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Induction of more aggressive tumoral phenotypes in LNCaP and PC3 cells by serum exosomes from prostate cancer patients

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Abstract

Prostate cancer (PCa) is the second most frequent and sixth most fatal cancer in men worldwide. Despite its high prevalence, our understanding of its etiology and the molecular mechanisms involved in the progression of the disease is substantially limited. In recent years, the potential participation of exosomes in this process has been suggested. Therefore, we aim to study the effect of exosomes isolated from the serum of patients with PCa on various cellular processes associated with increased tumor aggressiveness in two PCa cell lines: LNCaP-FGC and PC3. The exosomes were isolated by filtration wand ultracentrifugation. Their presence was confirmed by immunodetection of specific markers and their size distribution was analyzed by Dynamic Light Scattering (DLS). The results obtained demonstrated that serum exosomes from PCa patients increased migration of PC3 cells and neuroendocrine differentiation of LNCaP-FGC cells regardless of the grade of the tumor. PCa serum exosomes also enhanced the secretion of enzymes related to invasiveness and resistance to chemotherapeutics, such as extracellular matrix metalloproteases 2 and 9, and gamma-glutamyltransferase in both cell lines. Altogether, these findings support the pivotal participation of exosomes released by tumoral cells in the progression

Abbreviations: CC, colon cancer; CRPC, castration resistant prostate cancer; DLS, dynamic light scattering; DMSO, dimethyl sulfoxide; FBS, fetal bovine serum; GGT, gamma-glutamyltransferase; GSH, glutathione; ICAM-1, intracellular adhesion molecule 1; ISUP, International Society of Urological Pathology; LNCaP clone FGC, LNCaP-FGC; MMPs, matrix metalloproteinases; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazole bromide; NDS, normal donkey serum; NED, neuroendocrine differentiation; NEPCa, neuroendocrine prostate cancer; PBS, phosphate-buffered saline; PCa, prostate cancer; PSMA, prostate-specific membrane antigen; TEM, transmission electron microscopy.

of PCa. Future studies on the molecular mechanisms involved in the observed changes could provide crucial information on this disease and help in the discovery of new therapeutic targets.

KEYWORDS
LNCaP-FGC, PC3, prostate cancer, serum exosomes, tumor progression

What's new?

While exosomes have received increased attention for their potential roles in prostate cancer, the interaction between exosomes derived from the serum of prostate cancer patients and prostate cancer cells remains unexplored. In our study, serum exosomes increased the migration of PC3 cells, representative of castration-resistant prostate cancer, and the neuroendocrine differentiation of LNCaP cells, representative of the castration-sensitive stage of the disease. In both cell lines, serum exosomes also enhanced the secretion of enzymes related to invasiveness and resistance to chemotherapeutics. The findings support the pivotal role of these extracellular vesicles in the progression of the disease.

1 | INTRODUCTION

Prostate cancer (PCa) is the second most frequent and the sixth most deadly cancer in men worldwide and GLOBOCAN estimates suggest that both prevalence and mortality will tend to increase in the coming decades.[1] The main hallmark of this type of cancer is the progression to a castration-resistant and metastatic disease, a state related to a considerable low survival rate (29%), especially in comparison to the survival rate of patients with localized disease (99%).[2,3] Despite being such a common disease, little is known about its etiology and risk factors, which are limited to advanced age, family history and race.[2,4] In addition, prostate-specific antigen (PSA), the main biomarker for the diagnosis and screening of PCa, has proved to be unspecific. This lack of knowledge hampers the discovery of therapeutic targets and the possible development of drugs against them.

In the last decade, exosomes have drawn the scientific community's attention due to their role in cell communication. Exosomes are extracellular microvesicles of 30 to 150 nm that arise by invagination of the endosome membrane and are released by all cells studied to date. They can be found in most body fluids, such as urine or serum, and transport various types of molecules including lipids, proteins (such as the tetraspanins CD9 and CD63) and nucleic acids (like mRNAs or miRNAs).[5,6] These vesicles participate in intercellular communication by their fusion with the membranes of the recipient cells, which may affect both cells from local or distal locations, by transport in the bloodstream to target distal cells. Moreover, the content of exosomes varies depending on the cell type from which they are released and their physiological context[6]; in this regard, it has been shown that tumor prostate cells secrete exosomes with different content than healthy counterparts[7,8] The uptake of the exosomes can induce changes in target cells such as promoting cell differentiation and growth, modulating the immune response in tumors, and affecting the migratory and invasive capacity of tumoral cells.[8,9]

Specifically, in PCa, exosomes secreted by tumor cells can promote the invasive capacity of tumor cells, enhance androgen independence and favor metastasis in bone tissue and the development of chemoresistance.[10,11] In fact, there are several groups working on the development of diagnostic tools for PCa based on the characteristics and/or content of these exosomes.[12–14]

Some of the proteins that have aroused particular interest in PCa are prostate-specific membrane antigen (PSMA), matrix metalloproteinases (MMPs) and gamma-glutamyltransferase (GGT). PSMA is a transmembrane protein expressed in the brain, which is involved in folate uptake. It is also found in the prostate, an organ where its physiological function is not fully understood. Remarkably, PSMA expression is considerably increased in parallel with the progression of PCa. In this pathological scenario, the involvement of PSMA in the activation of the mitogen-activated protein kinase (MAPK) pathway has been described, promoting cancer cell survival and tumor progression.[15]

MMPs are zinc and calcium-dependent endopeptidases capable of degrading several components of the extracellular matrix, such as collagen, fibronectin or laminin. In PCa, the role of type IV gelatinases or collagenases, a group composed of MMP-2 and MMP-9, has been emphasized, due to its correlation with more aggressive cellular phenotypes.[16] In fact, some in vitro studies have shown a positive correlation between the expression of these MMPs and a greater invasive and migratory capacity of LNCaP and PC3.[17,18]

Finally, GGT is a membrane protein with enzymatic activity involved in the degradation of glutathione (GSH), a tripeptide of glutamate, cysteine and glycine whose main function is to act as an intracellular antioxidant.[19] In vivo assays with tumors induced in athymic mice with GGT-transfected PC3 cells showed an increased growth rate compared to tumors with untransfected PC3 cells.[20] Moreover, higher serum GGT levels have been associated with a worse response to chemotherapy in patients with metastatic castration-resistant PCa (CRPC),[21] so the involvement of this enzyme in tumor progression seems particularly relevant.

To recapitulate, there is an urgent need to deepen our understanding of the molecular mechanisms involved in the progression of prostate cancer at stages where the efficacy of the available therapies is insufficient. Furthermore, this knowledge could have a direct impact on the development of new targeted therapies which could increase

TABLE 1 Summary of the characteristics of the sample groups.

