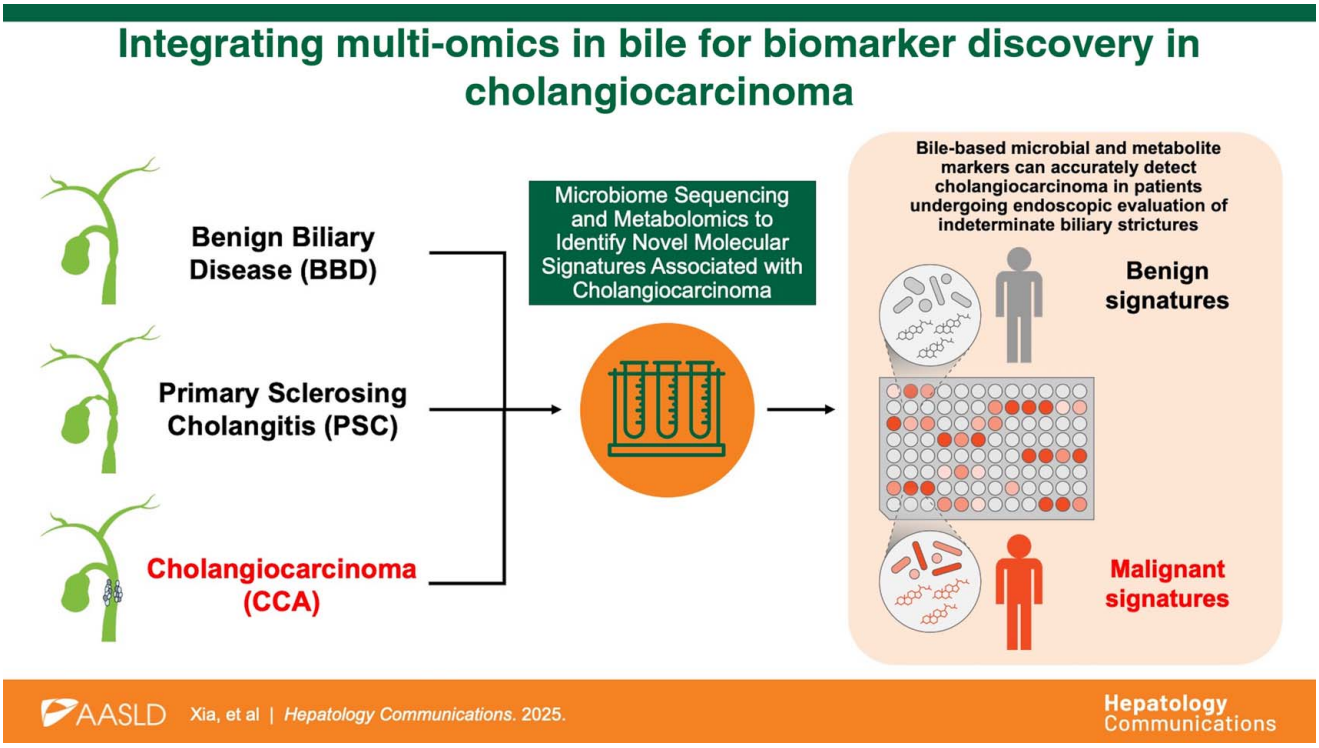
Integrating multi-omics in bile for biomarker discovery in cholangiocarcinoma

VISUAL ABSTRACT



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ORIGINAL ARTICLE

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Integrating multi-omics in bile for biomarker discovery in cholangiocarcinoma

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Abstract

Background: Cholangiocarcinoma (CCA) is an aggressive cancer with a poor prognosis. Histopathology evaluation of brushings and biopsies obtained during endoscopic retrograde cholangiopancreatography (ERCP) currently remains the main method of diagnosis, which has limited sensitivity for malignancy detection. Our study aimed to identify human bile-derived biomarkers to improve CCA diagnosis. Bile samples were collected from patients during ERCP for primary sclerosing cholangitis, CCA, or benign biliary disease.

Methods: Bile samples were collected from patients undergoing ERCP for biliary obstruction due to primary sclerosing cholangitis, newly identified malignant strictures concerning for CCA, or benign biliary disease. Using 16S sequencing, metabolomics, and bile acid quantification, we aimed to identify distinctive microbial and metabolite signatures associated with CCA.

Results: Multi-omics analyses revealed distinct microbial and metabolite signatures associated with CCA. From these findings, we identified and validated microbial and metabolite markers capable of accurately detecting CCA with improved sensitivity and specificity for malignancy detection compared to current cytology-based methods.

Abbreviations: AASLD, American Association for the Study of Liver Diseases; BA, bile acid; BBD, benign biliary disease; CA, cholic acid; CCA, cholangiocarcinoma; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; DPMCF, Duke University Proteomics and Metabolomics Core Facility; ERCP, endoscopic retrograde cholangiopancreatography; GMCF, Genomics and Microbiome Core Facility; LCA, lithocholic acid; PLS-DA, partial least squares discriminant analysis; PSC, primary sclerosing cholangitis; ROC, receiver operating characteristic; TUDCA, tauroursodeoxycholic acid; UDCA, ursodeoxycholic acid.

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Conclusions: These findings highlight the potential of multi-omics bile–based diagnostic panels to enhance endoscopic detection of biliary malignancies, offering a promising tool for evaluating indeterminate biliary strictures and advancing precision in ERCP diagnostics.

Keywords: cholangiocarcinoma, ERCP, indeterminate biliary strictures, metabolomics, microbiome, primary sclerosing cholangitis

INTRODUCTION

Cholangiocarcinoma (CCA) is a devastating malignancy that is associated with poor prognosis and a high mortality rate, with a 5-year survival rate of ~10%.[1] This malignancy develops mainly in bile ducts affected by chronic inflammation, such as in patients with primary sclerosing cholangitis (PSC).[2] Recent epidemiologic studies have highlighted an increase in the incidence of CCA globally, underscoring the need for improvement in methods of precise early diagnosis.[3,4]

Despite advances in pancreaticobiliary endoscopy, the diagnosis of perihilar and extrahepatic CCA is challenging, with 70% of patients presenting with advanced disease.[5] The current clinical standard of diagnosis is through brush cytology performed at endoscopic retrograde cholangiopancreatography (ERCP) with a limited sensitivity of 35%–48%.[6] Differentiating benign and malignant biliary strictures is still a clinical challenge requiring a multidisciplinary approach of imaging, endoscopic procedures, and pathologic evaluation.[7] Approximately 20% of biliary strictures remain undiagnosed and are classified as “indeterminate strictures.” Patients with these strictures usually undergo multiple rounds of invasive procedures before a definitive diagnosis can be reached.[7,8] In addition, screening modalities for CCA utilizing a combination of imaging studies and serum tumor markers have shown a lack of effectiveness in the evaluation of focal high-grade strictures, also known as dominant strictures, in PSC patients.[9–12] Improved diagnostic modalities at the time of endoscopic evaluation is needed to identify at risk patients earlier with the hope of curative resection and reduction of overall mortality.

Recent studies have shown a growing association between the role of the gut microbiome in human hepatobiliary malignancies and PSC.[13–15] Several recent studies have identified the presence of microbial communities from bile samples in patients with PSC and choledocholithiasis.[16,17] In addition, metabolomics, the analysis of the metabolic profile of cells, biofluids, and tissues, is an emerging liquid biopsy modality for early detection of cancers and identification of tumor-associated biomarkers.[18] Basic and translational research in cancer metabolism has shown that tumorigenesis is associated with specific cellular and extracellular metabolic alterations.[19] Therefore, changes in biliary tract microbial and metabolite composition can potentially serve as biomarkers aiding in early and accurate detection of bile duct epithelial dysplasia and its progression to CCA.

