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Characterization of biomarkers on small Extracellular Vesicles during SARS-CoV2 infections in lung transplant patients

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Abstract

Extracellular vesicles (EVs), including small EVs (sEVs), have emerged as crucial biomarkers for various diseases. This study investigated the potential of sEVs as biomarkers for SARS-CoV-2 infection in lung transplant patients, focusing on their role in organ transplants. Plasma samples from five lung transplant recipients diagnosed with SARS-CoV-2 and five healthy controls were analyzed. The sEVs were isolated using an Invitrogen Exosome Isolation Kit, characterized by Nanosight NS300 for particle size (< 200 nm), and quantified for protein concentration using the BCA assay. Extracted protein from the sEVs was subjected to SDS-PAGE, Coomassie staining, and western blotting to detect SARS-CoV-2 spike and nucleocapsid proteins. The results showed that the isolated sEVs from infected lung transplant patients contained significantly higher levels of SARS-CoV-2 spike and nucleocapsid proteins compared to those from the control group. Western blot analysis revealed strong bands corresponding to the spike and nucleocapsid proteins in infected patients, with statistical significance ($p < 0.0001$ for spike protein and $p < 0.05$ for nucleocapsid protein). These findings indicate that sEVs may serve as biomarkers for monitoring SARS-CoV-2 infection in lung transplant patients. Further studies with larger sample sizes are needed to validate these results and explore the broader therapeutic applications of sEVs in disease contexts. This study supports the potential utility of sEVs in detecting SARS-CoV-2 infection and monitoring transplant status, warranting additional investigation into their diagnostic value.

Keywords

sEVs, SARS-CoV-2 biomarkers, Nucleocapsid protein, SARS-CoV-2 Spike, Exosomes, Organ transplant rejection, Exosomal biomarkers, SDS-PAGE, Western Blot Analysis, BCA assay

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Introduction

The term “extracellular vesicle” was first used in the literature in 1971 by a study that involved electron microscopic analysis of the freshwater alga ‘Ochromonas danica’ (1). At that time, several terms were used to designate EVs, such as shedding vesicles, membrane fragments, plasma membrane vesicles, microvesicles and exosomes. A significant advancement for EVs occurred in 1996, when Raposo et al. (2) reported the participation of EVs in immune responses by observing their role in activating the adaptive immune response (3). Initially considered ‘cellular debris,’ EVs are now studied for their roles in cellular mechanisms and as disease biomarkers (4). EVs are secreted by cells from multicellular and unicellular organisms. EVs originate from plasma membranes either ectosomal or endosomal in origin. EVs are released from all types of cells undergoing stress including during transformation, activation, drug treatments, surgeries and infections.

According to the recent Minimal Information for Studies of Extracellular Vesicles (MISEV 2023) extracellular vesicles are lipid bilayer membrane that can be categorized as small (sEVs; 200-nm in diameter) or as apoptotic bodies (>1000-nm in diameter) (5-7). EVs are shown to carry a variety of biological molecules including proteins and peptides, lipids, nucleic acids, small metabolites, and antigens to infectious agents. The composition of EVs depends on their cellular origin and the functional state of that cell. Their genesis involves different mechanisms and they possess different sizes and buoyant densities (8). Exosomes are formed through the endolysosomal pathway following invagination of endosomal membranes to form multivesicular bodies and are released after fusion of multivesicular bodies with the plasma membrane (9). Small EVs (< 200 nm) are endogenous in origin and carry DNA, RNA, metabolites, proteins, and peptides, depending on their origin. International guidelines for EV isolation and characterization are regularly updated with advances in research. A representative diagram of an sEV is shown in Figure 1.

