Second year report of the on-going study "A combined bile and urine proteomic test for the detection of cholangiocarcinoma in patients with primary sclerosing cholangitis" supported by PSC Partners

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INTRODUCTION

Detection of cholangiocarcinoma (CC) remains a diagnostic challenge particularly in patients with primary sclerosing cholangitis (PSC) who are at risk for CC development. We recently established diagnostic peptide marker models in bile and urine to detect both local and systemic changes during CC progression by capillary electrophoresis mass spectrometry. In a subsequent case-control phase II study on 87 patients (36 CC including 5 with CC on top of PSC, 33 PSC and 18 other benign disorders) we combined both bile proteome analysis (BPA) and urine proteome analysis (UPA) by logistic regression modelling to a composite bile and urine diagnostic classifier, which we named BPA/UPA-test.

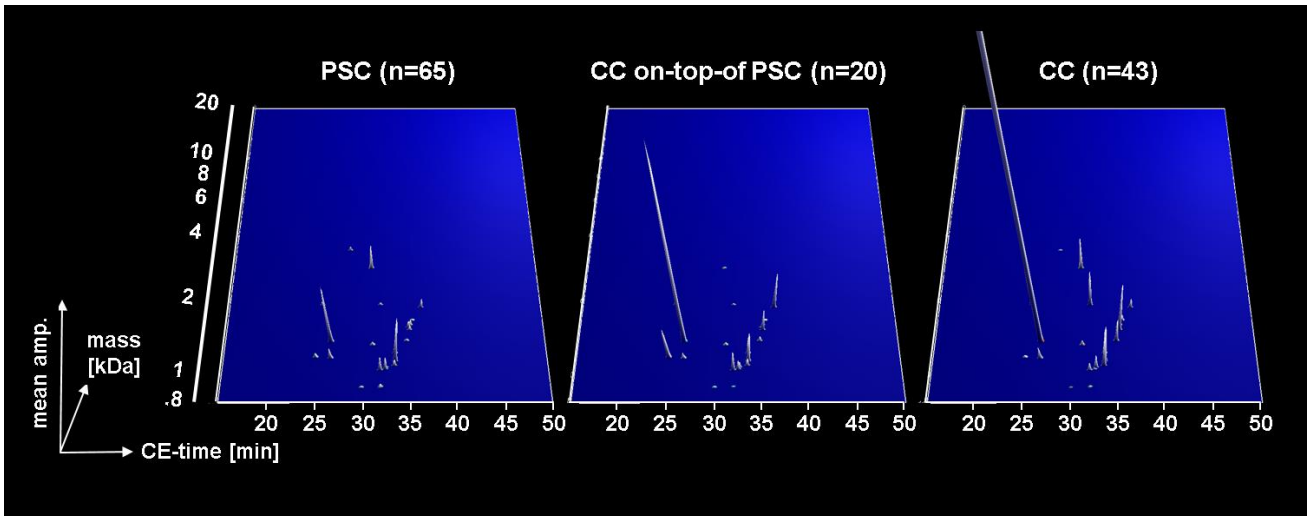
Within this follow-up study financially supported by PSC partners our aim was to validate our BPA/UPA-test, but also the single proteome analyses in bile and urine in a prospective patient set. Furthermore, we wanted to search for additional peptide marker candidates in bile and urine most specific for the differentiation of CC on-top-of PSC from PSC. At the end of this 2-year project the later peptides will be evaluated in respect to their ability to further improve sensitivity of CC detection in PSC patients upon addition to the bile or urine peptide marker models.

RESULTS AND DISCUSSION

During the project phase bile samples from 53 patients and urine samples from 70 patients referred to the gastroenterological department of the Hannover Medical School in Germany were analysed by capillary electrophoresis mass spectrometry. From these patients a definite clinical diagnosis was made for 28 patients. Nineteen patients were diagnosed as PSC, five as CC on-top-of PSC and four as pure CC. All patients are under further clinical surveillance or therapy and new patients will continuously enrolled in this study.

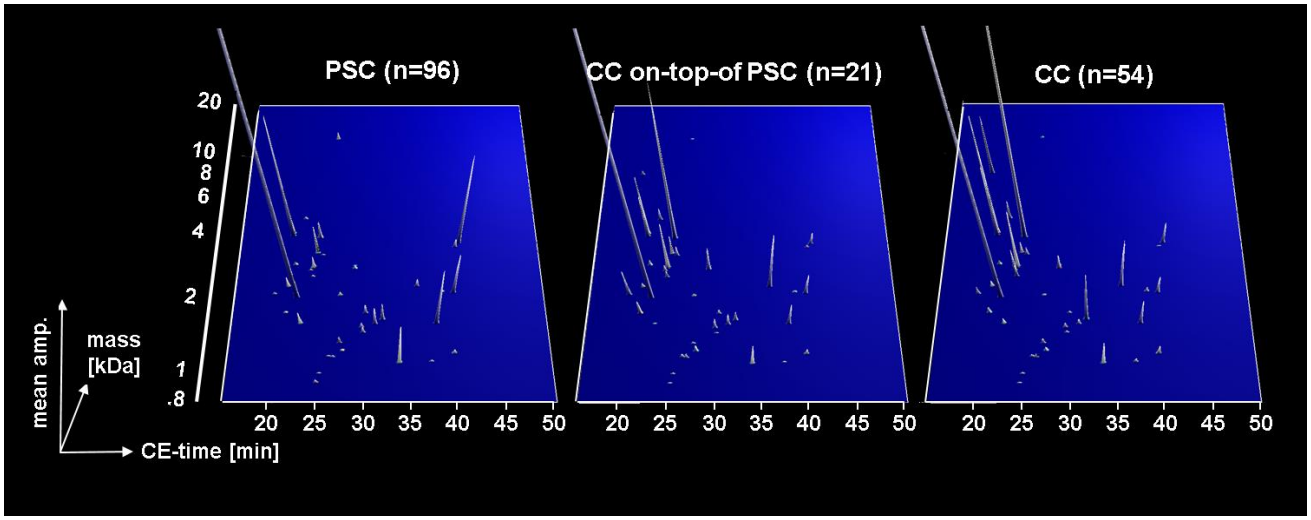
At the current state, we were able to record the group-specific biliary marker profiles of 65 PSC, 43 CC and 20 CC on-top-of PSC patients, all with well established clinical diagnosis. Figure 1 displays the compiled CE-MS profiles of the 22 bile peptides included in the already established bile proteomic model (for details see [1]) for the PSC patients on the left, CC on-top-of PSC patients in the middle and CC patients on the right.

