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Salivary Biomarker Evaluation of Chronic Pancreatitis Patients Reveals Alterations in Human Proteins, Cytokines, Prostaglandin E_2 Levels, and Bacterial Diversity

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Objectives: Chronic pancreatitis (CP) is a chronic fibroinflammatory condition of the pancreas difficult to diagnose in early stages. Novel biomarkers useful to facilitate early diagnosis or treatment responses may be found in biofluids. Although saliva can be easily and noninvasively collected from patients, useful salivary biomarkers from CP patients have not yet been identified.

Methods: Here, we analyzed the proteome by quantitative proteomics, cytokine/chemokine levels by Luminex analysis, prostaglandin E_2 (PGE_2) levels by a mass spectrometry-based assay, and bacterial species diversity by 16S ribosomal ribonucleic acid sequencing in saliva samples from confirmed CP patients and healthy controls.

Results: Our results indicate the presence of various differentially expressed proteins, cytokines/chemokines, and a loss of oral bacterial diversity in the saliva of CP patients. The PGE_2 levels trend toward elevation in CP patients. Area under the receiver operating characteristic curve models for proteomic, cytokine, and PGE_2 assays ranged from 0.59 to 0.90.

Conclusions: Collectively, our studies identify a range of putative CP biomarkers and alterations in human saliva requiring further validation. The biomarker discovery approaches we used might lead to identification of biomarkers useful for CP diagnosis and monitoring.

Key Words: chronic pancreatitis, saliva biomarkers, quantitative proteomics, Luminex analysis of cytokines/chemokines, prostaglandin E_2, bacterial diversity

(*Pancreas* 2022;51: 723–732)

Chronic pancreatitis (CP) is a painful, debilitating disease of the pancreas with limited treatments. Its causes include both environmental (excessive alcohol drinking, smoking) and genetic (involving, eg, mutant *PRSS1*, *SPINK1*, *CFTR*, or other gene loci) mechanisms. At early stages, the pancreas becomes fibrotic, and ongoing structural changes are present along with abdominal pain. Later stages are accompanied by loss of pancreas exocrine and endocrine function, continued pain, and further systemic alterations. Later stages can be detected by clinical imaging, but diagnosis of early CP remains problematic. Improved diagnosis and patient monitoring could be achieved through the identification and use of novel disease biomarkers. Biofluids and liquid biopsy represent ideal sources of biomarkers as they can be collected noninvasively and tested at reasonable expense.

Among potentially useful biofluids, saliva is especially straightforward to collect. However, its potential as a source of valuable disease biomarkers of CP remains unproven. Saliva has been used as a source of biomarkers in a limited number of studies. One previous study examined microbial genes in the saliva by a microarray approach and found distinct patterns of bacterial species colonizing the oral cavity in individuals with CP or pancreatic cancer versus controls.[1] Like human plasma or serum, the human saliva proteome contains some abundant proteins, such as the salivary amylase, that dominate proteomic data sets.[2] To discover new ways of diagnosing CP earlier in the course of the disease, we analyzed saliva from CP patients and matched controls with no history of pancreatitis, pancreatic tumors or cysts, or chronic abdominal pain.

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Received for publication November 12, 2021; accepted August 26, 2022.

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The content of this article is solely the responsibility of the authors and does not necessarily represent the official views of the National Cancer Institute, the National Institute of Health, or the National Institute of Diabetes and Digestive and Kidney Diseases. This study was supported by the Consortium for the Study of Chronic Pancreatitis, Diabetes, and Pancreatic Cancer and by grants from the National Cancer Institute and the National Institute of Diabetes and Digestive and Kidney Diseases under the following award numbers: U01DK108326, Baylor College of Medicine; U01DK108314, Cedars-Sinai Medical Center; U01DK108288, Mayo Clinic; U01DK108300, Stanford University; and U01DK108328, University of Texas MD Anderson Cancer Center. This study was also supported by the Department of Defense (W81XWH-19-1-0888 and W81XWH-20-1-0317). The work by J.Z. was funded (in part) by research training awards from the Cancer Prevention and Research Institute of Texas for the Cancer Prevention Post-Graduate Training Program in Integrative Epidemiology (RP160097; PI: M. Spitz) and the Systems Epidemiology of Cancer Training Program (RP210037; PI: A. Thrift). R.T.W. is the recipient of an award from Cedars-Sinai Precision Health.

The authors declare no conflict of interest.

Supplemental digital contents are available for this article. Direct URL citations appear in the printed text and are provided in the HTML and PDF versions of this article on the journal's Web site (www.pancreasjournal.com).

DOI: 10.1097/MPA.0000000000002113

Samples were collected at the Mayo Clinic (Rochester, Minn) and assayed at the Mayo Clinic and 3 other centers participating in the study, where different analytical methods and end points were applied. Methods included unbiased quantitative shotgun proteomics to assess changes in human proteins, Luminex assays to specifically examine cytokines, chemokines and growth factors, a mass spectrometry–based assay of the bioactive lipid prostaglandin E_2 (PGE_2), and 16S ribosomal ribonucleic acid (RNA) sequencing to explore changes in oral bacterial species diversity.

MATERIALS AND METHODS

Saliva Collection and Distribution

Saliva was collected at the Mayo Clinic from consented patients according to the following instructions. Subjects were to avoid brushing their teeth, using mouth washes or rinses, or eating for ≤1 hour before collection; avoid chewing gum or cigarette use for ≤30 minutes before collection; and avoid ingestion of alcohol for ≤12 hours before collection. They drooled into a specimen cup for 5 minutes, providing at least 1 mL of saliva apart from foam. Samples were immediately placed on ice, then aliquoted into cryovials, labeled with barcodes, and frozen at −80°C within 4 hours of collection. Samples were collected from 38 definite CP patients with unequivocal CP (Cambridge grade 3 or 4), and/or parenchymal and/or ductal calcifications by computed tomography, magnetic resonance imaging, or magnetic resonance cholangiopancreatography, or pancreatic histology diagnostic of CP as per the PROCEED study,[3] as well as 43 healthy control subjects. Chronic pancreatitis patients exhibited multiple etiologies, including many (22/38 = 57.89%) idiopathic cases, several (8/38 = 21.05%) alcohol-related cases, and a few (3/38 = 7.89%, 2/38 = 5.26%) biliary and hereditary cases, respectively, and a few others (1 each hypertriglyceridemia-related, smoking-related, or drug-induced). Samples were labeled as "A" or "B" type samples, respectively (similarly, males and females were designated as either sex groups 1 or 2) to ensure that investigators performing further analyses were blinded to the disease status and sex of the patient corresponding to each sample. Controls and CP patients were matched in age (median, 58 and 56 years, respectively), with overall range of 18 to 69 years. Females comprised 48% of controls and 44% of CP cases. Saliva samples collected from suspected CP and pancreatic ductal adenocarcinoma patients were not subjected to all the analyses presented here and were therefore excluded from this report. Samples stored at −80°C were retrieved, sorted, and either used to measure PGE_2 assays at the Mayo Clinic or shipped on dry ice by rapid overnight transit to investigators at Cedars-Sinai Medical Center (CSMC; Los Angeles, Calif), Stanford University (Palo Alto, Calif), and Baylor College of Medicine (BCM; Houston, Texas) for other analyses. The study protocol, including saliva sample collection procedures, was approved at Mayo Clinic (Mayo Clinic Institutional Review Board #16-009434). Proteomic analysis at CSMC was performed with approval from the CSMC IRB, as documented in #Pro00048082.