In the current report, blood was collected from lung transplant patients infected with SARS-CoV-2, and biomarkers on small extracellular vesicles were analyzed. Exosomes were analyzed from blood samples rather than nose-swabs and PCR tests because PCR testing can yield false-negative results (10, 11). Prior work has shown that infections may be trackable using exosomes throughout the course of infection and beyond the typical two-week infection period (12, 13). Exosomes may offer improved sensitivity and specificity as diagnostic tools (14). They may serve as confirmation of a SARS-CoV-2 infection prior to a lung transplant. In this work, protocols for plasma isolation, sEV isolation, purification, and characterization are described. Differences in sEVs from controls (non-infected patients) and infected patients were evaluated, and the associated biomarkers were quantitatively analyzed. The objective was to identify biomarkers on sEVs during SARS-CoV-2 infections in lung transplant patients.

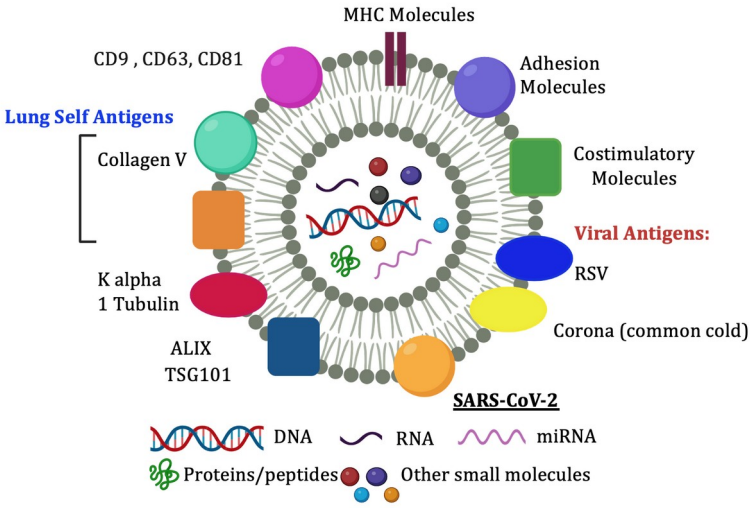

Figure 1. Structure of small Extracellular Vesicles with the antigens present (adapted from Transplantation. 2024 Feb 1; 108 (2):374-385).

Materials

Heparinized BD Vacutainer tubes (BD Biosciences, Cat# 367874) were used for blood collection. The Total Exosome Isolation Reagent was sourced from Invitrogen, Thermo Fisher Scientific. The NuPAGE SDS-PAGE gels and buffer were from Invitrogen, Cat# NP0001, Thermo Fisher Scientific. Protein quantification was performed using the Micro BCA Protein Assay Kit (Thermo Scientific, Cat# 23235). The NuPAGE Transfer Buffer was from Life Technologies, Cat# NP0006-1. The Transfer membrane was from Amersham Hybond-P PVDF membrane (Cytiva, Cat# 10600021). The Blocking agent was Bovine Serum Albumin (BSA) (Research Products International, Cat# A30075). The Primary antibodies were Anti-SARS-CoV-2 Spike protein antibody (Invitrogen, Cat# MA5-35948), and Anti-SARS-CoV-2 Nucleocapsid protein antibody (Invitrogen, Cat# MA5-35941). The Secondary antibody was Pierce Horseradish Peroxidase (Thermo Fisher Scientific, Cat# 31490). The Western blot washing buffer was TBS-T (Tris-buffered saline with Tween-20) (Sigma-Aldrich, Cat# 91414). The Detection reagent was Western Lightning Plus-ECL Chemiluminescent Substrate (Sigma-Aldrich, Cat# WBKLS0500). ImageJ (NIH) was used to image the Western Blot. The Power Blotter System was from Invitrogen, Cat# PB0012. The blots were exposed using a LI-COR Odyssey Imager (LI-COR Biosciences).

Methods

Isolation of Plasma from blood

This study is a retrospective analysis of samples from five SARS-CoV-2 negative controls and five adult lung transplant recipients diagnosed as SARS-CoV-2 positive. The samples from the SARS-CoV-2 negative controls were pooled and labeled as the control

group. The samples from the SARS-CoV-2 patients were separately analyzed. All patients were eligible for the study, had undergone lung transplantation at St. Joseph's Hospital, Phoenix, Arizona, and plasma samples were available for analysis. This study was approved by the Institutional Review Board at St. Joseph's Hospital (IRB# PHXB16-0027-10-18). Blood samples were centrifuged at 400 RCF for 45 minutes. The plasma was then aliquoted into smaller tubes, labeled, and stored in a deep freezer until further use.