	Diagnosis				
	NC	G6	G7	G8	G9
Age group (years)	65.1 ± 1.8	67 ± 1.4	67.5 ± 1.4	63.9 ± 3.3	75 ± 3.3
Serum PSAt (ng/mL)	7.5 ± 1.1	5.5 ± 0.8	11.31 ± 2.5	173.6 ± 91.3	656.5 ± 390.6
Group size (n)	17	13	17	10	11

Note: They are expressed as mean ± SEM.
Abbreviations: G6, Gleason 6; G7, Gleason 4 + 3; G8, Gleason 8; G9, Gleason 9; NC, non-cancer; PSAt, total prostate-specific antigen.

patient survival. Likewise, considering the potential involvement of exosomes in this process, the main objective of this work is to evaluate the effect of serum exosomes from patients with PCa on different processes related to tumor aggressiveness in two prostate cancer cell lines: LNCaP, representative of castration sensible stage of the disease and PC3, representative of CRPC.

2 | MATERIALS AND METHODS

2.1 | Samples from patients

The Urology Service of the Hospital Universitario Príncipe de Asturias collected serum samples from patients without PCa and patients diagnosed with PCa prior to the start of any treatment (Table 1). These samples were stored at −80°C until processing at the Biochemistry and Molecular Biology Unit of the University of Alcala. Specimen collection: The fasting venous blood of the subjects was collected by EDTA anticoagulant tube. Then the samples were centrifuged at 2000*g* for 10 minutes to separate the plasma.

2.2 | Isolation of prostate-derived exosomes

A first aliquot of the serum was taken and stored at −80°C for further study. After its thawing, the serum was then subjected to three successive centrifugations from which the pellet was discarded: 500*g* for 10 minutes, 2000*g* for 10 minutes and 16 000*g* for 15 minutes, all of them at 4°C. After this process, the supernatant was filtered through a 0.22 μm filter. Then, ultracentrifugation at 140 000*g* for 2 hours at 4°C was performed. The supernatant was discarded and the sediment containing the exosomes was resuspended in phosphate-buffered saline (PBS), divided into different aliquots and stored at −80°C until use.

2.3 | Transmission electron microscopy (TEM)

Exosomes extracted after isolation are dissolved in PBS and adsorbed on carbon-formvar-coated nickel grids. For the morphological and immunocytochemical study, they are fixed with 0.5% glutaraldehyde-2% paraformaldehyde, after washing the grid is blocked with 3% normal donkey serum (NDS) and incubated overnight with the corresponding primary antibody (anti CD-63, diluted 1:50). After washing with PBS, the sections were incubated with 10 nm gold labeled IgG goat anti-mouse (Biocell), at 1:100 dilution for 1 hour at room temperature and finally they are counterstained with 1% uranyl acetate saturated solution. Exosomes are visualized under a Zeiss EM-10 transmission electron microscope.

2.4 | Dynamic light scattering

The size distribution of the isolated exosomes was measured by dynamic light scattering (DLS) instrument (ZetaSizer Nano ZS, Malvern, UK). After the laser and temperature equilibrium of the device were stabilized, 90 μL of exosomes sample (previously diluted 10-fold) were transferred into a cuvette and introduced into the DLS instrument to start measuring. For reproducibility and standardization, the parameter values were fixed as follows; Laser Wavelength (nm): 633, Temperature Controlled: yes, Peak Radius Low Cutoff (nm): 0.4, Peak Radius High Cutoff (nm): 10 000, Auto-attenuation Time Limit(s): 10, Calculate Polydispersity: yes, Set temperature(C): 15, Wait (min): 2. The measurement with DLS was conducted with three acquisition of 40 seconds. N represents the number of extracts from each diagnosis, and n represents the number of data collected from each samples (NC: N = 19, n = 3; G6: N = 16, n = 3; G7: N = 21, n = 3; G8: N = 9, n = 3; G9: N = 10, n = 3).

2.5 | Cell cultures

Two human prostate cancer cell lines were obtained from the American Type Culture Collection (ATCC). They exhibit different features of PCa progression from early stages to androgen independence. LNCaP clone FGC (LNCaP-FGC; passages 3-14, ATCC CRL-1740, certified by STRS analysis; RRID: CVCL_1379) is an androgen-responsive cancer cell line and PC3 (passages 7-17, ATCC CRL-1435, certified by STRS analysis; RRID: CVCL_0035) is an androgen-unresponsive cell line that may be analogous to recurrent PCa that have achieved androgen independence. LNCaP-FGC and PC3 cells were grown and maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS). All experiments were performed with mycoplasma-free cells. All culture media contained 1% penicillin/streptomycin/amphotericin B

(Life Technologies). Culture was carried out in a humidified 5% CO_2 environment at 37°C.

2.6 | Treatments

The amount of exosomes used in the treatments was based on previous results of the group.[22] Exactly 5 µg of exosomes were used in the assays with LNCaP-FGC cells, whereas 10 µg of exosomes were used with PC3 cells.

2.7 | MTT/cell viability assay

A total of 8000/10 000 cells (PC3/LNCaP-FGC) per well were seeded in P-96 plates and maintained for 24 hours to allow adhesion. At that time, treatments are added for 24 hours, and after this time, 0.3 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazole bromide (MTT) was added for 90 minutes at 37°C in dark conditions. The crystals produced after the addition of MTT were solubilized in dimethyl sulfoxide (DMSO) and the absorbance was measured at 570 nm with the Multiscan FC plate reader (Thermo Fisher, Walthman, MA). The results are expressed as the percentage absorbance with respect to the control.

2.8 | Cell cycle

Exactly 100 000 cells per well were seeded in P-6 plates and maintained for 24 hours to allow adhesion. They were then deprived with serum-free medium and, after 24 hours, the treatments were added for 24 hours. After this time, the medium was collected, and cells were lifted with trypsin and centrifuged at 1500 rpm for 5 minutes. The cell pellet was fixed and permeabilized with cold 70% ethanol for 24 hours at 4°C. After that, cells were centrifuged to remove ethanol by decanting, washed with PBS, resuspended and incubated for 30 minutes in PBS containing 0.2 mg/mL RNase and 20 µg/mL propidium iodide. Finally, they were analyzed by flow cytometry with MACSQuant Analyzer 10 Flow Cytometer (Miltenyi Biotec, Bergisch Gladbach, Germany) and the distribution of cells between the different phases of the cycle was evaluated with MACSQuantify software (Miltenyi Biotec, Bergisch Gladbach, Germany).

