later grew in abundance in the postoperative period in some patients. This growth was possibly related to postop antibiotic exposure. Importantly, we found that negative controls (containing no patient sample) also contained a low abundance of *Klebsiella*. As noted, however, detailed analysis of negative controls (see Figure S1, Supplemental Digital Content 1) suggested that contamination was not the source of these taxa within patient samples.

By comparing PD samples across multiple body sites (Figure 4 and Table S3 in Supplemental Digital Content 2), we found that bile samples were enriched with sequences from the genera *Fusobacterium* and *Streptococcus* and pancreas samples were enriched with the pathogen *Acinetobacter*. Fecal samples were enriched with *Ruminococcus*, *Akkermansia*, *Bacteroides*, and *Parabacteroides*. There were no taxa that were specifically enriched or depleted in the jejunal samples.

Associating microbiota profiles with clinical features

To better understand relationships between clinical variables and the microbiome, we examined associations between microbiome data and four specific patient variables of interest: preoperative biliary stent placement, neoadjuvant chemotherapy, postoperative pancreatic leak, and death at 1 year. Each of these univariate comparisons was performed with LEfSe¹⁴. We found that pancreatic leak was strongly associated with increased abundance of *Klebsiella* and a decreased abundance of the commonly observed commensals *Parabacteroides* and *Ruminococcus* in postoperative fecal samples (Figure 5 and Table S4 in Supplemental Digital Content 2). Pancreatic leak was also associated with decreased abundance of the commensal anaerobe *Bifidobacterium* within pancreatic fluid (Figures 5A and 5B). Additionally, we found that preoperative stent placement for biliary obstruction was associated with increased abundance of *Acinetobacter* and *Sphingobium* in pancreatic samples and decreased abundance of *Haemophilus* within bile (see Table S4 in Supplemental Digital Content 2). Neoadjuvant therapy was associated with decreased *Bifidobacterium* in pancreatic fluid, increased *Bacteroides* and *Megasphaera* within bile, and increased *Clostridium* and *Enterococcus* within fecal samples. Interestingly, death at 1 year was associated with decreased *Klebsiella* within fecal samples.

Discussion

The convergence of personalized medicine and awareness about the human microbiome has naturally led to efforts to identify microbial factors associated with clinical outcomes¹⁵. Important studies have shown that configuration of the microbiota can be linked to tumor

responsiveness during chemotherapy^{3,16}, survival after stem cell transplantation¹⁷, and weight loss after Roux-en-Y gastric bypass¹⁸. In line with these concepts, we theorized that an individual's short- and long-term outcomes after PD may be similarly affected by the status of the microbiota. In particular, the notion that the microbiota contribute to the pathogenesis of pancreatic fistula represents a novel way of thinking about an old problem. Some evidence for this concept was provided in a recent article demonstrating the frequent presence of bacteria in fluid from peritoneal drains of patients that later developed POPF¹⁹.

The most striking finding from this study was an unusually high abundance of the gram-negative pathogen *Klebsiella* in samples from PD patients, particularly among fecal samples from PD patients that proceeded to develop a pancreatic fistula. Interestingly, an abundance of *Klebsiella pneumoniae* in fecal samples has been identified as a putative triggering factor involved in the pathogenesis of Crohn's disease and ankylosing spondylitis²⁰. The mechanism linking *Klebsiella* with autoimmunity involves molecular mimicry, whereby patients harboring *Klebsiella* generate cross-reactive antibodies against HLA antigens and collagen molecules²¹. We speculate that leakage from a pancreaticojejunal anastomosis could involve microbiota-induced generation of cross-reactive antibodies that contribute to collagen degradation. Such a mechanism would be analogous to a process recently described in anastomotic leakage after colon surgery². Notably, we also observed that fecal samples from patients with a leak harbored very little if any *Parabacteroides* and *Ruminococcus*, commensal anaerobes with anti-inflammatory properties²². We propose that adverse events after PD may reflect a high abundance of pro-inflammatory microbes as well as a corresponding absence of anti-inflammatory commensals. Prior studies have indicated that alterations of the gut microbiota can be strongly associated with disturbances of systemic immunity and host metabolism^{23,24}.

Surprisingly, we found highly similar microbial communities within the pancreas, bile, and jejunum of PD patients. This may be explained by bacterial spread during a preoperative procedure (namely, ERCP), or reflux from the proximal GI tract into the diseased biliary tree and pancreas. At each of these locations, we observed a preponderance of *Klebsiella*. *Klebsiella* species (most commonly *K. pneumonia* and *K. oxytoca*) are ubiquitous gram-negative organisms that cause nosocomial infections in humans²⁵ and commonly harbor genes for antibiotic resistance²⁶. Several prior studies of the microbiology of the jejunum¹⁸, biliary tree^{8,27–31}, and pancreas⁷ also identified an abundance of *Klebsiella* in patients with gallstone disease, cholangitis, and cancer. This pattern of results suggests that conditions within the pancreas and biliary tree are favorable for colonization by *Klebsiella* species, particularly in the setting of disease. Many pancreas, bile, and jejunal samples in this study were also enriched with Fusobacteria. Species from the phylum Fusobacteria (particularly *F. nucleatum*) are commonly observed in the oral cavity of individuals with periodontal disease, and recent mechanistic evidence has linked *F. nucleatum* to carcinogenesis³². Thus, as suggested by epidemiologic links between the oral microbiota and pancreatic cancer development^{33,34}, it may be that *Fusobacterium* contributes to pancreatic cancer pathogenesis in some patients.

