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Prognostic Utility of Blood-Derived CD133+ Extracellular Vesicles in Patients with Metastatic Colorectal Cancer

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ABSTRACT

Colorectal cancer (CRC) continues to rank among the most frequently diagnosed and deadliest cancers globally. Despite improvements in therapy that have extended the survival of patients with metastatic colorectal cancer (mCRC), there is still a shortage of effective biomarkers to inform treatment choices and guide clinical management. In this investigation, we employed a refined flow cytometry workflow to detect, quantify, and classify extracellular vesicles (EVs) in peripheral blood obtained from 54 individuals with mCRC and 48 healthy control participants matched by age and sex. Clinical outcomes, including overall survival (OS) and overall response rate (ORR), were assessed in patients who received first-line fluoropyrimidine-based chemotherapy.

The study revealed that mCRC patients exhibited markedly elevated circulating levels of total EVs as well as CD133+ and EPCAM+ EV subsets relative to healthy controls. Survival analyses indicated that patients with higher pre-treatment concentrations of total EVs and CD133+ EVs experienced significantly worse OS ($p = 0.001$ and $p = 0.0001$, respectively). Additionally, increased baseline levels of CD133+ EVs were associated with a diminished ORR to initial systemic therapy ($p = 0.045$).

Overall, these results highlight the potential value of blood-based EV profiling—particularly CD133+ EVs—as a source of new prognostic biomarkers that could enhance risk assessment and support more tailored therapeutic strategies for individuals with mCRC.

Keywords: Extracellular vesicles, Circulating biomarkers, Colorectal cancer

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Introduction

Colorectal cancer (CRC) remains a major contributor to cancer-related illness and death in Western populations [1]. At diagnosis, approximately one-fifth of CRC patients present with metastatic disease, and an additional 25% of those initially treated for localized tumors eventually develop distant metastases [2]. Therapeutic progress in metastatic colorectal cancer (mCRC) has substantially extended overall survival (OS) in recent years [3]. A range of systemic options—including chemotherapy, targeted agents, immunotherapies, and their combinations—have improved clinical outcomes [4]. Identifying reliable prognostic and predictive biomarkers is essential for recognizing high-risk individuals and guiding personalized therapeutic strategies in mCRC. Currently, treatment selection largely depends on molecular features of the tumor, such as RAS, RAF, and MMR status [5]. However, tissue-derived biomarkers may fail to capture full tumor heterogeneity and require invasive, resource-intensive sampling.

Liquid biopsy has emerged as a rapid, minimally invasive alternative or complement to tissue analysis [6]. This approach detects tumor-associated material in bodily fluids, including circulating tumor cells, nucleic acids, extracellular vesicles (EVs), and tumor-educated blood cells [6], offering new opportunities for individualized CRC management [7].

EVs are nanoscale, membrane-bound structures released by cells that mediate intercellular communication. They influence numerous processes involved in tumor progression [8]. Increasing data support EV involvement in

promoting cancer growth, metastatic niche formation, angiogenesis, therapy resistance, disruption of the blood–brain barrier, and immune escape [9]. Tumor-derived EVs carry distinct molecular profiles and are consistently detectable across a wide range of body fluids [10, 11]. These characteristics make circulating EVs promising candidates for cancer diagnosis, prognosis, and disease monitoring [12-14]. Analyses of EV molecular cargo have revealed cancer-specific proteins and various RNA species associated with CRC, suggesting potential utility for early detection and risk assessment [15].

Nonetheless, the prognostic and predictive relevance of EV subtypes defined by surface protein expression in CRC remains insufficiently explored. EV membrane proteins play key roles in determining EV targeting, circulation time, and uptake by recipient cells [16]. Consequently, characterizing EV surface phenotypes may provide important insights into their cellular origin and functional roles [17]. However, the substantial heterogeneity of EV surface proteins and enrichment of certain EV populations in circulation can hinder detection of rarer, disease-specific EV subsets when using bulk protein-based assays such as Western blotting or ELISA [18]. Sensitive analytical approaches requiring minimal sample preparation are therefore needed to support the clinical translation of EV-based biomarkers [19]. Recent studies have demonstrated the feasibility and clinical relevance of rapid phenotypic profiling of circulating EVs in CRC patients [20, 21].

In this work, we applied a patented polychromatic flow cytometry (PFC) technique to analyze intact circulating EVs carrying cancer-associated markers in patients with advanced CRC. We focused on EV populations expressing CD133 and EPCAM, based on evidence from earlier preclinical and clinical CRC research [22–27]. We compared EV concentrations between CRC patients and healthy controls and examined relationships between EV levels and clinicopathological features. Finally, we assessed the prognostic and predictive value of total EVs and CD133+ and EPCAM+ EV subsets in treatment-naïve patients with metastatic CRC.

Materials and Methods

Patients

This prospective observational study included adult individuals with a histologically or cytologically confirmed diagnosis of stage IV colorectal cancer who were scheduled to begin systemic treatment. Participants were enrolled at the Clinical Oncology Unit of SS Annunziata Hospital (Chieti, Italy) between January 2017 and August 2021. A control group of 48 healthy individuals, matched by age and sex, was also recruited.

All procedures were conducted according to the ethical principles outlined in the 1964 Declaration of Helsinki and subsequent amendments. Ethical approval was granted on 25 February 2016, and written informed consent was obtained from all study participants.

Blood collection

Peripheral blood samples were drawn at study enrollment, with a second sample collected 12 ± 6 weeks following the first treatment cycle. Blood was obtained via 21 G needles into two sodium-citrate anticoagulant tubes (Becton Dickinson Biosciences, San Jose, CA, USA). Samples were processed within 4 hours of venipuncture. To minimize EV release due to vascular injury, the first blood tube collected was discarded.

