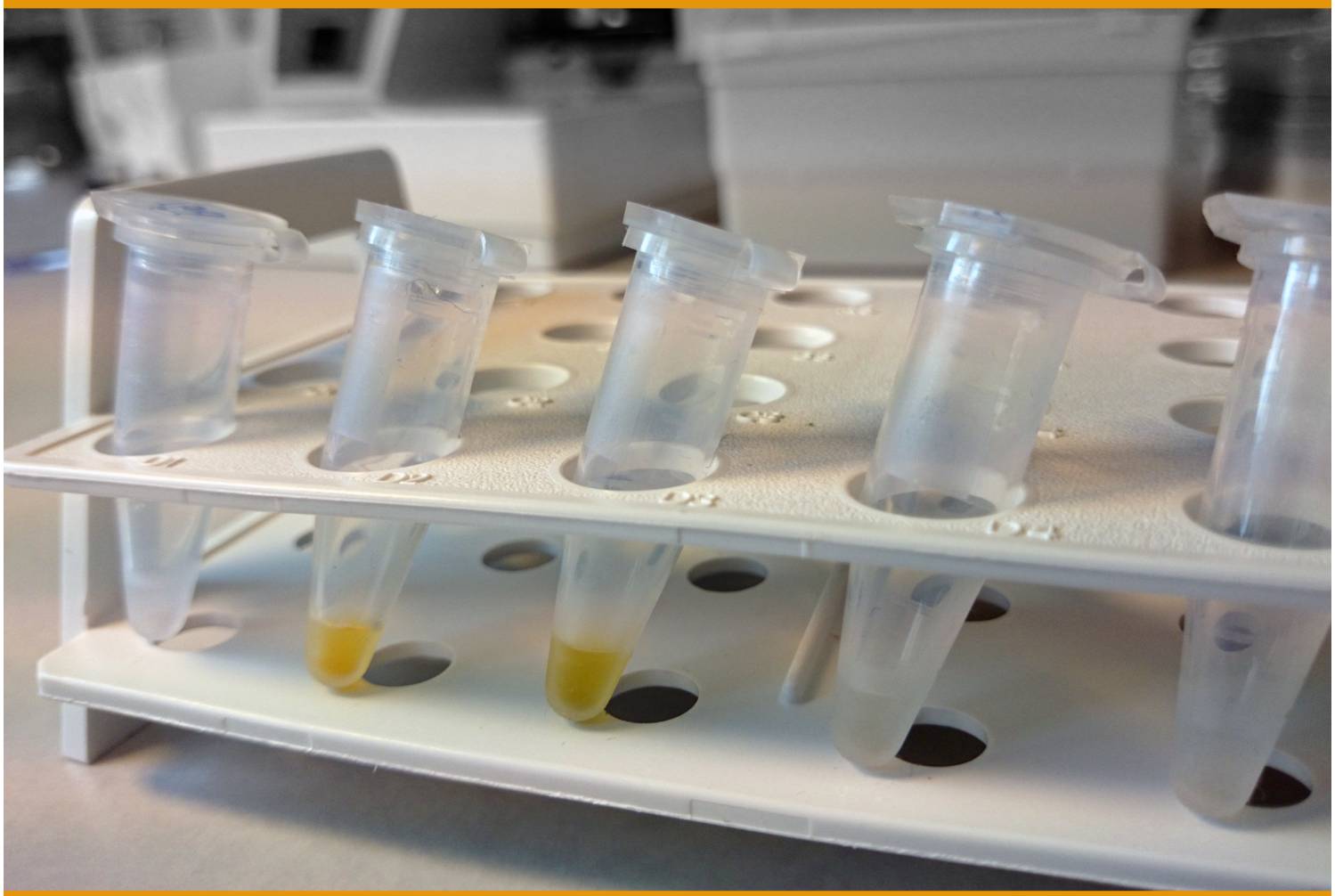
Proteomic analysis of human bile reveals potential biomarkers for cholangiocarcinoma

A Master's thesis in medicine

by Andreas Gustafsson



Proteomic analysis of human bile reveals potential biomarkers for cholangiocarcinoma

Master's thesis in Medicine

Andreas Gustafsson

Supervisors: Karolina S. Jabbar, M.D., doctoral student
and Gunnar C. Hansson, M.D., Ph.D.

Department of Medical Biochemistry, Institute of Biomedicine,
University of Gothenburg

UNIVERSITY OF GOTHENBURG

Programme in Medicine

Gothenburg, Sweden 2015

TABLE OF CONTENTS

ABSTRACT

Background: Cholangiocarcinoma (CC), is the second most common primary liver cancer in most parts of the world. In western countries, primary sclerosing cholangitis (PSC) is one of the most common causes of CC. CC has a low survival rate, between 2-30% depending on type and spread, and diagnosis often comes late in the development of the tumor, due to a late clinical presentation and unsatisfactory diagnostic methods. It is particularly difficult to determine if biliary strictures in patients with PSC are benign or malignant in nature. In recent years, there has been an increased interest in the use of proteomic analysis of bile for the discovery of potential biomarkers to aid in the diagnosis of CC.

Objective: The aim was to determine the existence of proteins whose relative abundance in bile differs depending on the underlying disease, in patients with biliary strictures. The main ambition was to discover one or more proteins with the ability to distinguish a benign biliary stricture from a malignant stricture in patients with PSC.

Methods: Bile samples from 37 patients were analyzed in this prospective exploratory study, including 5 patients with *de novo* CC, 4 with CC and PSC, 7 with PSC only, 6 with pancreatic cancer, and 15 controls. The bile was aspirated from patients undergoing endoscopic retrograde cholangiopancreatography. For one sample, bile was aspirated by percutaneous transhepatic cholangiography. Nano liquid chromatography tandem mass spectrometry was used for mass analysis. Proteins were identified by searches against a public database of human peptide sequences. Lastly, SPSS Statistical Package was used to calculate if there was a statistically significant difference in abundance of proteins between patient groups.

Results: A total of 6 proteins were identified where the relative abundance differed significantly between patient groups ($p < 0.05$). The most promising finding was that the

known tumor suppressor Receptor-type tyrosine-protein phosphatase eta (R-PTP-eta) was less abundant in patients with CC than in the control population and patients with PSC only.

Conclusions: The differential abundance of certain proteins present in human bile, such as R-PTP-eta, can be used to differentiate CC from other diseases of the bile ducts. None of the proteins described in this study were alone able to distinguish a benign biliary stricture from a malignant stricture in patients with PSC. Further studies are warranted to examine the usefulness of these proteins as clinical tumor markers for CC.

The abbreviations used in this report are: CC, Cholangiocarcinoma; PSC, Primary sclerosing cholangitis; MRCP, Magnetic resonance cholangiopancreatography; ERCP, Endoscopic retrograde cholangiopancreatography; CT, Computed tomography; CA 19-9, Serum carbohydrate antigen 19-9; CEA, Carcinoembryonic antigen; LC-MS/MS, Liquid Chromatography Tandem Mass Spectrometry; GuHCl, Guanidine HCl; R-PTP-eta, Receptor-type tyrosine-protein phosphatase eta.

INTRODUCTION

Cholangiocarcinoma. Bile duct cancer, or cholangiocarcinoma (CC) is a malignant tumor that originates in the epithelial cells that line the bile ducts. While CC is a relatively rare form of cancer, it is the second most prevalent primary liver cancer after hepatocellular carcinoma in most parts of the world[1]. Since CC rarely occurs before the age of 40[2], the incidence is consequently higher in older populations than in the Swedish population as a whole; the majority of Swedish patients diagnosed with CC in 2013 were 70 years of age or older[3]. In 2013, the incidence of CC per 100,000 Swedish inhabitants in the age group 40 years and older was 4.74[3,4]. The risk of developing CC is slightly higher for men than for women[1]. Over the past decades there has been a worldwide increase in the incidence and mortality of CC, according to epidemiological studies[1,5]. While the etiology of most cases of CC is unknown[2], there are still several known risk factors for developing the disease, all of which have chronic biliary inflammation in common[1]. In Western countries, primary sclerosing cholangitis (PSC) is one of the most common causes of CC[1].

CC can be classified according to its location as being either intra-hepatic or extra-hepatic, with the latter being the most common manifestation of the tumor[1]. The extra-hepatic tumors can in turn be divided into distal and hilar lesions. Hilar lesions are tumors with an origin at the confluence of the right and left hepatic ducts; these are often referred to as Klatskin tumors[6]. The distal lesions occur along the common bile duct[7] (Fig. 1).

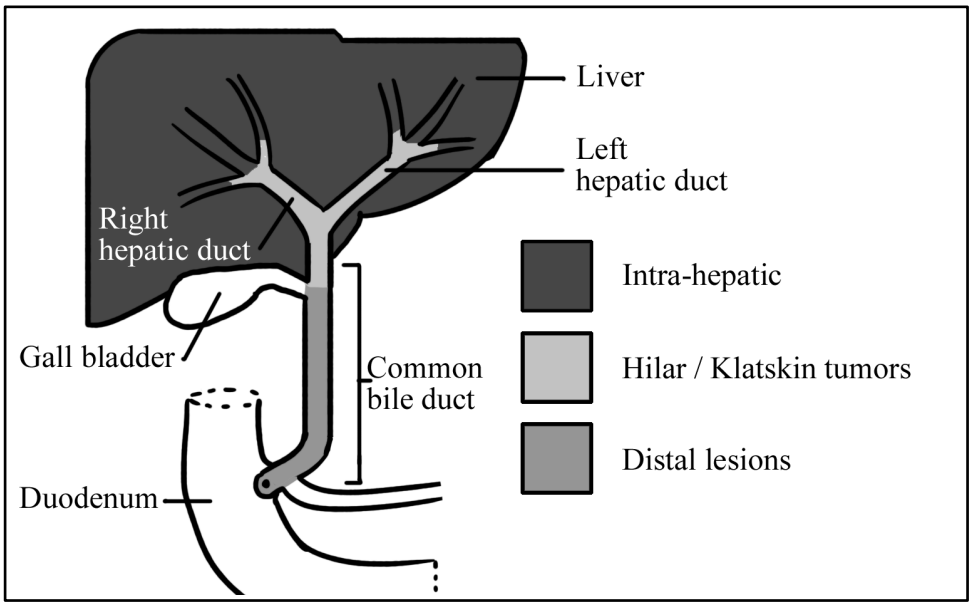


Figure 1. Anatomy of the bile ducts. The location of different forms of CC are marked in shades of gray (Gustafsson A, 2015).

The course of the disease, its clinical presentation, incidence, and risk factors differ somewhat depending on which of these subtypes the CC belongs to[1,2]. What all forms of CC have in common is that they are notoriously difficult to diagnose early on in their development, with a late and often unspecific clinical presentation, and a high mortality rate. Statistics from the U.S. National Cancer Institute show that the 5-year relative survival for patients diagnosed with either intra-hepatic or extra-hepatic CC in the years 2000 to 2006 was between 2-15% and 2-30% respectively, depending on how far the cancer had spread from its point of origin[8].