To further investigate the role of the bile microbiome and metabolome in CCA, we phenotyped human bile collected during ERCP from patients with CCA, benign biliary disease (BBD), and PSC to identify novel biliary microbial and metabolite signatures. We first profiled the bile microbiome in these patients through 16S sequencing and metabolite signatures through untargeted mass spectrometry of non-polar metabolites. We further investigated the bile acid (BA) composition of bile from these patients and its association with different disease states. From these multi-omics studies, we identified a 3-marker metabolite-based panel and a 4-marker microbiome and metabolite panel that can successfully differentiate CCA from patients with BBD and PSC, respectively, with high sensitivity and specificity. Next, we validated our diagnostic panels on a cohort of patients with indeterminate biliary strictures and PSC-dominant strictures. Overall, our findings demonstrate that multi-omics bile-based diagnostic panels can enhance current endoscopic evaluation of indeterminate biliary strictures.

METHODS

Patient population and sample collection

In this prospective single-center study, bile was obtained from patients undergoing ERCP from 2021 to 2024 at Northwestern Memorial Hospital for the indication of biliary obstruction due to malignant biliary stricture, BBDs (ie, choledocholithiasis, sphincter of Oddi dysfunction), PSC, and transplant-related stricture. This study was approved under the Institutional Review Board-approved study STU00203172 and STU215612. After consent was obtained before the procedure, a bile sample was aspirated after deep cannulation of the bile duct during ERCP before contrast injection with a sterile sphincterotome or balloon catheter. Antibiotics were applied intravenously after bile samples were obtained if indicated

for the procedure. Bile samples were then aliquoted to cryovials and snap-frozen in liquid nitrogen. Samples were then stored at −80 °C freezer until processing for 16S sequencing or metabolomics.

The diagnosis of PSC was confirmed in accordance with the current American Association for the Study of Liver Diseases (AASLD) guidelines using a combination of clinical, biochemical, and imaging features.[20] Diagnosis of malignancy was confirmed by histopathology findings on tissue biopsy samples.

DNA preparation and 16S sequencing

Microbial DNA was extracted from 250 μL of human bile using a modified QIAGEN DNeasy PowerSoil Pro kit, where the DNA binding spin column steps were replaced with DNA purification through Ampure magnetic beads (Beckman Coulter). In brief, after the cell lysis step, the lysates' 1:1 ratio of magnetic beads was mixed with the lysate. This mixture was then placed on the magnetic stand and washed twice with freshly made 80% ethanol. Residual ethanol was removed, and beads were air-dried for 2 minutes. The beads were removed from the magnetic stand and resuspended in 10 μL of sterile water. The pellet was then placed back on the magnetic stands, and the supernatant was placed in a new Eppendorf tube. The DNA extraction protocol was confirmed using human stool samples as positive controls and sterile water as negative controls.

16S sequencing was performed by the Genomics and Microbiome Core Facility (GMCF) at Rush University as previously described.[21] Please refer to Supplemental Methods for a detailed description, http://links.lww.com/HC9/C74.

Metabolite and BA quantification

Metabolites were extracted from 250 μL of human bile as previously described.[22] Metabolomics was performed in collaboration with the Northwestern Metabolomics Core Facility as previously described.[22] BA quantification was performed by the Duke University Proteomics and Metabolomics Core Facility (DPMCF) as previously described.[23] Please refer to Supplemental Methods for a detailed description, http://links.lww.com/HC9/C74.

Statistical analysis

Individual microbial, BA, and metabolite biomarkers were assessed with receiver operating characteristic (ROC) curves using GraphPad Prism. To find statistical models to differentiate benign and malignant strictures utilizing our multi-omics panels, linear regression analysis was performed in GraphPad Prism with a threshold set to 0.5.

RESULTS

Patient demographics

A total of 59 patients were included in our initial training cohort of patients with CCA (n = 13), BBD (n = 34), and PSC (n = 12) for multi-omics analysis. All patients underwent ERCP for indications including elevated liver enzymes, abnormal imaging, or a history of known PSC. The baseline characteristics of the cohorts differed, with PSC patients being the youngest and patients with CCA having the highest total bilirubin levels before ERCP at 4.9 mg/dL (Table 1). The stage of CCA in this cohort ranged from IB to IV (Supplemental Table S1, http://links.lww.com/HC9/C75). In all, 100% of the patients with BBD undergoing ERCP had native papilla, while 53.8% and 58.3% of the patients with CCA and PSC had native papilla, respectively (Table 1). While CCA and BBD had comparable percentages of female patients, the PSC cohort consisted of only 33.3% female patients (Table 1). Of the patients with BBD, 64.7% had choledocholithiasis on ERCP. In

TABLE 1 Patient demographics of the training cohort

	Cholangiocarcinoma (n = 13)	Benign biliary disease (n = 34)	Primary sclerosing cholangitis (n = 12)
Age (median with range)	70 (39–83)	60.5 (22–86)	39 (30–67)
% Female	53.8	61.8	33.3
Total bilirubin before ERCP (mg/dL)	4.9	1.7	1.5
% Native papilla	53.8	100	58.3
% Gallstone disease	NA	64.7	NA
% Cholangitis	15.4	11.8	0
% Ursodeoxycholic acid	7.7	0	25
% Antibiotics	15.4	26.5	58.3

Abbreviation: ERCP, endoscopic retrograde cholangiopancreatography.

addition, 15.4% and 11.8% of CCA and BBD patients, respectively, had the diagnosis of cholangitis before ERCP; none of the PSC patients had cholangitis (Table 1). Furthermore, 7.7% of CCA patients and 25% of PSC patients had ursodeoxycholic acid (UDCA) on their medication list before ERCP, while none of the patients with BBD had this medication (Table 1).

The biliary microbiome composition is distinct in patients with CCA and PSC compared with BBD

Before the advent of next-generation sequencing, low-biomass environments such as bile have been widely considered sterile. A recent study by Liwinski et al[17] demonstrated that the bile microbiome shared similarities with the oral microbiome. We also found that the bile and oral/gastric aspirates obtained during ERCP had similar microbial compositions that were predominantly oral/gastric in origin. Although non-significant, the biliary microbiome tended to be less diverse compared with their oral/gastric counterparts (Supplemental Figures S1A, B, http://links.lww.com/HC9/C75). We identified the 5 phyla of *Proteobacteria*, *Firmicutes*, *Actinobacteriota*, *Fusobacteriota*, and *Bacteriodota*, which essentially accounted for the entire microbiome in the bile of CCA, BBD, and PSC patients with native papilla (Figure 1A). A total of 412 genera were identified, the dominant genera seen in bile from all groups with native papilla were *Prevotella* and *Streptococcus* (Figure 1B). *Streptococcus* was the most abundant genus seen in bile of all 3 groups, comprising 35.3% of the bile microbiome in CCA and 26.2% of BBD and 26.4% of PSC (Figure 1B). Furthermore, *Prevotella* was much more abundant in the bile of CCA (19.3%) and PSC (14.4%) compared with BBD (6.8%), while *Enterococcus* was enriched in BBD (15.9%) compared with CCA (0.6%) and PSC (0.06%). Similar phyla and genera abundances were observed in the different disease groups, even after removing patients with a cholangitis diagnosis and UDCA use before ERCP from the cohorts (Figures 1A, B). Furthermore, when we included patients with non-native papilla, we also observed similar abundance patterns in CCA, BBD, and PSC groups (Supplemental Figures S1C, D, http://links.lww.com/HC9/C75).

Alpha-diversity based on Shannon entropy did not show significant differences between groups when including patients with non-native papilla (Supplemental Figure S1E, http://links.lww.com/HC9/C75). For patients with native papilla, we found that there was a significant difference in diversity between PSC and BBD groups and no differences between CCA and BBD or PSC groups (Figure 1C). This increase in diversity between PSC compared with BBD was observed even after accounting for patients with cholangitis and those taking UDCA (Figure 1C). The overall microbiota composition (β-diversity) of bile from patients with native papilla with CCA and PSC was significantly different compared with that of BBD (Figure 1D). These differences in composition were observed even after accounting for patients with cholangitis (Figure 1D). When patients taking UCDA before ERCP were removed, the microbiota composition was still significantly different between CCA and BBD, but the differences seen between the PSC and BBD groups were no longer observed (Figure 1D). In addition, after including patients with non-native papilla in the dataset, we did not observe any microbiome composition differences (Supplemental Figure S1F, http://links.lww.com/HC9/C75).