Flow cytometry detection of extracellular vesicles

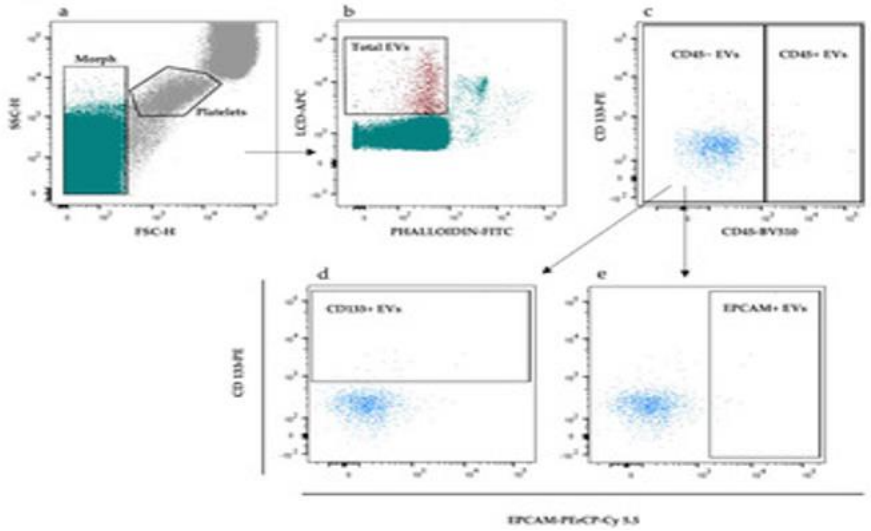
A lipophilic cationic dye (LCD) was combined in PBS; 5 µL of whole blood was added to this mixture. Before use, each antibody stock was centrifuged (21,000×g, 10 min) to reduce artifacts from aggregates and immune complexes. After a 45-minute incubation at room temperature in the dark, samples were diluted in PBS and analyzed by flow cytometry, acquiring 1 × 106 events per sample. EV quantification was performed using volumetric counting (FACSVerse, BD Biosciences, San Jose, CA, USA). The trigger threshold was set on the APC fluorescence channel as previously described [28, 29]. Signal height was used to capture FSC, SSC, and fluorescence parameters.

EV gating was defined using Megamix-Plus beads (Byocitex, Marseille, France) and the Rosetta Calibration System (Exometry, Amsterdam, The Netherlands) to ensure consistency over time [28, 29]. Fluorescence minus one (FMO) controls, combined with appropriate isotype controls, were used to determine gating precision and exclude nonspecific fluorescence [30]. Reagent-only, buffer-only, and Triton X-100 (1%) controls helped distinguish true EV signals from contaminants or debris. Compensation settings were established using CompBeads and single-stained controls. Data were processed using FACSuite v1.0.6.5230 and FlowJo v10 (BD Biosciences).

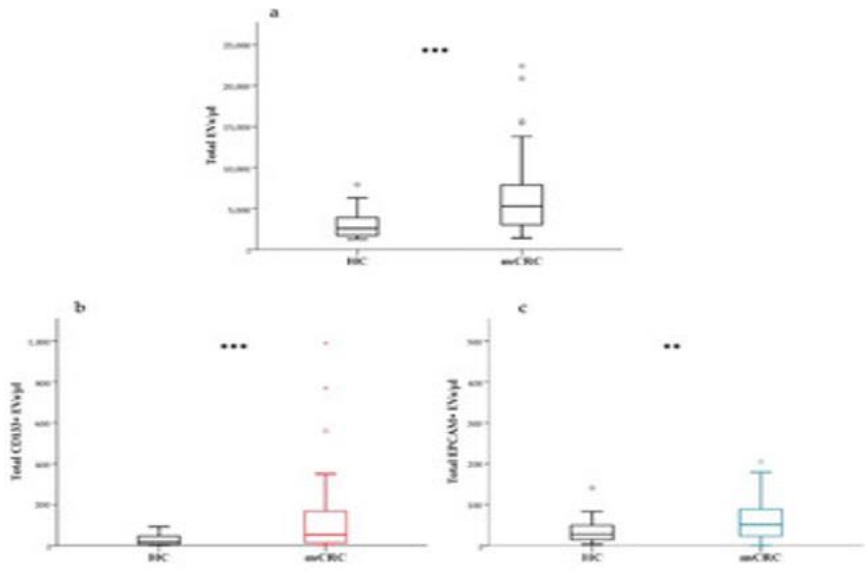
Flow cytometry subtyping of extracellular vesicles

Active, intact extracellular vesicles were defined as LCD+/phalloidin− events located within the scatter region corresponding to particles smaller than platelets **(Figure 1A(a))**. The physical and morphological profiles of EVs detected using this LCD-based method have been thoroughly described in earlier publications [28, 29]. As shown by Marchisio *et al*., more than 90% of circulating LCD+/phalloidin− particles exhibit diameters exceeding 160 nm.

Following identification, total EVs (LCD+/phalloidin−) were plotted on a CD45-H/CD133-H diagram **(Figure 1A(b))**, and CD45− events were subsequently gated **(Figure 1A(c))**. This CD45− fraction was then examined on a CD326-H/CD133-H dot plot to distinguish specific EV subsets. Through this analysis, CD133+ EVs (CD45−/CD133+) and EPCAM+ EVs (CD45−/EPCAM+) were identified as distinct populations **(Figure 1A(d, e)).**



A)



B)

Figure 1. (A) Overview of the gating workflow used to detect and classify extracellular vesicles (EVs) in peripheral blood. All acquired events were first visualized on a forward scatter-H vs. side scatter-H plot, where a "platelet-free area" (Morph) was delineated using platelets as the size reference. (a) This Morph region was then displayed on a phalloidin-H vs. lipophilic cationic dye (LCD)-H plot, and EVs were defined as LCD-positive and phalloidin-negative. (b) These total EVs were subsequently examined on a CD133-H/CD45-H plot, allowing separation of CD45− and CD45+ events (c). The CD45− subset was then analyzed on a CD133-H/EPCAM-H plot to identify the CD133+ EV population (CD45−/CD133+) (d) and the

EPCAM+ EV population (CD45−/EPCAM+) (e). (B) Box plots illustrating differences in total EVs (a), CD133+ EVs (b), and EPCAM+ EVs (c) between patients with mCRC (n = 54) and healthy subjects (n = 48). ** indicates $p < 0.01$; *** indicates $p < 0.001$. Extreme data points have been omitted.