Primary Sclerosing Cholangitis. As mentioned previously PSC is one of the most common causes of CC[1]. It is a chronic disorder characterized by inflammation and fibrosis of both the intra-hepatic and extra-hepatic bile ducts. Over time strictures may develop, which are abnormally narrowed ductal segments. There is a strong association with inflammatory bowel disease: approximately 70% of patients with PSC also have ulcerative colitis[9]. This association with ulcerative colitis, together with observations of lymphocytic infiltrates in the tissue surrounding the affected ducts, and circulating auto-antibodies in the plasma of PSC patients, suggests that PSC may be an immunologically mediated disease. How these findings relate to the pathogenesis of PSC still remains to be clarified[9,10].

While no more than 10% of CC can be attributed to PSC[2], the relative risk of developing CC is significantly increased in PSC patients compared with the general population. In a Swedish study with a cohort of 604 PSC patients, the frequency of CC was observed to be 13%[11]. Patients who develop CC as a result of underlying PSC are generally younger than patients who develop it *de novo*. The median age of patients diagnosed with PSC has been calculated as 41 years, and in those who also develop CC, the malignancy is frequently detected within the first 1-3 years after diagnosis of PSC[10].

The symptoms of PSC are cholestatic; the obstruction of bile flow from the liver to the duodenum causes systemic retention of bilirubin and other solutes which are normally eliminated in bile. In the case of PSC, this obstruction is due to stricturing of the bile ducts. The symptoms include jaundice, pruritus, dark urine and pale stools[9]. Other common symptoms are abdominal pain, fever, and fatigue. Magnetic resonance cholangiopancreatography (MRCP) is the preferred method for identifying PSC, having supplanted endoscopic retrograde cholangiopancreatography (ERCP) in this respect.

Nonetheless, ERCP is still used for investigations of symptomatic PSC patients where biliary obstruction or CC is suspected[10].

A feared complication of PSC is the formation of dominant strictures. These are defined as a stenosis ≤ 1.5 mm in the common bile duct or ≤ 1 mm in the hepatic duct. With current diagnostic methods, it is difficult to differentiate between benign and malignant dominant strictures[10]. Given the mortality rates of CC, this is crucial to determine in order to ensure a prompt and adequate treatment. The need for novel and accurate diagnostic methods for this purpose is great.

Diagnosing Cholangiocarcinoma. There are several factors that complicate the process of diagnosing CC. The first factor can be attributed to the insidious progress of the disease. Most patients are diagnosed with CC when the cancer is already at an advanced stage and often unresectable, having a survival of less than 12 months following diagnosis[7].

The second complicating factor lies with the symptoms themselves. Since they are so unspecific, there are many differential diagnoses to consider. Extra-hepatic CC is a cholestatic disease, and patients present with painless jaundice, dark urine, pale stools, pruritus, malaise, and weight loss[1,7]. Patients with intra-hepatic CC present with abdominal pain, malaise, weight loss, and decreased appetite. Sometimes an incidentally found abdominal mass is the only finding[1]. When a patient presents with these symptoms, the presence of CC or pancreatic malignancy must be suspected and evaluated, but many other diagnoses must also be considered and ruled out, e.g. gallbladder cancer, PSC, benign biliary strictures, and gallstones[7].

The third factor that complicates the diagnosing of CC is the lack of accurate diagnostic methods. There is currently no test that alone can diagnose bile duct malignancies with

confidence. Many different modalities are combined in clinical practice to achieve the best possible diagnostic accuracy: imaging techniques, endoscopy, cytology, and serum tumor markers[10]. Even so, in some difficult cases the results of these combined efforts are inconclusive.

Computed tomography (CT) is very sensitive for detecting intra-hepatic CC larger than 1 cm[1]. However, today CT has mostly been replaced with magnetic resonance imaging and MRCP for diagnosing and staging CC. This technique can reveal both the site and extent of tumor involvement as well as vascular involvement[1]. The sensitivity of MRCP for differentiating between benign and malignant biliary strictures ranges from 30-98%[12].

ERCP is one of the main tools in diagnosing CC. With this endoscopic technique, the bile ducts can be visualized in detail, and stenosing tumors along the bile ducts can be easily detected[1]. For the purpose of determining the etiology of a biliary stricture, ERCP has a diagnostic accuracy comparable to that of MRCP[12]. The downside is that ERCP, unlike MRCP and CT, is an invasive procedure; there is always the risk of complications such as bacterial cholangitis and pancreatitis. In a systematic survey of prospective studies of post-ERCP complications, Andriulli et al. reported a morbidity of 6.85% and a mortality of 0.33% that could be attributed to complications of the procedure[13].

During ERCP, brush cytology can be used to obtain cell samples for analysis. The diagnostic yield of this method is very low, from 9-24%[1]. Complicating matters further, it is difficult to evaluate cell samples from patients with PSC, due to the inflammatory, reactive changes often found in these patients[14]. Fluorescence in situ hybridization and digital image analysis is a more sensitive method, which is often combined with brush cytology to increase the diagnostic accuracy[1].

Tumor markers such as serum carbohydrate antigen 19-9 (CA 19-9), carcinoembryonic antigen (CEA), and cancer antigen 125 can be elevated in patients with CC[1,15]. Some studies have evaluated the possibility of using these and other existing tumor markers to diagnose CC, based on their presence in serum and bile, but they are not specific enough to be used clinically as tumor markers for CC[1,16].

Bile Proteomics. Bile proteomics is the scientific field devoted to identifying and quantifying the proteins that can be found in bile, more specifically human bile. The field has existed for decades, but has recently become more popular by building on the field of cancer proteomics: proteomic analysis of body fluids targeted at "the discovery of new clinically useful biomarkers" to detect cancer[17]. The principle behind cancer proteomics is that changes in the abundance of certain proteins occur during the development of malignant cells, which can be detected in the biological fluids in close contact with the malignant tissue[15,17], e.g. bile containing proteins secreted from a malignant biliary stricture. In the recent decade several proteomic studies have been conducted, aimed at discovering potential biomarkers for CC.

Liquid chromatography and tandem mass spectrometry (LC-MS/MS) is often used in proteomics for protein identification. It is an analytical technique that couples a liquid chromatograph to a tandem mass spectrometer to first separate the substances in a sample and then identify and quantify them. In the case of a sample containing peptides, the sequence of amino acids can be determined for each peptide, which can thus be identified. The process starts with liquid chromatography, where the peptides are eluted through a solid column by a liquid mobile phase. Peptides with a lower affinity to the column are eluted first, and by changing the chemical composition of the mobile phase, peptides with greater affinity to the column can be displaced and are eluted last. In this way the peptides in the sample can be separated based on their chemical properties. In tandem mass spectrometry two mass

analyzers are arranged in sequence. The peptides are first ionized and fragmented, subsequently the mass and charge of each fragment is analyzed, and in this way the peptide sequence is determined. The relative abundance of each ion is plotted in a mass spectrum as a function of the mass-to-charge ratio of the ion, represented graphically as peaks (Fig. 9). It is then possible to identify the components of a sample by the mass spectrum, in this example peptides in a sample of bile[18].

Objective. In this study the aim was to determine the existence of proteins whose relative abundance in bile differs depending on the underlying disease, in patients with biliary strictures. Such proteins could have potential as clinical tumor markers to diagnose CC at an earlier stage. It would be desirable to establish a tumor marker that has both a high sensitivity and a high specificity for differentiating between benign and malignant biliary strictures, particularly in patients with PSC.

METHODS

The scope of this master's thesis covers part of a larger ongoing study of the bile proteome which is being conducted at the Department of Medical Biochemistry, Institute of Biomedicine, University of Gothenburg, and at the Gastroenterologic Endoscopic ward at Sahlgrenska University Hospital, Gothenburg, Sweden. The study was approved by the Regional Ethical Review Board in Gothenburg, and written informed consent was obtained from all patients included in the study.

Collection of Bile

Bile samples were collected from patients undergoing ERCP at the Gastroenterologic Endoscopic ward, Sahlgrenska University Hospital. Additionally, plasma, urine and saliva

was also collected from some of the patients, for future analysis and evaluation of less invasive methods for detecting potential tumor markers. Indications for undergoing ERCP were: clinical symptoms related to bile duct disorder (e.g. jaundice), extraction of gallstones, investigation of a biliary stricture or suspected tumor, and the insertion or removal of stents in the bile ducts.

ERCP was performed with a common duodenoscope and according to clinical practice. Before the injection of contrast medium 1-3 ml of bile was aspirated by a catheter positioned in the extrahepatic bile duct. One of the patients included in this study had acquired a percutaneous transhepatic biliary drainage, and in that case bile was passively emptied through the drain. The bile sample was flushed into a plastic tube and put on ice, and within 1 h transported to a -80°C freezer for storage. Samples were thawed immediately before preparation of the bile for proteomic analysis.

Selection of Samples

The patient groups included in this study were: *de novo* CC, CC with concurrent PSC, PSC, pancreatic cancer, and lastly, patients with benign biliary diseases such as gallstones, pancreatitis, cholangitis, or benign strictures of the bile ducts. This latter category of patients with benign diseases served as controls in this study. In order to categorize the patients into the aforementioned groups, relevant clinical information was obtained through reading of their patient histories.

For this master's thesis, bile samples from 37 of the patients included in the larger study were selected for proteomic analysis. The subset of selected patients had been included in the study between January 2014 and January 2015. We included 5 patients with *de novo* CC (median age 62, range 57-80, 80% males), 4 patients with CC and concurrent PSC (median age 45,

range 33-70, 75% males), 7 patients with PSC only (median age 35, range 20-65, 71% males), 6 patients with pancreatic cancer (median age 67, range 52-75, 83% males), and 15 controls (median age 70, range 32-84, 60% males). 36 of the samples selected for analysis in this study were collected by ERCP, 1 sample was collected by percutaneous transhepatic biliary drainage. All bile samples that were analyzed had been collected distal to the biliary stricture (closer to the duodenum than the liver), in the cases where such a stricture was found. The only exclusion criterion was current malignancy other than CC and pancreatic cancer. One CC patient also had prostate cancer, but his disease was not spread and we reasoned that it would likely not affect the protein content of the bile, so he was included despite this. For a summary of the demographic and medical data of these patients, see Table 1 below.

Table 1.
Demographic and medical data for the 37 patients analyzed in this study.

Diagnosis	**Indication for ERCP**	**Age at ERCP (years)**	**Gender (M/F)**	**Other conditions**
CC	Bile obtained through percutaneous transhepatic biliary drainage.	57	M	-
CC (Klatskin tumor)	Investigation of stricture.	80	M	Renal failure.
CC (distal tumor)	Jaundice.	61	M	Prostate cancer.
CC (Klatskin tumor)	Cholestasis, abdominal pain.	62	F	Liver metastasis.
CC (Klatskin tumor)	Investigation of stricture.	70	M	-
CC and PSC (Klatskin tumor)	Investigation of stricture.	33	M	Ulcerative colitis. Colectomized.
CC and PSC (Klatskin tumor)	Jaundice, pruritus.	40	M	Ulcerative colitis. Colectomized.
CC and PSC (Klatskin tumor)	Investigation of stricture.	70	F	Ulcerative colitis. Prior thyroid cancer.
CC and PSC (intra-hepatic tumor)	Investigation of stricture.	50	M	Ulcerative colitis.
PSC	Investigation of stricture.	42	M	Ulcerative colitis. Cirrhosis. Colectomized.
PSC	Removal of stent.	29	F	-
PSC	Investigation of stricture.	36	M	-
PSC	Stenting of stricture.	65	M	Ulcerative colitis.
PSC	Concrements on ultrasound.	26	M	Ulcerative colitis.
PSC	Investigation of strictures.	20	M	Ulcerative colitis.
PSC	Dilatation of stricture.	35	F	Ulcerative colitis.
Pancreatic cancer	Pancreatic cancer on CT.	75	M	Diabetes. Hypertension.
Pancreatic cancer	Jaundice.	52	M	-
Pancreatic cancer	Investigation of stricture.	64	M	-
Pancreatic cancer	Investigation of stricture.	60	M	Pancreatitis.
Pancreatic cancer	Change of stent.	70	F	Cholecystectomized.