To identify microbial markers of CCA and PSC, we next performed differential abundance analysis of our 16S rRNA dataset on the level of genus. Previous studies have demonstrated that different differential abundance analysis methods can produce substantially different results.[24] To increase the sensitivity of identification of microbial markers of CCA, we opted to use differential abundance tools of edgeR and Wilcoxon rank-sum, which have been previously reported to output a higher number of significant amplicon sequence variants compared with other differential abundance analysis tools. We also utilized the MetagenomeSeq method, which has been reported to output an intermediate number of significant amplicon sequence variants.[24] To decrease the risk of false positives and increase reproducibility, we compiled the microbes that were found to be significantly differentially abundant through multiple analytic tools when comparing CCA to BBD and PSC. Using this method, we found the genera *Stomatobaculum*, *Tannerella*, and *Helicobacter* to be differentially abundant in bile collected from patients with CCA compared with BBD (Figure 1E). In addition, we found the genera *Peptostreptococcus*, *Leptotrichia*, *Tetragenococcus*, *Stenotrophomonas*, *Haemophilus*, *Anaeroglobus*, and *Neisseria* to be differentially abundant in bile collected from patients with CCA compared with PSC (Figure 1F). Differential abundance analysis was also performed between CCA, BBD, and PSC groups using linear discriminant analysis effect size (LEfSe) with 17 differentially abundant genera identified (Supplemental Table S2, http://links.lww.com/HC9/C75). These findings suggest that the biliary microbial signatures can be used to identify malignant biliary strictures, complementary to other testable markers.

The bile metabolome is distinct in CCA compared with PSC and BBD

To identify metabolite-based biomarkers for CCA, we performed comprehensive, unbiased polar metabolomics of bile collected from patients with CCA, PSC, and BBD. A total of 305 unique polar metabolites were

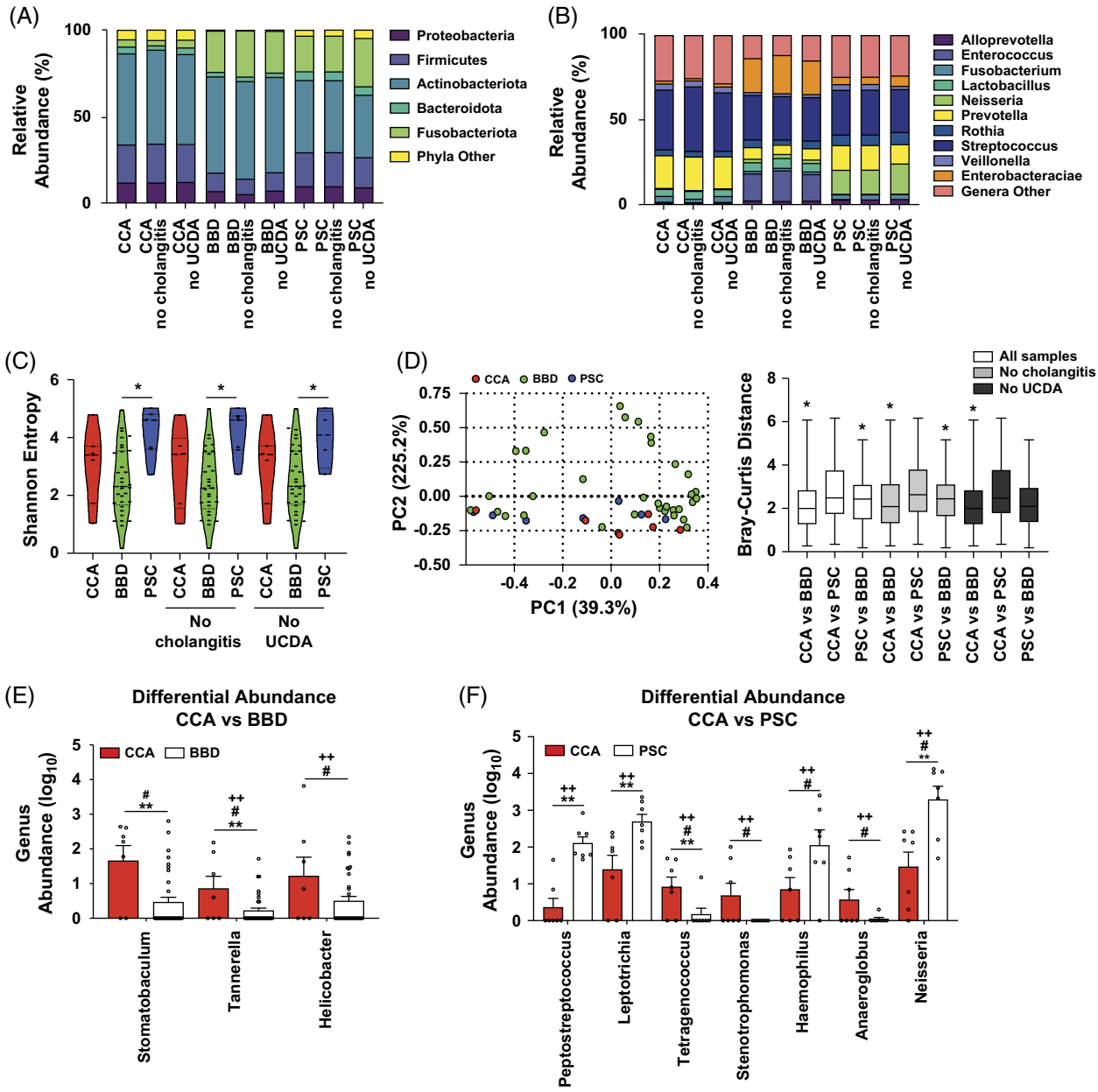

FIGURE 1 Bile microbiome composition is distinct in patients with CCA compared with patients with BBD and PSC. (A) Relative abundance distributions of the top 5 phyla in bile samples from patients with CCA, BBD, and PSC. (B) Relative abundance distributions of the top 10 genera in bile samples from patients with CCA, BBD, and PSC. (C) Alpha-diversity was calculated using the Shannon diversity index, comparing CCA, BBD, and PSC. (D) Principal coordinate analysis plot and Beta-diversity calculated using Bray–Curtis distance. (E) Differentially abundant genus comparing CCA and BBD identified through Wilcoxon rank-sum, MetagenomesSeq, and EdgeR. (F) Differentially abundant genus comparing CCA and PSC identified through Wilcoxon rank-sum, MetagenomesSeq, and EdgeR. *$p < 0.05$ for PERMANOVA for indicated comparisons. **FDR < 0.05, #FDR < 0.05. ++FDR < 0.05 for differential abundance analysis calculated by Wilcoxon rank-sum, MetagenomesSeq, and EdgeR, respectively. Abbreviations: BBD, benign biliary disease; CCA, cholangiocarcinoma; FDR, false discovery rate; PSC, primary sclerosing cholangitis.

detected in bile. Initial analysis of raw metabolomics data discovered 67 and 73 unique differentially abundant metabolites in bile from patients with CCA and PSC, respectively, when compared with BBD. Of those differentially abundant metabolites, 38 of them overlapped between the 2 comparisons (Figures 2A, B). Partial least squares discriminant analysis (PLS-DA) of bile metabolomics revealed separation between CCA samples from both PSC and BBD (Figure 2C). Orthogonal PLS-DA performed when comparing bile metabolomics of CCA to BBD and PSC showed clear clustering of CCA samples from non-malignant samples of BBD and PSC (Figure 2D). The significantly upregulated and downregulated metabolites in CCA compared with both BBD and PSC were initially identified (Figures 2E, F). Next, the metabolites from