Statistical analysis

All statistical procedures were carried out using SPSS v25.0 (IBM SPSS, Chicago, IL, USA) and MedCalc v14.8.1 (MedCalc Software, Ostend, Belgium). Since no assumption of normal distribution was made, non-parametric methods were used for group comparisons. The Mann–Whitney U test was applied to compare continuous variables, while associations between EV concentrations and clinicopathological parameters were examined using Spearman's correlation coefficients.

Univariate and multivariate Cox proportional hazards models were constructed to estimate hazard ratios (HRs) with corresponding 95% confidence intervals (CIs). To enhance analytical power, EV concentrations were treated as continuous variables [31], and log-transformed to address distribution skewness. Survival-based cutoffs for EV levels were generated using the Charité Cutoff Finder tool [32]. Median overall survival (mOS) was calculated using Kaplan–Meier estimators, and differences between survival curves were assessed with the log-rank test.

Overall response rate (ORR) was defined as the proportion of patients achieving complete response (CR) or partial response (PR), enabling classification into responder and non-responder groups. ORRs were compared using Fisher's exact test. ROC curve analysis (CR + PR vs. SD + PD) was performed to evaluate the predictive strength of baseline EV concentrations.

Relative changes in EV levels were calculated as percentage variation:

% change = {[(log2 EV concentration at week 12 / log2 EV concentration at baseline) − 1] × 100}.

Patients were categorized into two groups: those showing a ≥25% decrease and those with stable or increased EV levels (<25% decrease to <25% increase, or ≥25% increase). Internal validation was conducted using the SPSS bias-corrected and accelerated bootstrap method with 1000 resamples and a 95% confidence interval. A p-value < 0.05 was considered statistically significant.

Results and Discussion

Elevated total and CD133+ extracellular vesicle levels in patients with metastatic CRC

Concentrations of total EVs, CD133+ EVs, and EPCAM+ EVs were quantified in blood samples from patients with metastatic colorectal cancer (n = 54) and compared with levels measured in age- and sex-matched healthy controls (n = 48). Baseline medians for each EV category are presented in **Table 1** and visualized in **Figure 1B**. Patients with mCRC displayed significantly higher circulating levels of all three EV populations than healthy individuals. Median concentrations (95% CI) were as follows:

- Total EVs/µL: 5264.0 (4123.0–6314.0) in mCRC vs. 2548.0 (2100.7–3051.4) in controls
- CD133+ EVs/µL: 52.6 (32.4–96.1) in mCRC vs. 18.4 (11.6–32.2) in controls
- EPCAM+ EVs/µL: 50.9 (38.6–67.2) in mCRC vs. 27.0 (19.3–42.0) in controls

All differences were statistically significant **(Table 1)**.

Table 1. Comparison of total and subtype EV concentrations between mCRC patients (n = 54) and healthy controls (HCs) (n = 48).

	mCRC	**HCs**	**p-Value**
Age (%)			
≥65	35 (61.4)	22 (38.6)	0.07
<65	19 (42.2)	26 (57.8)	
Sex (%)			
Male	39 (54.9)	16 (51.6)	0.66
Female	12 (20.3)	15 (48.4)	
Median Total EVs/µL (95% CI)	5264.0 (4123.0–6314.0)	2548.0 (2100.7–3051.4)	0.000003
Median CD133+ EVs/µL (95% CI)	52.6 (32.4–96.1)	18.4 (11.6–32.2)	0.0002
Median EPCAM+ EVs/µL (95% CI)	50.9 (38.6–67.2)	27.0 (19.3–42.0)	0.007

Researchers looked at whether blood EV levels were linked to different clinical and pathological features such as age, sex, ECOG performance status, primary tumor site, tumor grade, K-RAS mutation, and the presence of liver or lung metastases, as well as the number of metastatic locations. None of these factors showed a meaningful relationship with total EV levels or with any of the specific EV subtypes measured.

In patients with mCRC, EV measurements were also compared before and after treatment. The pre-treatment group included baseline samples from patients who had not yet received therapy (n = 36), while post-treatment levels were taken from samples collected during or after first-line therapy (n = 41). Although there was a tendency for total EVs and CD133+ EVs to decline once treatment began, the differences between pre- and post-treatment levels were not statistically significant.

Total and CD133+ EVs concentrations are associated with overall survival in treatment naïve patients

Next, the study evaluated whether baseline EV levels were related to overall survival in patients starting their first line of therapy. This analysis included 33 treatment-naïve individuals receiving fluoropyrimidine-based chemotherapy. The median follow-up was 11.0 months (95% CI 8.0–19.0), and 24 patients (72.7%) were still alive at the time of assessment. The one-year overall survival rate was 81%.

To explore how EV levels affected survival, both univariate and multivariate Cox regression models were used. The univariate model first examined several possible predictors of overall survival, such as ECOG PS, age, number of metastatic sites, tumor grade, and primary tumor location. Only the variables that were statistically significant ($p < 0.05$) were entered into the multivariate analysis.

