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Serum Metabolites as Diagnostic Biomarkers for Cholangiocarcinoma, Hepatocellular Carcinoma, and Primary Sclerosing Cholangitis

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Early and differential diagnosis of intrahepatic cholangiocarcinoma (iCCA) and hepatocellular carcinoma (HCC) by noninvasive methods represents a current clinical challenge. The analysis of low-molecular-weight metabolites by new high-throughput techniques is a strategy for identifying biomarkers. Here, we have investigated whether serum metabolome can provide useful biomarkers in the diagnosis of iCCA and HCC and could discriminate iCCA from HCC. Because primary sclerosing cholangitis (PSC) is a risk factor for CCA, serum metabolic profiles of PSC and CCA have also been compared. The analysis of the levels of lipids and amino acids in the serum of patients with iCCA, HCC, and PSC and healthy individuals (n = 20/group) showed differential profiles. Several metabolites presented high diagnostic value for iCCA versus control, HCC versus control, and PSC versus control, with areas under the receiver operating characteristic curve (AUC) greater than those found in serum for the nonspecific tumor markers carbohydrate antigen 19-9 (CA 19-9) and alpha-fetoprotein (AFP), commonly used to help in the diagnosis of iCCA and HCC, respectively. The development of an algorithm combining glycine, aspartic acid, SM(42:3), and SM(43:2) permitted to accurately differentiate in the diagnosis of both types of tumors (biopsy-proven). The proposed model yielded 0.890 AUC, 75% sensitivity, and 90% specificity. Another algorithm by combination of PC(34:3) and histidine accurately permitted to differentiate PSC from iCCA, with an AUC of 0.990, 100% sensitivity, and 70% specificity. These results were validated in independent cohorts of 14-15 patients per group and compared with profiles found in patients with nonalcoholic fatty liver disease/nonalcoholic steatohepatitis. *Conclusion:* Specific changes in serum concentrations of certain metabolites are useful to differentiate iCCA from HCC or PSC, and could help in the early diagnosis of these diseases. (Hepatology 2019;70:547-562).

Cholangiocarcinoma (CCA) includes a heterogeneous group of biliary cancers with a dismal prognosis.[1] Their incidence is increasing worldwide, representing a significant health problem. The bad outcome of CCAs is due to their late diagnosis and refractory nature. According to the anatomical location, CCAs are classified as intrahepatic (iCCA), perihilar, or distal. In particular, iCCA accounts for 10%-15% of all CCAs,[2] and, as opposed to other primary liver cancers, its mortality rate has risen in most Western countries over the past decades.[3] iCCA is generally asymptomatic in early stages and is commonly diagnosed in advanced phases, when symptoms arise and the disease is found disseminated,

Abbreviations: AFP, α-fetoprotein; AUC, area under the receiver operating characteristic curve; CA 19-9, carbohydrate antigen 19-9; CCA, cholangiocarcinoma; HCC, hepatocellular carcinoma; iCCA, intrahepatic cholangiocarcinoma; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; OPLS, orthogonal partial least squares; PCA, principal component analysis; PSC, primary sclerosing cholangitis; UDCA, ursodeoxycholic acid; UHPLC, ultra-high-performance liquid chromatography.

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which limits current potentially curative options based on surgery or liver transplantation.[4,5] Therefore, only 25%-30% of patients with iCCA are eligible for surgery at the time of diagnosis. A combination of radiological, biochemical, and histological approaches is required to confirm the iCCA diagnosis. Moreover, histological analyses of tumor biopsies are required to differentially diagnose iCCA from the most common type of primary liver cancer, hepatocellular carcinoma (HCC), which is fundamental for providing adequate first-line treatments as indicated by international guidelines.[6] However, tumor biopsy is not indicated in the majority of cases because of the advanced disease stage and concomitant risks. The etiology of most iCCAs is unknown, although chronic cholestatic liver diseases, such as primary sclerosing cholangitis (PSC), are risk factors.[5] Currently, early diagnosis of CCA in patients with PSC by noninvasive methods is challenging and highly compromises patient outcome.[7]

Therefore, there is an urgent need for accurate noninvasive biomarkers for the early and differential diagnosis of iCCA and HCC. Cancer cells are highly metabolically active,[8] which is both cause and consequence of their pathogenesis, but this may also represent an opportunity for diagnosis and treatment. The analysis of the metabolome in body fluids is emerging as a diagnostic strategy that could be associated with the progression of the disease.[9] In this context, the aim of the present study was to determine serum metabolomic profiles in the search of accurate

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diagnostic biomarkers that could be used to distinguish iCCA from HCC or PSC, and also for the early diagnosis of these diseases.

Material and Methods

STUDY POPULATION AND ELIGIBILITY

Serum samples from patients with iCCA, HCC, and PSC and healthy individuals (n = 20/group in the "discovery cohort" and n = 14-15/group in the "validation cohort") and from patients with nonalcoholic fatty liver disease (NAFLD) and nonalcoholic steatohepatitis (NASH; n = 20/group) were obtained from the Donostia University Hospital (San Sebastian, Spain), the Biobank of "Clínica Universidad de Navarra" (Pamplona, Spain), the National DNA Bank-Carlos III of University of Salamanca (Salamanca, Spain), the Biobank of the University Hospital "Vírgen del Rocío-Instituto de Biomedicina de Sevilla" (Seville, Spain), and the Medical University of Warsaw (Warsaw, Poland). The research protocol was approved by the Ethics Committee for Clinical Research of each supporting institution, and all subjects provided written consent for their samples to be used for biomedical research.

Inclusion criteria for patients with iCCA and HCC included the following: 1) diagnosis confirmed by histopathology; 2) availability of serum samples obtained just after diagnosis and before patients had received any type of treatment; and 3) availability of complete demographic and clinical information. Exclusion criteria were as follows: 1) "mixed" tumors, as diagnosed by histopathology; 2) no definite diagnosis of iCCA or HCC; and 3) the presence of other types of malignancy. The histologic diagnoses of iCCA or HCC were established by expert liver pathologists at each participating hospital, and tumors were classified according to the 7th edition of the American Joint Committee on Cancer Staging Manual[10] and the Barcelona Clinic Liver Cancer (BCLC) staging system,[11] respectively.

Patients with PSC were diagnosed by the presence of bile duct alterations (multifocal strictures and segmental dilatations) by cholangiography and by the exclusion of secondary sclerosing cholangitis causes, following the guidelines of the European Association for the Study of the Liver.[12] All patients with PSC were treated with ursodeoxycholic acid (UDCA).

Inclusion criteria for patients with NAFLD or NASH were as follows: 1) diagnosis by histopathology NAFLD Activity Score (NAS score); 2) absence of acute or chronic disease except obesity or type 2 diabetes; and 3) alcohol consumption <20 g/day for women and <30 g/day for men. Exclusion criteria were other causes of liver disease, such as viral hepatitis.

For all subjects, blood was obtained under fasting conditions and serum was collected and stored at -80°C until analysis. Patient information and laboratory test values were collected retrospectively from the patients' records.

