Synergistic effects of extracellular vesicle phenotyping and AFP in hepatobiliary cancer differentiation

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Abbreviations: Annexin V, Annexin V; AUC, area under the curve; CA 19-9, carbohydrate antigen 19-9; CCA, cholangiocarcinoma; CRC, colorectal carcinoma; ENS-CCA, European Network for the Study of Cholangiocarcinoma; EpCAM, epithelial cell adhesion molecule; ESMO, European Society for Medical Oncology; EVs, extracellular vesicles; FACS, fluorescence-activated cell scanning; FBS, fetal bovine serum; GbCA, gallbladder carcinoma; HCC, hepatocellular carcinoma; ISEV, International Society for Extracellular Vesicles; MISEV, minimal information for studies of extracellular vesicles; nm, nanometer; NPV, negative predictive value; NSCLC, non-small cell lung carcinoma; PPV, positive predictive value; QM, quality management; ROC, receiver operating characteristic; TICs, tumour-initializing cells.

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INTRODUCTION

Biliary tract cancers are considered rare diseases on a worldwide scale, yet incidence rates are rising. Gallbladder cancer (GbCA) and cholangiocellular carcinoma (CCA) are characterized by high mortality rates owing to the tumor’s aggressiveness and lack of early diagnosing possibilities.1,2 Currently, no GbCA or CCA-specific serum, bile, urine or other non-invasive marker is available for reliable early detection, monitoring or screening.3 If diagnosed in time, surgical resection of the gallbladder and bile duct represents the only curative option.4 In most cases, GbCA and CCA progress asymptotically until a metastatic and inoperable stage is reached, resulting in 5-year survival rates of around 5% for GbCA and 20% for CCA.2,5 Despite multiple imaging techniques for staging of biliary tract malignancies, less than 10% of GbCA and only about 50% of CCA are resectable at the time of diagnosis.2

Recently, circulating extracellular vesicles (EVs) have been considered as a minimally invasive screening tool for early cancer diagnosis.6-11 According to the MISEV2018 guidelines, circulating EVs can be classified into small EVs (sEVs), typically with a diameter below 100 nm, and large EVs (lEVs) with typical diameters ranging between 100 and 1000 nm.12 If not specified otherwise the term ‘EVs’ is subsequently used to describe large EVs throughout the manuscript. Essentially, the two types differ in size and mode of cellular release. Whereas small EVs are generated within the endomembranous system of the cell and reside within so-called multi-vesicular bodies before their release, large EVs are shed

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Keypoints

- No reliable diagnostic serum biomarkers for biliary cancer, that are fatal diseases with high mortality rates, are available.
- Extracellular vesicles could be a new clinically relevant serum biomarker for biliary cancer screening/differentiation.
- Combination of extracellular vesicle levels and AFP values enhances the screening/diagnostic capacity for biliary cancer detection.
directly from the plasma membrane of their parental cell.\textsuperscript{13} By isolating circulating EVs from peripheral blood and analysing them by fluorescence-activated cell scanning (FACS), it is possible to create disease-specific EV profiles. Tumour-associated EVs have been investigated in many forms of cancer, that is, glioblastoma and hepatocellular carcinoma (HCC).\textsuperscript{8,34} Therefore, EVs may be considered a novel type of minimally invasive liquid biopsy as highlighted recently by others and our group.\textsuperscript{8,11,15,16}

Considering the fatality of GbCA and CCA that is due to insufficient diagnostic measures, the need for novel early and accurate cancer diagnosis tools is omnipresent.\textsuperscript{16,17} By making use of circulating EV profiling, we aim to find surface antigen combinations for biliary cancer-derived EVs and for EVs associated with the tumour microenvironment that might aid in early diagnosis of GbCA and CCA.

2 | MATERIALS AND METHODS

2.1 | Mice

Animals were obtained from Charles River (Sulzfeld) and housed in pathogen-free conditions in an assigned mouse cabinet (Bioscape) at the Department of Medicine II at Saarland University. All experimental procedures were performed on male 7-9-week-old wildtype C57Bl/6 mice, fed with standard diet, with the approval of the ethics and animal care committee Homburg.

2.2 | Preparation of organ single cell suspensions and FACS measurement

Murine single cells were digested and stained for flow cytometry as described earlier.\textsuperscript{18} Briefly, mouse organs were removed, cut into pieces and enzymatically digested for 60-90 minutes at 37\textdegree{}C. After digestion, cells were collected and red blood cells were lysed in liver and lung using ACK lysis buffer (Life Technologies). Single cell suspensions were counted on a MACSQuant\textsuperscript{®} Analyzer 10 (Miltenyi Biotec). For each staining, 3 x 10\textsuperscript{5} (liver), 1 x 10\textsuperscript{5} (gallbladder), 1 x 10\textsuperscript{5} (colon) or 4 x 10\textsuperscript{5} (lung) single living cells were incubated with antibodies against CD45 (103116, BioLegend), ASGPR1 (AF2755, R&D Systems), EpCAM (118225, BioLegend), CD133 (130-102-210, Miltenyi Biotec), gp38 (127410, BioLegend) and CD44 (130-102-904, Miltenyi Biotec). ASGPR1 was only included for liver, not for other organs. Liver cells were stained with a secondary antibody against goat IgG (A11055, Invitrogen). Detailed information about all applied antibodies can be found in Table S1. All cells were analysed using the MACSQuant Analyzer (Miltenyi Biotec). Cohort sizes within the progenitor cell- and tumour-associated cohorts were eventually not coherent due to flow cytometric measurement errors.