Proteomic Sample Preparation of Saliva Proteins

Proteomic studies were performed at CSMC. Twenty-five (A) and 25 (B) (~0.25 mL) samples were thawed, triethylammonium bicarbonate (TEAB) buffer (100 mM, pH 8.5) was added to achieve a 300 µL volume, then samples were microprobe sonicated on ice for 1 minute (3 bursts of 20 seconds) to reduce viscosity. Samples were spun at 14,000 rpm for 15 minutes at 4°C, and any pelletable material such as bacteria was removed. Protein in the samples was then precipitated using 10% trichloroacetic acid and incubated overnight at −20°C to maximize yield. Samples were spun at 14,000 rpm at 4°C for 30 minutes to recover protein pellets, redissolved in 100 µL TEAB, and protein concentration assessed by MicroBCA Assay (Thermo Fisher Scientific, Carlsbad, Calif) according to the manufacturer's instructions. At this point, the sample number was reduced to 20 controls and 20 CP patients to remove some samples with very low protein levels. Equal amounts of protein (25 µg) were transferred to fresh Eppendorf Protein LoBind tubes. Tris(2-carboxyethyl)phosphine HCl was added to 12.5 mM, and the samples reduced at 55°C for 1 hour, then 25 mM iodoacetamide was added and the samples alkylated in the dark at room temperature (RT) for 30 minutes. Dithiothreitol (10 mM) was added, and samples shaken on a thermomixer at 300 rpm at RT for 15 minutes to react excess iodoacetamide. Trypsin (predissolved in TEAB supplemented with 2.5 mM $CaCl_2$) was added to a final trypsin-protein ratio of 1:32, and the tubes were incubated for 16 hours, standing vertically in a 37°C water bath.

Tandem Mass Tag Labeling and Peptide Fractionation for Quantitative Proteomics

Peptides were labeled with 10-plex TMT[4] (Thermo Fisher Scientific) according to manufacturer's instructions and combined into nineplex samples according to the scheme shown in Supplemental Figure 1 (http://links.lww.com/MPA/A970). Briefly, tandem mass tag (TMT) reagents were dissolved with 41 µL of acetonitrile and the peptides of each patient sample labeled individually or as a mixed reference sample (all 40 samples combined in equal amounts). The reactions were performed with mixing (400 rpm for 1 hour at 25°C); then 8 µL of 5% hydroxylamine was added to quench. Peptide mixtures were combined into 5 nineplexes, each with (4) A, (4) B, and the reference (labeled with TMT-126). Excess free label and salts were removed by desalting on OASIS HLB (1 mL) tips as described in McDowell et al[5] using a vacuum manifold for washing steps and a benchtop centrifuge (400*g* for 3 minutes) for flow-through and elutions (collected in 15-mL tubes). The samples were flowed-through twice before washing. Eluted peptide mixtures were dried in a SpeedVac concentrator, then redissolved and fractionated into 8 fractions using high pH reverse-phase fractionation kit (Thermo Fisher 84868) according to manufacturer's instructions. These were catenated by combining fractions 1-4-7, 2-5-8, and 3-6. Peptide concentrations were determined using the Pierce quantitative colorimetric peptide assays (Thermo Fisher 23275). Samples were stored at −80°C until mass spectrometric analysis.

Proteomic Analysis and Data Processing

Proteomic analysis using LC-SPS-MS3 (liquid chromatography–synchronous precursor selection–mass spectrometry 3) was performed essentially as described.[6] Combined peptide fractions were separated by low-pH reversed-phase liquid chromatography and analyzed by (LC-SPS-MS3) using an EASY-nLC1200 chromatogram connected to a Fusion Lumos mass spectrometer (both Thermo Fisher Scientific). Briefly, peptides were loaded onto a 2-cm trap column (PepMap 100 C18, 75 µm inner diameter, 3 µm particles, 100 Å pore size) and separated by a 50-cm EASY-Spray column (PepMap RSLC C_{18}, 75 µm inner diameter, 2 µm particles, 100 Å pore size) heated to 55°C (all columns from Thermo Fisher Scientific). For low-pH reversed-phase liquid chromatography separation, the mobile phase consisted of 0.1% formic acid in water (phase A) or 80% acetonitrile (phase B). The 4-hour LC gradient was 3%–33% B over 180 minutes, 33%–100% B over 40 minutes, and 100% B over 20 minutes at a flow rate of 200 nL/min. Subsequently, SPS-MS3 analysis was conducted with parameters set for Fourier transform MS1, that is, orbitrap resolution (120,000), scan range (400–1400), maximum injection time (100 milliseconds), data

type (centroid), charge state (2–5), mass tolerance (7 ppm), and internal calibration using m/z 371.10123; for Ion trap MS2, mass range (400–1400), dependent scan number (10), isolation window (0.4 m/z), activation type (rapid collision–induced dissociation), collision energy (35%), maximum injection time (120 milliseconds), and data type (centroid); for MS3, mass range (400–2000), precursor ion exclusion (low m/z 50, high m/z 5), isolation window (m/z 0.7), MS2 isolation window (m/z 2), higher-energy collisional dissociation collision energy (55%), orbitrap resolution (50,000), maximum injection time (150 milliseconds), and data type (centroid). Raw files were processed using Maxquant[7] to identify and quantitate proteins using MS3 reporter ions, TMT10plex for the isobaric labels, and a reporter mass tolerance of 0.003 Da. A false discovery rate of 1% was applied to filter peptide-spectrum matches, peptides, and protein groups.