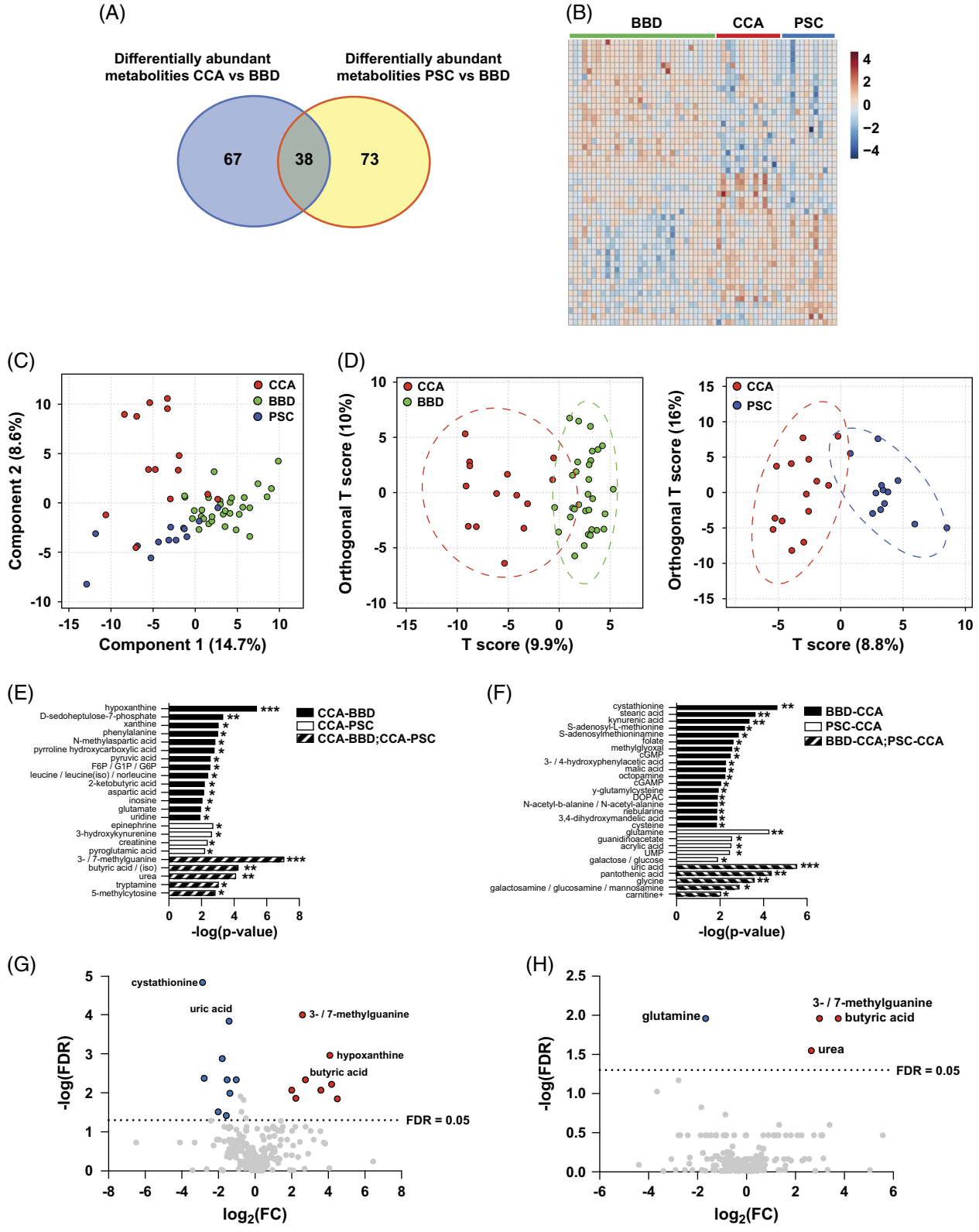

FIGURE 2 The bile metabolome is distinct in CCA compared with PSC and BBD. (A) Venn diagram depicting overlap in statistically significantly differentially abundant metabolites in CCA and PSC compared with BBD. (B) Heatmap depicting the top 50 differentially expressed bile metabolites in BBD, CCA, and PSC. (C) PL-SDA was performed on bile metabolomics data of CCA, BBD, and PSC. (D) Orthogonal PLS-DA was performed on bile metabolomics data of CCA compared to BBD and PSC, respectively. (E, F) Significantly upregulated and downregulated bile metabolites in CCA compared with both BBD and PSC. (G, H) Volcano plot of all the significant (FDR <0.05) bile metabolites in CCA compared with BBD and PSC, respectively. *FDR <0.05, **FDR <0.01, ***FDR <0.001 for ANOVA comparisons. Abbreviations: BBD, benign biliary disease; CCA, cholangiocarcinoma; FDR, false discovery rate; PL-SDA, partial least squares discriminant analysis; PSC, primary sclerosing cholangitis.

initial comparisons were further screened for those with at least a 2-fold increase/decrease and an FDR < 0.05 and used as potential biomarkers (Figures 2G, H). We found that 3/7-methylguanine, butyric acid, and urea were all highly increased in bile from CCA patients when compared with both BBD and PSC (Figures 2G, H and Supplemental Table S3, http://links.lww.com/HC9/C75). Cystathionine and glutamine were the top down-regulated metabolites found in the bile of CCA patients compared with BBD and PSC, respectively (Figures 2G, H and Supplementary Table S3, http://links.lww.com/HC9/C75). Our findings demonstrated that phenotyping the biliary metabolome can facilitate biomarker discovery to potentially aid in the diagnosis of biliary malignancies.

Bile from patients with CCA exhibits differential abundance in primary, secondary, and conjugated BA fractions compared with patients with BBD and PSC

PSC patients have been previously shown to have altered biliary and serum BA profiles, and certain BA fractions were predictive of future hepatic decompensation.[25,26] Furthermore, previous in vitro studies have shown that conjugated BAs may play a role in stimulation of CCA tumorigenesis.[27] Thus, we quantified 16 conjugated and unconjugated BAs from bile collected from patients with CCA, PSC, and BBD (Figure 3A and Supplemental Table S4, http://links.lww.com/HC9/C75). Bile from patients with CCA had significantly lower total BA concentration compared with both BBD and PSC (Figure 3B). When looking at total concentrations of individual species, CCA patients had lower concentrations of cholic acid (CA) and chenodeoxycholic acid (CDCA) compared with BBD and lower concentrations of UDCA compared with PSC (Supplemental Figure S2A, http://links.lww.com/HC9/C75). Like previous findings, the total BA concentrations also tended to be lower in the PSC group compared with BBD (Figure 3B).[26] Bile from CCA patients also had higher proportions of primary BAs and lower proportions of secondary BAs compared with both BBD and PSC (Figure 3C). Among the primary BA fractions, CCA patients had significantly elevated fractions of total CA and lower fractions of total CDCA (Figure 3D). The total fraction of secondary BAs from bile from patients with CCA, PSC, and BBD was comparable (Figure 3E). Interestingly, lithocholic acid (LCA), which has been shown previously in murine studies to cause cholestasis and hepatic carcinogenesis, was significantly elevated in CCA patients compared with both BBD and PSC (Figure 3E).[28] The proportions of deoxycholic acid (DCA) and ursodeoxycholic acid (UDCA) did not differ between the groups (Figure 3E). To control for possible group differences due to patients taking UDCA, we repeated our analysis after removing all patients with UDCA on their medication list before ERCP. Overall, we observed mostly similar findings compared with the previous study. However, some of the significant differences between CCA and PSC groups for total BA concentration, LCA and hyodeoxycholic acid fractions were no longer observed after removal of patients taking UDCA (Supplemental Table S5, http://links.lww.com/HC9/C75).

The total conjugated fractions of total BAs and primary BAs for CCA, PSC, and BBD were similar (Figure 3F and Supplemental Figure 2B, http://links.lww.com/HC9/C75). Within the primary BAs, CCA patients exhibited decreased levels of conjugated CDCA compared with both PSC and BBD (Figure 3F). In addition, CCA patients had significantly decreased levels of conjugated secondary BAs compared with both BBD and PSC (Figure 3F). The conjugated BA concentrations for BBD and PSC were not significantly different (Figure 3F). To evaluate whether certain BAs have utility as potential biomarkers for CCA, all 16 BA abundances were normalized and screened for those with significant differential abundance and FDR < 0.05 when CCA was compared with BBD and PSC, respectively (Figures 3G, H). We found that unconjugated CDCA levels were consistently elevated in CCA compared with both BBD and PSC (Figures 3G, H). LCA, DCA, and CA were also elevated in CCA compared with BBD but not PSC (Figures 3G, H). Tauroursodeoxycholic acid (TUDCA) levels were significantly decreased in CCA compared with PSC patients (Figure 3H). Thus, BAs unique to CCA can help differentiate malignant strictures from BBD and PSC.