2.3 | Human study cohort

The Ethics commissions of (a) the State Chambers of Medicine in Rhineland-Palatinate, Germany approval number: 837.151.13 (8836-F)); (b) Saarland, Germany (167/11); (c) San Sebastian, Spain (PI2014187); (d) Warsaw, Poland (KB/41/A/2016 and AKB/145/2014) and (e) Cluj-Napoca, Romania (3042/07.03.2018) approved this study. All patients gave their informed consent.

Patients that received chemotherapy or were subjected to any other anti-tumour therapy during the time blood samples were taken were excluded. The characteristics of the patients are summarized in Table 1. GbCA patients who had undergone previous cholecystectomy were excluded from the current study.

2.4 | Isolation of extracellular vesicles and subsequent FACS analysis

Human blood samples were collected in Clotting Activator S-Monocuvettes (7.5 mL, Sarstedt) and were allowed to coagulate at RT for 30-60 minutes. Subsequently, samples were centrifuged for 20 minutes at 1500 g. Isolated serum was collected and stored at -80\textdegree{}C.

All large EV isolation and staining procedures were performed according to previously established and published protocols.\textsuperscript{8,19} Briefly, 1 mL patient serum was successively centrifuged at 2000 g and 20 000 g. Small EVs were isolated using the Total Exosome Isolation Reagent (Invitrogen by Thermo Fisher Scientific) following the manufacturer’s specifications. Isolated EVs were incubated with Annexin V (AnnV)-FITC (130-093-060, Miltenyi Biotec) and were subsequently stained with antibodies against EpCAM (130-097-324), CD133 (130-107-453), gp38 (130-106-954) and CD44v6 (130-111-425, all Miltenyi Biotec). Detailed information about all applied antibodies can be found in Table S2. All samples were analysed using the MACSQuant Analyzer (Miltenyi Biotec). Cohort sizes within the progenitor cell- and tumour-associated cohorts were eventually not coherent due to flow cytometric measurement errors.

2.5 | LC-MS analysis

Details can be obtained from Supporting information.

2.6 | Human cancer cell lines

Information about the used cancer cell lines and details on staining protocols for FACS analysis can be obtained from Supporting information.

2.7 | Nanoparticle tracking analysis

Details can be obtained from Supporting information.
2.8 | Data processing and analysis

FACS data were analysed using FlowJo 10 for MAC OSX (Tree Star Inc). Statistical analysis was performed using GraphPad Prism 5 (GraphPad Software Inc). Figures were created using GraphPad Prism 5 and Adobe Illustrator (Adobe Systems Inc).

2.9 | Statistical analysis

All EV profiles depict the population median with interquartile range (IQR) and whiskers representing 1.5 × IQR according to Tukey. Multiple cohorts (>2) were assessed by Kruskal-Wallis non-parametric tests followed by Dunn's multiple comparison post hoc tests. Each degree of freedom (df) is indicated in the corresponding figure legend. To assess the diagnostic benchmarks of EV populations, we calculated sensitivity, specificity, positive predictive values (PPV) and negative predictive values (NPV), and area under ROC curve (AUC) values. Overall, P < .05 was considered significant. The total experimental strength was calculated with the G*Power program (Heinrich-Heine-Universität Düsseldorf, version 3.1.9.2) for different effects (effect size f: 0.25, 0.45 and 0.65). An α error of 0.05 was assumed. In detail, our validation study (Figure 3A,C) were associated with a test strength of >0.98 (1-β err prob) (f = 0.25, 3 df) each and (Figure 3E,G) with 0.85 (1-β err prob) (f = 0.25, 3 df) each respectively.

3 | RESULTS

3.1 | Selecting potential biomarkers for biliary cancer diagnosis

As published by us 2017 in the Journal of Hepatology, where we provide several useful large EV surface antigen combinations allowing us to differentiate liver tumour entities from other non-hepatic
malignancies, now we had aimed to distinguish hepatobiliary cancer entities from each other, in particular biliary cancers (CCA and GbCA) from HCC. As a starting point we used our published hepatobiliary EV antigen combination consisting of Annexin V, CD133 (Prominin-1), CD326 (EpCAM) but minus ASGPR1. Furthermore, we added CD44v6 to the combination, acknowledging several reports that had indicated that CCA might be associated with CD44v6 expression. Besides quantifying EVs derived directly form biliary cancer cells, we addressed the question if EVs shed from podoplanin+ (gp38) liver progenitor cells, as published by us, could indirectly indicate biliary cancer presence.