Table 1 (continued).
Demographic and medical data for the 37 patients analyzed in this study.

Diagnosis	Indication for ERCP	Age at ERCP (years)	Gender (M/F)	Other conditions
Gallstones	Jaundice.	65	M	Cholecystitis.
Gallstones	Concrements on MRCP.	57	F	-
Gallstones	Removal of stent.	32	M	-
Gallstones	Suspected gallstone.	74	M	Cholangitis. Chronic obstructive pulmonary disease.
Gallstones	Cholestasis.	57	M	Prior liver resection.
Gallstones	Control after stone extraction.	78	M	Hypertension. Nephrectomized.
Gallstones	Concrements on ultrasound.	39	F	-
Gallstones	Jaundice, abdominal pain.	83	M	Angina pectoris. Hypertension.
Benign stricture (distal)	Abdominal pain, jaundice.	84	M	Myocardial infarction.
Benign stricture (hilar)	Stenting of stricture.	74	F	Cholangitis.
Benign stricture	Cholestasis, abdominal pain, weight loss.	71	F	Cholecystitis.
Benign stricture (distal)	Concrements on ultrasound.	70	F	Pancreatitis.
Cholecystitis	Cholestasis.	70	F	-
Pancreatitis	Suspected gallstone.	52	M	Metabolic syndrome.
No current hepatobiliary disorder	Investigation of suspected stricture.	70	M	Ulcerative colitis. Liver transplanted for prior PSC.

Bile Sample Preparation

Filter Aided Sample Preparation. To prepare the bile for Nano LC-MS/MS analysis, a modified version of the Filter Aided Sample Preparation protocol, first described by Wisniewski et al. in 2009, was used[19]. First a portion (60 µl) of thawed bile was delipidated by addition of Cleanascite (Biotech Support Group, LLC., Monmouth Junction, NJ). The delipidated bile sample was then diluted in H_2O at a ratio of 1:1. We continued with 12 µl of this dilution for each sample. Proteins were reduced through incubation with a buffer containing 6 M guanidine HCl (GuHCl), 0.1 M Tris HCl (pH 8.0), 5 mM ethylenediaminetetraacetic acid (EDTA), and 0.1 M dithiothreitol for 20 h at 37°C. Following centrifugation, the supernatant was loaded onto a ultrafiltration device with a molecular weight cut-off of 30 kDa and centrifuged at 7,000 *g* until the sample had passed through the filter. After washing with 200 µl of 6 M GuHCl, proteins were alkylated on-filter by addition of 50 µl of a solution containing 0.1 M Tris (pH 8,5) and 0.05 M iodoacetamide. Buffer

exchange was achieved by adding 100 µl of 50 mM ammonium bicarbonate (pH 8) and centrifuging the filter; this step was repeated once.

Double Digestion with Lys-C and trypsin. The sample was sequentially digested using the two proteases Lys-C and trypsin. Each sample was ultimately divided into two fractions: one containing peptides digested with Lys-C, and the other containing peptides digested with Lys-C and trypsin. Lys-C (Wako Pure Chemical Industries, Ltd.) was loaded onto the filter, which was left to incubate for 4 h at 37°C. The Lys-C digest was eluted by centrifugation at 10,000 *g*, followed by addition of 0.5 M NaCl and centrifugation for 20 min at 10,000 *g*. The eluted Lys-C fraction was stored at 4°C overnight, while the filter was moved to a new collecting tube, to which trypsin (Promega, Madison, WI) and 50 mM ammoniumbicarbonate was added. This trypsin fraction was digested overnight at 37°C, and eluted as described above.

Desalting and Concentration of Peptides. The Lys-C and trypsin fractions were acidifed and desalted on separate C_{18} StageTips, using a modified version of the protocol described in 2003 by Rappsilber et al[20]. Each fraction was eluted and subsequently dried under vacuum. The vacuum dried peptides were redissolved in 0.2% formic acid, in order to protonate the peptides for analysis under positive ionization conditions in the mass spectrometer. The redissolved sample was centrifuged to sediment any larger particles that may have been eluted together with the sample. The top 15 µl of the sample were pipetted into a vial for mass spectrometry and stored at -20°C until analysis.

Soon after Nano LC-MS/MS analysis was started, there were issues with high protein concentrations in the samples, which interfered with the mass analysis. This was solved by diluting all of the samples in 0.2% formic acid by a factor of 10 before continuing with the analysis.

Analysis

Nano LC-MS/MS Analysis. Nano LC-MS/MS was used to identify the peptide sequences in the bile samples. Chromatographic separation of peptides was performed on an EASY-nLC 1000 (Thermo Fisher Scientific) with a silica column. The peptides were eluted over 108 min using a stepwise gradient (Table 2), starting with 98% mobile phase A (0.2% formic acid in 99.8% water) and 2% mobile phase B (0.2% formic acid in 80% acetonitrile and 19.8% water). The flow rate was constant at 200.00 nl/min. Mass spectrometrical analysis was performed on a Q Exactive instrument (Thermo Fisher Scientific), under positive ionization conditions.

Table 2.
The gradient used for Nano LC-MS/MS analysis.

Time (min)	Duration (min)	Mobile phase B (%)
0	5	2
5	30	5
35	20	15
55	15	25
70	30	60
100	8	100

Data Analysis. Raw data from the Nano LC-MS/MS analysis was compared against the human proteome using MaxQuant, version 1.4.1.2. The software was set up to search the Uniprot database UP000005640 (68,485 sequences, downloaded May 11, 2015). Fixed modifications were carbamidomethyl on cysteine residues (from alkylation), variable modifications were acetyl (protein N-term) and oxidation of methionine. Mass tolerance was 4.5 ppm, signifying the maximum mass by which a peptide can differ from its theoretical mass in the database and still be identified as a match. In order to achieve a false discovery rate of < 1% a search against a decoy database was performed. The peptide intensities (equivalent to peptide abundance) were normalized with Intensity-based absolute quantification, where the raw peptide intensities are divided by the number of theoretically observable peptides from digestion with trypsin[21].

The data was exported to a Microsoft Excel file. Microsoft Excel was used to perform t-tests for each protein, comparing the average normalized peptide intensity between patient groups. Using the results of the t-tests, a list was compiled with the 50 proteins that showed the greatest difference in relative abundance; 43 of these proteins showed significant differences in abundance ($p < 0.05$) and 7 proteins did not show significant differences in abundance, but were included anyway because of the clinical importance of the diseases the proteins could differentiate between (CC and PSC vs. PSC, and *de novo* CC vs. CC and PSC) (Table 5 in the Appendix). Due to time limitations, only 8 of the most interesting proteins were chosen for further data analysis. The decision to include a protein for further analysis was based on: the p-value calculated by the t-test, the amount of different t-tests the protein showed differential abundance in, how great the difference in average peptide abundance was between patient groups, and the clinical importance of the diseases the protein could differentiate between.

The final data analysis was performed with SPSS Statistical Package (IBM, SPSS Statistics for Windows, version 22.0). To calculate whether the difference in peptide intensity between patient groups was statistically significant or not at a significance level of 5%, non-parametric analyses were performed for these proteins (Mann-Whitney U-test and Kruskal-Wallis test). Box-plots for each protein of interest were obtained, depicting the differential peptide abundance (Fig. 2-7). Perseus, version 1.4.1.3, was used for principal component analysis of all proteins identified with MaxQuant, to test if any of them clustered according to diagnosis. This analysis translates correlated proteins into so-called components, to provide an objective assessment of how much the samples resemble each other.

RESULTS

A total of 910 proteins were identified in the bile with Nano LC-MS/MS analysis. The amount of protein identifications varied greatly within patient groups (see Table 4 in the Appendix). t-tests performed on these proteins showed 43 proteins whose relative abundance differed significantly between patient groups. Table 5 in the Appendix compiles the 50 proteins that showed the greatest difference in relative abundance after t-tests, including 7 proteins that did not show significant differences in abundance, but might nevertheless be interesting to study.

t-tests showed no significant difference in protein abundance between the patient groups of PSC only and "CC and PSC". However, since t-tests indicated that the protein Osteopontin was more abundant in PSC only than in the "CC and PSC" group ($p = 0.054$), this protein was deemed to be of interest for non-parametric testing in any case. t-tests did not show any significant difference in protein abundance between the patient groups of *de novo* CC and "CC and PSC" either, but Mucin-5B and Mucin-1 were both more abundant in *de novo* CC than in the "CC and PSC" group and in the control population ($p = 0.052$ and 0.11, respectively). The average peptide abundance of Mucin-1 was also slightly higher in *de novo* CC than in pancreatic cancer; the same was not observed for Mucin-5B. For these reasons, Mucin-1 was also deemed to be of interest for non-parametric testing.

Out of the 8 proteins chosen for analysis using SPSS Statistical Package, non-parametric tests showed that the relative peptide abundance differed significantly between patient groups for 6 proteins. These were: Receptor-type tyrosine-protein phosphatase eta (R-PTP-eta), Mucin-1, Cathelicidin antimicrobial peptide, Cytosolic non-specific dipeptidase, Ceruloplasmin, and Vitamin D-binding protein. The 2 proteins chosen for analysis that did not differ significantly

in peptide abundance between patient groups were Osteopontin and UPF0556 protein C19orf10.

R-PTP-eta was present in 2 patients with *de novo* CC, 2 patients with CC and PSC, 2 patients with pancreatic cancer, 3 patients with PSC only, and in 8 controls. It was less abundant in patients with CC (including those with concurrent PSC) than in the control population (p = 0.017) (Fig. 2A) and patients with PSC only (p = 0.034) (Fig. 2B). The median abundance of the protein was 4 times less for CC patients than for controls, and 13.5 times less for CC patients than for PSC patients.

