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PSA reactivity in extracellular microvesicles to commercial immunoassays

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ABSTRACT

Aims: Characterization of PSA in extracellular microvesicles (EVs) and its reactivity to commercial methods.
Materials and methods: EVs derived from serum of 47 prostate cancer (PCa) patients, 27 benign prostatic hyperplasia (BPH) patients and 42 healthy controls were analyzed. EVs isolation and quantification of PSA immunoreactive to total (ev-T-PSA) or free (ev-F-PSA) PSA immunoassays, were performed using commercial assays. PSA in CD81+ or CD63+ EVs was determined directly in serum by an immunocapture-ELISA (IC-ELISA).
Results: Ev-T-PSA immunoreactive to Elecsys assay was detected in all samples. Median T-PSA ev/srm ratio was 2.20 % (Q1-Q3: 0.80–4.00 %), although in some samples this ratio reached 59 %. T-PSA ev/srm ratio was higher in those samples with serum T-PSA below 4 μg/L than in those exceeding that cut-off ($p < 0.001$). T-PSA ev/srm ratio was lower in PCa patients compared to healthy controls and BPH patients ($p < 0.001$). Elecsys immunoassays detected higher concentrations of ev-T-PSA and ev-F-PSA than Immulite ($p < 0.001$). PSA was detected by IC-ELISA more intensely in CD81+ EVs than in CD63+ EVs, and ev-T-PSA correlated with PSA+ CD63+ ($p < 0.001$) but not with PSA+ CD81+.
Conclusion: EVs-bound PSA is another form of circulating PSA whose measurement could be easily performed in clinical laboratories by automated immunoassays.

1. Introduction

Prostate cancer (PCa) is the second most frequent cancer in men, accounting for about 7 % of cancer deaths worldwide, and about half of the men older than 70 years will eventually suffer PCa [1]. It is a heterogeneous disease, ranging from small, indolent, low-grade tumors, to large, aggressive and life-threatening ones [2]. Prostate specific antigen (PSA), a member of the kallikrein family of serine proteases, plays a fundamental role in PCa management. PSA circulates in blood as free PSA (F-PSA), or complexed with either alpha1-antichimiotrypsin (C-PSA) or with alpha2-macroglobulin, being the latter of them a hidden isoform and therefore, not recognized by commercial immunoassays [3]. Also, free PSA has been shown to exist in three molecular forms: proPSA, benign PSA (BPSA) and intact PSA (iPSA) [4]. Most PSA assays have been designed to obtain an equimolar response of F-PSA and C-PSA with maximum recognition of the different isoforms [5]. However, harmonization of PSA methods is limited and discrepant results can be obtained using different assays.

Although PSA is prostate tissue specific, it is far from being cancer specific and high concentrations of PSA are also found in benign pathologies such as benign prostatic hyperplasia (BPH) or prostatitis [5,6]. Although it was proposed as screening test for PCa, its use has resulted in overdiagnosis and unnecessary invasive and/or costly diagnostic tests such as prostatic biopsy [7]. In addition, it cannot differentiate high or

Abbreviations: 4K score, Four-kallikrein score test; AUC, Area under curve; BBS, Borate Buffer Saline; BPH, Benign prostatic hyperplasia; BPSA, Benign PSA; CI, Confidence interval; C-PSA, Complexed PSA; EVs, Extracellular microvesicles; F-PSA, Free PSA; HRP, Horseradish peroxidase; IC-ELISA, Immunocapture ELISA; iPSA, Intact PSA; ISUP, International Society of Urological Pathology; NTA, Nanoparticle Tracking Analysis; PBS, Phosphate Buffer Saline; PCa, Prostate cancer; PHI, Prostate Health Index test; PSA, Prostate Specific Antigen; PSMA, Prostate Specific Membrane Antigen; PTEN, Phosphatase and tensin homolog gene; Q1-Q3, Interquartile range; ROC, Receiver Operating Characteristic; SDS, Sodium dodecyl sulfate; *SEC*, Size exclusion chromatography; Srm, Serum; TBS, Tris Buffer Saline; TBS-T, Tris Buffer Saline – Tween; TMB, 3,3′,5,5′-Tetramethylbenzidine; T-PSA, Total PSA.

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low cancer staging and thus, its screening utility has been questioned [4,8,9]. In order to improve PSA sensitivity and specificity, different alternative approaches have been investigated trying to avoid the aforementioned consequences [10]. Some of these proposals include the use of age-adjusted reference ranges for PSA interpretation, the percent-free PSA, PSA-density, or PSA velocity, the Prostate Health Index test (PHI), the four-kallikrein score test (4 K score) [11], and urinary PCA3 [12].

All cells secrete extracellular microvesicles (EVs) that participate in local and systemic cell-to-cell communication, acting as carriers of bioactive molecules such as proteins, lipids, and nucleic acids from the origin cells [13]. Exosomes are a EVs type of 40–200 nm diameter that originate in the endosome [14]. Active secretion of EVs seems to be especially abundant in tumor cells participating in crucial steps such as tumor proliferation, epithelial-mesenchymal transition (EMT), tumor migration and metastases, induction of angiogenesis and immunosuppression [14]. The knowledge about circulating EVs importance as liquid biopsy in cancer patients is growing and also as a delivery system for some known tumor markers [15]. These biomarkers transported in EVs can help in the diagnosis, prognosis and disease monitoring [16]. Some of the proteins transported in EVs, such as the tetraspanins CD9, CD63 or CD81, have been used to identify these microvesicles [17,18]. Proteomic analysis of their content has described many proteins as potential tumor biomarkers in PCa [19,20] such as PCA3 analysis in urinary exosomes [21] or phosphatase and tensin homolog gene (PTEN) [22] and survivin [23] analysis in blood EVs. Prostatic EVs can carry Prostate Specific Membrane Antigen (PSMA), a cell surface antigen highly expressed in prostate, especially in advanced PCa, which has been used to specifically isolate these prostatic EVs from plasma for further analysis [24]. In addition, prostate EVs can express PSA [25] that could be a potential diagnostic biomarker [26]. PSA has been previously detected in urinary extracellular vesicles in PCa and BPH patients [27]. Urinary vesicle-associated PSA extraction ratio is associated to changes in *N*-glycosylation patterns relating with the participation of *N*-linked glycoforms in the formation of EVs from tumor cells. However, the processes of EVs isolation, characterization, and biomarker analysis are not standardized yet and different results can be obtained depending on the methodology used for those objectives [28].

