Supporting Information for the article:

Liquid biopsy protein biomarkers of cholangiocarcinoma risk, early diagnosis and survival mirroring tumor cells

Ainhoa Lapitz,[1] Mikel Azkargorta,[2,3] Piotr Milkiewicz,[4,5] Paula Olaizola,[1] Ekaterina Zhuravleva,[6] Marit M. Grimsrud,[7] Christoph Schramm,[8,9,10] Ander Arbelaiz,[1] Colm J. O'Rourke,[6] Adelaida La Casta,[1] Malgorzata Milkiewicz,[11] Tania Pastor,[1] Mette Vesterhus,[7,12] Raul Jimenez-Agüero,[1] Michael T. Dill,[13,14] Angela Lamarca,[15] Juan W. Valle,[15] Rocio I.R. Macias,[3,16] Laura Izquierdo-Sanchez,[1] Ylenia Pérez Castaño,[1,17] Francisco Javier Caballero-Camino,[1] Ioana Riano,[1] Marcin Krawczyk,[18] Cesar Ibarra,[19] Javier Bustamante,[19] Luiz Miguel Nova-Camacho,[20] Juan M. Falcon-Perez,[3,21,22] Felix Elortza,[2,3] Maria J. Perugorria,[1,3,23] Jesper B. Andersen,[6] Luis Bujanda,[1,3] Tom H. Karlsen,[7] Trine Folseraas,[7,24,*] Pedro M. Rodrigues,[1,3,22*] Jesus M. Banales[1,3,22,25*]

*Authors share co-seniorship.

[1]Department of Liver and Gastrointestinal Diseases, Biodonostia Health Research Institute – Donostia University Hospital, University of the Basque Country (UPV/EHU), 20014, San Sebastian, Spain; [2]Proteomics Platform, CIC bioGUNE, Basque Research and Technology Alliance (BRTA), ProteoRed-ISCIII, Bizkaia Science and Technology Park, 48160, Derio, Spain; [3]National Institute for the Study of Liver and Gastrointestinal Diseases (CIBERehd), ISCIII, 28220, Madrid, Spain; [4]Liver and Internal Medicine Unit, Department of General, Transplant and Liver Surgery, Medical University of Warsaw, 02-097, Warsaw, Poland; [5]Translational Medicine Group, Pomeranian Medical University, 70-204, Szczecin, Poland; [6]Biotech Research and Innovation Centre, Department of Health and Medical Sciences, University of Copenhagen, 2200, Copenhagen, Denmark; [7]Norwegian PSC Research Center, Department of

Transplantation Medicine, Division of Surgery, Inflammatory Medicine and Transplantation, Oslo University Hospital, Rikshospitalet, Oslo, Norway; [8]European Reference Network Hepatological Diseases (ERN RARE-LIVER), Hamburg, Germany; [9]1st Department of Internal Medicine, University Medical Center Hamburg-Eppendorf, Hamburg, Germany; [10]Martin Zeitz Centre for Rare Diseases, University Medical Centre Hamburg-Eppendorf, Hamburg, Germany; [11]Department of Medical Biology, Pomeranian Medical University in Szczecin, 70-111 Szczecin, Poland; [12]Department of Clinical Science, University of Bergen, Bergen, Norway; [13]Department of Gastroenterology, Infectious Diseases and Intoxication, Heidelberg University Hospital, 69120, Heidelberg, Germany; [14]Experimental Hepatology, Inflammation and Cancer, German Cancer Research Center (DKFZ), 69120, Heidelberg, Germany; [15]Department of Medical Oncology, The Christie NHS Foundation Trust/Division of Cancer Sciences, University of Manchester, Manchester, UK; [16]Experimental Hepatology and Drug Targeting (HEVEPHARM), University of Salamanca, Biomedical Research Institute of Salamanca (IBSAL), 37007, Salamanca, Spain; [17]Osakidetza Basque Health Service, Bidasoa IHO, Bidasoa Hospital, Department of Digestive System, Irun, Spain; [18]Department of Medicine II, Saarland University Medical Centre, Saarland University, 66421, Homburg, Germany; [19]Osakidetza Basque Health Service, Ezkerraldea-Enkarterri-Cruces IHO, Cruces University Hospital, Barakaldo, Spain; [20]Osakidetza Basque Health Service, Donostialdea IHO, Donostia University Hospital, Department of Pathology, San Sebastian, Spain; [21]Center for Cooperative Research in Biosciences (CIC bioGUNE), Basque Research and Technology Alliance (BRTA), Exosomes Laboratory, 48160, Derio, Spain; [22]Ikerbasque, Basque Foundation for Science, 48013, Bilbao, Spain; [23]Department of Medicine, Faculty of Medicine and Nursing, University of the Basque Country, UPV/EHU, Leioa, Spain; [24]Section of Gastroenterology, Department of Transplantation Medicine, Oslo University Hospital, Oslo, Norway; [25]Department of Biochemistry and Genetics, School of Sciences, University of Navarra, Pamplona, Spain.

Keywords

Cholangiocarcinoma; primary sclerosing cholangitis; protein biomarkers; extracellular vesicles; liquid biopsy; mass spectrometry; single-cell RNA-sequencing.

Corresponding authors:

Prof. Jesus M. Banales, PhD

Department of Liver and Gastrointestinal Diseases, Biodonostia Health Research Institute – Donostia University Hospital, Paseo del Dr. Begiristain s/n, E-20014, San Sebastian, Spain. E-mail: jesus.banales@biodonostia.org; Phone: +34 943006067.

or

Dr. Pedro M. Rodrigues, PhD

Department of Liver and Gastrointestinal Diseases, Biodonostia Health Research Institute – Donostia University Hospital, Paseo del Dr. Begiristain s/n, E-20014, San Sebastian, Spain. E-mail: pedro.rodrigues@biodonostia.org; Phone: +34 943006067.

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Study population

This international multicentric collaborative study includes samples retrospectively collected from different European University Hospitals and Research Centres, including Donostia University Hospital (San Sebastian, Spain), Oslo University Hospital Rikshospitalet (Oslo, Norway), Medical University Hospital of Warsaw (Warsaw, Poland), University Medical Center Hamburg-Eppendorf (Hamburg, Germany), Heidelberg University Hospital (Heidelberg, Germany), The Christie NHS Foundation Trust (Manchester, UK) and Salamanca University Hospital (Salamanca, Spain). As indicated in the European Association for the Study of the Liver (EASL) guidelines,[1] PSC diagnosis was based on standard clinical, biochemical, cholangiographic and histological criteria. Samples from patients with isolated PSC were obtained after confirming the absence of CCA development for more than 5 years after diagnosis. Serum from patients with PSC-CCA was obtained <2 months prior to CCA diagnosis or when tumor development was already confirmed. Serum from patients within the PSC to CCA group was obtained from patients with PSC with no clinical evidences of tumor presence at sampling, but who developed CCA during follow-up (sampling: 3-54 months before CCA diagnosis). For all patients with CCA, tumor development was confirmed by histology/cytology. Disease status was categorized as: *i*) local disease (LD), *ii*) locally-advanced disease (LAD), or *iii*) metastatic disease (MD). Patients with other types of biliary tract tumors (gallbladder or ampullary tumors), mixed HCC-iCCA or patients with both concomitant HCC and iCCA tumors were excluded from the analysis. Samples from patients with HCC were collected from individuals with histological confirmation of the tumor, excluding the ones with other type of malignancies.

In the “Orbitrap cohort”, serum samples from individuals with isolated PSC (n=18), concomitant PSC and CCA (PSC-CCA; n=14) and healthy individuals (n=19) were

included in the analysis. In the “timsTOF cohort”, additional samples from patients with isolated PSC (n=27), PSC-CCA (n=30) and healthy individuals (n=37), as well as samples from patients with PSC to CCA (n=25), CCA arising in patients without PSC (n=56) and HCC (n=34) were included, along with some samples from the “Orbitrap cohort”, reaching a total number of 257 in the “timsTOF cohort”. Demographical, clinical and biochemical features of both cohorts are summarized in Supplementary Table 1.

Serum samples from the Orbitrap/timsTOF cohorts, as well as additional ones from new patients, were used to test the efficacy of the biomarkers when measured in total serum by enzyme-linked immunosorbent assay (ELISA). In total, samples from patients with isolated PSC (n=46), PSC to CCA (n=24), PSC-CCA (n=43), non-PSC CCA (n=67), HCC (n=39) as well as from healthy individuals (n=32) were included. This cohort is summarized in Supplementary Table 2.

The study protocol was approved by the Euskadi Drug Research Ethics Committee (CEIm-E: PI2019116) and each participating Center obtained a local ethical approval (or equivalent). Informed consent was obtained from all individuals.

Isolation and characterization of extracellular vesicles (EVs) from human serum samples

EV isolation and characterization procedures were guided by the International Society for Extracellular Vesicles statement[2] and carried out as we previously described, by serial differential ultracentrifugation steps.[3] Briefly, 1 mL of serum was diluted in phosphate buffered saline (PBS) (DPBS, Gibco) in ultracentrifugation tubes (thick wall polycarbonate centrifuge tubes, Beckman coulter), which were centrifuged in a TLA110 rotor (Beckman coulter) at 10,000xg for 30 min to remove cell debris and large EVs. The supernatant was then subsequently ultracentrifuged at 100,000xg for 75 min. Pelleted EV fraction was then washed with PBS and ultracentrifuged at 100,000xg for another 75 min. Finally, the pelleted EV fraction was resuspended in 20 μL of PBS and then stored at -80ºC for further analysis. All relevant data on isolation and characterization

techniques have been submitted to the EV-TRACK knowledgebase (EV-TRACK ID: EV210077).[4]

Transmission Electron Microscopy (TEM)

For the characterization of EVs, PBS-resuspended EV isolate was negatively stained and evaluated by TEM. EV samples were directly adsorbed onto a glow-discharged (60 seg low discharging using a PELCO easy-glow device) carbon-coated copper grid (300 mesh). Afterwards, grids were fixed with 2% paraformaldehyde (PFA) in PBS 0.2M pH 7.4 for 20 min and washed with distilled water. Then, contrast staining was made by incubating the grids with 4% uranyl acetate at 4ºC for 15 min. TEM images were obtained by using TECNAI G2 20 C-TWIN high-resolution transmission electron microscope, at an acceleration voltage of 200 kV.

Nanoparticle tracking analysis (NTA)

To evaluate the size distribution and particle concentration of the isolate, EV samples were diluted 250-fold in PBS and later measured by using a NanoSight LM10 System (Malvern, UK) equipped with fast video capture and analyzed with the Nanoparticle Tracking Analysis (NTA) Version 2.2 particle-tracking software. Each EV preparation was measured twice and NTA post-acquisition settings were kept constant for all samples. Each recorded 1 min video was analyzed to obtain the mean and mode vesicle size as well as particle concentration.

Protein quantification, electrophoresis and immunoblotting

Protein concentration of isolated EVs, total serum, EV-depleted serum, as well as of whole cell extract (WCE) from normal human cholangiocyte (NHC) cultures was measured using the Micro BCA™ Protein Assay Kit according to the manufacturer's instructions (Thermo Scientific, USA).

In order to evaluate the expression of characteristic EV markers tetraspanins CD63 and CD81, 20 μg of serum EV fraction, total serum, EV-depleted serum and WCE samples were denaturalized by adding non-reducing Protein Loading Buffer [50 mM Tris-HCl, 2% SDS, 10% glycerol and 0.1% bromophenol blue, without β-mercaptoethanol or dithiothreitol (DTT)] and by heating the samples at 95ºC for 5 min. For the detection of the negative control endoplasmic reticulum chaperone BiP (GRP78), 10 μg of protein was used and 500 mM of 2-mercaptoethanol (Sigma-Aldrich) were included in the loading buffer (reducing conditions). Similarly, for the detection of candidate protein biomarkers, 10 μg of isolated serum EV fraction, EV-depleted and total serum were used while, for immunoblots including only total serum, 50 μg of protein were loaded in reducing conditions. Then, proteins were separated in a 12.5% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and electro-transferred onto a 0.45 μm pore-size nitrocellulose membrane (GE Healthcare). After blocking with 5% skim milk powder/Tris-buffered saline with 0.1% Tween 20 (TBS-T) (Sigma Aldrich) for 1 h at room temperature, membranes were incubated overnight at 4ºC with the appropriate primary antibodies (Supplementary Table 3) in blocking solution. Membranes were then washed 3 times with TBS-T and incubated with horseradish peroxidase-conjugated secondary antibodies at a dilution of 1:5000 (in blocking solution) for 1 h at room temperature. Membranes were washed with TBS-T and the signal was detected using the SuperSignal™ West Dura Extended Duration Substrate (Thermo Scientific, USA). Finally, the emitted chemiluminescence was visualized and captured with the iBright FL1500 Western Blot Imaging System (Thermo Fisher Scientific).

High-throughput proteomic analysis of serum EVs

Protein extraction and filter aided sample preparation (FASP)

All samples were resuspended in “cell lysis buffer” containing 7M Urea 2M Thiourea 4% CHAPS and incubated for 30 min under agitation. Next, FASP of the samples was

performed mainly as previously described.[5] This protocol is based on the use of standard filtration devices allowing buffer exchange and acting as a reactor for the digestion. Each digestion step was followed by 20 min centrifugations at 13,000 rpm in order to remove the buffer from the filter. Samples were loaded onto Amicon Ultra 0.5 mL 30K centrifugal units (Millipore), washed twice in UA solution (8 M Urea, 100 mM Tris-HCl pH 8.5), reduced (20 min incubation in 100 mM DTT prepared in UA solution) and alkylated (20 min incubation in 50 mM Iodoacetamide prepared in UA solution). Then, 3 additional washes were carried out in UA, followed by 3 additional washes in 50 mM AMBIC. Protein was quantified using Bio-Rad protein assay (Bio-Rad), and trypsin was added to a trypsin:protein ratio of 1:10. The mixture was incubated overnight at 37ºC. Peptides were recovered from the filter units. Samples were speed-vacuumed in a RVC2 25 speed-vac concentrator (Christ). Zip-tip peptides were resuspended in 0.1% formic acid (FA) prior to mass spectrometry (MS) analysis.

Mass spectrometry analysis

- Orbitrap MS

Peptide separation was performed on a nanoACQUITY UPLC System (Waters) connected to a LTQ Orbitrap XL (Thermo Electron). An aliquot of each sample was loaded onto a Symmetry 300 C18 UPLC Trap column (180 µm x 20 mm, 5 µm (Waters). The precolumn was connected to a BEH130 C18 column (75 µm x 200 mm, 1.7 µm; Waters) and equilibrated in 3% acetonitrile and 0.1% FA. Peptides were eluted directly into the nanoelectrospray capillary (Proxeon Biosystems) at 300 nL/min, using a 120 min linear gradient of 3–50% acetonitrile.

The LTQ Orbitrap XL ETD automatically switched between MS and MS/MS acquisition in DDA mode. Full MS scan survey spectra (m/z 400–2000) were acquired in the orbitrap with mass resolution of 30000 at m/z 400. After each survey scan, the six most intense ions above 1000 counts were sequentially subjected to collision-induced dissociation (CID) in the linear ion trap. Precursors with charge states of 2 and 3 were

specifically selected for CID. Peptides were excluded from further analysis for 60 seconds using the dynamic exclusion feature.

