uPAR⁺ extracellular vesicles: a robust biomarker of resistance to checkpoint inhibitor immunotherapy in metastatic melanoma patients

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ABSTRACT

Background Emerging evidence has highlighted the importance of extracellular vesicle (EV)-based biomarkers of resistance to immunotherapy with checkpoint inhibitors in metastatic melanoma. Considering the tumor-promoting implications of urokinase-type plasminogen activator receptor (uPAR) signaling, this study aimed to assess uPAR expression in the plasma-derived EVs of patients with metastatic melanoma to determine its potential correlation with clinical outcomes.

Methods Blood samples from 71 patients with metastatic melanoma were collected before initiating immunotherapy. Tumor-derived and immune cell-derived EVs were isolated and analyzed to assess the relative percentage of uPAR⁺ EVs. The associations between uPAR and clinical outcomes, sex, BRAF status, baseline lactate dehydrogenase levels and number of metastatic sites were assessed.

Results Responders had a significantly lower percentage of tumor-derived, dendritic cell (DC)-derived and CD8⁺ T cell-derived uPAR⁺ EVs at baseline than non-responders. The Kaplan-Meier survival curves for the uPAR⁺ EV quartiles indicated that higher levels of melanoma-derived uPAR⁺ EVs were strongly correlated with poorer progression-free survival ($p < 0.0001$) and overall survival ($p < 0.0001$). We also found a statistically significant correlation between lower levels of uPAR⁺ EVs from both CD8⁺ T cells and DCs and better survival.

Conclusions Our results indicate that higher levels of tumor-derived, DC-derived and CD8⁺ T cell-derived uPAR⁺ EVs in non-responders may represent a new biomarker of innate resistance to immunotherapy with checkpoint inhibitors. Moreover, uPAR⁺ EVs represent a new potential target for future therapeutic approaches.

BACKGROUND

Metastatic melanoma (MM) is one of the most aggressive types of cancer. In MM, identification of the BRAF mutation status provides guidance for treatment decisions.^{1 2} First-line therapy in patients with BRAF-V600 mutation is targeted therapy with a combination of BRAF-V600 inhibitors and MEK inhibitors

or immunotherapy with immune checkpoint inhibitors (ICIs) eg, anti-programmed cell death protein 1 (PD-1), anti-programmed death-ligand 1 (PD-L1) and anti-cytotoxic T-lymphocyte-associated protein 4 (CTLA-4).^{3 4} Patients without BRAF mutations are candidates for ICI therapy. Both targeted therapy and immunotherapy have provided substantial survival benefits in patients with MM; however, most patients develop resistance, and a durable response is limited to 30%–40%.⁵ Thus, there is an urgent clinical need to search for novel biomarkers for a better selection of patients who can respond to selected therapy. If these biomarkers can be detected by liquid biopsy through a simple blood test, their value increases significantly.

Among the prognostic/predictive biomarkers, high PDL-1 expression is associated with better clinical outcomes of ICI therapy,⁶ whereas high levels of lactate dehydrogenase (LDH), which is found in approximately 50% patients with MM, frequently correlate with unresponsiveness to targeted immunotherapy^{7 8} and with a worse prognosis in patients with MM.⁹ However, no other prognostic or predictive biomarkers have been identified in MM, although good performance status, low tumor burden and complete response (CR) has been reported to be associated with better overall response and survival to both targeted therapy and immunotherapy.

The detection and analysis of cell-free circulating nucleic acids, exosomes and microvesicles released into the peripheral blood from tumors represent a change in the paradigm of personalized medicine in cancer.^{10 11} Some circulating exosomes released from tumor cells carry specific biomarkers as their content reflects the nature and status of their cells of

origin. Further, exosomes transfer to adjacent cells in the tumor microenvironment (TME) or to distant recipient cells, modulating the intracellular signaling pathways, gene expression and phenotype of cells.¹² Exosomes derived from melanoma cells have been shown to influence the cells in the TME by shifting the phenotype and function of normal immune cells from an antitumor state to a protumor state and by playing a role in modulating the response to immunotherapy.¹³

Regarding response to therapy, circulating extracellular vesicle (EV)-based biomarkers derived from tumor or immune cells have demonstrated promising prospects in predicting the response to antitumor therapy with ICIs in patients with MM.^{14–16} In patients with MM treated with ipilimumab, the higher baseline levels of T-cell-derived PD-1⁺ and CD28⁺ exosomes were associated with response to therapy in a previous study.¹¹ Recently, the release of PD-L1-expressing exosomes from cancer cells has emerged as a novel mechanism of resistance to ICIs, confirming that the identification of exosome-based biomarkers is an important tool for the prediction of response to immunotherapy in patients with melanoma.¹⁴ However, there is a pitfall in exosome studies, as reported in the 2018 international society for extracellular vesicles (ISEV) guideline position paper, due to the lack of pure exosome separation with current techniques and in the definition of the commonly used term ‘exosomes’, which should be replaced with the term EVs, consisting mainly of exosomes and microvesicles with similar properties.¹⁷

