Liquid biopsy-based protein biomarkers for risk prediction, early diagnosis and prognostication of cholangiocarcinoma

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INTRODUCTION

- **Cholangiocarcinoma (CCA)** includes a heterogeneous group of malignancies with dismal prognosis.
- The etiology is unknown but pathologies such as **primary sclerosis cholangitis (PSC)** increase the odds of CCA development (up to 20% life-time risk).
- There is an urgent need of accurate non-invasive biomarkers for the early diagnosis of CCA, particularly in high-risk populations.
- Extracellular vesicles (EVs), small membranous spheres found in biofluids, have recently emerged as a potential source of biomarkers.

AIM

Identify new accurate non-invasive protein biomarkers in serum EVs to predict CCA development, to early diagnose CCA, as well as to estimate prognosis of patients with CCA.

Understand the potential origin of serum EV biomarkers for their liquid biopsy application.

METHODS

- **Isolation of serum EVs** from patients with isolated PSC (n=45), PSC without clinical evidences of malignancy at sampling who developed CCA overtime (PSC to CCA; n=25), concomitant PSC-CCA (n=42), CCAs from non-PSC etiology (n=56), hepatocellular carcinoma (HCC, n=34) and healthy individuals (n=55).
- EV characterization by transmission electron microscopy (**TEM**), **immunoblot** and nanoparticle tracking analysis (**NTA**).
- Proteomic analysis of EVs by **mass spectrometry (MS)**.
- Evaluation of the diagnostic efficacy of proteins by **receiver operating characteristic** (ROC) curves.
- Validation of the diagnostic capacity of biomarkers in total serum by enzyme-linked immunosorbent assay (**ELISA**)
- Expression analysis of biomarker candidates in **human multi-organ transcriptomes**, in single-cell RNA-sequencing (**scRNA-seq**) of **healthy livers** and in scRNA-seq of **CCA tumors.**
- Evaluation of the use of serum EV protein levels as **survival predictors**.

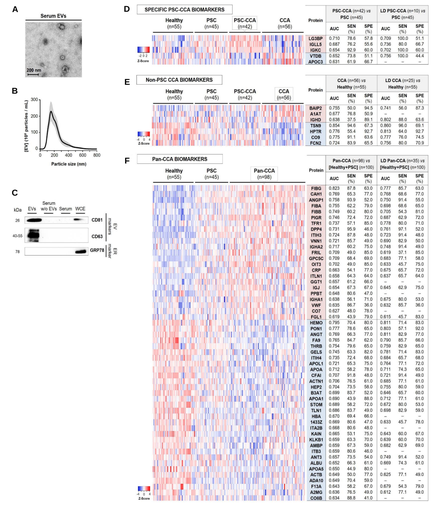
RESULTS

Table 1. *Demographic and clinical features of the study cohort (analyzed by MS)*