METABOLOMIC ANALYSES

The semi-quantification of the serum metabolic profiles was performed, as previously described.[13] Briefly, two ultra-high-performance liquid chromatography (UHPLC)-time-of-flight-mass spectrometry (MS)–based platforms analyzing methanol and chloroform/methanol serum extracts were combined with the amino acids measurement by a UHPLC-single-quadrupole-MS–based analysis. Identified ion features in the methanol extract platform included acyl carnitines, bile acids, free sphingoid bases, monoacylglycerophospholipids, monoetherglycerophospholipids, fatty acids, and oxidized fatty acids. The coverage of the chloroform/methanol extract included glycerolipids, diacylglycerophospholipids, acyl-ether-glycerophospholipids, cholesteryl esters, and sphingolipids. Lipid nomenclature and classification follows the LIPID MAPS convention (www.lipidmaps.org). Metabolite extraction procedures for each platform, as well as the chromatographic separation conditions and mass spectrometric detection conditions, have been previously described in detail.[13] Metabolomics data were preprocessed using the TargetLynx application manager for MassLynx 4.1 (Waters Corp., Milford, MA). Intra- and inter-batch normalization was performed by inclusion of multiple internal standards and pool calibration response correction, following a previously described procedure.[14]

STATISTICAL ANALYSIS

Data are shown as mean ± SD. Differences between groups were determined using the Student *t*-test or the Bonferroni method of multiple range test, where

appropriate. Volcano plot analysis was performed as an effective and easy-to-understand graph that summarizes fold-change and significance. It is a scatterplot of the negative $\log_{10}$-transformed *P* values from the *t* test against the $\log_2$ fold-change. Multivariate principal component analysis (PCA) and orthogonal partial least squares (OPLS) modeling were performed with the software SIMCA 14.1 (Umetrics, Malmo, Sweden). All calculations were performed using the statistical software package R v.3.4.0 (R Development Core Team, 2017; https://cran.r-project.org).

To find statistical models to differentiate iCCA versus HCC or PSC, a generalized linear model (GLM) was used to differentiate iCCA versus HCC or PSC, and leave-one-out cross validation (LOOCV) was performed to confirm the model. Box-Cox transformations were applied to the biomarker metabolite levels for correcting non-normally distributed data and used to calculate the classification algorithm. The diagnostic accuracy of the model to identify patients in each comparison was assessed using the area under the receiver operating characteristic curve (AUC).

Results

CHARACTERISTICS OF THE STUDY POPULATION

The demographic and clinical features of participants of the discovery cohort are summarized in Table 1A. No differences between the metabolic profiles of the samples due to the hospital of origin were found, as demonstrates the unsupervised PCA in Supporting Fig. 1A. Despite differences in mean age of patients among groups, and in gender in the control group compared with the other groups, no differences between serum metabolic profiles of the samples due to gender or age were found, as shown in the unsupervised PCAs in Supporting Figs. S1B, S1C, respectively. Moreover, no difference among patients due to the presence or absence of liver cirrhosis was found (Supporting Fig. S1D). PCA analysis showed that patients with iCCA clustered together irrespective of the stage (Supporting Fig. S1E), whereas the effect of tumor stage could not be analyzed in patients with HCC because most of them presented a BCLCA stage (Supporting Fig. S1F). A random distribution of the samples of patients and controls was observed (Supporting Fig. S1G). Statistical comparisons of the liver biochemical data (Table 1A) revealed higher levels of transaminases, alkaline phosphatase, and gamma-glutamyl transpeptidase in the serum of patients with PSC compared with healthy controls and, in some cases, also compared with patients with liver cancers. An important variability in the levels of nonspecific tumor biomarkers CA 19-9 and α-fetoprotein (AFP) was found in the serum of patients with iCCA and HCC, respectively. The demographic and clinical characteristics of patients of the validation cohort (Table 1B) were, in general, quite similar to those described for the discovery cohort. Supporting Fig. 1H shows that patients clustered together in both discovery and validation cohorts.

METABOLOMIC PROFILING IN SERUM FROM PATIENTS WITH ICCA, HCC, AND PSC AND HEALTHY INDIVIDUALS

A total of 424 metabolites in serum samples were identified in both cohorts (discovery and validation) and included in the subsequent univariate and multivariate data analyses. Notably, one analysis complements the other, and although their results do not always coincide, the use of both methods offers important advantages in data mining.[15] Supporting Fig. S2 shows the heatmaps representing fold-changes and *P* values generated for the different two-group comparisons in the discovery cohort, in the validation cohort, and considering all samples together. Changes in the levels of several compounds belonging to different families of metabolites, such as amino acids, saturated, monounsaturated and polyunsaturated fatty acids, diglycerides, triglycerides, cholesteryl esters, bile acids, steroids, lysophosphatidylethanolamines, lysophosphatidylcholines, phosphatidylinositols, ceramides, sphingomyelins, and monohexosylceramides, were found in all comparisons. The most interesting changes for each comparison are described in detail below.

COMPARATIVE SERUM METABOLOMIC CONTENT IN THE DISCOVERY COHORT

The unsupervised PCA model showed no separation in the serum metabolomic profiles between iCCA and control subjects (Supporting Fig. S3A),

TABLE 1A. Demographic and Clinical Characteristics of the Study Population

Variable	Control (n = 20)	iCCA (n = 20)	HCC (n = 20)	PSC (n = 20)
Age, mean ± SD	54.6 ± 15.5	68.4 ± 9.8*	58.8 ± 7.1*	33.6 ± 9.6*†
Age, range	25-72	46-81	49-71	19-54
Males, n (%)	8 (40)	12 (60)	19 (95)	16 (80)
Underlying disease, n (%)				
Cirrhosis	0 (0)	5 (25)	19 (95)	8 (40)
Hepatitis B virus	0 (0)	0 (0)	1 (5)	0 (0)
Hepatitis C virus	0 (0)	2 (10)	13 (65)	0 (0)
Alcoholic	NA	5 (25)	7 (35)	NA
Diabetes	3 (15)	2 (10)	7 (35)	NA
Ulcerative colitis	0 (0)	0 (0)	0 (0)	16 (80)
Number of nodules, n (%)				
Single	—	15 (75)	17 (85)	—
Two	—	2 (10)	2 (10)	—
More than two	—	2 (10)	1 (5)	—
Unknown	—	1 (5)	0 (0)	—
Nodule size (cm), n (%)				
<5	—	11 (55)	19 (95)	—
5-10	—	4 (20)	1 (5)	—
>10	—	2 (10)	0 (0)	—
Unknown	—	3 (15)	0 (0)	—
Tumor stage, n (%)				
BCLCA	—	—	18 (90)	—
BCLCB	—	—	1 (5)	—
BCLCC	—	—	1 (5)	—
I	—	9 (45)	—	—
II	—	2 (10)	—	—
III	—	3 (15)	—	—
IV	—	6 (30)	—	—
Tumor grade, n (%)				
Well differentiated	—	5 (25)	7 (35)	—
Moderately differentiated	—	10 (50)	13 (65)	—
Poorly differentiated	—	1 (5)	0 (0)	—
Unknown	—	4 (20)	0 (0)	—
Biochemistry, mean ± SD				
ALT (IU/L)	27.4 ± 14.1	42.0 ± 40.2	50.5 ± 34.8	107 ± 89*†
AST (IU/L)	23.6 ± 8.2	51.8 ± 50.3	46.6 ± 23.8	97.1 ± 73.4*
GGT (IU/L)	31.0 ± 20.1	134 ± 117*	118 ± 84.7*	281 ± 311*
Alkaline phosphatase (IU/L)	68.8 ± 18.9	165 ± 101*	77.9 ± 25.4†	335 ± 256*
Total bilirubin (mg/dL)	0.7 ± 0.3	1.5 ± 2.9	0.9 ± 0.7	4.5 ± 10.5
CA 19-9 (IU/mL)	9.8 ± 7.4	1,746 ± 4,921	29.3 ± 28.6	51.9 ± 121
AFP (ng/mL)	2.8 ± 2.1	3.2 ± 2.4	117 ± 267	NA

*$P < 0.05$ compared with control.
†$P < 0.05$ compared with iCCA, by the Bonferroni method of multiple-range testing.
Abbreviations: AFP, alpha-fetoprotein; ALT; alanine aminotransferase, AST; aspartate aminotransferase, BCLC, Barcelona Clinic Liver Cancer; GGT, gamma-glutamyl transpeptidase; CA19-9, carbohydrate antigen 19-9; HCC, hepatocellular carcinoma; iCCA, intrahepatic cholangiocarcinoma; NA, not available; PSC, primary sclerosing cholangitis.