Podoplanin (gp38) and Prominin-1 (CD133) are both transmembrane glycoproteins that are typically expressed on progenitor cells in the liver tumour microenvironment, whereas CD44 variant 6 (CD44v6) and epithelial cell adhesion molecule (EpCAM, CD326) are both transmembrane cell adhesion proteins that can be found on the surface of various carcinomas.

First, we compared in vitro expression levels of the selected markers EpCAM, CD44v6, CD133 and gp38 by FACS on CCA tumour cell lines (TFK-1, EGI-1 and CCC-5) and HCC tumour cell lines (HuH7, HepG2 and Hep3B), addressing the question if our selection of surface EV antigens could separate CCA from HCC at the cellular level (Figure 1A, B). Unfortunately, to our knowledge no GbCA cell line is commercially available. Gating strategy, performance of antibodies and their corresponding isotype controls are depicted in Figure S2. The expression profiles of the three tumour cell lines within each cohort (HCC or CCA) were similar, with EpCAM being universally present on all investigated tumour cell lines in relatively high levels (Figure 1A). Gp38 could not be detected on any tumour cell line, which is in agreement with available data since it is rather expressed on cancer stem cells and several types of squamous cell carcinomas, malignant mesothelioma and brain tumours. So far no expression of gp38 was reported on HCC cell lines. With exception of the CCA tumour cell line EGI-1, high CD133 expression was almost exclusively limited to HCC cell lines. CD133 was significantly (P ≤ .001) higher expressed by 6.6-fold in HCC than CCA cell lines (HCC: mean of 92% ± 3.625 SEM and CCA: mean of 14% ± 6.665 SEM). On the contrary, all three CCA cell lines expressed CD44v6 in high levels, CD44v6 expression was significantly (P ≤ .001) elevated in CCA cell lines by 58.5-fold (HCC: mean of 1.3% ± 0.223 SEM and CCA: mean of 76% ± 5.117 SEM, respectively), whereas no expression on HCC cell lines could be detected arguing strongly for being a suitable biliary cancer antigen that could be very likely utilized in our EV biliary cancer related surface antigen combination. Even though EpCAM was highly expressed on all cell lines, the cohort comparison revealed a significantly (P ≤ .001) lower EpCAM expression on CCA cell lines, with a mean of 99% ± 0.389 SEM and a mean of 96% ± 0.542 SEM on HCC and CCA cells respectively (Figure 1B).

Our in vitro tumour cell line data was supported by already published data that was upon our request analysed for EpCAM, CD133 and CD44 in EVs extracted from CCA tumour cell lines (TFK-1 and EGI-1, each n = 3), additionally complemented by EVs derived from human primary cholangiocytes (NHC; n = 3) and analysed by liquid chromatography–mass spectrometry. Corresponding statistics are provided in Table S3. In detail, EpCAM was detected in EVs derived from tumour cell lines and primary cholangiocytes, but was shown to be significantly (P ≤ .01) more abundant in CCA-derived EVs than in non-malignant primary cell EVs. CD133 was particularly enriched in EVs derived from EGI-1 cells, whereas CD44 was predominantly restricted to TFK-1-derived EVs. Importantly, CD133 and CD44 were less abundant in EVs derived from non-malignant primary cholangiocytes. Unfortunately, it was not possible to analyse any CD44 variants retrospectively.

3.2 Identifying possible parental cell populations expressing the candidate markers

After verifying the presence and differential expression of our candidate markers on malignant cells in vitro, we aimed to identify possible physiological donor cell populations in vivo that express one or more of the markers simultaneously on their surface and could thus be a source for circulating EVs presenting the respective markers. For FACS analysis, wild type C57Bl/6J mouse organs were enzymatically digested to single cell suspensions and subsequently stained with a panel of antibodies (CD45, CD31, ASGPR1 (liver only), CD133, gp38, EpCAM and CD44). Corresponding isotype performances are depicted in Figure S3. The general gating strategy applied to all organs is exemplarily summarized in Figure 1C. In short, after excluding cellular debris, cell clusters, dead cells (PI), nucleated hematopoietic cells (CD45+) and endothelial cells (CD31+) and hepatocytes (ASGPR1+, liver only), mesothelial cells were additionally excluded based on their high gp38 expression profile. Double positive CD133+gp38+ progenitor cells were detected in every organ except for the colon (Figure 1D, upper panel). Additionally, triple positive CD133+gp38+EpCAM+ cells could be found to various degrees in all organs except for the colon (Figure 1D, lower panel). CD44 could clearly be detected in colon but was weakly expressed in liver, gallbladder and lung (Figure 1E, upper panel). Accordingly, double positive CD44+CD133+ cells were rare in all mouse organs with a slightly increased abundance in murine gallbladder cells (Figure 1E, lower panel). In sum, our marker selection comprising the combinations CD133+gp38-, CD133+gp38+EpCAM-, CD44+ and CD44+CD133- were found to be expressed under steady state conditions in wild type mice.