		Healthy	PSC	PSC to CCA	PSC-CCA	CCA	HCC
		Orbitrap (n=19)	timsTOF (n=55)	Orbitrap (n=18)	timsTOF (n=45)	timsTOF (n=25)	Orbitrap (n=14)	timsTOF (n=42)	timsTOF (n=56)	timsTOF (n=34)
GENERAL DEMOGRAPHICS										
Age	mean ± SD (years)	**60** ± 6	57 ± 10	**35** ± 13	**38** ± 13	**48** ± 14	**53** ± 12	**49** ± 14	**66** ± 10	**63** ± 9
Sex	Male, n (%)	5 (26.3)	**22** (40.0)	15 (83.3)	**34** (75.6)	**21** (84.0)	10 (71.4)	**28** (66.7)	**40** (71.4)	33 (97.1)
CLINICAL PARAMETERS, n (%)										
IBD	No	-	-	**2** (11.1)	**6** (13.3)	6 (24.0)	0 (0.0)	**8** (19.0)	54 (96.4)	**34** (100)
	UC	-	-	**10** (55.6)	**30** (66.7)	**16** (64.0)	**13** (92.9)	**28** (66.7)	1 (1.8)	0 (0.0)
	Crohn	-	-	3 (16.7)	4 (8.9)	3 (12.0)	0 (0.0)	5 (11.9)	1 (1.8)	0 (0.0)
	Unspecified	-	-	3 (16.7)	5 (11.1)	0 (0)	1 (7.1)	1 (2.4)	0 (0)	0 (0.0)
Cirrhosis	Yes	-	-	0 (0.0)	0 (0.0)	9 (36.0)	1 (7.1)	4 (9.5)	4 (7.1)	**34** (70.6)
	No	-	-	15 (83.3)	**43** (95.6)	**16** (64.0)	**12** (85.7)	**38** (90.5)	**50** (89.3)	8 (23.5)
	NA	-	-	3 (16.7)	2 (4.4)	0 (0.0)	1 (7.1)	0 (0)	2 (3.6)	2 (5.9)
HBV / HCV	Yes	-	-	0 (0)	0 (0)	1 (4.0)	0 (0)	2 (4.8)	3 (5.4)	**26** (58.6)
	No	-	-	**18** (100)	**45** (100)	**24** (96.0)	**14** (100)	**39** (92.9)	**53** (94.6)	**14** (41.2)
	NA	-	-	0 (0)	0 (0)	0 (0)	0 (0)	1 (2.4)	0 (0)	0 (0)
Choledochal cysts	Yes	-	-	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	No	-	-	**18** (100)	**45** (100)	**25** (100)	**14** (100)	**42** (100)	**56** (100)	**34** (100)
	NA	-	-	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Liver fluke	Yes	-	-	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	No	-	-	**18** (100)	**45** (100)	**25** (100)	**14** (100)	**42** (100)	**56** (100)	**34** (100)
	NA	-	-	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
CCA subtype	iCCA	-	-	-	-	6 (24.0)	3 (21.4)	**16** (39.1)	**21** (37.5)	-
	pCCA	-	-	-	-	**15** (60.0)	7 (50.0)	**19** (45.2)	**18** (32.1)	-
	dCCA	-	-	-	-	2 (8.0)	4 (28.6)	3 (7.1)	**17** (30.4)	-
Disease status	LD	-	-	-	-	**7** (28.0)	**9** (23.8)	**25** (44.6)	-	
	LAD	-	-	-	-	**11** (44.0)	7 (50.0)	**15** (35.7)	**11** (19.6)	-
	MD	-	-	-	-	2 (8.0)	2 (14.3)	**8** (19.0)	**20** (35.7)	-
	No	-	-	-	-	4 (16.0)	5 (35.7)	**16** (39.1)	**37** (66.1)	-
CCA-related surgery	Liver transplant	-	-	-	-	**18** (72.0)	6 (42.9)	**8** (19.0)	0 (0.0)	-
	Tumor resection	-	-	-	-	2 (8.0)	3 (21.4)	**17** (40.5)	**19** (33.9)	-
	Exploratory	-	-	-	-	1 (4.0)	0 (0.0)	1 (2.4)	0 (0.0)	-
BIOCHEMICAL PARAMETERS										
ALT (IU/L)	mean ± SD	-	-	**108** ± 85.5	**91.2** ± 68.1	**105** ± 77.6	**129** ± 209	**121** ± 164	**88.8** ± 123.5	**52.6** ± 81.0
	NA, n (%)	-	-	-	-	-	1 (7.1)	2 (4.8)	-	-
AST (IU/L)	mean ± SD	-	-	**69.8** ± 69.3	**60.3** ± 48.9	**92.2** ± 61.8	**140** ± 209	**103** ± 135	**67.7** ± 68.4	**48.3** ± 50.0
	NA, n (%)	-	-	-	-	-	-	1 (2.4)	-	1 (2.9)
GGT (IU/L)	mean ± SD	-	-	**301** ± 234	**281.1** ± 266	**283.6** ± 244.7	**250** ± 249	**320** ± 296	**601.8** ± 899.5	**136.7** ± 247.2
	NA, n (%)	-	-	-	-	-	-	1 (2.4)	-	1 (2.9)
Total bilirubin (mg/dL)	mean ± SD	-	-	**1.0** ± 0.9	**1.2** ± 1.3	**5.2** ± 6.4	**9.4** ± 9.4	**6.28** ± 7.4	**3.0** ± 5.2	**0.64** ± 0.4
	NA, n (%)	-	-	-	-	-	-	-	-	1 (2.9)
ALP (IU/L)	mean ± SD	-	-	**238** ± 161	**236** ± 172	**398.2** ± 255.7	**381** ± 340	**425.5** ± 296	**321.3** ± 373.9	**116.7** ± 99.4
	NA, n (%)	-	-	-	-	-	-	-	-	1 (2.9)
CA19-9 (IU/mL)	mean ± SD	-	-	**31.5** ± 34.6	**27.6** ± 39.8	**187.4** ± 500.5	**1815** ± 3152	**2858** ± 7296	**3037** ± 8230	**21.8** ± 15.0
	NA, n (%)	-	-	3 (16.7)	4 (8.9)	0 (0)	1 (7.1)	2 (4.8)	4 (7.1)	5 (14.7)
AFP (ng/mL)	mean ± SD	-	-	**4.7** ± 4.4	**3.9** ± 3.5	**4.0** ± 4.2	**12.9** ± 21.0	**8.1** ± 15.4	**14.4** ± 37.3	**5.5** ± 5.9
	NA, n (%)	-	-	8 (44.4)	**27** (60.0)	2 (8.0)	2 (14.3)	9 (21.4)	**34** (60.7)	8 (23.5)