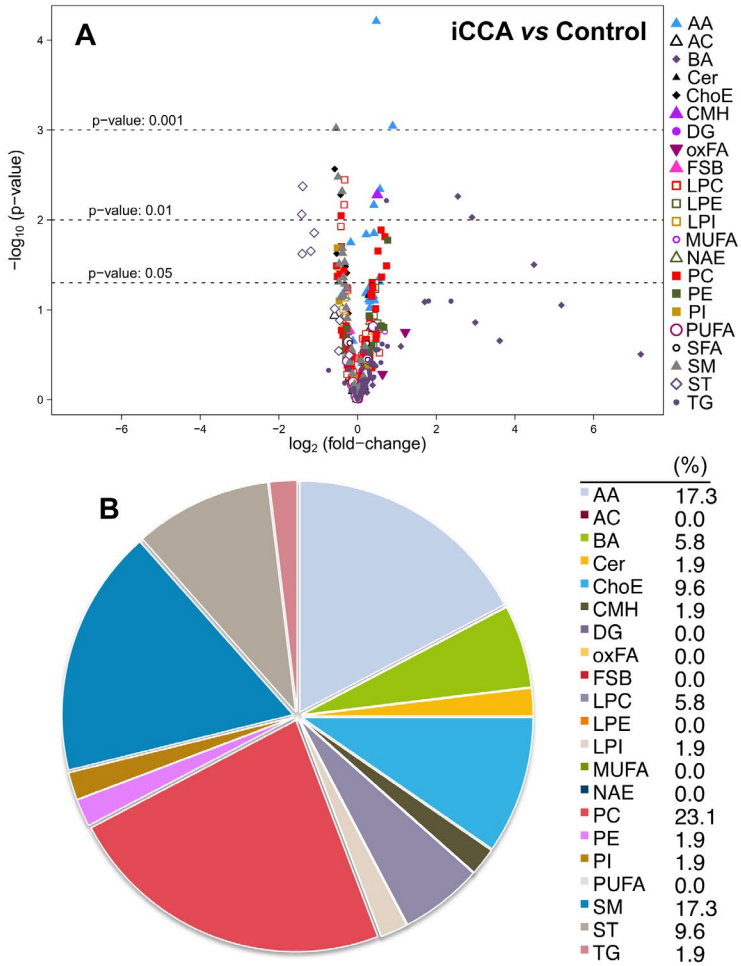
and the supervised OPLS model showed a low predictive ability (Supporting Fig. S3B), because the Q2X value was low (0.258). However, as shown in the volcano plot generated for this comparison (Fig. 1A), a total of 52 metabolites were altered in serum from patients with iCCA, where

TABLE 1B. Demographic and Clinical Characteristics of the Validation Cohort

Variable	Control (n = 15)	iCCA (n = 15)	HCC (n = 14)	PSC (n = 15)
Age, mean ± SD	52.5 ± 9.9	70.7 ± 5.4*	62.1 ± 9.2	38.3 ± 8.2*†
Age, range	32-68	61-81	46-75	30-61
Males, n (%)	7 (46.7)	8 (53.3)	13 (92.9)	9 (60)
Underlying disease, n (%)				
Cirrhosis	0 (0)	2 (13.3)	10 (71.4)	2 (13.3)
Hepatitis B virus	0 (0)	0 (0)	0 (0)	0 (0)
Hepatitis C virus	0 (0)	0 (0)	5 (35.7)	0 (0)
Alcoholic	0 (0)	6 (40)	9 (64.3)	0 (0)
Diabetes	0 (0)	5 (33.3)	4 (28.6)	NA
Ulcerative colitis	0 (0)	0 (0)	0 (0)	12 (80)
Number of nodules, n (%)				
Single	—	11 (73.3)	8 (57.2)	—
Two	—	3 (20)	3 (21.4)	—
More than two	—	1 (6.7)	3 (21.4)	—
Nodule size (cm), n (%)				
<5	—	9 (60)	9 (64.3)	—
5-10	—	5 (33.3)	3 (21.4)	—
>10	—	1 (6.7)	2 (14.3)	—
Tumor stage, n (%)				
BCLCA	—	—	10 (71.4)	—
BCLCB	—	—	2 (14.3)	—
BCLCC	—	—	2 (14.3)	—
I	—	6 (40)	—	—
II	—	3 (20)	—	—
III	—	2 (13.3)	—	—
IV	—	4 (26.7)	—	—
Tumor grade, n (%)				
Well differentiated	—	4 (26.7)	3 (21.4)	—
Moderately differentiated	—	3 (20)	9 (64.3)	—
Poorly differentiated	—	3 (20)	0 (0)	—
Unknown	—	5 (33.3)	2 (0)	—
Biochemistry, mean ± SD				
ALT (IU/L)	29.7 ± 12.6	36.3 ± 37.1	41.4 ± 16.0	92.5 ± 103.4*†
AST (IU/L)	18.8 ± 5.9	33.5 ± 13.5	60.4 ± 41.9	52.0 ± 40.0*
GGT (IU/L)	25.4 ± 19.0	293 ± 240	133 ± 65	268 ± 233*
Alkaline phosphatase (IU/L)	54.5 ± 14.5	203 ± 169	109 ± 39	238 ± 273*†
Total bilirubin (mg/dL)	0.6 ± 0.3	1.9 ± 4.2	1.3 ± 1.9	0.9 ± 0.5
CA 19-9 (IU/mL)	6.9 ± 7.6	535 ± 1,332	NA	24.1 ± 56.8
AFP (ng/mL)	2.4 ± 0.9	2.0 ± 0.9	250 ± 496	NA

*$P < 0.05$ compared with control.
†$P < 0.05$ compared with iCCA, by the Bonferroni method of multiple-range testing.
Abbreviations: AFP, alpha-fetoprotein; ALT; alanine aminotransferase, AST; aspartate aminotransferase, BCLC, Barcelona Clinic Liver Cancer; GGT, gamma-glutamyl transpeptidase; CA19-9, carbohydrate antigen 19-9; HCC, hepatocellular carcinoma; iCCA, intrahepatic cholangiocarcinoma; NA, not available; PSC, primary sclerosing cholangitis.

phosphatidylcholines > amino acids ≈ sphingomyelins and sterols were the most abundant metabolite families found to be altered (Fig. 1B). Fig. 1C shows the nine metabolites with the highest AUC, sensitivity, and specificity also found altered in the validation cohort. All of them presented higher values than CA 19-9, the unspecific tumor biomarker clinically used in the diagnosis of CCA.