Luminex Assays Measuring Secreted Factors

Luminex assays (ProcartaPlex Human Immune Monitoring 65-plex; Thermo Fisher, Santa Clara, Calif) were performed on saliva samples (24 controls and 24 CP) by the Human Immune Monitoring Center at Stanford University according to the manufacturer's recommendations with modifications as described. In brief, samples were combined with mixed antibody-linked beads in a 96-well plate, then incubated for 1 hour at RT and overnight at 4°C. All incubation steps were performed on an orbital shaker at 500 to 600 rpm. Plates were then washed 3 times in a Biotek ELx405 plate washer and mixed biotinylated detection antibodies added for 75 minutes at RT. Then, streptavidin-phycoerythrin reagent was added, and the plates were incubated for a further 30 minutes at RT. After a final wash as aforementioned, reading buffer was added to the wells. Each sample (50 μL of 3-fold diluted stock) was measured in duplicate. Plates were read using a Luminex 200 instrument with a lower bound of 50 beads/sample. An internal quality check was performed using Custom AssayChex control beads (Radix Biosolutions, Georgetown, Tex) across all wells of the assay.

Prostaglandin E_2 Assay

Analysis of PGE_2 was performed by LC-MS/MS (liquid chromatography–tandem mass spectrometry) at the Immunochemical Core Lab, Mayo Clinic. Saliva was combined with deuterated internal standard, indomethacin, formic acid, and acetonitrile before liquid-liquid extraction with ethyl acetate hexane. Extracts were injected onto a reversed-phase high-performance LC analytical column with C12 guard cartridge and analyzed via an Agilent 6490 tandem mass spectrometer (Agilent Technologies, Santa Clara, Calif) with electrospray ionization in negative mode. Interassay imprecision coefficient of variance (CV) was found to be 4.5% to 10.5% for samples ranging from 133 to 530 pg/mL, and lower limit of quantitation was defined as 77 pg/mL with a CV of 14.6%.

Microbiome Sequencing and Analysis

These studies were performed at BCM. Total genomic DNA was extracted from saliva with the DNeasy PowerSoil Kit (Qiagen, Inc, Redwood City, Calif) using methods optimized to maximize the yield of bacterial DNA.[8,9] Individual KAPA Hyper (Kapa Biosystems, Inc, Wilmington, Mass) libraries constructed from each DNA sample were sequenced on the HiSeq X platform (Illumina, Inc, San Diego, Calif) using the 2 × 150 bp paired-end protocol. Demultiplexed raw FASTQ files were quality filtered and mapped against a combined PhiX (sequencing control) and hg38 host reference genome database[10] to remove unwanted reads. The final set of filtered reads was processed using MetaPhlAn2,[11] which generates species-level normalized relative abundances per sample per taxon using marker genes for taxonomy assignment.

Statistical Methods

Group comparisons of CP versus controls were tested via 2-sample Student *t* test (log-transformed values of proteomics, Luminex, and PGE_2 data). Beta diversity of microbiome data was analyzed using permutational analysis of variance and the Mann-Whitney *U* test (microbiome data). Comparisons of taxa abundance were performed with linear discriminant analysis effect size methods. Box plots (median, 25th and 75th percentiles) were used for visual representation of data in both groups. Differentially expressed proteins ($P < 0.10$) were expressed as log-2 transformed fold changes (CP/control). Receiver operating characteristic curves and the area under the curve (AUC) were used to assess the discriminatory ability of biomarkers or combinations of biomarkers. Two different approaches were used for a combined proteomic approach. First, the 3 most differentially expressed proteins (excluding histones) were selected to create a combined test. Area under the curve was calculated using logistic regression. Second, we also developed an in silico predictive model using an elastic-net regularized logistic regression. Bootstrap resampling was performed with 1000 replicates to estimate AUC correcting for overfitting. Regularization and mixing parameters were chosen maximizing AUC with the smallest possible set of proteins. Relative protein weights were calculated based on regression coefficients, and Pearson correlation coefficient was also calculated for selected proteins.

Area under the curve was also calculated for the 6 upregulated cytokines determined by Luminex analysis and for the PGE_2 data. Luminex data were subjected to multivariate analysis. Duplicates of median fluorescence units (MFUs) were compiled into group (CP or control) means to calculate the fold change of cytokines as CP/control. Fold-changes and compiled MFU group values were log2-transformed to facilitate comparison, and differences evaluated by 2-sided Student *t* test. A penalized (Lasso) regression model was used to select cytokines for a multivariable model for CP, using cross-validation to determine an appropriate penalty parameter. The association between the differentially expressed cytokines measured across the 48 samples was evaluated using Pearson correlation coefficient. Associations between bacterial alpha diversity and age were explored using linear regression within each group. Principal component analysis (PCA) was applied to bacterial diversity using unweighted Jaccard distance (a measure of dissimilarity) and by weighted Bray-Curtis distance (a measure of relative abundance). Unless otherwise noted, a $P < 0.05$ significance level was used. Given the exploratory nature of these analysis and the expectation that findings must be validated in future studies, no adjustments for multiple hypothesis testing were made, unless otherwise noted. Several software packages were used: R (R Foundation for Statistical Computing, Vienna, Austria), version 3.5.3; IBM SPSS statistics version 24 (Armonk, NY); GraphPad Prism version 6.00 for Windows (La Jolla, Calif), and Atima (https://atima.research.bcm.edu).

RESULTS

Quantitative Proteomic Analysis of Human Proteins in Saliva

Five nineplex peptide mixtures, each fractionated into 3 samples, were run consecutively, with satisfactory calibration runs performed before and after using a trypsin-digested albumin standard. In the consolidated LC-MS/MS data set, a total of 544 human proteins were identified (see Supplemental Table 1, http://

links.lww.com/MPA/A970). Of these, 247 were detected across all (20 A, 20 B) experimental subjects, and a further 67 proteins were detected across 16/20 A/B expression ratios (Supplemental Table 2, http://links.lww.com/MPA/A970); these data were further examined using Perseus to determine the A/B expression ratios and their associated statistical significance. By this analysis, 28 proteins (14 elevated and 14 decreased) achieved significance ($P < 0.1$) with various fold changes. Five elevated and 3 decreased proteins achieved a significance level of $P < 0.05$ and a fold change of 1.5 (see Fig. 1 and Supplemental Table 2, http://links.lww.com/MPA/A970). The proteins found substantially increased or decreased in expression levels in CP patients included lysozyme C (LYZ), the most elevated at ~1.9-fold. Other substantially elevated proteins were high mobility group protein B2 (HMGB2) and histone H1B (HIST1H1B), both elevated ~1.8-fold. Lymphocyte-specific protein 1 (LSP1) was elevated ~1.5-fold. The 3 proteins found significantly decreased in the CP patients (Supplementary Table 2, http://links.lww.com/MPA/A970) were α-2-macroglobulin–like protein 1 (A2ML1, 0.66-fold), bactericidal/permeability-increasing fold-containing family B member 1 (BPIFB1, also 0.66-fold), also known as LPLUNC1, and IgGFc-binding protein (FCGBP, 0.52-fold). Twenty-eight proteins that were either elevated or decreased in the CP saliva samples are depicted in Figure 1A.