Figure 1. *Group specific marker profiles using the already established pattern for PSC (left), CC on-top-of PSC (middle) and CC (right) in bile proteome analysis (BPA)*



Moreover, we extended our urine proteome analysis to now include CE-MS analyses of 96 PSC, 54 CC and 21 CC on-top-of PSC patients. In figure 2 the complied urine proteome marker specific profiles (as first introduced in [2]) are presented on the left for the PSC, in the middle of for the CC on-top-of PSC and on the right for the CC patients.

Figure 2. *Group specific marker profiles using the already established urinary peptide marker pattern for PSC (left), CC on-top-of PSC (middle) and CC (right) in urine proteome analysis (UPA)*

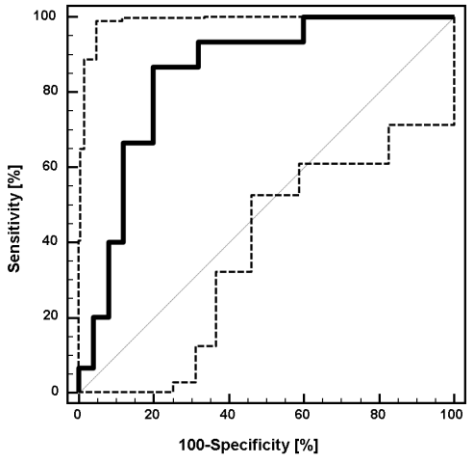


In both bile and urine proteome analysis it became evident, that the peptide marker profiles, also designated as group-specific marker signatures or fingerprints, for the CC on-top-of PSC patients include features of both the pure PSC and the pure CC group and therefore resembles an intermediate marker pattern. However, there are also some markers in CC on-top-of PSC that are regulated differentially compared to pure CC cases. This accounts more strongly for the BPA than for the UPA marker pattern.

Recently, we combined classification by the diagnostic marker sets in bile and urine to a logistic regression function based on the estimated correlation coefficients of 1.83 for bile and 2.64 for urine proteome analysis. Since the correlation coefficients express the change in the logged odds of having CC more weight is given in the logistic regression model to a positive test result in urine than bile proteome analysis. Since the regression model was established using a logistic fitting algorithm to identify the best correlation values, it is mandatory according to recent guidelines for the conduct of clinical proteomic studies to validate a multivariate diagnostic pattern on an independent set of patient samples. This was done for all patients currently enrolled in this prospective study for whom a well-established clinical outcome is already available. This accounts for 15 CC cases including 8 with CC on-top-of PSC and 25 PSC controls. As presented in figure 3, classification by the BPA/UPA logistic regression model resulted in an 'area under the curve' (AUC)-value of 0.85 ($p<0.0001$) and a 95% confidence interval (CI) in the range of 0.70 to 0.94 in 'Receiver Operating Characteristic' (ROC) analysis. At the cut-off of >-0.5 that was predetermined during establishment of the logistic regression model a sensitivity of 87% and a specificity of 84% was detected on this prospective set of

patient samples. However, more patients must be included in ROC analysis for a more accurate and detailed determination of the model's diagnostic performance.

Figure 3. *Receiver operating characteristic (ROC) curve for the classification of the prospective patient cohort consisting of 25 PSC and 15 CC (including 8 with CC on-top-of PSC) patients with the BPA/UPA logistic regression model.*

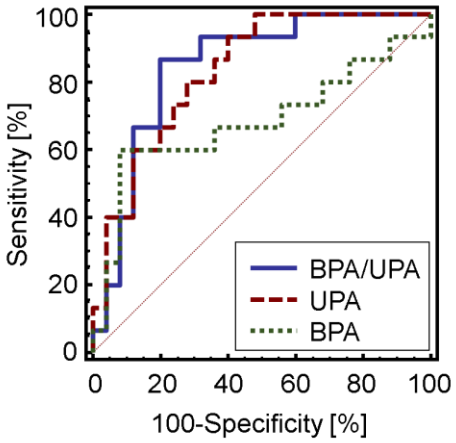


Test variable	Logit score BPA/UPA	
Classification criterion	Clinical diagnosis	
Total number of samples		40
Positive group :	CC on top of PSC (n=8) and CC (n=7)	15
Negative group:	PSC	25

Area under the ROC curve (AUC)	0.85
Standard error	0.06
95% Confidence Interval	0.70 to 0.94
z statistic	5.41
Significance level P (Area=0.5)	<0.0001
Sensitivity/specificity @ >-0.5	87/84 %

As presented in figure 4, the combined BPA/UPA-test resulted in a significant improvement in test accuracy compared to single BPA analysis on the same set of patients (difference in the AUC's of 0.16). In comparison to single UPA analysis the difference in the AUC's is only marginal (0.01), but inclusion of BPA in this case results in a more balanced tradeoff in the sensitivity and specificity of the test (87% versus 81% sensitivity, 85% versus 79% specificity). This makes the combined BPA/UPA-test more favorable over single BPA or UPA in patients with biliary strictures of unknown origin referred to the endoscopic unit.

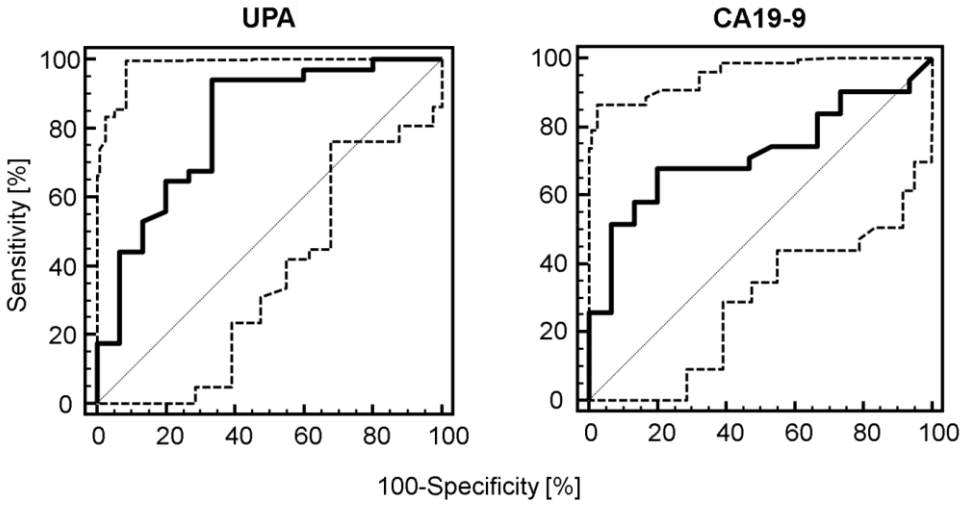
Figure 4. *Comparison of receiver operating characteristic (ROC) curves for classification of the prospective patient cohort consisting of 25 PSC and 15 CC (including 8 with CC on-top-of PSC) patients by the combined BPA/UPA-test and single bile (BPA) or urine (UPA) proteome analysis.*



