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Diagnostic performance of circulating EpCAM+ and CD45+ extracellular vesicles in platinum-sensitivity in high-grade serous ovarian cancer: a pilot study

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Abstract

Background Platinum-resistant high-grade serous ovarian cancer (HGSOC) is a fatal disease. The main goal of current study is to develop new strategies to predict platinum resistance, moving towards personalized therapy. Currently, there are no validated biomarkers able to predict at diagnosis platinum resistance. Circulating tumor-derived extracellular vesicles (EVs) represent a liquid biopsy of the tumor of origin and reflect its biological profile. EpCAM-expressing EVs are largely used biomarkers of epithelial cancers in translational research. Aim of this study was to quantify, by nano-flow cytometry, circulating EpCAM+ EVs in patients with histologically confirmed diagnosis of HGSOC FIGO stage III/IV, before and after intravenous administration of the first dose of chemotherapy with paclitaxel and carboplatin, and correlate EVs levels to platinum-sensitivity. As comparison, leukocyte-derived EVs were also assessed using the pan-leukocyte marker CD45.

Results Patients with platinum-sensitive HGSOC showed significantly lower pre-chemotherapy circulating levels of both EpCAM+ and CD45+ EVs compared to platinum-resistant cases ($p<0.01$). Platinum-sensitive patients displayed significantly higher levels of circulating EpCAM+ and CD45+ EVs 21 days post administration of the first dose of chemotherapy compared to pre-treatment levels ($p<0.05$ and $p<0.01$, respectively). Platinum-resistant and platinum-refractory patients showed significantly higher $EpCAM^{+}CD45^{+}$ EVs levels than platinum-sensitive patients ($p<0.01$, $p<0.05$, respectively).

Conclusions Tumor-derived EVs are valuable candidate biomarkers for early prediction of platinum resistance and need to be investigated in larger prospective clinical studies.

Keywords EpCAM, CD45, HGSOC, Chemoresistance, Extracellular vesicles

Background

High-grade serous ovarian carcinoma (HGSOC) is the most common histotype of ovarian cancer and accounts for most deaths from ovarian cancer. The high fatality rate [1] has shown little improvement for decades due to the lack of screening tests and the advanced stage at diagnosis in over 70% of women [2]. Overall, the 5-year survival

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rate is about 48% [3]. Currently, debulking surgery and platinum-based chemotherapy represent the gold standard treatment of the HGSOC. Although HGSOC is often sensitive to platinum-based chemotherapy and other DNA-damaging agents, resistance to platinum therapy continues to be a major issue and most patients who initially have a platinum-sensitive disease, through multiple recurrences, later acquire platinum-resistance [4]. Platinum resistance is commonly defined on the basis of the duration of the response to platinum-containing chemotherapy. Patients who initially respond to platinum-based chemotherapy and relapse 6 months or longer after the initial treatment are classified as platinum sensitive, while patients who relapse within 6 months after the start of platinum-based chemotherapy are considered platinum resistant. Within the platinum-resistant group, a subgroup of patients presenting with the worst prognosis is defined as platinum-refractory, with a disease that progresses during or within 1 month of platinum-containing first-line chemotherapy [5].

It would be of utmost importance to identify HGSOC patients non-responsive to platinum treatment beforehand, to avoid drug-induced toxicity and side effects without clinical benefit. Identification of predictive biomarkers for HGSOC platinum-sensitivity at diagnosis would help recognizing which patients are most likely to benefit from standard care and which patients are platinum-resistant or refractory and need more personalized and effective treatment strategies.

Extracellular vesicles (EVs) are lipid membrane vesicles secreted by the cell which are categorized based on size, biogenesis and release pathway. They are known to be secreted by all human cell types and to be present in body fluids [6]. Tumor cells produce large quantities of EVs whose surface cargo resembles that of parent cell. Thus, tumor-derived EVs are representative of the tumor cells from which they originate and, because they circulate freely in the plasma, are thought of as potential "liquid tumor biopsies" that require a minimally invasive biofluid sampling and may provide clinicians with a real-time snapshot of the disease [6, 7].