Isolation of exosomes

Exosomes were isolated from 700 µL of plasma obtained from both groups using the Invitrogen Exosome Isolation kit, followed by 0.22-micron filtration. All samples were centrifuged at 15,294 RCF for 90 minutes. After removing debris such as tissue fragments, 200–300 µL of Total Exosome Precipitation solution was added. After vortexing, the samples were incubated at room temperature for 30 minutes. The tubes were then centrifuged at 2,655 RCF for 20 minutes. The pellets were resuspended in phosphate-buffered saline (PBS) at a volume twice the pellet size, vortexed, and incubated at room temperature for an additional 10 minutes. The protocol is illustrated in Figure 2.

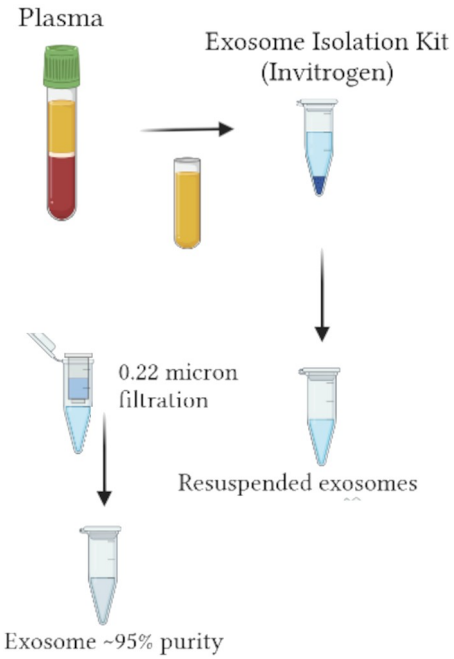

Figure 2. Schematic protocol for the isolation of exosomes.

Exosome size determination

Exosome size was measured using a Nanosight NS300 (Malvern, Great Malvern, UK), per the manufacturer's instructions. The instrument was calibrated with standard polystyrene nanospheres prior to use.

BCA Assay for protein estimation

The micro-BCA assay was performed according to the manufacturer's instructions. The detection range for protein concentration in this assay is 20 to 2,000 µg/mL. Optical density values obtained were used to calculate the protein concentration in 20 µL of buffer. This volume was later used for the SDS-PAGE analysis. The use of the BCA assay when it quantifies total protein in the exosome is to allow the loading of equal amounts of protein from each sample onto the gel for SDS-PAGE and western blotting. This advances our objective of identification and quantification of the two proteins of interest because this ensures that differences in SDS-PAGE band intensity reflect true differences in the proteins of interest, rather than variations in sample loading (although, see the Limitations section).

Sodium Dodecyl Sulphate PolyAcrylamide Gel Electrophoresis (SDS-PAGE)

All wells of the gel were loaded with equal protein content, volume adjusted based on the protein estimation by BCA. Samples were reconstituted at equal concentrations for SDS-PAGE (sample + buffer + sample loading dye = 20 µL in volume). The tubes were heated at 95 °C for 5 minutes and briefly centrifuged for 10 seconds to prevent changes in concentration due to vapor escape. SDS is a strong ionic detergent that disrupts lipid membranes by solubilizing membrane proteins and lipids, while the heat aids in protein denaturation and in the release of internal contents. This approach is commonly used to lyse extracellular vesicles, including exosomes, for protein extraction prior to electrophoresis (15). Given that intact exosomes (30–200 nm) are too large to migrate through the polyacrylamide gel, the observed bands, such as those for SARS-CoV-2 Spike and Nucleocapsid proteins, represent proteins released from lysed exosomes rather than from intact exosome migration.