3.3 Quality management (QM) for FACS analysis of EVs

We thoroughly tested the quality of every reagent used for EV analysis and could not detect any accountable contamination (Figure S4A). In agreement with the guidelines provided by the International Society for Extracellular Vesicles (ISEV), typically, fractions of large EVs isolated by centrifugation result in cross-contaminations.
with small EVs and vice versa.\textsuperscript{26} Complying with our QM, we tested the sensitivity of our FACS analysis assessing the numeric effect of a given small EV cross-contamination on our FACS-based large EV phenotypic analysis. In short, staining and FACS measurement parameters including gating strategy as utilized for large EV analysis (see Figure S5A) were applied to serum small EVs (Figure S4B). They were counted and confirmed in size by nanoparticle tracking analysis (NTA) prior to FACS, revealing a median diameter of 87.4 nm (D50), ranging from 35.7 (D10) to 139.8 nm (D90) (Figure S4C). The FACS sensitivity was set and confirmed by an initial number of employed small EVs for FACS measurement of $1.175 \times 10^5$, from which only a total of 130 events were positive for AnnV, an established EV marker, ruling out any substantial influence of small EV cross-contaminations on large EV quantification in this explicit experimental setting (Figure S4B). Large EVs that were employed to conduct the following diagnostic experiments were confirmed in size by NTA and revealed a median diameter of 209.0 nm (D50), ranging from 153.8 (D10) to 323.9 nm (D90) (Figure S4D). Note: small EVs were only used for QM. The whole study is based on large EVs. Thus, if not specified otherwise the term 'EVs' is subsequently used to describe large EVs throughout the manuscript.

3.4 | Explorative study – EVs discriminate biliary cancer from healthy controls

With our selection of surface antigens proven present in vivo, we aimed to confirm their pertinence in a pathophysiologically relevant setting. Serum EVs were isolated from 10 patients with biliary cancer (5 CCA and 5 GbCA) and from 10 healthy controls by differential centrifugation and stained using antibodies against CD133, gp38, EpCAM and CD44v6. All stainings included AnnV, a common EV marker. Stained samples were subsequently analysed by flow cytometry (FACS) using the gating strategy described in Figure S5A. For every combination of surface markers, the gates for each antibody were applied successively (Figure S5B,C). Importantly, all antibodies were titrated against their matching isotype prior use (Figure S5D). Statistical analysis by two-tailed Mann-Whitney U tests revealed that EVs from patients with biliary cancer were significantly elevated as compared to healthy controls in all four investigated EV populations (Figure 2A-D). In detail, AnnV\textsuperscript{CD133}\textsuperscript{gp38} EV levels of biliary cancer patients showed a 7.1-fold increase as compared to healthy donors (P ≤ .01; biliary CA: median 20.9, healthy CTRL: median 3.0) (Figure 2A). AnnV\textsuperscript{EpCAM}\textsuperscript{CD133}\textsuperscript{gp38} EV levels of biliary cancer patients were 5.7-fold increased compared to healthy controls (P ≤ .001; biliary CA: median 13.6, healthy CTRL: median 2.4) (Figure 2B). AnnV\textsuperscript{CD44v6} EV levels of biliary cancer patients showed a 2.5-fold elevation as compared to healthy donors (P ≤ .05; biliary CA: median 93.4, healthy CTRL: median 37.7) (Figure 2C) and AnnV\textsuperscript{CD44v6}\textsuperscript{CD133} EV levels of biliary cancer patients were 2.3-fold elevated compared to healthy controls (P ≤ .01; biliary CA: median 28.4, healthy CTRL: median 12.3) (Figure 2D).

3.5 | Validation study – progenitor cell-associated and tumour-associated EVs for biliary cancer diagnosis