Extensive evidence has highlighted the involvement of urokinase-type plasminogen activator receptor (uPAR), a main factor of the fibrinolytic system, in tumor progression and metastasis.^{18,19} Furthermore, uPAR has been associated with immunity and inflammation²⁰ and has crucial implications for tumor development.^{21,22} uPAR is considered a negative prognostic marker for many tumors and its circulating levels correlate with response to therapy, such as hormone treatment in patients with estrogen receptor-positive breast cancer and bevacizumab treatment in patients with MM.^{23,24} Recently, Zhou *et al* demonstrated an increase in exosomal uPAR mRNA expression in the plasma of patients with gefitinib-resistant non-small-cell lung cancer (NSCLC) compared with that in patients with gefitinib-sensitive NSCLC,²⁵ underlining the importance of uPAR as an EV-based biomarker. However, limited information is available regarding its involvement in EV signaling and there are no data on the implication and role of uPAR⁺EVs in the development of MM and in response to ICI therapy.

The relationship between the burden of blood uPAR⁺ EVs and clinical outcomes in patients treated with ICIs has not been investigated. Thus, we performed a baseline assessment of various EV populations, including melanoma uPAR⁺ EVs, in a single-center cohort of patients with MM treated with anti-PD1 to verify whether uPAR⁺ EVs can play a role as predictive markers of ICI response or mediate immunotherapy resistance.

METHODS

Patients and study design

The prospective study enrolled patients from the IRCCS Istituto Tumori Giovanni Paolo II in Bari, Italy. Written informed consent was obtained from all patients enrolled in the study. A total of 71 patients with MM were enrolled in this study. All patients were treated with the ICIs nivolumab (38 patients), pembrolizumab (27 patients) or nivolumab plus ipilimumab (6 patients). Patients were recruited between January 2017 and January 2020. The main patient characteristics evaluated included sex, age at diagnosis, metastatic disease, origin of primary melanoma, BRAF genotype evaluation, Eastern Cooperative Oncology Group performance status, M stage, number of metastatic sites and LDH level before ICI administration (table 1).

Objective tumor responses were assessed by instrumental analysis using CT or MRI. Clinical responses were assessed and reported as CR, partial response (PR), stable disease (SD) and progressive disease (PD) based on the Response Evaluation Criteria in Solid Tumors V.1.1.²⁶ We also evaluated the overall response rate as CR plus PR; the disease control rate (DCR), defined as CR plus PR plus SD lasting >6 months; progression-free survival (PFS) and overall survival (OS). PFS was defined as the time from the first treatment cycle to disease progression or death, and OS was defined as the time from the first treatment cycle to death or the last follow-up.

Pembrolizumab was administered at 2 mg/kg every 3 weeks or at a flat dose of 200 mg every 3 weeks, and nivolumab was administered at 3 mg/kg every 2 weeks or at a flat dose of 240 mg every 2 weeks or at 480 mg every 4 weeks. In the combination of nivolumab and ipilimumab, the first dose was administered at 1 mg/kg, the other doses were administered at 3 mg/kg or 1 mg/kg every 3 weeks for a total of four doses.

A total of 48% (n=34) patients had received previous systemic therapy including anti-BRAF/anti-MEK in 23 patients with BRAF mutation, chemotherapy in three patients, and ipilimumab in five patients. A total of 28% (n=20) patients had received subsequent therapeutic lines after anti-PD-1/anti-CTLA4 therapy.

We collected blood samples before initiating ICI therapy in all patients.

Blood and plasma sample collection

Peripheral blood was obtained in a sodium citrate tube before therapy (baseline) (76 patients), at the best response (19 patients) and at tumor progression (12 patients). After blood collection, the blood samples were incubated for 30 min at 4°C and centrifuged at 2500 rpm for 15 min.

Peripheral blood mononuclear cell (PBMC) isolation

PBMCs were separated from 5 to 8 mL of peripheral blood using Ficoll-Hypaque gradient centrifugation. They were then resuspended in phosphate-buffered saline (PBS) in a 1:1 ratio, carefully layered on top of the Ficoll-Hypaque

Table 1 Summary of clinical characteristics and outcomes to anti PD-1 immunotherapy of the study cohort (n=71)

Age at diagnosis, median, years	56 (32–86)
Age at metastatic disease, median, years	60 (33–86)
Gender, n (%)	
Male	35 (49.3)
Female	36 (50.7)
Stage at diagnosis, n (%)	
I/II	19 (26.8)
III	24 (33.8)
IV	16 (22.5)
Primary unknown	12 (16.9)
Origin of melanoma, n (%)	
Cutaneous	51 (71.8)
Non-cutaneous	20 (28.2)
BRAF status, n (%)	
Mutated	34 (47.9)
wt	37 (52.1)
Previous systemic therapy for metastatic disease, n (%)	
Yes	34 (47.9)
No	37 (52.1)
Stage at metastatic disease*, n (%)	
M1a	14 (19.7)
M1b	9 (12.7)
M1c	31 (43.7)
M1d	17 (23.9)
No of metastatic sites*, n (%)	
1–2	39 (54.9)
≥3	32 (45.1)
PS (ECOG)*, n (%)	
0	32 (45.1)
1	33 (46.5)
2	6 (8.4)
LDH, n (%)	
1 xULN	35 (49.3)
2xULN	28 (39.4)
>2xULN	7 (9.9)
Unspecified	1 (1.4)
Best response, n (%)	
ORR	29 (40.8)
DCR	33 (46.5)
CR	8 (27.6)
PD	38 (53.5)
PFS median, months	4
OS median, months	11

CR, complete response; DCR, disease control rate; ECOG, Eastern Cooperative Oncology Group ; LDH, lactate dehydrogenase; ORR, overall response rate; OS, overall survival; PD, progressive disease; PFS, progression-free survival; PS, Performance Status ; ULN, upper limit of normal.

and processed as previously described.²⁷ The isolated cells were stored at -195°C .