Combination of biliary microbiome, metabolome, and BA markers results in improved predictive performance of cholangiocarcinoma

To identify clinically relevant diagnostic markers of CCA, we next evaluated the prediction accuracy of differentially abundant bacterial, metabolomic, and BA markers. Considering that patients with PSC has higher risk of CCA compared with the general population, we conducted separate marker validation comparing CCA patients with BBD and PSC.[20] For microbial marker validation, we used our training cohort of bile from patients with CCA, BBD, and PSC patients with native papilla. We found that of the significantly differentially abundant bacterial genera, only *Stomatobaculum* could significantly discriminate CCA from BBD patients with native papilla with an AUC of 0.779 (Figure 4A and Supplemental Figure 3A, http://links.lww.com/HC9/C75). ROC curve analysis of differentially abundant bacterial genera when comparing CCA and PSC showed that *Peptostreptococcus*, *Leptotrichia*, *Tetragenococcus*, and *Neisseria* were all capable of discriminating, with

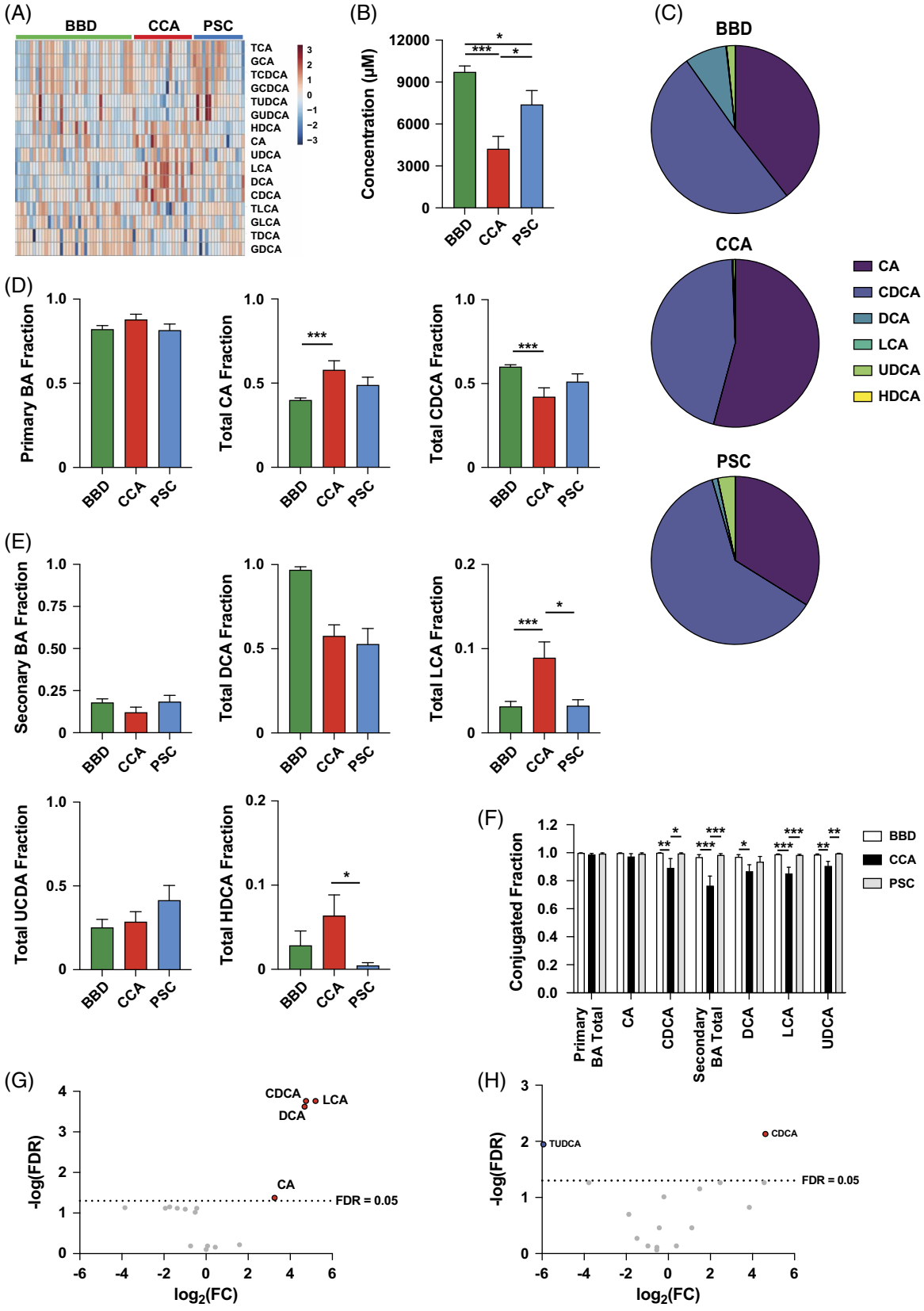

FIGURE 3 Bile from patients with CCA exhibits differential abundance in primary, secondary, and conjugated BA fractions compared with patients with BBD and PSC. (A) Heatmap depicting all quantified BAs in bile from patients with BBD, CCA, and PSC. (B) Total BA concentrations (μM) in BBD, CCA, and PSC. (C) BA composition by BA family type in BBD, CCA, and PSC. (D) Total primary BA fractions and specific fractions of CA and CDCA fractions in the bile of patients with BBD, CCA, and PSC. (E) Total secondary BA fractions and specific fractions of DCA, LCA, UCDA, and HDCA in the bile of patients with BBD, CCA, and PSC. (F) Breakdown of the conjugated fractions of the major primary and secondary

BAs. (G) Volcano plot of all significant (FDR <0.05) BAs in CCA compared with BBD. (H) Volcano plot of all significant (FDR <0.05) BAs in CCA compared with PSC. *FDR <0.05, **FDR <0.01, and *** FDR <0.001 for ANOVA comparisons. Abbreviations: BA, bile acid; BBD, benign biliary disease; CA, cholic acid; CCA, cholangiocarcinoma; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; FDR, false discovery ratio; HDCA, hyodeoxycholic acid; LCA, lithocholic acid; PSC, primary sclerosing cholangitis; UDCA, ursodeoxycholic acid.

Peptostreptococcus having the best AUC of 0.999 (Figure 4B). For validation of metabolite and BA markers, our training cohort consisted of bile collected from patients with CCA, BBD, and PSC patients who had undergone ERCP with both native and non-native papilla. Of the most differentially abundant and statistically significant ($p<0.05$) metabolite markers when comparing CCA and BBD, we found that 3/7-methylguanine and butyric acid were best at discriminating cancer from benign disease with AUC of 0.893 and 0.899, respectively (Figure 4C). Similarly, we also found that 3/7-methylguanine, butyric acid could delineate