We next examined the gene ontology categories (biological processes, cellular compartments, and molecular functions) most closely associated with the elevated proteins (Fig. 1B, upper panel) and decreased proteins (Fig. 1B, lower panel), using ToppGene Suite (Cincinnati Children's Hospital Medical Center, Cincinnati, Ohio).[12] Box plots for the 4 top differentially overexpressed proteins LYZ, HMGB2, HIST1H1B, and LSP1 are depicted in Figure 1C.

The value for AUC for LSP1, 0.75 (not shown), suggested that the accuracy of a test of LSP1 in the saliva might be fair as a diagnostic biomarker of CP. Shown in Figure 1D, we also calculated the AUC derived from a combined test of 3 analytes, LSP1, HMGB2, and LYZ. A cutoff of 1.37-fold as the expression ratio for these 3 proteins yielded an AUC value of 0.86.

In Supplemental Figure 2A (http://links.lww.com/MPA/A970) a diagram with our predictive model for the proteome illustrates the relationships between AUC and elastic-net parameters for a number of potential models. As this analysis shows, the overfitted-corrected AUC is more conservative than our previous analysis requiring a larger number of proteins to achieve a maximum AUC of 0.64. We chose the parameters of regularization = 0 and mixing = 0.7 resulting in an AUC of 0.60 achieved with 50 proteins (Supplemental Fig. 2A, http://links.lww.com/MPA/A970). Supplemental Figure 2B (http://links.lww.com/MPA/A970), shows a plot of the weights associated with each of the 50 proteins such that LYZ, HMGB2, and LSP1 are among them, and Supplemental Figure 2C (http://links.lww.com/MPA/A970), shows a correlogram of selected proteins.

Multiplex Luminex Analysis of Saliva Samples

We tested the saliva samples for a panel of cytokines and chemokines by Luminex assays. In this case, 24 controls were compared with 24 CP patients, as described in the Materials and Methods. Data showed that the cytokines interferon gamma–induced protein 10 or C-X-C motif chemokine ligand 10 (IP-10/CXCL10), growth-regulated oncogene alpha/C-X-C motif chemokine ligand 1 (GROA/CXCL1), stromal cell–derived factor 1/C-X-C motif chemokine ligand 12 (SDF-1/CXCL12), interleukin (IL) 21, tumor necrosis factor–related apoptosis-inducing ligand (TRAIL), and Fas ligand (FASLG) were significantly and selectively elevated in CP saliva samples (shown in Fig. 2A). There were no analytes found decreased significantly. Scatterplots of the data showing elevated cytokines are shown in Figure 2B. Further analysis revealed positive correlations between several analytes, with high Pearson correlation coefficients between SDF-1/CXCL12 and IL-21 among others. In Figure 2C, Pearson correlation coefficients calculated and tabulated for the data corresponding to the 6 cytokines selectively elevated in CP saliva are depicted. A linear plot of the highly correlated data of SDF1/CXCL12 with IL-21 is shown in Figure 2D. Penalized regression selected 8 variables to distinguish between CP and controls, with the largest coefficients assigned to FASL and IP-10/CXCL10. The resulting model had an AUC of 0.90 (Supplemental Fig. 3, http://links.lww.com/MPA/A970).

PGE_2 Analysis of Saliva Samples

Analysis of pancreatic juice, a relatively invasive procedure, revealed that PGE_2 levels are elevated in CP patients in the pancreatic juice.[13] It was therefore of interest to examine whether saliva, a much more accessible biofluid, could provide a similar result. Indeed, measures of PGE_2 in 27 controls and 25 CP patients exhibited values trending toward elevation in the CP cases. The mean values, as shown by the scatterplot in Figure 3, were 94.0 (standard deviation [SD], 21.47) for controls (n = 27) and 245.4 (SD, 129.9) (n = 25) for CP, with $P = 0.24$. Receiver operating characteristic analysis of PGE_2, which yielded an area under the receiver operating characteristic curve of 0.589, is shown in Supplemental Figure 4 (http://links.lww.com/MPA/A970).

Bacterial Species Identification and Diversity Analysis Using 16S Ribosomal Ribonucleic Acid Sequencing

Ribonucleic acid sequencing of 16S RNA was used to identify bacterial species in the saliva of CP patients and controls, and these data were analyzed to compare the diversity and species identification patterns between the 2 groups. In Figure 4A, the comparison of bacterial species alpha diversity is depicted by observed diversity, an indicator of richness, that is, an approximation of the number of taxa, and Shannon diversity, a specialized index that determines how evenly distributed the individual species are between identified taxa. By the observed alpha diversity (Fig. 4A, left panel), the control samples were higher, at ~70 operational taxonomic units (OTU), than CP patients at ~66 OTU. This comparison nearly achieved statistical significance, with a $P = 0.06$. In Shannon diversity (Fig. 4A, right panel), both controls and CP patients saliva were close to values of 3.0, with the comparison evaluated with $P = 0.63$, as shown in Figure 4A.

We next examined the bacterial alpha diversity as a function of age, shown in Figure 4B. Observed diversities of the controls and CP patients were plotted in OTU (Fig. 4B, left panel) or Shannon diversity index (Fig. 4B, right panel) versus age. When applying PCA to bacterial diversity by unweighted Jaccard distance, a measure of dissimilarity, approximately 14.6% (Fig. 4C, left panel) of the variation could be explained by a single principal component. By weighted Bray-Curtis principal coordinates analysis, a measure of relative abundance (Fig. 4C, right panel), only 19.2% and 14.3% of the variability were explained by components 1 and 2, respectively.

