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Characterization of the perioperative changes of exosomal immune-related cytokines induced by prostatectomy in early-stage prostate cancer patients

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ABSTRACT

Background: Myeloid-derived suppressor cells (MDSCs) are relevant in prostate cancer microenvironment collaborating in tumor development. The main tumor marker used in this disease, prostate-specific antigen (PSA), does not provide information related to this tumor microenvironment. Cancer cells secrete exosomes carrying bioactive molecules contributing to MDSCs recruitment and induction. The aim of this study was to characterize the perioperative changes of exosomal cytokines relevant in MDSCs recruitment induced by prostatectomy in prostate cancer patients.

Methods: Blood was drawn from 26 early-stage prostate cancer patients before and after radical prostatectomy and from 16 healthy volunteers. Serum exosomes were separated by precipitation. Cytokines related with MDSC cell recruitment and activation CCL2, CXCL2, CXCL5, CXCL8, CXCL12, MIF, S100A9 and TGF-ß were measured in serum and serum-derived exosomes using immunometric assays.

Results: All cytokines were detected both in serum and exosomes, except for CXCL12, which was detected only in serum. Exosomes were enriched specially in MIF, TGF-ß and CXCL2. Presurgical MIF levels in exosomes correlated negatively with serum PSA. Also, presurgical TGF-ß decreased both in serum and exosomes as Gleason score rises. Patientś presurgical exosomes had increased CCL2, CXCL5 and TGF-ß levels than exosomes from healthy controls. These differences were not observed when cytokines were analyzed in serum, except for TGF-ß. Cytokine levels of CCL2, CXCL5 decreased in patients' postsurgical exosomes, while TGF-ß further increased. On the contrary, S100A9 levels were lower in patientś presurgical exosomes but increased after radical prostatectomy.

Conclusions: Blood exosomal content in cytokines constitute an attractive source to evaluate MDSCs immunomodulators providing additional information related to tumor microenvironment in prostate cancer.

Abbreviations: PCa, Prostate cancer; PSA, prostate-specific antigen; MDSCs, myeloid-derived suppressor cells; MIF, migration inhibitory factor.

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1. Introduction

Prostate cancer (PCa) is the most common cancer in men and a major cause of death [1]. It usually presents as a localized tumor that is managed by observation, prostatectomy or radiation therapy, and for advanced tumors, the mainstay of treatment consists on hormonal therapy [2,3]. The main circulating biomarker for this disease is prostate-specific antigen (PSA), which presents some limitations, especially in tumor diagnosis [4]. In addition, PSA does not provide information on the tumor microenvironment, which could be of utmost importance for determining the prognosis and the optimal management.

A crucial point in PCa evolution to advanced stages is the creation of an inflammatory microenvironment by which immune cells are recruited and "reprogrammed" to provide a milieu that favors tumor growth and suppresses the immune response [5]. Within the immune cells present in the tumor niche, myeloid-derived suppressor cells (MDSCs) are especially relevant, due to their strong immunosuppressive function [6–9]. MDSCs can also collaborate in tumor development by promoting angiogenesis, cancer cell survival, invasion and metastasis. Recruitment of MDSCs into tumor sites is regulated by different immunomodulatory molecules produced by tumor cells [6].

Exosomes are virus-size vesicles of 50–200 nm produced in the endosome multivesicular bodies and released actively by normal and malignant cells [10]. Circulating exosomes are present in biological fluids such as blood or urine, from where they can be isolated [11,12]. These microvesicles carry proteins and nucleic acids, some of them characteristic and representative of the cells where they originated from [13]. In addition, exosomes play important roles in cell-to-cell communication by transferring these proteins and RNAs to the cells they fuse with. Cancer cells secrete exosomes carrying bioactive molecules that participate in the modeling of tumor microenvironment contributing to cell proliferation, angiogenesis, immunosuppression, and the creation of the metastatic niche, allowing the malignant cells to thrive [14].