2.9 | Migration assays

Cells (25×10^4 cells per well) were seeded in P-24 plates and left until they form a monolayer of confluent cells, at which time the wound was made with a plastic tip, the medium was aspirated and treatments (10 µg of exosomes) were added. To assess cell migration, photos were taken at different times (0, 4, 8 and 24 hours) using a Nikon Optiphot-2 phase contrast inverted microscope. The results were presented as the percentage of the wound length closed. At the end of the assay, the media were collected and stored at −20°C for further study.

2.10 | Neuroendocrine differentiation

Cells (3×10^4 per well) were seeded onto P-24 plates and allowed to adhere for 24 hours. Treatments (5 µg of exosomes) were added, and pictures are taken at 24 and 48 hours with a Nikon Optiphot-2 phase contrast inverted microscope. Serum-deprived cells were used as a positive control for neuroendocrine differentiation (NED). At the end of the assay, media were collected and stored at −20°C for further study. Neurodifferentiated cells were those that present at least one dendrite that doubles the size of the cell soma.

2.11 | Protein quantification

After isolation of exosomes and collection of media, the protein concentration of each sample was assessed with the BCA PierceTM Protein Assay Kit (ThermoFisher Scientific), following the manufacturer's instructions.

2.12 | Western blotting

Serum samples and exosomes isolated from serum were studied by this technique. For this purpose, 30 µg were mixed with loading buffer containing 50 mM Tris-HCl (pH 6.8), 10% glycerol, 0.01% SDS, 0.01% bromophenol blue and 0.7 M β-mercaptoethanol, and denatured for 5 minutes at 95°C. They were then separated by electrophoresis on 12% acrylamide/bisacrylamide gels under denaturing conditions (SDS-PAGE) at room temperature. They were transferred to a PVDF membrane (Bio-Rad) at 10 V for 50 minutes. The membrane was stained with 0.25% Ponceau red solution to confirm the correct transfer of proteins to the membrane and washed with PBS to remove the stain. The membrane was then blocked with 5% skim milk in PBS for 1 hour at room temperature. After blocking, it was incubated overnight at 4°C in motion with the following primary antibodies: anti-CD9 (1:200), anti-CD63 (1:200) (Santa Cruz), anti-PSMA (1:50 000) (Abcam) or anti-neuron specific enolase (1:6000) (ICN Biomedicals). Subsequently washed with PBS and incubated with the corresponding horseradish peroxidase-conjugated secondary antibody (1:5000) 1 hour at room temperature in motion, washed with PBS and incubated with Excellent Chemiluminescent Substrate Detection Kit chemiluminescence reagent (Elabscience) for 1 minutes. The membrane was then exposed to ultraviolet light with the ChemiDoc MP Imaging System (Bio-Rad) apparatus to detect the signal and analyzed with Image Lab software (Bio-Rad).

2.13 | Zymography assays

The gelatinase activity of the media collected after the treatments was evaluated by Zymography. For this, 15 µg of protein mixed with loading buffer composed of 50 mM Tris-HCl (pH 6.8), 10% glycerol, 0.01% SDS and 0.01% bromophenol blue was loaded onto a 0.1%

gelatin copolymerized acrylamide/bisacrylamide gel (Sigma Aldrich) and cold electrophoresis was run. This was followed by two 30 minutes washes with 50 mM Tris-HCl (pH 7.4) and 2.5% Triton X-100, two 10 minutes washes with 50 mM Tris-HCl (pH 7.4) and the gel was incubated overnight at 37°C with a buffer containing 50 mM Tris-HCl (pH 7.4), 10 mM $CaCl_2$, 0.15 M NaCl, 0.1% Triton X-100 and 0.02% sodium azide. After incubation, the gels were stained for at least 1 hour with 50% methanol, 10% acetic acid and 0.25% Coomassie blue R-250 0.25%. Finally, they were discolored with a deinking solution containing 20% methanol and 7.5% acetic acid, at which time the white bands originating from the gelatinases were visualized on a blue background. The gels were photographed with the ChemiDoc MP Imaging System and analyzed with Image Lab software (Bio-Rad). The results are shown as the percentage of optical intensity with respect to the control.

2.14 | Gamma-glutamyl transferase activity

The gamma-glutamyl transferase (GGT) activity secreted into the medium after treatments was assessed with the Spinreact Quantitative Determination of Gamma-Glutamyl Transferase kit according to the manufacturer's instructions. The results are expressed as a percentage with respect to the control.

2.15 | Statistical analysis

Statistical analysis was performed with GraphPad Prism 8 software (GraphPad Software Inc., San Diego, CA). For this purpose, the normality of these variables was tested with Shapiro-Wilk test and one-way analysis of variance (ANOVA) and the Bonferroni test for multiple comparisons between the groups studied were performed. Data are presented as mean ± SEM. Statistical significance was considered at $P < .05$.

3 | RESULTS

3.1 | Isolation and identification of serum exosomes

The presence of exosomes after isolation was confirmed by immunodetection of CD9 and CD63 antigens, both of which are considered exosome-specific markers.[6] As shown in Figure 1A, CD9 and CD63 proteins expression were higher in the exosome fraction compared to serum in all samples except those from patients with Gleason 6 prostate cancer. In addition, the presence of prostatic exosomes was corroborated by immunodetection of PSMA (Figure 1B).

The presence of exosomes was also confirmed by transmission electron microscopy observing vesicles with a diameter between 30 and 100 nm (Figure 1C, left). Exosome size distributions showed that the majority population of microvesicles (60%), which probably corresponds to exosomes, has a size between 140 and 170 nm hydrodynamic radius (Figure 1C, right). A population of between 20 and 25 nm was also observed.

3.2 | Effect of patient exosome treatment on cell viability

As a first approach, the effect of exosomes isolated from patient serum on the viability of LNCaP-FGC and PC3 cells was evaluated. As shown in Figure 1D, the different treatments did not produce significant changes in the viability of LNCaP-FGC cells compared to the control. However, they induced a noticeable effect on PC3 cells, causing a significant reduction in their viability after treatment with exosomes from patients without PCa (89.9%) and from patients with grade G6 PCa (92.1%) when compared to the control. This reduction was more significant after treatment with exosomes from patients without cancer.

3.3 | Effect of patient exosome treatment on cell cycle

The influence of serum exosomes isolated from patients on the cell cycle of LNCaP-FGC and PC3 cells was studied by flow cytometry. The results included in Figure 2 showed no significant changes after the treatment in comparison to the control.