Progenesis LC-MS (version 4.0.4265.42984, Nonlinear Dynamics) was used for the label-free differential protein expression analysis. Once the Raw files were imported, one of the runs was used as the reference to which the precursor masses in all other samples were aligned to. Only features comprising charges of 2+ and 3+ were selected. The raw abundances of each feature were automatically normalized and logarithmized against the reference run. A peak list containing the information of the detected different features was generated and exported to the Mascot search engine (Matrix Science Ltd.). The generated “mgf” file was searched against the Uniprot/Swissprot human database using 10 ppm and 0.5 Da tolerances for precursor and fragment ions, respectively. The list of identified peptides was imported in Progenesis LC-MS and the previously quantified features were matched to the corresponding peptides.

The relative quantification of non-conflicting peptides exported from Progenesis LC-MS was analyzed with the proBatch package in R in order to normalize peptide quantification data.[6] Briefly, raw data matrix was transformed onto log scale and quality was examined by visualizing the global quantitative pattern plotting the sample mean and boxplots. Next, the total intensity of the samples was scaled, normalizing the distribution of the raw intensities to be the same in all samples by applying quantile normalization. Protein quantification was performed after quantile normalization of the peptide data. For this approach, protein quantification was calculated by the average of the antilogarithm corrected value of the peptides, which are unique for each protein. Finally, protein abundance was transformed into $\log_2$ scale.

- timsTOF MS

Samples were analyzed in a timsTOF Pro with PASEF (Bruker Daltonics) coupled online to a Evosep ONE liquid chromatograph (Evosep). 200 ng were directly loaded onto the Evosep ONE and resolved using the 60 samples-per-day protocol.

Protein identification and quantification was carried out using PEAKS X software (Bioinformatics solutions), and their identification was contrasted with a database consisting of *Homo sapiens* entries (Uniprot/Swissprot), with precursor and fragment tolerances of 20 ppm and 0.05 Da. Only proteins identified with at least two peptides at FDR<1% were considered for further analysis. Protein abundances inferred from PEAKS were loaded onto Perseus platform,[7] log_2 transformed and 10% imputed. Proteins that were not detected in at least 75% of the samples of at least one group were excluded from the analysis. Log_2 transformed and imputed protein data were uploaded to R studio, and by the proBatch package, values were quantile normalized and batch corrected in order to minimize sample origin as well as MS set variability.[6]

Diagnostic biomarker selection of the identified serum EV-proteins

To assess the potential usefulness of serum EV-proteins for the diagnosis of CCA in patients with or without PSC etiologies, area under the receiver-operating characteristic curve (AUC) values were calculated for each biomolecule using SPSS software version 22.0 (IBM, Ehningen, Germany).

Considering the variability between liquid chromatography (LC)-MS runs and between different LC-MS systems, and in order to increase the reliability of the results and select the most robust candidate biomarkers, different LC-MS systems and runs were used and compared. To evaluate the etiological specificity of the identified candidate biomarkers, we used the proteomic dataset obtained in the timsTOF cohort and we have considered all common proteins identified in the timsTOF MS, combined with the ones detected in the Orbitrap MS and/or in the dataset previously published (Orbitrap MS 2017[3]; Supplementary Fig. 2A).

To classify candidate protein biomarkers into specific diagnostic biomarkers for CCA in patients with PSC (PSC-CCA diagnostic biomarkers), diagnostic biomarkers for CCA regardless its etiology (diagnostic biomarkers for all CCAs: Pan-CCA) and diagnostic biomarkers for CCA of non-PSC etiologies (non-PSC CCA diagnostic

biomarkers) distinct criteria were established aiming to prevent false positive results. They are summarized in Supplementary Fig. 2B and described in detail below:

a) specific PSC-CCA biomarkers were selected from pair-wise comparisons of PSC-CCA to PSC and to non-PSC CCA, based on AUC p-values, excluding the ones that were significant between patients with non-PSC CCA and healthy individuals. The obtained candidates were cross-validated using only the "Orbitrap cohort", finally selecting the ones with significant alterations in patients with PSC-CCA compared to patients with PSC and/or a non-malignant control group [Healthy individuals + PSC], based on AUC p-values;

b) biomarkers specific for non-PSC CCA were selected from pair-wise comparisons of non-PSC CCA to Healthy and to PSC-CCA groups, based on AUC p-values, excluding the ones that were significant between patients with PSC-CCA and PSC. The obtained candidates were cross-validated using only the dataset previously published in 2017,[3] which includes patients with non-PSC CCA, finally selecting the ones with significant alterations in patients with non-PSC CCA compared healthy individuals, based on AUC p-values;

c) biomarkers for the general diagnosis of CCA regardless its etiology (Pan-CCA) were selected from pair-wise comparisons of Pan-CCAs to the non-malignant group comprised of healthy individuals and patients with PSC, based on AUC p-values, but excluding the ones that were specific for both PSC-CCA or non-PSC CCA according to the criteria in a) and b). The obtained candidates were cross-validated using the "Orbitrap cohort" and also in the dataset previously published in 2017,[3] selecting the ones with significant alterations in patients with PSC-CCA compared to patients with PSC and/or a non-malignant control group (healthy individuals + PSC), and/or in patients with non-PSC CCA compared healthy individuals, based on AUC p-values;

d) biomarkers specific for iCCA were selected from pair-wise comparisons of iCCA to HCC and to the non-malignant control (healthy and PSC) groups, based on AUC p-values;
e) biomarkers specific for HCC were selected from pair-wise comparisons of HCC to iCCA and healthy individuals, based on AUC p-values.

The diagnostic parameters, including sensitivity (SEN), specificity (SPE), positive predictive value (PPV), negative predicting value (NPV), accuracy index (AI) and odds ratio (OR), were calculated by 2X2 contingency tables to patient groups dichotomized using the optimal cut-off value for every comparative established by the top Youden Index (YI) of each ROC curve. Additionally, multivariable binary logistic regression was accomplished to make logistic model protein biomarker combinations and to evaluate the diagnostic power of the logistic functions. Logistic regression models were firstly designed in a training cohort, comprised of 70% of the patients included in the study, and then validated in a testing cohort, with the remaining 30% of patients as well as in a cohort only containing samples with CCA at LD stage. In order to compare the diagnostic value of the developed logistic models with serum levels of CA19-9 or other markers of liver damage and cholestatic liver injury (ALT, AST, total bilirubin, GGT, ALP), ROC curves were statistically compared with the method described by DeLong *et al*. (1988) by using the MedCalc® v20.115 software.[8]

Measurement of protein biomarkers in total serum by enzyme-linked immunosorbent assay (ELISA)

Biomarkers to be tested by ELISA were selected among Pan-CCA biomarkers, selecting the ones with increased levels in individuals with CCA and with reliable, reproducible and commercially-available ELISA kits that required low serum volumes (<7uL). Protein levels of FGL1, CRP, FIBRINOGEN, VWF, PIGR, OIT3 and FRIL were quantified in human total serum using ELISA, according to manufacturer's instructions. Dilutions for

the assays, as well as the references of each of the ELISA kits used are listed in Supplementary Table 4.

Gene expression analysis of circulating diagnostic biomarkers in human tissues

To evaluate the potential origin of serum EV protein biomarkers, the expression of those protein-coding genes was analyzed in human tissue bulk RNA datasets, in healthy liver single-cell RNA-seq (scRNA-seq) dataset, as well as in scRNA-seq data from patients with CCA.

Human multi-organ gene expression analysis of candidate biomarkers

Three transcriptomics datasets (HPA, GTEx and FANTOM5) downloaded from the Human Protein Atlas were used to estimate the relative gene expression levels of candidate biomarkers and assess the organ/tissue origin of the defined protein biomarkers derived from serum EVs (http://www.proteinatlas.org / v20.proteinatlas.org).[9] The Human Protein Atlas (HPA) consortium RNA-seq dataset consists of mRNA sequencing of different 37 human tissues and 6 hematological cell types including B-cells, T-cells, NK-cells, monocytes, granulocytes and dendritic cells.[7] The Genotype-Tissue Expression (GTEx) project gathers RNA-seq data from 34 different human tissues and organs.[10] The Functional Annotation of Mammalian Genomes 5 (FANTOM5) project brings together mammalian cell-type specific transcriptomes from 45 different human tissues and organs by using the Cap Analysis of Gene Expression (CAGE) strategy.[11] RNA Consensus tissue gene data summarizes normalized transcript expression levels of 61 tissues based on transcriptomic data from the previous mentioned three sources: HPA, GTEx and FANTOM5. The normalized RNA Consensus data includes the following human tissue and organs: adipose tissue, adrenal gland, amygdala, appendix, B-cells, basal ganglia, bone marrow, breast, cerebellum, cerebral cortex, cervix, colon, corpus callosum, dendritic cells, ductus deferens, duodenum, endometrium, epididymis, esophagus, fallopian tube, gallbladder,

granulocytes, heart muscle, hippocampal formation, hypothalamus, kidney, liver, lung, lymph node, midbrain, monocytes, natural killer (NK)-cells, olfactory region, ovary, pancreas, parathyroid gland, pituitary gland, placenta, pons and medulla, prostate, rectum, retina, salivary gland, seminal vesicle, skeletal muscle, skin, small intestine, smooth muscle, spinal cord, spleen, stomach, T-cells, testis, thalamus, thymus, thyroid gland, tongue, tonsil, total peripheral blood mononuclear cells (PBMCs), urinary bladder and vagina.

Single-cell RNA sequencing analysis (scRNA-seq)

Single-cell transcriptome profiling from healthy liver samples as well as from CCA tumors from two different studies were downloaded from GSE115469, GSE151530 and GSE125449 GEO datasets, respectively.

The normal liver dataset (GSE115469) comprises the transcriptional profile of 8,444 cells obtained from the fractionation of fresh hepatic tissue from five healthy human livers.[12] From the scRNA-seq data published in GSE125449, a total number of 5,376 cells were chosen that were originally isolated from 10 tumor biopsies from patients with iCCA.[13] Sequencing data from 4,961 cells coming from 14 fresh liver tumor biopsies from 12 patients with iCCA were analyzed in the GSE151530 dataset.[14]

Regarding data processing, for the normal liver single-cell sequencing (GSE115469), data filtering and clustering was preserved as in the original study. In relation to CCA datasets, all these three datasets were processed with Seurat version 4.0.0. Briefly, features reported at least in 3 cells and cells with at least 500 features were considered. Cells with UMI counts below 700 or mitochondrial content above 35% in GSE 1515130 or above 20% in GSE125449 were removed. Outliers were defined per sample with scatter (more than 3 median absolute deviations from the median value) and removed. Doublets/multiplets were predicted and removed with scDblFinder, default settings. Data was scaled with LogNormalize transformation and scale factor 10,000 according to default Seurat settings. Variable genes for PCA were identified using the

Seurat function FindVariableFeatures using the vst method with 2000 features selected in GSE 1515130 or with 2244 features selected in GSE125449. Data was scaled, UMI numbered and mitochondrial gene content were regressed out. For the tSNE plots, top 30 PCA components were selected to perform dimension reduction (dims.use = 1:30, resolution = 1) in GSE1515130 and top 20 in GSE125449 (dims.use = 1:20, resolution = 0.8).

Known cell marker genes used for classification of the main cell types were unchanged among all scRNA-seq studies: hepatocytes (*HPX*, *LBP*, *HPR* and *SERPINA10*); cholangiocytes (*KRT19*, *KRT7* and *FXYD2*); endothelial cells (*CD34*, *JAM2* and *VWF*); hepatic stellate cells (*COL1A2*, *LUM* and *DCN*); macrophages (*CD68*, *CD163* and *MSR1*); B cells (*CD19*, *FCRL5* and *CD79A*); NK cells (*KLRF1* and *NCAM1*) and T cells (*CD3D*, *CD3E* and *CD3G*) (Supplementary Fig. 11B and 12A).

Immunofluorescence

Immunofluorescence (IF) was performed in formalin-fixed paraffin-embedded (FFPE) human tissue sections.[15] Briefly, tissue slides were incubated in xylene and rehydrated in graded series of ethanol. Following antigen retrieval with Tris-EDTA (TE) buffer (pH 9.0), samples were immersed in 0.25% NH_3/EtOH treatment to reduce autofluorescence and blocked with 10% goat serum. Next, tissue sections were immersed in 3% H_2O_2 and primary antibodies (Supplementary Table 3) were incubated overnight at 4ºC. Afterwards, slides were incubated with Alexa Fluor 488 and 594 secondary antibodies. Tissue sections were then immersed in 0.3% Sudan Black for 1 hour. Coverslips were mounted on slides using VECTASHIELD® mounting medium with DAPI (Vector laboratories). Images were obtained at 10X with an Eclipse 80i Nikon microscope with the Digital sight DS-U2 camera controller (Nikon) using NIS-Elements.

Survival analysis

The potential association of serum EV protein abundance with the overall survival (OS) of patients with CCA regardless disease etiology (Pan-CCA) was evaluated by univariable and multivariable Cox proportional hazard analysis and by the Log-rank (Mantel-Cox) test as explained in Figure 8A.

First, the continuous variables (*i.e.*, EV protein abundance) were categorized using the Survminer 0.4.8 R package. This package determines the optimal cut-point using the maximally selected rank statistics from the 'maxstat' R package.[16] This categorical classification according to high or low abundance of selected EV-proteins in serum was employed to evaluate if protein dichotomic abundance was associated with OS time by calculating the Hazard Ratio of the Cox proportional-hazards model. Univariable Cox regression was conducted including patients with CCA regardless etiology from which serum samples were collected up to 2 months before or after definite CCA diagnosis. For the Cox proportional hazard regression analysis, the R packages Survival 3.2-7 and RegParallel 1.6.0 were used and unadjusted p-value <0.05 of the Hazard Ratio was considered as cut-off.