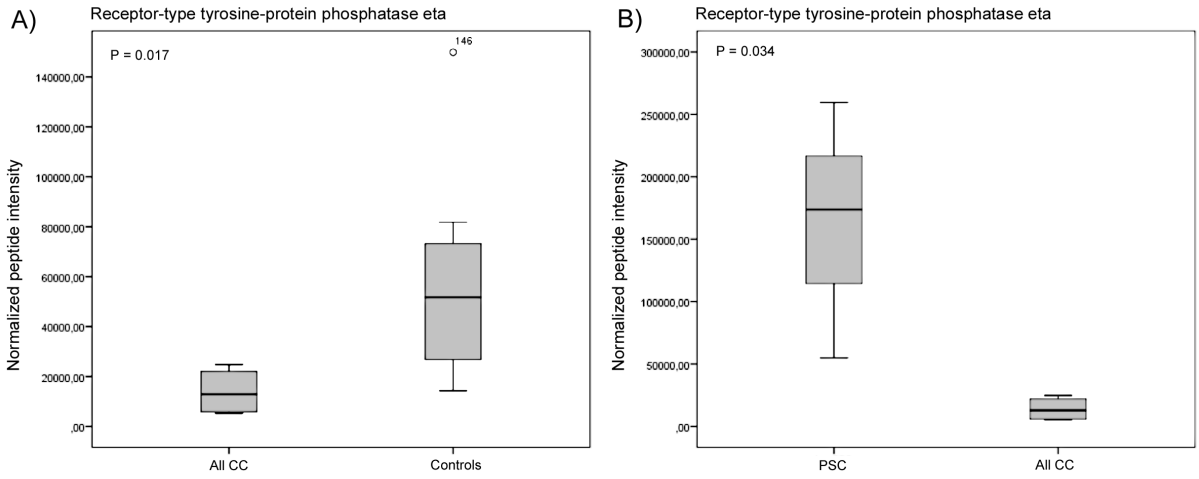


Figure 2. R-PTP-eta was less abundant in patients with CC (including those with concurrent PSC) than in the control population (A) and patients with PSC only (B).

Mucin-1 was found in 3 patients with *de novo* CC, 3 patients with CC and PSC, 4 patients with pancreatic cancer, 4 patients with PSC only, and in 8 controls. It was more abundant in patients with *de novo* CC than in patients with CC and concurrent PSC, p = 0.050 (this figure was rounded up by SPSS, so we regarded the finding as significant) (Fig. 3). The median abundance of the protein was 10 times greater for patients with *de novo* CC than for patients with CC and PSC.

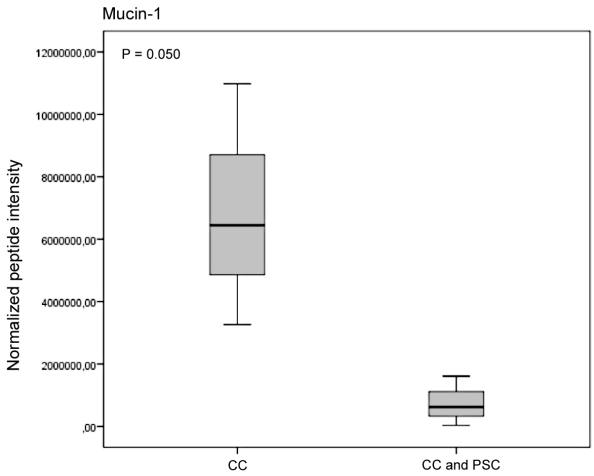


Figure 3. Mucin-1 was more abundant in patients with *de novo* CC than in patients with CC and concurrent PSC. The p-value 0.050 was rounded up by SPSS, so we regarded this finding as significant.

Cathelicidin antimicrobial peptide was present in 3 patients with *de novo* CC, 3 patients with CC and PSC, 3 patients with pancreatic cancer, 5 patients with PSC only, and in 9 controls. It was more abundant in patients with PSC only than in patients with CC (including those with concurrent PSC) (p = 0.045) (Fig. 4A) and the control population (p = 0.039) (Fig. 4B). The median abundance of the protein was 35.5 times greater for PSC patients than for CC patients, and 22.5 times greater for PSC patients than for controls.

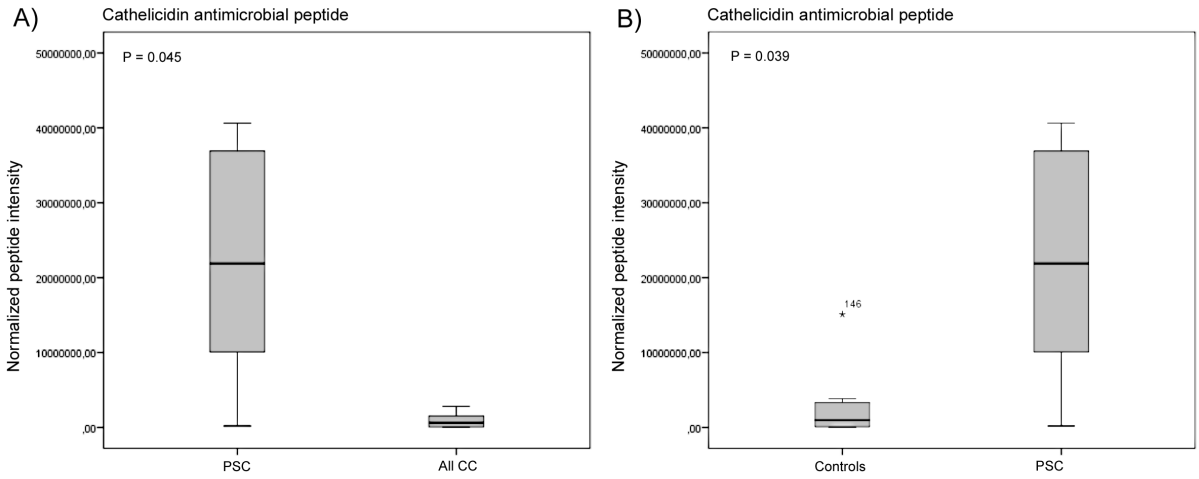


Figure 4. Cathelicidin antimicrobial peptide was more abundant in patients with PSC only than in patients with CC (including those with concurrent PSC) (A) and the control population (B).

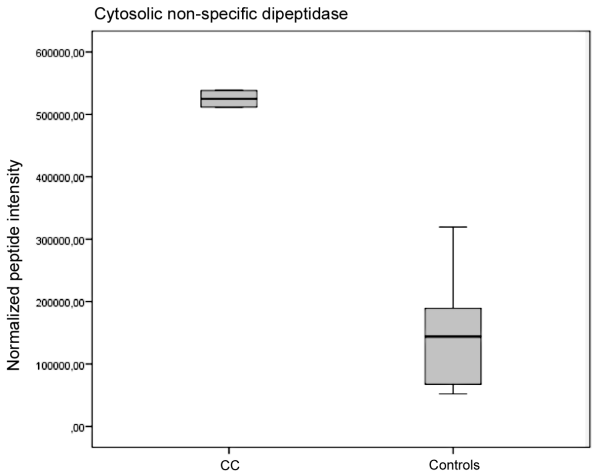


Figure 5. Cytosolic non-specific dipeptidase was more abundant in patients with *de novo* CC than in the control population.

Cytosolic non-specific dipeptidase was present in 2 patients with *de novo* CC, 3 patients with CC and PSC, 5 patients with PSC only, and in 6 controls. It was more abundant in patients with *de novo* CC than in the control population (p = 0.046) (Fig. 5). The median abundance of the protein was 3.6 times greater for patients with *de novo* CC than for controls.

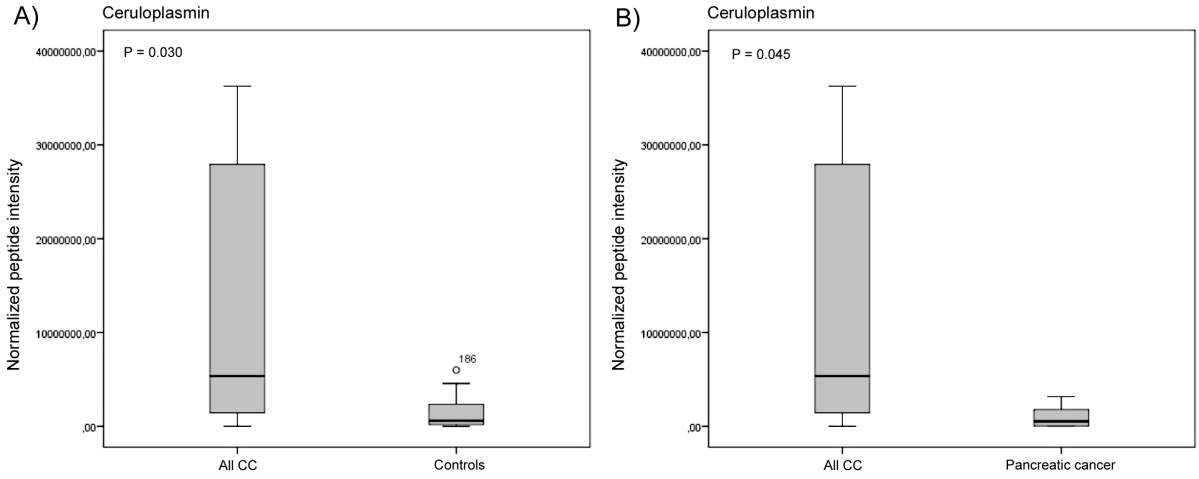


Figure 6. Ceruloplasmin was more abundant in patients with CC (including those with concurrent PSC) than in the control population (A) and patients with pancreatic cancer (B).

Ceruloplasmin was present in all 37 samples. It was more abundant in patients with CC (including those with concurrent PSC) than in the control population (p = 0.030) (Fig. 6A)

and patients with pancreatic cancer (p = 0.045) (Fig. 6B). The median abundance of the protein was 8.9 times greater for CC patients than for controls, and 10 times greater for CC patients than for patients with pancreatic cancer. It was also more abundant in patients with PSC (including those with concurrent CC) than in the control population (p = 0.046).

Vitamin D-binding protein was present in all 37 samples. It was more abundant in patients with CC (including those with concurrent PSC) than in the control population (p = 0.030) (Fig. 7A). It was also more abundant in patients with PSC (including those with concurrent CC) than in the control population (p = 0.027) (Fig. 7B). The median abundance of the protein was 1.9 times greater for CC patients than for controls, and 6.9 times greater for PSC patients than for controls.

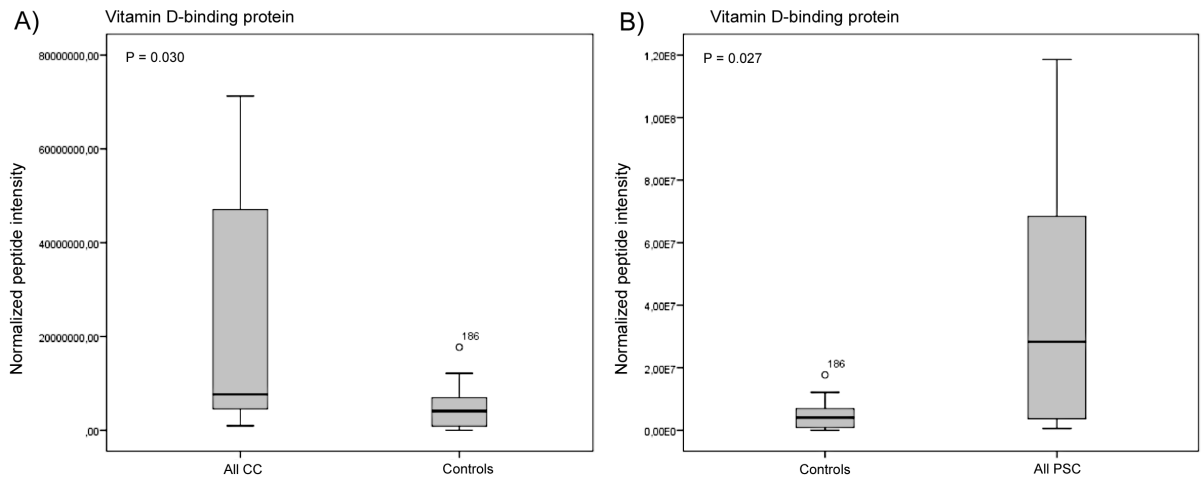


Figure 7. Vitamin D-binding protein was more abundant in patients with CC (including those with concurrent PSC) than in the control population (A). It was also more abundant in patients with PSC (including those with concurrent CC) than in the control population.