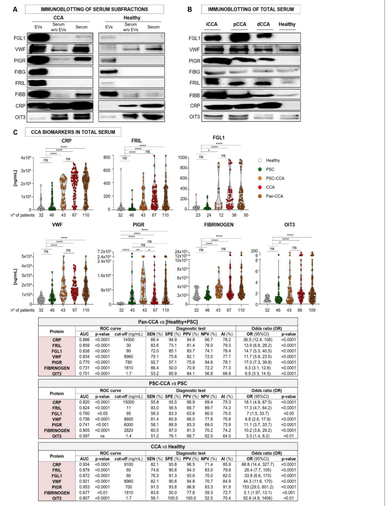
* Healthy (18/55 samples previously analysed in the Orbitrap MS), PSC (18/45 samples analysed also in the Orbitrap MS), PSC-CCA (12/42 samples analysed in the Orbitrap MS)

Figure 1. *Serum EV-protein biomarkers for CCA diagnosis according to tumor etiology*



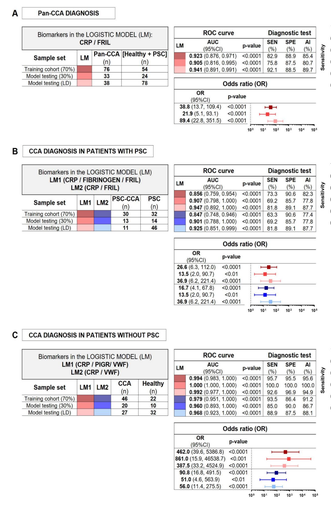
Characterization of serum EVs by (**A**) TEM, (**B**) NTA and (**C**) immunoblotting. Biomarkers for the specific diagnosis of (**D**) CCA in patients with PSC, (**E**) CCA in patients without PSC and (**F**) CCA regardless etiology (Pan-CCA biomarkers). Enriched proteins are colored in red and proteins with lower abundance in blue.

Figure 2. *EV-protein biomarkers are detected using total serum and aid the diagnosis of CCA*



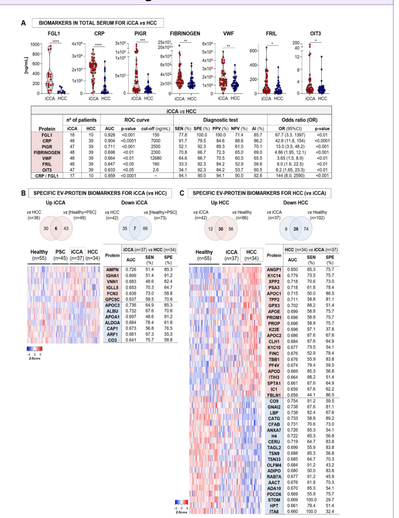
(**A**) Immunoblots of selected biomarkers in serum subfractions of patients with CCA and healthy individuals. (**B**) Immunoblots of biomarkers in total serum of patients with iCCA, pCCA or dCCA and healthy individuals. (**C**) Biomarker levels measured by ELISA in serum samples from patients with PSC, PSC-CCA, non-PSC CCA and healthy individuals and their individual diagnostic values.

Figure 3. *Logistic models combining ELISA-validated serum protein biomarkers for accurate CCA diagnosis*



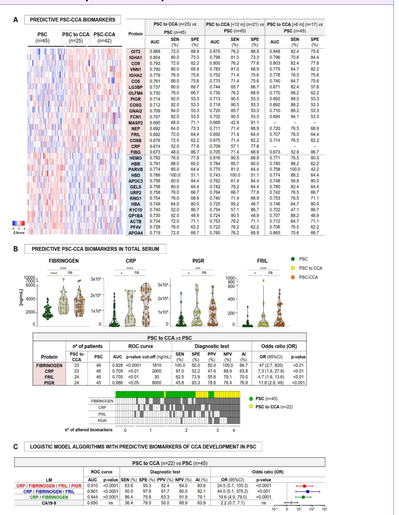
Logistic models combining ELISA-validated serum protein biomarkers enables the accurate diagnosis of CCA in patients with or without PSC. Binary logistic regression models in the training (70%), as well as in testing 30% and local disease (LD) cohorts for CCA diagnosis (**A**) regardless disease etiology, (**B**) in patients with PSC and (**C**) in patients without PSC.