EV isolation

EVs were isolated from the plasma by ultracentrifugation. In particular, 5 mL of plasma was collected and centrifuged at $2600\times g$ for 15 min to remove cell debris and apoptotic bodies, as previously described.²⁸ The supernatant fractions were diluted with an equal volume of PBS and filtered using 200 nm pore size filters to eliminate larger EVs. The resulting plasma was ultracentrifuged twice at $100\,000\times g$ for 70 min, and the pellet was resuspended in PBS. The pooled EVs were stored at -80°C .

Nanoparticle tracking analysis

Samples were analyzed using NanoSight NS300 (Malvern Panalytical) according to the manufacturer's instructions (NanoSight NS300 User Manual, MAN0541-02-EN, 2018).²⁹ Briefly, 5–10 μL of each EV sample was diluted, resulting in a particle per frame rate of 10–50 particles/frame. Three 30 s videos were recorded of flowing particles using the CMOS camera and the embedded 532 nm CW green laser. NanoSight software (Nanoparticle Tracking Analysis-NTA 3.4 Build 3.4.003) was used to analyze the videos (screen gain and detection threshold settings yielded 10–100 distinct particle cores with fewer than five false positives). All measurements were performed with an optimized setting by the same experienced operator to achieve comparable results.

PBMC characterisation by flow cytometry

The isolated PBMCs were preincubated for 30 min at 2°C – 8°C in the dark, with 5 μL of Super Bright Complete Staining Buffer to prevent non-specific polymer interactions. Then, the cells were labeled with antihuman antibodies and stored for another 30 min at 2°C – 8°C in the dark. After staining, the labeled PBMCs were washed with PBS, collected and analyzed using an Attune NxT Acoustic Focusing Cytometer (ThermoFisher) equipped with four lasers (405 nm (violet), 488 nm (blue), 561 nm (yellow) and 637 nm (red)) for sample reading. The final data were analyzed using Attune NxT Analysis Software (ThermoFisher).³⁰

EVs characterisation by flow cytometry

Each EVs sample was preincubated with 5 μL of Super Bright Complete Staining Buffer. Then, the EVs were labeled with antihuman antibodies. After staining, EVs were collected and analyzed using an Attune NxT acoustic focusing cytometer (ThermoFisher), as described above.

Primary labeled antibodies

The following primary labeled antibodies were obtained from eBiosciences (Thermo Fisher Scientific, Waltham, Massachusetts, USA): anti-human-CD9 (FITC), anti-human-CD63 (PE-CYN7), anti-human-CD81 (APC), anti-human-CD87 (PERCP-EF710), anti-human-CD146 (PE), anti-human-CD1a (eFluor-450), anti-human-CD8

(PE-CYN5), anti-human-CD14 (PE-EF610) and anti-human-CD19 (EF506).

The biomarkers chosen to evaluate the melanoma samples were selected based on a preliminary characterization performed on EVs; these were released from a panel of melanoma cell lines after incubation for 24 hours in a complete medium with serum without EVs. For each cell line, the percentage of EVs that were positive for one of three well-known biomarkers of melanoma—S100,^{31–33} CD146^{31 34 35} and Pmel17^{31 33}—in addition to the three tetraspanins CD9, CD63 and CD81 ('classic' biomarkers of EVs) was measured by flow cytometry (FCM). The results showed variable expression of S100, CD146 and Pmel17. Analysis of the dot plots to detect the double positives (CD146⁺/S100⁺; CD146⁺/Pmel17⁺ and S100⁺/Pmel17⁺) indicated that all the EVs positive for S100 or Pmel17 were also positive for CD146⁺, while some EVs positive for CD146 were not positive for S100 or Pmel17. Therefore, the EVs labeled with CD146 (median value, 92% of the total EVs) also included those double labeled for the other two markers of melanoma and were the most representative population; therefore, they were suitable for the analysis of melanoma EVs in the plasma.

Statistical analysis

Statistical significance was calculated using two-tailed t-tests, analysis of variance, Kruskal-Wallis tests, Dunn tests and Mann-Whitney U tests. Statistical significance was set at $p < 0.05$ (* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$). Statistical analyses were performed using GraphPad Prism V.5.0 software (GraphPad Software, San Diego, California, USA). Survival analyses were performed using R V.3.62. Kaplan-Meier survival analyses and Coxhazard regression analyses were performed using the R package 'survival'. A pairwise comparison of Kaplan-Meier curves was also performed using the R package 'survminer'. Kaplan-Meier curves were depicted using the R package 'survminer', and the forest plot for multivariate Cox analyses was drawn using the R package 'forest model'. The R package 'ggplot2' was used to depict bar plots. The proportion test was used to compare the clinical responses.