Previous works have shown that tumor exosomes can transport cytokines and growth factors, such as TGF-β and PGE-2, which contribute to immunosuppression by induction of MDSCs differentiation that, in turns, favors tumor progression [15,16]. Additionally, immunomodulatory factors transported by exosomes can modulate the tumor microenvironment, as described for macrophage migration inhibitory factor (MIF) in pancreatic cancer [17], or TGF-ß in prostate cancer [18]. Therefore, exosomes and their transported molecules could be potential cancer biomarkers [12,17]. In fact, proteomic studies have identified numerous protein biomarkers from urinary microvesicles or PCa cell lines [19–21].

Here, we report the first study that characterizes the changes induced by prostatectomy in serum exosomal cytokines, by analyzing sequential blood samples from localized PCa patients treated with radical prostatectomy. We focused in chemokines and factors (globally referred to as cytokines) that are known to be relevant in MDSCs recruitment: CCL2, CXCL2, CXCL5, CXCL8 and CXCL12, MIF, S100A9 and TGF-ß [6]. We compared the levels of different cytokines between patients and a group of healthy controls, and we also followed the intra-patient evolution of them before and after prostatectomy [22].

2. Material and methods

2.1. Study design

We included patients with localized PCa treated with radical prostatectomy. Tumor staging was classified according to the AJCC Cancer Staging Manual [23] and Gleason score and grading was stablished according to International Society of Urological Pathology (ISUP) [24]. For each patient, two 10-mL serum samples were collected: before and after surgery, with an average pre-surgical interval of 1 day (range: 1–4 days) and post-surgical interval of 4 days (range: 3–7 days), time enough to exclude exosome's release due to surgery trauma [25]. As previously described, peripheral blood was drawn and centrifuged at 3500 rpm at room temperature. A second high speed centrifugation at 13,000 rpm was performed and samples were subsequently aliquoted and stored at –80 °C until further analysis [11].

We also included a control group of healthy age-matched male volunteers with no previous prostatic history [22]. The study was approved by our institutiońs Ethical Committee and all participants signed an informed consent.

2.2. Exosome separation

Exosomes were separated from serum using the ExoQuick™ precipitation solution (System Biosciences, Mountain View, CA, USA) according to manufacturer's instructions. Particles' characteristics separated with this method were analyzed by both western blot and particle-based approaches as we have previously described [11]. Briefly, 250 μL of serum were mixed with 63 μL of ExoQuick solution and incubated 30 min at 4 °C. After centrifugation at 1500*g* for 30 min, pellets were resuspended in 200 μL of PBS.

2.3. Cytokine analysis

CCL2, CXCL2, CXCL5, CXCL8, CXCL12, MIF and S100A9 were measured in both serum and exosomes simultaneously using a custom magnetic bead-based Luminex assay (R&D Systems, MN, USA) in a Luminex® 200 analyzer (Luminex xMAP Technology, Luminex, Austin, TX, USA) according to manufacturer's instructions. Data were analyzed with the xPONENT 3.1 software. The median fluorescence intensity was computed for each bead type in the sample. A minimum threshold of 50 events was stablished for each analyte. TGF-β was analyzed by a commercial enzyme-linked immunosorbent assay (ELISA) (Human TGF-β1 Quantikine ELISA; R&D Systems) according to manufacturer's instructions.

Detection limits were: 9.9 ng/L for CCL2, 7.86 ng/L for CXCL2, 8.2 ng/L for CXCL5, 1.8 ng/L for CXCL8, 17 ng/L for CXCL12, 38.8 ng/L for MIF, 6.39 ng/L for S100A9 and 4.61 ng/L for TGF-β.

A correction factor was applied to adjust concentrations to initial volume of serum. Due to the limitation of blood volume, some cytokines could not be analyzed in all samples.