As PSA is the main PCa biomarker, the aim of this work was to characterize the presence of this biomarker in EVs and to introduce a standardized methodology for EVs-bound PSA quantification. For this reason, we have analyzed the PSA molecular forms present in EVs, their reactivity with commercial immunoassays and their presence in relation to soluble PSA concentrations in three differentiate groups: patients with PCa or BPH and healthy individuals.

2. Materials and methods

2.1. Samples and patients selection

A cohort of 47 PCa patients, 27 BPH patients and 42 healthy male controls, was analyzed in this study (Table 1). In addition, samples from six healthy women were anonymously used as negative controls. PCa patients were selected according to their precedent plasma PSA values, and PCa grading was established based on the International Society of Urological Pathology (ISUP) grade [29].

No clinical evidence of prostate cancer or other prostatic pathologies was found in any of the healthy participants. None of BPH patients had received treatment with 5α-reductase inhibitors. The protocol was approved by the local Ethics Committee (Ref: 2021.039) and all participants signed an informed consent.

Blood samples were collected into 5 mL BD Vacutainer serum collection tubes (Beckton Dickinson). To obtain serum, tubes were centrifuged at 2000x*g* for 10 min after clotting formation. Serum samples were then aliquoted and stored at −80 °C until further analysis.

Table 1
Clinical characteristics of the participants of the study. Age data are reported as median and interquartile range.

Healthy controls	**n**	42 (36.2 %)
	Age (years)	59 (54–67)
Benign prostatic hyperplasia patients	**n**	27 (23.3 %)
	Age (years)	65 (56–70)
Prostate cancer patients	**n**	47 (40.5 %)
	Age (years)	70 (65–75)
	Gleason	
	≤7	18
	>7	23
	Unknown	6
	ISUP	
	<3	8
	≥3	33
	Unknown	6
	Tumor Histology	
	Adenocarcinoma	43
	Neuroendocrine	3
	Unknown	1
	Stage	
	I	1
	II	13
	III	9
	IV	24

*Abbreviations: ISUP: International Society of Urological Pathology.

2.2. Extracellular microvesicles isolation

Upon thawing, serum samples were centrifuged at 300x*g* for 10 min. After being spined again at 16,000x*g* for 30 min, 100 μL of EVs-containing supernatant were collected and applied to size exclusion chromatography (*SEC*) for EVs isolation. For that purpose, we used exo-spin mini columns (Cell Guidance System), previously stabilized and prepared with Phosphate Buffer Saline (PBS) [28]. EVs were finally eluted from the column with 180 μL of PBS and diluted to a final volume of 200 μL.

2.3. Extracellular microvesicles characterization and quantification

2.3.1. Immunocapture ELISA (IC-ELISA)

Plates (Thermo Fisher Scientific) were coated with 100 μL of capturing mouse monoclonal antibodies, either anti-CD63 (clone TEA3/18, Immunostep S.L,) or anti-CD81 (clone M38, Abcam) at 6 μg/mL in Borate Buffer Saline (BBS) and incubated overnight at 4 °C. After washing, plates were blocked with PBS 1 % casein (Bio-Rad, Hercules, California, USA) at 37 °C for 2 h, followed by washing. Then serum samples were added to each well and incubated overnight at room temperature. After washing, plates were incubated for 1 h at 37 °C with detection biotin-conjugated antibody, either 4 μg/mL of mouse anti-PSA (clone A67-B/E3, Thermo Fisher), 0.5 μg/mL of anti-CD81 (clone M38), or 0.5 μg/mL of isotype IgG1 antibody (Clone MOPC-21, Biolegend). After washing, Streptavidin-horseradish peroxidase (HRP, 1:2000 dilution, Biolegend) was added and incubated 1 h at room temperature. After washes, the reaction was developed using 3,3′,5,5′-Tetramethylbenzidine substrate (Single Component TMB Peroxidase ELISA Substrate, Bio-Rad), and stopped with 2M H_2SO_4 solution. All washing steps were performed with PBS-Tween 0.05 %. Sample absorbances measured at 450 nm were corrected from background by subtracting the corresponding absorbances with IgG1 antibody. As positive control, commercially available exosomes from the PSA-expressing cell line LNCaP (HansaBioMed Life Sciences) were used.

2.3.2. Western blot

Fifteen μg of EVs protein were boiled in SDS-containing and non-reducing loading buffer (4x Laemmli Sample Buffer, Bio-Rad) and subjected to electrophoresis on Mini-Protean TGX precast 12 % gels (Bio-Rad). Proteins were transferred to nitrocellulose blotting membranes,

then blocked in TBS containing 0.05 % (w/v) Tween-20 (TBS-T) and 5 % (w/v) skimmed milk for 1 h, and incubated overnight at 4 °C with mouse monoclonal anti-CD63 antibody (clone E-12, 1:500 dilution, Santa Cruz Technology) or anti-PSA antibody (clone A67-B/E3, 1:500 dilution, Santa Cruz Biotechnology). After washing with TBS-T, membranes were incubated with an anti-mouse IgG-HRP conjugated antibody (1:5000 dilution, Amersham Biosciences) for 30 min at room temperature and revealed with the ECL Western Blotting Detection Reagent (Amersham Biosciences). Pictures were taken with a Carestream Gel Logic 2200 Pro equipment (Carestream).

2.3.3. Nanoparticle Tracking analysis

Particle size and concentration of isolated microvesicles from five PCa patients were determined by Nanoparticle Tracking Analysis (NTA) in a NanoSight LM20 (Malvern Panalytical). Concentrations were reported in particles/mL and sizes in nm.