Epithelial cancer cells usually express epithelial cell adhesion molecule (EpCAM) [8] thereby making EpCAM-expressing EVs (EpCAM+EVs) a proxy for tumor cells, while leukocyte-derived EVs express the common leukocyte marker CD45, thereby making CD45+EVs a proxy for either activated or damaged leukocytes. Moreover, it has been reported that cell–cell fusion in the ovarian cancer microenvironment leads to development of tumor cells carrying both EpCAM and CD45 on the cell surface and that this phenotype is less sensitive to platinum-based standard chemotherapy [9].

In this study, we analyzed circulating levels of EpCAM+ and CD45+ EVs in women with HGSOC at diagnosis and after 21 days from first administration of chemotherapy with paclitaxel and carboplatin, in relation to the disease chemosensitivity and prognosis.

Materials and methods

Patients

This study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Ethics Committee of the Fondazione Policlinico A.Gemelli IRCCS, Rome, Italy (ID: 2564 date of approval 18/07/2019).

Patients with histologically confirmed diagnosis of HGSOC FIGO stage III/IV, referred to the Unit of Gynecologic Oncology of the Fondazione Policlinico A. Gemelli IRCCS of Rome, Italy, from August to October 2019 and undergoing six cycles of standard platinum-based chemotherapy, once every 3 weeks, were enrolled in this study. Exclusion criteria were: age <18 years, unable to give written informed consent, presence of synchronous neoplasms. Written informed consent was obtained from all recruited patients.

Sample collection

All patients with HGSOC enrolled in this study underwent venous blood sampling, by venipuncture from the antecubital fossa, before and after 21 days from the first cycle of chemotherapy with paclitaxel and carboplatin. Blood samples were collected in a VACUETTE® test tube with sodium citrate for plasma (2 mL). Each blood sample was double centrifuged at 1800 g for 15 min at 20° to remove cellular components. Plasma samples were then frozen at −80 °C, until further use.

Flow cytometric analysis of EVs

To analyze circulating EVs, staining was performed according to the International Society for Extracellular Vesicles (ISEV) guidelines [10]. To identify tumor-derived and leukocyte-derived EVs, we used anti-CD326 (EpCAM) PE (phycoerythrin)-conjugated murine monoclonal antibody (mAb) (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and anti-CD45 APC (allophycocyanin)-conjugated mAb (Becton Dickinson, Franklin Lakes, New Jersey, USA), respectively. The mAbs were used at a final dilution of 1:40. A cocktail of the mAbs was prepared on each experimental day and centrifuged at 13,000xg at +4 °C for 15 min. Plasma samples were thawed, v/v diluted with 100 nm-filtered phosphate buffered saline (PBS) and incubated in the dark at 4 °C for 1 h with the mAb cocktail. Appropriate isotype controls were also used. As additional staining control, samples were run after disruption of EVs membranes

with 1% Triton-X- 100, to verify disappearance of positive events. The set-up of the instrument was performed as detailed elsewhere [11]. Briefly, the best gains for the photodiodes, which detect fluorescence and VSSC-A signals, were determined using the 8-Peak Rainbow Beads (BD Biosciences, CA, USA) and Megamix-Plus FSC beads (BioCytek Marseille, France), respectively. EVs were analyzed by a CytoFLEX (Beckman Coulter, Brea, CA, USA) cytometer equipped with violet (405 nM), blue (488 nM), yellow-green (561 nM), and red (638 nM) laser excitation sources. EVs concentration was calculated using the volumetric measurement featured in the CytoFLEX.

Statistical analysis

Data are shown as the mean ± standard deviation (SD) or percentage (%), depending on the type of variable. All data were analyzed for distribution characteristics using the Shapiro-Wilk test and analyzed by Chi-squared test, Student's t-test, Mann-Whitney U (Wilcoxon) or Kruskal-Wallis statistics, as appropriate, performed with Prism software version 10. For all analyses, $p < 0.05$ was considered significant. The sensitivity and specificity of EpCAM+ and/or CD45+ EVs to diagnose platinum sensitivity were analyzed using the Receiver Operating Characteristics (ROC) curve and its area under the curve (AUC) value with 95% confidence intervals (95% CI). Statistical significance of the predictive value was assessed in comparison to the test with zero predictive value (AUC= 0.5).