The results of principal component analysis did not show clustering according to diagnosis for any of the identified components.

Table 3.
Potential biliary biomarker candidates.

Protein	Differentiates	p-value
R-PTP-eta	CC from controls;	0.017;
	CC from PSC.	0.034
Mucin-1	*de novo* CC from CC with concurrent PSC.	0.050
Cathelicidin antimicrobial peptide	PSC from controls;	0.039;
	PSC from CC.	0.045
Cytosolic non-specific dipeptidase	*de novo* CC from controls.	0.046
Ceruloplasmin	CC from controls;	0.030;
	CC from pancreatic cancer;	0.045;
	PSC (incl. concurrent CC) from controls.	0.046
Vitamin D-binding protein	CC from controls;	0.030;
	PSC (incl. concurrent CC) from controls.	0.027

DISCUSSION

Nano LC-MS/MS followed by comparative proteomic analysis of bile samples from a cohort of 37 patients with either malignant biliary strictures, benign biliary strictures, or no biliary strictures, revealed several proteins with the potential to differentiate CC from other diseases of the bile ducts (Table 3). Unfortunately, none of the proteins described in this study were alone able to distinguish a benign biliary stricture from a malignant stricture in patients with PSC.

R-PTP-eta. Since the main aim of this study was to examine whether it was possible to use biliary proteins to differentiate benign from malignant biliary strictures, the most interesting finding was perhaps R-PTP-eta, which was less abundant in patients with CC than in the control population and patients with PSC only (Fig. 2AB). The protein is a member of the protein tyrosine phosphatase family of signaling molecules that regulate cell growth, differentiation, mitotic cycle, and oncogenic transformation[22]. It is encoded in humans by the gene PTPRJ, which is known to be a tumor suppressor for various cancer types. Ruivenkamp et al. have demonstrated that loss of heterozygosity of PTPRJ is an early event in the development of colorectal cancer. One copy of the gene is also deleted in a large percentage of human lung and breast carcinomas[23]. Furthermore, PTPRJ is involved in the development

of papillary thyroid carcinoma, where both loss of heterozygosity and a generally decreased expression of PTPRJ protein have been shown in thyroid tumors[24]. It seems plausible then that decreased expression of PTPRJ protein likewise plays a role in the development of bile duct malignancies. R-PTP-eta was not able to differentiate between benign and malignant biliary strictures in PSC patients, either due to the limitations of the present study, or because its abundance really does not differ much between PSC patients with or without CC. Further studies more targeted at R-PTP-eta and with larger populations are warranted to examine this. In any case, R-PTP-eta could possibly be useful to help determine the etiology of biliary strictures in patients where a diagnosis of PSC has been ruled out.

Mucin-1. Mucin-1 was less abundant in patients with CC and concurrent PSC than in patients with *de novo* CC (Fig. 3). It is a membrane-bound protein of the mucin family, encoded in humans by the MUC1 gene. Mucin-1 is involved in cell-adhesion and cell signaling[25]. Overexpression has been linked with several epithelial cancers, including pancreatic ductal adenocarcinoma and CC[26,27]. Tréhoux et al. recently showed that inhibiting the expression of Mucin-1 *in vitro* in pancreatic cancer cells decreases their proliferative, migratory, and invasive properties[26]. In the current study, Mucin-1 was only able to differentiate *de novo* CC from CC with concurrent PSC, and in clinical practice the diagnosis of PSC usually predates a diagnosis of CC, so its usefulness as a clinical biomarker is unclear. All the same, the differential abundance of Mucin-1 hints at a different pathogenesis for CC depending on whether it developed *de novo* or in a patient with PSC.

Cathelicidin antimicrobial peptide. Cathelicidin antimicrobial peptide was more abundant in patients with PSC only than in the control population and patients with CC (Fig. 4AB). The protein is encoded by the gene CAMP, and belongs to a family of antimicrobial peptides. Besides antibacterial activities, it has antifungal and antiviral activities, and functions in cell

chemotaxis, immune mediator induction, and inflammatory response regulation[28]. Cathelicidin antimicrobial peptide is expressed in the human biliary epithelium and is secreted into the bile ducts in response to bacterial infection. Additionaly, bile salts induce cathelicidin antimicrobial peptide expression in the human biliary epithelium[29]. Cathelicidin antimicrobial peptide was unable to differentiate between benign and malignant biliary strictures in PSC patients. High amounts of the protein in bile appears to be specific for PSC, independent of an underlying malignancy. Considering its high abundance in bile from PSC patients, as well as the fact that it is secreted into the bile ducts in response to bacterial infection, this could suggest that the presence of bacteria in the bile ducts plays a role in the pathogenesis of PSC.

Other Proteins of Interest. Cytosolic non-specific dipeptidase was more abundant in patients with *de novo* CC than in the control population (Fig 5). It is encoded in humans by the CNDP2 gene. It has been associated with a number of different cancer types. For example, the expression of Cytosolic non-specific dipeptidase in colon tumors has been shown to be significantly higher than in normal colon mucosa[30], whereas loss of the CNDP2 gene has been associated with adverse prognosis in patients with resected pancreatic cancer, as shown by Lee et al[31]. Interestingly, in this study the protein was not present in any of the 6 bile samples from patients with pancreatic cancer.

Ceruloplasmin was more abundant in patients with CC than in the control population and patients with pancreatic cancer (Fig. 6AB). It was also more abundant in patients with PSC (including those with concurrent CC) than in the control population. It is a metalloprotein that binds copper in plasma, and is also involved in oxidation-reduction, cellular iron homeostasis, and transmembrane transport. The protein is encoded in humans by the gene CP[32]. Plasma levels of Ceruloplasmin are reduced in the majority of patients with Wilson disease, a disease

characterized by accumulation of copper in the liver. The affected gene in Wilson disease has been identified as ATP7B, and in a study of ATP7B knockout mice, copper accumulation led to changes in bile duct morphology consistent with CC[33]. It is unclear if this somehow relates to the finding of increased biliary Ceruloplasmin in CC patients in the current study. Ceruloplasmin could not differentiate between CC and PSC in this study. Like R-PTP-eta, Ceruloplasmin might however be useful for determining the etiology of biliary strictures in patients where a diagnosis of PSC has been ruled out.

Vitamin D-binding protein was more abundant in patients with CC (including those with concurrent PSC) than in the control population (Fig. 7A). It was also more abundant in patients with PSC (including those with concurrent CC) than in the control population (Fig. 7B). The protein is encoded in humans by the GC gene and belongs to the albumin gene family. It is found in many body fluids and on the surface of many cell types. The protein binds to vitamin D and its plasma metabolites and transports them to target tissues[34]. It is also related to inflammatory response regulation, as it enhances macrophage chemotaxis and activation. Different phenotypes of Vitamin D-binding protein appear to have different effects on the risk of developing cancer. In a Norwegian longitudinal study, Jorde et al. have found an association between the 1f/1f phenotype of Vitamin D-binding protein and a reduced risk of cancer, which they speculate could depend on its effect on macrophage activation. Another phenotype of the protein is associated with an elevated risk of gastrointestinal cancer[35]. In short, there appears to be an association between cancer and Vitamin D-binding protein, but not much has been reported on this subject. Both CC and PSC are diseases with a pronounced inflammatory aspect, so it is not surprising that the abundance of Vitamin D-binding protein is increased in these patient groups, considering its role in inflammatory response regulation. Since the protein is elevated in both of these diseases, it would probably not be a very good biomarker for diagnosing biliary strictures.

Previous Studies of the Bile Proteome and Cholangiocarcinoma. Most proteomic studies of human bile have used sodium dodecylsulfate polyacrylamide gel electrophoresis and liquid chromatography mass spectrometry[36], and have analyzed bile samples from only a small number of patients. The results of one of the earliest mass spectrometric studies of the human bile proteome were reported in 2004 by Kristiansen et al. By fractioning bile from a CC patient with gel electrophoresis and analysing it with LC-MS/MS they were able to identify 87 unique proteins[15]. In 2011, Lankisch et al. described a model for distinguishing CC from PSC, using capillary electrophoresis mass spectrometry to identify disease-specific peptide patterns, with a reported specificity of 78% and a sensitivity of 84%[36]. However, many of the proteins included in their biomarker model, such as hemoglobin, albumin, actin, and keratin, are found in high abundance in bile and are unspecific, or are likely contaminants. In 2014, Farina et al. reported that they had analyzed 41 bile samples with high-performance LC-MS/MS, followed by immunoblot assessment of proteins overexpressed in their cancer samples. They found that Carcinoembryonic cell adhesion molecule 6 best detected cancer, and proposed a combined panel of biliary Carcinoembryonic cell adhesion molecule 6 and serum CA 19-9 to diagnose malignant biliary stenoses[37]. Farina et al. did not attempt to distinguish between different types of cancer with their biomarker, so the specificity of their proposed biomarker for detecting CC was not reported. Also in 2014, Navaneethan et al. reported that protein biomarkers in bile may differentiate malignant from benign biliary strictures. They had used liquid chromatography with mass spectrometry to identify the differential abundance of biliary proteins in bile samples from 24 patients. One of the proteins they identified as more abundant in CC samples than in PSC samples was Ceruloplasmin precursor[38], somewhat contrary to the findings of the current study, where Ceruloplasmin was most abundant in patients with PSC.

Other studies have tried to further the understanding of the pathogenesis of CC by analyzing the genetic profile of bile duct tumors. Osteopontin is a secreted glycoprotein with an important role in malignant cell attachment and tumor invasion. The gene encoding Osteopontin was identified in 2008 by Hass et al. to be the most overexpressed gene in CC. Surgical specimens from 10 patients, all containing histopathologically verified cholangiocellular carcinoma, were analyzed by oligonucleotide arrays[39]. In the current study, high levels of Osteopontin were found in bile from patients with *de novo* CC and patients with pancreatic cancer, but the differential abundance was not statistically significant between any patient groups.

Some studies have aimed to develop novel biliary tumor markers for CC without the use of proteomic analysis. Matsuda et al. demonstrated in 2010 that an assay of sialylated Mucin-1 in bile samples could diagnose CC with higher sensitivity than routine biliary cytology, but with a reported specificity of only 76.3%[27]. This corroborates the results of the current study, which strengthen the association of Mucin-1 to CC. Although Mucin-1 was not able to differentiate CC from controls, it could differentiate *de novo* CC from PSC-related CC, suggesting that there may be an association between low levels of Mucin-1 and PSC-related CC. In a cross-sectional study by Ince et al. published in 2014, they concluded that a combined assay of existing serum tumor markers may not be useful in differentiating benign from malignant strictures, due to the low specificity of the tumor markers which they had evaluated. Serum CEA was found to be elevated in patients with CC, with a sensitivity of 68.6% and a specificity of 81.6%. Biliary CA 19-9 had the highest sensitivity of biliary tumor markers at 74%, but also the lowest specificity at 34.1%[16].

