uPAR\textsuperscript{+} 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\textsuperscript{+} 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\textsuperscript{+} T cell-derived uPAR\textsuperscript{+} EVs at baseline than non-responders. The Kaplan-Meier survival curves for the uPAR\textsuperscript{+} EV quartiles indicated that higher levels of melanoma-derived uPAR\textsuperscript{+} 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\textsuperscript{+} EVs from both CD8\textsuperscript{+} T cells and DCs and better survival.

**Conclusions** Our results indicate that higher levels of tumor-derived, DC-derived and CD8\textsuperscript{+} T cell-derived uPAR\textsuperscript{+} EVs in non-responders may represent a new biomarker of innate resistance to immunotherapy with checkpoint inhibitors. Moreover, uPAR\textsuperscript{+} 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.\textsuperscript{1,2} First-line therapy in patients with BRAF\textsuperscript{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).\textsuperscript{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\%.\textsuperscript{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,\textsuperscript{6} whereas high levels of lactate dehydrogenase (LDH), which is found in approximately 50\% patients with MM, frequently correlate with unresponsiveness to targeted immunotherapy\textsuperscript{7,8} and with a worse prognosis in patients with MM.\textsuperscript{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. 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. 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.

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. 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. 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. 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.

Extensive evidence has highlighted the involvement of urokinase-type plasminogen activator receptor (uPAR), a major factor of the fibrinolytic system, in tumor progression and metastasis. Furthermore, uPAR has been associated with immunity and inflammation and has crucial implications for tumor development. 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. 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, 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. 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 patient 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
EV isolation
EVs were isolated from the plasma by ultracentrifugation. In particular, 5 mL of plasma was collected and centrifuged at 2600×g for 15 min to remove cell debris and apoptotic bodies, as previously described. 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×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). 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 nonspecific 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).

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-EP710), anti-human-CD146 (PE), anti-human-CD1a (eFluor-450), anti-human-CD8

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

| Characteristic                              | Value                  |
|---------------------------------------------|------------------------|
| 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)              |
| 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)              |
| 2 xULN                                      | 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.
(PE-CY5), 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, CD146, and Pmel17—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 Cox hazard 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 plot’. 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 ultracentrifugation 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.

FCM characterization of the EVs consisted of the analysis of the expression levels of three exosome-related tetraspanins—CD9, CD63 and CD81. 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, 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. 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.

**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
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
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 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.

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 clinical 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, 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,4 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 (2) 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.
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**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 niches and,

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**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) | 1.86^{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.
Confirmed that there was a statistically significant higher in responders and non-
sized that high level of such EVs might be a predictor of
noma cells, CD8+ T cells and DCs. Furthermore, univar-
evans CD9, CD63 and CD81,42 and the anal-
ysis 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, mono-
cytes 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 hypothe-
sized 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
divided that the OS and PFS were significantly poorer in
patients with MM with high levels uPAR+ EVs from mel-
anoma cells, CD8+ T cells and DCs. Furthermore, univar-
ate 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 pretreat-
ment 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, their emerging role as biomarkers of response to
immunotherapy, and the tumor-promoting implications related to uPAR signaling, 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 demon-
strated 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 microenvi-
ronment 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 transmigra-
tion of immature DCs.36 Thus, functionally, we can spec-
ulate 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.4They demonstrated that uPAR over-
expression drives a glycolytic and invasive phenotype in
melanoma cells; 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+ circu-
lating 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.
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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REFERENCES
1 Laurenzana A, Margheri F, Biagioni A, et al. EGFR/uPAR interaction as drug target to overcome vemurafenib acquired resistance in melanoma cells. EBioMedicine 2019;39:194–206.
2 LoRusso PM, Schaller K, Kosman J. Targeted therapy and immunotherapy: emerging biomarkers in metastatic melanoma. Pigment Cell Melanoma Res 2020;33:390–402.
3 Hodi FS, O’Day SJ, McDermott DF, et al. Improved survival with ipilimumab in patients with metastatic melanoma. N Engl J Med Overseas Ed 2010;363:711–23.
4 Anon. Abstracts. Pigment Cell Melanoma Res 2020;33:148–255.
5 Robert C, Schachter J, Long GV, et al. Pembrolizumab versus ipilimumab in advanced melanoma. N Engl J Med 2015;372:2521–32.
6 Morrison C, Paba S, Conroy JM, et al. Predicting response to checkpoint inhibitors in melanoma beyond PD-L1 and mutational burden. J Immunother Cancer 2018;6:902.
7 Guida M, Bartolomeo N, De Risio I, et al. The management of oligoprogression in the landscape of new therapies for metastatic melanoma. Cancers 2019;11:1559.
8 Diem S, Kasenda B, Span L, et al. Serum lactate dehydrogenase as an early marker for outcome in patients treated with anti-PD-1 therapy in metastatic melanoma. Br J Cancer 2016;114:256–61.
9 Agarwala SS, Keilholz U, Gilles E, et al. Ldh correlation with survival in advanced melanoma from two large, randomised trials (Oblimersen GMS01 and EORTC 18951). Eur J Cancer 2009;45:1807–14.
10 Alegre E, Zubiri L, Perez-Gracia JL, et al. Circulating melanoma exosomes as diagnostic and prognosis biomarkers. Clin Chim Acta 2016;454:28–32.
11 Tucci M, Mannavola F, Passarelli A, et al. Exosomes in melanoma: a role in tumor progression, metastasis and impaired immune system activity. Oncotarget 2019;8:20526–37.
12 Fernández-Lázaro D, García Hernández JL, García AC, et al. Liquid biopsy as novel tool in precision medicine: Origins, properties, identification and clinical perspective of cancer's biomarkers.