The Jaccard and Bray-Curtis distance measures of beta diversity were also measured to determine their contributions to the overall variability observed between control and CP patients' saliva samples (Fig. 4D). Both Jaccard (left panel) and Bray-Curtis distances (right panel) were higher in comparisons of CP versus CP than between controls or between controls and CP, as shown in

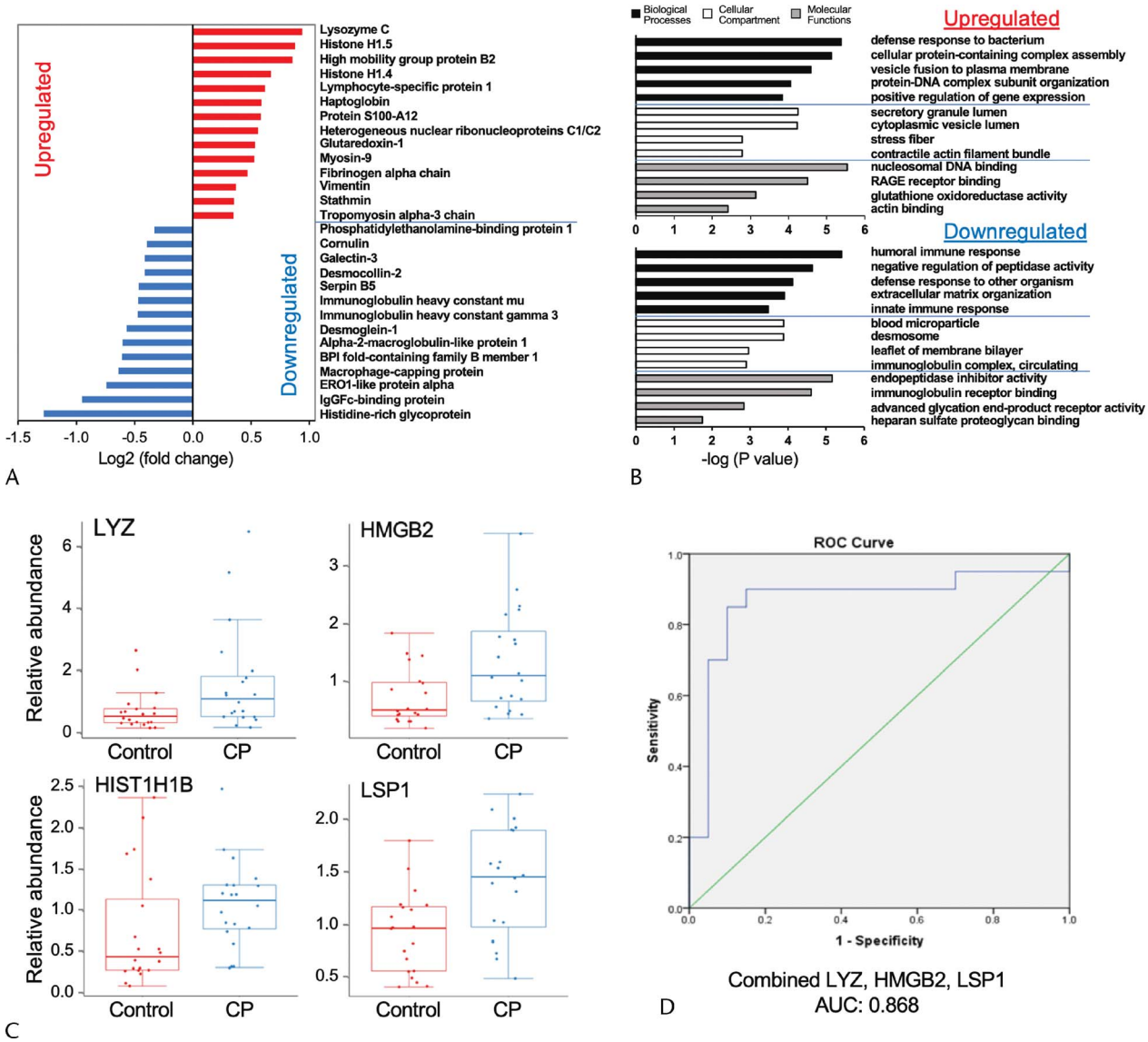


FIGURE 1. Quantitative proteomic analysis of human proteins in chronic pancreatitis (CP) patients versus healthy volunteers. A, Proteins changed up (red bars) or down (blue bars) in CP, with a cutoff of statistical significance of $P < 0.1$. The log2-transformed fold change is indicated on the *x*-axis. B, Prominent gene ontology categories revealed by ToppGene analysis of the elevated proteins (upper panel) or the decreased proteins (lower panel). The legend indicates the associated biological processes, cellular components, and molecular functions, with the –log of the *P* value indicated on the *x*-axis. C, Box plots of prominently elevated proteins in the saliva of CP patients. Associated *P* values were LYZ, 0.017; HIST1H1B, 0.017; HMGB2, 0.006; and LSP, 0.004. D, AUC analysis for a combined test of LYZ, HMGB2, and LSP1, with a cutoff of 1.4-fold elevation of each protein.

Figure 4D. Finally, the relative levels of certain specific taxa present in the saliva were examined and their relative abundances measured in the 2 groups, as shown in Figure 4E.

DISCUSSION

Endoscopic ultrasound and a range of other imaging techniques are clinically available and typically used to diagnose CP,[14,15] but these are costly to implement for routine investigation. Cost-effective and rapid means are needed to accurately identify CP patients early in the disease course, when it may be possible to introduce measures that prevent or slow the progression of the disease.[16] Biomarker testing has potential to enable CP diagnosis, but has not been achieved yet. Recently, it was reported that microbial RNA from oral bacteria can be identified in pancreas tissues.[17] However, biomarkers of pancreatic disease are currently being sought in biofluids that do not require invasive procedures. Whereas a wide variety of potential CP biomarkers were recently evaluated,[18] practically all detected in blood samples, many of the potential saliva biomarkers we identified here have not been examined to date in other sample types.

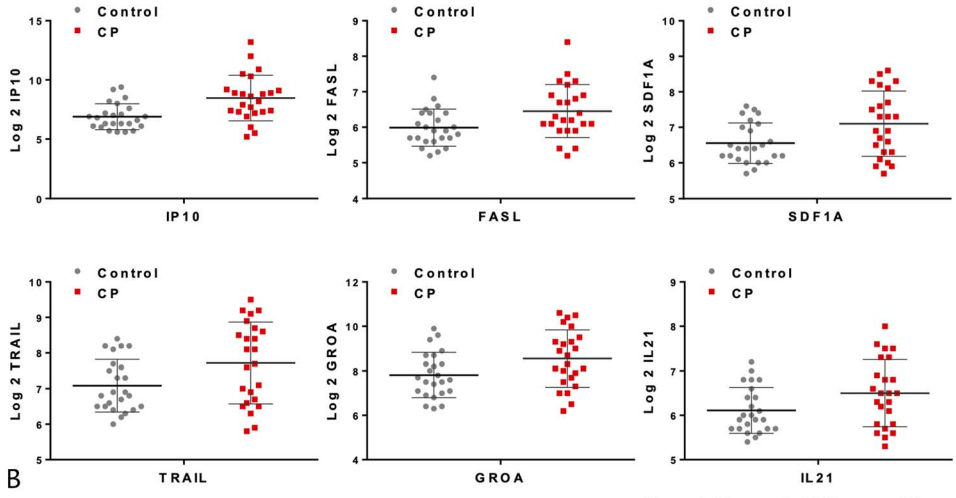
Potential human salivary proteomic biomarkers of pancreatic ductal adenocarcinoma were recently reported.[19] Here we examined the potential of saliva to provide protein biomarkers of CP and performed other measures to provide insight into the pathogenesis of pancreatic diseases.