Discussion of the Study Design and Methods

Study Design. In this study bile from 37 patients was analyzed. Since the bile analyzed in this study was collected from patients who all had medical reasons for undergoing ERCP, an invasive intervention with strict indications, it was not possible to include a significant number of healthy controls. Instead, patients with benign biliary diseases (other than PSC) were assigned as controls. This may not be a weakness of the study since the patients who were included were a demographically good match for the patients in a future clinical setting where a tumor marker could be used for diagnosing biliary strictures of unknown etiology. To study patients free from biliary diseases might therefore not be relevant. In some cases the diagnosis was still uncertain before Nano LC-MS/MS analysis, since clinical investigations were either still in progress, or the patient had died and investigations had been inconclusive. Such patients were categorized as having CC or PSC when that respective disease was strongly suspected based on the results of several diagnostic measures. This is of course a potential source of error, as some patients included in the "CC and PSC" group may have had only one of these diseases. A final decision on how to categorize these difficult cases was reached after discussions with an experienced clinician.

As this was an exploratory study 37 patients is quite a large number compared to earlier studies of the bile proteome. However, the hypothesis that there is a differential abundance of biliary proteins between patient groups could likely have been tested with more confidence if the sample size of patients with *de novo* CC and CC with concurrent PSC had been larger. It was unfortunately not possible to analyze more of such patients in this study, due to limitations of time and the number of patients with those respective diagnoses included in the larger study. Hopefully, the results of this study are valid enough that further studies, more targeted at the proteins described here and with larger study populations, can better elucidate the usefulness of these proteins as clinical biomarkers for CC.

As described previously, many differences exist in how the subtypes of CC behave clinically[1,2]. In this study, all cases of *de novo* CC were in practice treated as having the same disease, as were the "CC and PSC" cases, regardless of the subtype of cancer these patients had. The conclusions drawn from the results of the current study may therefore not be generalizable to all subtypes of CC, as most of the patients whose bile was analyzed had Klatskin type lesions. For future larger studies of the bile proteome it would be prudent, if possible, to include representatives of all subtypes of CC. Furthermore, a more equal gender distribution might also be advantageous for future studies. The current study did not attempt to examine if the gender of a patient is a factor that affects the composition of biliary proteins, and it would have been difficult to do so given the small sample sizes.

Optimization of Methods. To evaluate the efficacy of the experimental method, Nano LC-MS/MS analyses were performed continuously on the bile samples and the results were discussed. The first month of the study was spent optimizing the protocol for bile sample preparation and the gradient of the liquid chromatography mobile phase. Problems were encountered with obstruction of the column, as well as inadequate mass spectra, due to the large amount of proteins in the samples that we analyzed (Fig. 8). The sheer amount of sample also gave rise to long centrifugation times during ultrafiltration. After each Nano LC-MS/MS analysis the raw data was converted to peak lists and searched with Mascot software against a database of human peptide sequences. These searches indicated how many proteins were identified in each bile sample; information that was useful when deciding how to optimize the method.

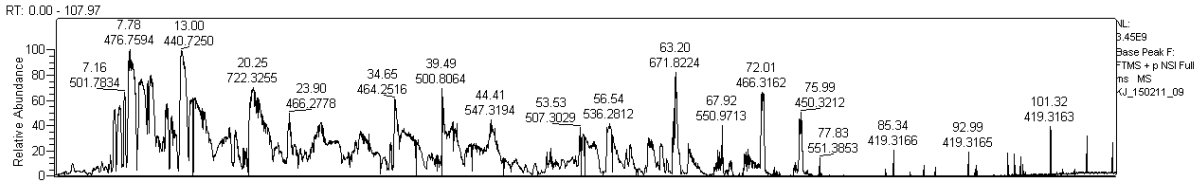


Figure 8. Mass spectrum for bile sample from a patient with CC, before the sample preparation method had been optimized. Note the wide and indistinct peaks.

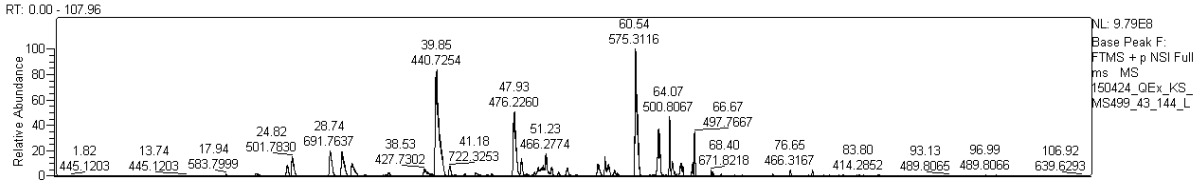


Figure 9. Mass spectrum for bile from the same patient as in Fig. 8, after the method had been optimized to decrease the protein concentration in the samples. The same mobile phase gradient was used in this analysis and the one shown in Fig. 8.

Bile Sample Preparation. The chemical composition of bile poses certain difficulties when it comes to proteomic analysis, all of which need to be considered during sample preparation. The purpose of sample preparation of bile is to remove as much unwanted material as possible, mainly bile salts and lipids, while retaining the proteins for analysis. Proteins amount to only 7% of the total solute concentration of bile[17]. The first course of action during optimization of the sample preparation protocol was to lower the volume of bile loaded onto the ultrafiltration device, from an initial volume of 60 µl down to 6 µl (diluted in 6 µl H2O). Even smaller bile volumes were evaluated, but these were difficult to work with and yielded too few protein identifications to be acceptable. Initially, ultrafiltration devices with a molecular weight cut-off of 10 kDa were used. Mainly hoping to shorten centrifugation times, ultrafiltration devices with a molecular weight cut-off of 30 kDa were evaluated. The 30 kDa filters not only shortened centrifugation times, but also yielded a slight increase in the amount of peptide identifications when compared with samples prepared on filters with a 10 kDa molecular weight cut-off. 30 kDa filters were thus chosen instead.

Bile contains large amounts of lipids that need to be removed, as they may interfere with the proteomic analysis[40]. The lipid removal reagent Cleanascite was used for delipidation. There was some concern whether the bile samples were being properly delipidated or not. The efficacy of lipid removal by Cleanascite has been shown to be in the range of 61 to 70% in one study conducted on human serum samples[41]. The ratio of Cleanascite to sample was increased from 1:4 to 1:3, to achieve a greater lipid removal. A protocol where the delipidation process was performed twice was also attempted. However, this double delipidation yielded few peptide identifications after analysis and was deemed unsatisfactory.

Mucins are present in varying amounts in human bile. They are large glycoproteins that are difficult to denature, due to their abundant glycosylations[42]. In this study, some of the chemical reagents used in the original FASP protocol were substituted with others, e.g. the use of GuHCl instead of urea. GuHCl is a stronger protein denaturant than urea, with a general effectiveness of 2 to 2.5-fold that of urea[43]. This property is helpful for preparation of samples containing mucins, in order to ensure denaturation.

Bile also contains bile salts, which were removed by desalting the samples on C_{18} StageTips. Advantages of this method is that the StageTips accommodate large volumes of sample to be loaded and desalted, while a low and concentrated volume of sample can be eluted[20].

The last major alteration to the sample preparation protocol was the change from digestion with only trypsin to double digestion with Lys-C and trypsin. This method has proven advantages over digestion where only trypsin is used, in terms of the number of peptide identifications by mass spectrometric measurements[44].

It was found that the protein concentration of the prepared samples exceeded the maximum capacity of the mass spectrometer, as manifested in the high electrical signal produced during

ion detection. This high protein concentration was probably the culprit in the obstruction of the liquid chromatograph column. Consequently, the prepared samples of bile were diluted by a factor of 10 in 0.2% formic acid. Unfortunately, lowering the peptide concentration in the samples came at the cost of less peptide identifications, particularly of the low-abundance proteins. Because the most abundant proteins in bile number only a handful, while an overwhelming majority of biliary proteins are much less abundant, the high-abundance proteins prevent the identification of these less abundant proteins due to dynamic range issues[15]. In this study, albumin was the most abundant protein in the majority of bile samples, followed by hemoglobin, a blood contaminant. It is possible to use methods such as lectin affinity chromatography or immunoglobulin depletion to remove the high-abundance proteins from bile[15]. Some of the drawbacks of depletion, as summed up by Frantzi et al., are "issues concerning … reproducibility, [the] co-depletion of proteins of interest, [and the] requirement of higher amount of starting material"[45]. Due to time limitations the desalting process was not optimized in this study, but the samples would probably have been less concentrated if a smaller volume of sample had been loaded onto the StageTips. Decreasing this volume is almost certainly preferable over dilution of the final sample, since inaccuracies can be introduced during the dilution process if all samples are not treated equally.

Changes to the Liquid Chromatography Mobile Phase Gradient. In the first method the chemical composition of the mobile phase was changed over time with a linear gradient. The gradient started with 10% mobile phase B, increasing to 30% at 90 min and reaching 100% at 103 min. With a starting concentration of 10% mobile phase B, most of the peptides were eluted during the first 30 min of the method, yielding indistinct peaks in the mass spectra. Next, three different methods were evaluated, all of which had a lower starting concentration of mobile phase B, to allow a more gradual elution of peptides and a better separation of peaks. The second method was 103 min long and started with a 5% concentration of mobile

phase B, increasing slowly to 25% at 95 min and 100% at 103 min. The third method had a similar gradient steepness, but was only 60 min long. The fourth method (Table 2) was 108 min long, with the gradient shaped in a stepwise fashion. The third and fourth methods were compared with each other by analyzing the same four bile samples with each method. The third method yielded an average of 320 protein identifications (range 140-402) and the fourth yielded an average of 341 protein identifications (range 148-447). The fourth method was used for the final large-scale Nano LC-MS/MS analysis of all 37 samples.

Nano LC-MS/MS Analysis. Tandem mass spectrometry is often used for proteomic analysis. It is a fast method of fragmenting large amounts of peptides and determining their peptide sequences with high sensitivity and resolution[46]. It is advantageous to normalize the peptide intensities before LC-MS/MS analysis by spiking all samples with a defined quantity of a peptide with a known peptide sequence as an internal standard[45], to ensure that the protein contents are correctly quantified, and that no systematic differences in protein concentration are introduced during sample preparation. Since the samples were not normalized in this study, this is another potential error source.

Data Analysis. The statistical analyses were performed with either of two non-parametric tests: the Mann-Whitney U-test for comparing two patient groups with each other, and the Kruskal-Wallis test for comparing more than two patient groups with each other. Non-parametric tests were used because the sample sizes of the patient groups were quite small, ranging from 4-15 patients. Further, it was not assumed that the abundance of each tested protein was normally distributed between samples, rendering parametric tests unsuitable for this study.