Figure 4. *Serum EV-protein biomarkers for the differential diagnosis of iCCA vs HCC*



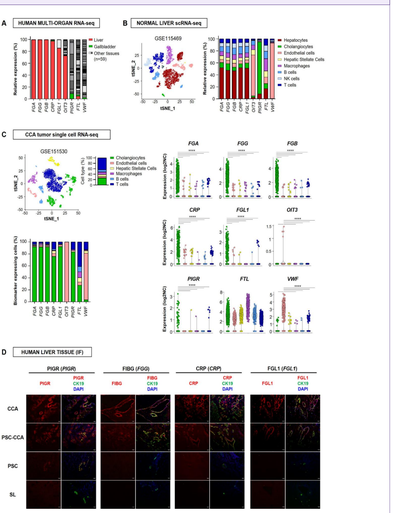
(**A**) Levels and diagnostic values of FGL1, CRP, PIGR, FIBRINOGEN, VWF, FRIL and OIT3 measured by ELISA in serum samples from patients with iCCA and HCC. Heatmaps, Venn diagrams and diagnostic values of specific EV-proteins for the diagnosis of (**B**) iCCA and (**C**) HCC.

Figure 5. *Serum proteins allow the prediction of CCA development in patients with PSC*



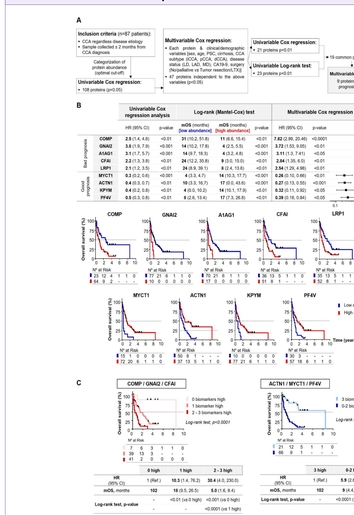
(**A**) Specific EV-proteins for the differential identification of patients with PSC who progressed to CCA over-time (PSC to CCA) and non-malignant PSC. (**B**) Levels and diagnostic values of FIBRINOGEN, CRP, PIGR and FRIL in total serum from PSC to CCA, PSC-CCA patients and non-malignant PSC. (**C**) Binary logistic regression models for the prediction of CCA development in patients with PSC.

Figure 6. *Potential origin of serum EV-protein biomarkers*



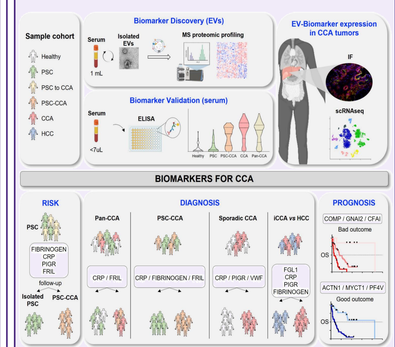
(**A**) Expression of candidate serum biomarkers in 61 human tissues/organs from the Human Protein Atlas. (**B**) tSNE plot and expression of candidate biomarkers in each liver cell type from normal liver scRNA-seq (GSE115469). (**C**) tSNE plot and cell type proportion from iCCA tumors (GSE151530). Biomarker expressing-positive cells and relative expression within iCCA tumor cells. (**D**) Immunofluorescence images of biomarkers and co-localization with CK19+-positive cells.

Figure 7. *Association of serum EV-protein levels with patients' outcome*



(**A**) Strategy used to define prognostic biomarkers. (**B**) Multivariable analysis of serum EV-proteins with independent prognostic value to clinical variables. (**C**) Kaplan Meier curve, Cox regression analysis and Log-rank test of patients with CCA according to the "bad prognostic" (COMP/GNAI2/CFAI) and "good prognostic" (ACTN1/MYCT1/PF4V) panels.

CONCLUSIONS



Serum EVs contain protein biomarkers for:

- the prediction of CCA development in PSC
- the early tumor detection in individuals with PSC, in individuals without PSC and also for CCAs regardless of disease etiology
- the differential diagnosis between iCCA and HCC
- the prognostic estimation of individuals with CCA

Serum EV biomarkers are amenable to be **detected using total serum.**

Most of these candidate biomarkers are preferentially expressed in malignant cholangiocytes within CCA tumors, representing a novel tumor cell-derived **liquid biopsy** for personalized medicine.

DISCLAIMER

The findings presented in this abstract have been recently published as **Liquid biopsy-based protein biomarkers for risk prediction, early diagnosis and prognostication of cholangiocarcinoma** in *Journal of Hepatology* [2023 Mar 1; S0168-8278(23)00159-9. doi: 10.1016/j.jhep.2023.02.027]. We acknowledge and thank the journal for allowing us to present this work at the AMMF 2023 European Cholangiocarcinoma Conference.

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