Group comparison	CC vs.PSC
Sample size (case/control)	40 (15/25)
Area under ROC curve (AUC) / 95% Confidence interval (95% CI)	
Combined BPA/UPA	0.85 / 0.70-0.94
Single UPA	0.84 / 0.68-0.93
Single BPA	0.68 / 0.52-0.82

Besides patients with strictures referred to the gastroenterological department for endoscopic examination, another patient set consisting of those without stenosis referred to the clinic for explorative laparotomy was analyzed by the single UPA test for its better characterization. The 59 patients with CC in this cohort are well characterized in respect to the stage and localization (intra- or extrahepatic) of CC, including those with or without lymph node metastasis and diffuse or focused CC, the later with tumor areas in the range of 1 to 12 cm in diameter as case group. A total of 21 patients with adenoma (n=x) or benign biliary diseases (n=x) matched for the same localization of the disorder within the biliary tract served as appropriate controls. By analyzing this patient set, it became clear that differences of the UPA test exist in the recognition of extrahepatic and intrahepatic forms of CC. As presented in figure 5, analysis of the extrahepatic group including 34 patients with CC and 15 patients with benign biliary diseases resulted in an AUC of 0.82, whereas CA19-9 in this patient set only reached an AUC of 0.72.

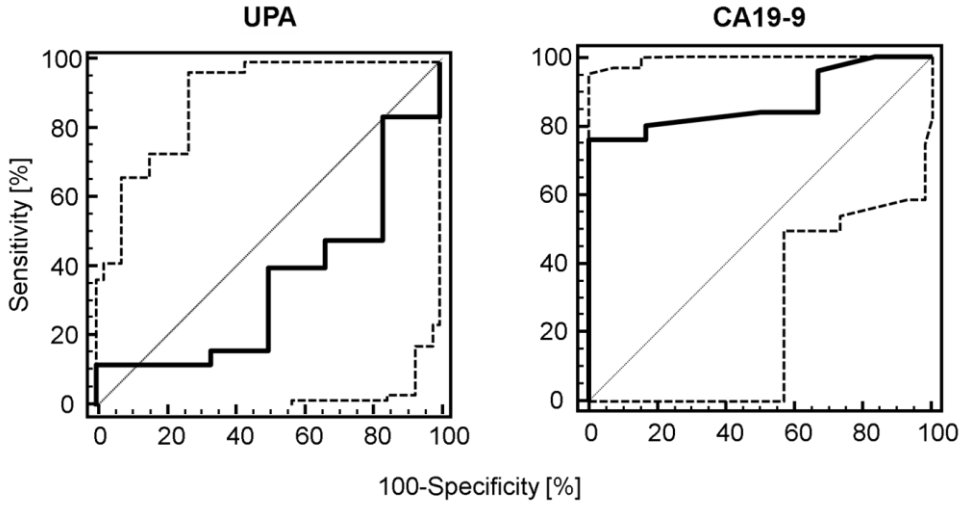
Figure 5. *Receiver operating characteristic (ROC) curve for the diagnosis of extrahepatic CC by non-invasive single UPA and by measurement of CA19-9 serum levels in patients without biliary strictures referred to the clinic for explorative laparotomy due to a suspicious ultrasound and/or magnetresonance imaging result. The patient set consists of 34 extrahepatic CC patients and 15 patients with benign biliary lesions as revealed by histological examination after explorative laparotomy.*



ROC characteristic	UPA	CA19-9
Area under ROC curve	0.82	0.72
95% confidence interval	0.67 to 0.91	0.57 to 0.84
Sensitivity / specificity at cut-off	94 / 67 at -0.98	68 / 80 at 39 U/dL

In contrast, as shown in figure 6, the UPA test failed to detect intrahepatic CC, since classification by UPA results in an AUC below 0.5. In the case of intrahepatic CC, CA19-9 demonstrated a satisfactorily high AUC of 0.87. Due to these results, we postulate that extra- and intrahepatic CC display two different disease etiologies. This is in line with a recent report about CC heterogeneity of Alvaro et al. [3]. In respect to our non-invasive urine test we have now the indication that it is only applicable to extrahepatic, perihilar types of CC. Therefore, the localization of the suspected area in the biliary tract must be previously investigated by ultrasound before proteome analysis can be applied for the differentiation of CC from benign strictures.

Figure 6. *Receiver operating characteristic (ROC) curve for the diagnosis of intrahepatic CC by non-invasive single UPA and by measurement of CA19-9 serum levels in patients without biliary strictures referred to the clinic for explorative laparotomy due to a suspicious ultrasound and/or magnet resonance imaging result. The patient set consists of 25 intrahepatic CC patients and 6 patients with benign biliary lesions as revealed by histological examination after explorative laparotomy.*



ROC characteristic	UPA	CA19-9
Area under ROC curve	0.35	0.87
95% confidence interval	0.11 to 0.60	0.70 to 0.96
Sensitivity / specificity at cut-off	48 / 17 at -0.89	76 / 100 at 30 U/dL

Next, we started to search for additional CC-specific peptides in bile and urine that are especially useful for the discrimination of CC on-top-of PSC from PSC. By inclusion of the 8 CC on-top-of PSC patients enrolled in this prospective trial to the already available patient samples used in our previous studies, a total of 20 biliary and 21 urinary peptide profiles of CC on-top-of PSC patients could be used for this task.