2.4. Statistical analysis

Due to their non-Gaussian distribution determined with the Kolmogorov-Smirnov and Shapiro-Wilks tests, concentrations were expressed as median and Interquartile range (IQR). The non-parametric Mann-Whitney U, Wilcoxon test and Dunn's multiple comparison tests were applied to assess the statistical differences in cytokines levels. Correlation analysis was performed using the Spearman correlation test. Statistical analysis was performed with IBM SPSS Statistics, version 20 (IBM Corp., Armonk, N.Y., USA). A two-tailed P-value < 0.05 was considered to be statistically significant.

3. Results

3.1. Clinical characteristics of patients

Twenty-six PCa patients treated with radical prostatectomy and sixteen healthy volunteers were included. Patients' characteristics are indicated in Table 1. Patients had not received previous treatment for their PCa. The median follow-up of the study was 25 months (IQR: 3–57 months).

3.2. Presence of cytokines in exosomes

We detected all the cytokines studied in exosomes from both healthy

Table 1
Clinical characteristics of healthy controls and patients at baseline. Age and pre-surgical PSA levels are expressed in median ± standard deviation.

HEALTHY CONTROLS	
Age (years)	57 ± 16
PSA (μg/L)	1.18 ± 1.15
PATIENTS	
Age (years)	64 ± 6.5
Histology	
Adenocarcinoma	100 (100%)
PSA (μg/L)	7.71 ± 3.01
< 10	20 (77%)
≥ 10	6 (23%)
TNM classification	
IIa	7 (26.9%)
IIb	11 (42.3%)
III	8 (30.8%)
Gleason group	
Group I (Gleason 6)	13 (50%)
Group II (Gleason 3 + 4)	7 (27%)
Group III (Gleason 4 + 3)	3 (11.5%)
Group IV (Gleason 8)	1 (3.8%)
Group V (Gleason > 8)	2 (7.7%)

controls and PCa patients at baseline, except for CXCL12, which was only detected in serum (Table 2). Considering all samples, there was a significant positive correlation between all cytokines' concentrations found in serum and serum-derived exosomes (Fig. 1).

Next, we investigated if exosomes were more loaded with some cytokines in relation to others. For this reason, we calculated the ratio exosomes/serum levels for each cytokine [12]. We found that exosomes were significantly enriched ($p < 0.05$) in MIF (median: 0.54; IQR: 0.42–0.98), TGF-β (median: 0.54; IQR: 0.42–0.60) and CXCL2 (median: 0.45; IQR: 0.37–0.63) in relation to the other cytokines measured (Fig. 2). There were no significant differences in the ratio exosomes/serum levels for each cytokine between controls and patients.

Table 2
Cytokines concentrations obtained in serum and exosomes from controls and prostate cancer patients. Data are expressed as median and interquartile range. $P < 0.05$ was considered statistically significant. [a] means significant differences related to controls; [b] means significant differences related to patients at baseline. n.d: not detected.

	Healthy Controls	Patients	
		Baseline	Post-surgery
CCL2 (ng/L)			
Serum	308 (230–484)	425 (282–755)	453 (331–627)
Exosomes	122 (104–126)	131 [a] (126–137)	128 [a,b] (122–132)
CXCL2 (ng/L)			
Serum	702 (470–880)	482 (359–629)	714[b] (594–1,202)
Exosomes	282 (224–318)	282 (267–299)	287 (281–299)
CXCL5 (ng/L)			
Serum	568 (434–1,292)	767 (596–1,392)	750[b] (501–1,012)
Exosomes	114 (97–164)	183 [a] (114–287)	121[b] (89–172)
CXCL8 (ng/L)			
Serum	22 (16–44)	21 (14–27)	18 (14–26)
Exosomes	6 (6–10)	7 (6–8)	6 (5–7)
CXCL12 (ng/L)			
Serum	388 (304–579)	342 (296–417)	286[b] (263–366)
Exosomes	n.d.	n.d.	n.d.
MIF (ng/L)			
Serum	1,620 (974–2,734)	3,617 [a] (2,299–6,279)	4,128 [a] (2,412–8,690)
Exosomes	1,288 (1,056–1,812)	1,768 (1,396–2,324)	1,716 (1,202–2,142)
S100A9 (ng/L)			
Serum	964 (617–2,105)	545 (303–1,125)	1033[b] (727–1,963)
Exosomes	285 (182–490)	155 [a] (111–220)	199[b] (162–332)
TGF-β (ng/L)			
Serum	21 (18–25)	31 [a] (21–49)	27[b] (15–38)
Exosomes	9 (7–13)	16 [a] (12–28)	18 [a,b] (9–20)