2.3.4. Determination of albumin and total protein content

Albumin and total protein concentrations were determined in serum and EVs, in order to study the effectiveness and purity of the isolation process. Albumin was measured in a c702 module of a Cobas 8000 analyzer (Roche Diagnostics) by immunoturbidimetry. Total protein concentrations were measured by Nanodrop® spectrophotometer ND-1000 (Thermo Scientific).

2.4. PSA quantification

In both serum (srm-) and isolated EVs (ev-), concentrations of total PSA (T-PSA) and F-PSA were determined in 2 different autoanalyzers using their corresponding commercial immunoassays designed for serum quantifications. In the Cobas 8000 (Roche Diagnostics), T-PSA and F-PSA were measured in a c602 module using Elecsys® total PSA and Elecsys® free PSA reagent kits based on electrochemiluminiscent immunoassays in a "sandwich" configuration. In Immulite 2000 XPi (Siemens Healthineers), T-PSA and F-PSA concentrations were quantified by the corresponding chemiluminescent immunoassays, Immulite® 2000 PSA and Immulite® 2000 Free PSA, also with a "sandwich" configuration.

C-PSA was calculated by subtracting the F-PSA concentration from the T-PSA one. T-PSA and F-PSA ev/srm ratios (%) were calculated by dividing the concentrations of T-PSA or F-PSA measured in EVs by those in serum, and expressed as percentage:

- T-PSA ev/srm ratio (%) = ev-T-PSA/srm-T-PSA x100
- F-PSA ev/srm ratio (%) = ev-F-PSA/srm-F-PSA x100

The ratio of T-PSA between both commercial methods was calculated by dividing T-PSA concentrations measured with Immulite by T-PSA concentrations measured with Elecsys, in both serum and EVs samples.

Detection limits are 0.016 μg/L for F-PSA and 0.010 μg/L for T-PSA in Elecsys assays, and 0.070 μg/L and 0.040 μg/L respectively, in Immulite ones. Quantification limits of Elecsys methods are 0.018 μg/L for F-PSA and 0.014 μg/L for T-PSA, and of Immulite methods 0.070 μg/L and 0.040 μg/L, respectively.

2.5. Statistical analysis

Non-parametric statistical analysis was performed using Graphpad Prism version 6. Data were represented as median and interquartile range or range. For comparisons, Mann-Whitney's U and Wilcoxon tests were used, while for correlations Spearman's test was performed. Agreement assessing between methods was performed with Bland-Altman test. Diagnostic efficiency was evaluated by Receiver Operating Characteristic (ROC) curves and the area under curve (AUC) analysis. A two-tailed p-value of < 0.05 was considered to be statistically significant.

3. Results

3.1. Detection of PSA in extracellular microvesicles

The first aim of our study was to analyze the presence of PSA at the EVs surface. For that purpose, we tuned up an IC-ELISA, based on EVs capture with an antibody against a EVs surface molecule, either CD63 or CD81, and subsequent detection with a biotinylated antibody specific to PSA (Supplementary Data 1A). Using this method, PSA was detected in CD63+ EVs from the PCa cell line LNCaP and in CD63+ EVs present in serum from male participants (Fig. 1), whereas serum samples from six women, used as negative control, produced absorbance signals near background. Similarly, PSA could also be detected in CD81+ EVs, even with more intense signals in serum samples ($p = 0.003$) and in LNCaP exosomes (16 % higher signal in CD81+ EVs compared to CD63+ EVs). However, there was no correlation between PSA signals obtained in CD81+ EVs and those in CD63+ EVs ($p = 0.619$), and neither between the signal corresponding to double positive CD63+ CD81+ EVs and PSA+ CD63+ nor PSA+ CD81+ EVs ($p = 0.111$ and $p = 0.266$, respectively). Finally, we observed a positive correlation between srm-T-PSA measured with the Elecsys immunoassay and PSA+ CD63+ EVs ($r = 0.500$; $p = 0.021$) and a negative correlation in the case of PSA+ CD81+ EVs ($r = -0.697$; $p = 0.004$), and no significant correlation with CD63+ CD81+ EVs ($r = 0.268$; $p = 0.185$).

In order to further study PSA in EVs, we isolated EVs from serum by *SEC*. To assess the efficiency of this isolation method, we analyzed the particle size and concentration of the vesicles obtained from five patients' samples. The median size of the isolated particles was 181 nm (Q1-Q3: 179–185 nm) (Supplementary Data 2A), and concentrations varied between $8.99 \pm 0.472 \times 10^9$ and $42.80 \pm 0.901 \times 10^9$ particles/mL. The presence of contaminants in the purified EVs was checked by measuring albumin concentrations before and after the *SEC* procedure, noting an albumin clearance of 99.1 ± 0.4 %. In addition, western blot analysis showed the characteristic EVs surface marker CD63 (Supplementary Data 2B). Finally, after reproving with an anti-PSA antibody, we also observed a band in each lane. Consequently, we could confirm that we were able to isolate PSA-containing EVs from serum with this method.

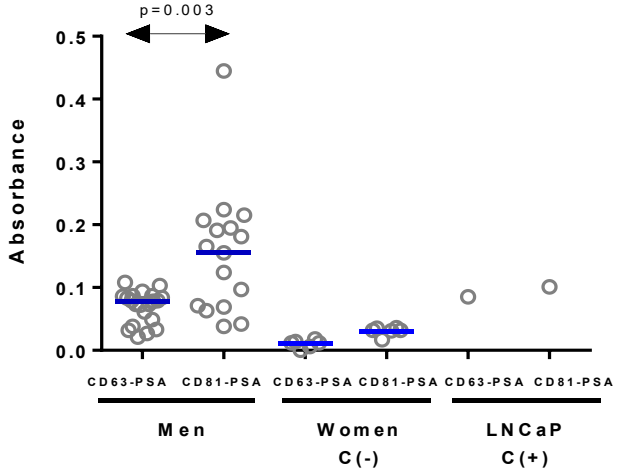

Fig. 1. Immunocapture ELISA (IC-ELISA) of PSA+ extracellular microvesicles (EVs) using anti-CD63 or anti-CD81 for capture and anti-PSA for detection. Analysis were performed in human serum samples from men. Serum samples from women were used as negative control, C(-), and exosomes from PSA-expressing LNCaP cell line as positive control, C(+). Blue lines represent median. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.2. Extracellular microvesicles PSA quantification with commercial kits