In bile proteome analysis, all available CE-MS profiles were first used to perform a search for new peptide clusters. By applying a frequency threshold of 0.3, which means that a peptide must be detected with 30% frequency in at least one group, 1381 new peptides could be identified and added to the already existing peptide list containing 1439 peptide entities resulting in a total number of 2820 CE-MS-resolved biliary peptide entities. Using this extended list of peptides, a statistical comparison between the CC on-top-of PSC case and PSC control group was performed to validate the previously selected bile peptide marker candidates (see Supplementary table 1 of the previous report) but also to identify new peptide marker candidates. However, no bile peptides could be identified with a significant p-value after false discovery correction. Only peptide 24144 had a p-value of 0.058 and was therefore close to the significance level after false discovery correction by the method of Benjamini and Hochberg [4] if all CC cases were compared to all PSC and non-PSC benign stricture controls (data not shown). If the Wilcoxon rank sum p-values of the peptide markers were used as selection criterion for the comparison of CC on-top-of PSC versus PSC patients, a total number of 47 bile peptides could be selected for further analysis. From these 47 peptide markers presented in table 1, 11 were already identified in the previous statistical comparison. From the 36 newly identified marker candidates 22 were derived from the new peptide clusters (peptide-ID's in table 1 starting from 30000). As presented in table 2, only 10 out of the 47 bile peptide markers showed consistent differences in amplitude signals and distribution frequencies between CC and CC on-top-of PSC cases and PSC and other benign strictures controls. None of these peptide marker candidates is already part of the previously established biliary marker model. Therefore, all ten new biliary peptide markers were selected as candidates to improve our multidimensional classification model for bile proteome analysis.

In comparison to bile, urine proteome analysis revealed a higher number of differentially regulated peptides between CC on-top-of PSC and PSC patients. Comparison of the proteomic patterns of the two patient groups was carried out using 21 CC on-top-of PSC and 96 PSC patients. By applying again a threshold of 30% in peptide frequency, 290 urinary peptides were identified to be differentially regulated in total between PSC with against those without CC progression even after false discovery adjustment by the Benjamini and Hochberg method [4]. The huge list of differentially regulated urinary peptides was further restricted by applying the more stringent false discovery rate-correction methods of Benjamini and Yekutieli [5] and Bonferroni [6]. This resulted in the list of 39 highly significant peptide markers that are presented in table 3 for their CE-MS physicochemical, sequence and intergroup statistical comparison characteristics. From the 39 Bonferroni-adjusted markers presented in table 3, 36 were already identified in the previous statistical comparison with lower case and control samples reported in our first project report. These together with the three additional markers were subsequently analyzed in respect to their distribution frequency in the relevant CC case and PSC as well as other benign biliary stricture control groups. As presented in table 4, 33 out of the 39 markers showed consistent differences in amplitude signals and distribution frequencies between CC and CC on-top-of PSC cases and PSC and other benign strictures controls. Out of these, five were already part of our previously established urinary peptide marker pattern. Therefore, the remaining 28 peptides were selected as new peptide marker candidates to improve the diagnosis of CC by our multidimensional classification model for urine proteome analysis.

Table 1. List of biliary peptides (n=47) with significant Wilcoxon p-value in the comparison of biliary peptide profiles of 65 PSC and 20 CC on-top-of PSC patients.

CE-MS characteristics			Intergroup statistical comparison CC on-top-of PSC -vs- PSC		CE-MS characteristics			Intergroup statistical comparison CC on-top-of PSC -vs- PSC	
Peptide ID	Molecular mass [Dalton]	CE-migration time [min]	Wilcoxon p-value	AUC	Peptide ID	Molecular mass [Dalton]	CE-migration time [min]	Wilcoxon p-value	AUC
1149	932.46	30.77	4.28E-02	0.64	24144	6236.97	23.74	4.70E-03	0.65
1535	958.57	22.78	4.75E-02	0.64	30077	881.52	18.91	1.79E-03	0.64
2175	1004.56	32.09	2.64E-02	0.64	30098	898.51	22.68	1.42E-03	0.64
2891	1056.61	32.39	2.28E-02	0.63	30172	974.58	23.75	3.24E-03	0.65
3457	1094.62	25.28	2.07E-02	0.63	30179	985.64	23.27	9.40E-03	0.62
4079	1143.64	33.11	7.85E-03	0.68	30188	990.58	22.27	3.27E-02	0.61
4100	1145.65	24.48	2.78E-02	0.64	30212	1014.58	24.90	1.57E-02	0.62
4662	1194.65	27.15	1.23E-02	0.65	30220	1024.63	24.52	4.91E-02	0.61
4815	1210.67	33.72	4.86E-02	0.60	30232	1036.59	24.42	2.18E-02	0.62
5072	1240.73	25.95	2.02E-02	0.63	30235	1042.61	23.89	3.08E-02	0.62
6064	1346.70	34.19	1.69E-02	0.64	30253	1065.59	32.54	2.76E-02	0.62
6091	1349.71	34.22	4.28E-02	0.62	30282	1102.62	24.73	2.60E-02	0.63
6899	1441.77	35.01	3.15E-02	0.64	30443	1333.69	33.93	2.92E-02	0.62
7417	1501.86	27.96	3.54E-02	0.61	30503	2081.08	36.79	5.69E-04	0.65
7723	1539.85	35.26	4.52E-02	0.62	30716	2575.51	20.40	2.04E-03	0.66
8631	1647.96	23.26	3.64E-03	0.65	30722	2596.55	21.98	3.80E-03	0.65
9222	1721.92	35.86	1.07E-02	0.64	30895	4799.98	28.63	1.30E-02	0.63
10675	1909.03	24.70	4.80E-02	0.61	30918	8288.93	19.97	7.41E-04	0.68
10955	1952.06	31.74	4.28E-02	0.62	30928	1369.71	34.18	1.05E-02	0.62
12746	2215.12	32.12	2.92E-02	0.63	31269	1840.04	30.43	2.28E-02	0.61
12763	2217.17	31.78	1.82E-02	0.64	31286	1852.95	36.31	1.15E-02	0.62
14199	2431.38	27.92	7.94E-03	0.63	31305	1875.98	30.41	3.70E-02	0.62
16014	2725.45	28.49	2.82E-02	0.62	31307	1878.00	36.44	2.70E-02	0.63
16936	2902.65	29.59	2.19E-03	0.65					

Table 2. Group-specific distribution of the 47 biliary peptide markers with significant Wilcoxon p-values for the differentiation of CC on-top-of PSC from PSC. Peptides with consistent regulation of mean amplitude signal and frequency distribution differences between CC on-top-of PSC (n=20) and CC (n=43) as case and PSC (n=65) and non-PSC benign biliary disorders (BBD, n=53) as control groups are marked in bold.