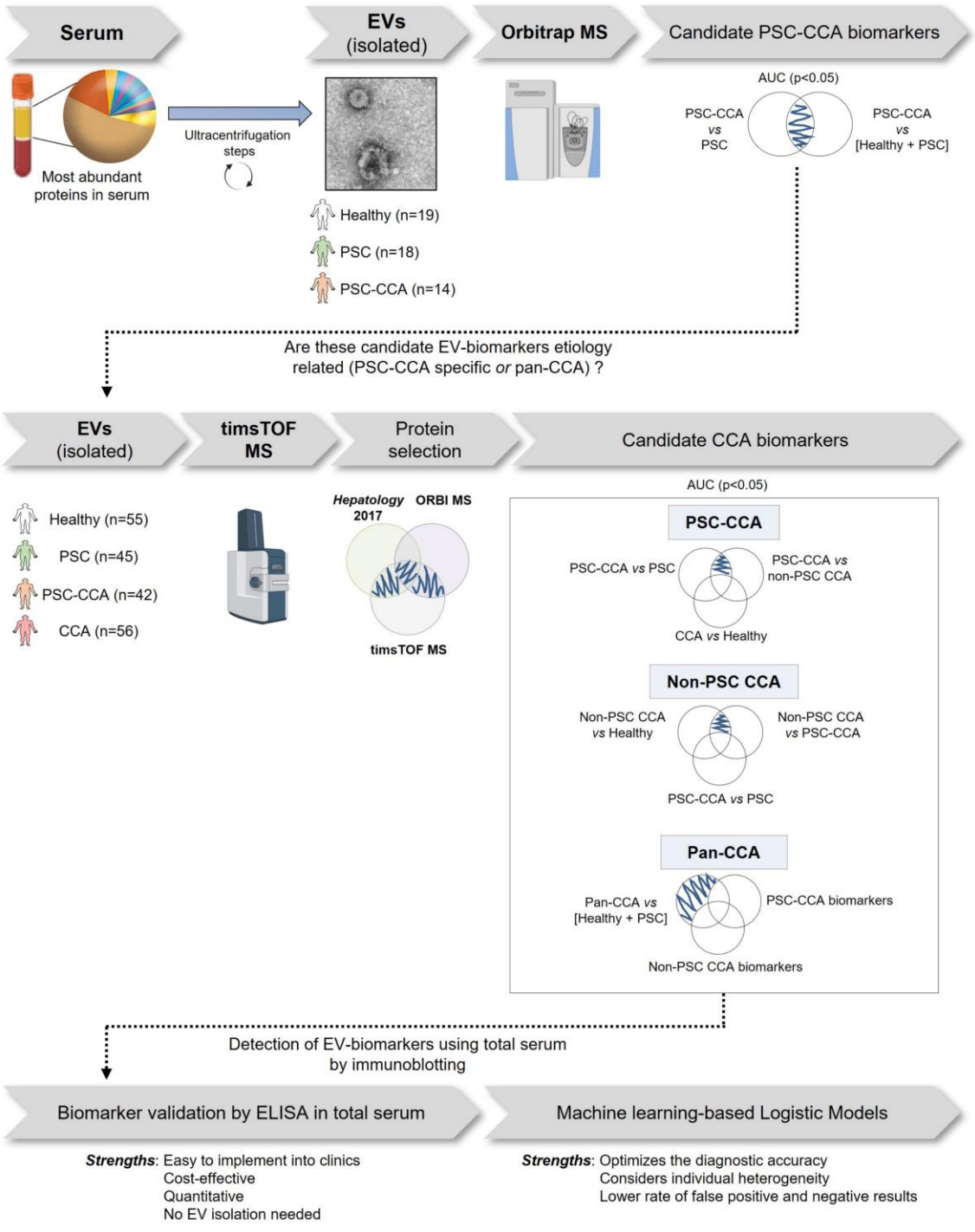
Multivariable Cox regression was then conducted in order to identify the proteins that were associated with OS, independently of demographical and clinical variables including sex, age, diagnosis of PSC, presence of cirrhosis, CCA subtype (iCCA, pCCA, dCCA), disease status [local disease (LD), locally-advanced disease (LAD), metastatic disease (MD)], serum CA19-9 levels and surgery (no/palliative vs tumor resection/liver transplantation) by calculating the Hazard Ratio of the Cox proportional-hazards model, with an unadjusted p-value <0.05. After identifying the proteins with independent prognostic value, univariable Cox regression and Log-rank (Mantel-Cox) statistical tests were used to identify common proteins associated with the risk of death and with OS, with an unadjusted p-value <0.01 used as a cut-off for both tests. Finally, with the final list of proteins, multivariable Cox regression was used to identify proteins that have a

prognostic value independent to any of the other biomarkers selected (unadjusted p-value <0.01 as the cut-off).

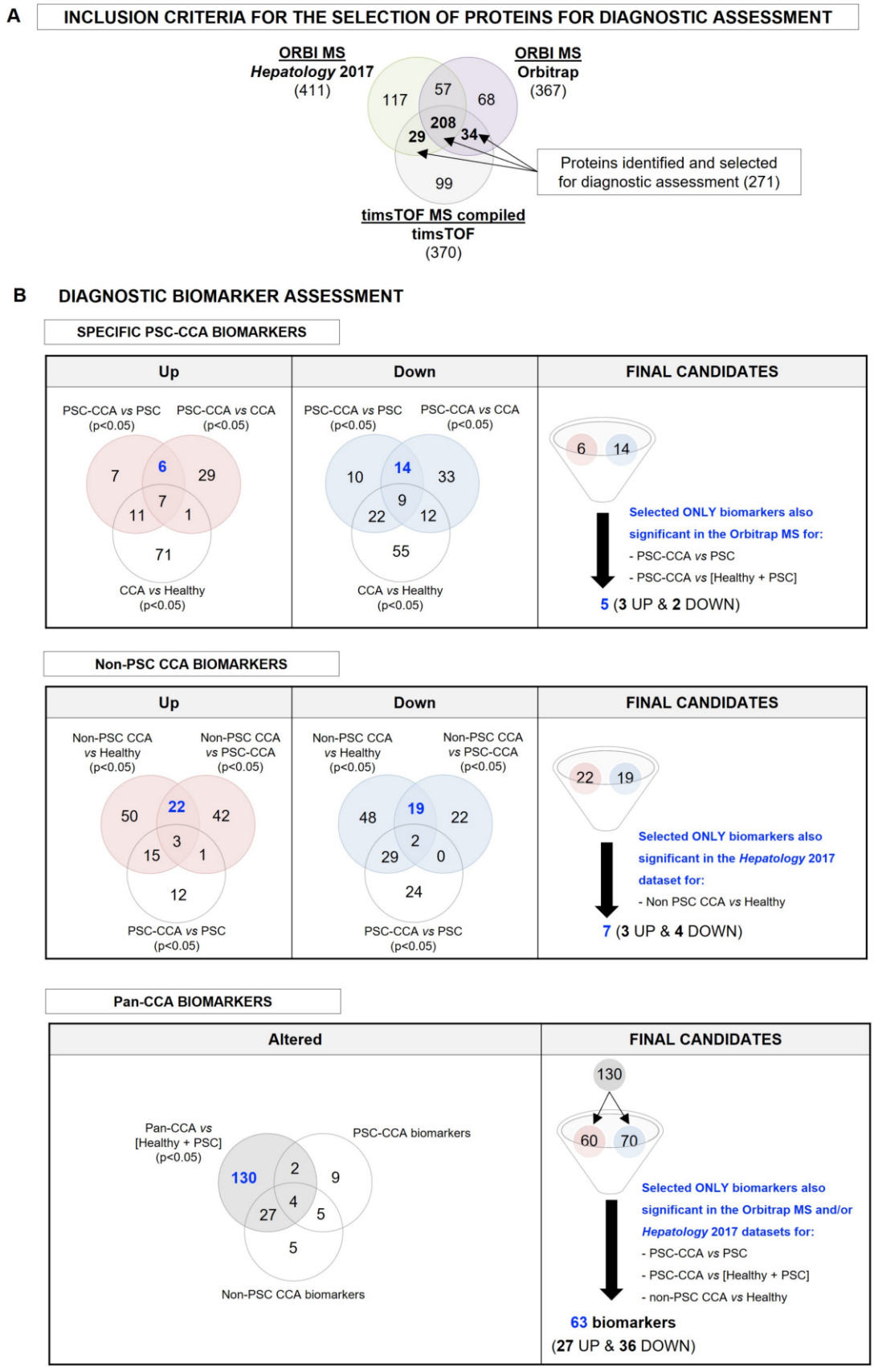
Statistical analysis

Statistical analyses were performed using R Studio with the R version 4.0.2 (2020-06-22), IBM SPSS Statistics software version 22 (IBM, Ehningen, Germany), MedCalc® version 20.115 (MedCalc Software Ltd, Ostend, Belgium) and GraphPad Prism version 8.0 (GraphPad Software, San Diego, CA, USA). All values were tested to evaluate if they follow a Gaussian distribution by the normality tests D'Agostino-Pearson Omnibus and Shapiro-Wilk. When comparing two groups, non-parametric Mann-Whitney or parametric t-Student tests were conducted. For comparisons between more than two groups, non-parametric Kruskal-Wallis test followed by a posteriori Dunns test or the parametric one-way analysis of variance (ANOVA) test followed by a posteriori Tukey's post hoc test were used. Differences were considered significant when $p < 0.05$.

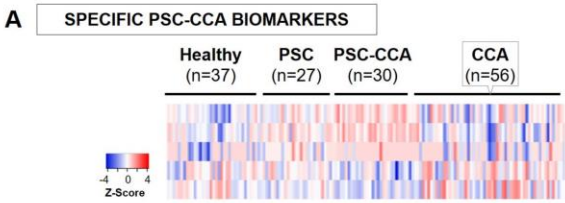
Supplementary figures



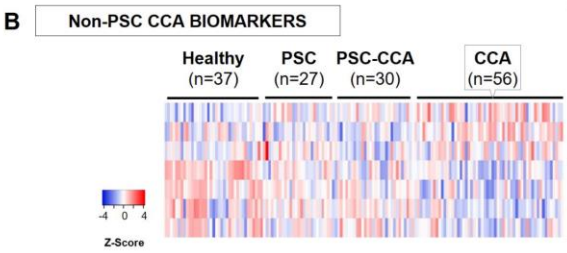
Supplementary Fig. 1: Workflow of the strategy used for diagnostic biomarker discovery.



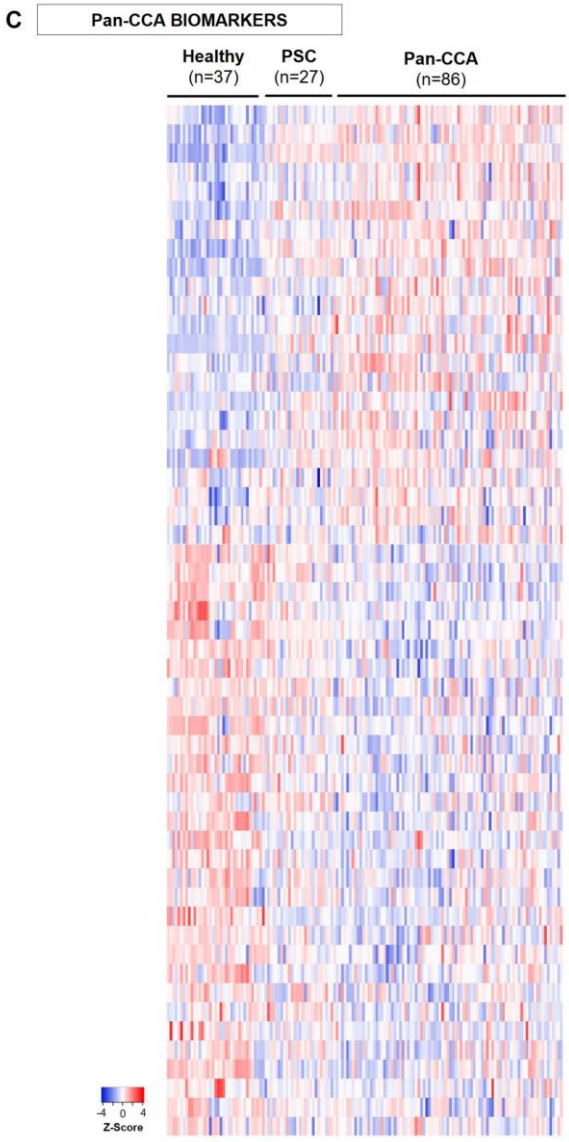
Supplementary Fig. 2: **Criteria for protein selection and biomarker classification**. **(A)** Selection of proteins to be evaluated in the timsTOF cohort. **(B)** Criteria for the classification of diagnostic biomarkers specific for PSC-CCA, non-PSC CCA and CCA regardless disease etiology (Pan-CCA).



Protein	PSC-CCA (n=30) *vs* PSC (n=27)			LD PSC-CCA (n=6) *vs* PSC (n=27)		
	AUC	SEN (%)	SPE (%)	AUC	SEN (%)	SPE (%)
LG3BP	0.744	76.7	63.0	0.710	100.0	51.9
IGLL5	0.712	63.3	74.1	0.858	100.0	74.1
IGKC	0.774	93.3	81.5	0.815	100.0	81.5
VTDB	0.698	70.0	66.7	0.889	100.0	66.7
APOC3	0.658	70.0	63.0	0.778	100.0	59.3

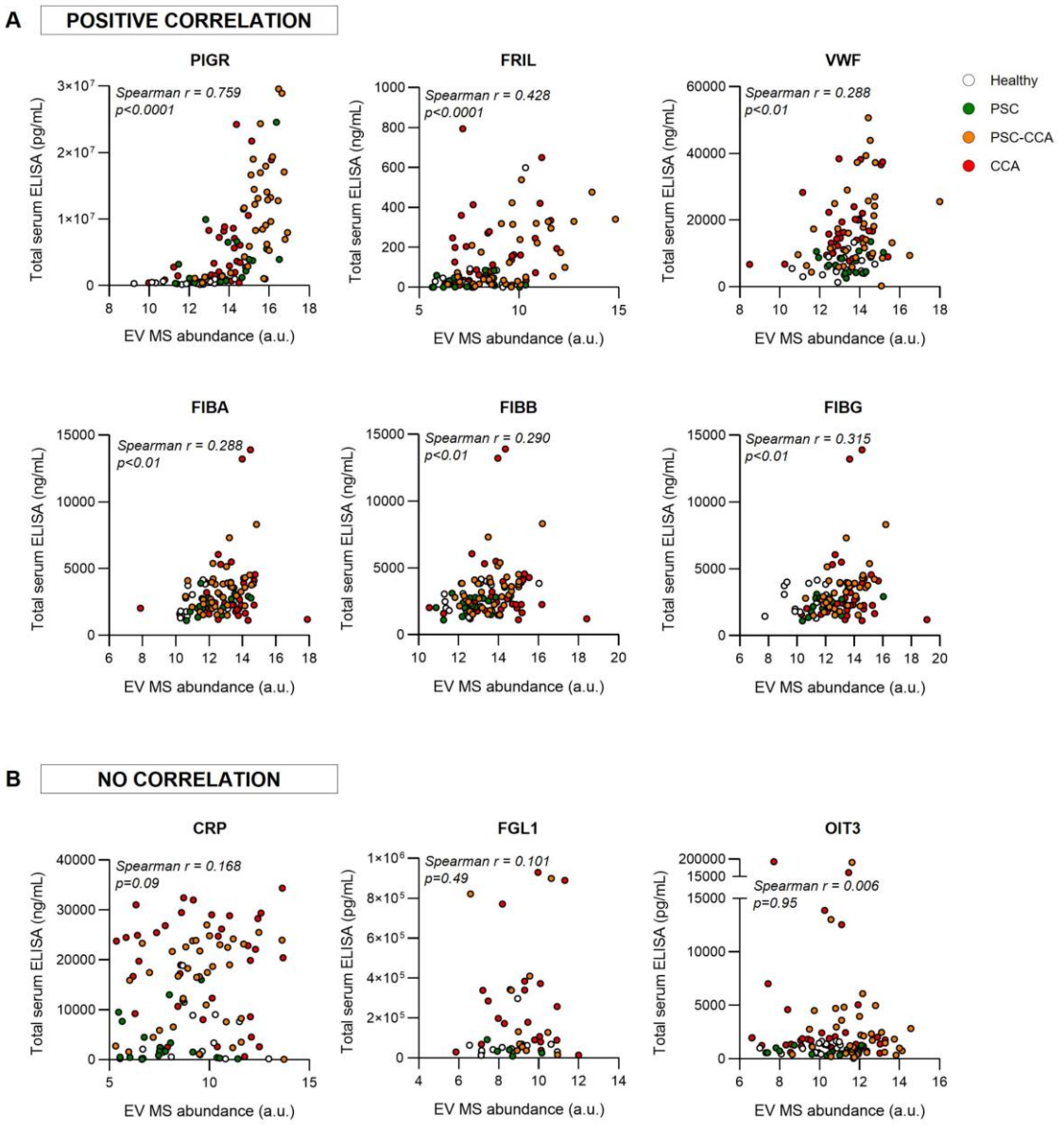


Protein	CCA (n=56) *vs* Healthy (n=37)			LD CCA (n=23) *vs* Healthy (n=37)		
	AUC	SEN (%)	SPE (%)	AUC	SEN (%)	SPE (%)
BAIP2	0.750	50.0	97.3	0.714	47.8	97.3
A1AT	0.660	82.1	45.9	-	-	-
IGHD	-	-	-	0.788	65.2	86.5
TSN9	0.863	94.6	64.9	0.868	95.7	67.6
HPTR	0.774	55.4	89.2	0.794	60.9	89.2
CO9	0.726	91.1	62.2	0.725	82.6	62.2
FCN2	0.699	83.9	62.2	0.713	78.3	67.6