We studied if ev-PSA was detectable by commercial immunoassays commonly used in routine clinical laboratories, designed for serum PSA quantification (Supplementary Data 1B). Using the Elecsys commercial assays from Roche Diagnostics, we observed in exosomes derived from PSA-expressing LNCaP cell line, a concentration of 35.3 μg/g exosomes with the T-PSA assay, and of 31 μg/g exosomes with the F-PSA assay (87.8 % of reactive PSA for F-PSA assay). Although this is not real free PSA, as it is EVs-bound, we denominate it ev-F-PSA for analogy with its reactivity.

As well, PSA was determined using Elecsys immunoassay in serum EVs after *SEC*-based isolation from patients' samples. We could detect ev-T-PSA in all samples analyzed (median: 0.076 μg/L; Q1-Q3: 0.050–0.170 μg/L), which correlated significantly with srm-T-PSA ($r = 0.557$; $p < 0.001$) (Fig. 2A). Ev-F-PSA concentrations were near the limit of quantification of the Elecsys assay for F-PSA, but we could measure ev-F-PSA in 84 % of them (median: 0.042 μg/L; Q1-Q3: 0.020–0.074 μg/L). In those samples with detectable ev-F-PSA, correlation with srm-F-PSA was not significant ($r = 0.125$; $p = 0.264$) (Fig. 2B).

There was a significant correlation between ev-T-PSA concentrations and PSA+ CD63+ EVs ($r = 0.711$; $p < 0.001$) (Fig. 2C), but not with PSA+ CD81+ EVs ($p = 0.147$) nor with CD63+ CD81+ EVs ($p = 0.272$).

3.3. Comparison of extracellular microvesicles PSA immunoreactivity to different commercial immunoassays

In order to know if PSA reactivity in EVs was maintained between different assay kits, we performed a comparative study between Elecsys and Immulite immunoassays for T-PSA and F-PSA in a sub-group of 27 participants. In this sub-group, median srm-T-PSA concentration was 15.64 μg/L (range: 0.88–192.40 μg/L) and median srm-F-PSA concentration 1.67 μg/L (range: 0.25–35.44 μg/L) measured with Elecsys kits, and 18.9 μg/L (range: 0.7–128.0 μg/L) and 1.39 μg/L (range: 0.19–39.60 μg/L), with Immulite, respectively (Fig. 3A). We could detect ev-T-PSA in all those 27 patients using Elecsys methodology (median: 0.080 μg/L; range: 0.036–1.202 μg/L). However, we could detect ev-T-PSA only in six samples (22 %) using Immulite methodology and with concentrations near the limit of detection (median: 0.040 μg/L; range: 0.040–0.674 μg/L; $p < 0.001$). We assessed the agreement between Immulite and Elecsys methods to measure srm-T-PSA and ev-T-PSA with Bland-Altman plots, representing the difference between methods against their mean. We observed that Elecsys method overestimated both magnitudes in comparison to Immulite assay (Fig. 3B and 3C). Ev-F-PSA was detected in 67 % of these samples with Elecsys assay kit (median: 0.026 μg/L; range: 0.016–0.144 μg/L) and in none of them with the Immulite free PSA assay kit ($p < 0.001$). The ratio of T-PSA quantified between Immulite and Elecsys was significantly lower in EVs (median: 0.00; range: 0.00–0.76) than in serum (median: 0.88; range: 0.63–1.21; $p < 0.001$) (Fig. 3D). This indicates that the immunoreactivity against ev-T-PSA and ev-F-PSA is higher when using Elecsys assays compared to Immulite ones. For this reason, in the following experiments we only used Elecsys assay kits.

3.4. Extracellular microvesicles PSA in relation to serum PSA

The T-PSA ev/srm ratio was lower than 5 % in most samples (median: 2.20 %; Q1-Q3: 0.80–4.00 %), although in some of them ev-T-PSA represented up to 59 % of srm-T-PSA. We could observe higher ratios for F-PSA (median 5.09 %; Q1-Q3: 0.30–13.20 %) in comparison with calculated C-PSA (median: 0.89 %; Q1-Q3: 0.40–2.00 %), although not