RESULTS

Clinical outcomes

For the evaluation of uPAR⁺ EVs, we enrolled 71 patients with MM who were candidates for ICI therapy. Regarding clinical outcomes, 29 (40.8%) of 71 patients had a response, with 8 patients achieving CR and 21 patients achieving PR. The DCR, including CR, PR and SD >6 months, was 46.5% ($n = 33$), whereas the PD rate was 53.5% ($n = 38$). The median PFS of the entire population was 4 months, and the median OS was 11 months. Patient characteristics and outcomes of ICI therapy are summarized in [table 1](#).

EV purification and characterisation

EVs from the plasma of each patient enrolled in the study were isolated by ultracentrifugation³⁶ and characterized for size and concentration by NTA. In [figure 1A](#), three examples of NTA characterization of plasma-purified EVs are shown. More than 80% of the EVs samples were smaller than 200 nm. The histograms in [figure 1A](#) are representative of all the plasma samples. Therefore, we have generically used the term EVs to denote a mixture of small EVs composed mainly (>80%) of EVs measuring <200 nm, compatible with exosomes measuring 40–200 nm.³⁷

FCM characterization of the EVs consisted of the analysis of the expression levels of three exosome-related tetraspanins—CD9, CD63 and CD81.³⁶ As shown in [figure 1B](#), EVs from all plasma samples were mainly positive for CD81 (median value: 84.17% vs 66.56% and 52.72% for CD9⁺ and CD63⁺, respectively). Moreover, we analyzed the distribution of the double-positive EVs, CD9⁺/CD63⁺, CD9⁺/CD81⁺, and CD63⁺/CD81⁺ and the results, reported in [figure 1C](#) along with the median values, showed that CD63⁺/CD81⁺ was the highest representative population in plasma-derived EVs (median value: 35.58% vs 12.40% and 11.25% for CD9⁺/CD63⁺ and CD9⁺/CD81⁺, respectively). All the data shown in this manuscript refer to EVs positive for at least one of the three tetraspanins.

The awareness of the great heterogeneity in the cells of origin of these EVs suggested classifying them as originating from melanoma cells and immune cells, including CD8⁺ T cells, B cells, monocytes and dendritic cells (DCs). In [figure 1D](#), the distribution of EVs of different origin isolated from the plasma of patients with MM responding and not responding to ICI therapy showed that only EVs originating from melanoma, CD8⁺ T cells and DCs were statistically higher in non-responders than in responders.

Since the levels of PDL-1⁺ EVs in the plasma of patients with MM have been hypothesized as biomarkers of ICI resistance,¹⁴ we preliminarily characterized the PDL-1⁺ EVs in the plasma of patients with MM before initiating ICI therapy. The results, reported in [figure 1E](#), showed that the level of PDL-1⁺ EVs was higher in responders than in non-responders, in contrast with the literature results.¹⁴ Thus, we deepened the analysis by focusing on the percentage of PDL-1⁺ EVs released by melanoma and immune cells in responders and non-responders. The sum of PDL-1⁺ EVs released by melanoma, CD8⁺ T cells, B cells, monocytes and DCs in responders and non-responders is shown in [figure 1F](#), demonstrating that these EVs are statistically higher in non-responders than in responders, in agreement with the results reported by Chen.¹⁴

Baseline immune cell populations, uPAR-positive cells and uPAR⁺ EVs in the plasma of patients with MM

Before the analysis of the levels of uPAR⁺ EVs released in the plasma of 71 patients with MM enrolled in the study, we compared the baseline circulating frequencies of immune cells as well as their uPAR expression between responders and non-responders. PBMCs were isolated from the blood

Figure 1 EVs characterization by NTA and FCM analysis. (A) Representative NTA histograms reporting size and concentration of EVs purified from plasma samples at basal level of 3 mM patients. Scatter plots with median showing the percentage of (B) CD9⁺EVs, CD81⁺EVs and CD63⁺EVs and (C) double positive CD9⁺/CD81⁺ EVs, CD9⁺/CD63⁺ EVs, and CD81⁺/CD63⁺ EVs in 71 patients analyzed by FCM. (D) Scatter plot with median showing the percentage of uPAR⁺ EVs derived from melanoma cells (CD146⁺), T cells (CD8⁺) and B cells (CD19⁺), monocytes (CD14⁺) and DC (CD1a⁺) in responders (n=38) and non-responders (n=33). (E) Scatter plot of PDL-1⁺ EVs population in responders (n=38) and non-responders (n=33) and (F) of the sum of PDL-1⁺ EVs originating from melanoma cells, CD8⁺ T cells, B cells, monocytes and DCs in responders (n=190) and non-responders (n=165) (**p<0.01, ***p<0.001). DC, dendritic cell; EVs, extracellular vesicle; FCM, flow cytometry; NTA, nanoparticle tracking analysis.

of six non-responders and five responders. The immune cell populations of uPAR⁺ cells and the uPAR⁺ EVs released by them were analyzed by FCM, revealing no difference between the immune cell populations in both responders and non-responders (figure 2A). CD8⁺T cells were more abundant than B lymphocytes and monocytes, while DCs were less abundant in all patients analyzed (figure 2A,B). Moreover, determination of uPAR positivity in immune cells showed that uPAR was expressed in most immune cells (figure 2B). In addition, only CD8⁺ T cells and DCs released statistically different levels of uPAR⁺ EVs between responders and non-responders to ICI (figure 2C).