3.4 | Effect of patient exosome treatment on PC3 cell migration

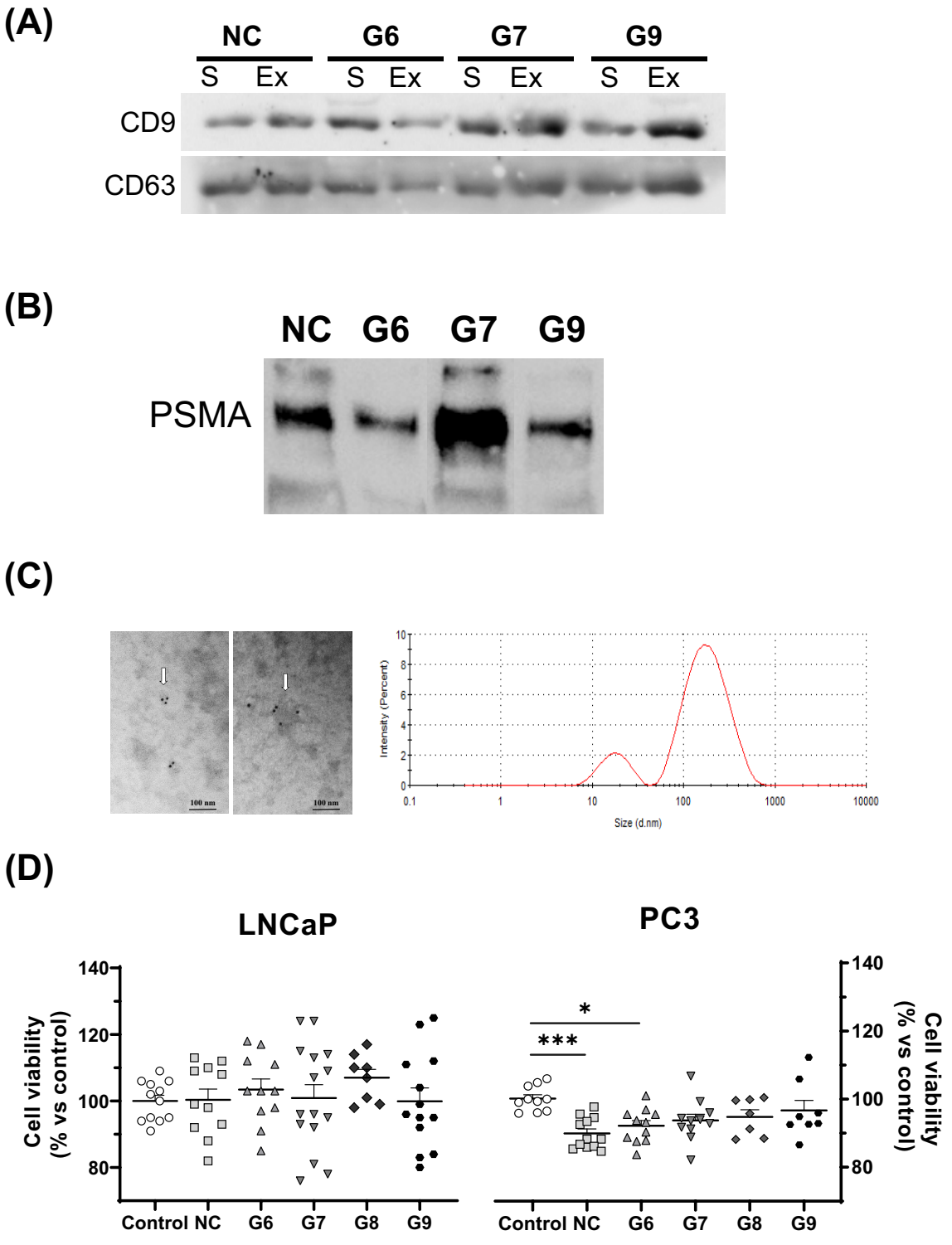
Subsequently, the effect of exosomes on PC3 cell migration was evaluated using the wound closure assay. The results shown in Figure 3 demonstrated a greater wound closure at 8 and 24 hours after exosome treatments from PCa patients compared to the control and with exosome treatment of patients without tumor. Thus, after 8 hours treatment, exosomes from patients with PCa G6, G7, G8 and G9 presented a wound healing of 41.9%, 56.9%, 38.4% and 47%, respectively, a significantly higher percentage when compared to the control (25.6%) and to exosomes from patients without PCa (22.9%). The wound healing at 24 hours was 94.2% for PC3 cells treated with exosomes from patients with PCa G6, 92.1% for G7, 91.1% for G8 and 86.3% for G9, significantly higher than 72.2% of control cells and 71.7% of PC3 cells treated with exosomes from patients without PCa.

3.5 | Effect of treatment with patient exosomes on neuroendocrine differentiation of LNCaP-FGC cells

In this section, the effect of exosomes isolated from serum of patients with and without PCa on the NED of LNCaP-FGC cells was assessed.

As shown in Figure 4, treatment with exosomes from patients diagnosed with PCa at any of the stages analyzed increased the percentage of neuroendocrine cells at 24 and 48 hours significantly compared to control cells and to cells treated with exosomes from NC patients.

Specifically, exosomes from G6 grade patients resulted in 16.2% and 18.1% of LNCaP-FGC cells presenting dendrites at 24 and 48 hours, respectively, G7 in 16.4% and 19.6%, G8 in 11.9% and 15.3% and G9 in 13.9% and 15.9% compared to 6.5% and 8% of control, 8.7% and 8.4% of the treatment with exosomes from NC patients



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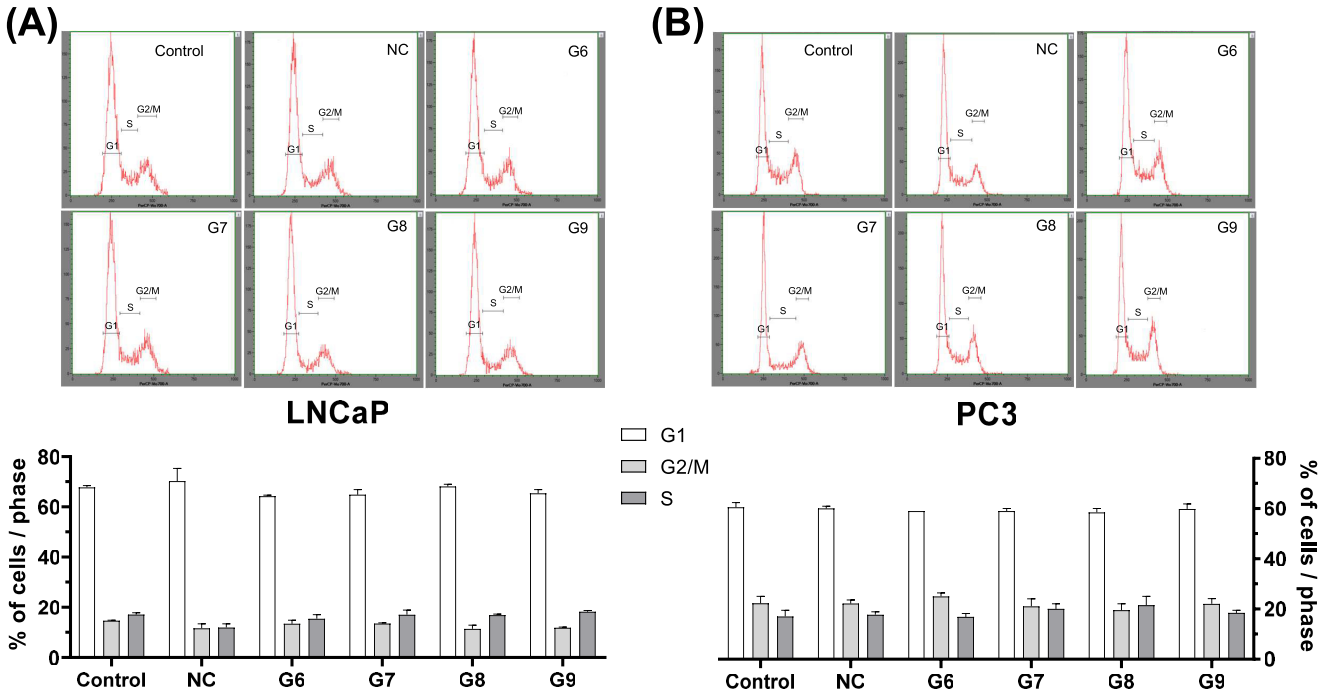