Proteomic analysis of saliva samples indicated that several proteins were elevated in the saliva of CP patients. Among the

Luminex analytes elevated in CP vs Control:

Analyte	Gene name	Description	Log2 FC	FC	*P*
IP10	CXCL10	C-X-C motif chemokine ligand 10	2.562	5.905	0.001
FASL	FASLG	Fas ligand	0.571	1.485	0.016
SDF1A	CXCL12	C-X-C motif chemokine ligand 12	0.713	1.639	0.016
TRAIL	TNFSF10	tumor necrosis factor superfamily member 10	0.848	1.800	0.027
GROA	CXCL1	C-X-C motif chemokine ligand 1	0.891	1.854	0.033
IL21	IL21	interleukin 21	0.486	1.401	0.044

A



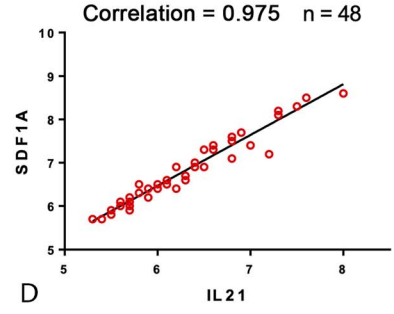
B

Pearson correlation coefficients

	SDF1A	IL21	GROA	IP10	FASL	TRAIL
SDF1A	1	0.975	0.852	0.588	0.855	0.894
IL21		1	0.775	0.542	0.865	0.854
GROA			1	0.666	0.661	0.929
IP10				1	0.425	0.700
FASL					1	0.734
TRAIL						1

All correlations are significant at the 0.01 level (2-tailed)

C



D

FIGURE 2. Luminex analysis of saliva from healthy controls and CP patients. A, List of substantially elevated proteins found in the comparison of control versus CP. Raw Luminex data from a multiplex assay were performed using 24 control and 24 CP saliva samples as described in the Materials and Methods. Fold changes (FC) were determined from raw values of MFUs for each determination by combining the data from each group into mean values. Fold change converted to log2 for comparison is defined as the ratio between the groups, that is, CP/control. Multivariate analysis and Student *t* test were used to determine the statistical significance, using the log2 transformed MFU values of control and CP (n = 24 in each group). B, Scatter dot plots of each of the analytes found selectively elevated in CP. C, A correlation matrix depicting statistical correlation (Pearson correlation coefficients) between the 6 analytes found selectively elevated in CP. D, A linear plot of data corresponding to the highest 2 correlated analytes, IL-21, and SDF-1/CXCL12.

human proteins we identified, LYZ is an immune defense protein synthesized by salivary glands and immune cells as an antibacterial protein serving immune functions to protect the oral cavity.[20] The LYZ protein is also expressed by monocytes and macrophages, which together with neutrophils are immune cell types known to participate in pancreatitis progression.[21] Human HMGB2 is closely related (83% identical in protein sequence) to HMGB1 and shares its DNA-binding characteristics, but its potential role as an inflammatory mediator is not as well established as HMGB1, which is being studied intensively as a damage-associated molecular pattern protein with a proinflammatory role in pancreatic disease and was previously found elevated in serum of pancreatitis patients.[22] The source of HMGB2 is unknown, but it could derive from damaged cells at disease sites. Systemically mobilized histone proteins were recently implicated as mediators of acute pancreatitis.[23] Whereas LYZ and HP (haptoglobin) are secreted proteins, HIST1H1B and HMGB2 are nuclear proteins. Both elevated secreted and nuclear proteins fit a pattern that was established in pancreatic fluid markers in CP.[24] The roles of damage-associated molecular pattern such as HMGB1/2 and histones in the activation of NLRP2 inflammasome-mediated pathways have emerged as an important process in CP.[25–27] The protein S100A12 (also known as ENRAGE) is a receptor for advanced glycation end-products–binding protein of the calgranulin family, associated with proinflammatory activity.[28] Nonetheless, further studies will be required to establish the pathophysiologic role in CP and relevance of salivary elevation of these factors.

As shown in Figure 1D, the hypothetical AUC for our data describing a triplicate test of LSP1, HMGB2, and LYZ was 0.868. Thus, such a test could hypothetically be seen as an excellent

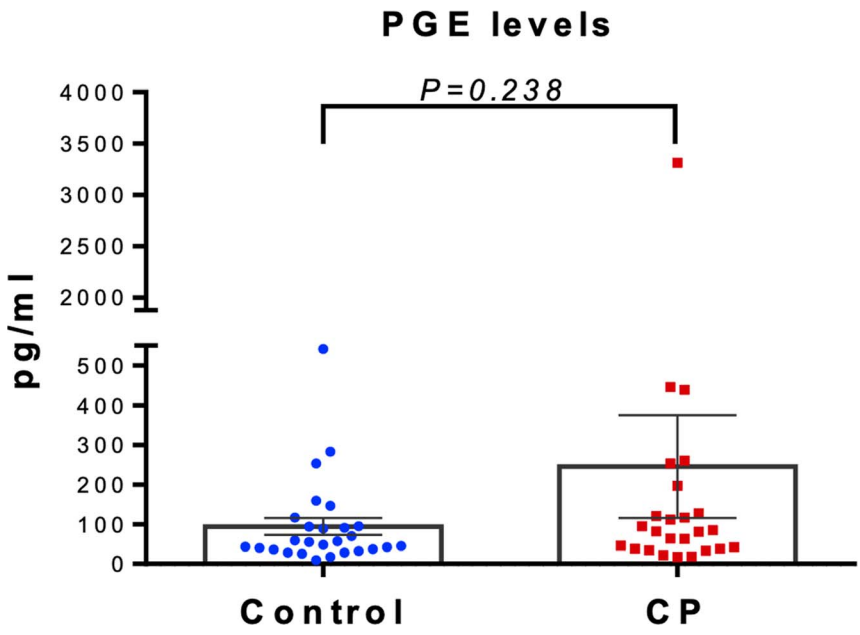


FIGURE 3. Prostaglandin E_2 analysis was carried out between 26 control and 27 CP saliva. These data were analyzed for fold change, and a 2-tailed Student *t* test was carried out to determine the statistical significance of the values obtained. A scatter dot plot is shown. *P* value for comparison of control and CP values was calculated as 0.238.