High levels of uPAR⁺EVs indicate poor clinical outcomes of immunotherapy

The uPAR⁺ EVs isolated from the plasma of 71 patients with MM were analyzed, showing no differences between

responders or non-responders (figure 3A). Conversely, when the analysis was performed by discriminating by cells of origin, a statistically significant difference was found between responders and non-responders for uPAR⁺ EVs from melanoma cells, CD8⁺ T cells and DCs (figure 3B), but not from monocytes and B cells (data not shown). The sum of uPAR⁺ EVs released from melanoma cells, CD8⁺ T cells and DCs, as shown in figure 3C, remained statistically significant between responders and non-responders, with a median value of 0.31% vs 12.5%, respectively.

We then correlated the levels of uPAR⁺ EVs of different origins according to their median PFS and OS (4 and 11 months, respectively). The Kaplan-Meier survival curves for the uPAR⁺ EV quartiles are shown in figure 4A,B. We demonstrated that higher levels of melanoma cell-derived

Figure 2 PBMCs characterization by FCM analysis. Scatter plots with medians showing the percentage of immune cell populations (A), uPAR⁺ immune cells (B) and uPAR⁺ EVs derived from PBMC (C) in responders and non-responders obtained by FCM analysis (** p<0.01). EVs, extracellular vesicle; FCM, flow cytometry; uPAR, urokinase-type plasminogen activator receptor.

uPAR⁺ EVs were strongly correlated with poorer PFS (p<0.0001) and OS (p<0.0001). We also found a statistically significant correlation among lower levels of uPAR⁺

EVs from both CD8⁺ T cells and DCs, and better survival. Conversely, uPAR⁺ EVs from other immune cells did not correlate with outcomes (data not shown).

Figure 3 uPAR⁺ EVs derived from different cell types released in the plasma of patients clustered by response to therapy. (A) Scatter plot with median showing the percentage of uPAR⁺ EVs released in the plasma of responders (n=38) and non-responders (n=33). (B) Scatter plots with medians showing the FCM analysis of uPAR⁺ EVs (%) derived from melanoma cells and immune cells of non-responders (n=33) and responders (n=38) and (C) showing the sum uPAR⁺ EVs released from melanoma cells, CD8⁺ T cells and DCs of responders (n=114) and non-responders (n=99) (***p<0.001). DC, dendritic cell; EVs, extracellular vesicle; uPAR, urokinase-type plasminogen activator receptor.

Figure 4 Evaluation of PFS and OS in patients with uPAR⁺EVs derived from melanoma cells and immune cells. Kaplan-Meier survival curve analysis according to uPAR⁺ EVs quartiles, with uPAR⁺EVs from melanoma cells, CD8⁺ T cells and DCs as respect to PFS (A) and OS (B). For each analysis, a pairwise comparison of curves has been performed. P values are shown in tables below the graph. The distribution of the best responses stratifying patients by quartiles of uPAR⁺EVs from melanoma cells (C), CD8⁺ T cells (D) and DCs (E), respectively. (F) Multivariate Cox hazard regression analysis for OS. DC, dendritic cell; EVs, extracellular vesicles; OS, overall survival; PFS, progression-free survival; uPAR, urokinase-type plasminogen activator receptor.

Figure 4C–E shows the distribution of the best responses, with patients stratified by quartiles of uPAR⁺EVs from melanoma cells, CD8⁺ T cells and DCs. Only the proportions of non-responders were statistically compared in the quartiles. Regarding uPAR⁺ EVs from melanoma cells, the proportion of PD patients significantly increased ($p<0.0001$) along the quartile stratification (5.5%, 55.5%, 70.5% and 83.3%). Similar results were observed when we focused on uPAR⁺ EVs from CD8⁺ T cells (11.1%, 55.5%, 70.5%, 77.7%, $p=0.0002$) and DCs (22.2%, 55.5%, 64.7%, 72.2%, $p=0.01$).

We then assessed the correlation of PFS and OS with uPAR⁺EVs and several clinic pathological features. To further investigate these correlations, patients were classified according to uPAR⁺ EV level quartiles. Univariate analysis revealed a significantly shorter PFS and OS in patients with elevated levels of melanoma cell-derived uPAR⁺ EVs, with progressively higher significance in the higher quartiles (HR 6.6, 95% CI 2.8 to 15.9, $p=1.8-5$ and HR 34.9, 95% CI 4.5 to 267.8, $p=0.00061$, respectively, in the fourth quartile). We also found a similar strong correlation between uPAR⁺ EVs from CD8⁺ T cells and clinical outcomes (HR 5.0, 95% CI 2.1 to 11.4, $p=0.00015$ and HR 13.8, 95% CI 3.1 to 60.9, $p=0.00053$ for PFS and OS, respectively, in the fourth quartile). A similar but weaker correlation was found between OS and uPAR⁺ EVs from DCs and B cells. Among the clinical features, having more than three metastatic sites was also significantly associated with a poorer OS (HR 2.0, 95% CI 1.0 to 3.6, $p=0.025$) (table 2).