We observed substantive differences between the microbiota identified in postoperative fecal samples from PD patients and well-established profiles of the microbes within fecal samples

from participants in the American Gut project. This result may reflect the simple fact that PD samples were collected after patients had received broad-spectrum antibiotics and had been unable to eat regular food for several days. However, it may also suggest that pancreatic cancer patients harbor distinct patterns of colonic microbiota, as has been reported for colorectal cancer patients (most notably, an increased abundance of *F. nucleatum*)^{35,36}. Thus, the microbial composition of postoperative fecal samples may have predictive value in identifying patients at high risk for pancreatic cancer. Future studies will be required to tease out these possibilities. Regardless, it is clear that the postoperative fecal microbiota deviates sharply from the patterns of microbes generally regarded as normal in healthy individuals. Notably, there is a depletion of strict anaerobes (*Bacteroides*, *Roseburia*, and *Faecalibacterium*) that are known to contribute to overall health partly by exerting systemic anti-inflammatory effects. Additionally, there is an apparent expansion of some taxa from the phylum Proteobacteria, and it is well recognized that a bloom of Proteobacteria in the gut represents an unstable configuration of the gut microbiota³⁷.

In conclusion, we have shown that clinically relevant populations of bacteria exist in pancreas, bile, and jejunal contents of patients undergoing PD, and we have shown that the gut microbiome is highly abnormal in the postoperative period. Although the human microbiome is generally resilient in healthy volunteers³⁸, it is not yet known whether microbiome abnormalities in hospitalized patients are transient or permanent. A limitation of the current study was the large number of swabs which did not generate 16S rRNA gene sequencing data – potential explanations for these sequencing failures include low microbial biomass within those specimens, variation in the amount of fluid captured on individual swabs, and the possible presence of inhibitors that interfered with PCR reactions. Further investigation with a larger sample size should make it possible to evaluate these possibilities and improve experimental protocols. It will also be possible to formally test the hypothesis that differences in these communities impacts surgical complications such as PF formation. If this were true, then there may be a role for preoperative and postoperative testing of the microbiome. Discovery of abnormal microbiome profiles may lead to interventions to specifically modify or “rescue” the microbiome on a personalized basis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Bacterial load per sample assessed via ddPCR counts

A highly conserved region of the bacterial 16S rRNA gene was targeted for biomass quantification via droplet digital PCR. Dots represent counts from technical replicates, which are plotted behind a standard boxplot. Samples were analyzed from six patients with complete sample sets, i.e. patients for whom quality DNA sequencing reads were obtained from all body sites (fecal, pancreas, bile, and jejunal samples). Different colors represent samples from different patients.

Figure 2. Alpha diversity comparisons of microbial communities within samples from pancreaticoduodenectomy patients and adult citizen scientists participating in the American Gut Project

Shown are the Chao1 indicators for each sample group. Diversity is highest in fecal samples from both PD patients and AG participants. Lower diversity is observed in the pancreas, bile, and jejunum samples.

Figure 3. Beta diversity comparisons of microbial communities within samples from pancreaticoduodenectomy patients and AG participants

Displayed are principal component analyses of weighted UniFrac distances between (A) all samples analyzed, including fecal samples from AG participants and (B) intraoperative samples from the pancreas, bile, and jejunum of PD patients. Axis labels indicate the proportion of variance explained by each principal coordinate axis. In panel (A), fecal samples from AG participants and fecal samples from PD patients cluster separately within PCoA space (PERMANOVA, $p < 0.001$, $R = 0.02$). As shown in panel (B), samples from

the pancreas, bile, and jejunum overlap significantly within PCoA space rather than clustering according to body site.

Figure 4. Mean relative abundances of bacterial taxa in samples from pancreaticoduodenectomy patients and AG participants

Taxonomic assignments are shown at the level of (A) phylum and (B) genus. Note the expansion of *Klebsiella* (phylum Proteobacteria) in all PD samples relative to fecal samples from AG citizen scientists. A complete list of taxa found to be enriched or depleted within PD samples is provided in Supplemental Table 4.

Figure 5. Abundance of bacterial taxa in fecal samples from PDA patients with and without postoperative pancreatic fistula

Shown is the abundance of (A) *Klebsiella* and (B) *Parabacteroides* in fecal samples from 227 healthy adult volunteers, 42 PDA patients without fistula, and 8 PDA patients with fistula. For both genera, the mean relative abundance among patients with and without fistula was significantly different ($p < 0.05$).