indicator of the presence of CP. However, performing AUC testing on a set of differentially expressed markers using the same data used to determine the markers, as we have done here, is known as overfitting as it tends to overinflate AUC values. Results of a less biased approach, the regression modeling selected, for example, 50 proteins with predicted AUC of 0.60, a much weaker performance (see Supplemental Fig. 2, http://links.lww.com/MPA/A970). Albeit the modeling also selects LYZ, HMGB2, and LSP1, whether the presence of these proteins or other markers in the saliva has predictive value or potential to detect CP requires further clinical validation.

One protein we found decreased in CP saliva, BPIFB2, appears to play important protective roles in mucosa. Expression of this protein has been localized to the oral/nasal/respiratory passages.[29] One of the most decreased proteins in our data set is FCGBP, a mucin-like protein that forms dimers with other known mucous barrier-protective proteins such as trefoil factor.[30] This protein may regulate epithelial mesenchymal transition, a process important for development of cancer malignancy and metastases.[31] The FCGPB protein has previously been detected in the saliva,[32] where it may serve as a protective factor in the oral cavity. The importance of replenishing such decreased protective factors could be considered as preventive therapies against worsening of pancreatic disease.

In our Luminex analysis, interferon gamma–induced protein of 10 kDa (IP-10, also known as CXC chemokine ligand 10 or IP-10/CXCL10), TRAIL, and growth-regulated alpha protein (GROA, also called CXC chemokine ligand 1 [GROA/CXCL1]) were elevated significantly in CP patients compared with healthy controls. IP-10, which we found elevated in the saliva, was previously found elevated in the blood of CP patients, where among several factors it alters the quality of life in the disease.[33] The IP-10/CXCL10 chemokine was previously implicated as a mediator of progression of pancreatitis-like disease in a viral model.[34]

The classic proinflammatory cytokine GROA/CXCL1 is involved in chemoattraction of immune cells and expression of inflammatory pain mediators in different cell types and is clearly involved in pancreatitis as a chemoattractant for neutrophils or other leukocytes.[35,36] It was shown to be elevated in severe acute pancreatitis,[37] and mechanistically, its elevation was attributed to an IL-6 signal transducer and activator of T cells 3 signaling pathway relevant to acute lung injury.[38,39] The cytokines/chemokines GROA/CXCL1 and IP-10/CXCL10 are also previously detected in the saliva in both oral disease and other systemic diseases and cancer.[40] Hypothetically, IP-10/CXCL10 is a cytokine/chemokine involved in progression of AP to CP, and its expression is induced by interferon gamma, which was reported as consistently elevated in blood and/or pancreas tissue in CP.[41] It is also widely hypothesized to be involved in diabetes mellitus and has been contemplated as a therapeutic target in this disease.[42] Interleukin 21 is associated with autoimmune disease[43] and more specifically with immune thrombocytopenic purpura. In immune thrombocytopenic purpura, IL-21 is elevated and SDF-1/CXCL12 also trends toward an increase.[44] Immune thrombocytopenic purpura was recently associated with increased pancreatitis risk.[45] SDF1/CXCL12 was also hypothesized to be important in CP.[36] C-X-C motif chemokines and other factors were recently hypothesized to play roles in progression of CP to pancreatic cancer.[46] Based on these observations, the cytokines selectively observed to be elevated in CP saliva in this study support various previous studies consistent with their involvement in CP, as well as in other inflammatory conditions. Further research is needed to determine whether their specific combination could be advantageous for testing or monitoring therapeutic responses.

Various local and immunomodulatory factors such as interleukins were previously identified in blood and pancreas tissue[41] in CP. Levels of PGE_2 can be a useful inflammatory marker including in the saliva, for example, for periodontal disease. Indeed, whether or not a saliva test for PGE_2 or other factors will be useful as an indicator of CP could be confounded by the presence of diseases of the oral cavity. Nevertheless, anti-inflammatory drugs such as indomethacin that decrease PGE_2 production may prove