In the univariate results, patients with higher total EV levels had a significantly greater risk of death (HR (95% CI) = 1.77 (1.24–2.54)) (p = 0.002) (**Table 2)**. Higher CD133+ EV levels showed a similar association with poorer survival (HR (95% CI) = 1.72 (1.24–2.39)) (p = 0.001) **(Table 3)**. EPCAM+ EV levels, however, did not show a significant link to survival outcomes **(Table 3)**. The multivariate model confirmed that both total EVs and CD133+ EVs were independent predictors of shorter survival, and these findings were further supported by bootstrap validation.

Table 2. Univariate Cox proportional hazards model predicting OS in the treatment-naive cohort (n = 33).

Variable	Univariate Analysis		Bootstrap Results (1000 Replicas)			
	HR (95% CI)	p	Bias	SE	95% CI	p
Total EVs [a]	1.77 (1.24–2.54)	0.002	0.15	0.58	0.30 to 1.78	0.001 [b]
EPCAM EVs [a]	1.31 (0.94–1.84)	0.11	0.33	0.64	−0.03 to 2.02	0.32
CD133+ EVs [a]	1.72 (1.24–2.39)	0.001	0.12	0.30	0.38 to 1.42	0.001 [c]
ECOG PS						
1	1 [reference]					
0	0.15 (0.03–0.72)	0.02	−0.46	1.98	−8.37 to −0.08	0.003
Age (years)						
≥65	1 [reference]					
<65	0.48 (0.12–1.94)	0.30	−0.21	1.04	−4.02 to 0.68	0.13
No. of metastatic sites						
>1	1 [reference]					
1	0.95 (0.25–3.57)	0.94	−0.05	0.87	−1.73 to 1.41	0.93
Grading						
1–2	1 [reference]					
3	3.47 (0.37–32.4)	0.27	−0.18	3.91	−3.22 to 13.5	0.10 [b]
Primary site						
Left-sided colon/rectum	1 [reference]					
Right-sided colon	2.75 (0.675-11.57)	0.17	0.01	1.81	−3.21 to 3.09	0.10 [c]

[a] continuous variable (log-transformed); [b] based on 927 samples; [c] based on 998 samples; abbreviations—HR: hazard ratio; SE: standard error; CI: confidence interval.

Table 3. Multivariate Cox proportional hazards model predicting OS in the treatment-naive cohort (n = 33).

Variable	Multivariate Analysis		Bootstrap Results (1000 Replicas)			
	HR (95% CI)	p	Bias	SE	95% CI	p
Total EVs	1.80 (1.06–3.09)	0.03	1.14	3.37	−0.10 to 11.30	0.01 [b]
CD133+ EVs	1.67 (1.02–2.74)	0.04	0.73	3.21	−0.07 to 7.43	0.006 [b]
ECOG PS						
1	1 [reference]					
0	0.06 (0.01–0.55)	0.01	−4.17	16.55	−36.16 to 1.65	0.02 [a]

[a] continuous variable (log-transformed); [b] based on 998 samples; abbreviations—HR: hazard ratio; SE: standard error; CI: confidence interval.

The link between overall survival (OS) and blood levels of total EVs and CD133+ EVs was further assessed using Kaplan–Meier survival curves **(Figure 2)**. For this analysis, EV concentrations were divided into high and low groups based on cut-off values generated by the Charité Cutoff Finder (Total EVs: 7007 EVs/µL; CD133+ EVs: 163.6 EVs/µL), as described in the Methods section **(Figures 2A(b) and 2B(b))**.

Patients whose total EV levels exceeded 7007 EVs/µL had a clearly poorer prognosis, showing a median OS of 9 months (95% CI 6.1–11.9). In contrast, the median OS had not yet been reached in the group with lower total EV levels by the time of analysis (p = 0.001) **(Figure 2A(a))**. A similar pattern was observed for CD133+ EVs: individuals with CD133+ EV concentrations above 163.6 EVs/µL experienced significantly shorter survival compared to those with lower levels (p = 0.0001) **(Figure 2B(a))**.

The study also examined whether the relationship between CD133+ EVs and OS varied according to K-RAS mutation status. Higher CD133+ EV levels were linked with reduced survival in both K-RAS–mutant and K-RAS wild-type patients, indicating that this association was independent of mutation status.