Results

Clinical results

Twenty-eight patients with diagnosis of HGSOC were enrolled. The clinical characteristics of the study population are shown in Table 1.

Clinical characteristics of HGSOC patients, according to platinum- sensitivity, -resistance or -refractory, are shown in Table 2. As expected, platinum-sensitive patients underwent maintenance therapy more frequently and showed longer platinum-free survival (PFS), disease-free survival (DFS) and overall survival (OS) than platinum-resistant and -refractory patients. Furthermore, all platinum-resistant and -refractory patients displayed a BRCA1/2 wild type status.

Pre-chemotherapy plasma EpCAM+ and CD45+ EVs are lower in platinum-sensitive compared to platinum-resistant HGSOC patients

The plasma levels of EpCAM+, CD45+ and EpCAM+CD45+ EVs in platinum-sensitive HGSOC patients (n=17) at diagnosis were significantly lower than in platinum-resistant (n=6) patients (p<0.01; Fig. 1a-c

Table 1 Clinical characteristics of the study population

	HGSOC (n=28)
Age at diagnosis	62.2 ± 9.5
BMI (Kg/m2)	23.5 ± 2.7
Previous cancer	2 (7.1%)
Breast cancer	1 (50%)
Gastrointestinal Stromal Tumors	1 (50%)
Thyroid cancer	0 (0%)
Comorbidity	
Hypertension	7 (25%)
Hypercholesterolemia	4 (14.3%)
Hypertrigliceridemia	2 (7.1%)
Heart disease	1 (3.6%)
Depression	1 (3.6%)
Diabetes mellitus	2 (7.1%)
BRCA 1 mut	6 (21.4%)
BRCA 2 mut	6 (21.4%)
BRCA wt	16 (57.2%)
Surgery	19 (67.9%)
PDS	8 (28.6%)
IDS	11 (39.3%)
TR	4 (14.3%)
≤ 1 cm	3 (75%)
> 1 cm	1 (25%)
Fist line chemotherapy	
Carboplatin+Taxol	14 (50%)
Carboplatin+Taxol+Bevacizumab	10 (35.7%)
Carboplatin+Taxol+Gemcitabin+Bevacizumab	4 (14.3%)
Maintenance therapy	20 (71.4%)
Niraparib	2 (10%)
Bevacizumab	6 (30%)
Olaparib	5 (25%)
Bevacizumab+Olaparib	2 (10%)
Others	5 (25%)
FIGO stage	
IIIC	20 (71.4%)
IVA	3 (10.7%)
IVB	5 (17.9%)
Prognosis	
Recurrence	24 (85.7%)
PFI (months)	12.5 ± 10.9
DFS (months)	25.8 ± 15.2
OS (months)	39.9 ± 19.4

Results are expressed as mean ± SD, % or Median (min-max) according to type of variables

HGSOC High-grade serous ovarian cancer, *BMI* body mass index, *BRCA* breast cancer genes, *mut* mutated, *wt* wild type, *PDS* Primary debulking surgery, *IDS* Interval debulking surgery, *TR* tumor residual, *FIGO* International Federation of Gynaecology and Obstetrics, *PFI* platinum-free interval, *DFS* disease free survival, *OS* overall survival

Table 2 Comparison of clinical characteristics of HGSOC patients in relation to platinum-sensitivity