Peptide-ID	Mean Amp (SD) Freq.				Fold change MW/Freq			
	PSC	Non-PSC BBD	CC	CC on-top-of PSC	CC on-top-of PSC -vs- PSC	CC on-top-of PSC -vs- Non-PSC BBD	CC -vs- PSC	CC -vs- Non-PSC BBD
1149	453 (2410) 42	257 (1322) 47	54 (148) 37	161 (207) 70	0.36 / 1.67	0.63 / 1.49	0.12 / 0.88	0.21 / 0.79
1535	145 (336) 45	349 (1123) 47	179 (344) 47	755 (1933) 65	5.21 / 1.44	2.16 / 1.38	1.23 / 1.04	0.51 / 1
2175	115 (415) 32	243 (927) 38	171 (462) 28	269 (483) 55	2.34 / 1.72	1.11 / 1.45	1.49 / 0.88	0.7 / 0.74
2891	**211 (1352) 22**	**201 (616) 34**	**251 (760) 37**	**309 (961) 50**	**1.46 / 2.27**	**1.54 / 1.47**	**1.19 / 1.68**	**1.25 / 1.09**
3457	51 (133) 31	154 (522) 38	343 (788) 42	2 (8) 5	0.04 / 0.16	0.01 / 0.13	6.73 / 1.35	2.23 / 1.11
4079	242 (664) 49	428 (1465) 38	378 (792) 44	72 (299) 15	0.3 / 0.31	0.17 / 0.39	1.56 / 0.9	0.88 / 1.16
4100	210 (671) 28	1564 (5748) 30	683 (2765) 30	1519 (4399) 50	7.23 / 1.79	0.97 / 1.67	3.25 / 1.07	0.44 / 1
4662	175 (363) 38	193 (485) 43	328 (619) 40	9 (35) 10	0.05 / 0.26	0.05 / 0.23	1.87 / 1.05	1.7 / 0.93
4815	19 (91) 15	189 (813) 38	40 (199) 19	46 (96) 35	2.42 / 2.33	0.24 / 0.92	2.11 / 1.27	0.21 / 0.5
5072	29 (108) 17	315 (1156) 30	284 (1170) 23	92 (160) 40	3.17 / 2.35	0.29 / 1.33	9.79 / 1.35	0.9 / 0.77
6064	221 (575) 32	69 (367) 28	410 (1571) 19	13 (56) 5	0.06 / 0.16	0.19 / 0.18	1.86 / 0.59	5.94 / 0.68
6091	1205 (7479) 35	220 (606) 43	334 (835) 37	3 (7) 15	0 / 0.43	0.01 / 0.35	0.28 / 1.06	1.52 / 0.86
6899	214 (629) 43	846 (5246) 43	775 (2504) 49	33 (101) 15	0.15 / 0.35	0.04 / 0.35	3.62 / 1.14	0.92 / 1.14
7417	**154 (1093) 14**	**92 (389) 15**	**224 (931) 16**	**284 (1042) 35**	**1.84 / 2.5**	**3.09 / 2.33**	**1.45 / 1.14**	**2.43 / 1.07**
7723	386 (1143) 34	726 (2026) 36	439 (1057) 30	73 (292) 10	0.19 / 0.29	0.1 / 0.28	1.14 / 0.88	0.6 / 0.83
8631	43 (157) 14	939 (5099) 30	466 (1755) 16	387 (1147) 45	9 / 3.21	0.41 / 1.5	10.84 / 1.14	0.5 / 0.53
9222	202 (1062) 17	88 (290) 30	292 (1325) 30	103 (252) 50	0.51 / 2.94	1.17 / 1.67	1.45 / 1.76	3.32 / 1
10675	**511 (2826) 20**	**125 (741) 17**	**725 (2759) 21**	**6058 (26381) 40**	**11.86 / 2**	**48.46 / 2.35**	**1.42 / 1.05**	**5.8 / 1.24**
10955	302 (1025) 25	411 (1199) 38	1609 (5781) 26	493 (1085) 50	1.63 / 2	1.2 / 1.32	5.33 / 1.04	3.91 / 0.68
12746	**1314 (5990) 35**	**431 (1581) 26**	**119 (498) 12**	**33 (139) 10**	**0.03 / 0.29**	**0.08 / 0.38**	**0.09 / 0.34**	**0.28 / 0.46**
12763	102 (271) 32	290 (800) 30	785 (4979) 21	10 (45) 5	0.1 / 0.16	0.03 / 0.17	7.7 / 0.66	2.71 / 0.7
14199	49 (196) 11	701 (3545) 15	983 (4653) 21	593 (1415) 35	12.1 / 3.18	0.85 / 2.33	20.06 / 1.91	1.4 / 1.4
16014	82 (325) 17	91 (193) 28	230 (965) 19	985 (3344) 40	12.01 / 2.35	10.82 / 1.43	2.8 / 1.12	2.53 / 0.68
16936	24 (112) 11	190 (778) 13	832 (4147) 16	262 (802) 40	10.92 / 3.64	1.38 / 3.08	34.67 / 1.45	4.38 / 1.23
24144	**1227 (6350) 15**	**6463 (41108) 28**	**222942 (822767) 47**	**47308 (202618) 45**	**38.56 / 3**	**7.32 / 1.61**	**181.7 / 3.13**	**34.5 / 1.68**
30077	3 (12) 8	104 (742) 9	8 (32) 7	34 (67) 35	11.33 / 4.38	0.33 / 3.89	2.67 / 0.88	0.08 / 0.78
30098	3 (15) 8	195 (1176) 11	57 (235) 14	84 (184) 35	28 / 4.38	0.43 / 3.18	19 / 1.75	0.29 / 1.27
30172	**13 (49) 14**	**69 (422) 11**	**126 (722) 19**	**298 (591) 40**	**22.92 / 2.86**	**4.32 / 3.64**	**9.69 / 1.36**	**1.83 / 1.73**
30179	79 (359) 9	215 (750) 23	42 (246) 7	172 (551) 35	2.18 / 3.89	0.8 / 1.52	0.53 / 0.78	0.2 / 0.3
30188	**13 (52) 15**	**28 (117) 17**	**43 (129) 28**	**195 (520) 35**	**15 / 2.33**	**6.96 / 2.06**	**3.31 / 1.87**	**1.54 / 1.65**
30212	17 (97) 12	176 (912) 25	83 (268) 16	92 (273) 35	5.41 / 2.92	0.52 / 1.4	4.88 / 1.33	0.47 / 0.64
30220	323 (1238) 17	66 (421) 8	508 (3261) 9	535 (1435) 40	1.66 / 2.35	8.11 / 5	1.57 / 0.53	7.7 / 1.13
30232	59 (342) 17	669 (3587) 30	19 (59) 28	370 (1140) 40	6.27 / 2.35	0.55 / 1.33	0.32 / 1.65	0.03 / 0.93
30235	53 (165) 20	190 (605) 40	84 (234) 33	153 (316) 45	2.89 / 2.25	0.81 / 1.13	1.58 / 1.65	0.44 / 0.83
30253	88 (319) 31	250 (872) 28	198 (827) 26	28 (126) 5	0.32 / 0.16	0.11 / 0.18	2.25 / 0.84	0.79 / 0.93
30282	21 (58) 20	203 (1282) 17	75 (273) 19	94 (216) 45	4.48 / 2.25	0.46 / 2.65	3.57 / 0.95	0.37 / 1.12
30443	77 (270) 18	63 (237) 23	122 (573) 21	325 (695) 40	4.22 / 2.22	5.16 / 1.74	1.58 / 1.17	1.94 / 0.91
30503	8 (47) 6	37 (111) 17	1667 (10779) 16	216 (675) 35	27 / 5.83	5.84 / 2.06	208.38 / 2.67	45.05 / 0.94
30716	200 (1060) 12	80 (251) 17	189 (796) 16	257 (464) 45	1.29 / 3.75	3.21 / 2.65	0.95 / 1.33	2.36 / 0.94
30722	**1525 (11059) 12**	**425 (1866) 19**	**2710 (8816) 28**	**22073 (90661) 40**	**14.47 / 3.33**	**51.94 / 2.11**	**1.78 / 2.33**	**6.38 / 1.47**
30895	263 (1291) 14	753 (2615) 34	107 (411) 16	484 (1165) 40	1.84 / 2.86	0.64 / 1.18	0.41 / 1.14	0.14 / 0.47
30918	100 (360) 14	1541 (6629) 32	1089 (3010) 28	6611 (25488) 50	66.11 / 3.57	4.29 / 1.56	10.89 / 2	0.71 / 0.88
30928	107 (736) 11	202 (548) 25	2002 (8977) 26	142 (374) 35	1.33 / 3.18	0.7 / 1.4	18.71 / 2.36	9.91 / 1.04
31269	32 (116) 12	64 (338) 13	64 (251) 9	661 (2723) 35	20.66 / 2.92	10.33 / 2.69	2 / 0.75	1 / 0.69
31286	**19 (70) 11**	**119 (376) 19**	**645 (2126) 30**	**201 (650) 35**	**10.58 / 3.18**	**1.69 / 1.84**	**33.95 / 2.73**	**5.42 / 1.58**
31305	850 (3240) 32	866 (2556) 28	658 (1746) 30	6 (25) 10	0.01 / 0.31	0.01 / 0.36	0.77 / 0.94	0.76 / 1.07
31307	**80 (245) 23**	**87 (415) 21**	**322 (1022) 28**	**554 (1163) 45**	**6.93 / 1.96**	**6.37 / 2.14**	**4.03 / 1.22**	**3.7 / 1.33**