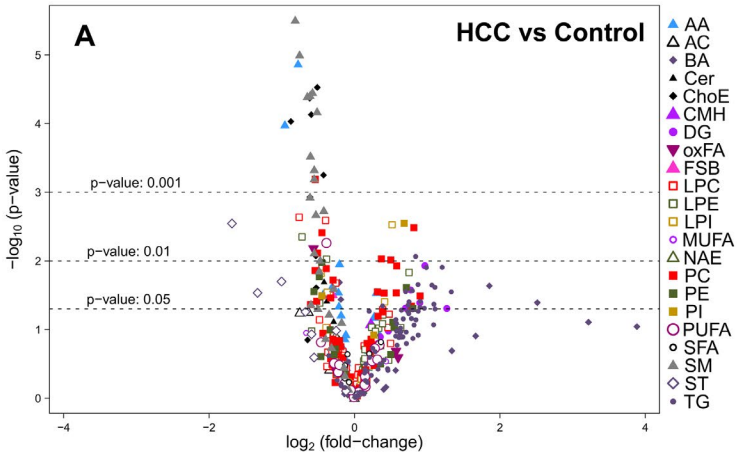
C iCCA *vs* Control

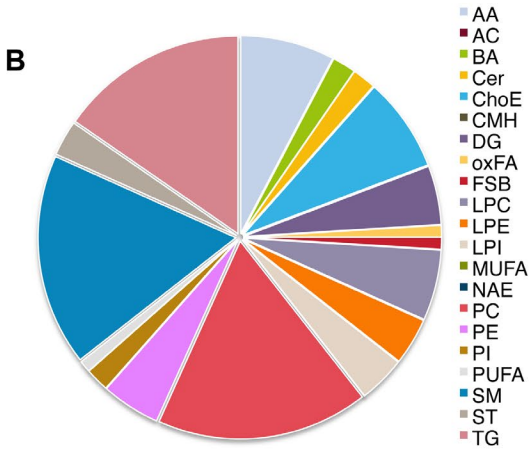
	Metabolite	AUC	SEN (%)	SPE (%)	Log2 (FC)
BA	GCA	0.857	65	90	2.78
	GCDCA	0.823	70	90	2.47
ST	Androsterone sulfate II	0.808	95	58	-1.41
	DHEAS	0.790	65	85	-1.39
	ChoE(22:6)	0.763	65	85	-0.58
	ChoE(20:4)	0.760	80	75	-0.43
CMH	CMH(d18:1/16:0)	0.798	75	80	0.50
PC	PC(16:0/16:0)	0.773	55	100	0.60
SM	SM(d18:2/20:0)	0.755	65	75	-0.49
	CA 19-9	0.720	75	80	7.48

FIG. 1. Comparative serum metabolomic profiles of patients with iCCA and controls in the discovery cohort. (A) Volcano plot ($-\log_{10}$[*P* value] and $\log_2$[fold-change]) of the serum metabolic ion features of patients with iCCA compared with controls. (B) Percentage of metabolite classes significantly different in the serum of patients with iCCA compared with healthy individuals. (C) Diagnostic capacity of the nine selected metabolites also similarly found altered in the validation cohort. Abbreviations: AA, amino acids; AC, acylcarnitines; AUC, area under the receiver operating characteristic curve; BA, bile acids; Cer, ceramides; ChoE, cholesteryl esters; CMH, monohexosylceramides; DG, diglycerides; DHEAS, dehydroepiandrosterone sulfate; FC, fold-change; FSB, free sphingoid bases; GCA, glychocolic acid; GCDCA, glychochenodeoxycholic acid; LPC, lysophosphatidylcholines; LPE, lysophosphatidylethanolamines; LPI, lysophosphatidylinositols; MUFA, monounsaturated fatty acids; NAE, N-acyl ethanolamines; oxFA, oxidized fatty acids; PC, phosphatidylcholines; PE, phosphatidylethanolamines; PI, phosphatidylinositols; PUFA, polyunsaturated fatty acids; SEN, sensitivity; SFA, saturated fatty acids; SM, sphingomyelins; SPE, specificity; ST, steroids; TG, triglycerides.

The unsupervised PCA model generated for the comparison of HCC versus control revealed a potential separation between groups (Supporting Fig. S3C), and the supervised OPLS model separated both groups with moderate predictive ability (Q2X = 0.499) (Supporting Fig. S3D). The volcano plot (Fig. 2A) showed that 104 metabolites were altered in the serum from patients with HCC, mainly phosphatidylcholines ≈ sphingomyelins ≈ triglycerides > amino acids, cholesteryl esters > lysophosphatidylcholines (Fig. 2B). The 13 metabolites with the highest AUC, sensitivity, and specificity in this comparison, and similarly found altered in the validation cohort, are shown in Fig. 2C. Most of them showed higher values than the biomarker AFP.

The unsupervised PCA model performed including patients with HCC and iCCA (Supporting Fig. S3E) showed no clear separation of samples, and the supervised OPLS discriminant analysis (Supporting Fig. S3F) showed a negative predictive ability





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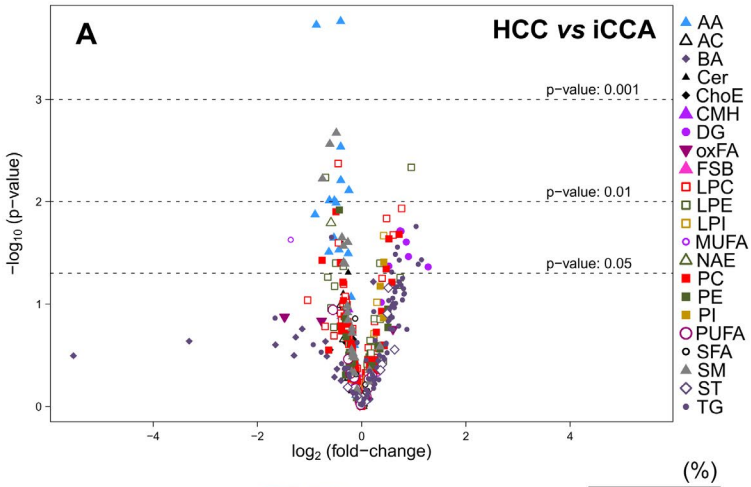
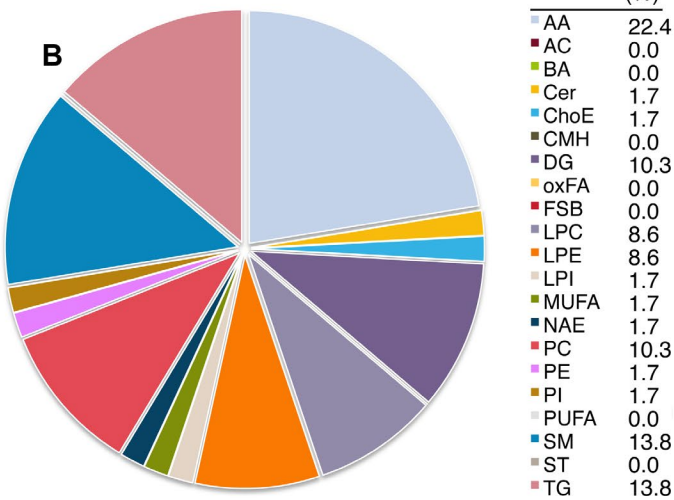
HCC vs Control	Metabolite	AUC	SEN (%)	SPE (%)	Log 2 (FC)
SM	SM(36:2)	0.895	90	85	-0.82
	SM(d18:2/20:0)	0.880	65	100	-0.75
	SM(d18:1/18:0)	0.858	80	85	-0.58
	SM(42:1)	0.813	65	90	-0.55
	SM(d18:1/22:0)	0.803	65	90	-0.55
AA	Taurine	0.873	75	90	-0.78
ChoE	ChoE(20:4)	0.873	80	90	-0.62
	ChoE(18:2)	0.858	65	95	-0.51
	ChoE(22:6)	0.828	70	90	-0.87
	ChoE(16:0)	0.800	80	80	-0.43
BA	Glycocholic acid	0.858	80	80	2.44
PC	PC(16:0/22:6)	0.800	65	90	-0.54
LPI	LPI(16:0)	0.785	90	80	0.44
	AFP	0.808	80	70	5.30

FIG. 2. Comparative serum metabolomic profiles of patients with HCC and controls in the discovery cohort. (A) Volcano plot ($-\log_{10}$[*P* value] and $\log_2$[fold-change]) of the serum metabolic ion features of patients with HCC compared with controls. (B) Percentage of metabolite classes significantly different in the serum of patients with HCC compared with healthy individuals. (C) Diagnostic capacity of the 13 selected metabolites also similarly found altered in the validation cohort. Abbreviations: AA, amino acids; AC, acylcarnitines; AUC, area under the receiver operating characteristic curve; BA, bile acids; Cer, ceramides; ChoE, cholesteryl esters; CMH, monohexosylceramides; DG, diglycerides; FC, fold-change; FSB, free sphingoid bases; LPC, lysophosphatidylcholines; LPE, lysophosphatidylethanolamines; LPI, lysophosphatidylinositols; MUFA, monounsaturated fatty acids; NAE, N-acyl ethanolamines; oxFA, oxidized fatty acids; PC, phosphatidylcholines; PE, phosphatidylethanolamines; PI, phosphatidylinositols; PUFA, polyunsaturated fatty acids; SEN, sensitivity; SFA, saturated fatty acids; SM, sphingomyelins; SPE, specificity; ST, steroids; TG, triglycerides.