	Sensitive (*n*=17)	Resistant (*n*=6)	Refractory (*n*=5)	*p* value
Age at diagnosis (yrs)	62.6 ± 10.1	60.5 ± 8.6	62.8 ± 10.2	0.8
BMI (Kg/m2)	23.3 ± 2.8	25.2 ± 2.1	22.2 ± 2.6	0.2
Previous cancer	1 (5.9%)	0 (0%)	1 (20%)	0.4
Breast cancer	0 (0%)	0 (0%)	1 (100%)	-
Gastrointestinal Stromal Tumors	1 (100%)	0 (0%)	0 (0%)	-
Thyroid cancer	0 (0%)	0 (0%)	0 (0%)	-
Comorbidity				
Hypertension	4 (23.5%)	1 (16.7%)	2 (40%)	0.6
Hypercholesterolemia	2 (11.8%)	1 (16.7%)	1 (20%)	0.9
Hypertrigliceridemia	1 (5.9%)	1 (16.7%)	0 (0%)	0.5
Heart disease	1 (5.9%)	0 (0%)	0 (0%)	0.7
Depression	1 (5.9%)	0 (0%)	0 (0%)	0.7
Diabetes mellitus	0 (0%)	1 (16.7%)	1 (20%)	0.2
BRCA 1 mut	5 (29.4%)	0 (0%)	0 (0%)	0.1
BRCA 2 mut	5 (29.4%)	0 (0%)	0 (0%)	0.1
BRCA wt	7 (41.2%)	6 (100%)	5 (100%)	**< 0.01**
Surgery				
PDS	7 (41.2%)	1 (16.7%)	0 (0%)	0.1
IDS	7 (41.2%)	1 (16.7%)	3 (60%)	0.3
TR	2 (11.8%)	0 (0%)	2 (40%)	0.1
0[a]	15 (88.2%)	6 (100%)	3 (60%)	0.1
≤ 1 cm	2 (11.8%)	0 (0%)	1 (20%)	-
> 1 cm	0 (0%)	0 (0%)	1 (20%)	-
Fist line chemotherapy				
Carboplatin Taxol	8 (47%)	3 (50%)	3 (60%)	0.9
Carboplatin Taxol Bevacizumab	7 (41.2%)	1 (16.7%)	2 (40%)	0.5
Others	2 (11.8%)	2 (33.3%)	0 (0%)	0.2
Maintenance therapy	14 (82.3%)	5 (83.3%)	1 (20%)	**< 0.05**
Niraparib	2 (14.3%)	0 (0%)	0 (0%)	0.6
Bevacizumab	4 (28.6%)	2 (40%)	0 (0%)	0.7
Olaparib	4 (28.6%)	1 (20%)	0 (0%)	0.8
Bevacizumab+Olaparib	1 (7.1%)	0 (0%)	1 (100%)	**< 0.01**
Others	3 (21.4%)	2 (40%)	0 (0%)	0.6
FIGO stage				
IIIC	14 (82.3%)	4 (66.6%)	2 (40%)	0.2
IVA	1 (5.9%)	1 (16.7%)	1 (20%)	0.6
IVB	2 (11.8%)	1 (16.7%)	2 (40%)	0.3
Prognosis				
Recurrence	14 (82.3%)	6 (100%)	4 (80%)	0.5
PFI (months)	18.5 ± 10.9	5 ± 0	3.2 ± 1.3	**< 0.0001**
DFS (months)	30.3 ± 15.6	15.7 ± 8.2	9.5 ± 3.5	**< 0.05**
OS (months)	44.9 ± 17.6	22.5 ± 9.2	14.2± 3.6	**< 0.01**

Results are expressed as mean ± SD, % or Median (min-max) according to type of variables

HGSOC High-grade serous ovarian cancer, *BMI* body mass index, *BRCA* breast cancer genes, *mut* mutated, *wt* wild type, *PDS* Primary debulking surgery, *IDS* Interval debulking surgery, *TR* tumor residual, *FIGO* International Federation of Gynaecology and Obstetrics, *PFI* platinum-free interval, *DFS* disease free survival, *OS* overall survival