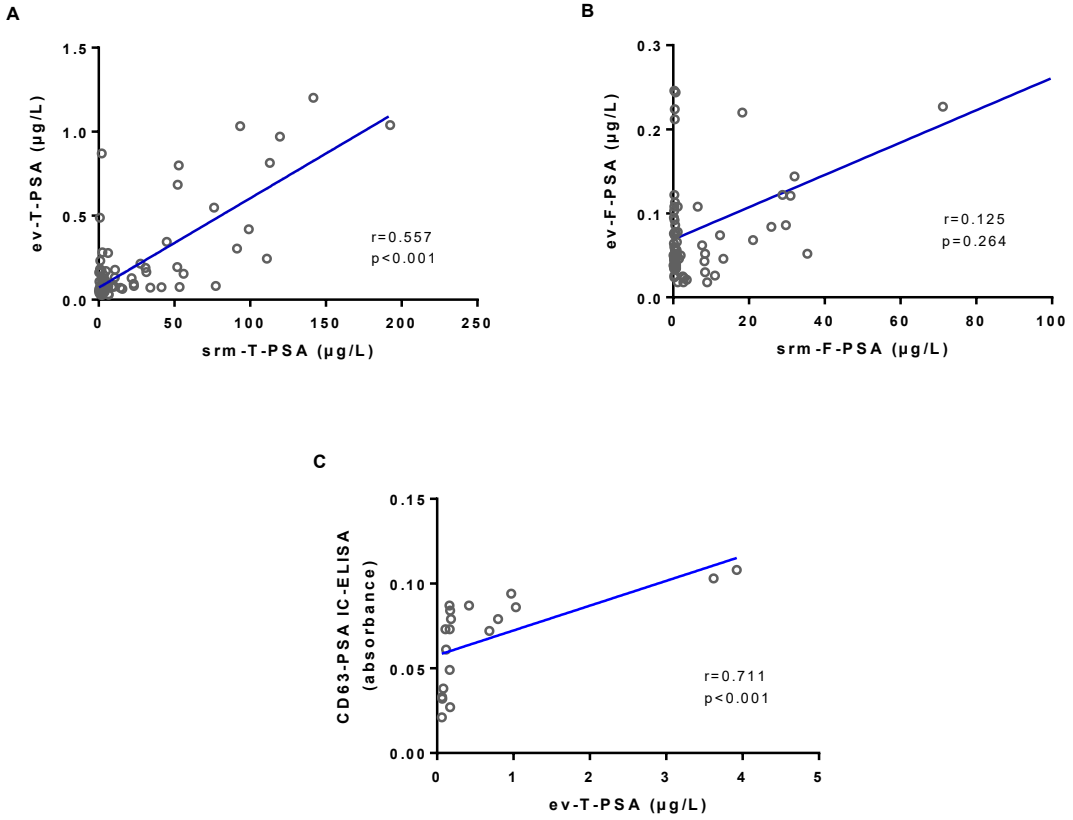

Fig. 2. Total (T-) and free (F-) PSA measured in extracellular microvesicles (ev-) and serum (srm-) with commercial immunoassays. (a) Correlation between ev-T-PSA and srm-T-PSA measured with Elecsys assay; (b) Correlation between ev-F-PSA and srm-F-PSA measured with Elecsys assay; (c) Correlation between ev-T-PSA measured with Elecsys assay and PSA+ CD63+ EVs measured by IC-ELISA.

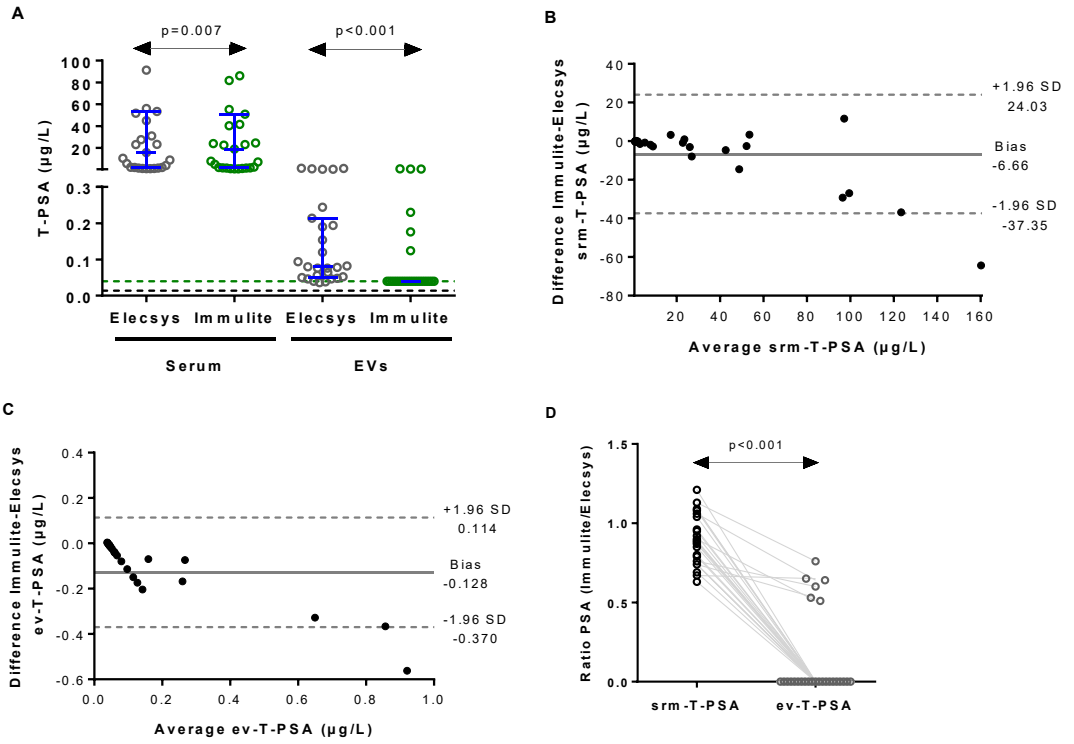

Fig. 3. Comparison of total (T-) PSA immunoreactivity in extracellular microvesicles (ev-) and serum (srm-) between Elecsys and Immulite commercial immunoassays. (a) Comparison of srm-T-PSA and ev-T-PSA concentrations measured with Elecsys and Immulite assays. Blue lines represent median and interquartile range. Dotted lines (black for Elecsys and green for Immulite) indicate quantification limits; (b) Bland-Altman plot of the difference between srm-T-PSA concentrations measured by Immulite and Elecsys methods; (c) Bland-Altman plot of the difference between ev-T-PSA concentrations measured by Immulite and Elecsys methods; (d) Comparison of the immunoreactivity of srm-T-PSA and ev-T-PSA measured with Immulite and Elecsys assays, expressed as Immulite/Elecsys ratio. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

significant. When classifying T-PSA ev/srm ratios based on srm-T-PSA concentrations, these ratios were significantly lower (median: 0.76 %; Q1-Q3: 0.41–1.30 %) in samples with srm-T-PSA higher than 4 µg/L than in those with srm-T-PSA below that cut-off (median: 3.90 %; Q1-Q3: 2.48–6.90 %; $p < 0.001$) (Fig. 4). Similarly occurred with the ratios for F-PSA and C-PSA ($p < 0.001$ and $p = 0.013$, respectively).