FIGURE 2 Effect of exosomes (5 μg for LNCaP treatment/10 μg for PC3 treatment) from PCa patients on the cell cycle of LNCaP (A) and PC3 (B) cells. The percentage of cells in each phase is shown, representing mean ± SEM of four independent experiments. Control, untreated cells; G6, Gleason 6; G7, Gleason 4 + 3; G8, Gleason 8; G9, Gleason 9; NC, non-cancer.

and 14.9% and 13.7% of serum-deprived cells, which are a positive control for NED. This correlates with the results obtained from the study of neuron-specific enolase (NSE) expression by Western blotting (Figure 4C).

3.6 | Effect of treatment with patient exosomes on enzyme secretion

The release of enzymes into the extracellular medium is another process related to tumor aggressiveness, since an increase in such secretion can affect both the structure of the extracellular matrix in the case of metalloproteases, and the availability of certain biomolecules with respect to GGT.

First, the gelatinase activity of the media of LNCaP-FGC and PC3 cells was studied 48 and 24 hours, respectively, after treatment by Zymography assay. Figure 5 shows that treatment with exosomes from G7 PCa patients produced a significant increase in pro-MMP-9 secretion in LNCaP-FGC cells (174%) compared to the control (100%). With respect to PC3, the exosomes from patients with PCa ≥ G8 caused an increase in the inactive and active forms of this metalloprotease (141% and 128%, respectively) compared to control (100%). It is worth mentioning the reduction of MMP-9 secretion after treatment with exosomes from NC, G6 and G7 patients compared to the control, although it did not reach a statistical significance.

Regarding MMP-2 isoforms, the same pattern of response was observed in both cell lines, presenting a significant increase in pro-MMP-2 secretion after treatment with exosomes from G7 PCa patients (145% LNCaP-FGC, 163% PC3) when compared to control (100%) and to treatment with exosomes from non-tumor patients in the case of PC3 (99%).

FIGURE 1 Characterization of patient serum exosomes and their effect on viability of LNCaP and PC3 cells. (A) Immunodetection of CD9 and CD63 antigens in sera and exosomes from such as sera. Total protein for detection of CD9 and CD63 was 30 μg. A representative experiment of the different Gleason grades is shown. Ex, exosomes, G6, Gleason 6; G7, Gleason 4 + 3; G9, Gleason 9; NC, non-cancer; S, serum.
(B) Immunodetection of PSMA antigen in serum exosomes. Total protein for detection of PSMA was 40 μg. A representative experiment of the different Gleason grades is shown. G6, Gleason 6; G7, Gleason 4 + 3; G9, Gleason 9; NC, non-cancer. (C) Left panel: Transmission electron microscopy (TEM) of exosomes from human serum. Immuno-gold labeled exosome with uranyl acetate staining. Exosomes are indicated by arrows. Scale bar = 100 nm; Right panel: size distribution histogram of exosomes obtained by dynamic light scattering (DLS). A representative experiment of the Gleason grade 6 is indicated. (D) Effect of exosomes (5 μg for LNCaP treatment/10 μg for PC3 treatment) from PCa patients on the viability of LNCaP and PC3 cells. Absorbance relative to control is shown, representing mean ± SEM of seven independent experiments. Control, untreated cells; G6, Gleason 6; G7, Gleason 4 + 3; G8, Gleason 8; G9, Gleason 9; NC, non-cancer. *$P < .05$; ***$P < .001$ compared to control.