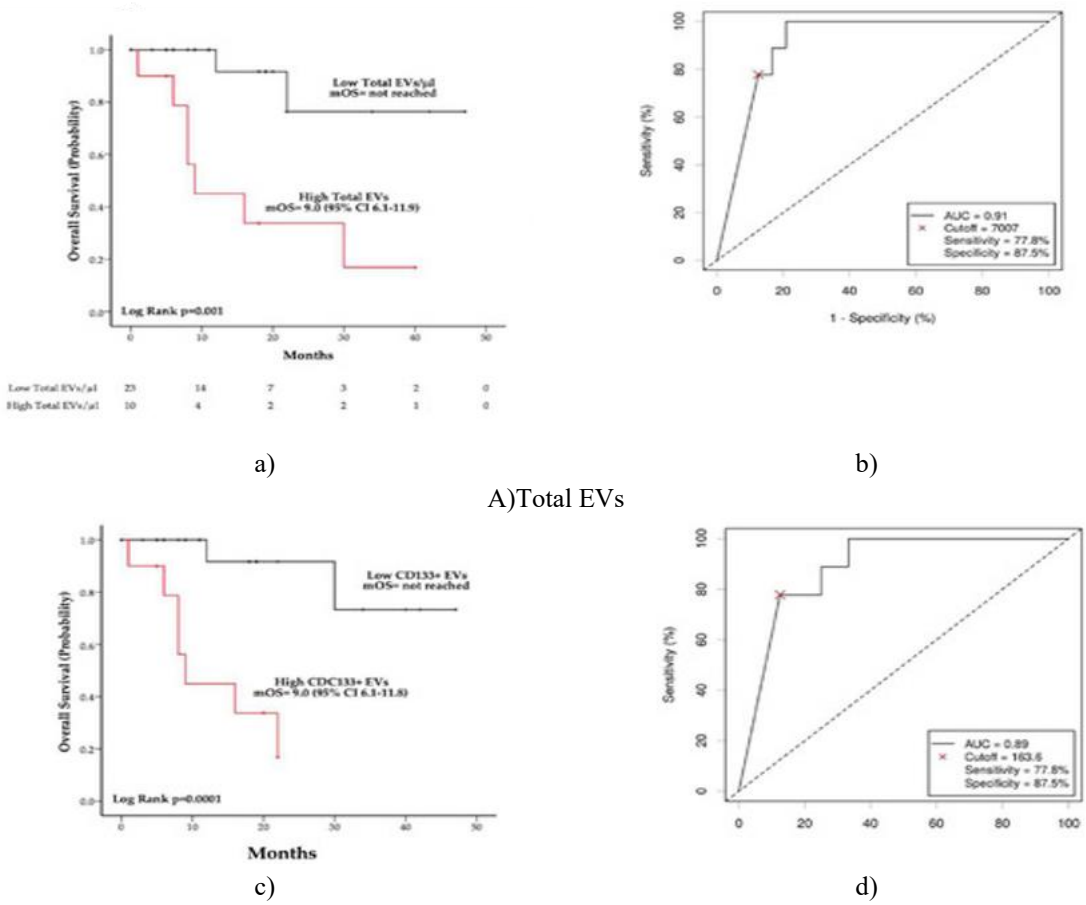


Figure 2. Kaplan–Meier (KM) curves depict the relationship between overall survival (OS) and circulating levels of total EVs (panel A(a)) and CD133+ EVs (panel B(a)). Panels A(b) and B(b) show ROC analyses used to determine the optimal EV cut-off values.

Association of blood CD133+ EVs with treatment response

We next assessed whether baseline EV levels could predict response to first-line therapy in the same patient cohort. Out of 33 patients, 30 had evaluable responses. The overall response rate (ORR) in this subgroup was 46.7%, with a median progression-free survival (PFS) of 12.0 months (95% CI 7.5–16.5).

Comparing EV concentrations between responders and non-responders revealed that patients achieving a response had significantly lower pre-treatment CD133+ EV levels (p = 0.03) **(Figure 3a)**. In contrast, total EVs and EPCAM+ EV levels did not differ significantly between these two groups.

ROC analysis confirmed the predictive potential of CD133+ EVs, yielding an AUC of 0.728 (95% CI 0.542–0.913; p = 0.03) **(Figure 3c)**. Stratifying patients based on the CD133+ EV cut-off of 163.6 EVs/µL, those with higher levels (>163.6 EVs/µL) exhibited a lower ORR (20%) compared to patients with lower levels (≤163.6 EVs/µL), who had an ORR of 60% (p = 0.045) **(Figure 3b)**.

Additionally, patients with elevated baseline CD133+ EVs experienced shorter PFS compared to those with lower levels, and this difference was statistically significant (p = 0.0003) **(Figure 3d)**. These findings highlight the potential of CD133+ EVs as a biomarker for predicting both treatment response and disease progression in mCRC.

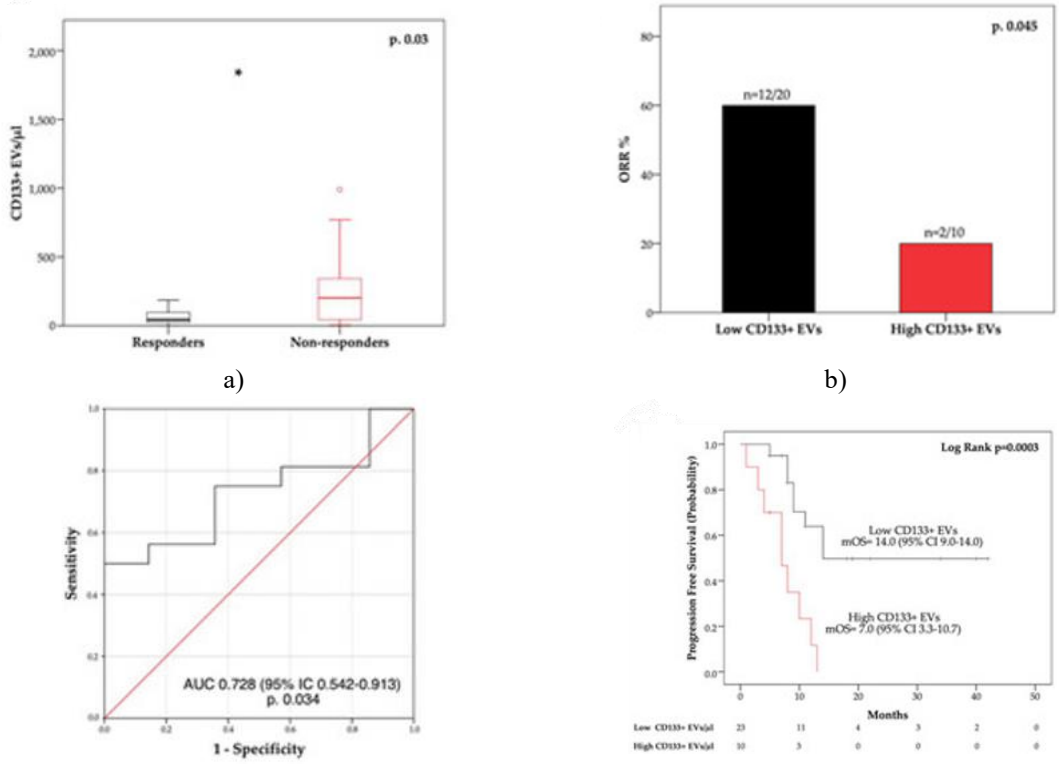


Figure 3. Baseline blood levels of CD133+ EVs and treatment response are presented. (a) Boxplots show the differences in CD133+ EV concentrations between responders and non-responders. (b) Histograms illustrate the overall response rates for patients with high versus low CD133+ EV levels (*, p < 0.05). (c) ROC curve analysis indicates the predictive value of CD133+ EVs for treatment response. (d) Kaplan–Meier curves compare progression-free survival (PFS) between patients with high and low baseline CD133+ EV concentrations.