(Q2X = −0.013). In addition, a total of 58 metabolites were different upon comparing the serum of patients with both types of liver cancer (Fig. 3A). Different levels of amino acids > sphingomyelins ≈ triglycerides > diglycerides and other metabolite species were found when comparing HCC versus iCCA (Fig. 3B); more precisely, this included seven amino acids, three sphingomyelins, and eight glycerolipids. Fig. 3C shows six metabolites with the highest values of AUC, sensitivity, and specificity for this comparison, and that were also found similarly altered in the validation cohort. The six independent metabolites showed higher AUC diagnostic values than CA 19-9. However, the ability of AFP to discriminate iCCA from HCC was higher than that of CA 19-9, and the AUC value was also a little higher than the values of some of the aforementioned metabolites analyzed independently. Of note, one of the selected candidate metabolites for the differential diagnosis of iCCA versus HCC (i.e., hypotaurine) improved its diagnostic values when comparing early-stage iCCA (I-II) versus HCC (Fig. 3D), and also showed higher





C HCC *vs* iCCA

	Metabolite	AUC	SEN (%)	SPE (%)	Log2 (FC)
AA	Hypotaurine	0.725	55	90	-0.89
	Phe-Phe	0.675	80	55	-0.63
PC	PC(O-18:0/18:2)	0.730	65	85	0.72
PE	PE(14:0/0:0)	0.705	75	60	-0.44
Cer	Cer(d18:1/16:0)	0.685	70	80	-0.26
SM	SM(d18:2/16:0)	0.683	50	90	-0.26
	AFP	0.785	75	75	5.11
	CA 19-9	0.575	100	35	-5.90

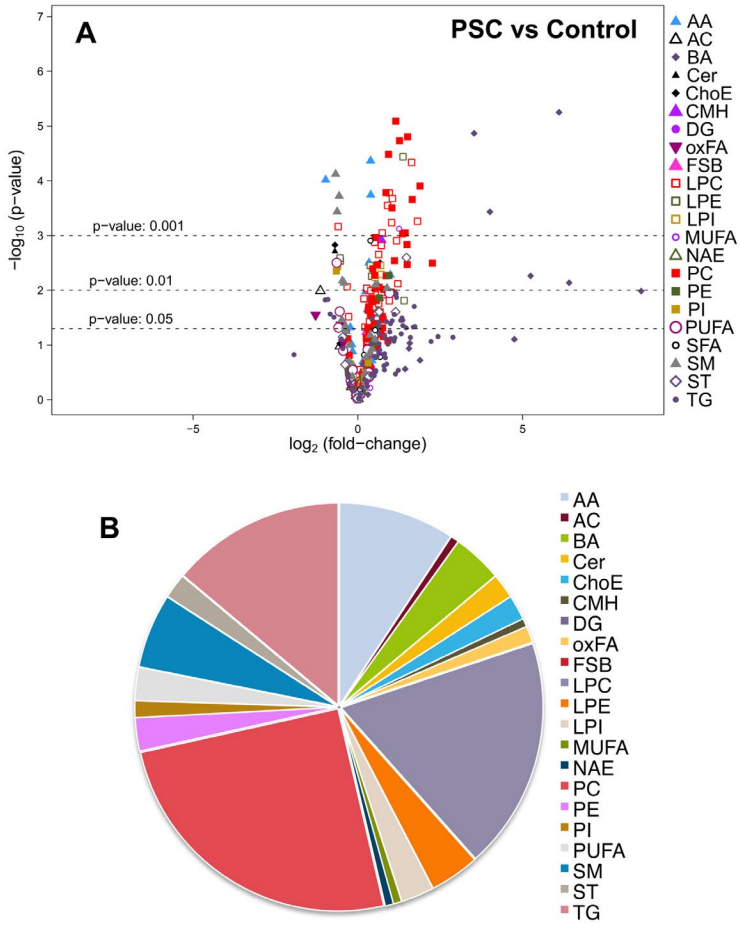
D HCC *vs* iCCA (I-II stage)

	Metabolite	AUC	SEN (%)	SPE (%)	Log2 (FC)
AA	Hypotaurine	0.805	64	90	-1.07
	AFP	0.809	75	82	5.48
	CA 19-9	0.586	82	50	-0.84

FIG. 3. Comparative serum metabolomic profiles of patients with HCC and iCCA in the discovery cohort. (A) Volcano plot ($-\log_{10}$[*P* value] and $\log_2$[fold-change]) of the serum metabolic ion features of patients with iCCA compared with HCC. (B) Percentage of metabolite classes significantly different in the serum of patients with HCC versus iCCA. (C) Diagnostic capacity of the six selected metabolites also similarly found altered in the validation cohort. Abbreviations: AA, amino acids; AC, acylcarnitines; AUC, area under the receiver operating characteristic curve; BA, bile acids; Cer, ceramides; ChoE, cholesteryl esters; CMH, monohexosylceramides; DG, diglycerides; FC, fold-change; FSB, free sphingoid bases; LPC, lysophosphatidylcholines; LPE, lysophosphatidylethanolamines; LPI, lysophosphatidylinositols; MUFA, monounsaturated fatty acids; NAE, N-acyl ethanolamines; oxFA, oxidized fatty acids; PC, phosphatidylcholines; PE, phosphatidylethanolamines; PI, phosphatidylinositols; PUFA, polyunsaturated fatty acids; SEN, sensitivity; SFA, saturated fatty acids; SM, sphingomyelins; SPE, specificity; ST, steroids; TG, triglycerides.

diagnostic values than CA 19-9 (AUC [95% confidence interval], 0.586 [0.385-0.765]) and similar to AFP (AUC [95% confidence interval], 0.809 [0.673-0.942]) in this comparison.

As a secondary aim, we investigated the serum metabolic profile of patients with PSC, which allowed PSC to be distinguished from iCCA. The unsupervised PCA model revealed a degree of separation between PSC and control individuals (Supporting Fig. S3G), whose significance increased following a supervised OPLS discriminant analysis to potentiate the differences between groups (Supporting Fig. S3H; model predictive ability Q2X = 0.565). The volcano plot generated for this comparison (Fig. 4A) showed that patients with PSC had higher levels of bile acids, phosphatidylethanolamines, phosphatidyl- and lysophosphatidyl-cholines, and lysophosphatidylinositols, together with a decrease in some species of fatty acids, sphingomyelins, and triglycerides, where phosphatidylcholines ≈ lysophosphatidylcholines > triglycerides