In multivariate Cox regression analysis of OS, including the uPAR⁺ EV quartiles from the different cell types and the number of metastatic sites, both uPAR⁺ EVs from CD8⁺ T cells and those from DCs were independently associated with OS (figure 4F). In addition, uPAR⁺ EVs from melanoma cells and B lymphocytes were nearly statistically significant. No significant results were found in Cox hazard regression analysis for PFS (online supplemental figure S1).

Correlation between uPAR⁺ EVs and LDH levels

Since LDH is a tumor marker accepted as a validated prognostic and predictive factor in patients with melanoma,³⁸ we investigated if the circulating uPAR⁺ EVs were consistent with the levels of LDH before the administration of ICIs. We categorized the study patients into the normal LDH group ($n=37$) and higher LDH group ($n=33$) according to the normal range. As shown in online supplemental figure S2A, we found a small statistically significant increase in uPAR⁺ EVs from CD8⁺ T cells and from DCs in the higher LDH group, with a median uPAR⁺ EVs expression value of 1.12% vs 10.23% and 0.29% vs 7.27% in patients with normal and high LDH levels, respectively. Conversely, in monocytes, a statistically significant reduction was evident in uPAR⁺ EVs from non-responders compared with those from responders. No difference was observed in the EV populations of melanoma cells and B cells.

Therefore, we decided to further investigate the possible correlation between uPAR⁺EVs and LDH levels as a function of the response to immunotherapy. Among patients with normal LDH levels, we observed a significantly lower level of uPAR⁺ EVs isolated from melanoma cells, T cells, and DCs of responders (DCR) than of non-responders (PD) (online supplemental figure S2B). The median value of uPAR⁺ EVs in PD vs DCR was 17.64% vs 8.24% (EVs from melanoma cell), 9.98% vs 0.07% (EVs from T cells), and 3.82% vs 0.07% (EVs from DCs). Although a decrease in the percentage of uPAR⁺ EVs from monocytes and B cells was also observed, these results were not statistically significant (data not shown). Even in patients with higher LDH plasma levels, we confirmed the trend of a lower level of uPAR⁺ EVs from the DCR patients compared with PD patients (data not shown), which was statistically significant when the uPAR⁺ EVs were from melanoma cells (15.21% in PD vs 11.39% in DCR) (online supplemental figure S2C).

BRAF status, number of metastatic sites and sex did not affect the release of uPAR⁺ EVs

Since an important mechanism of reduced sensitivity to BRAF inhibitors, driven by elevated levels of uPAR in melanoma cells, has been recently described,¹ we investigated uPAR⁺ EVs as a function of BRAF mutation status. As reported in table 1, half of the patients enrolled in the study had BRAF mutation. However, FCM analysis showed that there were no differences in the percentage of uPAR⁺ EV expression between BRAF wild type and BRAF mutation patients in all cell populations analyzed (online supplemental figure S3).

Furthermore, since uPAR is recognized as one of the greatest proinvasive factors in tumors,¹ we investigated the correlation between uPAR⁺ EVs and the number of metastatic sites. The patients were divided into two categories—(1) those with one or two metastatic sites and ii) those with three or more metastatic sites (table 1). As shown in online supplemental figure S3, we did not find any correlation between uPAR⁺ EVs and the number of metastatic sites, despite poorer OS being reported in patients with three or more metastatic sites (HR=2.001, 95% CI 1.087 to 3.685, $p=0.025$) than in those with one or two metastatic sites (table 2).

Currently, sex is one of the most intriguing prognostic factors in melanoma. In recent years, several biological mechanisms that could contribute to sex disparities in melanoma outcomes have been proposed. In particular, significant differences exist in melanoma mortality between men and women. Women experience longer survival and have a better outcome and response to immunotherapy than men.³⁹ Therefore, we evaluated whether there could be a correlation between uPAR⁺ EVs derived from different cell populations and sex. As shown in online supplemental figure S3, no correlation was found between uPAR⁺ EVs and sex in the different EV populations.