For SDS-PAGE, 950 mL of distilled water was mixed with 50 mL of SDS running buffer and added to the electrophoresis chamber. A total of 18 µL of each sample was loaded into individual wells adjacent to the molecular weight ladder. The approximate detection limit for SDS-PAGE is 2–5 ng per protein band. The gel was run for approximately 2 hours, or until the dye front reached the bottom.

One section of the gel was stained with Coomassie Brilliant Blue to confirm successful electrophoresis and band resolution. A separate, unstained replica gel was used for protein transfer onto a polyvinylidene difluoride (PVDF) membrane. The PVDF membrane was activated by soaking in methanol for 30 seconds, then incubated in TBST buffer. One section of the gel was positioned between the membranes, and two 0.75 mm-thick sheets of blotting paper were placed below and above the gel. This assembly was placed into a Power Blotter system and run for two cycles of 11 minutes each. The experimental layout is presented in Figure 3.

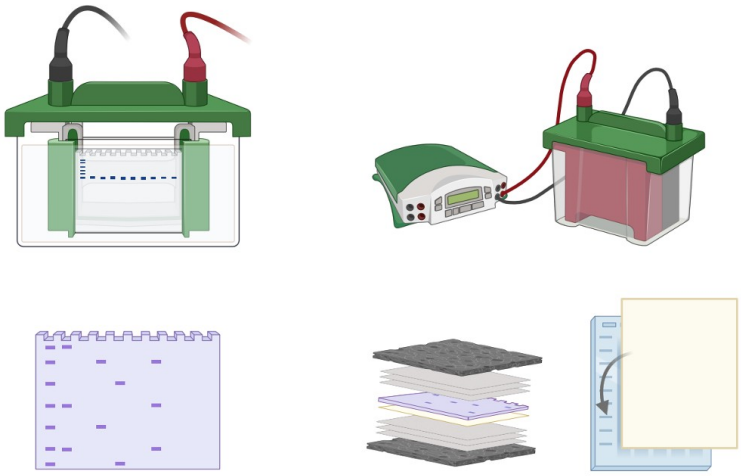

Figure 3. Experimental layout for SDS-PAGE and steps prior to Western blotting.

Characterization of exosomes using western blot

A total of 15 µg of exosomal protein was resolved by polyacrylamide gel electrophoresis, and the proteins were transferred onto a PVDF membrane. The western blot assay has a detection sensitivity of ~ 10 pg per lane. Lysed Exosomes from all patients, including pooled exosomes from five SARS-CoV-2 negative controls and individual exosomes from five SARS-CoV-2 positive lung transplant recipients (n = 5), were characterized for the presence of SARS-CoV-2 Spike protein (S1/S2) and nucleocapsid protein.

The membrane was blocked with 5% BSA and probed with the exosome-specific marker Light Chain IgG. SARS-CoV-2 spike protein S2 (Thermo Fisher Scientific) and SARS-CoV-2 nucleoprotein antibody (Thermo Fisher Scientific) were used as primary antibodies (Abs); secondary Abs conjugated with horseradish peroxidase (HRP), specific to the primary Abs, were employed. The blots were washed with PBS-Tween (Thermo Fisher Scientific), developed using a chemiluminescent HRP substrate (WBKLS0500, Millipore Sigma), and exposed using the Odyssey CLx Imaging System (LI-COR Biosciences). The band intensity of the target proteins was quantified using ImageJ software and normalized to Light Chain IgG. Western blot analysis was not performed using signal-to-noise ratios; instead, it was conducted using an internal control, Light Chain IgG. Each band in every sample lane was normalized to this internal control before analysis.

Statistical analysis

Data analysis was performed using Prism 8.0 software (GraphPad, Inc.). Optical densities of sEVs containing SARS-CoV-2 Spike and nucleocapsid proteins were compared using the Student's t-test. P values < 0.05 were considered statistically significant in all

comparative analyses. The mean optical densities of exosomal viral antigens were calculated after normalization with the exosome-specific marker Light Chain IgG.