C

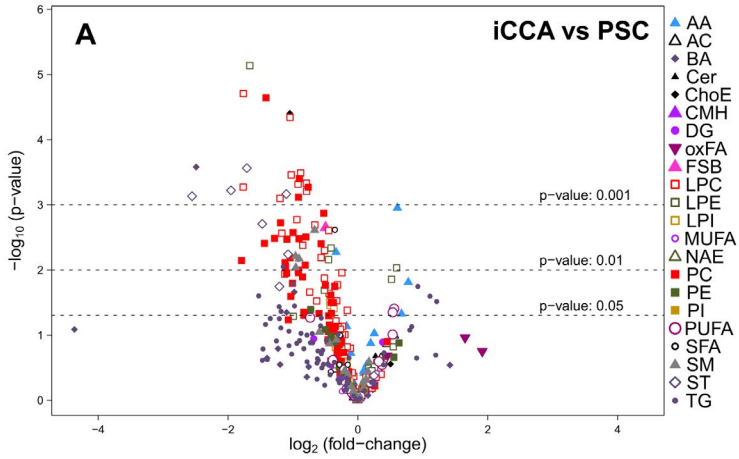
PSC *vs* Control

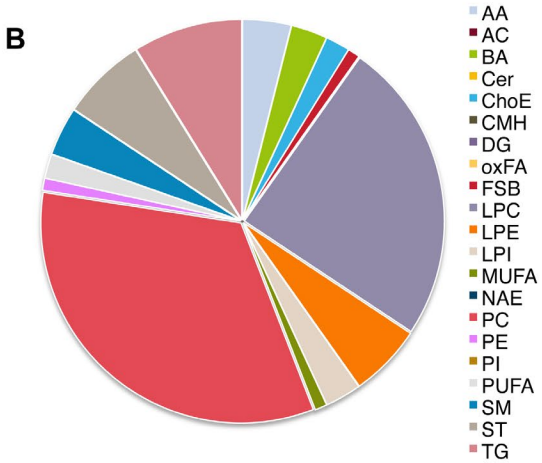
	Metabolite	AUC	SEN (%)	SPE (%)	Log2 (FC)
PC	PC(34:3)	0.930	80	95	1.51
	PC(31:0)	0.928	90	85	1.65
	PC(30:0)	0.923	90	80	1.89
	PC(14:0/16:1)	0.903	95	70	2.26
	PC(O-16:0/14:0)	0.893	90	90	1.26
	PC(O-16:0/16:0)	0.885	80	90	0.93
	PC(P-16:0/14:0)	0.883	80	90	1.15
	PC(32:1)	0.883	85	75	1.49
	PC(16:0/17:0)	0.870	85	80	1.44
	PC(16:0/16:0)	0.868	70	100	0.85
	PC(15:0/18:2)	0.858	70	90	1.04
	PC(33:1)	0.850	65	95	1.36
	PC(14:0/18:2)	0.830	95	75	1.50
BA	Glycocholic acid	0.920	80	95	3.87
PE	PE(16:1/0:0)	0.895	85	90	1.32
AA	D(-)-2-aminobutyric acid	0.888	80	95	-0.98
LPC	PC(0:0/18:3)	0.880	70	95	1.84
	PC(18:3/0:0)-I	0.875	75	90	1.64
	PC(17:1/0:0)	0.865	100	60	0.96
	PC(16:1/0:0)	0.845	70	90	0.85
	PC(0:0/14:0)	0.825	90	70	1.12
	PC(14:0/0:0)	0.815	90	70	0.96
	PC(0:0/17:1)	0.803	60	95	0.53
CMH	CMH(d18:1/16:0)	0.828	75	90	0.69

FIG. 4. Comparative serum metabolomic profiles of patients with PSC and controls in the discovery cohort. (A) Volcano plot ($-\log_{10}$[*P* value] and $\log_2$[fold-change]) of the serum metabolic ion features of patients with PSC compared with controls. (B) Percentage of metabolite classes significantly different in the serum of patients with PSC compared with healthy individuals. (C) Diagnostic capacity of the 24 selected metabolites also similarly found altered in the validation cohort. Abbreviations: AA, amino acids; AC, acylcarnitines; AUC, area under the receiver operating characteristic curve; BA, bile acids; Cer, ceramides; ChoE, cholesteryl esters; CMH, monohexosylceramides; DG, diglycerides; FC, fold-change; FSB, free sphingoid bases; LPC, lysophosphatidylcholines; LPE, lysophosphatidylethanolamines; LPI, lysophosphatidylinositols; MUFA, monounsaturated fatty acids; NAE, N-acyl ethanolamines; oxFA, oxidized fatty acids; PC, phosphatidylcholines; PE, phosphatidylethanolamines; PI, phosphatidylinositols; PUFA, polyunsaturated fatty acids; SEN, sensitivity; SFA, saturated fatty acids; SM, sphingomyelins; SPE, specificity; ST, steroids; TG, triglycerides.

> amino acid were the most abundant species altered in PSC (Fig. 4B). Also, 151 metabolites were found to be altered in the serum of these patients. Fig. 4C depicts the 24 metabolites with the highest AUC, sensitivity, and specificity in this comparison, which were also found similarly altered in the validation cohort. Because patients with PSC had received treatment with UDCA, levels of conjugated UDCA were higher in patients with PSC as compared with healthy controls.

Finally, the unsupervised PCA performed with iCCA and PSC samples did not show a clear separation between these groups (Supporting Fig. S3I), but a potential separation between both groups was obtained when the supervised OPLS model was generated (Supporting Fig. S3J), with a moderate predictive ability (Q2X = 0.554). The volcano plot generated for this comparison (Fig. 5A) showed 102 metabolites altered in the serum of patients with iCCA versus PSC, mainly phosphatidylcholine and lysophosphatidylcholine species (Fig. 5B), which were lower in iCCA samples. Fig. 5C shows the 12 metabolites with the highest values of AUC, sensitivity, and





C iCCA *vs* PSC

	Metabolite	AUC	SEN (%)	SPE (%)	Log2 (FC)
PC	PC(34:3)	0.970	100	80	-1.42
	PC(14:0/16:1)	0.855	65	95	-1.79
PE	PE(16:1/0:0)	0.913	90	90	-1.58
LPC	PC(18:3/0:0)-I	0.890	75	95	-1.69
	PC(0:0/18:3)	0.875	80	95	-1.79
	PC(17:1/0:0)	0.860	90	70	-0.84
	PC(16:1/0:0)	0.843	70	90	-0.79
	PC(18:2/0:0)	0.840	60	100	-1.07
	PC(14:0/0:0)	0.823	65	90	-0.93
	PC(0:0/14:0)	0.823	90	65	-1.10
	PC(0:0/15:0)	0.775	70	85	-0.71
ChoE	ChoE(18:3)	0.877	90	75	-1.05
	CA 19-9	0.601	40	90	-5.07

D iCCA (I-II stage) *vs* PSC

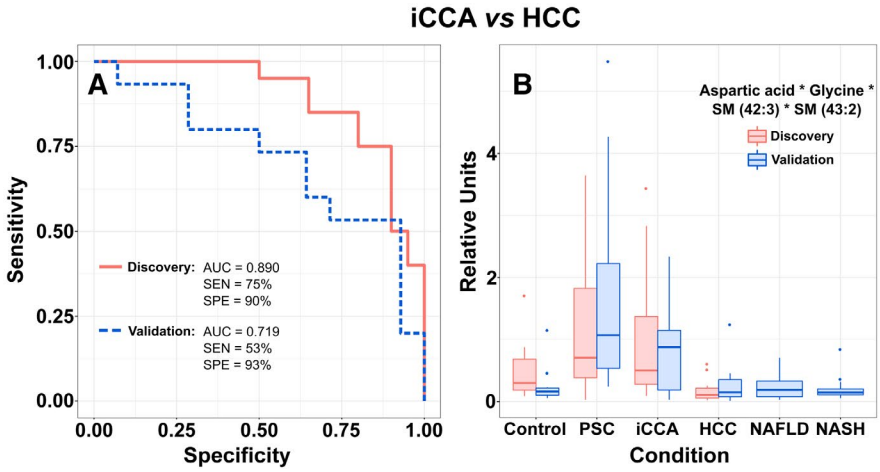
	Metabolite	AUC	SEN (%)	SPE (%)	Log2 (FC)
PC	PC(34:3)	0.986	100	91	-1.69
	PC(14:0/16:1)	0.914	85	91	-2.22
LPC	PC(18:3/0:0)-I	0.882	85	82	-1.64
	PC(17:1/0:0)	0.859	100	64	-0.85
	PC(16:1/0:0)	0.841	100	64	-0.83
	PC(0:0/18:3)	0.864	80	91	-1.74
	PC(18:2/0:0)	0.855	60	100	-1.12
	PC(14:0/0:0)	0.800	90	82	-0.85
LPE	PE(16:1/0:0)	0.936	95	91	-1.46
ChoE	ChoE(18:3)	0.885	89	82	-1.14
	CA 19-9	0.484	45	70	0.008