Table 3. List of urinary peptides (n=39) with significant p-value in the comparison of biliary peptide profiles of 96 PSC and 21 CC on-top-of PSC patients after false discovery correction by the method of Bonferroni [6].

CE-MS characteristics			Intergroup statistical comparison CC on-top-of PSC -vs- PSC				
Peptide ID	Molecular mass [Dalton]	CE-migration time [min]	unadjusted Wilcoxon p-value	AUC	FDR-adj. p-value according to Benjamini and Hochberg [4]	FDR-adj. p-value according to Benjamini and Yekutieli [5]	FDR-adj. p-value according to Bonferroni [6]
21709	1156.61	27.15	2.14E-08	0.72	1.30E-05	1.03E-04	3.38E-05
26163	1226.53	21.02	3.14E-05	0.79	1.27E-03	1.01E-02	4.95E-02
37056	1409.58	22.04	5.04E-06	0.82	3.32E-04	2.63E-03	7.96E-03
37949	1425.59	22.32	9.18E-06	0.81	4.84E-04	3.84E-03	1.45E-02
38752	1438.45	36.76	6.58E-06	0.81	3.90E-04	3.10E-03	1.04E-02
40091	1449.64	21.86	5.23E-07	0.85	1.03E-04	8.20E-04	8.26E-04
48580	1588.71	30.15	4.72E-06	0.82	3.24E-04	2.57E-03	7.45E-03
50212	1613.82	23.99	2.92E-05	0.79	1.21E-03	9.64E-03	4.61E-02
50638	1620.70	22.66	2.38E-06	0.81	2.28E-04	1.81E-03	3.76E-03
50904	1624.55	37.73	9.15E-06	0.81	4.84E-04	3.84E-03	1.45E-02
53216	1654.78	23.13	1.41E-07	0.83	4.99E-05	3.96E-04	2.23E-04
58143	1751.80	31.43	2.27E-06	0.67	2.28E-04	1.81E-03	3.58E-03
61332	1819.80	23.36	2.13E-06	0.83	2.28E-04	1.81E-03	3.36E-03
62146	1838.93	20.91	1.24E-06	0.66	1.95E-04	1.55E-03	1.95E-03
69769	1991.94	22.05	1.55E-05	0.80	6.99E-04	5.55E-03	2.45E-02
70413	2007.95	22.10	1.05E-05	0.81	5.34E-04	4.24E-03	1.66E-02
70896	2019.88	19.75	3.39E-06	0.73	2.55E-04	2.03E-03	5.36E-03
75025	2090.90	19.77	4.49E-06	0.73	3.22E-04	2.56E-03	7.09E-03
75410	2100.01	19.48	2.74E-06	0.71	2.28E-04	1.81E-03	4.33E-03
80817	2203.11	22.00	1.90E-07	0.73	4.99E-05	3.96E-04	2.99E-04
83257	2246.02	26.93	2.59E-06	0.67	2.28E-04	1.81E-03	4.09E-03
85503	2286.12	19.42	1.15E-05	0.67	5.50E-04	4.37E-03	1.82E-02
86426	2306.03	19.53	1.80E-07	0.85	4.99E-05	3.96E-04	2.84E-04
89909	2368.06	34.02	6.67E-06	0.65	3.90E-04	3.10E-03	1.05E-02
98089	2559.18	19.41	1.13E-05	0.80	5.50E-04	4.37E-03	1.79E-02
100537	2603.28	20.07	2.35E-05	0.65	1.00E-03	7.96E-03	3.71E-02
104954	2682.14	22.49	2.90E-06	0.83	2.29E-04	1.82E-03	4.58E-03
105836	2708.26	23.38	8.19E-09	0.69	1.29E-05	1.03E-04	1.29E-05
106195	2716.37	20.19	8.00E-06	0.69	4.51E-04	3.58E-03	1.26E-02
109937	2796.24	28.56	8.72E-07	0.80	1.53E-04	1.22E-03	1.38E-03
112839	2873.33	28.56	2.47E-08	0.72	1.30E-05	1.03E-04	3.90E-05
118597	3021.35	23.42	1.65E-05	0.80	7.25E-04	5.76E-03	2.61E-02
124688	3185.47	25.47	2.40E-06	0.80	2.28E-04	1.81E-03	3.79E-03
126982	3256.53	33.03	2.04E-06	0.83	2.28E-04	1.81E-03	3.23E-03
128811	3307.50	26.03	1.22E-05	0.67	5.67E-04	4.51E-03	1.93E-02
130661	3356.52	22.03	6.67E-06	0.65	3.90E-04	3.10E-03	1.05E-02
140112	3657.67	40.71	3.33E-07	0.86	7.51E-05	5.97E-04	5.26E-04
146614	3927.69	19.75	2.39E-06	0.71	2.28E-04	1.81E-03	3.78E-03
176869	5921.67	20.47	2.75E-06	0.69	2.28E-04	1.81E-03	4.34E-03