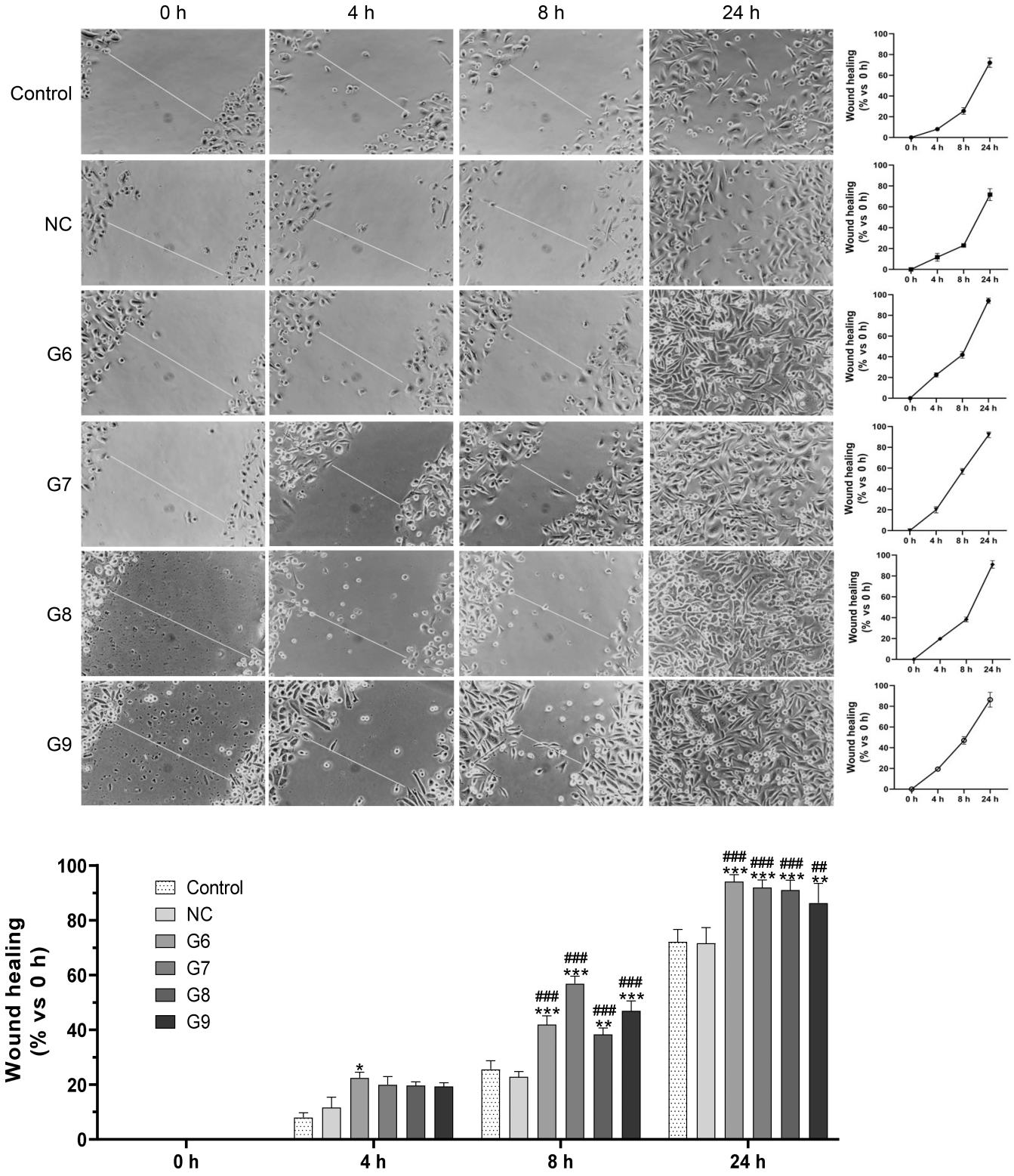

FIGURE 3 Effect of PCa patient exosomes (10 μg) on PC3 cell migration. The percentage of remaining wound at different times (4, 8 and 24 hours) compared to the initial wound (0 hour) is shown, representing mean ± SEM of six independent experiments. Control, untreated cells; G6, Gleason 6; G7, Gleason 4 + 3; G8, Gleason 8; G9, Gleason 9; NC, non-cancer. $^{*}P < .05$; $^{**}P < .01$; $^{***}P < .001$ comparing with control; $^{\#\#}P < .01$; $^{\#\#\#}P < .001$ comparing with treatment with exosomes from NC patients.

Second, the GGT activity of LNCaP-FGC cell media is evaluated 48 hours after treatment with exosomes from patients without tumor (NC) or from patients with PCa at different stages. As shown in Figure 5B, a significant increase in GGT activity was observed when treating with exosomes from patients diagnosed with PCa compared to the control (100%), to the treatment with exosomes from patients

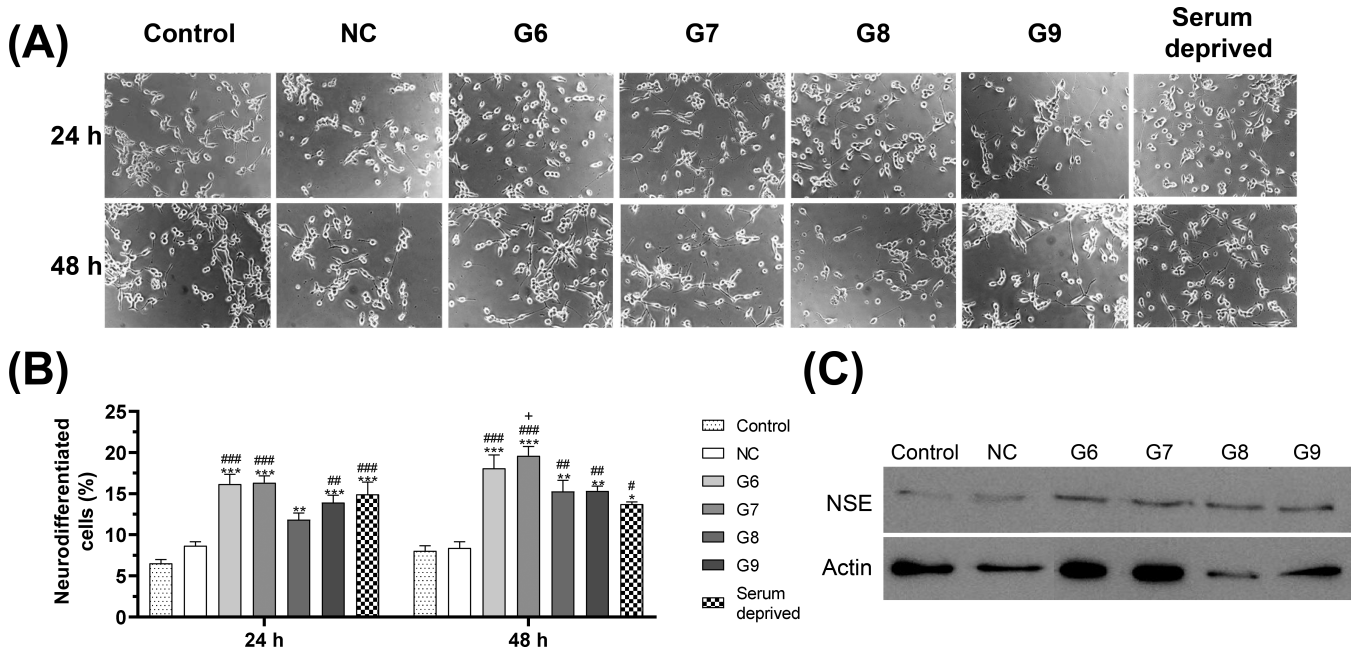

FIGURE 4 Effect of serum exosomes from PCa patients (5 μg) on neuroendocrine differentiation (NED) of LNCaP cells. (A) Shown are representative images of one of the six experiments performed of the control, exosome treatment for 24 or 48 hours of patients without tumor (NC) and exosome treatment of stages G6, G7, G8, G9 and serum-deprived cells, which are a positive control for NED. (B) At the bottom is shown quantitatively the percentage of neuroendocrine cells with each treatment at both study times, representing mean ± SEM of eight independent experiments, $^{*}P < .05$; $^{**}P < .01$; $^{***}P < .001$ comparing with control; $^{\#}P < .05$; $^{\#\#}P < .01$; $^{\#\#\#}P < .001$ comparing with treatment with exosomes from NC patients; $^{+}P < .05$ comparing with serum-deprived cells. (C) Immunodetection of neuron-specific enolase (NSE) antigen in LNCaP cells after treatment with exosomes for 24 hours from sera patients. Total protein for detection of NSE was 80 μg. A representative experiment of the different Gleason grades is shown. G6, Gleason 6; G7, Gleason 4 + 3; G8, Gleason 8; G9, Gleason 9; NC, non-cancer.

without tumor (132%) and to serum-deprived cells (125%). This increase correlated with the Gleason grade of the patient, being 177% with G6 exosomes, 186% with G7, 203% with G8 and 168% with G9.