Based on the results of our explorative study we next evaluated EV levels of the four surface antigen combinations on EVs in a large validation study, additionally including several cancer cohorts as negative control, that is, hepatocellular carcinoma (HCC), colorectal carcinoma (CRC) and non-small cell lung carcinoma (NSCLC) and patients with cirrhosis. Patient characteristics can be obtained from Table 1. Sample preparation of patient serum and analysis of EV surface antigens were performed as described in the explorative study. EV levels of the individual cohorts can be obtained from Figure S6. The group analysis between healthy donors, patients with cirrhosis, biliary cancer (GbCA and CCA), HCC and non-biliary cancer (HCC, CRC and NSCLC) entities revealed that EV levels were significantly elevated in biliary cancers as compared to every control group in all four EV populations (Figure 3A,C,E,G). In detail, AnnV\textsuperscript{CD133}\textsuperscript{gp38} EV levels of biliary cancer patients were 3.0-fold increased compared to healthy controls (P ≤ .01; biliary CA: median 24.3, healthy CTRL: median 8.2), 3.2-fold increased compared to HCC subjects (P ≤ .001; HCC: median 7.7) and 3.6-fold increased compared to non-biliary cancer patients (P ≤ .001; non-biliary CA: median 6.7) (Figure 3A). AnnV\textsuperscript{EpCAM}\textsuperscript{CD133}\textsuperscript{gp38} EV levels of biliary cancer
patients showed a 2.2-fold elevation as compared to healthy controls (P ≤ .01) and non-biliary cancer patients (P ≤ .001), respectively (biliary CA: median 12.3, healthy CTRL and non-biliary CA: median 5.5, respectively), and a 1.9-fold elevation as compared to HCC patients (P ≤ .001; HCC: median 6.4) (Figure 3C). AnnV⁺CD44v6⁺ EV levels of biliary cancer patients were 2.2-fold elevated compared to healthy controls (P ≤ .001; biliary CA: median 65.4, healthy CTRL: median 29.5), 2.9-fold elevated compared to HCC subjects (P ≤ .001; HCC: median 22.4) and 2.3-fold elevated compared to non-biliary cancer patients (P ≤ .001; non-biliary CA: median 28.5) (Figure 3E). AnnV⁺CD44v6⁺CD133⁺ EV levels of biliary cancer patients showed a 2.4-fold increase as compared to healthy controls (P ≤ .01; biliary CA: median 23.8, healthy CTRL: median 9.9), a 3.7-fold increase as compared to HCC subjects (P ≤ .001; HCC: median 6.5) and a 4.2-fold increase as compared to non-biliary cancer patients (P ≤ .001; non-biliary CA: median 5.7) (Figure 3G). Considering the clinical importance of differential HCC/CCA diagnosis, ROC curves for all four EV populations were computed showing diagnostic AUC values ranging from 0.68 to 0.81 for biliary CA vs HCC (Figure 3A,C,E,G). We additionally evaluated the potential of EV profiling to differentially diagnose the biliary cancers GbCA and CCA (data not shown) but obtained no discriminatory findings. Furthermore, EV profiling did not yield a significant discrimination between CCAs of intra- or extrahepatic origin (Figure 3B,D,F,H).

**FIGURE 3** Validation study – CD133, gp38, EpCAM and CD44v6 positive extracellular vesicles discriminate biliary cancer from healthy controls. EVs were isolated and characterized by FACS from serum of indicated biliary cancer patients (biliary CA, comprising GbCA and CCA patients) and healthy donors (healthy CTRL). Corresponding gating strategy and isotype controls are provided in Figure S5 and summarized patient characteristics can be found in Table 1. A, AnnV⁺CD133⁺gp38⁺ EV profile for biliary (biliary CA) and non-biliary cancer patients (non-biliary CA) as well as for negative controls (HCC, cirrhosis and healthy CTRL). ‘Biliary CA’ combines GbCA and CCA patients. ‘Non-biliary CA’ comprises the cancer cohorts HCC, CRC and NSCLC. EV values for the individuals cohorts can be found in Figure S6. Data shown represent medians with interquartile range (IQR), whiskers represent 1.5 × IQR (Tukey) with outliers plotted as dots. (A-D) EV profiles for the populations AnnV⁺CD133⁺gp38⁺ (A), AnnV⁺EpCAM⁺CD133⁺gp38⁺ (B), AnnV⁺CD44v6⁺ (C) and AnnV⁺CD44v6⁺CD133⁺ (D) are depicted. Statistical significance was assessed by two-tailed Mann-Whitney U tests with P ≤ .05 considered statistically significant (* = P ≤ .05, ** = P ≤ .01, *** = P ≤ .001).
3.6 | Combining AFP and EV surface screening yields a diagnostically powerful biomarker for biliary cancer diagnosis as compared to HCC

Next, we addressed the question if our antigen combinations could be of diagnostic benefit when combined with other serum tumour markers that are already under investigation, especially in the context of differential HCC and CCA diagnosis. Therefore, we correlated serum AFP values, a serum tumour marker widely investigated in HCC diagnosis and surveillance, with serum EV levels of all four combinations for HCC and biliary cancer patients. Computed r-values (Spearman) ranging from −0.17 to 0.24 for HCC subjects and from −0.13 to 0.08 for biliary cancer patients revealed no significant correlation (P > .05) between the two parameters (Figure 4A). Consequently, AFP and EV levels can be considered as two independent biomarkers. In a following step we evaluated the diagnostic performance of the two markers separately and in a combined approach by calculating sensitivity, specificity and positive and negative predictive values (Table 2). To assess the diagnostic potential of progenitor cell-derived EV populations (AnnV⁺CD133⁺gp38⁺ and AnnV⁺EpCAM⁺CD133⁺gp38⁺ EVs) and tumour-associated EV populations (AnnV⁺CD44v6⁺ and AnnV⁺CD44v6⁺CD133⁺ EVs) for detecting biliary cancers individually, we combined the respective EV populations into two separate cohorts and compared the results to serum AFP levels for each cohort. For combined analysis of AFP and EV populations biliary cancer patients were considered positive if they fulfilled the requirements for at least one of the parameters, for example, AFP below 20 ng/mL or EV levels above the respective cut-off or both, and vice versa for patients with HCC. Twenty ng/mL represents the screening cut-off for HCC surveillance as