Results and Discussion

Exosome size determination

Exosomes from SARS-CoV2 infected patients displayed multi-modal size distributions (Figure 4(B through F), with a majority of particles below 400 nm with a peak in size distribution ~ 100 nm. The exosome distribution from non-infected patients was mono-modal, with a peak ~ 50 nm (Figure 4A).

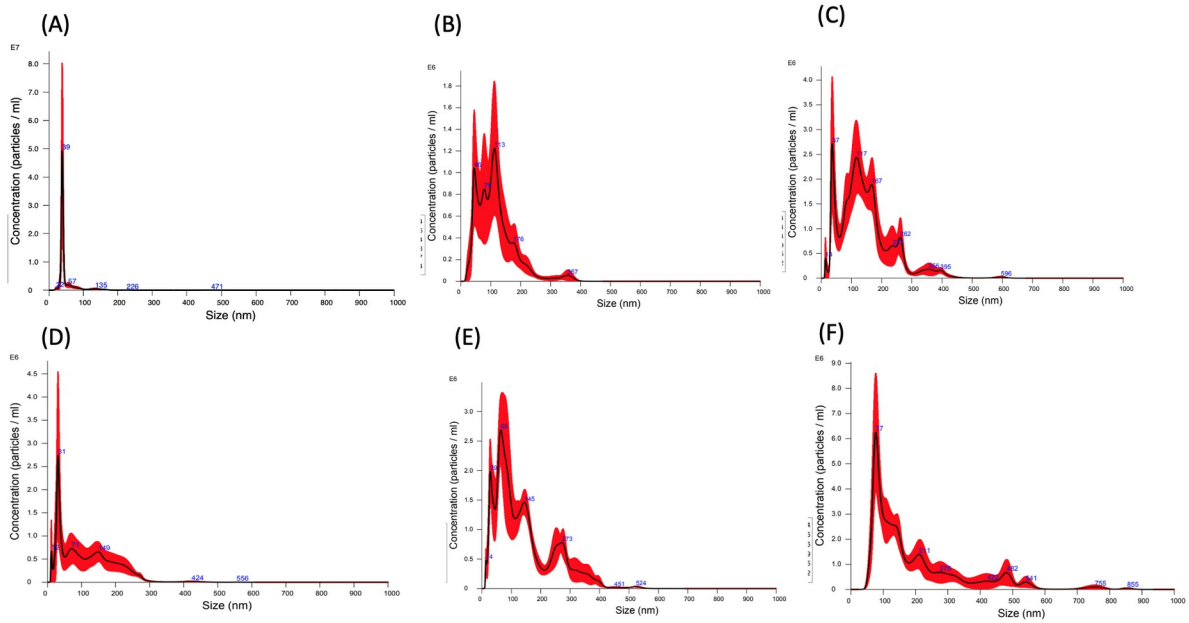

Figure 4. NanoSight NS300 images. Panels A–C correspond to the Control, SARS-CoV-2 Positive Sample 1, and SARS-CoV-2 Positive Sample 2, respectively; panels D–F correspond to SARS-CoV-2 Positive Sample 3, SARS-CoV-2 Positive Sample 4, and SARS-CoV-2 Positive Sample 5, respectively.

BCA Assay for protein estimation

Following isolation and size validation, small extracellular vesicles (sEVs) were measured for protein content to enable further analysis. The BCA standard curve encompassing the range of sample protein concentrations was linear (Figure 5). The total protein concentrations for P1 through P5 (µg/µL) were determined to be 16.73, 20.02, 21.24, 26.96, and 7.40 µg/µL respectively. The average protein concentration was 18.47 µg/µL, with a standard deviation of 6.44 µg/µL.