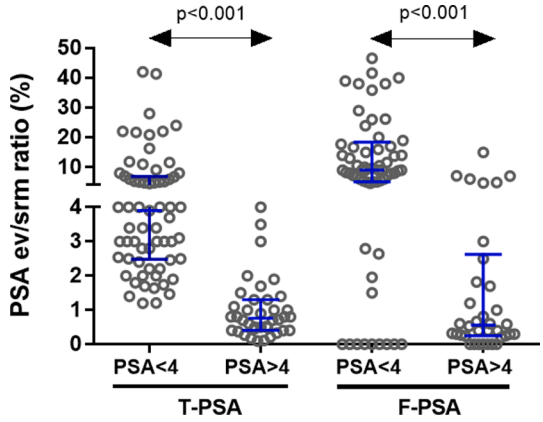

Fig. 4. Total (T-) and free (F-) PSA extracellular microvesicles (ev)/serum (srm) ratios, expressed in percentage, classified according to serum PSA concentrations (cut-off of 4 µg/L). Blue lines represent median and interquartile range. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.5. Extracellular microvesicles PSA in patients

Finally, we wanted to study whether the presence of ev-PSA changes in PCa patients. In our study groups, both T-PSA and F-PSA serum concentrations were significantly higher in PCa patients compared to controls and BPH patients ($p < 0.001$; Table 2). Similarly, ev-T-PSA was higher in patients in relation to controls ($p < 0.001$) and BPH ($p = 0.002$) (Table 2 and Fig. 5A). When analyzing ev-F-PSA, there were significant differences between BPH and PCa ($p = 0.006$), but not between controls and PCa patients ($p = 0.199$)(Fig. 5A). On the contrary, T-PSA ev/srm ratios in PCa group were significantly lower compared to healthy controls ($p < 0.001$) and to BPH ($p < 0.001$) (Fig. 5B), and significant differences were also observed in F-PSA ev/srm ratios between PCa and controls ($p = 0.005$) and BPH ($p < 0.001$).

We performed ROC curves analysis to study the efficiency of the T-PSA ev/srm ratio in PCa diagnosis and compared to srm-F-PSA/srm-T-PSA (Table 2). When analyzing PCa patients against controls, the T-PSA ev/srm ratio ROC curve provided an AUC much higher than the AUC for srm-F-PSA/srm-T-PSA. Further, in the analysis of PCa patients against BPH patients the AUC of T-PSA ev/srm ratio curve was even higher when comparing to srm-F-PSA/srm-T-PSA.

There were not significant differences between controls and PCa, when analyzing PSA+ CD63+ or PSA+ CD81+ EVs by IC-ELISA ($p = 0.123$ and $p = 0.838$, respectively), and neither in total circulating CD63+ CD81+ EVs ($p = 0.539$).

To further analyze possible T-PSA ev/srm ratio changes, we studied five PCa patients with progressive disease during different therapeutic approaches (chemotherapy, immunotherapy, radiotherapy and hormonal treatments) (Fig. 5C and Supplementary Data 3). We observed that in four patients whose srm-T-PSA increased with the advance of the

Table 2
Comparison of EVs-related biomarkers between healthy controls (controls), BPH patients (BPH) and PCa patients (patients). A p-value of < 0.05 was considered to be statistically significant.

	Controls Median (Q1-Q3)	BPH Median (Q1-Q3)	Patients Median (Q1-Q3)	p-value (Controls vs BPH)	p-value (Controls vs Patients)	p-value (BPH vs Patients)	AUC (CI95%; p-value) (Controls vs patients)	AUC (CI95%; p-value) (BPH vs Patients)
srm-T-PSA (μg/L)	1.59 (1.03–2.11)	1.46 (0.95–2.47)	27.09 (6.44–55.45)	$p = 0.838$	$p < 0.001$	$p < 0.001$	0.986 (0.968–1.005;$p < 0.001$)	0.983 (0.960–1.006;$p < 0.001$)
srm-F-PSA (μg/L)	0.39 (0.27–0.61)	0.45 (0.32–0.64)	3.45 (0.80–10.49)	$p = 0.390$	$p < 0.001$	$p < 0.001$	0.886 (0.799–0.974;$p < 0.001$)	0.877 (0.784–0.969;$p < 0.001$)
srm-F-PSA/srm-T-PSA (%)	26.60 (20.00–35.28)	31.10 (26.00–39.00)	12.88 (7.15–27.89)	$p = 0.152$	$p < 0.001$	$p < 0.001$	0.739 (0.616–0.863;$p < 0.001$)	0.779 (0.658–0.899;$p < 0.001$)
ev-T-PSA (μg/L)	0.05 (0.04–0.08)	0.07 (0.05–0.11)	0.14 (0.07–0.33)	$p = 0.019$	$p < 0.001$	$p = 0.002$	0.821 (0.722–0.921;$p < 0.001$)	0.724 (0.594–0.853;$p = 0.003$)
ev-F-PSA (μg/L)	0.03 (0.02–0.04)	0.06 (0.05–0.08)	0.04 (0.02–0.07)	$p < 0.001$	$p = 0.199$	$p = 0.006$	0.576 (0.440–0.712;$p = 0.269$)	0.720 (0.593–0.846;$p = 0.003$)
ev-F-PSA/ev-T-PSA (%)	64.00 (35.25–80.75)	91.00 (71.00–100.00)	27.00 (6.50–39.75)	$p < 0.001$	$p < 0.001$	$p < 0.001$	0.744 (0.616–0.873;$p < 0.001$)	0.981 (0.954–1.008;$p < 0.001$)
T-PSA ev/srm ratio (%)	3.00 (2.00–5.77)	4.00 (3.00–8.00)	0.54 (0.37–1.30)	$p = 0.012$	$p < 0.001$	$p < 0.001$	0.918 (0.849–0.987;$p < 0.001$)	0.958 (0.909–1.007;$p < 0.001$)
F-PSA ev/srm ratio (%)	6.65 (2.13–10.40)	15.00 (9.00–29.00)	0.59 (0.25–3.00)	$p < 0.001$	$p = 0.005$	$p < 0.001$	0.688 (0.553–0.823;$p = 0.007$)	0.912 (0.830–0.994;$p < 0.001$)

*Abbreviations: EVs: extracellular microvesicles; BPH: benign prostatic hyperplasia; PCa: prostate cancer; srm-T-PSA: serum total PSA; srm-F-PSA: serum free PSA; ev-T-PSA: extracellular microvesicles total PSA; ev-F-PSA: extracellular microvesicles free PSA; T-PSA ev/srm ratio: total PSA extracellular microvesicles/serum ratio; F-PSA ev/srm ratio: free PSA extracellular microvesicles/serum ratio; AUC: area under curve.

disease, the T-PSA ev/srm ratio decreased, while in other patient in which serum PSA did not change significantly (7.7 % increase), T-PSA ev/srm ratio increased noticeably (263 %).