**FIGURE 4** Combined analysis of AFP levels and EV profiling reliably discriminates HCC from biliary cancer. A, Correlation between AFP levels and EV counts from different populations for HCC (left panel) or biliary CA patients (right panel) are depicted. Two-tailed Spearman’s correlation (r), P values and cohort sizes (n) are indicated for each population. B, Displayed are AFP values for HCC and biliary CA patients. In the left panel all patients with EV profiles for progenitor cell-associated EV populations (AnnV⁺CD133⁺gp38⁺ and AnnV⁺EpCAM⁺CD133⁺gp38⁺) are included, whereas in the right panel all patients with EV profiles for tumour-associated EV populations (AnnV⁺CD44v6⁺ and AnnV⁺CD44v6⁺CD133⁺) are included. Indicated in red are patients that based on AFP levels are not classified as HCC patients (AFP < 20 ng/mL) but can positively be identified as HCC by AnnV⁺CD133⁺gp38⁺ EVs (left panel, EVs < 10.43) or AnnV⁺CD44v6⁺ EVs (right panel, EVs < 34). Indicated in blue are biliary CA patients that are not classified as such according to AFP levels (AFP > 20 ng/mL) but can be identified as biliary CA by AnnV⁺CD133⁺gp38⁺ EVs (left panel, EVs > 10.43) or AnnV⁺CD44v6⁺ EVs (right panel, EVs > 34). Corresponding diagnostic values can be found in Table 2. Dotted line indicates diagnostic cut-off of 20 ng/mL for AFP. Statistical significance was assessed by two-tailed Mann-Whitney U tests with P ≤ .05 considered statistically significant (* = P ≤ .05, ** = P ≤ .01, *** = P ≤ .001, n.s. = non significant)
TABLE 2  Diagnostic performance of the indicated EV populations individually and combined with AFP in biliary cancers (GbCA and CCA) as compared to HCC. Depicted are diagnostically relevant cut-offs (AFP: ng/mL, EVs: number per 10^4). AUROC, P-value, Cut-off, Sensitivity [%], Specificity [%], PPV [%], NPV [%] values obtained in Table 2 and indicates patients that, based on combined AUROC were not calculated.

| Progenitor cell-associated EVs (biliary CA: n = 73, HCC: n = 59) | AUROC | P-value | Cut-off | Sensitivity [%] | Specificity [%] | PPV [%] | NPV [%] |
|---------------------------------------------------------------|-------|---------|---------|-----------------|----------------|---------|---------|
| AnnV'CD133^gp38^                                            | 0.74  | <.0001  | 10.43   | 72.6            | 59.3           | 68.8    | 63.6    |
| AnnV'EpCAM'CD133^gp38^                                      | 0.68  | <.0001  | 7.83    | 71.2            | 59.3           | 68.4    | 62.5    |
| AFP                                                          | 0.89  | <.0001  | 20.00   | 98.6            | 54.2           | 72.7    | 97.0    |
| AFP and AnnV'CD133^gp38^                                     | *     | *       | 20.00 and 10.43 | 100.0          | 76.3           | 83.9    | 100.0   |
| AFP and AnnV'EpCAM'CD133^gp38^                               | *     | *       | 20.00 and 7.83  | 100.0          | 76.3           | 83.9    | 100.0   |

| Tumor-associated EVs (biliary CA: n = 60, HCC: n = 29) | AUROC | P-value | Cut-off | Sensitivity [%] | Specificity [%] | PPV [%] | NPV [%] |
|-------------------------------------------------------|-------|---------|---------|-----------------|----------------|---------|---------|
| AnnV'CD44v6^                                          | 0.81  | <.0001  | 34.00   | 91.7            | 69.0           | 85.9    | 80.0    |
| AnnV'CD44v6'CD133^                                     | 0.75  | <.0001  | 10.24   | 81.7            | 58.6           | 80.3    | 60.7    |
| AFP                                                    | 0.95  | <.0001  | 20.00   | 98.3            | 79.3           | 90.8    | 95.8    |
| AFP and AnnV'CD44v6^                                    | *     | *       | 20.00 and 34.00 | 100.0          | 100.0          | 100.0   | 100.0   |
| AFP and AnnV'CD44v6'CD133^                               | *     | *       | 20.00 and 10.24 | 100.0          | 96.6           | 98.4    | 100.0   |