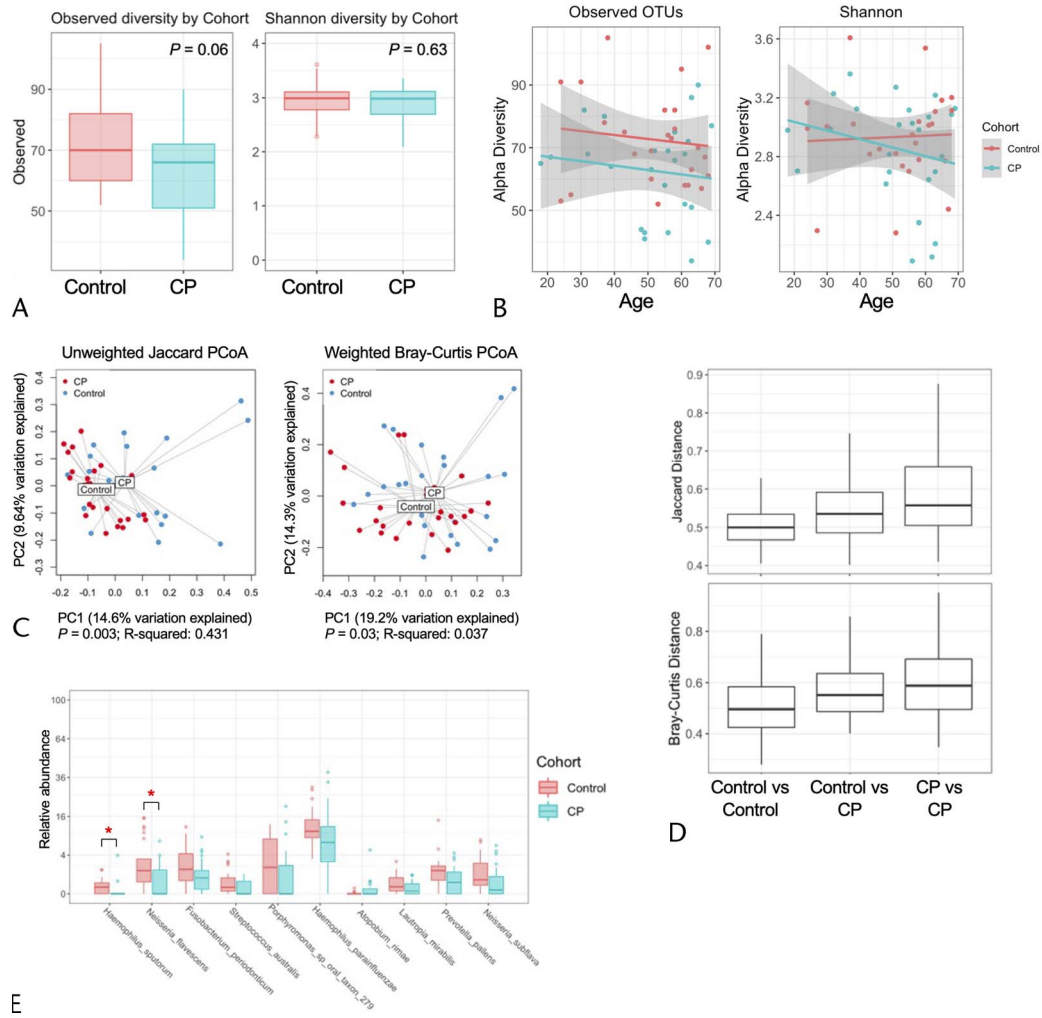


FIGURE 4. Bacterial diversity was evaluated between control and CP saliva samples. 16S rRNA sequencing was performed to identify bacterial diversity, mainly at the species level. A, Alpha diversity by OTU (left) and by Shannon diversity (right). B, Alpha diversity versus age for observed OTU (left) and Shannon index (right). C, Principal component analyses by Jaccard analysis (left) and by Bray-Curtis analysis (right) CP symbols are crimson, controls are blue. D, Measures of Jaccard distance (left) and Bray-Curtis distance (right) across each cohort and between cohorts, shown as box plots (*False Discovery Rate-adjusted $P < 0.05$). E, Comparison of the relative abundance of various bacterial species between controls and CP. In A, B, and E, The controls are depicted in pink/red, and CP samples in aqua symbols and lines.

a useful approach to mitigate the severity of CP. High levels of PGE_2 in pancreatic juice were shown to correlate with the presence of CP.[13] The role of PGE_2 as a mediator of both AP and CP has been shown in animal studies.[47] Our PGE_2 assays indicated a trend toward increasing in CP diseased patients, with a goqod level of consistency achieved, primarily with CV's between 5% and 12% of the values obtained. However, the diversity was such that statistical significance could not be established. These data imply that PGE_2 tests alone would not be an adequate test for CP identification. Nevertheless, they are consistent with a model in which PGE_2 plays a role in CP.

Disturbances of microbiota in disease are being intensely studied as they may prove to be causative factors as well as indicators. Impairment of (exocrine) pancreas function in CP implies nutritional deficiencies including low protein intake.[48] Reduced levels of protein in turn can lead to altered microbiota, especially in the gut.[49] Indeed, recent studies indicate alterations in gut flora is associated with CP, such as small intestinal overgrowth and dysbiosis.[50,51] Previous studies showed that the oral microflora can be altered in CP and pancreatic cancer.[1,52] Oral microbial profiles have also been examined in periodontal disease, which sometimes correlates with other disease states.[53]

In our bacterial (16S RNAseq) analysis, alpha diversity showed a trend toward a decreased richness in CP cases compared with controls as judged by the observed diversity. The *P* value for this assessment was 0.06, or very close to significance. In contrast, Shannon diversity (evenness) did not indicate differences, with a *P* value of 0.62. Taken together, our data did not strongly support an overt difference in alpha diversity.

Data from observed diversity suggested that both control and CP patients lost alpha diversity gradually with age, with controls remaining more diverse than CP patients at all times. In contrast, plotting Shannon diversity by age showed a different pattern, with CP patients exhibiting a pronounced loss in diversity over a wide age range, and controls maintaining relatively stable or even modestly increased diversity with age. Thus, Shannon diversity showed a trend toward decreasing with age in CP patients. However, the overlap in these data imply that bacterial alpha diversity does not associate much with age in CP.

The results of PCA are consistent with several variables contributing to the variability. Overall, whereas a single component of variability (eg, their control vs CP status) could account for a portion of the variability in either case, most of the variation remained unexplained by either analysis. These data confirm previous indications that oral bacterial communities vary with pancreatic disease, as exemplified by CP. The Jaccard and Bray-Curtis distance measures of beta diversity implied that the salivary microbiome is more similar in disease-free individuals than in CP patients. Examination of the relative levels of specific taxa revealed the loss of some commensal species in CP patients. Specific commensal species selectively diminished in CP patients (with False Discovery Rate-adjusted $P < 0.05$) included *Haemophilus sputorum*, *Neisseria flavescens*, and *Fusobacterium periodonticum*. It is conceivable that loss of certain oral bacterial commensal species could indicate or even play roles in systemic inflammatory diseases or CP.

Limitations of the current study include the small sample sizes. It is a survey of 4 small exploratory studies using different analytical methods and only partially overlapping sample utilization. The application of multiple testing such as false discovery rate analysis is not uniformly applicable. Based on these considerations, we describe the significant differentially expressed molecules identified here as candidate biomarkers to emphasize the need for their further validation in future preclinical or clinical studies.

In summary, this study assembles results from multiple different CPDPC (Consortium for the Study of Chronic Pancreatitis, Diabetes, and Pancreatic Cancer) investigation sites suggesting that saliva is a valuable source of biomarkers. By these approaches, we found several proteins and cytokines differentially expressed in CP, some novel and others with previously implicated involvement in CP, as well as distinct alterations in relative abundance of particular bacterial species. Taken together, the differentially expressed candidate biomarkers presented here should be investigated further to evaluate their presence in the saliva as a biomarker strategy. Their further study may provide valuable insights into the pathogenesis of CP and associated diseases.

ACKNOWLEDGMENTS

The authors acknowledge the assistance with mass spectrometry from Biomarker Discovery Platform, Wei Yang, Bo Zhou, and Michael R. Freeman of CSMC. They also acknowledge the Human Immune Monitoring Center (https://iti.stanford.edu/himc.html) and Stanford Diabetes Research Center (https://sdrc.stanford.edu/) for the technical assistance with Luminex assays. They deeply appreciate many helpful scientific discussions with Darwin L. Conwell, Zobeida Cruz-Monserrate, and Hanno Steen.

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