With respect to the GGT activity of PC3 cell media, a significant increase was observed after treatment with exosomes of patients with PCa grade G6 (203%) and G7 (220%) when compared to the control (100%) and to exosomes from patients without PCa (128%). Our results show an increase in GGT expression in both cell lines upon treatment with exosomes from PCa patients.

4 | DISCUSSION

In recent years, the development of new therapies for PCa and the analysis of the most common molecular alterations through next-generation sequencing have advanced prostate cancer research. Despite these advances, there are still many unknowns that need to be answered. For example, the description of new biomarkers that allow us to distinguish cases of indolent disease from those of aggressive disease, and that will help to determine the prognosis and select the most appropriate therapy for each patient.[23-25] One of the areas that has captured the interest of the scientific community is exosomes and their use as biomarkers.[26,27] In fact, the effect of certain exosomal molecules on various cellular processes related to tumor aggressiveness has been assessed. Likewise, the effect of exosomes derived from aggressive cell lines, such as PC3, on less aggressive others, such as LNCaP-FGC, has been demonstrated. This has made it possible to describe the active involvement of these microvesicles in the acquisition of more aggressive tumor characteristics, such as the development of chemoresistance or cell growth under conditions of nutrient starvation.[22,28-30] However, the interaction between serum exosomes and PCa cells has not been studied to date. Therefore, in our study, we propose to evaluate the effect of exosomes isolated from the serum of PCa patients on two PCa cell lines: LNCaP-FGC and PC3.

Firstly, we have performed the immunodetection of specific exosome markers after isolation, thus confirming the success of the process. Among these markers, PSMA is noteworthy. Our results revealed that PSMA expression increases in the exosomal fraction with disease progression and are in agreement with previous results demonstrating that PSMA expression in prostate cancer is correlated with the stage and Gleason score.[31] In addition, it has been described the involvement of PSMA in the activation of the MAPK pathway, promoting cancer cell survival and tumor progression.[15]

Secondly, the effect of these serum exosomes on cell viability was then evaluated. Our results show a slight but significant loss of viability of PC3 cells after treatment with exosomes from non-PCa patients and patients with grade G6 PCa, while no significant differences are observed after treatment in LNCaP-FGC cells. These