[a] 0 absence of tumor residual after debulking surgery

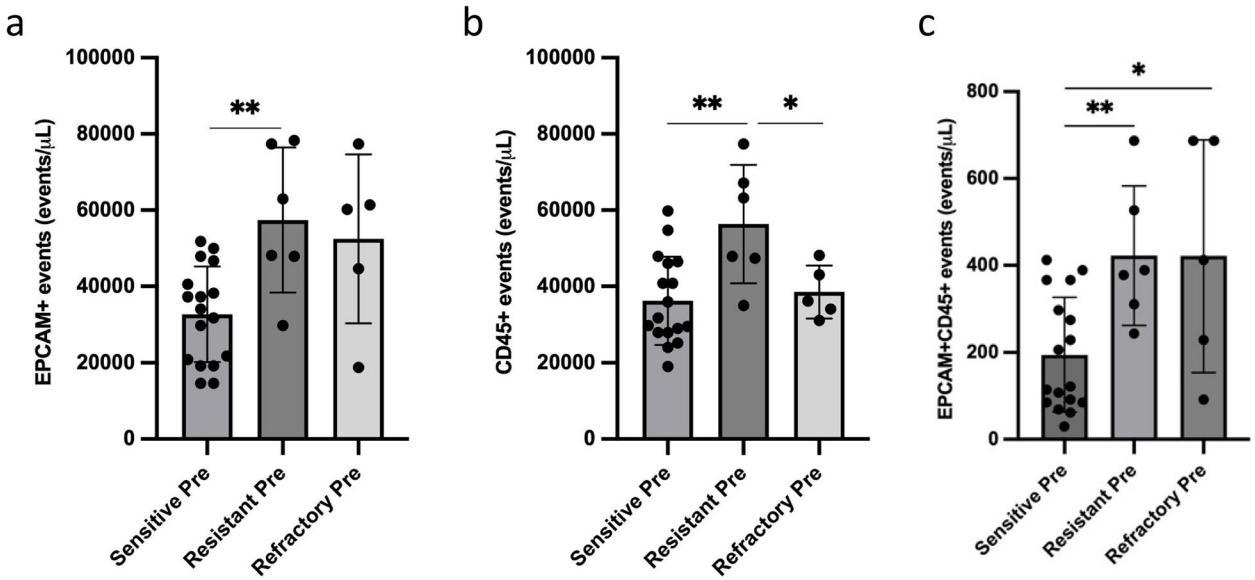

Fig. 1 **a**-**c** Scatter plots representing pre-chemotherapy plasma levels of EpCAM+ EVs (**a**), CD45+ EVs (**b**) and double positive EpCAM+CD45+ EVs (**c**) in platinum-sensitive (n=17), platinum-resistant (n=6) and platinum-refractory (n=5) patients. **a** Pre-chemotherapy plasma levels of EpCAM+ EVs across patients' groups. **b** Pre-chemotherapy plasma levels of CD45+ EVs across patients' groups. **c** Pre-chemotherapy plasma levels of double-positive EpCAM+CD45+EVs across patients' groups. Results are expressed as mean +/- SD or median, according to type of variables. Pre: pre-chemotherapy; Post: post chemotherapy; * p<0.05; ** p<0.01

and Fig. S1). Plasma CD45+ EVs were less abundant in platinum-refractory (n=5) than platinum-resistant patients (p<0.05; Fig. 1b). Platinum-refractory HGSOC patients showed higher plasma levels of EpCAM+CD45+ EVs at diagnosis compared to platinum-sensitive patients (p<0.05; Fig. 1c).

EpCAM+ and CD45+ EVs plasma levels increase in platinum-sensitive HGSOC patients three weeks post-chemotherapy

Plasma levels of EpCAM+EVs and CD45+EVs significantly increased three weeks post-chemotherapy in platinum-sensitive HGSOC patients compared to pre-treatment levels (p<0.05 and p<0.01, respectively; Fig. 2a and d). No significant differences of EpCAM+ and CD45+ EVs were observed between pre- and post-chemotherapy, in platinum-resistant or platinum-refractory HGSOC patients (Fig. 2b-c and e-f). No significant differences were found in $EpCAM^{+}CD45^{+}$ EVs plasma levels between before-after chemotherapy in platinum-sensitive, -resistant or –refractory HGSOC patients (Fig. 2g-i).

Diagnostic performance of plasma EpCAM+ and CD45+EVs levels in platinum-sensitivity

The predictive value of plasma levels of EpCAM+ and CD45+ EVs pre-chemotherapy for platinum sensitivity were assessed by ROC analysis. The area under the curve (AUC) was 0.82 (95% CI 0.64–0.99; p=0.005) and 0.76 (95% CI 0.59–0.94; p=0.021), respectively (Fig. 3a-b). The sensitivity and specificity of EPCAM+ EVS were 76.5% and 81.8% (cut off= 42594 events/µl). For CD45+ EVs, the sensitivity was 53% and the specificity 91% (cut off= 32976 events/µl). The analysis of the diagnostic value of double positive EpCAM+CD45+ EVs plasma levels at diagnosis in platinum-resistance/refractory showed an AUC of 0.79 (95% CI 0.61–0.98; p=0.01) (Fig. 3c) with a sensitivity of 59% and a specificity of 91% (cut off= 217.6 events/µl).