Protein	Pan-CCA (n=86) *vs* [Healthy+PSC] (n=64)			LD Pan-CCA (n=29) *vs* [Healthy+PSC] (n=64)		
	AUC	SEN (%)	SPE (%)	AUC	SEN (%)	SPE (%)
FIBG	0.824	62.8	87.5	0.772	93.1	50.0
CAH1	0.772	47.7	95.3	0.787	58.6	89.1
ANGP1	0.757	94.2	56.3	0.771	96.6	56.2
FIBA	0.758	59.3	82.8	0.690	41.4	95.3
FIBB	0.755	60.5	79.7	0.707	55.2	79.7
PIGR	0.739	73.3	70.3	0.699	79.3	59.4
TFR1	0.729	60.5	82.8	0.767	82.8	65.6
DPP4	0.741	89.5	53.1	0.784	93.1	57.8
ITIH3	0.727	88.4	48.4	0.737	93.1	45.3
VNN1	0.722	83.7	53.1	0.711	82.8	53.1
IGHA2	0.720	60.5	78.1	0.772	72.4	75.0
FRIL	0.701	46.5	85.9	-	-	-
GPC5C	0.727	67.4	75.0	0.677	82.8	57.8
OIT3	0.700	36.0	96.9	0.637	96.6	28.1
CRP	0.652	60.5	71.9	0.647	62.1	75.0
ITLN1	0.668	62.8	68.7	0.650	65.5	68.8
GGT1	0.680	59.3	75.0	0.628	69.0	62.5
IGJ	0.644	58.1	71.9	0.649	65.5	75.0
PPBT	0.645	73.3	57.8	-	-	-
IGHA1	0.617	53.5	71.9	0.671	79.3	54.7
VWF	0.614	87.2	35.9	-	-	-
CO7	0.634	47.7	78.1	-	-	-
FGL1	0.601	38.4	84.4	-	-	-
HEMO	0.795	70.9	81.2	0.809	72.4	81.2
PON1	0.789	62.8	85.9	0.830	65.5	93.7
ANGT	0.766	64.0	78.1	0.803	82.8	76.6
FA9	0.753	87.2	59.4	0.744	86.2	59.4
THRB	0.755	80.2	67.2	0.762	72.4	78.1
GELS	0.773	60.5	89.1	0.797	69.0	89.1
ITIH4	0.733	62.8	78.1	0.686	55.2	76.6
APOL1	0.715	60.5	79.7	0.746	65.5	82.8
APOA	0.706	68.6	67.2	0.683	55.2	84.4
CFAI	0.710	93.0	48.4	0.709	89.7	50.0
ACTN1	0.712	70.9	67.2	0.651	72.4	57.8
HEP2	0.700	38.4	92.2	0.724	41.4	92.2
B3AT	0.709	73.3	60.9	0.630	100.0	23.4
APOA1	0.681	41.9	90.6	0.684	44.8	90.6
STOM	0.699	59.3	73.4	0.662	82.8	50.0
TLN1	0.677	58.1	71.9	0.679	82.8	56.3
HBA	0.673	64.0	71.9	-	-	-
1433Z	0.676	83.7	45.3	0.643	51.7	75.0
ITA2B	0.668	74.4	56.2	-	-	-
KAIN	0.661	65.1	65.6	-	-	-
KLKB1	0.651	62.8	68.7	0.636	69.0	59.4
AMBP	0.663	75.6	56.2	0.668	79.3	53.1
ITB3	0.660	70.9	57.8	-	-	-
ANT3	0.647	74.4	51.6	0.757	96.6	50.0
ALBU	0.646	67.4	59.4	0.662	72.4	59.4
APOA5	0.667	46.5	82.8	-	-	-
ACTB	0.656	74.4	53.1	0.630	75.9	53.1
ADA10	0.644	67.4	62.5	-	-	-
F13A	0.613	44.2	79.7	0.689	58.6	79.7
A2MG	0.621	76.7	45.3	-	-	-
CO8B	0.645	88.4	39.1	-	-	-

Supplementary Fig. 3: Diagnostic capacity of serum EV-protein biomarkers for the diagnosis of CCA according to cancer etiology using only the new set of samples included in the timsTOF cohort (not analyzed in the orbitrap MS). Biomarkers for the specific diagnosis of CCA in patients with PSC (specific PSC-CCA biomarkers), CCA in patients without PSC (non-PSC CCA biomarkers) and CCA regardless etiology (Pan-CCA biomarkersEnriched proteins are colored in red and proteins with lower abundance in blue. "-", non-significant AUC.



Supplementary Fig. 4: Correlation of levels of candidate biomarkers measured in total serum by ELISA and in serum EVs, by proteomics. Correlation analysis of protein biomarker levels (PIGR, FRIL, VWF, FIBA, FIBB, FIBG, CRP, FGL1, OIT3) in total serum *vs* isolated EVs from healthy individuals and patients with PSC, PSC-CCA or non-PSC CCA.

Pan-CCA *vs* [Healthy+PSC]												
Protein	Pan-CCA (n)	Healthy + PSC (n)	ROC curve			Diagnostic test					Odds ratio (OR)	
			AUC	p-value	cut-off (ng/mL)	SEN (%)	SPE (%)	PPV (%)	NPV (%)	AI (%)	OR (95%CI)	p-value
CRP	**39**	**44**	0.886	<0.0001	14000	69.2	93.2	90.0	77.4	81.9	30.8 (7.9, 119.2)	<0.0001
FRIL	**39**	**44**	0.912	<0.0001	30	92.3	84.1	83.7	92.5	88.0	63.4 (15.2, 264.6)	<0.0001
FGL1	**17**	**31**	0.906	<0.0001	50	94.1	74.2	66.7	95.8	81.3	46.0 (5.2, 404.7)	<0.001
VWF	**39**	**44**	0.791	<0.0001	8500	82.1	72.7	72.7	82.1	77.1	12.2 (4.3, 34.9)	<0.0001
PIGR	**39**	**44**	0.700	<0.01	750	92.3	56.8	65.5	89.3	73.5	15.8 (4.2, 59.1)	<0.0001
FIBRINOGEN	**39**	**44**	0.870	<0.0001	1810	92.3	70.5	73.5	91.2	80.7	28.6 (7.5, 109.7)	<0.0001
OIT3	**39**	**44**	0.656	<0.05	1.78	53.9	79.6	70.0	66.0	67.5	4.5 (1.7, 11.9)	<0.01

Supplementary Fig. 5: Diagnostic capacity of serum protein biomarkers for Pan-CCAs using new independent samples (not analyzed by MS) by ELISA. Number of measured samples in CRP, FRIL, VWF, PIGR, FIBRINOGEN and OIT3: PSC-CCA (n=6), CCA (n=33), Healthy (n=16) and PSC (n=28). Number of measured samples in FGL1: PSC-CCA (n=1), CCA (n=16), Healthy (n=13) and PSC (n=18).

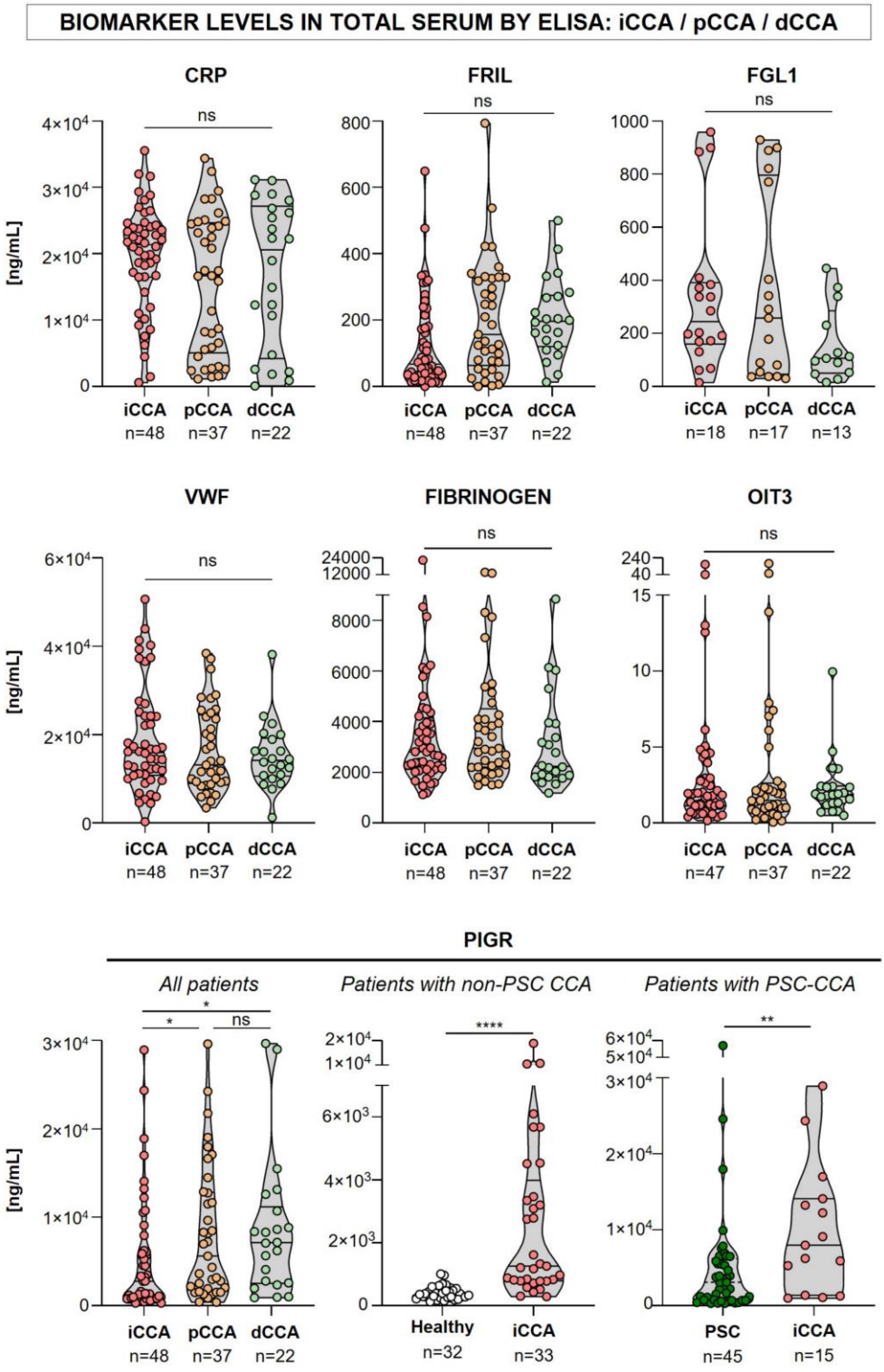
LOCAL DISEASE (LD) CCA

LD Pan-CCA *vs* [Healthy + PSC]												
Protein	Pan-CCA (n)	Healthy + PSC (n)	ROC curve			Diagnostic test					Odds ratio (OR)	
			AUC	p-value	cut-off (ng/mL)	SEN (%)	SPE (%)	PPV (%)	NPV (%)	AI (%)	OR (95%CI)	p-value
CRP	39	78	0.900	<0.0001	12000	79.5	91.0	81.6	89.9	87.2	39.3 (13.1, 117.9)	<0.0001
FRIL	39	78	0.855	<0.0001	34	82.1	74.4	61.5	89.2	76.9	13.3 (5.1, 34.7)	<0.0001
FGL1	24	47	0.821	<0.0001	100	70.8	89.4	77.3	85.7	83.1	20.4 (5.7, 73.3)	<0.0001
VWF	39	78	0.872	<0.0001	8800	92.3	74.4	64.3	95.1	80.3	34.8 (9.6, 125.5)	<0.0001
PIGR	39	77	0.778	<0.0001	775	92.3	57.1	52.2	93.6	69.0	16.0 (4.5, 56.5)	<0.0001
FIBRINOGEN	39	78	0.692	<0.001	2020	76.9	55.1	46.2	82.7	62.4	4.1 (1.7, 9.8)	<0.01
OIT3	38	78	0.732	<0.0001	1.09	81.6	62.8	51.7	87.5	69.0	7.5 (2.9, 19.2)	<0.0001

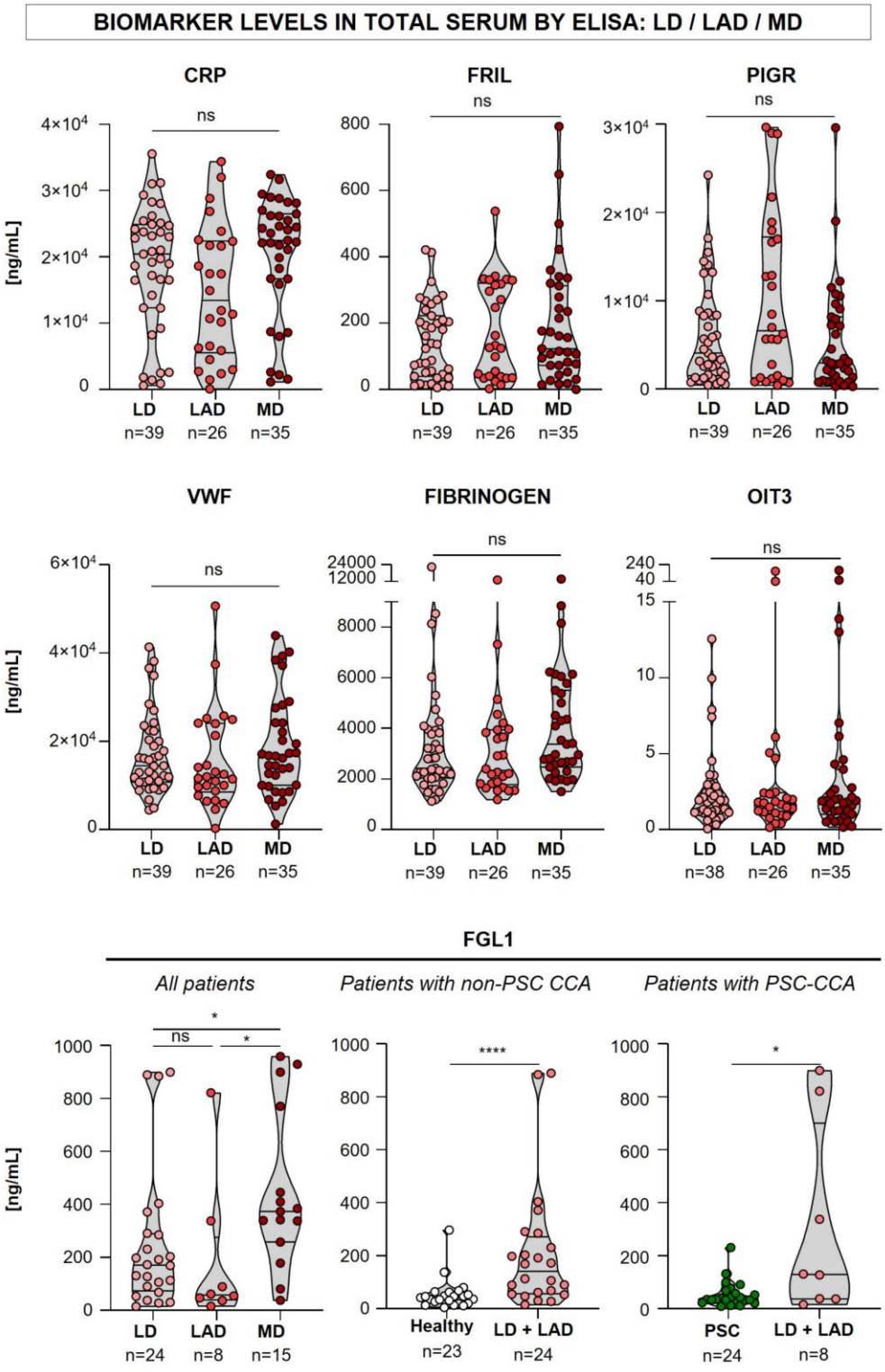
LD PSC-CCA *vs* PSC												
Protein	PSC-CCA (n)	PSC (n)	ROC curve			Diagnostic test					Odds ratio (OR)	
			AUC	p-value	cut-off (ng/mL)	SEN (%)	SPE (%)	PPV (%)	NPV (%)	AI (%)	OR (95%CI)	p-value
CRP	11	46	0.901	<0.0001	12000	81.8	87.0	60.0	95.2	86.0	30.0 (5.2, 173.7)	<0.0001
FRIL	11	46	0.812	<0.01	34	72.7	76.1	42.1	92.1	75.4	8.5 (1.9, 37.6)	<0.01
FGL1	4	24	0.813	<0.05	100	75.0	87.5	50.0	95.5	85.7	21.0 (1.6, 273.4)	<0.05
VWF	11	46	0.796	<0.01	8800	90.9	65.2	38.5	96.8	70.2	18.8 (2.2, 159.9)	<0.01
PIGR	11	45	0.770	<0.01	8000	54.6	93.3	66.7	89.4	85.7	16.8 (3.17, 89.0)	<0.001
FIBRINOGEN	11	46	0.842	<0.0001	2770	72.7	82.6	50.0	92.7	80.7	12.7 (2.7, 58.5)	<0.01
OIT3	11	46	0.631	ns	2.0	54.6	82.6	42.9	88.4	77.2	5.7 (1.4, 23.4)	<0.05