FIG. 5. Comparative serum metabolomic profiles of patients with iCCA and PSC in the discovery cohort. (A) Volcano plot ($-\log_{10}$[*P* value] and $\log_2$[fold-change]) of the serum metabolic ion features of patients with iCCA compared with PSC. (B) Percentage of metabolite classes significantly different in the serum of patients with iCCA as compared with PSC. (C) Diagnostic capacity of the 12 selected metabolites also similarly found altered in the validation cohort. Abbreviations: AA, amino acids; AC, acylcarnitines; AUC, area under the receiver operating characteristic curve; BA, bile acids; Cer, ceramides; ChoE, cholesteryl esters; CMH, monohexosylceramides; DG, diglycerides; FC, fold-change; FSB, free sphingoid bases; LPC, lysophosphatidylcholines; LPE, lysophosphatidylethanolamines; LPI, lysophosphatidylinositols; MUFA, monounsaturated fatty acids; NAE, N-acyl ethanolamines; oxFA, oxidized fatty acids; PC, phosphatidylcholines; PE, phosphatidylethanolamines; PI, phosphatidylinositols; PUFA, polyunsaturated fatty acids; SEN, sensitivity; SFA, saturated fatty acids; SM, sphingomyelins; SPE, specificity; ST, steroids; TG, triglycerides.

specificity in both the discovery and also the validation cohort. All of them presented better diagnostic values than CA 19-9, and most of them improved their differential diagnosis values when comparing early-stage iCCA (I-II) versus PSC (Fig. 5D).

VALIDATION OF THE BIOMARKERS ACCURACY

The results obtained with the selected metabolites in the discovery cohort were confirmed in an independent validation cohort, as shown in Supporting Fig. S4, S5. Diagnostic models were built through GLM. An algorithm by combining glycine, aspartic acid, SM(42:3), and SM(43:2) permitted to differentiate both types of tumors with an AUC of 0.890, 75% sensitivity, and 90% specificity (Fig. 6A). Fig. 6B shows the box plot diagram of the selected combination of metabolite biomarkers in the serum of patients and controls in the two independent cohorts. In the validation study, we also included groups of patients with NAFLD or NASH to confirm the



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N	iCCA *vs* HCC Models	Cohort	AUC	SEN (%)	SPE (%)
2	Aspartic acid* PC(O-24:1/0:0)	Discovery	0.918	85	90
		Validation	0.614	60	71
2	Aspartic acid* Glycine	Discovery	0.888	65	100
		Validation	0.605	53	79
2	SM(42:3)* SM(43:2)	Discovery	0.803	80	70
		Validation	0.767	80	71
3	Cer(d18:1/16:0)* SM(43:2)* SM(d18:2/16:0)	Discovery	0.775	55	85
		Validation	0.805	73	86
6	SM(43:2)* PC(O-16:0/20:3)* PC(O-18:0/18:2)* SM(d18:2/16:0)* Cer(d18:1/16:0)* SM(42:3)*	Discovery	0.900	80	90
		Validation	0.981	93	93
7	SM(d18:2/16:0)* SM(42:3)* SM(43:2)* PC(O-18:0/18:2)* PC(O-16:0/20:3)* Cer(d18:1/16:0)* DG(32:1)	Discovery	0.908	85	85
		Validation	0.738	60	93

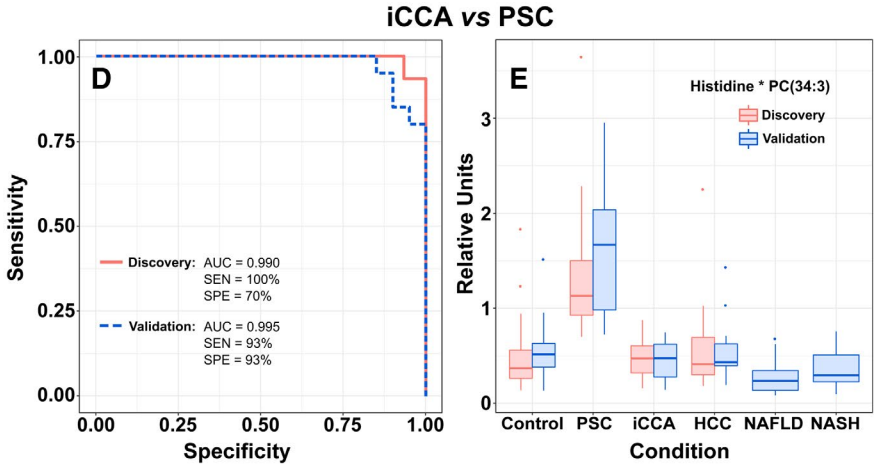

FIG. 6. Diagnostic prediction capacity of the selected metabolites in iCCA versus HCC, and iCCA versus PSC in discovery and validation cohorts. (A,B) Combination of aspartic acid, glycine, SM(42:3), and SM(43:2) in serum iCCA versus HCC. (C) Values of AUC, sensitivity, and specificity of other algorithms to differentiate iCCA versus HCC. (D,E) Combination of histidine and PC(34:3) in serum iCCA versus PSC (0.990 AUC, 100% sensitivity, 70% specificity, 76.9% positive predictive value, and 100% negative predictive value). Linear discriminant (A,C,D) analysis was carried out through LOOCV. (B,E) Box plot diagrams for each combination, respectively. Abbreviations: AUC, area under the receiver operating characteristic curve; N, number of metabolites in the algorithm; SEN, sensitivity; SPE, specificity. The asterisks indicate combination of metabolites.

accuracy (sensitivity and specificity) and usefulness of the selected metabolites. Fig. 6C shows the results in the discovery and validation cohorts of six additional different diagnostic models by combining metabolites that permitted to differentiate iCCA and HCC. High values of AUC, sensitivity, and specificity were