Multiple comparisons were tested during analysis, so the number of false positive results is expected to be higher than if only a single hypothesis had been tested. This can be corrected

by adjusting the significance level to a lower value, but in this study the significance level was not adjusted, since this is an exploratory study aimed at discovering a few potential biomarker candidates among over 900 identified biliary proteins.

Principal component analysis was used to group the 910 proteins identified with MaxQuant according to their abundance. The analysis did not show clustering according to diagnosis for any of the components. This could be due to large differences between samples in the amount of protein identifications, or how much contamination with blood there was.

Only a very limited number of proteins were selected for non-parametric analyses, and it is possible that the proteins described in the results section of this report are not the best biomarkers for CC, but they are a start. The list that can be found as Table 5 in the Appendix contains many additional interesting proteins, some of which might even be better biomarker candidates than the ones discussed earlier. It is clear that much remains to be studied in the search for biomarkers for CC.

Conclusions

For six of the proteins analyzed with non-parametric tests in this study, the relative abundance in human bile differed depending on the underlying disease of the patient (Table 3). R-PTP-eta was able to differentiate patients with CC (including patients with concurrent PSC) from patients with PSC only, as well as from patients with other benign diseases of the bile ducts (Fig. 2AB). It is expressed by the PTPRJ gene, which is a known tumor suppressor[23]. Mucin-1 was able to differentiate patients with *de novo* CC from patients with CC and concurrent PSC (Fig. 3), which hints at a different pathogenesis for CC depending on if it develops *de novo* or in a patient with PSC. The copper-binding protein Ceruloplasmin was able to differentiate CC from pancreatic cancer (Fig. 6B). None of the biliary proteins described in

this study were able to alone differentiate between a benign and malignant stricture in patients with PSC. The methods used in this study for preparation of bile samples yielded an acceptable amount of protein identifications, but tended to cause obstruction of the liquid chromatograph column. These methods should be optimized further, for example by evaluating the possibility of using even smaller volumes of bile during the desalting process. Further studies specifically targeted at the proteins described in this study, and with larger populations, are warranted to examine if these proteins have potential as clinically useful tumor markers for CC. Perhaps an immunoassay combining some of these proteins could provide an acceptable diagnostic accuracy for determining the etiology of bile duct strictures, even in patients with PSC. Such a fluid-based biomarker assay could be used both for detecting bile duct malignancies in symptomatic patients, and for screening high-risk populations, such as asymptomatic PSC patients.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Gallvägscancer är en relativt ovanlig sjukdom, men är ändå den näst vanligaste typen av levercancer i Sverige: 237 personer insjuknade år 2013. Det är en mycket svårdiagnosticerad sjukdom med hög dödlighet, och i Sverige är den vanligaste riskfaktorn för sjukdomen just primär skleroserande kolangit. I den aktuella studien analyserades galla som samlats in på Sahlgrenska Universitetssjukhuset i Göteborgs Gastroenterologiska avdelning under perioden januari 2014 - januari 2015, från patienter som genomgick en röntgenundersökning som kallas ERCP, där en böjlig slang leds genom munnen och tarmen och slutligen in i gallvägarna, där en röntgenbild av gallvägarna tas efter injektion av kontrastmedel. Sammanlagt analyserades galla från 37 patienter. Patienterna som ingick i studien delades in i olika grupper beroende på vilken sjukdom de hade vid undersökningstillfället. Grupperna var: patienter med gallvägscancer med eller utan samtidig primär skleroserande kolangit, patienter

med cancer i bukspottskörteln, patienter med endast primär skleroserande kolangit, och patienter med andra godartade gallvägstillstånd, t.ex. gallsten. De olika proteinerna som fanns i gallan identifierades och deras mängd uppmättes med hjälp av en teknik som kallas vätskekromatografi med masspektrometri, varefter skillnaden i proteinhalter jämfördes mellan de olika sjukdomsgrupperna med statistiska analysmetoder. I dagsläget finns det inga laboratorieprover för att utesluta eller ställa diagnosen gallvägscancer. Resultaten från denna studien visar att det finns proteiner som möjligen kan fungera som tumörmarkörer i ett sådant laboratorieprov, men det krävs fortsatta studier på dessa proteiner innan ett sådant prov kan användas för rutinmässig diagnostik av gallvägscancer.

REFERENCES

1. Ustundag Y, Bayraktar Y. Cholangiocarcinoma: A compact review of the literature. *World J Gastroenterol*. 2008 Nov; 14(42):6458-6466.

2. Tyson GL, El-Serag HB. Risk factors for cholangiocarcinoma. *Hepatology*. 2011 Jul; 54(1):173–184.

3. Official Statistics of Sweden – Health and Medical Care. Cancer Incidence in Sweden 2013. *Socialstyrelsen*. 2014 Dec.

4. Statistics Sweden. Population by region, marital status, age and sex. Year 1968 - 2014. Retrieved 2015 Apr 28 from http://www.statistikdatabasen.scb.se/pxweb/sv/ssd/START__BE__BE0101__BE0101A/BefolkningNy/?rxid=3f323202-9d47-417b-8c29-90c2e8f9dfe3

5. Patel T. Worldwide trends in mortality from biliary tract malignancies. *BMC Cancer*. 2002 May; 2:10.

6. Serrablo A, Tejedor L. Outcome of surgical resection in Klatskin tumors. *World J Gastrointest Oncol*. 2013 July; 5(7):147-158.

7. Anderson CD, Pinson CW, Berlin J, Chari RS. Diagnosis and Treatment of Cholangiocarcinoma. *The Oncologist*. 2004 Feb; 9(1):43-57.

8. American Cancer Society (2014). Survival statistics for bile duct cancers. Retrieved from http://www.cancer.org/cancer/bileductcancer/detailedguide/bile-duct-cancer-survival-by-stage

9. Crawford JM, Liu C. Liver and Biliary Tract. In: Kumar V, Abbas AK, Fausto N, Aster JC, editors. Robbins and Cotran Pathologic Basis of Disease. 8th ed. Philadelphia, PA: *Saunders Elsevier*. 2010; 834-881.

10. Eaton, JE, Talwalkar JA, Lazaridis KN, Gores GJ, Lindor KD. Pathogenesis of Primary Sclerosing Cholangitis and Advances in Diagnosis and Management. *Gastroenterology*. 2013 Sep; 145(3):521-536.

11. Bergquist A, Ekbom A, Olsson R, Kornfeldt D, Lööf L, Danielsson A, et al. Hepatic and extrahepatic malignancies in primary sclerosing cholangitis. *J Hepatol*. 2002 Mar; 36(3):321-7.

12. Park MS, Kim TK, Kim KW, Park SW, Lee JK, Kim JS, et al. Differentiation of extrahepatic bile duct cholangiocarcinoma from benign stricture: findings at MRCP versus ERCP. *Radiology*. 2004 Oct; 233(1):234-40.

13. Andriulli A, Loperfido S, Napolitano G, Niro G, Valvano MR, Spirito F, et al. Incidence Rates of Post-ERCP Complications: A Systematic Survey of Prospective Studies. *Am J Gastroenterol*. 2007 Aug; 102(8):1781–1788.

14. Furmanczyk PS, Grieco VS, Agoff SN. Biliary brush cytology and the detection of cholangiocarcinoma in primary sclerosing cholangitis: evaluation of specific cytomorphologic features and CA19-9 levels. *Am J Clin Pathol*. 2005 Sep; 124:355-360.

15. Kristiansen TZ, Bunkenborg J, Gronborg M, Molina H, Thuluvath PJ, Argani P. A proteomic analysis of human bile. *Mol Cell Proteomics*. 2004 Jul; 3(7):715-28.

16. Ince AT, Yildiz K, Baysal B, Danalioglu A, Kocaman O, Tozlu M, et al. Roles of serum and biliary CEA, CA19-9, VEGFR3, and TAC in differentiating between malignant and benign biliary obstructions. *Turk J Gastroenterol*. 2014 Apr; 25(2):162-9.

17. Farina A, Dumonceau JM, Lescuyer P. Proteomic Analysis of Human Bile and Potential Applications for Cancer Diagnosis. *Expert Rev Proteomics*. 2009; 6(3):285-301.

18. Burtis CA, Ashwood ER, and Bruns DE, editors. Tietz Fundamentals of Clinical Chemistry. 6th ed. St. Louis, MO: *Saunders Elsevier*. 2008; 112-139.

19. Wisniewski J, Zougman A, Nagaraj N, Mann M. Universal sample preparation method for proteome analysis. *Nature Methods*. 2009 May; 6(5):359-362.

20. Rappsilber J, Ishihama Y, Mann M. Stop and go extraction tips for matrix-assisted laser desorption/ionization, nanoelectrospray, and LC/MS sample pretreatment in proteomics. *Anal Chem*. 2003 Feb; 75(3):663-70.

21. MaxQuant documentation (2014). Frequently Asked Questions. Retrieved 2015 May 16 from http://141.61.102.17/maxquant_doku/doku.php?id=maxquant:faq

22. National Center for Biotechnology Information (US) (2015). PTPRJ protein tyrosine phosphatase, receptor type, J[Homo sapiens]. Retrieved 2015 May 17 from http://www.ncbi.nlm.nih.gov/gene/5795?report=full_report&format=text

23. Ruivenkamp C, Hermsen M, Postma C, Klous A, Baak J, Meijer G, Demant P. LOH of PTPRJ occurs early in colorectal cancer and is associated with chromosomal loss of 18q12–21. *Oncogene*. 2003 May; 22(22):3472–3474.

24. Iuliano R, Palmieri D, He H, Iervolino A, Borbone E, Pallante P, et al. Role of PTPRJ genotype in papillary thyroid carcinoma risk. *Endocr Relat Cancer*. 2010 Dec; 17(4):1001-6.

25. National Center for Biotechnology Information (US) (2015). MUC1 mucin 1, cell surface associated[Homo sapiens]. Retrieved 2015 May 18 from http://www.ncbi.nlm.nih.gov/gene/4582?report=full_report&format=text

26. Tréhoux S, Duchêne B, Jonckheere N, Van Seuningen I. The MUC1 oncomucin regulates pancreatic cancer cell biological properties and chemoresistance. Implication of p42-44 MAPK, Akt, Bcl-2 and MMP13 pathways. *Biochem Biophys Res Commun*. 2015 Jan; 456(3):757-62.

27. Matsuda A, Kuno A, Kawamoto T, Matsuzaki H, Irimura T, Ikehara Y, et al. Wisteria floribunda agglutinin-positive mucin 1 is a sensitive biliary marker for human cholangiocarcinoma. *Hepatology*. 2010 Jul; 52(1):174-82.

28. National Center for Biotechnology Information (US) (2015). CAMP cathelicidin antimicrobial peptide[Homo sapiens]. Retrieved 2015 May 17 from http://www.ncbi.nlm.nih.gov/gene/820?report=full_report&format=text

29. D'Aldebert E, Biyeyeme MJ, Mergey M, Wendum D, Firrincieli D, Coilly A, et al. Bile salts control the antimicrobial peptide cathelicidin through nuclear receptors in the human biliary epithelium. *Gastroenterology*. 2009 Apr; 136(4):1435-43.

30. Xue C, Zhang Z, Yu H, Yu M, Yuan K, Yang T, et al. Up-regulation of CNDP2 facilitates the proliferation of colon cancer. *BMC Gastroenterol*. 2014 May; 14:96.