3.3. Differences in exosomal and serum cytokines between healthy controls and pre-surgical samples from PCa patients

Cytokine concentrations in exosomes and serum from healthy controls and patients at baseline are shown in Table 2. CCL2, CXCL5 and TGF-β concentrations in exosomes were significantly higher in PCa patients, as compared with controls, whereas S100A9 levels were lower ($p < 0.05$) (Table 2). CXCL2, CXCL8 and MIF exosome levels were similar between healthy controls and patients. Interestingly, we did not observe significant differences in serum cytokines between controls and PCa patients, except for MIF and TGF-β, which showed higher concentrations in PCa patients as compared with controls (Table 2).

3.4. Correlations between serum and exosomal cytokines concentrations and clinical variables

We correlated baseline serum and exosomal cytokine levels with clinical and analytical variables from PCa patients. MIF levels in exosomes correlated negatively with serum PSA levels ($r = -0.585$; $p < 0.05$) (Fig. 3A). We also observed a negative correlation between Gleason score and serum TGF-β ($r = -0.626$; $p = 0.001$) and exosomal TGF-β ($r = -0.444$; $p < 0.05$) levels (Fig. 3B).

3.5. Sequential evolution of exosomal and serum cytokines in PCa patients before and after radical prostatectomy

Following radical prostatectomy, exosomal S100A9 and TGF-ß concentrations increased significantly compared with pre-surgical situation, whereas CCL2 and CXCL5 decreased ($p < 0.05$ for both comparisons, Table 2). Median exosomal levels of cytokines in post-surgical samples were similar to healthy controls, except for CCL2 and TGF-β that remained significantly higher ($p < 0.05$ for both comparisons).

Also, post-surgical serum CXCL2 and S100A9 levels increased significantly compared with baseline situation, while CXCL5 and TGF-ß decreased ($p < 0.05$ for both comparisons, Table 2). Finally, although not detected in exosomes, serum CXCL12 decreased significantly following prostatectomy, as compared with baseline samples ($p < 0.05$).

4. Discussion

In prostate cancer, cytokines are involved in a wide range of processes implicated in metastasis, as epithelial–mesenchymal transition (EMT), angiogenesis, premetastatic niche creation or mechanisms of tumor escape of the immune system like MDSCs recruitment at the tumor site [9,26]. Our results show that these immunomodulatory proteins fundamental for MDSCs recruitment at the tumor site can be detected in circulating exosomes.

These exosomes were specially enriched in MIF, TGF-ß and CXCL2, as compared with other cytokines. Exosomes may shield these molecules, protecting them from exogenous proteases and facilitating their interaction with target cells [27]. Specifically, this has been confirmed for TGF-ß, which is transported by exosomes on their surface and exerts higher potency, as compared with free molecules [27].

In this study, we have observed that exosomes from PCa patients had higher levels of CCL2, CXCL5 and TGF-ß, as compared with healthy controls, and remained higher even after surgery, except for CXCL5 concentrations, which returned to control levels. Elevated exosome levels of TGF-β has been also observed in patients with acute myeloid leukemia, in which modifications in exosomal TGF-β content correlated with response to chemotherapy [28]. The increased levels of CCL2 observed in patients could be related to the conditions within the tumor site, such as a hypoxic environment that could favor their expression, as compared to non-neoplasic epithelia [29], and the release of exosomes loaded with these immunomodulatory molecules [30]. These cytokines have a relevant role in MDSCs recruitment during tumor development [15,31,32], and even promote growth of prostate bone tumor metastases