Diagnostics 2020;10:215.

13 Pretti MAM, Bernardes SS, da Cruz JGV, et al. Extracellular vesicle-mediated crosstalk between melanoma and the immune system: impact on tumor progression and therapy response. J Leukoc Biol 2020;108:1101–15.
14 Chen G, Huang AC, Zhang W, et al. Exosomal PD-L1 contributes to immunosuppression and is associated with anti-PD-1 response. Nature 2018;560:382–6.
15 Huber V, Vallacchi V, Fleming V, et al. Tumor-Derived microRNAs induce myeloid suppressor cells and predict immunotherapy resistance in melanoma. J Clin Invest 2018;128:5055–16.
16 Del Re M, Marconcini R, Pasquini G, et al. Pd-L1 mRNA expression in plasma-derived exosomes is associated with response to anti-PD-1 antibodies in melanoma and NSCLC. Br J Cancer 2018;118:820–4.
17 Thery C, Witwer KW, Aikawa E, et al. Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the International Society for extracellular vesicles and update of the MISEV2014 guidelines. J Extracell Vesicles 2018;7:1353750.
18 Beleva E, Grudeva-Popova J. From Virchow’s triad to metastasis: circulating hematopoietic factors as predictors of risk for metastasis in solid tumors. J Buon 2013;18:25–33.
Open access

19 Mahmoud N, Mihalciou C, Rabbani SA. Multifaceted role of the urokinase-type plasminogen activator (uPA) and its receptor (uPAR): diagnostic, prognostic, and therapeutic applications. Front Oncol 2018;8:24.

20 Del Rosso M, Margheri F, Serrati S, et al. The urokinase receptor system, a key regulator at the intersection between inflammation, immunity, and coagulation. Curr Pharm Des 2011;17:1924–43.

21 Heissig B, Eiamboonsert S, Salama Y, et al. Cancer therapy targeting the fibrinolytic system. Adv Drug Deliv Rev 2016;99:172–9.

22 Mondino A, Blasi F, uPA and uPAR in fibrinolysis, immunity and pathology. Trends Immunol 2004;25:450–5.

23 Maduni J. The urokinase plasminogen activator system in human cancers: an overview of its prognostic and predictive role. Thromb Haemost 2018;118:2020–36.

24 Schuster C, Akslen LA, Stokowy T, et al. Predictive value of angiogenic proteins in patients with metastatic melanoma treated with bevacizumab monotherapy. J Pathol Clin Res 2019;5:53–62.