31. Lee JH, Giovannetti E, Hwang JH, Petrini I, Wang Q, Voortman J, et al. Loss of 18q22.3 involving the carboxypeptidase of glutamate-like gene is associated with poor prognosis in resected pancreatic cancer. *Clin Cancer Res*. 2012 Jan; 18(2):524–533.

32. National Center for Biotechnology Information (US) (2015). CP ceruloplasmin (ferroxidase)[Homo sapiens]. Retrieved 2015 May 17 from http://www.ncbi.nlm.nih.gov/gene/1356?report=full_report&format=text

33. Huster D, Finegold MJ, Morgan CT, Burkhead JL, Nixon R, Vanderwerf SM, et al. Consequences of copper accumulation in the livers of the Atp7b-/- (Wilson disease gene) knockout mice. *Am J Pathol*. 2006 Feb; 168(2):423-34.

34. National Center for Biotechnology Information (US) (2015). GC group-specific component (vitamin D binding protein)[Homo sapiens]. Retrieved 2015 May 17 from http://www.ncbi.nlm.nih.gov/gene/2638?report=full_report&format=text

35. Jorde R, Schirmer H, Wilsgaard T, Mathiesen EB, Njølstad I, Løchen ML, et al. The DBP Phenotype Gc-1f/Gc-1f Is Associated with Reduced Risk of Cancer. The Tromsø Study. *PLOS ONE*. 2015 May; 10(5):e0126359. doi:10.1371/journal.pone.0126359

36. Lankisch TO, Metzger J, Negm AA, Vosskuhl K, Schiffer E, Siwy J, et al. Bile Proteomic Profiles Differentiate Cholangiocarcinoma from Primary Sclerosing Cholangitis and Choledocholithiasis. *Hepatology*. 2011 Mar; 53(3):875-884.

37. Farina A, Dumonceau JM, Antinori P, Annessi-Ramseyer I, Frossard JL, Hochstrasser DF, et. al. Bile carcinoembryonic cell adhesion molecule 6 (CEAM6) as a biomarker of malignant biliary stenoses. *Biochim Biophys Acta*. 2014 May; 1844(5):1018-25.

38. Navaneethan U, Lourdusamy V, Venkatesh PG, Willard B, Sanaka MR, Parsi MA. Bile proteomics for differentiation of malignant from benign biliary strictures: a pilot study. *Gastroenterol Rep*. 2015 May; 3(2):136-43.

39. Hass HG, Nehls O, Jobst J, Frilling A, Vogel U, Kaiser S. Identification of osteopontin as the most consistently over-expressed gene in intrahepatic cholangiocarcinoma: Detection by oligonucleotide microarray and real-time PCR analysis. *World J Gastroenterol*. 2008 April; 14(16):2501-2510.

40. Boschetti E, Righetti PG. Low-Abundance Proteome Discovery: State of the Art and Protocols. Amsterdam: *Elsevier*. 2013 Mar.

41. Castro AR, Morrill WE, Pope V. Lipid removal from human serum samples. *Clin. Diagn. Lab. Immunol*. 2000 Mar; 7(2):197-9.

42. Beeley JG. Glycoprotein and Proteoglycan Techniques. In: Burdon RH. and van Knippenberg PH., editors. Techniques in Biochemistry and Molecular Biology. Vol. 16. Amsterdam: *Elsevier*. 1985; 34.

43. Dempsey CE, Piggot TJ, Mason PE. Dissecting Contributions to the Denaturant Sensitivities of Proteins. *Biochemistry*. 2005; 44:775-781.

44. Wisniewski J, Mann M. Consecutive Proteolytic Digestion in an Enzyme Reactor Increases Depth of Proteomic and Phosphoproteomic Analysis. *Anal Chem*. 2012 Feb; 84:2631−2637.

45. Frantzi M, Bhat A, Latosinska A. Clinical proteomic biomarkers: relevant issues on study design & technical considerations in biomarker development. *Clin Transl Med*. 2014 Mar; 3(1):7.

46. Michalski A, Damoc E, Hauschild JP, Lange O, Wieghaus A, Makarov A, et al. Mass Spectrometry-based Proteomics Using Q Exactive, a High-performance Benchtop Quadrupole Orbitrap Mass Spectrometer. *Mol Cell Proteomics*. 2011 Sep; 10(9):M111.011015.

APPENDIX: TABLES

Table 4.
The amount of protein identifications for each patient.

Diagnosis	Protein identifications (n)
CC	333
CC (Klatskin tumor)	536
CC (distal tumor)	571
CC (Klatskin tumor)	653
CC (Klatskin tumor)	299
CC and PSC (Klatskin tumor)	202
CC and PSC (Klatskin tumor)	487
CC and PSC (Klatskin tumor)	672
CC and PSC (intra-hepatic tumor)	416
PSC	478
PSC	723
PSC	575
PSC	613
PSC	711
PSC	238
PSC	291
Pancreatic cancer	296
Pancreatic cancer	189
Pancreatic cancer	197
Pancreatic cancer	421
Pancreatic cancer	172
Pancreatic cancer	335
Gallstones	389
Gallstones	162
Gallstones	423
Gallstones	253
Gallstones	265
Gallstones	496
Gallstones	346
Gallstones	509
Benign stricture (distal)	160
Benign stricture (hilar)	666
Benign stricture	611
Benign stricture (distal)	155
Cholecystitis	201
Pancreatitis	423
No current hepatobiliary disorder	142

Abbreviations: CC, cholangiocarcinoma; PSC, primary sclerosing cholangitis.

Table 5.
The 50 proteins that showed the greatest difference in relative abundance after t-tests.

Majority protein name	**Gene name**	**Present in (n) samples**	**t-test performed on**	**p-value**
Receptor-type tyrosine-protein phosphatase eta	PTPRJ	17	all CC vs. controls; all CC vs. PSC, PAC, controls; *de novo* CC vs. controls.	0.023; 0.007; 0.026
Thyroxine-binding globulin	SERPINA7	28	all CC vs. controls.	0.033
Ceruloplasmin	CP	37	all CC vs. controls; all CC vs. PAC.	0.038; 0.032
Vitamin D-binding protein	GC	37	all CC vs. controls; all CC vs. PAC.	0.0498; 0.041
Moesin	MSN	17	all CC vs. PSC, PAC, controls.	0.025
Tyrosine-protein phosphatase non-receptor type substrate 1	SIRPA	35	all CC vs. PSC, PAC, controls.	0.036
Porphobilinogen deaminase	HMBS	6	all CC vs. PSC, PAC, controls.	0.039
Cathelicidin antimicrobial peptide	CAMP	23	all CC vs. PSC, PAC, controls; CC-PSC vs. PSC.	0.041; 0.053
Protein IGLV8-61	IGLV8-61	17	all CC vs. PSC, PAC, controls.	0.047
Mucin-5B	MUC5B	36	*de novo* CC vs. CC-PSC.	0.052
Annexin	ANXA2	25	*de novo* CC vs. CC-PSC.	0.054
Hypoxia up-regulated protein 1	HYOU1	9	*de novo* CC vs. CC-PSC.	0.076
Desmocollin-1	DSC1	13	*de novo* CC vs. CC-PSC.	0.081
Complement C5	C5	25	*de novo* CC vs. CC-PSC.	0.091
Mucin-1	MUC1	22	*de novo* CC vs. CC-PSC.	0.106
Osteopontin	SPP1	29	CC-PSC vs. PSC	0.054
Protein IGKV3D-20	IGKV3D-20	30	all CC vs. PAC; *de novo* CC vs. CC-PSC.	0.014
Ig heavy chain V-III region VH26	IGHV3-23	25	all CC vs. PAC; *de novo* CC vs. PAC.	0.017; 0.045
Ig gamma-1 chain C region	IGHG1	37	all CC vs. PAC.	0.021
Protein IGHV4-34/59/61/39	IGHV4-34	36	all CC vs. PAC.	0.024
Protein IGLV4-69	IGLV4-69	28	all CC vs. PAC.	0.025
Hemoglobin subunit zeta	HBZ	26	all CC vs. PAC.	0.027
Ig lambda chain V-III region LOI	-	37	all CC vs. PAC.	0.030
Plastin-2	LCP1	30	all CC vs. PAC.	0.035
Ig heavy chain V-I region EU	-	37	all CC vs. PAC.	0.036
Alpha-1B-glycoprotein	A1BG	37	all CC vs. PAC.	0.038
Profilin-1	PFN1	22	all CC vs. PAC.	0.038

Table 5 (continued).
The 50 proteins that showed the greatest difference in relative abundance after t-tests.

Majority protein name	**Gene name**	**Present in (n) samples**	**t-test performed on**	**p-value**
2-hydroxyacylsphingosine 1-beta-galactosyltransferase	UGT8	25	all CC vs. PAC.	0.040
Ig heavy chain V-II region ARH-77	-	27	all CC vs. PAC.	0.040
Ig lambda chain V-I region NEWM	-	29	all CC vs. PAC.	0.040
Immunoglobulin lambda-like polypeptide 5	IGLL5	37	all CC vs. PAC.	0.045
Beta-2-microglobulin	B2M	22	all CC vs. PAC.	0.043
Ig lambda chain V-I region WAH	-	32	all CC vs. PAC.	0.043
Leucine-rich alpha-2-glycoprotein	LRG1	35	all CC vs. PAC.	0.044
Ig kappa chain V-IV region	IGKV4-1	34	all CC vs. PAC.	0.044
Complement C3	C3	37	all CC vs. PAC.	0.045
Vitronectin	VTN	29	all CC vs. PAC.	0.048
Serotransferrin	TF	37	all CC vs. PAC.	0.048
Ig gamma-3 chain C region	IGHG3	27	all CC vs. PAC.	0.049
UPF0556 protein C19orf10	C19orf10	18	*de novo* CC vs. PAC.	0.008
78 kDa glucose-regulated protein	HSPA5	28	*de novo* CC vs. PAC.	0.024
Cathepsin S	CTSS	21	*de novo* CC vs. PAC.	0.024
Calreticulin	CALR	35	*de novo* CC vs. PAC.	0.037
Keratin, type II cytoskeletal 8	KRT8	34	*de novo* CC vs. PAC.	0.038
Calmodulin	CALM1;CALM2;CALM3	36	*de novo* CC vs. PAC.	0.042
Heat shock 70 kDa protein 1A/1B	HSPA1A	21	*de novo* CC vs. PAC.	0.043
Superoxide dismutase [Mn], mitochondrial	SOD2	36	*de novo* CC vs. PAC.	0.044
Triosephosphate isomerase	TPI1	37	*de novo* CC vs. PAC.	0.044
Cytosolic non-specific dipeptidase	CNDP2	16	*de novo* CC vs. controls.	0.0001
Ras-related C3 botulinum toxin substrate 1;2;3	RAC1;RAC2;RAC3	10	*de novo* CC vs. controls.	0.049

Abbreviations: all CC, all cases of cholangiocarcinoma including those with concurrent PSC; PSC, primary sclerosing cholangitis; PAC, pancreatic cancer; CC-PSC, cholangiocarcinoma with concurrent PSC.