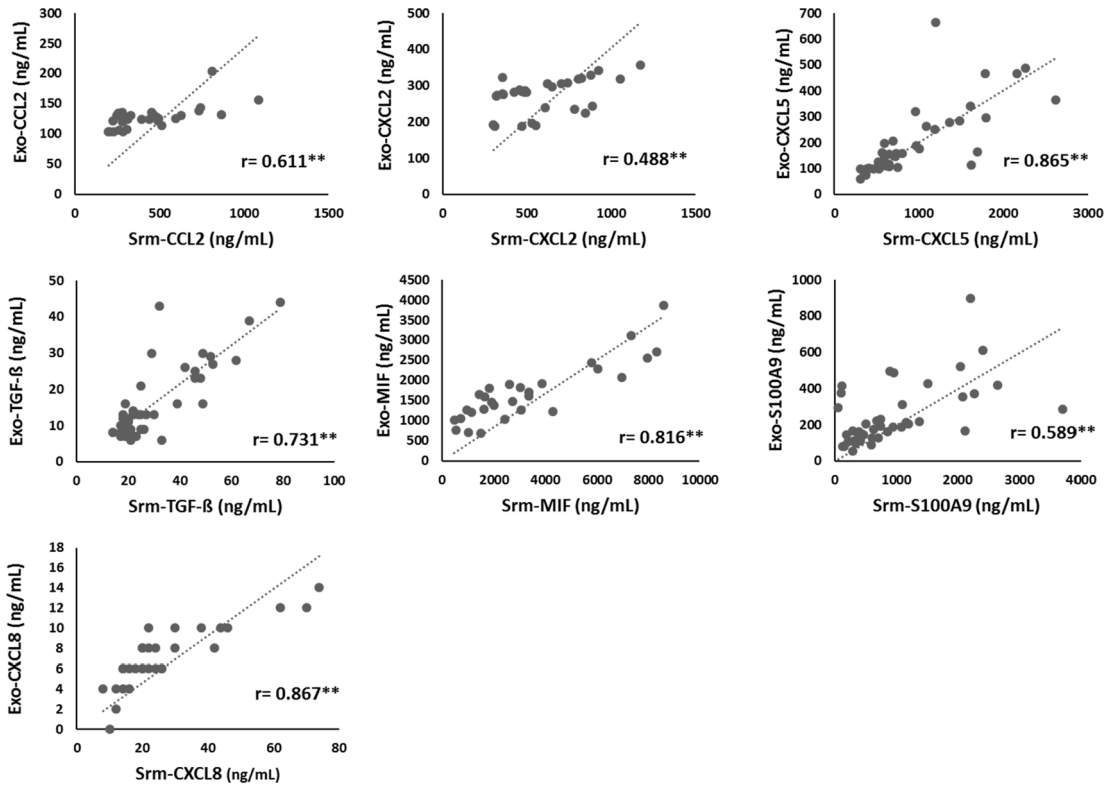

Fig. 1. Correlation between cytokines concentrations in serum (srm) and serum-derived exosomes (exo). ** $p < 0.01$.

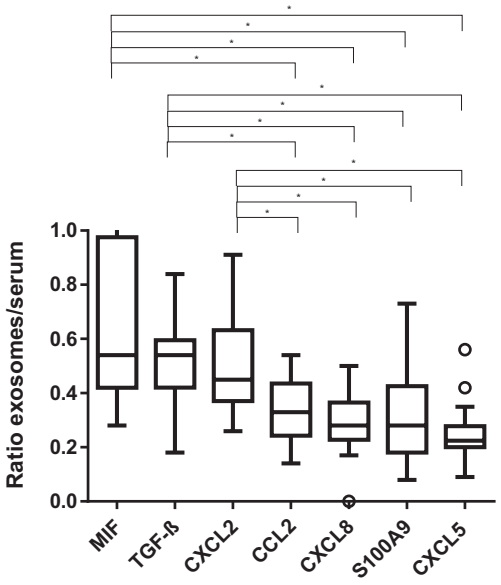

Fig. 2. Ratio of cytokines concentrations between exosomes and serum. Results are expressed as median and interquartile range. * $p < 0.05$.