recommended by the AASLD and EASL Clinical Practice Guidelines for the Management of Hepatocellular Carcinoma. For biliary cancer diagnosis, progenitor cell-associated EV populations showed sensitivities ranging from 71%-73% and positive predictive values (PPVs) of 68%, respectively, while specificities (59%, respectively) and negative predictive values (NPVs; 63%-64%) were less diagnostically relevant. AFP as a tumour marker by itself achieved very good diagnostic values with 98.6% sensitivity and a NPV of 97%, although lacking in specificity (54.2) and PPV (72.7%). Interestingly, by combining AFP and progenitor cell-associated EV levels, sensitivity and NPV were increased to 100%, respectively, while simultaneously increasing specificity to 76.3% and PPV to 83.9%. In respect to tumour-associated EV populations in biliary cancer diagnosis, they showed a better diagnostic performance than progenitor cell-associated EVs (sensitivities: 81.7%-91.7%, specificities: 58.6%-69.0%, PPVs: 80.3%-85.9%, NPVs: 60.7%-80.0%) and were only slightly surpassed by the diagnostic values for AFP in this cohort (sensitivity: 98.3%, specificity: 79.3%, PPV: 90.8%, NPV: 95.8%). Interestingly, sensitivity and NPV could be increased to 100%, when combining AFP levels with AnnV'CD44v6'CD133^ EVs, while simultaneously specificity and PPV reached very good diagnostic values of 96.9% and 98.4% respectively. Most importantly, sensitivity, specificity, PPV and NPV all achieved 100%, when combining AFP levels with AnnV'CD44v6^ EVs.

In Figure 4B AFP values of HCC and biliary cancer patients are displayed, separated into the two EV population cohorts (progenitor cell- or tumour-associated). It represents an illustration of the diagnostic values obtained in Table 2 and indicates patients that, based on AFP values, could additionally be identified as patients with HCC (red) or as biliary cancer patients (blue) by AnnV'CD133^gp38^ EVs (left panel) and AnnV'CD44v6^ EVs (right panel), thus highlighting the benefit of a combined analysis. Furthermore, we investigated, if combining CA19-9, a proposed tumour marker for bilo-pancreatic cancer diagnosis, and our EV populations in the same manner as with AFP could be of diagnostic benefit but did not obtain better results (data not shown). Additionally, we evaluated if EV levels correlated with TNM stage or extent of metastatic spread of HCC and biliary tumours but did not observe any significant correlations (data not shown).

4 | DISCUSSION

Recently, we showed that a minimally invasive, liquid biopsy-based approach involving large EVs is advantageous for detecting hepatobiliary malignancies, however, without being able to discriminate between them. Here, in this subsequent study the potential of large EVs as a diagnostic biomarker for biliary cancer was investigated, in order to detect and differentiate between those malignancies. Except for ultrasonography (US) in patients suffering from gallstones as a possible indication for a given GbCA risk, early detection presents difficult. Furthermore, GbCA diagnosis often only occurs incidentally during pathological assessment of routine cholecystectomy specimens due to benign diseases such as gallstones. Hence, biliary cancers are highly fatal diseases, characterized by high mortality and poor 5-year survival rates. Therefore, several specialist societies such as ENS-CCA and ESMO are strongly in favour of developing new tools for (early and specific) biliary cancer diagnosis. Podoplanin, alias gp38, is a novel yet not completely understood player in tumour immunology, tumour progression and recurrence besides being a liver progenitor cell marker. Since hepatic progenitor cells are activated in most chronic liver diseases and apparently are associated with hepatic carcinogenesis, increasingly appearing liver progenitor cells during chronic hepatic inflammation...
could potentially reveal the presence of hepatobiliary cancers. These liver progenitor cells were typically identified as double positive for CD133 and podoplanin.\textsuperscript{21} Furthermore, podoplanin is regarded as a potential marker for tumour-initializing cells (TICs) with stem cell-like properties, defined by their self-renewal, differentiation and tumour initiation capacities.\textsuperscript{37}EpCAM is highly overexpressed and associated with various cancer entities in regard to cancer prognosis and cancer targeting.\textsuperscript{38}We reported its feasibility as part of a diagnostic biomarker combination on large EVs.\textsuperscript{8,9,39}Moreover, simultaneous expression of EpCAM and CD133 has been found to be strongly increased in biliary cancer and to be related to progression, invasive and metastatic behaviour and prognosis.\textsuperscript{40}

Since both CD44v6 as well as CD133 are well-established tumour stem cell and cancer markers for gallbladder carcinoma, CCA and other cancers, their single as well as combined expression on EVs was additionally of interest.\textsuperscript{41-44}Antigen expression analyses on CCA cell line-derived EVs and CCA and HCC cell lines supported our hypothesis of gp38, CD133, EpCAM and CD44v6 being suitable markers (Figure 1A,B; Figure S1), hence we tested the indicated EV antigen combination being simultaneously positive for these. The human cancer cell line expression profiles indicated that CD44v6 might be of interest for CCA and HCC differentiation, since increased in biliary cancer and to be related to progression, invasive and metastatic behaviour and prognosis.\textsuperscript{40}

CD44v6 expression profile analysis of CD133, EpCAM and gp38 on EVs were not beneficial (data not shown). With an AUC value of 0.81 AnnV+CD44v6+ EVs were the most powerful biomarker for biliary cancer detection in this study. Our observation that patients with biliary cancer display elevated levels of AnnV+CD44v6+ EVs is consistent with previous findings that demonstrated by immunohistochemistry and real time PCR, that CD44v6 is not detected in healthy gallbladder mucosa, but strongly expressed in GbCA.\textsuperscript{46}High CD44v6+ EV levels in both GbCA and CCA concur with observations that linked increased CD44v6 expression in biliary epithelium of both gallbladder and bile ducts to cancer progression.\textsuperscript{47}We have to note that our selected EV antigens were not capable of distinguishing between intra- and extrahepatic CCA and between GbCA and CCA. This might be due to the fact that there exists a more optimal EV antigen combination for these discriminations, but we doubt that any EV surface antigen or antigen combinations will have the needed sensitivity to differentiate between intra- and extrahepatic CCA. From the surface antigenic view we do not expect any differences caused by a different location of the primary CCA tumour. We suppose that intra-vascular differences on protein, mRNA or miRNA levels might be noticeable due to an environment depending interaction of the EV donor CCA cells.