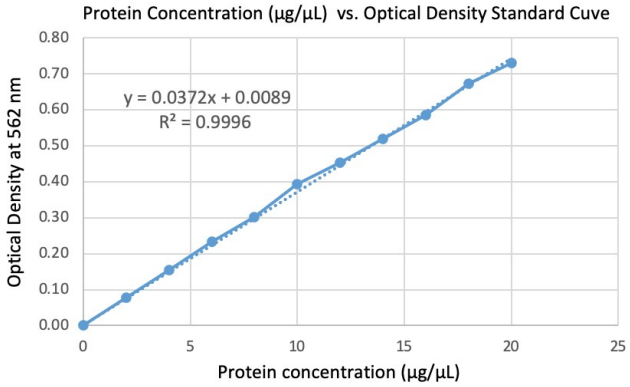

Figure 5. The BCA standard curve. The curve demonstrates a strong linear relationship between concentration and absorbance, with a regression coefficient (R^2) of 0.9996. The highest standard concentration used in the assay was 20 µg/µL.

The contents of the lysed sEVs were subjected to SDS-PAGE, followed by staining with Coomassie Brilliant Blue dye to visualize and validate the clarity of protein separation prior to transfer for western blotting. After transferring the gel contents onto a PVDF membrane, Ponceau S staining was performed to confirm successful transfer of protein bands. The results are shown in Figure 6.

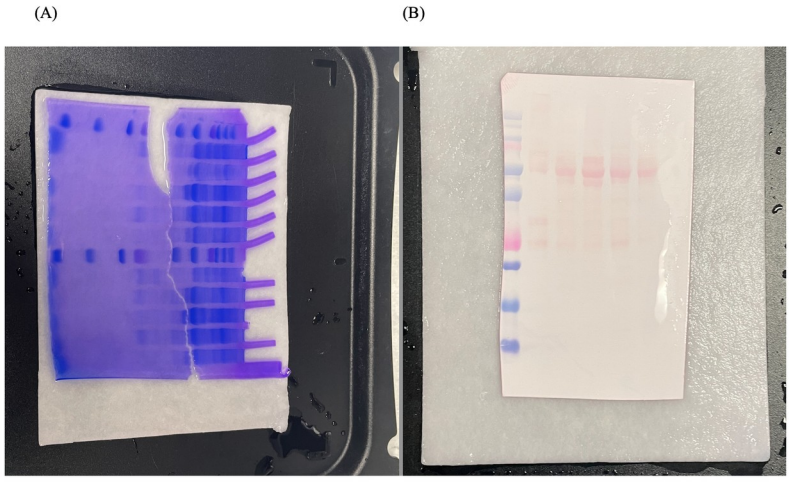

Figure 6. Coomassie Brilliant Blue Staining is in Panel A. Ponceau S Staining is in Panel B.

Upon confirmation of complete transfer and protocol optimization, the membranes were probed with appropriate antibodies, as described in the Materials section. Results are

presented in Figure 7. Panel A in Figure 7 shows the western blot results, showing bands corresponding to the expected molecular weights of the SARS-CoV-2 spike protein (~180 kDa) and nucleocapsid protein (~46 kDa). Both proteins were normalized to Light Chain IgG, which served as an internal control.

The data in Figure 7, Panel B demonstrate that the control group (a pool of SARS-CoV-2 negative samples) showed little to no detectable signal for the spike and nucleocapsid proteins. In contrast, the SARS-CoV-2 positive samples exhibited detectable signals for both proteins (except for P4 nucleocapsid). Band intensities were quantified using GraphPad Prism software, and statistical analysis revealed a significant difference in spike protein levels between the control and infected groups ($p < 0.0001$). Similarly, nucleocapsid protein levels differed significantly between groups ($p < 0.05$). The individual sample and control values for relative density are shown in Panel B.