We also evaluated how changes in EV concentrations during therapy relate to treatment response. Follow-up EV data were available for 18 of the 30 patients. In this analysis, responders and non-responders were evenly distributed between patients with stable/increasing versus decreasing levels of total, CD133+, or EPCAM+ EVs.

Discussion

Colorectal cancer (CRC) remains a leading cause of cancer-related mortality worldwide. Despite improvements in diagnosis, treatment, and management, only a limited number of CRC-specific biomarkers are currently applied in clinical practice. Blood-based biomarkers have garnered interest because they are minimally invasive and

provide dynamic information reflecting the tumor's overall phenotype and functional status [6]. Circulating extracellular vesicles (EVs) have emerged as a promising form of liquid biopsy. Tumor-derived EVs carry molecular signals into the bloodstream, serving as potential indicators of cancer progression, early diagnosis, prognosis, and treatment response [7]. In this study, we implemented a novel polychromatic flow cytometry (PFC) protocol to characterize blood EVs expressing putative CRC markers and to investigate their prognostic and predictive potential in stage IV CRC patients.

Our findings show a marked increase in total circulating EV levels in patients with metastatic CRC (mCRC) compared to healthy controls, consistent with prior reports linking elevated blood EVs to various cancers, including colon cancer [23, 25, 33–35]. This expansion of the EV pool may result from both increased secretion by cancer cells and tumor-driven EV release from non-malignant cells [10]. However, understanding the tumor-specific information carried by EVs requires detailed analysis of their phenotype and cargo [12]. Accordingly, we analyzed the expression of tumor-associated markers CD133 and EPCAM in circulating EVs. Patients with mCRC exhibited significantly higher CD133+ and EPCAM+ EV levels than healthy controls. Both CD133 and EPCAM are transmembrane proteins frequently overexpressed in colorectal cancer [36], and in vitro studies have shown that cancer cells can release EVs enriched for these markers [22, 37].

It is plausible that elevated CD133+ and EPCAM+ EVs in mCRC reflect increased secretion from cancer cells. Nevertheless, inflammation related to both CRC and non-CRC causes can also induce CD133 and EPCAM expression [38, 39], and enhanced CD133+ particles have been reported in patients with inflammatory conditions [40]. Tumor-associated inflammation and heightened cell turnover may therefore contribute to higher circulating CD133+ and EPCAM+ EV levels in CRC patients.

Survival analyses revealed that higher total EV concentrations were linked with worse outcomes in treatment-naïve mCRC patients, consistent with previous studies connecting elevated circulating EVs to lower overall survival in cancer [41, 42]. Increased EV levels may reflect a greater release of tumor-derived vesicles, which are known to promote cancer progression, metastasis, therapy resistance, and immune evasion [33, 43]. However, the prognostic significance of total EVs can be influenced by EVs from non-tumor sources; prior work has shown that higher platelet- or endothelial-derived EVs are associated with poor survival in advanced cancers [13, 44, 45]. These observations highlight the importance of characterizing EV subpopulations to clarify their biological and clinical relevance.

In this study, CD133+ EVs demonstrated independent prognostic value in advanced CRC, whereas EPCAM+ EVs did not correlate with overall survival. Elevated CD133+ EV levels were associated with shorter OS. Similar findings have been reported in advanced non-small cell lung cancer [23], and CD133 expression in tumors has previously been linked to recurrence, metastasis, and poor survival in CRC [46]. In vitro data also indicate that CD133-enriched EVs enhance tumor growth, metastasis, proliferation, and motility across several cancer types, including CRC [22, 47]. CD133 is recognized as a marker of colon cancer stem cells (CSCs) [48], and CSC-derived EVs have been shown to promote stem-like properties in differentiated cancer cells, confer chemoresistance, and stimulate metastasis and angiogenesis [49]. These mechanisms may explain the negative prognostic impact of high CD133+ EV levels in advanced CRC [50].

Conversely, CD45− EPCAM+ EV concentrations were not significantly associated with OS. EPCAM and CD45 are commonly used to isolate circulating tumor cells (CTCs), which possess prognostic relevance in CRC [51]. In a large cohort, Nanou *et al*. evaluated the prognostic role of CTCs and tumor-derived EVs (TdEVs), defining TdEVs as EpCAM+, cytokeratin+, DAPI−, CD45− microparticles. Both elevated TdEVs and CTCs were linked with worse overall survival, suggesting that more detailed phenotypic analysis of EPCAM+ EVs could improve their use as prognostic biomarkers in CRC [27].

We found that circulating CD133+ EVs were significantly associated with patients' response to systemic therapy. Specifically, higher baseline levels of blood CD133+ EVs correlated with a lower overall response rate (ORR) and shorter progression-free survival (PFS) in advanced CRC patients receiving fluoropyrimidine-based chemotherapy. This observation aligns with prior evidence linking tumor CD133 expression to drug resistance [52, 53]. Mechanistically, CD133 has been shown to activate PI3K and FLIP3 signaling pathways in cancer cells, inhibiting apoptosis and autophagy, thereby promoting chemoresistance [54, 55]. Additionally, two in vitro studies demonstrated that CD133-enriched EVs secreted by colon cancer cells can induce drug resistance in recipient cells through activation of key signaling pathways [22, 26]. Notably, some patients in our cohort received anti-EGFR therapy in combination with chemotherapy, and preclinical data suggest that colon cancer cells with wild-type KRAS can develop resistance to anti-EGFR agents after exposure to CD133+ microvesicles [22]. Taken

together, these findings support a functional role for CD133+ EVs in mediating chemoresistance, which is consistent with our clinical observations. While our results highlight a clear association between elevated CD133+ EV levels and poor therapeutic outcomes in mCRC, further validation through larger clinical studies and additional preclinical investigations is warranted.