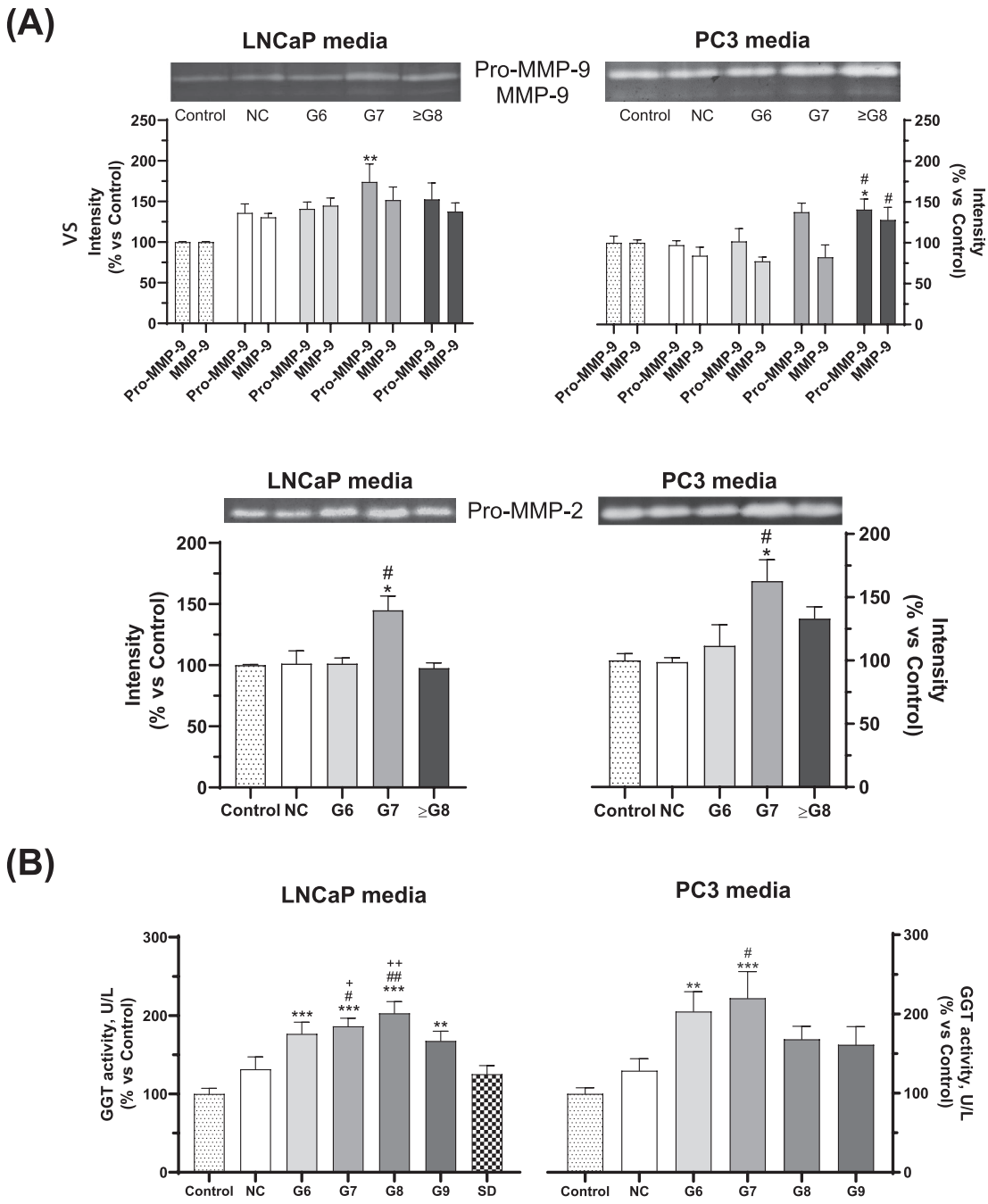

FIGURE 5 Effect of exosomes from PCa patients on the gelatinolytic activity of the metalloproteases MMP-9 (top) and pro-MMP-2 (bottom) (A) and on the enzymatic activity of GGT (B) into the media of LNCaP and PC3 cells. (A) Optical intensity of the relativized band vs control is shown representing mean ± SEM of six independent experiments. Control, untreated cells; G6, Gleason 6; G7, Gleason 4 + 3; ≥G8, Gleason 8-Gleason 10; NC, non-cancer. $^{*}P < .05$; $^{**}P < .01$ comparing with control; $^{\#}P < .05$ comparing with treatment with exosomes from NC patients; (B) GGT enzymatic activity relativized against control is shown representing mean ± SEM of six independent experiments. Control, untreated cells; G6, Gleason 6; G7, Gleason 4 + 3; G8, Gleason 8; G9, Gleason 9; NC, non-cancer; SD, serum deprived. $^{**}P < .01$; $^{***}P < .001$ comparing with control; $^{\#}P < .05$; $^{\#\#}P < .01$ comparing with the activity of media from exosome-treated cells from patients without tumor (NC); $^{+}P < .05$; $^{+}$ $^{+}P < .01$ comparing with the media of serum-deprived cells.

differences may be due to the expression of different receptors at the membrane level or to the alteration of signaling proteins, which may favor the differential activation of signaling cascades, triggering different cellular responses, as well as affecting the affinity of the exosomes for the cells themselves.[6] Nevertheless, the effect on PC3 cells is remarkable, since they are representative of CRPC for which current therapies are far from being fully effective.[2,24] In this sense, studying the mechanism responsible for this effect of exosomes from non-tumor patients could help to propose some therapeutic targets against which to direct adjuvant therapies for patients with CRPC.

Pang et al. (2021) showed the effect of serum-derived exosomes from patients diagnosed with colon cancer (CC) on the CC cell line SW480. They demonstrated that exosomes from CC patients significantly increase the migration of SW480 cells compared to exosomes from healthy patients.[32] Our results show a similar trend induced by exosomes from PCa patients, as these treatments significantly increased wound healing at 8 and 24 hours. In fact, the response induced by the treatment with exosomes from patients without PCa is practically identical to the response of control cells. This may suggest that exosomes derived from pathological contexts, in this case PCa, can promote the migration capacity of tumoral cells. These results agree with those observed by Li et al. in 2022, where they showed that intracellular adhesion molecule 1 (ICAM-1), whose presence has been described in PCa exosomes, is able to promote PCa DU145 cell migration by interacting with RelB, a component of the NF-κB pathway.[33] From a clinical point of view, it is interesting to note that castration-resistant cell lines, regardless of the degree of cell differentiation in prostate cancer patients, develop the ability to migrate. In this respect, it should be noted that the samples from the patients with more poorly differentiated International Society of Urological Pathology (ISUP) five grades were mostly metastatic.[34]

Neuroendocrine prostate cancer (NEPCa) is an aggressive variant of PCa that usually evolves from initially androgen-sensitive cells that acquire resistance to androgen deprivation.[27] Our group has previously described how PC3 cell-derived exosomes induced NED of LNCaP-FGC cells.[22] In our study, we were interested in the behavior of androgen-dependent cells (LNCaP) after treatment with exosomes from prostate cancer patients in relation to their transformation or neuroendocrine differentiation. The selective induction of neurodifferentiation in LNCaP cells could be due to the existence of androgen receptors and the absence of neuroendocrine markers. Whereas in PC3 cells, they do not have androgen receptors and are positive for neuroendocrine markers.[35,36] The results of this present work demonstrate that this process is also induced by serum exosomes from PCa patients, showing a significant overt increase at both 24 and 48 hours after treatment. These findings support the involvement of exosomes in the progression of PCa to more aggressive stages, such as NEPCa.

It is striking that even exosomes from patients with G6 PCa produce such a marked increase in PC3 cell migration and in the percentage of neuroendocrine cells in LNCaP-FGC, as this is a stage of low risk and little clinical relevance.[3,23] These results may indicate that these cells have reached the tipping point at the molecular level that allows the exosomes they release and their content to favor the progression of PCa, even though the G6 grade does not have major clinical implications in the short term.

Overexpression of MMP-9 and MMP-2 has been associated with increased invasiveness of PCa cells in vitro and in vivo.[17,37,38] Our observations are consistent with these findings, as exosomes from patients with PCa ≥ G8 increase both pro-MMP-9 expression and PC3 cell migration, and those from patients with PCa G7 promote both NED and pro-MMP-9 secretion from LNCaP-FGC cells and pro-MMP-2 expression in both cell lines.

GGT expression appears upregulated in PCa cells, and its overexpression is associated with poor prognosis.[19] This elevation increases cellular capacity for GSH production, as well as activates signaling through the glutamate receptor, which may promote cell survival through activation of the PI3K-Akt pathway.[39] Our results show an increase in GGT expression in both cell lines upon treatment with exosomes from PCa patients, in parallel with the acquisition of more aggressive features such as increased cell migration or neurodifferentiation. This evidence supports the correlation between increased GGT secretion and increased tumor aggressiveness.

Activation of the NF-κB pathway in PCa cell lines has been described to promote the expression of MMP-2 and MMP-9,[40] as well as the expression of GGT in chronic myeloid leukemia cells.[41] Therefore, the effect of serum exosomes from PCa patients on this pathway is likely and should be evaluated as it would explain many of the responses described in this work.

Our study shows, for the first time, the effect of serum-derived exosomes from PCa patients on the acquisition of several features related to more aggressive tumor phenotypes, supporting the pivotal role of these extracellular vesicles in the progression of the disease. Further investigation of the molecular mechanisms involved could provide very useful information for the understanding and future management of PCa, particularly in the discovery of therapeutic targets and in the development of personalized therapies.

AUTHOR CONTRIBUTIONS

Raquel Huertas-Lárez: Investigation, Writing – original draft, Data curation; **Laura Muñoz-Moreno:** Methodology, Supervision, Visualization, Writing – review & editing; **Jorge Recio-Aldavero:** Investigation, Data curation, Methodology; **Irene Dolores Román:** Methodology, Writing – review & editing; María Isabel Arenas: Investigation, Data curation, Methodology; **Ana Blasco:** Resources, Data curation; Ángeles Sanchís-Bonet: Resources, Funding acquisition; **Ana M. Bajo:** Conceptualization, Resources, Funding acquisition, Supervision. The work reported in the article has been performed by the authors, unless clearly specified in the text.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of our study are available from the corresponding author upon reasonable request.

ETHICS STATEMENT

The present study was approved by the Ethics Committee of Hospital Universitario Principe de Asturias de Alcala de Henares. Informed consent was obtained from all the participants before the start of the study.

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