Table 4. Group-specific distribution of the 39 urinary peptide markers with significant p-values for the differentiation of CC on-top-of PSC from PSC after false discovery correction by the method of Bonferroni [6]. Peptides with consistent regulation of mean amplitude signal and frequency distribution differences between CC on-top-of PSC (n=21) and CC (n=54) as case and PSC (n=96) and non-PSC benign biliary disorders (BBD, n=52) as control groups are marked in bold.

Peptide-ID	Mean Amp (SD) Freq.				Fold change MW/Freq			
	PSC	Non-PSC BBD	CC	CC on-top-of PSC	CC on-top-of PSC -vs- PSC	CC on-top-of PSC -vs- Non-PSC BBD	CC -vs- PSC	CC -vs- Non-PSC BBD
21709	**8 (48) 4**	**94 (518) 12**	**67 (142) 28**	**205 (387) 48**	**25.63 / 12**	**2.18 / 4**	**8.38 / 7**	**0.71 / 2.33**
26163	**482 (449) 90**	**306 (338) 81**	**284 (516) 63**	**197 (440) 48**	**0.41 / 0.53**	**0.64 / 0.59**	**0.59 / 0.7**	**0.93 / 0.78**
37056	**10924 (5016) 100**	**9422 (6085) 100**	**6801 (7595) 100**	**5556 (3699) 100**	**0.51 / 1**	**0.59 / 1**	**0.62 / 1**	**0.72 / 1**
37949	**3324 (1722) 98**	**3213 (2588) 96**	**1620 (2078) 85**	**1509 (1460) 90**	**0.45 / 0.92**	**0.47 / 0.94**	**0.49 / 0.87**	**0.5 / 0.89**
38752	**7304 (5339) 100**	**5801 (6235) 88**	**2952 (3401) 87**	**2724 (3964) 67**	**0.37 / 0.67**	**0.47 / 0.76**	**0.4 / 0.87**	**0.51 / 0.99**
40091	**6743 (3859) 100**	**4849 (3108) 96**	**2846 (3518) 94**	**2693 (1937) 95**	**0.4 / 0.95**	**0.56 / 0.99**	**0.42 / 0.94**	**0.59 / 0.98**
48580	**749 (734) 86**	**433 (680) 69**	**195 (375) 44**	**191 (472) 29**	**0.26 / 0.34**	**0.44 / 0.42**	**0.26 / 0.51**	**0.45 / 0.64**
50212	**180 (150) 83**	**215 (857) 63**	**41 (89) 28**	**53 (104) 33**	**0.29 / 0.4**	**0.25 / 0.52**	**0.23 / 0.34**	**0.19 / 0.44**
50638	**313 (362) 67**	**174 (235) 54**	**131 (322) 28**	**13 (61) 5**	**0.04 / 0.07**	**0.07 / 0.09**	**0.42 / 0.42**	**0.75 / 0.52**
50904	**1440 (929) 99**	**1117 (1313) 77**	**651 (1297) 57**	**580 (803) 62**	**0.4 / 0.63**	**0.52 / 0.81**	**0.45 / 0.58**	**0.58 / 0.74**
53216	**157 (425) 30**	**230 (545) 40**	**1676 (2626) 74**	**1079 (1317) 86**	**6.87 / 2.87**	**4.69 / 2.15**	**10.68 / 2.47**	**7.29 / 1.85**
58143	**6 (32) 4**	**60 (157) 21**	**141 (303) 33**	**258 (526) 38**	**43 / 9.5**	**4.3 / 1.81**	**23.5 / 8.25**	**2.35 / 1.57**
61332	**5824 (2752) 100**	**4162 (2433) 98**	**3790 (3814) 100**	**2680 (2052) 95**	**0.46 / 0.95**	**0.64 / 0.97**	**0.65 / 1**	**0.91 / 1.02**
62146	5 (35) 2	189 (934) 10	75 (238) 20	167 (408) 33	33.4 / 16.5	0.88 / 3.3	15 / 10	0.4 / 2
69769	**1723 (1634) 98**	**1732 (1432) 98**	**4241 (4180) 96**	**5972 (5821) 100**	**3.47 / 1.02**	**3.45 / 1.02**	**2.46 / 0.98**	**2.45 / 0.98**
70413	**3115 (2242) 98**	**3403 (3715) 98**	**7128 (6075) 100**	**9412 (9663) 100**	**3.02 / 1.02**	**2.77 / 1.02**	**2.29 / 1.02**	**2.09 / 1.02**
70896	**25 (89) 13**	**8 (36) 10**	**73 (206) 35**	**146 (207) 57**	**5.84 / 4.38**	**18.25 / 5.7**	**2.92 / 2.69**	**9.13 / 3.5**
75025	**95 (467) 14**	**51 (146) 23**	**487 (1065) 56**	**1132 (2911) 57**	**11.92 / 4.07**	**22.2 / 2.48**	**5.13 / 4**	**9.55 / 2.43**
75410	**21 (125) 9**	**13 (79) 6**	**138 (359) 30**	**910 (2047) 48**	**43.33 / 5.33**	**70 / 8**	**6.57 / 3.33**	**10.62 / 5**
80817	18 (94) 7	235 (1128) 15	130 (328) 24	348 (874) 52	19.33 / 7.43	1.48 / 3.47	7.22 / 3.43	0.55 / 1.6
83257	**17 (100) 4**	**6 (39) 4**	**81 (266) 11**	**820 (1886) 38**	**48.24 / 9.5**	**136.67 / 9.5**	**4.76 / 2.75**	**13.5 / 2.75**
85503	**36 (258) 5**	**66 (307) 13**	**491 (1869) 35**	**705 (2039) 38**	**19.58 / 7.6**	**10.68 / 2.92**	**13.64 / 7**	**7.44 / 2.69**
86426	**1066 (2493) 55**	**874 (1406) 54**	**5884 (10240) 80**	**8667 (12193) 95**	**8.13 / 1.73**	**9.92 / 1.76**	**5.52 / 1.45**	**6.73 / 1.48**