4. Discussion

Here we show that prostate circulating CD81+ or CD63+ EVs carry the specific tissue biomarker PSA [30,31]. We have observed that there are higher PSA concentrations in CD81+ EVs over CD63 + EVs, which could be related to a lower CD63 expression when compared to CD81 in PSA+ EVs [32,33], or to a different PSA loading among EVs subpopulations. In fact, most EVs do not express simultaneously CD63 and CD81 [34] and the heterogeneous tetraspanin expression in EVs may affect the performance of biomarkers analysis in microvesicles when CD63 or CD81 capture is the initial enrichment step for downstream analysis [33]. For example, as shown for flow cytometry analysis, there could be differences in the number of microvesicles captured by beads depending on the expression of these molecules [30]. Interestingly, and contrary to CD63, CD81 does not correlate with Gleason in PCa patients [35]. In relation to this, we found different sign of relationship between srm-T-PSA and either PSA+ CD63+ EVs or PSA+ CD81+ EVs. Additionally, we did not find differences in relation to PSA+ CD81+ or PSA+ CD63+ EVs between PCa patients and controls, similar results to Pang et al. [36], who used flow cytometry to analyze PSA in EVs captured with antibody cocktail of CD9,CD63 and CD81. However, Logozzi et al. [31] observed higher levels of PSA+ CD81+ EVs in PCa patients compared to controls and BPH patients. The discrepancy could be due to the different expression and selectivity of these tetraspanins used to capture EVs and the quantification methods that also lacks standards to compare [37].

PSA, as also proposed with the other prostate marker PSMA [24], can serve to identify prostate derived EVs that circulate mixed with EVs released by cells from other origin. Although we and other authors have previously analyzed biomarkers in total plasma EVs in PCa patients [23,38], the sensitivity to precise identification and analysis can be impaired as prostate specific vesicles are probably only a minor part of circulating EVs. This can be the reason for the lack of relationship between CD63+ CD81+ EVs and srm-T-PSA. Other authors, such as Vermassen et al. [27], have demonstrated the presence of extracellular vesicles-bound PSA in urine, and studied its utility in PCa diagnosis.

EVs use as clinical biopsy has been hampered by the lack of standardization of techniques for EVs isolation and downstream analysis, which makes comparisons between different laboratories very difficult [31,36]. In this work, we have utilized a commercial methodology that could be standardized and whose results can be easily assessed and compared between clinical laboratories. Within EVs isolation methods, *SEC* is the second most commonly used technique due to its simplicity, excellent EVs yield and purity and the availability of commercial kits [39,40]. Also, in relation to downstream analysis, common T-PSA assays can be used to determine PSA concentration in EVs, whose immunoreactivity correlates better with PSA in CD63+ EVs than in CD81+ EVs. Methods designed to measure F-PSA in serum use antibodies that react with PSA epitopes hidden when bound to alpha1-antichimotrypsin [3]. Interestingly, although not free, part of PSA molecules present in EVs surface can be recognized with these methods, indicating that, at least, some of the epitopes targeted by F-PSA kits remain available even when PSA circulates bound to EVs (Supplementary Data 1B). However, it should be noted the poor relationship of F-PSA between serum and EVs, that can be due to the different antibody accessibility. Furthermore, measured concentrations are near the limit of quantification, which could limit the potential utility of these commercial immunoassays for measuring ev-F-PSA without a methodical optimization.

Although ev-PSA is only a minor fraction of circulating PSA, in some samples can be half of the immunoreactive PSA. The immune reactivity against the ev-PSA (both total and free), was lower when using Immulite assays in relation to Elecsys methods, being the difference more