LD CCA *vs* Healthy												
Protein	Non-PSC CCA (n)	Healthy (n)	ROC curve			Diagnostic test					Odds ratio (OR)	
			AUC	p-value	cut-off (ng/mL)	SEN (%)	SPE (%)	PPV (%)	NPV (%)	AI (%)	OR (95%CI)	p-value
CRP	28	32	0.912	<0.0001	9000	82.1	93.8	92.0	85.7	88.3	69.0 (12.3, 388.2)	<0.0001
FRIL	28	32	0.862	<0.0001	54	75.0	87.5	84.0	80.0	81.7	21.0 (5.4, 81.2)	<0.0001
FGL1	20	23	0.839	<0.0001	66	80.0	87.0	84.2	83.3	83.7	26.7 (5.2, 136.8)	<0.0001
VWF	28	32	0.948	<0.0001	8500	92.9	87.5	86.7	93.3	90.0	91.0 (15.4, 539.3)	<0.0001
PIGR	28	32	0.964	<0.0001	700	89.3	93.8	92.6	90.9	91.7	125.0 (19.3, 808.0)	<0.0001
FIBRINOGEN	28	32	0.626	ns	1550	85.7	40.6	55.8	76.5	61.7	4.1 (1.2, 14.6)	<0.05
OIT3	27	32	0.819	<0.0001	1.67	55.6	100.0	100.0	72.7	79.7	80.6 (4.5, 1451.4)	<0.01

Supplementary Fig. 6: Diagnostic values of Pan-CCA, PSC-CCA and non-PSC CCA protein biomarkers measured by ELISA in patients with local disease (LD), compared to a non-malignant group [Healthy + PSC], PSC or healthy individuals, respectively. ns, non significant.



Supplementary Fig. 7. Serum candidate biomarker levels according to CCA subtype. Levels of CRP, FRIL, FGL1, VWF, FIBRINOGEN, OIT3 and PIGR measured by ELISA in serum samples from patients with iCCA, pCCA and dCCA. ns, non-significant; *p<0.05; **p<0.01 and ****p<0.0001 (Kruskal-Wallis and Mann-Whitney tests).

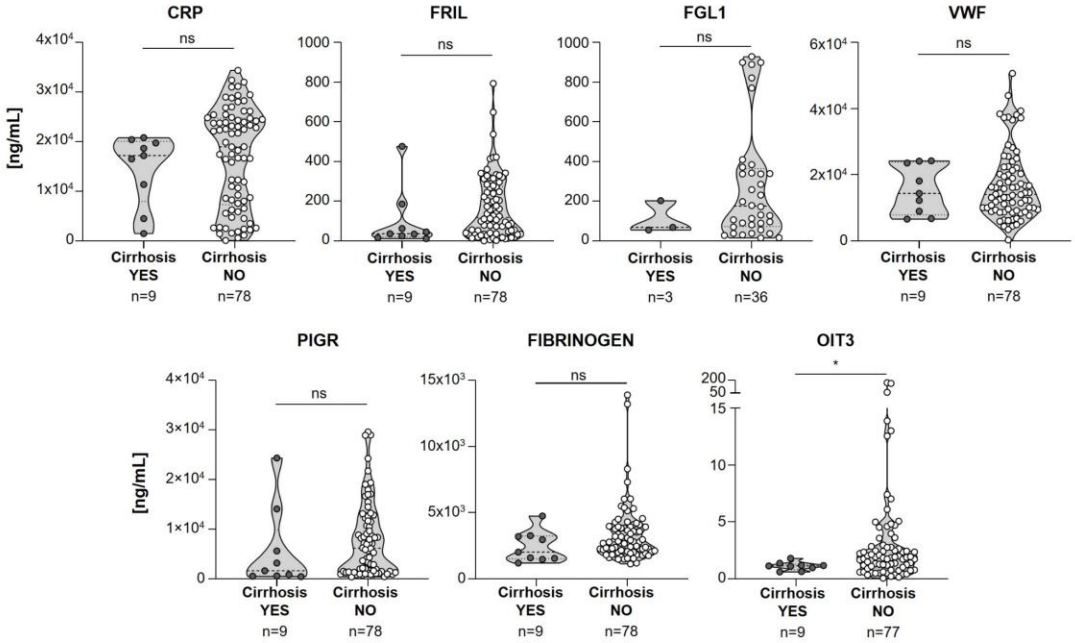


Supplementary Fig. 8. Serum candidate biomarker levels according to disease status. Levels of CRP, FRIL, FGL1, VWF, FIBRINOGEN, OIT3 and PIGR measured by ELISA in serum samples from patients with CCA stratified according the disease status in local disease (LD), locally-advanced disease (LAD) and metastatic disease (MD). ns, non-significant; *p<0.05 and ****p<0.0001 (Kruskal-Wallis and Mann-Whitney tests).

A **BIOMARKER LEVELS IN TOTAL SERUM BY ELISA: CA19-9**

Protein	CA19-9 IU/mL (continuous)		
	Spearman, r	95% CI	p-value
CRP	0.107	-0.099, 0.305	ns
FRIL	0.168	-0.030, 0.354	ns
FGL1	0.161	-0.134, 0.430	ns
VWF	0.199	0.002, 0.381	<0.05
PIGR	0.138	-0.061, 0.326	ns
FIBRINOGEN	0.290	0.099, 0.461	<0.01
OIT3	0.368	0.183, 0.527	<0.001

B **BIOMARKER LEVELS IN TOTAL SERUM BY ELISA: CIRRHOSIS (YES *vs* NO)**



C **DIAGNOSTIC VALUE OF CANDIDATE INDIVIDUAL BIOMARKERS *VS* CA19-9 LEVELS**

				Pan-CCA *vs* PSC				PSC-CCA *vs* PSC			
				ROC curve		DeLong test (*vs* CA19-9)		ROC curve		DeLong test (*vs* CA19-9)	
Biomarker	PSC (n)	PSC-CCA (n)	CCA (n)	AUC	p-value	difference between AUCs (95%CI)	p-value	AUC	p-value	difference between AUCs (95%CI)	p-value
CRP	40	40	65	0.869	<0.0001	0.096 (0.005, 0.186)	<0.05	0.813	<0.0001	0.062 (-0.077, 0.200)	ns
FRIL	40	40	65	0.861	<0.0001	0.088 (-0.008, 0.184)	p=0.07	0.811	<0.0001	0.059 (-0.065, 0.184)	ns
FGL1	21	12	37	0.825	<0.0001	0.051 (-0.079, 0.181)	ns	0.746	<0.01	0.202 (0.003, 0.401)	<0.05
VWF	40	40	65	0.789	<0.0001	0.016 (-0.089, 0.121)	ns	0.762	<0.0001	0.011 (-0.132, 0.154)	ns
PIGR	39	40	65	0.613	<0.05	0.160 (0.050, 0.271)	<0.01	0.728	<0.001	0.024 (-0.109, 0.157)	ns
FIBRINOGEN	40	40	65	0.750	<0.0001	0.023 (-0.084, 0.130)	ns	0.801	<0.0001	0.050 (-0.084, 0.184)	ns
OIT3	40	40	65	0.672	<0.001	0.104 (-0.011, 0.219)	ns	0.588	ns	0.163 (0.022, 0.305)	<0.05
CA19-9	40	40	65	0.773	<0.0001	-	-	0.752	<0.0001	-	-

				LD Pan-CCA *vs* PSC				LD PSC-CCA *vs* PSC			
				ROC curve		DeLong test (*vs* CA19-9)		ROC curve		DeLong test (*vs* CA19-9)	
Biomarker	PSC (n)	PSC-CCA (n)	CCA (n)	AUC	p-value	difference between AUCs (95%CI)	p-value	AUC	p-value	difference between AUCs (95%CI)	p-value
CRP	40	10	26	0.911	<0.0001	0.166 (0.036, 0.295)	<0.05	0.945	<0.0001	0.210 (-0.047, 0.467)	ns
FRIL	40	10	27	0.859	<0.0001	0.114 (-0.004, 0.232)	p=0.06	0.787	<0.001	0.053 (-0.127, 0.232)	ns
FGL1	21	4	19	0.823	<0.0001	0.015 (-0.154, 0.183)	ns	0.798	<0.05	0.119 (-0.208, 0.446)	ns
VWF	40	10	26	0.845	<0.0001	0.100 (-0.034, 0.233)	ns	0.838	<0.0001	0.103 (-0.160, 0.365)	ns
PIGR	39	10	26	0.634	<0.05	0.111 (-0.032, 0.254)	ns	0.797	<0.001	0.064 (-0.181, 0.310)	ns
FIBRINOGEN	40	10	26	0.718	<0.001	0.028 (-0.129, 0.185)	ns	0.825	<0.0001	0.090 (-0.156, 0.336)	ns
OIT3	40	10	21	0.697	<0.01	0.055 (-0.092, 0.201)	ns	0.609	ns	0.126 (-0.092, 0.345)	ns
CA19-9	40	10	26	0.745	<0.0001	-	-	0.735	ns	-	-

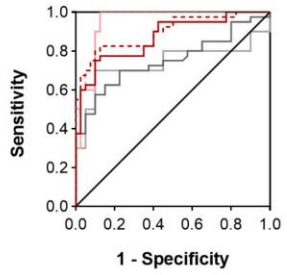
Supplementary Fig. 9. Association between the serum levels of candidate biomarkers with serum CA19-9 and the presence of cirrhosis. (A) Spearman correlation of CA19-9 with the levels of candidate biomarkers in total serum. **(B)** Levels of candidate biomarkers in total serum from patients with CCA, stratified according to the presence/absence of cirrhosis. *p<0.05 (Mann-Whitney test). **(C)** Comparative analysis of the diagnostic capacities of individual biomarkers with serum CA19-9 levels in Pan-CCA *vs* PSC, and PSC-CCA *vs* PSC, both in the full cohort and in patients with local disease (LD). ns, non-significant.

CCA DIAGNOSIS IN PATIENTS WITH PSC Comparison and combination with CA19-9

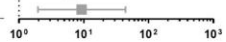
Biomarkers in the LOGISTIC MODEL 1 (LM1)
CRP / FIBRINOGEN / FRIL

Sample set	Tested variable	PSC-CCA (n)	PSC (n)
Model testing (samples with CA19-9 measured)	**LM1**	40	40
Samples with CA19-9 measured	**LM1 + CA19-9**	40	40
Model testing (LD)	**LM1**	10	40
LD PSC-CCA *vs* PSC	**LM1 + CA19-9**	10	40
Samples with CA19-9 measured	**CA19-9**	40	40
LD PSC-CCA *vs* PSC	**CA19-9**	10	40

	ROC curve		Diagnostic test			DeLong test (*vs* CA19-9)	
LM	AUC (95%CI)	p-value	SEN (%)	SPE (%)	AI (%)	difference between AUCs (95%CI)	p-value
	0.870 (0.792, 0.948)	<0.0001	75.0	87.5	81.3	0.119 (-0.008, 0.247)	p=0.07
	0.897 (0.828, 0.966)	<0.0001	80.0	90.0	85.0	0.145 (0.035, 0.256)	**<0.05**
	0.950 (0.893, 1.000)	<0.0001	90.0	87.5	88.0	0.215 (-0.013, 0.443)	p=0.07
	0.960 (0.911, 1.000)	<0.0001	90.0	90.0	90.0	0.225 (0.002, 0.448)	**<0.05**
	0.752 (0.642, 0.861)	<0.001	62.5	80.0	71.3	-	-
	0.735 (0.504, 0.966)	<0.05	70.0	80.0	78.0	-	-



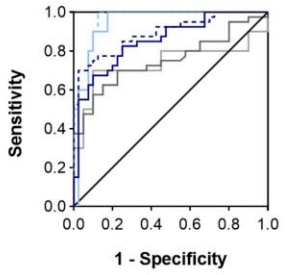
Odds ratio (OR)		
OR (95%CI)	p-value	
21.0 (6.5, 68.3)	<0.0001	
36.0 (9.9, 130.9)	<0.0001	
63.0 (6.5, 608.9)	<0.001	
81.0 (8.0, 815.9)	<0.001	
6.7 (2.4, 18.2)	<0.001	
9.3 (1.96, 44.4)	<0.01	



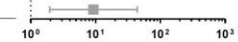
Biomarkers in the LOGISTIC MODEL 2 (LM2)
CRP / FRIL

Sample set	Tested variable	PSC-CCA (n)	PSC (n)
Model testing (samples with CA19-9 measured)	**LM2**	40	40
Samples with CA19-9 measured	**LM2 + CA19-9**	40	40
Model testing (LD)	**LM2**	10	40
LD PSC-CCA *vs* PSC	**LM2 + CA19-9**	10	40
Samples with CA19-9 measured	**CA19-9**	40	40
LD PSC-CCA *vs* PSC	**CA19-9**	10	40