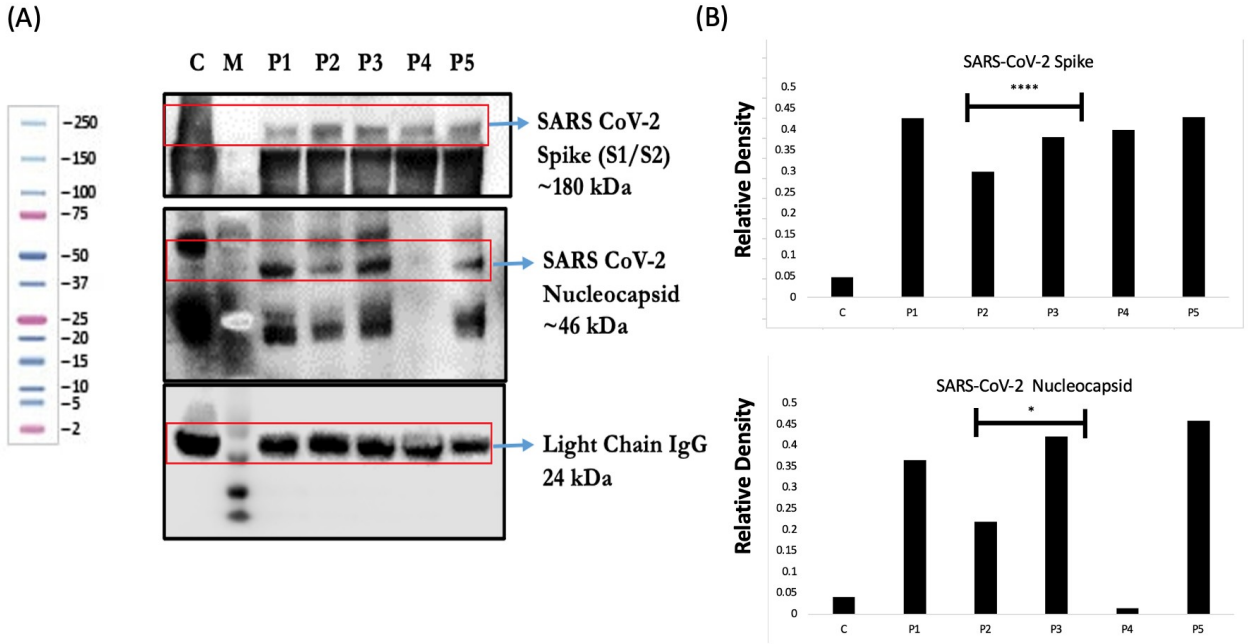

Figure 7. Western blot and densitometry analysis of sEV proteins from lung transplant recipients (LTxRs) with confirmed SARS-CoV-2 infection and non-infected controls. Panel A shows representative Western blots of sEV protein samples, with C representing a non-infected control, M denoting the molecular weight marker (protein ladder), and P1–P5 corresponding to samples from patients 1 through 5 with positive SARS-CoV-2 infection. Panel B presents the corresponding densitometry analysis of the protein bands. Statistical significance is indicated as follows: $p < 0.05$ (*), $p < 0.0001$ (****).

Limitations

The sample size was limited to 5 samples. Further studies with larger sample sizes are needed to validate these results, especially considering dissimilar exosome particle size distributions and ambiguity regarding the BCA assay for protein extraction.

There is no consensus in the literature on whether the BCA assay; when performed per the vendor's procedure; results in the lysis of exosomes. Some non-peer reviewed posts in the public domain use RIPA buffer prior to subjecting the (now extracted) protein in the exosomes to the BCA assay, while other posts claim that the BCA reagents are capable of exosome lysis without prior lysis buffer treatment. The vendor does not explicitly address protein extraction or lysis in the procedure. While the presence of color is indicative of lysis, there can be no assurance of complete lysis of the exosome population; or of particle size dependent lysis, unless a post-BCA analysis for particle size using the Nanosight instrument detects no particle population in the relevant size range. Therefore, variations in sample loading, when performing SDS-PAGE cannot be discounted.

Conclusion

Isolated sEVs from SARS-CoV-2 infected lung transplant patients contained significantly higher levels of SARS-CoV-2 spike and nucleocapsid proteins compared to those from the control group. These findings suggest that sEV-associated proteins may serve as potential biomarkers for SARS-CoV-2 infection and related pathological conditions in lung transplant patients.

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