Table 2 Univariate analysis related to PFS and OS

	Univariate PFS		Univariate OS	
	HR (95% CI)	P value	HR (95% CI)	P value
uPAR⁺ EVs from melanoma				
First quartile		Ref		Ref
Second quartile	2.166 (0.8816 to 5.319)	0.091963	15.69 (2.037 to 120.8)	0.008214
Third quartile	4.758 (2.0121 to 11.253)	0.000382	24.81 (3.259 to 188.9)	0.001930
Fourth quartile	6.695 (2.8076 to 15.966)	1.8e ^{-0.5}	34.99 (4.572 to 267.8)	0.000618
uPAR⁺ EVs from B cells				
First quartile		Ref		Ref
Second quartile	2.428 (1.1151 to 5.287)	0.0255	3.276 (1.2528 to 8.566)	0.0155
Third quartile	1.959 (0.8736 to 4.393)	0.1027	2.801 (1.0322 to 7.600)	0.0432
Fourth quartile	1.690 (0.7560 to 3.778)	0.2011	2.5360 (0.9342 to 6.884)	0.0678
uPAR⁺ EVs from CD8⁺T cells				
First quartile		Ref		Ref
Second quartile	1.759 (0.7364 to 4.201)	0.203706	6.945 (1.537 to 31.38)	0.11767
Third quartile	3.071 (1.3359 to 7.061)	0.008245	11.451 (2.596 to 50.51)	0.001281
Fourth quartile	5.014 (2.1778 to 11.543)	0.000151	13.803 (3.125 to 60.97)	0.000534
uPAR⁺ EVs from DC				
First quartile		Ref		Ref
Second quartile	1.258 (0.5514 to 2.869)	0.58578	1.981 (0.6763 to 5.806)	0.2125
Third quartile	1.725 (0.7695 to 3.868)	0.18554	3.331 (1.1819 to 9.385)	0.0228
Fourth quartile	3.285 (1.5054 to 7.167)	0.00281	3.642 (1.3060 to 10.154)	0.0135
BRAF				
wt		Ref		Ref
Mutated	1.179 (0.6765 to 2.056)	0.561	1.454 (0.7738 to 2.732)	0.245
No of metastatic sites				
1–2		Ref		Ref
≥3	1.547 (0.9001 to 2.66)	0.114	2.001 (1.087 to 3.685)	0.0259
LDH				
<ULN		Ref		Ref
>ULN	0.8219 (0.476 to 1.419)	0.482	0.9127 (0.4941 to 1.686)	0.771
Previous therapy for metastatic disease				
No		Ref		Ref
Yes	1.017 (0.5909 to 1.75)	0.952	1.499 (0.8137 to 2.762)	0.194

DC, dendritic cell; EVs, extracellular vesicles; HR, higher quartiles; LDH, lactate dehydrogenase; OS, overall survival; PFS, progression-free survival; ULN, upper limit of normal; uPAR, urokinase-type plasminogen activator receptor.

uPAR⁺ EVs in naive versus pretreated patients

As described in [table 1](#), 34% patients enrolled in this study had undergone previous systemic therapy for metastatic disease. Therefore, we investigated whether the expression of uPAR⁺ EVs could change as a function of previous pharmacological treatments.

In the EVs derived from all cell populations of non-responders, we observed an increasing trend of uPAR⁺ EVs in pretreated patients compared with naive patients, except for EVs from DCs. Conversely, an opposite

distribution was found in responders with reduced levels of uPAR⁺ EVs among pretreated patients (online supplemental figure S4).

DISCUSSION

Tumor-derived EVs are able to condition cells residing close to tumors and at distant sites to facilitate tumor progression. Melanoma cell-derived EVs educate normal cells, induce the formation of premetastatic niches⁴⁰ and,

when expressed low PDL-1 levels, are associated with better clinical outcomes following ICI treatments.^{14 41}

From this perspective, particularly if tumor tissues are not available, the parsing of EV profiles could have very promising implications for the early diagnosis of cancer and prediction and monitoring of a patient's response to antitumor therapy. Here, we isolated EVs from the plasma of patients with MM before initiating ICI therapy to investigate whether a well-known mediator of cancer invasiveness and progression, uPAR, was present on the surface of EVs released by cancer cells and immune cells.

First, we analyzed the percentage of CD8⁺ T cells, B cells, monocytes and DCs in PBMCs isolated from the blood of patients with MM as well as the percentage of uPAR expression in each immune cell population. However, we failed to reveal any differences between the percentage of each of them between responders and non-responders.

We then analyzed the levels of PDL-1⁺ and uPAR⁺ EVs in the plasma samples from 71 patients with MM before they initiated immunotherapy. Initially, the EVs from the plasma were characterized by their expression of the three tetraspanins CD9, CD63 and CD81,⁴² and the analysis showed that they were enriched mainly in CD81, as already reported by Muhsin-Sharafaldine,⁴³ and that they were coexpressed with CD63 in >50% of samples. EVs from blood samples of patients drawn before immunotherapy were characterized by their cell of origin (melanoma cells and immune cells such as CD8⁺ T cells and B cells, monocytes and DCs) and by the presence of PDL-1 and uPAR in responders and non-responders. The analysis showed a statistically significant increase in the release of EVs from cancer cells, CD8⁺ T cells and DCs in non-responders. We confirmed that there was a statistically significant higher release of PDL-1⁺ EVs from melanoma cells and immune cells in the plasma of non-responders than in the plasma of responders, as suggested by Chen *et al.*, who hypothesized that high level of such EVs might be a predictor of the lack of responses to ICI.¹⁴

Our findings showed that even if the basal levels of uPAR⁺ EVs in responders and non-responders were similar, responders had significantly lower basal levels of uPAR⁺ EVs from melanoma cells, CD8⁺ T cells and DCs than non-responders. The Kaplan-Meier survival curves indicated that the OS and PFS were significantly poorer in patients with MM with high levels uPAR⁺ EVs from melanoma cells, CD8⁺ T cells and DCs. Furthermore, univariate analysis confirmed the correlation of uPAR⁺ EVs with OS and PFS, demonstrating that the levels of these uPAR⁺ EVs were inversely correlated with therapy outcomes, with a progressively higher significance according at higher quartiles of EVs originating from melanoma cells, CD8⁺ T cells and DCs.