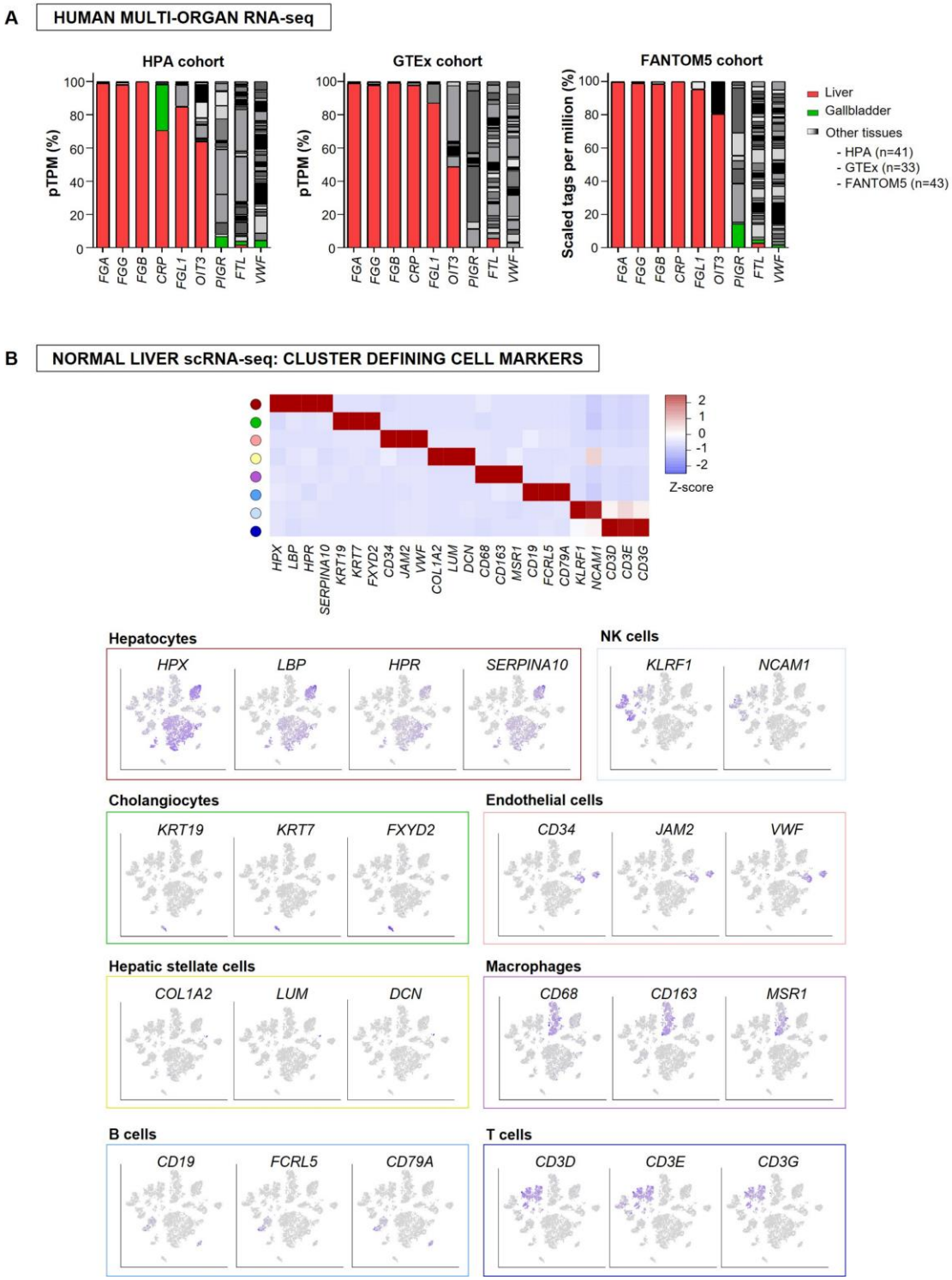
	ROC curve		Diagnostic test			DeLong test (*vs* CA19-9)	
LM	AUC (95%CI)	p-value	SEN (%)	SPE (%)	AI (%)	difference between AUCs (95%CI)	p-value
	0.853 (0.770, 0.935)	<0.0001	67.5	87.5	77.5	0.104 (-0.026, 0.233)	p=0.11
	0.891 (0.821, 0.962)	<0.0001	72.5	90.0	81.3	0.140 (0.029, 0.250)	**<0.05**
	0.943 (0.879, 1.000)	<0.0001	90.0	87.5	88.0	0.211 (-0.029, 0.452)	p=0.09
	0.965 (0.920, 1.000)	<0.0001	80.0	90.0	88.0	0.230 (0.002, 0.458)	**<0.05**
	0.752 (0.642, 0.861)	<0.001	62.5	80.0	71.3	-	-
	0.735 (0.504, 0.966)	<0.05	70.0	80.0	78.0	-	-



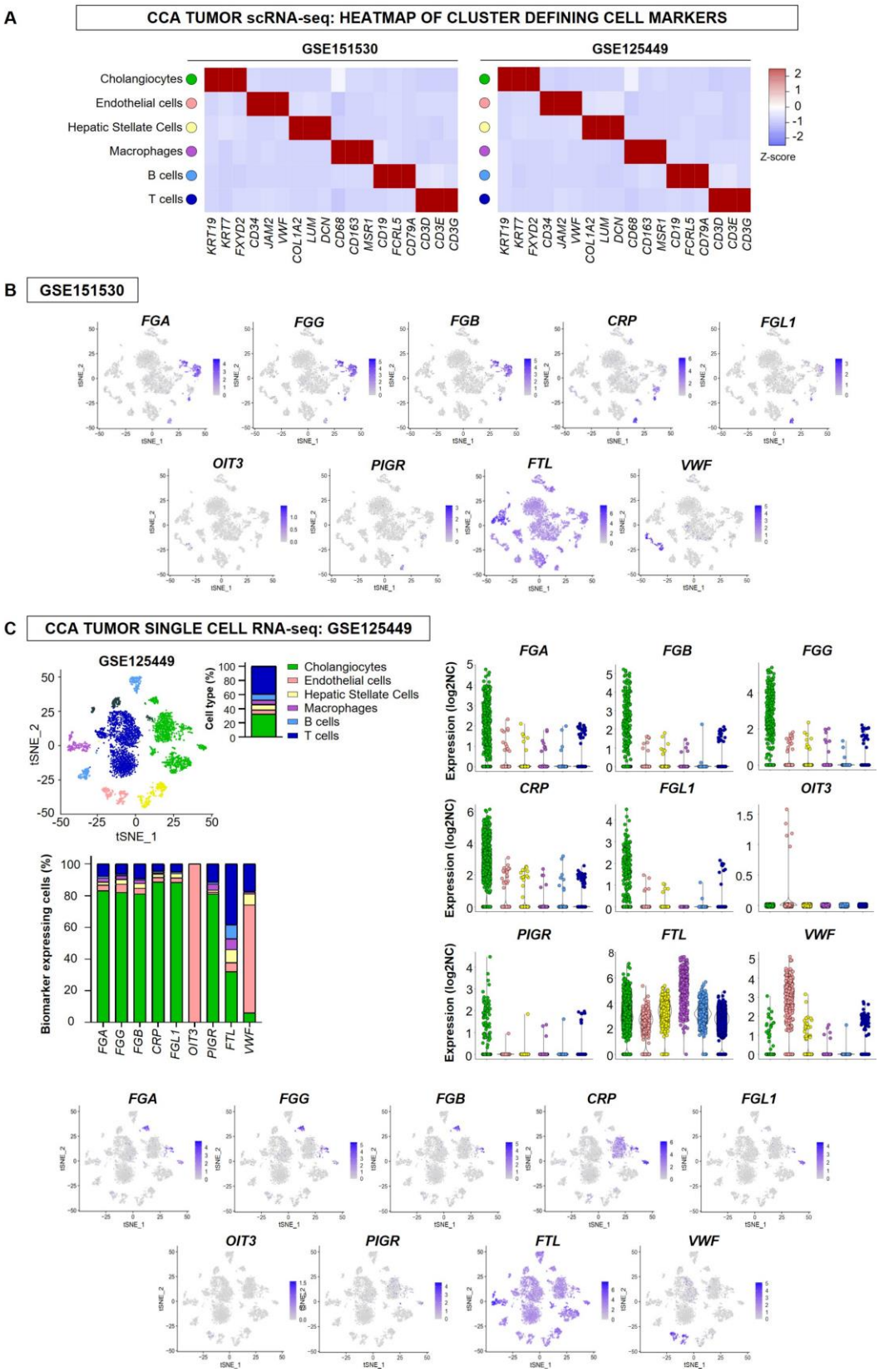
Odds ratio (OR)		
OR (95%CI)	p-value	
14.5 (4.6, 45.8)	<0.0001	
23.7 (6.8, 82.4)	<0.0001	
63.0 (6.5, 608.9)	<0.001	
36.0 (5.6, 231.8)	<0.001	
6.7 (2.4, 18.2)	<0.001	
9.3 (1.96, 44.4)	<0.01	



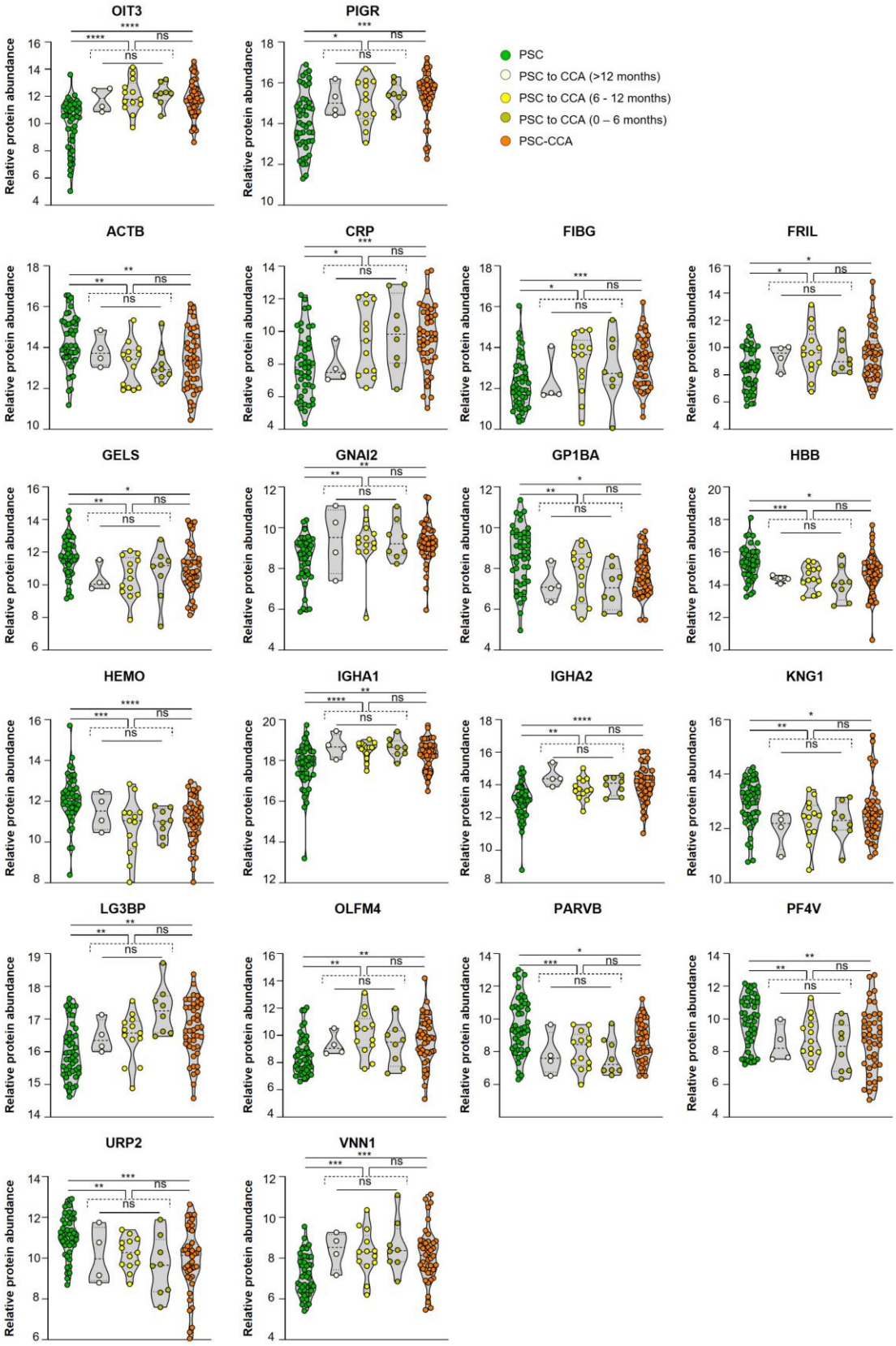
Supplementary Fig. 10. Comparison of the diagnostic capacity of logistic models alone or in combination with CA19-9 for PSC-CCA *vs* PSC. The AUC of each logistic model (LM1 and LM2) alone or combined with CA19-9, was compared to the single use of CA19-9 using the DeLong test.



Supplementary Fig. 11. Expression of candidate diagnostic biomarkers in the cohorts included in the HPA Consensus gene data and cluster-defining cell markers in normal liver scRNA-seq. (A) Analysis of candidate serum biomarkers in the three tissue cohorts which are summarized in the Consensus gene data of The Human Protein Atlas: HPA, GTEx and FANTOM5. **(B)** Heatmap and tSNE plots of cell type markers used to identify specific cell populations in normal liver scRNA-seq data.



Supplementary Fig. 12. Expression of the candidate biomarkers in CCA tumors at a single-cell level. (A) Heatmaps of cell type markers used to identify specific cell populations in 2 CCA tumor scRNA-seq cohorts; **(B)** tSNE plots of the candidate biomarkers in CCA tumor-constituting single-cell types from the GSE151530; **(C)** tSNE plots, normalized relative biomarker expression and comparison of biomarker-expressing cells within the single-cell types of iCCA tumors from the GSE125449.



Supplementary Fig. 13. Serum EV-protein abundance is already altered in patients with PSC more than 1 year before CCA diagnosis. Violin plot representation of EV candidate biomarkers of CCA risk in patients with PSC, PSC to CCA according to the time before CCA diagnosis (0-6 months, >6-12 months; >12 months) and concomitant PSC-CCA. ns, non-significant; *p<0.05; **p<0.01; ***p<0.001 and ****p<0.0001 (Kruskal-Wallis test).