Discussion

Circulating tumor-derived EVs are an appealing analytical target in the context of tumor patient management as they represent a liquid biopsy of their cells of origin and may therefore be regarded as a potential diagnostic and prognostic tool. The role of tumor-derived EVs as biomarkers in ovarian cancer patients is currently under intensive investigation using a variety of techniques [12].

By this approach, in this study we showed that platinum-resistant patients with HGSOC had higher levels of plasma EpCAM+ EVs pre-chemotherapy compared to platinum-sensitive patients, likely reflecting a more elevated tumor cell turnover in these tumors. This is a novel finding that extends in vitro studies showing that the more aggressive ovarian cancer cells secrete higher amounts of EVs and demonstrating an inherent link between EVs release and platinum-resistance [13, 14].

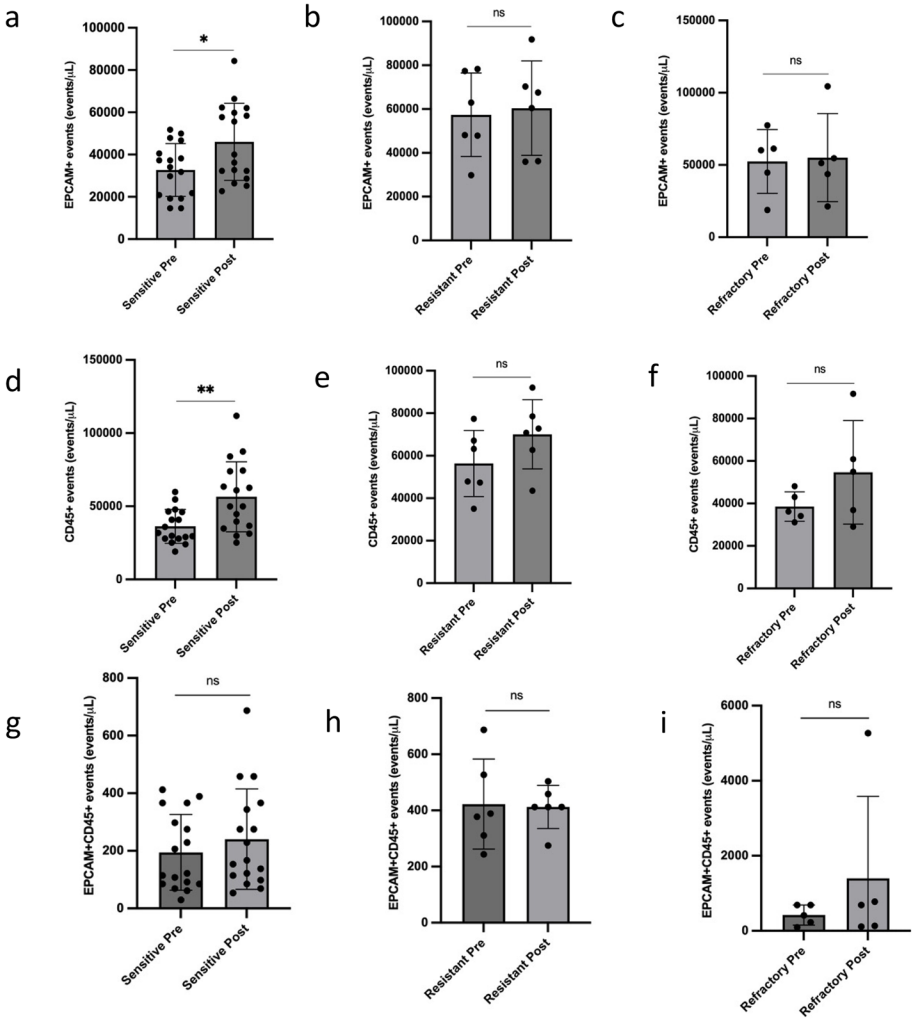

Fig. 2 Scatter plots representing pre- and post- chemotherapy plasma levels of EpCAM+ EVs (**a**-**c**), CD45+ EVs (**d**-**f**) and double positive EPCAM+CD45+ EVs (**g**-**i**) in platinum-sensitive (*n*=17), -resistant (*n*=6) and -refractory patients (*n*=5). **a**-**c** Pre- and post-chemotherapy plasma levels of EpCAM+ EVs across patients' groups. **d**-**f** Pre- and post-chemotherapy plasma levels of CD45+ EVs across patients' groups. **g**-**i** Pre- and post-chemotherapy plasma levels of double positive EpCAM+CD45+ EVs across patients' groups. Results are expressed as mean +/- SD or median, according to type of variables. Pre: pre-chemotherapy; Post: post chemotherapy; ns: not significant; * $p<0.05$; ** $p<0.01$