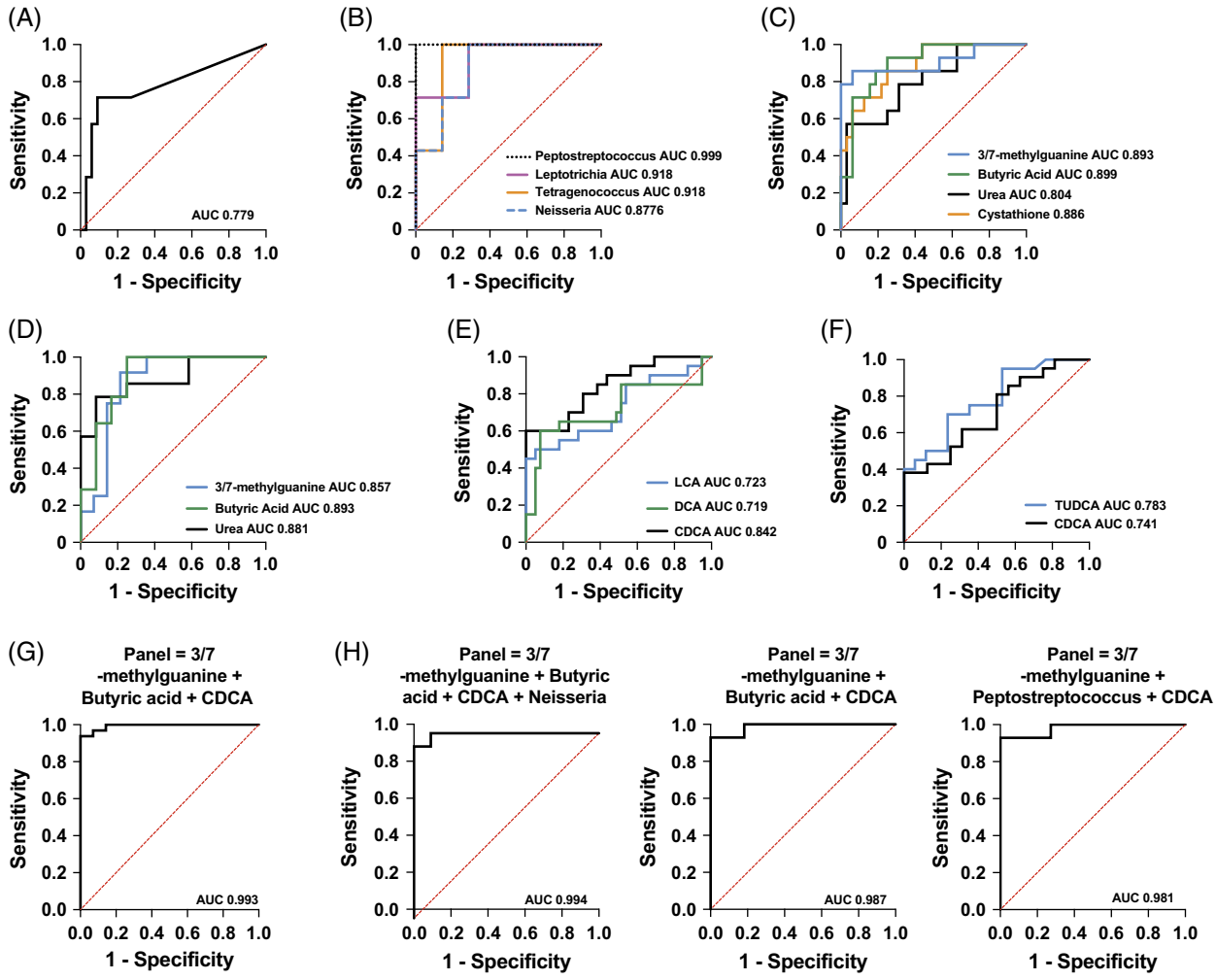

FIGURE 4 A combination of bile microbiome, metabolome, and BA markers results in improved predictive performance of cholangiocarcinoma. (A) ROC curve for discriminating patients with CCA and BBD using the relative abundance of the genus *Stomatobaculum* in bile ($p=0.0217$). (B) ROC curves for discriminating patients with CCA and PSC using the relative abundances of the genus *Peptostreptococcus* ($p=0.0017$), *Leptotrichia* ($p=0.009$), *Tetragenococcus* ($p=0.009$), and *Neisseria* ($p=0.0181$) in bile. (C) ROC curves for discriminating patients with CCA and BBD using relative abundance of bile metabolites of 3/7-methylguanine ($p<0.0001$), butyric acid ($p<0.0001$), urea ($p=0.0012$), and cystathione ($p<0.0001$). (D) ROC curves for discriminating patients with CCA and PSC using relative abundance of bile metabolites of 3/7-methylguanine ($p=0.002$), butyric acid ($p=0.0007$), and urea ($p=0.001$). (E) ROC curves for discriminating patients with CCA and BBD using relative abundance of LCA ($p=0.0056$), DCA ($p=0.0062$), and CDCA ($p<0.0001$) in bile. (F) ROC curves for discriminating patients with CCA and PSC using relative abundance of TUDCA ($p=0.0033$) and CDCA ($p=0.0273$) in bile. (G) ROC curve for discriminating patients with CCA and BBD using a panel consisting of abundances of 3/7-methylguanine, butyric acid, and CDCA in bile ($p<0.0001$). (H) ROC curve for discriminating patients with CCA and PSC using multi-omics panels as labeled (all $p<0.0001$). Abbreviations: BA, bile acid; BBD, benign biliary disease; CCA, cholangiocarcinoma; CDCA, chenodeoxycholic acid; LCA, lithocholic acid; PSC, primary sclerosing cholangitis; ROC, receiver operating characteristic.

patients with CCA from those with PSC (Figure 4D). Bile levels of urea were another marker that was effective in discriminating patients with CCA from PSC, with an AUC of 0.881 (Figure 4D). When examining BA markers for CCA, we found that concentrations of the primary unconjugated BA, CDCA, were capable of discriminating CCA from both BBD and PSC effectively with AUC of 0.842 and 0.741, respectively (Figures 4E, F). TUDCA exhibited slightly improved discrimination of CCA from PSC compared with CDCA, with an AUC of 0.783 (Figure 4F).

We next tested the performance of the combination of microbial, metabolic, and BA markers for the detection of CCA. For the detection of CCA compared with BBD, we found that a panel combining 3/7-methylguanine, butyric acid, and CDCA levels performed best with an AUC of 0.993 (Figure 4G). Diagnostic panels using a combination of 3/7-methylguanine, urea, and CDCA or urea, butyric acid, and CDCA also yielded high-performing results with AUCs of 0.973 and 0.975, respectively (Supplemental Figure 3B, http://links.lww.com/HC9/C75). In addition, we found that inclusion of the microbial marker *Stomatobaculum* into our panel yielded poorer results than the individual markers themselves (Supplemental Figure 3C, http://links.lww.com/HC9/C75). This is likely because the data used for the initial ROC curve with *Stomatobaculum* was based on patients with native papilla only, and the training cohort of the multi-omics panels included patients with both native and non-native papilla. For the detection of CCA compared with PSC, we identified a panel combining 3/7-methylguanine, CDCA, butyric acid, and *Neisseria* levels that had the highest performance with an AUC of 0.994 (Figure 4H). Panels containing 3/7-methylguanine, CDCA, and butyric acid or 3/7-methylguanine, CDCA, and *Peptostreptococcus* also successfully delineate cancer from PSC with AUC of 0.987 and 0.981, respectively (Figure 4H). Panels containing urea had lower performance discriminating cancer from PSC, but were overall still higher than individual markers (Supplemental Figure 3D, http://links.lww.com/HC9/C75). Since TUDCA exhibited slightly improved discrimination between CCA and PSC on initial ROC curves, we trialed panels containing TUDCA instead of CDCA as the BA component. These panels had similar but slightly lower performance in discriminating CCA from PSC (Supplemental Figure 3E, http://links.lww.com/HC9/C75). As mentioned previously, UCDA usage can be common in the PSC patient population and could affect biliary concentrations of UCDA and conjugates. For this reason, we chose to move forward with CDCA instead of TUDCA in our final diagnostic panel for PSC. Overall, a combination of microbiome, metabolite, and BA panels may serve as a novel tool to improve endoscopic detection of cholangiocarcinoma and cancer surveillance in PSC patients.