This evidence, together with the significant association of three or more metastatic sites with a poorer OS and the absence of a correlation with other parameters such as sex, BRAF mutation status and pharmacological pretreatment of patients, suggest that the percentage of uPAR⁺ EVs from melanoma cells, CD8⁺ T cells and DCs is related

to the intrinsic characteristics of each group of patients (responders and non-responders).

Considering that tumor-derived exosomes regulate the maturation, migration, and differentiation of immune cells,³³ their emerging role as biomarkers of response to immunotherapy,^{13 34} and the tumor-promoting implications related to uPAR signaling,³⁵ our results lend credence to the hypothesis that higher levels of uPAR⁺ EVs from tumor cells, DC and CD8⁺ T cells of non-responders may represent a condition that counters anti-tumor immunity systemically and implicates these as new biomarkers of innate resistance to ICIs.

As a receptor for uPA, uPAR overexpression, both as membrane-bound and soluble receptors, has been demonstrated to increase cell-surface proteolysis by increasing the ability of migrating cells to degrade barriers, such as the basal membrane, thus favoring the migration of immune cells and interactions with the extracellular matrix (ECM).³² The uPA/uPAR axis plays a crucial role in the differentiation of immune cells. The uPA/uPAR axis drives the differentiation of DCs to become fully mature antigen presenting cells,³⁶ which respond to microenvironment stimuli, and migrate through the ECM to reach the inflammation site. After maturation, uPAR is normally downregulated and less functional in DCs; however, uPA/uPAR reactivation is involved in the reverse transmigration of immature DCs.³⁶ Thus, functionally, we can speculate that the increase in the percentage of uPAR⁺ EVs from DCs in non-responders reflects the reactivation of a signaling in such cells, which could contribute to the recirculation of DCs and their removal from the TME. Similar to DCs, uPAR expression is important for the recruitment and priming of T cells.³⁷ However, although CD8⁺ T cell-derived EVs that express uPAR reflect cells may be prone to recruitment, they are dysfunctional cells. A possible explanation for this has been recently suggested by Laurenzana *et al.*⁴⁴ They demonstrated that uPAR overexpression drives a glycolytic and invasive phenotype in melanoma cells⁴⁴; hence, tumors which release uPAR⁺ EVs are considered glycolytic tumors that dramatically alter the TME by causing glucose deprivation, a condition that suppresses antitumor immune cell functions, considering that T cells are dependent on glycolytic metabolism for the maintenance of immune functions.^{40 41}

To date, only LDH has been suggested as a metabolic and immune surveillance prognostic biomarker.³⁸ High levels of LDH correlate with the metabolic switch of tumor cells from oxidative phosphorylation (OXPHOS) metabolism to glycolysis, through which LDH lowers the intratumoral pH, promoting an immunosuppressive TME with reduced infiltration of DCs, natural killer cells, cytotoxic CD8⁺ T cells and suppression of interferon-gamma expression.³⁹ Accordingly, we found a significant correlation between high amounts of CD8⁺/uPAR⁺ and CD1a⁺/uPAR⁺ circulating EVs and unresponsiveness to immunotherapy in patients who had high levels of blood LDH. However, we also found higher levels of uPAR⁺ EV in non-responders than in responders, irrespective of their blood LDH. The

percentage of melanoma cell-derived, DC-derived and T cell-derived uPAR⁺EVs in non-responders with normal LDH levels was higher than that in responders, suggesting no direct correlation between LDH values with uPAR⁺ EV profiles and responses to therapy. Therefore, similar to LDH blood values, we suggest that the burden of circulating uPAR⁺ EVs released by tumor cells, DC and CD8⁺T cells in the presence of normal LDH levels may identify tumors harboring a glycolytic trait at basal level, which is not reflected in the blood LDH level, but defines naturally refractory patients to immunotherapy.

Although further studies addressing the molecular mechanisms underlying the role of uPAR⁺ EVs as an innate resistance biomarker to immunotherapy with ICIs in patients with MM would be needed and could lead to novel anticancer approaches, the analysis reported herein might optimize the stratification of patients who should have a better response to this anticancer approach. The non-statistically valid results of the multivariate analysis could depend on a modest cohort size; thus, we intend to continue to recruit patients with MM to validate our endpoints in a broader case series. However, our hypothesis-driven prospective and retrospective design and the strength of the associations reported enhanced the intrinsic value of the results. Moreover, in the future, we would like to investigate whether the predictive role of uPAR⁺ EVs that originate from tumor cells, CD8⁺ T cells and DCs could be valid in other cancer diseases as a general biomarker that is easily detectable by liquid biopsy to perform patient stratification for immunotherapy.

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