A PREDICTIVE PSC-CCA BIOMARKERS — DeLong test of individual biomarkers

	FIBRINOGEN				CRP				FRIL				PIGR			
	ROC curve		DeLong test		ROC curve		DeLong test		ROC curve		DeLong test		ROC curve		DeLong test	
	AUC	p-value	difference between AUCs (95%CI)	p-value	AUC	p-value	difference between AUCs (95%CI)	p-value	AUC	p-value	difference between AUCs (95%CI)	p-value	AUC	p-value	difference between AUCs (95%CI)	p-value
CA19-9	**0.823** **0.660**	<0.0001 <0.05	0.163 (-0.003, 0.329)	p=0.05	**0.691** **0.660**	<0.01 <0.05	0.031 (-0.144, 0.207)	ns	**0.690** **0.672**	<0.01 <0.05	0.018 (-0.161, 0.197)	ns	**0.677** **0.672**	<0.05 <0.05	0.005 (-0.173, 0.182)	ns
Total bilirubin	**0.828** **0.727**	<0.0001 <0.001	0.101 (-0.027, 0.228)	ns	**0.709** **0.721**	<0.01 <0.01	0.012 (-0.163, 0.187)	ns	**0.705** **0.732**	<0.01 <0.001	0.028 (-0.104, 0.159)	ns	**0.686** **0.741**	<0.01 <0.001	0.055 (-0.102, 0.213)	ns
AST	**0.828** **0.588**	<0.0001 ns	0.240 (0.085, 0.395)	**<0.01**	**0.709** **0.560**	<0.01 ns	0.148 (0.007, 0.290)	**<0.05**	**0.705** **0.575**	<0.01 ns	0.130 (-0.057, 0.316)	ns	**0.686** **0.581**	<0.01 ns	0.106 (-0.030, 0.241)	ns
ALT	**0.828** **0.525**	<0.0001 ns	0.303 (0.154, 0.452)	**<0.0001**	**0.709** **0.510**	<0.01 ns	0.199 (-0.023, 0.421)	p=0.08	**0.705** **0.510**	<0.01 ns	0.195 (0.022, 0.368)	**<0.05**	**0.686** **0.517**	<0.01 ns	0.169 (0.021, 0.317)	**<0.05**
GGT	**0.828** **0.513**	<0.0001 ns	0.315 (0.143, 0.487)	**<0.001**	**0.709** **0.501**	<0.01 ns	0.208 (0.005, 0.411)	**<0.05**	**0.705** **0.505**	<0.01 ns	0.200 (0.020, 0.381)	**<0.05**	**0.686** **0.515**	<0.01 ns	0.171 (0.001, 0.342)	**<0.05**
ALP	**0.828** **0.617**	<0.001 ns	0.211 (0.053, 0.369)	**<0.01**	**0.709** **0.617**	<0.01 ns	0.092 (-0.057, 0.241)	ns	**0.705** **0.623**	<0.01 ns	0.082 (-0.094, 0.257)	ns	**0.686** **0.631**	<0.01 ns	0.055 (-0.061, 0.170)	ns

B PREDICTIVE PSC-CCA BIOMARKERS — DeLong test of biomarker panels

	CRP / FIBRINOGEN / FRIL / PIGR				CRP / FIBRINOGEN / FRIL				CRP / FIBRINOGEN			
	ROC curve		DeLong test		ROC curve		DeLong test		ROC curve		DeLong test	
	AUC	p-value	difference between AUCs (95%CI)	p-value	AUC	p-value	difference between AUCs (95%CI)	p-value	AUC	p-value	difference between AUCs (95%CI)	p-value
CA19-9	**0.897** **0.650**	<0.0001 ns	0.247 (0.109, 0.385)	**<0.001**	**0.887** **0.650**	<0.0001 ns	0.237 (0.096, 0.378)	**<0.001**	**0.824** **0.650**	<0.0001 ns	0.174 (0.025, 0.324)	**<0.05**
Total bilirubin	**0.910** **0.725**	<0.0001 <0.01	0.185 (0.0485, 0.321)	**<0.01**	**0.901** **0.725**	<0.0001 <0.01	0.176 (0.032, 0.320)	**<0.05**	**0.844** **0.725**	<0.0001 <0.01	0.120 (-0.034, 0.273)	ns
AST	**0.910** **0.579**	<0.0001 ns	0.331 (0.189, 0.473)	**<0.0001**	**0.901** **0.579**	<0.0001 ns	0.322 (0.171, 0.474)	**<0.0001**	**0.844** **0.579**	<0.0001 ns	0.266 (0.104, 0.427)	**<0.01**
ALT	**0.910** **0.514**	<0.0001 ns	0.396 (0.265, 0.526)	**<0.0001**	**0.901** **0.514**	<0.0001 ns	0.387 (0.249, 0.526)	**<0.0001**	**0.844** **0.514**	<0.0001 ns	0.331 (0.181, 0.481)	**<0.0001**
GGT	**0.910** **0.510**	<0.0001 ns	0.399 (0.241, 0.558)	**<0.0001**	**0.901** **0.510**	<0.0001 ns	0.391 (0.233, 0.549)	**<0.0001**	**0.844** **0.510**	<0.0001 ns	0.344 (0.169, 0.500)	**<0.0001**
ALP	**0.910** **0.618**	<0.0001 ns	0.291 (0.150, 0.432)	**<0.0001**	**0.901** **0.618**	<0.0001 ns	0.283 (0.134, 0.432)	**<0.001**	**0.844** **0.618**	<0.0001 ns	0.226 (0.069, 0.383)	**<0.01**

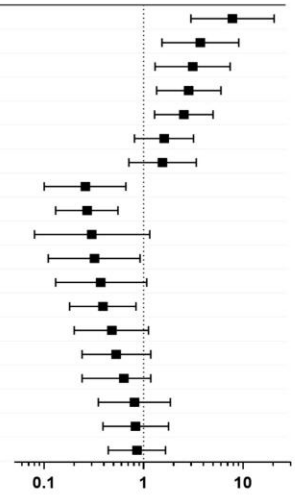
Supplementary Fig. 14. Comparison of the predictive capacity of individual protein biomarkers and logistic models to CA19-9 and common markers of cholestatic liver injury for PSC-CCA *vs* PSC. (A) The accuracy of each individual candidate was compared to the single use of CA19-9, total bilirubin, ALT, AST, GGT and ALP using the DeLong test. **(B)** The accuracy of each logistic model was compared to the single use of CA19-9, total bilirubin, ALT, AST, GGT and ALP using the DeLong test. ns, non-significant.

A

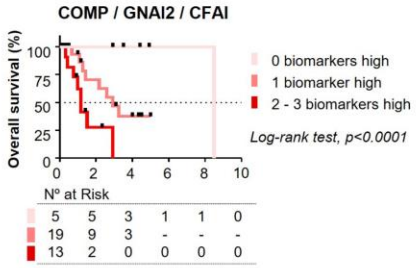
	Univariable analysis		
Clinical variables	HR	95% CI	p-value
Age, (continuous)	1.01	0.99, 1.03	ns
Sex, male (*vs* female)	1.06	0.63, 1.77	ns
CCA subtype, (*vs* iCCA)	-	-	**<0.05**
pCCA	0.72	0.41, 1.25	ns
dCCA	0.38	0.19, 0.77	**<0.01**
Disease status, (*vs* metastatic disease)	-	-	**<0.001**
Local disease	0.37	0.20, 0.66	**<0.001**
Locally advanced disease	0.33	0.15, 0.70	**<0.01**
PSC, Yes (*vs* No)	0.95	0.57, 1.57	ns
Cirrhosis, Yes (*vs* No)	1.18	0.54, 2.6	ns
Surgery, Yes (*vs* No/palliative)	0.24	0.13, 0.42	**<0.0001**
CA19-9, (continuous)	1.000031	1.000002, 1.000060	**<0.05**

B

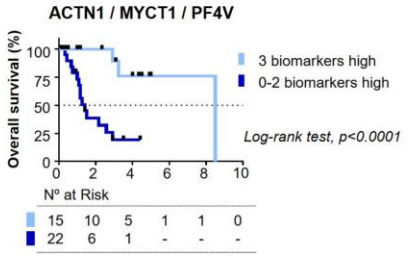
	Univariable Cox regression analysis		Multivariable Cox regression analysis	
	HR (95% CI)	p-Value	HR (95% CI)	p-Value
COMP	2.5 (1.4, 4.6)	<0.01	7.82 (2.99, 20.46)	<0.0001
GNAI2	3.8 (1.9, 7.9)	<0.001	3.72 (1.53, 9.05)	<0.01
A1AG1	3.1 (1.7, 5.7)	<0.001	3.11 (1.30, 7.41)	<0.05
CFAI	2.2 (1.3, 3.8)	<0.01	2.84 (1.35, 6.00)	<0.01
LRP1	2.1 (1.2, 3.5)	<0.01	2.54 (1.29, 4.98)	<0.01
OLFM4	2.6 (1.5, 4.4)	<0.001	1.61 (0.81, 3.18)	ns
FIBB	3.0 (1.5, 5.7)	<0.01	1.55 (0.71, 3.38)	ns
MYCT1	0.31 (0.17, 0.57)	<0.001	0.26 (0.10, 0.66)	<0.01
ACTN1	0.42 (0.25, 0.72)	<0.01	0.27 (0.13, 0.55)	<0.001
PZP	0.17 (0.053, 0.55)	<0.01	0.30 (0.08, 1.15)	ns
KPYM	0.4 (0.2, 0.81)	<0.01	0.32 (0.11, 0.92)	<0.05
ECM1	2.7 (1.4, 5.4)	<0.01	0.37 (0.13, 1.07)	ns
PF4V	0.47 (0.28, 0.78)	<0.01	0.39 (0.18, 0.84)	<0.05
FCN1	0.49 (0.29, 0.82)	<0.01	0.48 (0.20, 1.12)	ns
APOH	0.41 (0.22, 0.77)	<0.01	0.53 (0.24, 1.18)	ns
FCN3	0.37 (0.2, 0.69)	<0.01	0.63 (0.24, 1.18)	ns
IGHG3	3.1 (1.8, 5.4)	<0.0001	0.81 (0.35, 1.86)	ns
PSA1	0.49 (0.29, 0.82)	<0.01	0.83 (0.39, 1.78)	ns
APOC2	0.4 (0.24, 0.67)	<0.001	0.86 (0.44, 1.66)	ns



C **SURGERY: TUMOR RESECTION, LTX OR CEPHALIC DUODENO-PANCREATECTOMY**

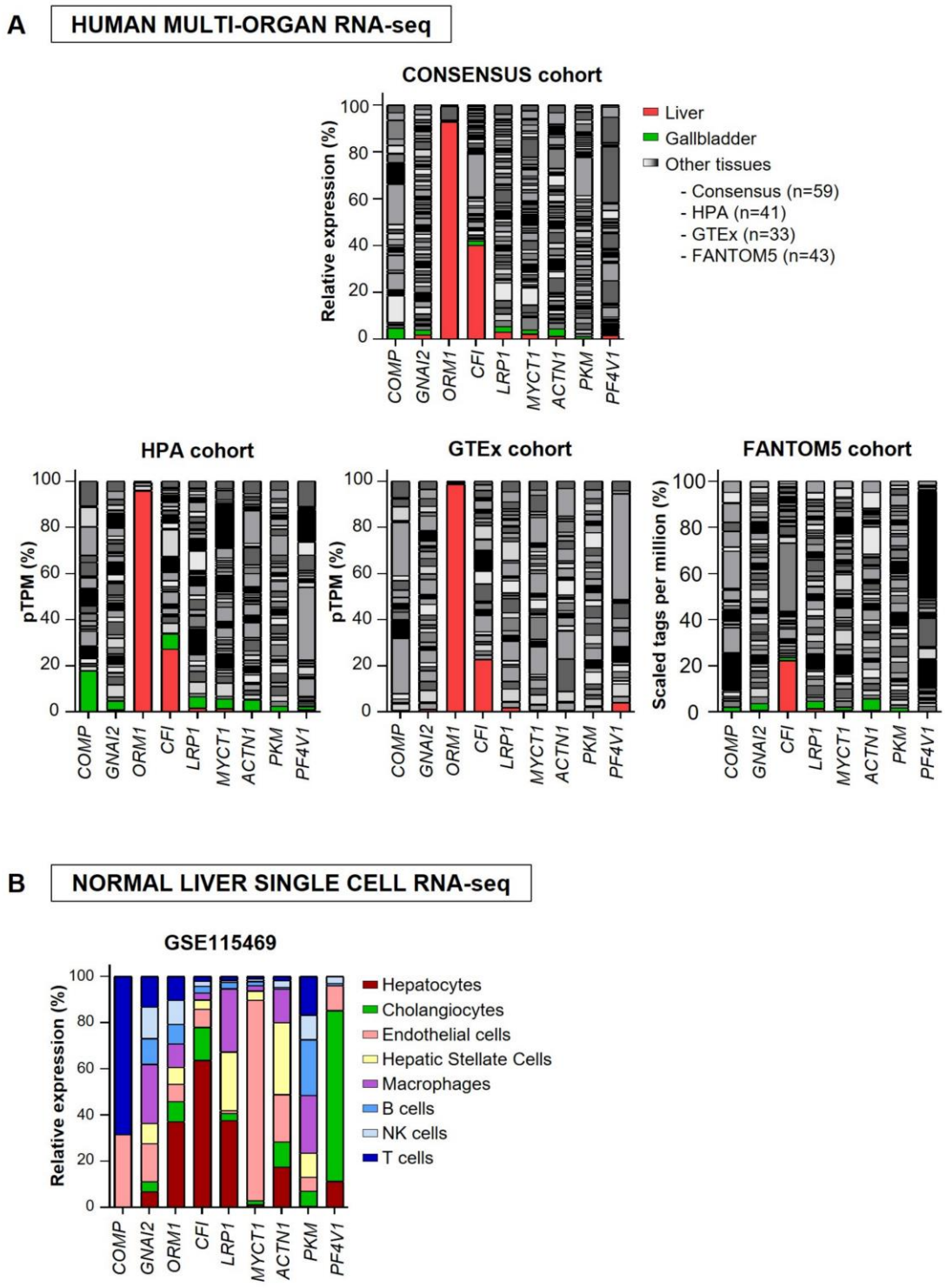


	0 high	1 high	2 - 3 high
Median survival, *months*	**102**	**35** (20.9, 49.1)	**14** (11.0, 17.0)
Log-rank test, p-Value	-	<0.05 (*vs* 0 high)	<0.01 (*vs* 0 high)
	-	-	<0. 05 (*vs* 1 high)