Combination of multi-omics diagnostic panels can predict malignancy in patients with indeterminate biliary strictures and PSC patients with dominant strictures

The evaluation of indeterminate biliary strictures remains challenging without a clear guideline consensus, highlighting the need for improved tools to enhance endoscopic diagnosis.[29] To evaluate whether our multi-omics panel can identify patients at risk for CCA, we collected bile samples during ERCP for patients with indeterminate biliary strictures and PSC patients with dominant stricture. We utilized our panel containing 3/7-methylguanine, butyric acid, and CDCA for the evaluation of 12 patients seen between 2023 and 2024 for the evaluation of indeterminate strictures. For our logistic regression model, we employed a standard cutoff of 0.5, where values above that are malignant and below that are benign. Bile samples were all collected before the final pathology diagnosis of these patients. Of these 12 patients, 5 had malignant strictures and 7 had benign strictures (Table 2 and Supplemental Table S6, http://links.lww.com/HC9/C75). Our 3-marker panel was able to accurately predict malignancy in all 5 patients and benign disease in the other 7 (Table 2). Four of the 5 patients with malignant strictures were diagnosed non-endoscopically by interventional radiology biopsy of lesions concerning for metastasis up to 5 months after initial ERCP. One patient was diagnosed through surgical pathology after left lobe liver resection (Table 2). All endoscopic brush cytology performed at the same endoscopic session as bile collection were negative for malignancy. The patients with benign disease consisted mainly of common bile duct strictures due to chronic pancreatitis and IgG4-related disease. Most of these patients had undergone at least 3 ERCPs and 2 endoscopic ultrasounds before final diagnosis (Table 2). Interestingly, one of the patients with initial concern for CCA was later found to have a malignant stricture due to metastatic rectal cancer. Our 3-marker panel was highly positive on bile collected from his initial ERCP (Table 2). Thus, we then utilized our 3-marker panel on bile collected from a cohort of patients with malignant strictures due to known metastatic cancer or benign transplant anastomotic strictures. We found that of the patients with strictures from metastatic disease, the 3-marker panel was effective in identifying malignancy in those with intraductal metastasis rather than strictures due to metastatic lesions causing extrinsic compression (Supplemental Table S7, http://links.lww.com/HC9/C75). None of the 12 patients with transplant anastomotic strictures were positive for malignancy from our 3-marker panel (Supplemental Table S7, http://links.lww.com/HC9/C75). In addition, whether the patient had a native or non-native papilla did not impact the performance of our diagnostic panel.

TABLE 2 Clinical characteristics and multi-omics panel results of the validation cohort of patients with indeterminate strictures and PSC patients with dominant strictures

Indeterminate strictures	Stricture location	Age	Sex	Native papilla	CA 19-9 level (U/mL)	First ERCP*	Date of bile collection	3-Marker panel logistic regression value	Bile panel prediction	Brush cytology at the time of bile collection	Date of cancer diagnosis	Modality of diagnosis	Total # of procedures before diagnosis
1	Common hepatic duct	83	M	No	45	10/18/2022	5/30/2023	0.99999	Malignant	Negative	11/21/2023	Cholangioscopy with biopsy	7 ERCPs, 1 EUS++
2	Common hepatic duct	44	F	Yes	9	2/27/2023	2/27/2023	0.00179	Benign	Negative	NA	Resolution of stricture on 4/3/23 ERCP	2 ERCPs
3	Left main hepatic duct	76	F	Yes	151	4/18/2023	4/18/2023	0.84872	Malignant	Negative	9/8/2023	Left lobe hepatectomy with/pathology positive for adenocarcinoma	3 ERCPs
4	Common hepatic duct	68	F	Yes	401.1	6/13/2023	6/13/2023	0.99999	Malignant	Negative	8/11/2023	Peritoneal nodule biopsy positive for adenocarcinoma	3 ERCPs, 1 EUS
5	Hilar stricture	70	M	Yes	25	7/7/2023	7/7/2023	0.00081	Benign	Negative	NA	IgG4# elevated to 681 on 8/2/23, strictures improved after prednisone	3 ERCPs, 1 EUS
6	Common hepatic duct	62	M	Yes	611	7/26/2023	7/26/2023	0.99999	Malignant	Negative	8/28/2024	Colon mass biopsy, liver mass biopsy	2 ERCPs
7	Common bile duct stricture	46	M	Yes	32	1/13/2023	1/13/2023	0.00175	Benign	Negative	NA	Multiple EUS biopsies with chronic pancreatitis	4 EUS, 8 ERCPs
8	Common hepatic duct	69	M	Yes	20779	2/27/2023	2/27/2023	0.99999	Malignant	Negative	2/28/2024	Liver lesion biopsy positive for adenocarcinoma	2 ERCPs
9	Common bile duct stricture	51	M	Yes	267	5/26/2023	5/26/2023	0.00192	Benign	Negative	NA	IgG4 670.6 on 9/17/23	2 EUS, 4 ERCPs
10	Common bile duct stricture	68	M	No	42	11/26/2021	3/17/2022	0.00053	Benign	Negative	NA	Multiple EUS biopsies with chronic pancreatitis	9 EUS,9 ERCPs
11	Common hepatic duct	40	M	No	9	9/3/2023	11/1/2023	0.0001	Benign	Negative	NA	All samplings thus far negative, IgG4 negative, CA 19-9 negative	6 ERCPs, 3 EUS
12	Common bile duct stricture	43	M	No	6.4	5/22/2023	1/29/2024	0.00008	Benign	Negative	NA	EUS lymph node biopsy with granulomas	4 ERCPs, 2 EUS

TABLE 2. (*continued*)

Indeterminate strictures	Stricture location	Age	Sex	Native papilla	CA 19-9 level (U/mL)	First ERCP*	Date of bile collection	3-Marker panel logistic regression value	Bile panel prediction	Brush cytology at the time of bile collection	Date of cancer diagnosis	Modality of diagnosis	Total # of procedures before diagnosis
PSC with dominant strictures	**ERCP indication**	**Age**	**Sex**	**Native papilla**	**CA 19-9 level (U/mL)**	**First ERCP**	**Date of bile collection**	**3-Panel logistic regression value**	**Bile panel prediction**	**Brush cytology at the time of bile collection**	**Date of cancer diagnosis**	**Modality of diagnosis**	**Total # of procedures before diagnosis**
1	Common bile duct stricture	35	M	Yes	86	3/2/2023	3/2/2023	0.877788831	Malignant	Negative EUS-guided lymph node biopsy	3/16/2023	Cholecystectomy with pathology findings of adenocarcinoma	1 EUS, 1 ERCP
2	Common hepatic duct stricture	33	M	No	3010	4/6/2023	7/6/2023	0.054207929	Benign	Negative	NA	All sampling negative, CA 19-9 downtrending	4 ERCPs
3	Left and right intrahepatic strictures	32	M	Yes	5	7/14/2023	7/14/2023	0.124469033	Benign	Negative	NA	Liver explant with cirrhosis	1 ERCP
4	Left main hepatic duct stricture	45	M	No	29	12/22/2022	7/25/2023	0.007878913	Benign	Negative	NA	All sampling negative	4 ERCPs, 2 EUS
5	Left main hepatic duct takeoff stricture	38	F	No	<2	1/30/2024	1/30/2024	0.138832049	Benign	Negative	NA	Resolution of stricture on ERCP in 6/12/2024	3 ERCPs, 1 EUS
6	Left main hepatic duct stricture	66	F	No	99	10/5/2022	4/25/2024	0.000747577	Benign	Negative	NA	All sampling negative	4 ERCPs
7	Common bile duct stricture	26	M	Yes	154	3/27/2024	3/27/2024	0.000806505	Benign	Negative	NA	Resolution of stricture on ERCP in 4/24/24	2 ERCPs
8	Hilar stricture	44	M	No	37	1/4/2023	5/24/2023	0.998703796	Malignant	Negative	3/16/2024	Explant positive for adenocarcinoma with intraductal growth in the background of cirrhosis	7 ERCPs, 2 EUS

Abbreviations: ERCP, endoscopic retrograde cholangiopancreatography; EUS, endoscopic ultrasound; F, female; M, male; PSC, primary sclerosing cholangitis.