25 Zhou J, Kwak KJ, Wu Z, et al. PLAUR confers resistance to gefitinib through EGFR/P- AKT/Survivin signaling pathway. Cell Physiol Biochem 2018;47:1905–24.

26 Eisenhauer EA, Therasse P, Bogaerts J, et al. New response evaluation criteria in solid tumours: revised RECIST guideline (version 1.1). Eur J Cancer 2009;45:228–47.

27 Vecchione A, Di Fonte R, Gerosa J, et al. Reduced PD-1 expression on circulating follicular and conventional FOXP3+ Treg cells in children with new onset type 1 diabetes and autoantibody-positive at-risk children. Clin Immunol 2020;211:108319.

28 Biagioni A, Laurennzana A, Menicacci B, et al. uPAR-expressing melanoma exosomes promote angiogenesis by VE-cadherin, EGFR and uPAR overexpression and rise of ERK1,2 signaling in endothelial cells. Cell Mol Life Sci 2021;78:3057–72.

29 Bachurski D, Schulders M, Nguyen P-H, et al. Extracellular vesicle measurements with nanoparticle tracking analysis - An accuracy and repeatability comparison between NanoSight NS300 and ZetaView. J Extracell Vesicles 2019;8:1596016.

30 Gorgun C, Reverberi D, Rotta G, et al. Isolation and flow cytometry characterization of Extracellular-Vesicle subpopulations derived from human mesenchymal stromal cells. Curr Protoc Stem Cell Biol 2019;48:e786.

31 Ordóñez NG. Value of melanocytic-associated immunohistochemical markers in the diagnosis of malignant melanoma: a review and update. Hum Pathol 2014;45:191–205.

32 Harpio R, Emansson R. S100 proteins as cancer biomarkers with focus on S100B in malignant melanoma. Clin Biochem 2004;37:512–8.

33 Viray H, Bradley WR, Schalper KA, et al. Marginal and joint distributions of S100, HMB-45, and Melan-A across a large series of cutaneous melanomas. Arch Pathol Lab Med 2013;137:1063–73.

34 Lei X, Guan C-W, Song Y, et al. The multifaceted role of CD146/MCAM in the promotion of melanoma progression. Cancer Cell Int 2015;15:3.

35 Rapanotti MC, Campione E, Spallone G, et al. Minimal residual disease in melanoma: circulating melanoma cells and predictive role of MCAM/MUC18/MelCAM/CD146. Cell Death Discov 2017;3:17005.

36 Kowal EJK, Ter-Ovanesyan D, Regev A, et al. Extracellular vesicle isolation and analysis by Western blotting. Methods Mol Biol 2017;1660:143–52.

37 Kalishwaralal K, Kwon WY, Park KS. Exosomes for non-invasive cancer monitoring. Biotechnol J 2019;14:1800430.

38 Diaz-Lagares A, Alegre E, Arroyo A, et al. Evaluation of multiple serum markers in advanced melanoma. Tumour Biol 2011;32:1155–61.

39 Nosratil MT, Wei ML. Sex disparities in melanoma outcomes: the role of biology. Arch Biochem Biophys 2014;563:42–50.

40 Peinado H, Alevčević K, Lavotshkin S, et al. Melanoma exosomes educate bone marrow progenitor cells toward a pro-metastatic phenotype through Met. Nat Med 2012;18:883–91.

41 Mathew M, Zade M, Mezghani N, et al. Extracellular vesicles as biomarkers in cancer immunotherapy. Cancers (Basel) [Internet] 2020;12:1–21 https://www.mdpi.com/2072-6694/12/10/2825.

42 Surman M, Stupieie E, Przybylko M. Melanoma-Derived extracellular vesicles: focus on their proteome. Proteomes 2019;7:21.

43 Muhsin-Sharafaldine M-R, Sauderson SC, Dunn AG, et al. Procoagulant and immunogenic properties of melanoma exosomes, microvesicles and apoptotic vesicles. Oncotarget 2016;7:56279–94.

44 Laurenzana A, Chilla A, Luciani C, et al. uPA/uPAR system activation drives a glycolytic phenotype in melanoma cells. Int J Cancer 2017;141:1190–200.