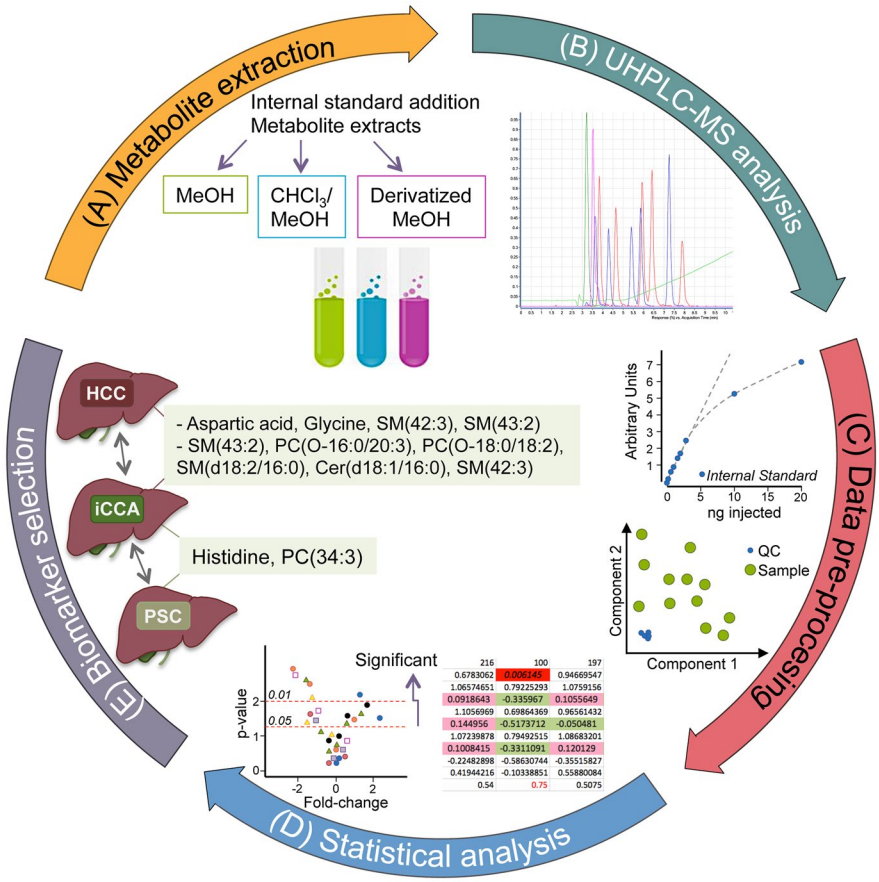

FIG. 7. Metabolic profiling workflow. (A) Metabolite extraction was accomplished by fractionating samples into pools of species with similar physicochemical properties using appropriate combinations of organic solvents, after the addition of an internal standard. (B) Three separate UHPLC-MS–based platforms were used to perform optimal profiling of UPLC-MS of the serum metabolome. (C) Data preprocessing generated a list of chromatographic peak areas for the metabolites detected in each sample injection. An approximated linear detection range was defined for each identified metabolite. Intra- and inter-batch normalization was carried out with internal standard correction and quality control calibration. (D) Once normalized, multivariate and univariate data analyses were performed. (E) Selection of the best candidate biomarkers for the differential diagnosis of HCC, iCCA, and PSC. The asterisks indicate combination of metabolites. Abbreviation: QC, quality control.

obtained, especially with the model including six metabolites, and all of them presented better values than AFP and CA 19-9.

Moreover, an algorithm consisting of PC(34:3) and histidine accurately permitted to differentiate between PSC and iCCA in the discovery cohort with an AUC of 0.990, 100% sensitivity, 70% specificity, and 85% accuracy, which was successfully confirmed in the validation cohort (Fig. 6D). Fig. 6E shows the box plot diagram of the combination of PC(34:3) and histidine in the serum of patients and controls of both cohorts, and also including NALFD and NASH groups. Data show that these metabolites were specifically increased in the serum of patients with PSC compared with all groups.

A summary of the metabolic profiling workflow for selecting the best biomarker candidates in the comparisons of iCCA versus HCC and iCCA versus PSC is presented in Fig. 7.

Discussion

The liver is essential in a large variety of metabolic processes that are critical for the maintenance of body homeostasis. Therefore, it seems reasonable to hypothesize that damage to this organ may have a reflection in serum, in terms of an imbalance in circulating metabolites due to the leakage of compounds

from injured cells and/or from cancer cells with altered metabolic activities. The heterogeneity among each type of cancer cell, and the fact that tumors can develop in livers with different underlying diseases, complicates the identification of accurate serum biomarkers. The analysis of nonspecific biomarkers, such as CA 19-9, may help in the noninvasive diagnosis of CCA. However, its specificity is low, because this biomarker is also elevated in one third of patients with PSC without biliary cancer, and in other liver diseases and tumors; in addition, there is an important heterogeneity in its elevation in patients with iCCA.[16] Moreover, the sensitivity of this cancer biomarker is compromised by the fact that ~10% of the population does not produce CA 19-9.[17] On the other hand, AFP provides low diagnostic capacity for HCC and is therefore not recommended for screening or diagnosis in Western guidelines.[18,19]

The identification of accurate noninvasive biomarkers remains a challenge. To date, few analyses based on metabolomics have been carried out in plasma/serum for the identification of biomarkers for liver diseases, including cancers and PSC.[20,21] The value of metabolomics, and particularly lipidomics, has been previously highlighted in the discovery of biomarkers for the diagnosis of NAFLD and NASH,[13,22,23] which are currently in clinical use. This technique allowed different metabolomics subtypes of NASH to be identified in animal models and patients.[24] Moreover, integration of genomics, transcriptomics, and metabolomics in tumor tissue has been proposed for the identification of molecular subtypes of HCC and iCCA with similar prognosis.[25] Our metabolomic study was conducted on serum searching for biomarkers that can differentially diagnose iCCA from HCC, the most frequent type of liver cancer, as well as for PSC, a condition with an enhanced risk of developing iCCA. Moreover, it is important to highlight that in this study the diagnosis of all liver tumors (iCCA or HCC) was biopsy-proven, and the serum samples had been obtained just after diagnosis and no treatment had been previously applied.

Among the metabolites that allowed to discriminate both types of cancer, some were amino acids with lower levels in HCC. In this line, reductions of serum phenylalanine, aspartate, glutamate, glycine, and serine levels were reported in the progression of hepatitis B to HCC.[26] Also, aberrant lipid metabolism has been described in patients with HCC and other chronic liver diseases.[21] In agreement with this study, we found lower levels of sphingomyelins in the serum from patients with HCC, and, interestingly, these molecules were not altered in patients with iCCA. We also found increased serum levels of bile acids in patients with liver tumors, although these changes also occur in other liver diseases accompanied by cholestasis, including NAFLD/NASH, and thus these molecules alone cannot be considered good biomarkers. Interestingly, the combination of two amino acids and two sphingomyelins permitted to discriminate patients with iCCA and HCC with an AUC of 0.890 and 0.720 in the discovery and validation cohorts, respectively, and other diagnostic models, especially the mode obtained with six metabolites, provided high values in both cohorts of patients (AUC of 0.900 and 0.929 in the discovery and validation cohorts, respectively). It is important to note that these diagnostic models maintained their specificity compared with other liver diseases such as NAFLD or NASH. Thus, the analysis of serum metabolites by UHPLC may allow the specific noninvasive diagnosis of both types of liver tumors, avoiding the inherent risks of tumor biopsy.

The diagnosis of PSC is difficult without the use of endoscopic retrograde cholangiopancreatography or magnetic resonance cholangiopancreatography techniques. Increased levels of circulating bile acids are a characteristic of PSC, as well as of other cholestatic disorders, as a result of compensatory mechanisms triggered by the intrahepatic accumulation of bile acids.[27] The accumulation of some bile acid species may induce cytotoxic effects and act as cocarcinogenic factors promoting bile duct proliferation/inflammation and reducing chemoprotection.[28] Previous studies have described differentially altered profiles of serum metabolites in PSC and primary biliary cirrhosis.[29,30] Here, we found elevated levels of primary bile acids in PSC compared with iCCA. However, obstructive cholestasis may occur depending on the localization of tumors, and high serum levels of bile acids can be found in some patients with iCCA.[31] Interestingly, increased levels of cholesterol and 38 glycerophospholipids, mainly phosphatidylcholines and lysophosphatidylcholines, in the serum of patients with PSC were found to be unaltered in patients with iCCA. Phosphatidylcholine species are important components of bile, playing a key role in the

formation of mixed micelles of bile acids and cholesterol, and protecting cholangiocytes from the harmful detergent properties of bile acids. Reduced levels of phosphatidylcholines have been found in bile of patients with PSC and in patients with PSC-related CCA.[32] Here, we also found a reduction of the essential fatty acid linoleate (18:2n6), which was previously reported to be elevated in the serum of patients with PSC, in addition to other free fatty acids.[30] Interestingly, in our study, the combination of one amino acid and one phosphatidylcholine permitted to accurately differentiate between patients with PSC and iCCA with AUCs of 0.990 and 0.995 in the discovery and validation cohorts, respectively.

In conclusion, serum metabolomic profiling is a promising noninvasive approach for the diagnosis of iCCA, HCC, and PSC and to distinguish patients with iCCA from those with HCC, as well as from PSC. Larger-scale validation studies in patients with different conditions and ethnicities are needed to determine the clinical diagnostic value of such biomarkers.

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