[32].

TGF-ß levels were also higher in serum from PCa patient than in healthy volunteers, even after prostatectomy. Other authors observed elevated TGF-ß plasma levels in PCa patients [33], and elevated post-surgery plasma TGF-ß levels predicted poor oncological outcomes in patients with localized PCa treated with prostate cryoablation [34]. MIF, although in high proportion in exosomes, was only significant elevated in PCa patients when analyzed in serum. Also, contrary to that observed in exosomes, we have not detected differences between controls and PCa patients when analyzing serum CCL2 and CXCL5. This suggests that exosomes samples can be better than serum for analyzing CCL2 and CXCL5 in this disease. Conversely, other authors observed higher CCL2 [29] and CXCL5 [32] serum levels compared with healthy controls. The discrepancy could be because we studied patients at earlier stages.

We have only observed a relationship between the levels of exosomal MIF and serum PSA, but not for the other exosomal cytokines studied. This suggests that these cytokines are biomarkers that evaluate tumors differently than PSA or tumor burden, similarly to that observed by others for CCL2 [29]. Although PSA can be detected in exosomes [13], the type of producing cells, the function, the way of release, and the circulation is very different. Moreover, there was an inverse correlation between exosomal TGF-β concentrations and Gleason score. Consistently, other authors have not found correlation between TGF-ß and clinicopathological parameters, although TGF-β was increased in patients with extraprostatic extension, seminal vesicle involvement, and lymph nodes metastases [35]. Furthermore, the presence of differential concentrations of immunomodulators in exosomes could herald a potentially more aggressive disease. This has been observed in pancreatic cancer, where MIF concentrations were especially high in exosomes from stage I patients who later developed liver metastases [17].

S100A9 protein participates in MDSCs accumulation at the tumor sites and enhances the suppressive functions of MDSCs [8]. In addition, MDSCs release exosomes enriched in this protein [36]. Although initially it was suggested that circulating S100A9 could be a potential biomarker to discriminate between malignant and benign prostate disease [37], further studies did not confirm neither this fact, nor its correlation with clinicopathological parameters [38]. Our data agree with the latter in relation to serum S100A9, and although exosomal S100A9 levels were lower in cancer patients compared with healthy controls, it did not correlate with Gleason score.

Circulating exosomes may originate from the tumor or from another cellular source [39]. Our data supports that exosomal CCL2 and CXCL5 may originate from the tumor, because their level decreased soon after prostatectomy, as opposed to S100A9 and TGF-ß, which increased