Conclusion

Using PFC-based immunophenotyping, we demonstrated that circulating levels of total EVs, CD133+ EVs, and EPCAM+ EVs are elevated in patients with advanced CRC compared to healthy controls, reflecting tumor-driven phenotypic alterations. Importantly, higher CD133+ EV concentrations were linked to reduced survival and lower response rates to systemic therapy, suggesting a potential mechanistic role in modulating treatment outcomes.
Overall, these findings provide novel insights into the prognostic and predictive utility of tumor-associated circulating EVs, supporting their potential application in liquid biopsy strategies to improve personalized management in stage IV CRC.

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Conflict of Interest: None

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Ethics Statement: Informed consent was obtained from all subjects involved in the study.

References

1. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin. 2018;68(6):394–424.
2. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2015. CA Cancer J Clin. 2015;65(1):5–29.
3. Jawed I, Wilkerson J, Prasad V, Duffy AG, Fojo T. Colorectal cancer survival gains and novel treatment regimens. JAMA Oncol. 2015;1(5):787.
4. Kopetz S. New therapies and insights into the changing landscape of colorectal cancer. Nat Rev Gastroenterol Hepatol. 2019;16(2):79–80.
5. Di Nicolantonio F, Vitiello PP, Marsoni S, Siena S, Tabernero J, Trusolino L, et al. Precision oncology in metastatic colorectal cancer. Nat Rev Clin Oncol. 2021;18(8):506–25.
6. Martins I, Ribeiro IP, Jorge J, Gonçalves AC, Sarmento-Ribeiro AB, Melo JB, et al. Liquid biopsies: applications for cancer diagnosis and monitoring. Genes. 2021;12(3):349.
7. Kolenčík D, Shishido SN, Pitule P, Mason J, Hicks J, Kuhn P. Liquid biopsy in colorectal carcinoma: clinical applications and challenges. Cancers. 2020;12(5):1376.
8. Xu R, Rai A, Chen M, Suwakulsiri W, Greening DW, Simpson RJ. Extracellular vesicles in cancer. Nat Rev Clin Oncol. 2018;15(10):617–38.
9. Tao SC, Guo SC. Role of extracellular vesicles in tumour microenvironment. Cell Commun Signal. 2020;18(1):163.
10. Becker A, Thakur BK, Weiss JM, Kim HS, Peinado H, Lyden D. Extracellular vesicles in cancer: mediators of metastasis. Cancer Cell. 2016;30(6):836–48.
11. Rahbarghazi R, Jabbari N, Sani NA, Asghari R, Salimi L, Kalashani SA, et al. Tumor-derived extracellular vesicles. Cell Commun Signal. 2019;17(1):73.
12. Möller A, Lobb RJ. The evolving translational potential of small extracellular vesicles in cancer. Nat Rev Cancer. 2020;20(11):697–709.