Plasma CD45+ EVs were less abundant in platinum-sensitive than resistant patients. It may be speculated that this reflected an ongoing immune cell activation attempting to counteract tumor growth. Conversely, the scarce immune response that characterizes the most aggressive OC tumors may explain the very low CD45+EVs plasma level observed in refractory tumors [15, 16].

It has been reported that tumor cell carrying both EpCAM and CD45 on the cell surface and that this phenotype is less sensitive to platinum-based standard chemotherapy, thereby pointing to EpCAM+/CD45+EVs as a valid marker of drug resistance [9]. Consistently to this observation, we found higher circulating levels EpCAM+/CD45+ EVs in resistant to refractory HGSOC patients compared to sensitive patients, supporting the hypothesis that this EVs phenotype is specific to more aggressive tumors.

To directly explore how chemotherapy administration influenced EVs release, we compared the EpCAM+, CD45+ and EpCAM+/CD45+ EVs plasma levels pre-chemotherapy and after 21 days from the first cycle of chemotherapy with carboplatin and taxol. Chemotherapy increased the plasma level of EpCAM+ and CD45+ EVs in platinum-sensitive patients. This result was expected, suggesting an enhanced shedding from tumor cells consistent to a higher susceptibility to platinum-based regimen. Indeed, it is well documented that EVs shedding increases upon exposure to various cell stressors [6].

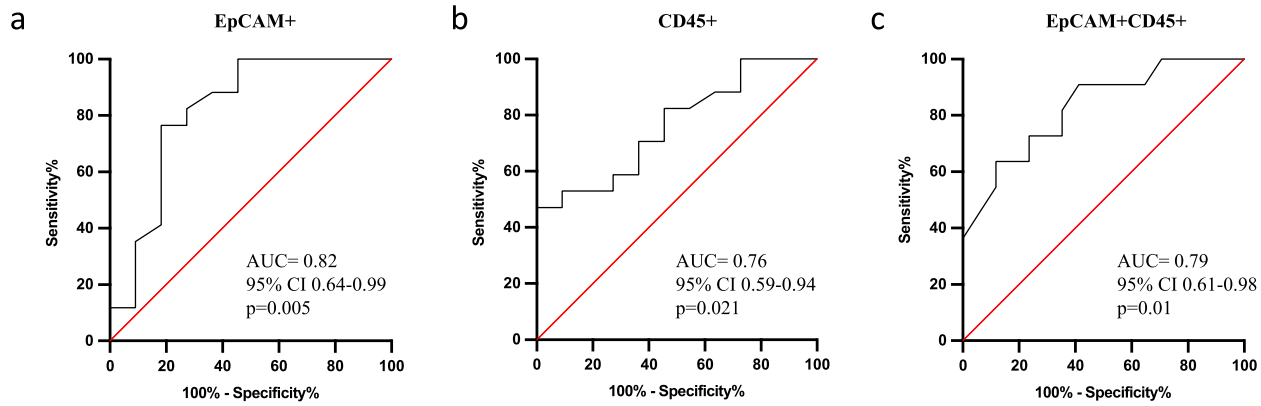

Fig. 3 AUC analysis assessing the predictive performance of pre-chemotherapy EV levels. **a**-**b** Performance of EpCAM+ (**a**) or CD45+ (**b**) EVs pre-chemotherapy levels in the prediction of platinum sensitivity. **c** Performance of EpCAM+CD45+ EVs pre-chemotherapy levels in the prediction of platinum resistance/refractory. Red line: reference line

On the other hand, migration and infiltration of ovarian tumor tissue by T-cell is a known mechanism of immunological recognition and response to tumor cells and correlates with improved overall survival in epithelial ovarian cancer ([15, 16, 6]. Thus, the increased shedding of CD45+ EVs in platinum-sensitive patients might be explained by a more intense activation of immune response against tumor and a more extended tumor infiltration by leukocyte, following chemotherapy, which is a known platinum-induced effect [17].