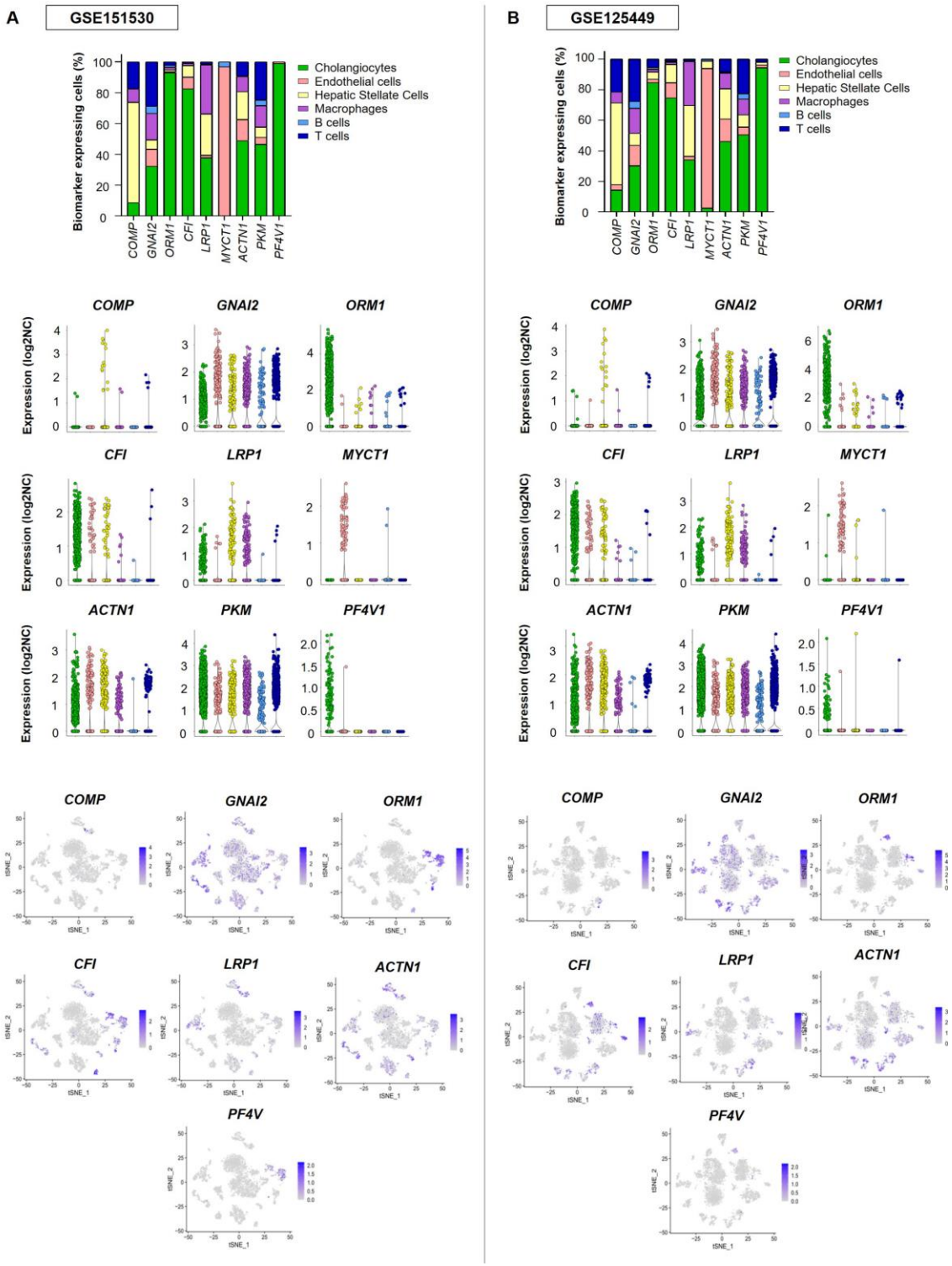


	3 high	0-2 high
Median survival, *months*	**102**	**15** (10.8, 19.2)
Log-rank test, p-Value	-	<0.001 (*vs* 0 high)

Supplementary Fig. 15. Serum EV-proteins as prognostic biomarkers for patients with CCA. (A) Univariable Cox regression analysis of the clinical/demographical parameters age, biological sex, anatomical CCA subtype, disease status, presence of PSC, presence of cirrhosis, surgery and serum CA19-9 levels in patients with CCAs regardless etiology; **(B)** Univariable and multivariable Cox regression analysis of serum EV proteins; **(C)** Kaplan Meier curve and log-rank test of patients with CCA undergoing surgery (tumor resection, liver transplantation or cephalic duodeno-pancreatectomy) according to the "bad prognostic" (COMP/GNAI2/CFAI) and "good prognostic" (ACTN1/MYCT1/PF4V) biomarker panels. ns, non-significant.



Supplementary Fig. 16. Expression of candidate prognostic biomarkers in human tissues and in normal liver cell types. (A) Analysis of candidate serum prognostic biomarkers in the Consensus cohort and also in three tissue cohorts summarized in the Consensus gene data of The Human Protein Atlas: HPA, GTEx and FANTOM5. **(B)** Normalized expression of candidate biomarkers in each liver cell type from normal liver scRNA-seq (GSE115469).



Supplementary Fig. 17. Expression of candidate prognostic biomarkers in human CCA tumors. Comparison of biomarker-expressing cells, relative biomarker expression within the single-cell types and tSNE plots of iCCA tumors from the **(A)** GSE151530 and **(B)** GSE125449.

Supplementary tables

Supplementary Table 1 – Demographic and clinical features of the study cohort analyzed by mass spectrometry (MS)

		Healthy		PSC		PSC to CCA	PSC-CCA		CCA	HCC
		Orbitrap (n=19)	timsTOF (n=55)	Orbitrap (n=18)	timsTOF (n=45)	timsTOF (n=25)	Orbitrap (n=14)	timsTOF (n=42)	timsTOF (n=56)	timsTOF (n=34)
GENERAL DEMOGRAPHICS										
Age	mean ± SD (years)	**60** ± 6	57 ± 10	**35** ± 13	**38** ± 13	**48** ± 14	**53** ± 12	**49** ± 14	**66** ± 10	**63** ± 9
Sex	Male, n (%)	**5** (26.3)	**22** (40.0)	**15** (83.3)	**34** (75.6)	**21** (84.0)	**10** (71.4)	**28** (66.7)	**40** (71.4)	**33** (97.1)
CLINICAL PARAMETERS, n (%)										
IBD	No	-	-	**2** (11.1)	**6** (13.3)	**6** (24.0)	**0** (0.0)	**8** (19.0)	**54** (96.4)	**34** (100)
	UC	-	-	**10** (55.6)	**30** (66.7)	**16** (64.0)	**13** (92.9)	**28** (66.7)	**1** (1.8)	**0** (0.0)
	Crohn	-	-	**3** (16.7)	**4** (8.9)	**3** (12.0)	**0** (0.0)	**5** (11.9)	**1** (1.8)	**0** (0.0)
	Unspecified	-	-	**3** (16.7)	**5** (11.1)	**0** (0)	**1** (7.1)	**1** (2.4)	**0** (0)	**0** (0.0)
Cirrhosis	Yes	-	-	**0** (0.0)	**0** (0.0)	**9** (36.0)	**1** (7.1)	**4** (9.5)	**4** (7.1)	**24** (70.6)
	No	-	-	**15** (83.3)	**43** (95.6)	**16** (64.0)	**12** (85.7)	**38** (90.5)	**50** (89.3)	**8** (23.5)
	NA	-	-	**3** (16.7)	**2** (4.4)	**0** (0.0)	**1** (7.1)	**0** (0)	**2** (3.6)	**2** (5.9)
HBV / HCV	Yes	-	-	**0** (0)	**0** (0)	**1** (4.0)	**0** (0)	**2** (4.8)	**3** (5.4)	**20** (58.8)
	No	-	-	**18** (100)	**45** (100)	**24** (96.0)	**14** (100)	**39** (92.9)	**53** (94.6)	**14** (41.2)
	NA	-	-	**0** (0)	**0** (0)	**0** (0)	**0** (0)	**1** (2.4)	**0** (0)	**0** (0)
Choledochal cysts	Yes	-	-	**0** (0)	**0** (0)	**0** (0)	**0** (0)	**0** (0)	**0** (0)	**0** (0)
	No	-	-	**18** (100)	**45** (100)	**25** (100)	**14** (100)	**42** (100)	**56** (100)	**34** (100)
	NA	-	-	**0** (0)	**0** (0)	**0** (0)	**0** (0)	**0** (0)	**0** (0)	**0** (0)
Liver fluke	Yes	-	-	**0** (0)	**0** (0)	**0** (0)	**0** (0)	**0** (0)	**0** (0)	**0** (0)
	No	-	-	**18** (100)	**45** (100)	**25** (100)	**14** (100)	**42** (100)	**56** (100)	**34** (100)
	NA	-	-	**0** (0)	**0** (0)	**0** (0)	**0** (0)	**0** (0)	**0** (0)	**0** (0)
CCA subtype	iCCA	-	-	-	-	**6** (24.0)	**3** (21.4)	**16** (30.1)	**21** (37.5)	-
	pCCA	-	-	-	-	**15** (60.0)	**7** (50.0)	**19** (45.2)	**18** (32.1)	-
	dCCA	-	-	-	-	**2** (8.0)	**4** (28.6)	**3** (7.1)	**17** (30.4)	-
Disease status	LD	-	-	-	-	**7** (28.0)	**3** (21.4)	**10** (23.8)	**25** (44.6)	-
	LAD	-	-	-	-	**11** (44.0)	**7** (50.0)	**15** (35.7)	**11** (19.6)	-
	MD	-	-	-	-	**2** (8.0)	**2** (14.3)	**8** (19.0)	**20** (35.7)	-
CCA-related surgery	No	-	-	-	-	**4** (16.0)	**5** (35.7)	**16** (30.1)	**37** (66.1)	-
	Liver transplant	-	-	-	-	**18** (72.0)	**6** (42.9)	**8** (19.0)	**0** (0.0)	-
	Tumor resection	-	-	-	-	**2** (8.0)	**3** (21.4)	**17** (40.5)	**19** (33.9)	-
	Exploratory	-	-	-	-	**1** (4.0)	**0** (0.0)	**1** (2.4)	**0** (0.0)	-
BIOCHEMICAL PARAMETERS										
ALT (IU/L)	mean ± SD	-	-	**108** ± 85.5	**91.2** ± 68.1	**105** ± 77.6	**129** ± 209	**121** ± 164	**88.8** ± 123.5	**52.6** ± 81.0
	NA, n (%)	-	-	-	-	-	**1** (7.1)	**2** (4.8)	-	-
AST (IU/L)	mean ± SD	-	-	**69.8** ± 69.3	**60.3** ± 48.9	**92.2** ± 61.8	**142** ± 209	**103** ± 135	**67.7** ± 68.4	**48.3** ± 50.0
	NA, n (%)	-	-	-	-	-	-	**1** (2.4)	-	**1** (2.9)
GGT (IU/L)	mean ± SD	-	-	**301** ± 234	**281.1** ± 266	**283.6** ± 244.7	**250** ± 249	**320** ± 296	**601.8** ± 899.5	**136.7** ± 247.2
	NA, n (%)	-	-	-	-	-	**1** (7.1)	**1** (2.4)	-	**1** (2.9)
Total bilirubin (mg/dL)	mean ± SD	-	-	**1.0** ± 0.9	**1.2** ± 1.3	**5.2** ± 6.4	**9.4** ± 9.4	**6.28** ± 7.4	**3.0** ± 5.2	**0.64** ± 0.4
	NA, n (%)	-	-	-	-	-	-	-	-	-
ALP (IU/L)	mean ± SD	-	-	**238** ± 161	**236** ± 172	**398.2** ± 255.7	**381** ± 340	**425.5** ± 296	**321.3** ± 372.9	**116.7** ± 99.4
	NA, n (%)	-	-	-	-	-	-	-	-	**1** (2.9)
CA19-9 (IU/mL)	mean ± SD	-	-	**31.5** ± 34.6	**27.6** ± 39.8	**187.4** ± 500.5	**1815** ± 3152	**2858** ± 7296	**2037** ± 8230	**21.0** ± 15.0
	NA, n (%)	-	-	**3** (16.7)	**4** (8.9)	**0** (0)	**1** (7.1)	**2** (4.8)	**4** (7.1)	**5** (14.7)
AFP (ng/mL)	mean ± SD	-	-	**4.7** ± 4.4	**3.9** ± 3.5	**4.0** ± 4.2	**12.9** ± 21.0	**8.1** ± 15.4	**14.4** ± 37.3	**5.5** ± 5.9
	NA, n (%)	-	-	**8** (44.4)	**27** (60.0)	**2** (8.0)	**2** (14.3)	**9** (21.4)	**34** (60.7)	**8** (23.5)

* Healthy (18/55 samples previously analysed in the Orbitrap MS), PSC (18/45 samples analysed also in the Orbitrap MS), PSC-CCA (12/42 samples analysed in the Orbitrap MS)

Supplementary Table 2 –Clinical features of the study cohort used for the ELISA validation of candidate biomarkers

		Healthy (n=32)	PSC (n=46)	PSC to CCA (n=24)	PSC-CCA (n=43)	CCA (n=67)	HCC (n=39)
Cirrhosis	Yes	-	**7** (15.2)	**9** (37.5)	**3** (7.0)	**6** (9.0)	**28** (71.8)
	No	-	**33** (71.7)	**15** (62.5)	**40** (93.0)	**38** (56.7)	**10** (25.6)
	NA	-	**5** (10.9)	**0** (0)	**0** (0)	**23** (34.3)	**1** (2.6)
CCA subtype	iCCA	-	-	**6** (25.0)	**15** (34.9)	**33** (49.3)	-
	pCCA	-	-	**14** (58.3)	**21** (48.8)	**16** (23.9)	-
	dCCA	-	-	**2** (8.3)	**4** (9.3)	**18** (26.9)	-
Disease status	LD	-	-	**7** (29.2)	**11** (25.6)	**28** (41.8)	-
	LAD	-	-	**10** (41.7)	**12** (27.9)	**14** (20.9)	-
	MD	-	-	**2** (8.3)	**10** (23.3)	**25** (37.3)	-
CA19-9 (IU/mL)	mean ± SD	-	**30.7** ± 52.8	**194.8** ± 509.9	**2710** ± 7269	**3892** ± 24001	**20.8** ± 12.7
	NA, n (%)	-	**6** (13.0)	**0** (0)	**3** (7.0)	**2** (3.0)	**31** (79.5)

* Samples previously analysed in serum EVs by mass spectrometry: Healthy (16/32), PSC (18/46), PSC to CCA (24/24), PSC-CCA (37/43), CCA (34/67), HCC (25/39)

Supplementary Table 3 – List of antibodies used for immunoblotting and immunofluorescence (IF)

Antibody	Company	Reference	Clone	Application
Mouse monoclonal anti-CD63	DHSB	H5C6	-	WB
Purified Mouse anti-CD81	BD Biosciences	555675	JXS-81	WB
Rabbit monoclonal anti-CK19	Abcam	ab52625	EP1580Y	IF
Mouse monoclonal anti-CRP	Abcam	ab50861	26D7	IF, WB
Mouse monoclonal anti-FGL1	Proteintech	67391-1-Ig	2B11C6	IF
Rabbit monoclonal anti-FGL1	Cell Signaling Technology	#47507	E8V9Q	WB
Rabbit monoclonal anti-FIBB	Abcam	ab189490	EPR18145-84	WB
Mouse monoclonal anti-FIBG	Abcam	ab119948	5A6	IF, WB
Mouse monoclonal anti-FTL	Santa Cruz Biotechnology	Sc-74513	D-9	WB
Purified mouse anti-GRP78	BD Biosciences	610979	40/Bip	WB
Rabbit polyclonal anti-OIT3	Thermo Fisher	bs-19567R	-	WB
Rabbit polyclonal anti-PIGR	Abcam	ab96196	-	IF, WB
Rabbit polyclonal anti-vWF	Abcam	ab6994	-	WB
Goat anti-rabbit IgG, Alexa Fluor™ Plus 488	Thermo Scientific	A32731	-	IF
Goat anti-mouse IgG, Alexa Fluor™ 488	Thermo Scientific	A28175	-	IF
Goat anti-rabbit IgG, Alexa Fluor™ 594	Thermo Scientific	A11012	-	IF
Donkey anti-mouse IgG, Alexa Fluor™ 594	Thermo Scientific	A21203	-	IF
Anti-mouse IgG, HRP-linked antibody	Cell Signaling Technology	#7076	-	WB
Anti-rabbit IgG, HRP-linked antibody	Cell Signaling Technology	#7074	-	WB

Supplementary Table 4 – List of ELISA kits and dilutions used in the assays

Protein	Company	Reference	Dilution
FGL1	Abcam	ab284622	1:100
CRP	Innovative Research	IHUCRPKT	1:1000
FIBRINOGEN	Innovative Research	IHFBGNKT	1:100
VWF	Abcam	ab223864	1:1000
PIGR	Abcam	ab282302	1:500
OIT3	Abbexa	abx054228	1:15
FRIL	Abbexa	abx151595	1:50

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Author names in bold designate shared co-first authorship.

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