For the 8 PSC patients undergoing ERCP for evaluation of dominant stricture, we evaluated multiple diagnostic panels, as they previously had similar discriminant performance with ROC curves. Due to this, we utilized a logistic regression model using the 3-marker panels, including 3/7-methylguanine, CDCA, and *Peptostreptococcus* or butyric acid, and the 4-marker panel, including 3/7-methylguanine, CDCA, butyric acid, and *Neisseria*. Only the logistic regression model based on the panel including 3/7-methylguanine, CDCA, and *Peptostreptococcus* could identify both PSC patients with underlying malignancy when employing a similar cutoff, which we used previously for indeterminate biliary strictures (Table 2). The other 2 panels only correctly identified 1 of the PSC patients with underlying malignancy (Supplemental Table S8, http://links.lww.com/HC9/C75). Interestingly, this PSC patient had imaging concerning for a gallbladder mass and underwent ERCP with no clear biliary stricture and EUS-guided periportal lymph node biopsy with benign pathology (Table 2). However, when he had a cholecystectomy for gallbladder removal, surgical pathology was positive for gallbladder adenocarcinoma. The second PSC patient with an underlying malignancy had a hilar stricture that had undergone multiple ERCPs and EUS-guided lymph node biopsies without a clear diagnosis of malignancy. The patient underwent liver transplantation due to decompensated cirrhosis, and his liver explant was found to have intraductal adenocarcinoma in the background of cirrhosis (Table 2). The 6 PSC patients with benign dominant strictures all underwent at least 2 ERCPs from 2022 to 2024, with biliary brushings cytologies all without evidence of malignancy. None of these patients had any significant clinical changes as of July 2024 (Supplemental Table S8, http://links.lww.com/HC9/C75).

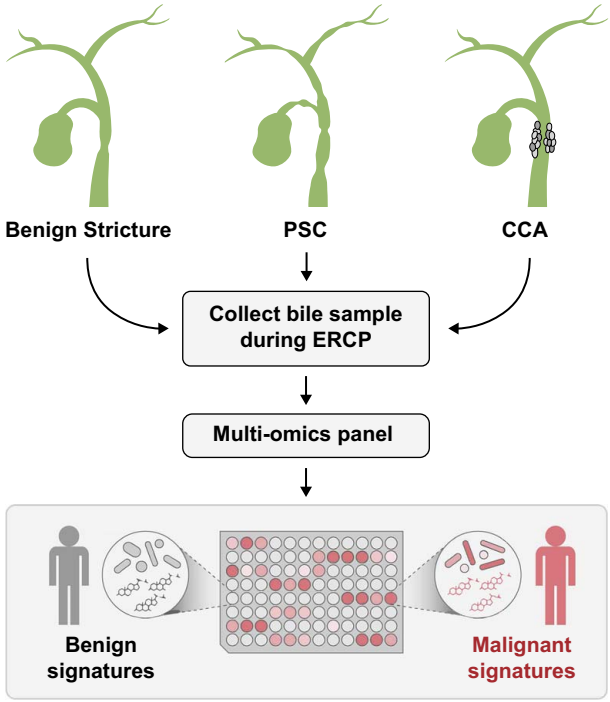

FIGURE 5 A schematic of multi-omics bile diagnostic panels that can be used as a method to enhance the current endoscopic evaluation of malignant biliary strictures. Abbreviations: CCA, cholangiocarcinoma; ERCP, endoscopic retrograde cholangiopancreatography; PSC, primary sclerosing cholangitis.

DISCUSSION

We have developed a multi-omics diagnostic panel that can reliably detect intraductal malignancy in patients undergoing ERCP for evaluation of indeterminate strictures (Figure 5). Overall, the negative and positive predictive values of our multi-omics panels are over 90%, which holds the potential to meet the current clinical need for more accurate and early detection of malignancy in indeterminate biliary strictures compared with current modalities of biliary biopsy and brush cytology.[7]

Endoscopy is still the main clinical modality used to distinguish benign and malignant biliary strictures.[29,30] Recently, studies utilizing next-generation sequencing to profile brush samples have been shown to increase clinical sensitivity to 73% but it is time-intensive and labor-intensive.[31] Furthermore, the amount of sample required to produce the quality of DNA needed for next-generation sequencing does not overcome the main issue of inadequate tissue acquisition common for most patients with indeterminate biliary strictures and PSC.[32] Our multi-omics diagnostic panels require only biliary fluid aspirate, which overcomes the difficulty of obtaining adequate tissue sampling. These panels were positive for all patients with malignant strictures during the time of ERCP, whereas biliary brushings or biopsies were negative for all patients during the same procedure. Although limited in size, our validation cohort was prospectively assembled and mirrors the diagnostic challenges faced in real-world endoscopic practice for indeterminate biliary strictures. No patients in the validation cohort with benign biliary strictures due to chronic pancreatitis, IgG4 disease, or transplant strictures tested positive with our diagnostic panels. Thus, our multi-omics panel can accurately risk-stratify patients undergoing endoscopic evaluation of indeterminate strictures.

Previous studies examining the bile microbiome have consistently identified microbial species associated with different biliary disease states.[17,33–36] In our cohort, we identified that the *Prevotella* genus was enriched in the bile of patients with PSC and CCA compared with BBD. Increased *Prevotella* has been linked to increased systemic inflammation, metabolic dysfunction, and the development of HCC in gut microbiome studies.[37,38] Longitudinal studies are needed to determine the role of

Prevotella in biliary dysplasia and tumorigenesis. Similar to previous studies, we observed a correlation between the biliary microbiome with gastric and oral microbial communities in patients with or without biliary sphincterotomy.[17,35] Future studies are needed to determine whether these community overlaps are due to duodenal fluid reflux into the biliary tract or bile reflux into the stomach.[39–41] Furthermore, we found that the biliary microbiome diversity and composition did not appear to be significantly affected by whether the patient had cholangitis before ERCP. In contrast, outpatient use of UDCA appeared to influence biliary microbial composition, underscoring a potential role of BAs in shaping the biliary microbiome. Overall, successful bacterial DNA extraction and control for contamination remain challenging for low-biomass microbiome studies in environments such as bile.[42] Similar to other studies on low-biomass environments, our use of magnetic bead-based DNA extraction has improved the reliability of our results by reducing erroneous signals and increasing DNA extraction yield.[43]

Basic and translational research utilizing non-targeted metabolomics has demonstrated unique serum and bile profiles of hepatobiliary malignancies and PSC.[25,26,44–46] Most of these studies have focused on non-polar metabolites such as lipids. Our study focused on the identification of unique polar metabolites that are hallmarks of metabolic reprogramming in tumorigenesis.[47] Untargeted lipidomics and metabolomics require cumbersome data analysis and equipment expertise that remains expensive and difficult to scale for current clinical practice.[48] Our diagnostics panels overcome this issue by identifying and focusing on specific microbiome and metabolite signatures unique to malignant strictures to assist in expedited clinical decision making.

There are ~12,000 new cases of CCA in the United States annually.[49] Currently, a major clinical gap remains in methods of precise, noninvasive early diagnostics of cholangiocarcinoma and cancer monitoring in PSC patients with dominant stricture. Our identification of microbiome and metabolite-based diagnostic panels holds the potential to enhance endoscopic evaluation of indeterminate biliary strictures and cancer surveillance in PSC, which currently is an unmet clinical need.

DATA AVAILABILITY STATEMENT

The repository accession code for 16S sequencing data is PRJNA1283195. Any additional information required to re-analyze the data reported in this work paper is available from the corresponding author upon request.

AUTHOR CONTRIBUTIONS

Jonathan Y. Xia designed the study, carried out the research, interpreted the results, wrote the manuscript and is responsible for the integrity of the work. Srinadh Komanduri, Rajesh N. Keswani, Jasmine Sinha, Terrance R. Rodrigues, and Arvind Rengarajan carried out the research and reviewed the manuscript. Chelsea Hepler performed the metabolomics experiments and interpreted the results. Arthur Prindle carried out the research and reviewed the manuscript. Peter Tran assisted in figure construction and reviewed the manuscript. A. Aziz Aadam designed the study, reviewed, and revised the manuscript.

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CONFLICTS OF INTEREST

Sri Komanduri consults and advises Lucid Diagnostics. He consults for Boston Scientific, Ethicon Endosurgery, Medtronic, Castle Biosciences, Aurora Medtech, and Merit Endotek. He advises EndoscopyNow. Rajesh Keswani consults and has received grants from Medtronic. He consults for Boston Scientific, Olympus, Neptune Medical, and Cook Medical. Jasmine Sinha consults for Boston Scientific. The remaining authors have no conflicts to report.

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