13. Brocco D, Lanuti P, Pieragostino D, Cufaro MC, Simeone P, Bologna G, et al. Blood circulating EV hallmarks in NSCLC responders. Cancers. 2021;13(3):585.
14. De Lellis L, Florio R, Di Bella MC, Brocco D, Guidotti F, Tinari N, et al. Exosomes in pancreatic cancer. Biomedicines. 2021;9(3):275.
15. Chang LC, Chiu HM, Wu MS, Shen TL. EVs in colorectal cancer. Int J Mol Sci. 2022;23(3):1379.
16. Hoshino A, Costa-Silva B, Shen TL, Rodrigues G, Hashimoto A, Tesic MM, et al. Tumour exosome integrins determine organotropic metastasis. Nature. 2015;527(7578):329–35.
17. Kowal J, Arras G, Colombo M, Jouve M, Morath JP, Primdal-Bengtson B, et al. Proteomic comparison of EV subtypes. Proc Natl Acad Sci USA. 2016;113(8):E968–77.
18. Gandham S, Su X, Wood J, Nocera AL, Alli SC, Milane L, et al. Technologies in EV research. Trends Biotechnol. 2020;38(10):1066–98.
19. Shao H, Im H, Castro CM, Breakefield X, Weissleder R, Lee H. New technologies for EV analysis. Chem Rev. 2018;118(4):1917–50.
20. Tian Y, Ma L, Gong M, Su G, Zhu S, Zhang W, et al. EV profiling by flow cytometry. ACS Nano. 2018;12(1):671–80.
21. Park J, Park JS, Huang CH, Jo A, Cook K, Wang R, et al. Rapid profiling of tumour EVs. Nat Biomed Eng. 2021;5(7):678–89.
22. Kang M, Kim S, Ko J. Roles of CD133 in microvesicle formation. FASEB J. 2019;33(4):4248–60.
23. Brocco D, Lanuti P, Simeone P, Bologna G, Pieragostino D, Cufaro MC, et al. CSC-derived EVs as biomarkers. J Oncol. 2019;2019:5879616.
24. Ostenfeld MS, Jensen SG, Jeppesen DK, Christensen LL, Thorsen SB, Stenvang J, et al. miRNA profiling in EpCAM+ EVs. J Extracell Vesicles. 2016;5(1):31488.
25. Wei P, Wu F, Kang B, Sun X, Heskia F, Pachot A, et al. Plasma EVs detected by SiMoA. J Extracell Vesicles. 2020;9(1):1809765.
26. Lucchetti D, Calapà F, Palmieri V, Fanali C, Carbone F, Papa A, et al. Exosomes from colon cancer cells. Am J Pathol. 2017;187(7):1633–44.
27. Nanou A, Miller MC, Zeune LL, de Wit S, Punt CJA, Groen HJM, et al. Tumour-derived EVs in metastatic cancer patients. Br J Cancer. 2020;122(6):801–11.
28. Simeone P, Celia C, Bologna G, Ercolino E, Pierdomenico L, Cilurzo F, et al. Flow cytometry calibration for EVs. Int J Mol Sci. 2020;21(21):7885.
29. Marchisio M, Simeone P, Bologna G, Ercolino E, Pierdomenico L, Pieragostino D, et al. Circulating EV subtype analysis. Int J Mol Sci. 2020;22(1):48.
30. Cossarizza A, Chang HD, Radbruch A, Akdis M, Andrä I, Annunziato F, et al. Guidelines for flow cytometry. Eur J Immunol. 2017;47(10):1584–797.
31. Altman DG, Royston P. Cost of dichotomising continuous variables. BMJ. 2006;332(7549):1080.
32. Budczies J, Klauschen F, Sinn BV, Győrffy B, Schmitt WD, Darb-Esfahani S, et al. Cutoff Finder tool. PLoS One. 2012;7(12):e51862.
33. Osti D, Del Bene M, Rappa G, Santos M, Matafora V, Richichi C, et al. EVs in glioblastoma plasma. Clin Cancer Res. 2019;25(1):266–76.
34. Liu S, Liao Y, Hosseinifard H, Imani S, Wen Q. Diagnostic role of EVs in cancer. Front Cell Dev Biol. 2021;9:2749.
35. Zhao L, Bi Y, Kou J, Shi J, Piao D. Procoagulant microparticles in colon cancer. J Exp Clin Cancer Res. 2016;35(1):54.
36. Shmelkov SV, Butler JM, Hooper AT, Hormigo A, Kushner J, Milde T, et al. CD133 expression in colon cancer cells. J Clin Invest. 2008;118(6):2111–20.
37. Tauro BJ, Greening DW, Mathias RA, Mathivanan S, Ji H, Simpson RJ. Two exosome populations from LIM1863. Mol Cell Proteomics. 2013;12(3):587–98.
38. Kazama S, Kishikawa J, Tanaka T, Hata K, Kawai K, Nozawa H, et al. CD133 and LGR5 in UC-associated CRC. In Vivo. 2019;33(4):1279–84.
39. Yasuda H, Tanaka K, Okita Y, Araki T, Saigusa S, Toiyama Y, et al. CD133, OCT4, NANOG in UC-associated CRC. Oncol Lett. 2011;2(6):1065–71.
40. Bobinger T, May L, Lücking H, Kloska SP, Burkardt P, Spitzer P, et al. CD133+ particles in CSF. Front Cell Neurosci. 2017;11:77.

41. Evans SM, Putt M, Yang XY, Lustig RA, Martinez-Lage M, Williams D, et al. Blood-borne EVs in glioblastoma. J Neurooncol. 2016;127(2):391–400.
42. De Luca L, D'Arena G, Simeon V, Trino S, Laurenzana I, Caivano A, et al. Circulating microvesicles in CLL. Leuk Lymphoma. 2017;58(7):1424–32.
43. Han L, Lam EWF, Sun Y. EVs in tumour microenvironment. Mol Cancer. 2019;18(1):59.
44. Wang CC, Tseng CC, Chang HC, Huang KT, Fang WF, Chen YM, et al. Circulating microparticles in NSCLC. Oncotarget. 2017;8(46):75952–67.
45. Wang CC, Tseng CC, Hsiao CC, Chang HC, Chang LT, Fang WF, et al. Endothelial microparticle as biomarker in NSCLC. Biomed Res Int. 2014;2014:1–11.
46. Ren F, Sheng WQ, Du X. CD133 as CSC marker in colorectal cancer. World J Gastroenterol. 2013;19(17):2603–11.
47. Rappa G, Mercapide J, Anzanello F, Pope RM, Lorico A. Characterization of CD133 exosomes. Mol Cancer. 2013;12(1):62.
48. Munro MJ, Wickremesekera SK, Peng L, Tan ST, Itinteang T. Cancer stem cells in colorectal cancer. J Clin Pathol. 2018;71(2):110–6.
49. Su C, Zhang J, Yarden Y, Fu L. CSC-derived EVs. Signal Transduct Target Ther. 2021;6(1):109.
50. Zhou Y, Xia L, Wang H, Oyang L, Su M, Liu Q, et al. CSCs in colorectal cancer progression. Oncotarget. 2018;9(23):33403–15.
51. Veyrune L, Naumann DN, Christou N. Circulating tumour cells as biomarkers in CRC. Int J Mol Sci. 2021;22(7):3437.
52. Paschall AV, Yang D, Lu C, Redd PS, Choi JH, Heaton CM, et al. CD133+CD24lo phenotype and 5-FU resistance. Oncotarget. 2016;7(51):78698–712.
53. Angelastro JM, Lamé MW. CD133 promotes drug resistance in glioma cells. Mol Cancer Res. 2010;8(8):1105–15.
54. Zobalova R, McDermott L, Stantic M, Prokopova K, Dong LF, Neuzil J. CD133+ cells resistant to TRAIL. Biochem Biophys Res Commun. 2008;373(4):567–71.
55. Wei Y, Jiang Y, Zou F, Liu Y, Wang S, Xu N, et al. PI3K/Akt activation via CD133-p85. Proc Natl Acad Sci USA. 2013;110(17):6829–34.