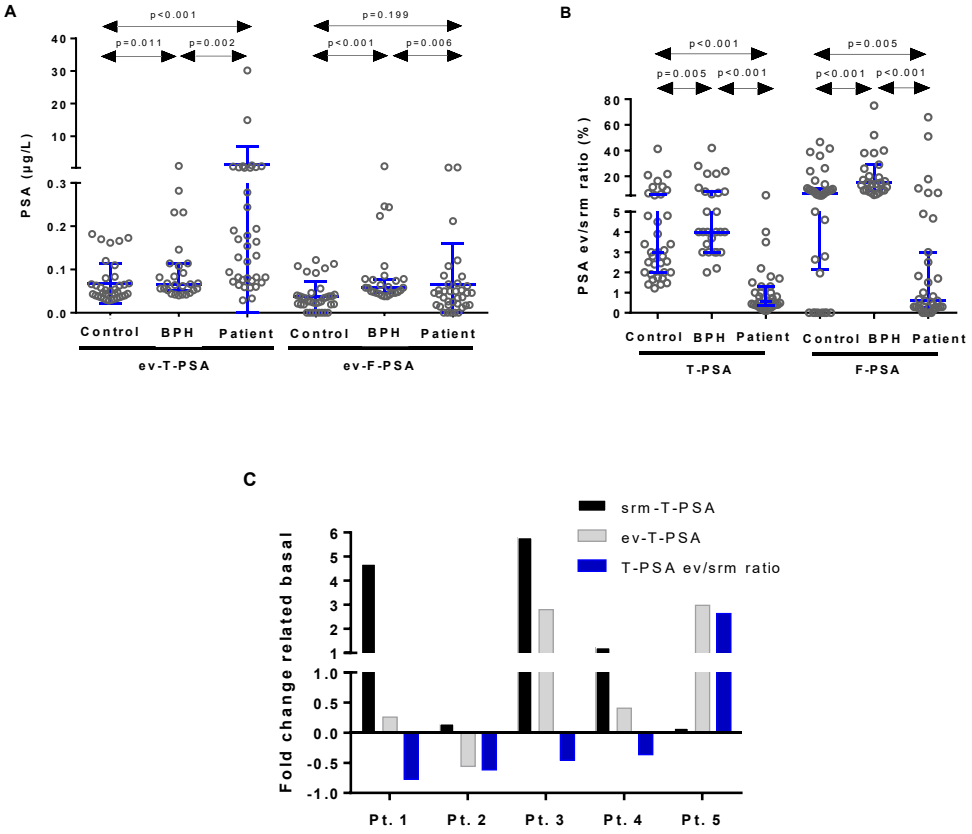

Fig. 5. Extracellular microvesicles (ev-) PSA analysis in healthy controls, benign prostatic hyperplasia patients and prostate cancer patients. (a) Comparison of total (T-) and free (F-) ev-PSA between controls (control), benign prostatic hyperplasia patients (BPH) and prostate cancer patients (patient); (b) Comparison of T-PSA ev/srm ratio, expressed in percentage, between controls, BPH and cancer patients; (c) Changes in the concentrations of srm-T-PSA, ev-T-PSA and T-PSA ev/srm ratio in 5 PCa patients (Pt) with progressive disease during treatment with immunotherapy combined with an androgen biosynthesis inhibitor (Pt.1), radiotherapy (Pt.2), chemotherapy (Pt.3 and 5) and androgen receptor inhibitor (Pt.4).

pronounced when measuring immunoreactive F-PSA. This could be probably due to the different capacity to access to a protein epitope included in EVs, the different antibodies used [41] and differences in the limit of detection and quantification of each method. For example, Ferraro et al. [5] showed a general trend for Roche and Abbott assays to overestimate and for Siemens and Beckman assays to underestimate serum T-PSA, and even a wider disagreement between F-PSA assays. The observed overestimation holds also true for EVs-bound PSA and even to a higher degree [42].

Using *SEC* for EVs isolation and commercial PSA immunoassays for quantification, we observed that ev-T-PSA concentrations are higher in PCa patients than in BPH or healthy controls. However, ev-F-PSA was very similar between groups. The difference could be due to the proteolytic inactivation in the lumen of the PSA included in EVs [43]. In addition, prostate cancer grading should be considered, as observed by Ferraro et al. [44], who showed that although no differences were found in srm-F-PSA when comparing ISUP $\geq$ 3 PCa patients to healthy controls, it was higher in ISUP 5 PCa patients compared to ISUP 3 or 4 PCa patients. The release of EVs carrying PSA from the prostate to circulation depends on the production and the structural tissue integrity, which can be deeply modified by the tumor microenvironment and cellular activity. In relation to this, Logozzi et al. [31] showed that low pH condition, characteristic of tumors, induced the release of EVs with PSA expression in their membranes. However, PSA incorporation into EVs and subsequent efflux into circulation are probably more complex compared to soluble PSA as reflected by the increase in both srm-T-PSA and ev-T-PSA concentrations, but decrease in T-PSA ev/srm ratio in advanced PCa. In addition, T-PSA ev/srm ratio seems to change during cancer follow-up according to disease aggressiveness, and may possible be associated to type of therapy. These data pointed that cancer aggressiveness affects PSA transport into EVs, although further studies should confirm this data.

Our results in relation to ev-T-PSA in PCa are a proof of concept performed in a small cohort including PCa patients, healthy controls and BPH patients. This is a potential limitation of the study and to analyze the utility of ev-T-PSA in PCa and, considering these results, it could be interesting to perform further studies including patients with PCa and different benign prostate diseases, especially those with T-PSA between 2 and 10 µg/L. Another potential limitation is that PSA commercial methods are not designed to measure ev-PSA. However, the methodology used here, standardized to some point and that can be easily used in other clinical laboratories, would allow performing multicentric studies that would facilitate studies with larger sample sizes.

In summary, our data show that prostate-derived EVs transport PSA at their surface, which can be measured with conventional methods originally designed for serum quantification. The measurement of PSA in EVs is also affected by the interchangeability between commercial assays. Nevertheless, complexed and free PSA are loaded differentially in EVs and their concentrations could help in the management of PCa patients. The underlying tumoral process leading to this change in EVs-bound PSA is currently unknown and warrant further investigation.

Policy and ethics: The study was conducted in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki) and sample collection and storage was approved by the local Ethics Committee of University of Navarra (protocol code: 2022.087 approved the 26th of may of 2022). Informed consent was obtained from all individuals included in this study.

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CRediT authorship contribution statement

Amaia Sandúa: Methodology, Validation, Formal analysis, Investigation, Writing – original draft, Visualization, Funding acquisition. **Miguel F. Sanmamed:** Investigation, Resources. **María Rodríguez:** Investigation, Resources. **Javier Ancizu-Marckert:** Investigation, Resources. **Alfonso Gúrpide:** Investigation, Resources. **José L. Perez-Gracia:** Investigation, Resources. **Estibaliz Alegre:** Conceptualization, Methodology, Writing – review & editing, Supervision. **Álvaro González:** Conceptualization, Methodology, Formal analysis, Writing – original draft, Writing – review & editing, Supervision, Project administration.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Miguel F Sanmamed received grants from Roche and BMS, not related to this work. María Rodríguez received speaker's bureau honoraria from BMS and ROCHE, support for attending meeting from Roche, Astrazeneca, Catalym, and BMS, and research materials from Roche and Highlight Therapeutics, not related to this work. José L Pérez-Gracia has received grants from Roche, BMS, MSD and Seattle Genetics, not related to this work, and is a member of speaker bureau and advisory boards from Roche, BMS, Ipsen, MSD and Seattle Genetics. He has received travel support from Roche, MSD and BMS. Álvaro González has received support from Roche Diagnostics for attending Euromedlab 2021.

Data availability

Data will be made available on request.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cca.2023.117303.

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