Next the question arose, if our EV-based phenotyping could be improved and if synergistic effects could be observed by taking advantage of other serological screening markers such as AFP (Figure 4). AFP is a widely investigated serum tumour marker for HCC, whose use for diagnostic purposes is not recommended by the AASLD and EASL Clinical Practice Guidelines for the Management of Hepatocellular Carcinoma, whereas it has proven beneficial for HCC surveillance at a cut-off of 20 ng/mL.\textsuperscript{27,28}In contrast to the recommendations of the AASLD/EASL guidelines, serum AFP levels by itself, at a cut-off of 20 ng/mL, showed a diagnostic capacity for differentially diagnosing HCC and biliary cancers in this study, surpassing the performance of our investigated EV populations (Table 2). However, importantly, the diagnostic performance of AFP could be enhanced, when combined with EV levels, especially when combining it with the tumour-associated EV populations AnnV+CD44v6+ and AnnV+CD44v6+CD133+. Remarkably, the combination of AnnV+CD44v6+ EVs and AFP values led to a perfect separation of biliary cancer and HCC patients, with sensitivity, specificity, PPV and NPV all achieving 100%. Biliary cancers are commonly associated with low AFP levels. Except for one patient, this observation was confirmed in our study. The patient in question might display a mixed hepatocellular cholangiocarcinoma, which would explain the slightly elevated AFP levels. According to the AASLD and EASL Clinical Practice Guidelines HCCs cannot reliably be detected by AFP values alone,\textsuperscript{27,28}which was confirmed in our study. However, depending on the EV population added to the analysis, all or almost all previously undetected HCCs with low AFP values could be correctly diagnosed. Thus, our synergistic approach illustrates the benefit of adding EV levels to AFP-based diagnosis. It might have particular clinical relevance for differential hepatobiliary cancer detection and should be followed up by a large multi-centre study. A current alternative serum biomarker for biliary cancer diagnosis as proposed by the ESMO Clinical Practice Guidelines is CA 19-9\textsuperscript{29}but it is associated with low sensitivity and specificity of 62% and 63%,
early cancer screening as discussed by others and us.\textsuperscript{9,16,49} Therefore, EV profiling represents a potent tool for its performance quickly without special need for long medical observation afterwards. Thus, EV profiling represents a potent tool for is performed quickly without special need for long medical observation. It requires minimum equipment and sample is required. For most patients, drawing blood is an uncritical and acceptable procedure. It requires minimum equipment and is performed quickly without special need for long medical observation afterwards. Therefore, EV profiling represents a potent tool for early cancer screening as discussed by others and us.\textsuperscript{9,16,49}

5 | CONCLUSION

In summary, our study provides valuable data arguing that EV phenotyping together with AFP assessment is a powerful diagnostic biomarker in detection and differentiation of hepatobiliary cancers. We presented four EV surface antigen combinations that confidently differentiated between patients with biliary cancer and HCC and whose performance could even be enhanced by combined AFP measurements. Considering the results of this and other studies, liquid biopsy-based differential diagnosis of hepatobiliary cancers might be in reach.

ETHICS APPROVAL AND CONSENT OF PARTICIPATION

The Ethics commissions of (i) the State Chambers of Medicine in Rhineland-Palatinate, Germany; many approval numbers: 837.151.13 (8836-F); (ii) Saarland, Germany (167/11); (iii) San Sebastian, Spain (PI2014187); (iv) Warsaw, Poland (KB/41/A/2016 and AKB/145/2014) and (v) Cluj-Napoca, Romania (3042/07.03.2018) approved this study. All patients gave their informed consent.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interests.

AUTHORS’ CONTRIBUTIONS

M.Ko contributed to conceptualization and supervision. SKU, HJ-H., HS and M.Ko contributed to methodology. M.Kr, A.Wi., JL, MA, TM, CK, BK, KJ, WP, Marek K., KZ, WH, Ł.K., MR, KG, WW, RK, JR-W., AS, RS, A.W6., MG-C., SG, ZS, JMB, CPS, FL, PM and M.Ko contributed to resources. SKU, HS, HJ-H. and M.A contributed to investigation. SKU, HS, M.Kr, MA HJ-H. and M.Ko contributed to formal analysis. M.Ko contributed to project administration. M.Ko contributed to funding acquisition. SKU, HS and M.Ko contributed to writing – original draft.

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SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section.