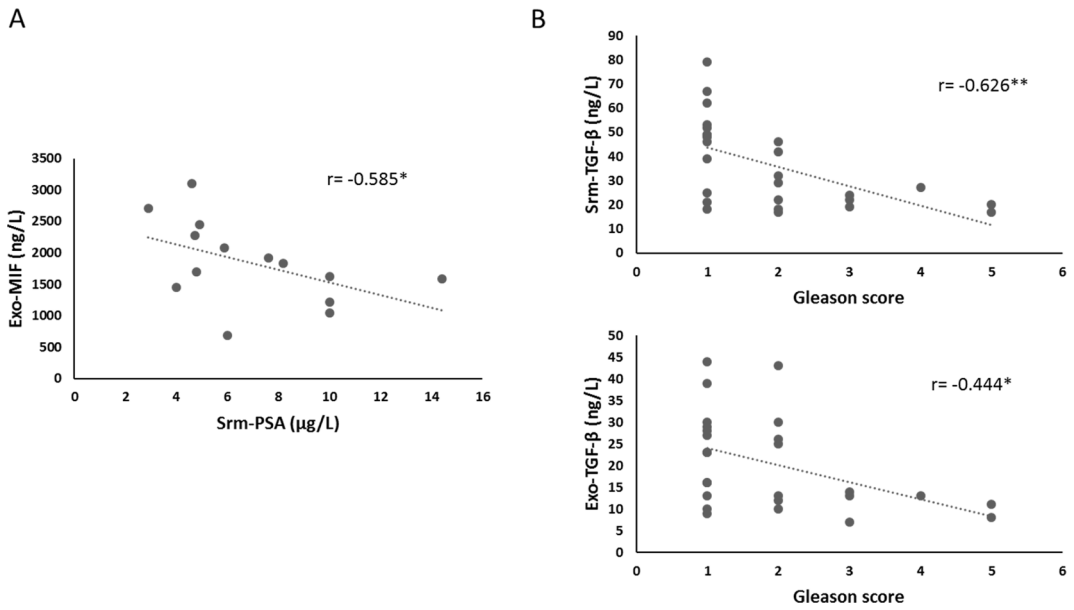

Fig. 3. Correlations between (A) serum (srm) PSA and exosome (exo) MIF and (B) between serum and exosome TGF-ß and Gleason score. * $p < 0.05$; ** $p < 0.01$.

following surgery. Unfortunately, up to now there are no procedures to isolate tumor exosomes from blood in clinical settings, and even the separation method can affect the measurement, which is especially noticeable in the case of CXCL12 [11].

The main limitation of our study is the sample size. Nevertheless, the availability of sequential samples before and after surgery allows performing intra-patient comparisons which increase the significance of our findings [22]. The type of surgery procedure (prostatectomy with or without lymphadenectomy) and the time elapsed between pre-surgery biopsy and blood analysis could be also interesting. However, the effect of exosome release during surgery in this study can be excluded, as cytokines half-life in blood circulation is usually even lower than that of exosomes (around 30 min) and the vast majority of extracellular vesicles are cleared from blood in 6 h [25]. Our results demonstrate that cytokines involved in MDSCs recruitment can be detected in circulating exosomes although further interaction studies between exosomes containing these cytokines and recipient cells should be conducted. We should also consider that, although manufacturers of separation kits claim to achieve specific exosomes' isolation, they are often contaminated with different subsets of microvesicles other than exosomes [40], as well as non-exosomal components in the exosomes extract as we and others have previously showed [11,41]. For this reason, the International Society of Extracellular Vesicles (ISEV) guidelines published in 2018 recommend using the general term "small extracellular vesicles" instead of exosomes [42]. Nevertheless, in this study we employ the term exosomes, as it is claimed by the isolation kit manufacturer used in this project.

4.1. Conclusions

In summary, we have analyzed for the first time the evolution of exosomal cytokines in pre and post-surgical blood samples from prostate cancer patients treated with radical prostatectomy. Our findings show that MDSCs immunomodulatory cytokines can be detected and monitored in exosomes from peripheral blood, and support that some of them may play a pivotal role in MDSCs recruitment at the tumor site which should be further confirmed in experimental prostate cancer models. In addition, some of these cytokines, particularly CCL2 and CXCL5 may hold interest as cancer biomarkers in PCa when measured in exosomes.

CRediT authorship contribution statement

Mónica Macías: Methodology, Formal analysis, Investigation. **Ángel García-Cortés:** Resources. **Marcos Torres:** Resources. **Javier Ancizu-Marckert:** Resources. **Juan Ignacio Pascual:** Resources. **Fernando Díez-Caballero:** Resources. **José Enrique Robles:** Resources. **David Rosell:** Resources. **Bernardino Miñana:** Resources. **Beatriz Mateos:** Investigation. **Daniel Ajona:** Methodology, Validation, Investigation. **Rodrigo Sánchez-Bayona:** Investigation. **Oihane Bedialauneta:** Resources. **Susana Chocarro:** Resources. **Ana Navarro:** Resources. **María P. Andueza:** Resources. **Alfonso Gúrpide:** Resources. **Jose Luis Perez-Gracia:** Conceptualization, Methodology, Formal analysis, Resources. **Estibaliz Alegre:** Conceptualization, Methodology, Formal analysis, Investigation. **Álvaro González:** Conceptualization, Methodology, Formal analysis, Resources, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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