89909	4 (24) 3	1327 (9362) 12	1645 (10460) 24	119 (322) 33	29.75 / 11	0.09 / 2.75	411.25 / 8	1.24 / 2
98089	**4851 (14326) 67**	**4285 (15821) 46**	**21377 (39474) 81**	**24892 (44084) 90**	**5.13 / 1.34**	**5.81 / 1.96**	**4.41 / 1.21**	**4.99 / 1.76**
100537	32 (217) 4	7420 (50945) 12	20978 (78931) 31	1631 (4958) 33	50.97 / 8.25	0.22 / 2.75	655.56 / 7.75	2.83 / 2.58
104954	**1089 (619) 98**	**815 (647) 96**	**593 (737) 72**	**412 (406) 67**	**0.38 / 0.68**	**0.51 / 0.7**	**0.54 / 0.73**	**0.73 / 0.75**
105836	**1 (11) 1**	**68 (179) 19**	**162 (353) 28**	**220 (377) 38**	**220 / 38**	**3.24 / 2**	**162 / 28**	**2.38 / 1.47**
106195	43 (191) 7	55288 (380476) 19	74697 (278117) 41	8851 (18995) 43	205.84 / 6.14	0.16 / 2.26	1737.14 / 5.86	1.35 / 2.16
109937	**61 (169) 29**	**53 (121) 27**	**340 (539) 57**	**569 (827) 81**	**9.33 / 2.79**	**10.74 / 3**	**5.57 / 1.97**	**6.42 / 2.11**
112839	**7 (54) 4**	**6 (23) 8**	**94 (267) 22**	**103 (182) 48**	**14.71 / 12**	**17.17 / 6**	**13.43 / 5.5**	**15.67 / 2.75**
118597	**1545 (1421) 85**	**1324 (1327) 83**	**592 (949) 54**	**384 (504) 48**	**0.25 / 0.56**	**0.29 / 0.58**	**0.38 / 0.64**	**0.45 / 0.65**
124688	**117 (293) 34**	**133 (426) 38**	**960 (1968) 65**	**1103 (1865) 81**	**9.43 / 2.38**	**8.29 / 2.13**	**8.21 / 1.91**	**7.22 / 1.71**
126982	**2745 (3595) 86**	**1656 (2411) 63**	**1026 (2050) 59**	**624 (2105) 38**	**0.23 / 0.44**	**0.38 / 0.6**	**0.37 / 0.69**	**0.62 / 0.94**
128811	**10 (56) 5**	**81 (196) 27**	**260 (737) 35**	**235 (561) 38**	**23.5 / 7.6**	**2.9 / 1.41**	**26 / 7**	**3.21 / 1.3**
130661	28 (208) 3	1716 (11632) 21	2936 (10083) 31	510 (1170) 33	18.21 / 11	0.3 / 1.57	104.86 / 10.33	1.71 / 1.48
140112	**1961 (2218) 90**	**879 (1291) 67**	**509 (1221) 35**	**280 (613) 33**	**0.14 / 0.37**	**0.32 / 0.49**	**0.26 / 0.39**	**0.58 / 0.52**
146614	**92 (392) 8**	**29 (89) 13**	**543 (2058) 24**	**331 (893) 52**	**3.6 / 6.5**	**11.41 / 4**	**5.9 / 3**	**18.72 / 1.85**
176869	**21 (96) 6**	**418 (1458) 13**	**5799 (22432) 26**	**699 (1399) 43**	**33.29 / 7.17**	**1.67 / 3.31**	**276.14 / 4.33**	**13.87 / 2**

Conclusions and future plans

Combined proteomic analysis in bile for local and urine for systemic changes during CC progression in PSC patients was successfully validated. Classification of 25 PSC, 8 CC on-top-of PSC and 7 CC prospectively collected patients during this project by a logistic regression combination of bile and urine classification scores resulted in an area under the receiver operating characteristics curve of 0.85. However, more samples are needed to more accurately detect the diagnostic performance of this diagnostic test. Besides that, additional peptide marker candidates could be identified both in bile and in urine specifically for the differentiation of CC on-top-of PSC versus PSC. In the next phase of the study it will be tested if these new marker candidates can improve classification performance of bile and/or urine proteome analysis upon inclusion in the already existing peptide marker panels. At this stage, our results clearly indicate that urine proteome analysis is applicable for the diagnosis of extrahepatic, but not intrahepatic types of CC. For the differentiation of extrahepatic CC from benign biliary strictures, peptide markers for CC progression are more readily detectable in urine than in bile. The latter finding might be attributed to the higher degree of complexity and intersample variability of bile compared to urine. In respect to the dissemination of these important results, a publication will be prepared where we will mention PSC partners as funding partner. Our results were only made possible by your financial support.

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