The assessment of the diagnostic performance of EpCAM+ and CD45+ EVs levels at diagnosis in platinum sensitivity provided promising results. In addition, assessment of circulating levels at diagnosis of EpCAM+CD45+ revealed a good diagnostic performance of platinum–resistance/refractory.

Conclusions

In conclusions, this pilot study provides preliminary results pointing to EpCAM+ and CD45+ EVs as possible predictive marker of platinum-sensitivity in HGSOC. The main limit of the study is the small population analyzed. Further research is needed to confirm this preliminary observation in a larger cohort of patients.

Abbreviations

HGSOC	High-grade Serous Ovarian Cancer
EVs	Extracellular Vesicles
EpCAM	Epithelial Cell Adhesion Molecule
FIGO	International Federation of Gynecology and Obstetrics
CD45	Leucocyte common antigen
ISEV	International Society for Extracellular Vesicles
PE	Phycoerythrin
mAb	Monoclonal Antibody
APC	Allophycocyanin
SD	Standard Deviation
ROC	Receiver Operating Characteristics
AUC	Area Under Curve
CI	Confidence Intervals
BMI	Body Mass Index
BRCA	Breast Cancer Genes
Mut	Mutated
Wt	Wild type
PDS	Primary Debulking Surgery
IDS	Interval Debulking Surgery
TR	Tumor Residual
PFI	Platinum-free Interval
DFS	Disease Free Survival
OS	Overall Survival
Fig	Figure

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13048-025-01656-9.

Supplementary Material 1: Fig. S1. Representative flow cytometry analysis of pre-chemotherapy plasma levels. (A-C) Representative bivariate R660H (y-axis) and 585H (x-axis) pseudocolor plots showing the flow cytometry approach for evaluation of EVs derived from leukocytes and tumour cells of pre-chemotherapy plasma levels in a platinum-sensitive patient. EVs staining positively for CD45 were identified as being of leukocyte origin, whereas EVs staining positively for EpCAM and double positive EpCAM+/CD45+ were identified as being of tumor origin.In all plots, regions 1, 2 and 3 display CD45+, EpCAM+CD45+ and EpCAM+ events, respectively. A) Unstained plasma control. B) CD45 (y-axis) and EpCAM (x-axis) antibodies: The counts of CD45+, EpCAM+CD45+ and EpCAM+ events/microL were computed using the volumetric measurement featured in the flow cytometer. In this sample, region 1 contained 91,600 events/µL, region 2 contained 5267 events/µL and region 3 contained 104.424 events/µL. C) Same sample as in B run after incubation with 1% Triton-X-100 to verify disappearance of positive events.

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Clinical trial number

Not applicable.

Authors' contributions

Conceptualization, C.T. and A.Fatt; methodology, C.T., M.O., A.Buz. and A. Fer.; software, M.O. and C.T.; validation, C.T., A.Fatt. and A.B; formal analysis, C.T. and M.O.; investigation, C.T., M.O.,G.B., F.S., and A.P.; resources, A.Fag. and G.S.; data

curation, M.O., C.N., G.B., F.S. and A.P.; writing—original draft preparation, C.T. and M.O.; writing—review and editing, C.T., M.O., A.Fatt. A.B. and G.C.; visualization, A.Fag. and G.S.; supervision, A.Fag. and G.S.; project administration, C.T.; funding acquisition, G.S. and A.Fag. All authors have read and agreed to the published version of the manuscript.

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Data availability
Data is provided within the manuscript.

Declarations

6Ethics approval and consent to participate
This study was approved by the Ethics Committee of the Fondazione Policlinico Universitario A. Gemelli IRCCS, Italy (IRB ID 2564, date of approval 18/07/2019). Written informed consent was obtained from all recruited patients.

Consent for publication
All authors have read and agreed to the published version of the manuscript.

Competing interests
The authors declare no competing interests.

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