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**Extracellular vesicles from long Covid patients promote
RUNX2-mediated cellular stress via dysregulated miR-204
and p53 pathway activation**

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Sharazed Zouari
PhD thesis
Verona, 2025

“For all the questions that led to answers, and all the answers that led to more questions”.

ABSTRACT

People living with long COVID, also known as post-acute sequelae of SARS-CoV-2, often report very different symptoms from one another. The most common ones are constant tiredness, trouble catching their breath, and sometimes a strange discomfort in the legs when they try to be active. These things make day-to-day life harder, but even with so many cases being reported now, a clear picture of why it occurs is still lacking. As of today, extracellular vesicles (EVs), are known to be small particles released by cells, can play roles in tissue repair and in carrying bits of genetic or protein material between cells. What is still unclear is what makes EVs from people with long COVID unique, and how they affect other cells.

In this study, EVs from sera of long COVID patients were isolated and examined in detail using nanoparticle tracking analysis (NTA), atomic force microscopy (AFM), and flow cytometry. Next, their effects were tested on different cell types: lung cells, mesenchymal stem cells (MSCs), endothelial cells from the umbilical vein (HUVECs), and aortic smooth muscle cells (ASMCs). Stress signals were induced by EVs, increasing SESN1, SESN2, and p53 levels. In addition, examining mitochondrial function and performing targeted transfections was done to determine what molecular pathways were activated or inhibited. The results were straightforward. EVs derived from long COVID patients prompted lung cells to overly express RUNX2 and activate p53/p21 pathways alongside multiple other signals associated with fibrosis and cellular stress. Moreover, EVs diminished mitochondrial function, especially in a crowded cellular environment, reducing maximal respiration and ATP yield. Proteomic interaction analysis suggested that RUNX2 was functioning in several key pathways and was post-transcriptionally regulated by miR-204. Moreover, EVs elevated RUNX2, SESN1, p53, and p21 in endothelial, smooth muscle, and stem cells, further activating cellular stress responses. Overall, EVs from long COVID patients induce a stressed, fibrotic state in cells while disrupting mitochondrial energetics. This represents one aspect of the many effects of this condition and, nevertheless, improves the understanding of the effects of long COVID.

RIASSUNTO

Le persone che vivono con il long COVID, noto anche come sequele post-acute da SARS-CoV-2, riportano spesso sintomi molto diversi l'uno dall'altro. I più comuni sono stanchezza costante, difficoltà a riprendere fiato e talvolta uno strano fastidio alle gambe quando cercano di essere attivi. Queste cose rendono la vita quotidiana più difficile, ma anche con così tanti casi riportati oggi, manca ancora un quadro chiaro del perché si verifichi. Ad oggi le vescicole extracellulari (EVs), note per essere piccole particelle rilasciate dalle cellule, possono avere un ruolo nella riparazione dei tessuti e nel trasporto di frammenti di materiale genetico o proteico tra le cellule. Ciò che non è ancora chiaro è cosa renda uniche le EVs delle persone con long COVID e come esse influenzino altre cellule.

In questo studio, le EVs dal siero di pazienti con long COVID sono state isolate ed esaminate in dettaglio utilizzando la nanoparticle tracking analysis (NTA), la microscopia a forza atomica (AFM) e la citofluorimetria a flusso. Successivamente, i loro effetti sono stati testati su diversi tipi cellulari: cellule polmonari, cellule staminali mesenchimali (MSCs), cellule endoteliali della vena ombelicale (HUVECs) e cellule muscolari lisce aortiche (ASMCs). I segnali di stress sono stati indotti dalle EVs, aumentando i livelli di SESN1, SESN2 e p53. Inoltre, è stata esaminata la funzione mitocondriale ed effettuate trasfezioni mirate per determinare quali vie molecolari fossero attivate o inibite. I risultati sono stati chiari. Le EVs derivate da pazienti con long COVID hanno indotto le cellule polmonari a sovra esprimere RUNX2 e ad attivare le vie p53/p21 insieme a molteplici altri segnali associati a fibrosi e stress cellulare. Inoltre, le EVs hanno ridotto la funzione mitocondriale, specialmente in un ambiente cellulare affollato, diminuendo la respirazione massimale e la produzione di ATP. L'analisi delle interazioni proteomiche ha suggerito che RUNX2 funziona in diverse vie chiave ed è regolato post-trascrizionalmente da miR-204. Inoltre, le EVs hanno aumentato RUNX2, SESN1, p53 e p21 nelle cellule endoteliali, muscolari lisce e staminali, attivando ulteriormente le risposte di stress cellulare. Complessivamente, le EVs di pazienti con long COVID inducono uno stato cellulare stressato e fibrotico, mentre compromettono l'energetica mitocondriale. Questo rappresenta un aspetto dei molti effetti di questa condizione e, comunque, migliora la comprensione del long COVID.

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1. Introduction

Even though more than five years have passed, SARS-CoV-2 is still spreading and causing serious health problems worldwide. It is becoming increasingly clear how the virus affects the body and various organs. COVID-19 can cause lasting damage to multiple organs even after the infection has resolved. [1]. These long-term issues can involve the lungs and other parts of the body [1]. People with long COVID, also called post-acute sequelae of SARS-CoV-2 infection (PASC), often feel tired all the time, get short of breath, have trouble thinking or remembering things, and experience other problems that make daily life harder [2–4]. Research shows that roughly 31% to 69% of people who had COVID-19 may end up with long COVID symptoms [5]. Despite more cases being reported, the underlying causes of long COVID remain unclear. Some studies suggest that COVID-19 can change what is inside EVs [6]. EVs are tiny particles with a membrane that cells release to send signals to other cells. They carry proteins, fats, and genetic material. EVs come in two main types: exosomes, about 30–150 nm in size, made inside the cell and released when small internal bodies fuse with the cell membrane; and microvesicles, about 100–1000 nm, formed when the cell membrane buds out and pinches off. These types differ in size, origin, and content, giving them different roles in the body [7].

EVs might help regulate the immune system, repair tissues, and carry viral particles. In COVID-19, EVs have been shown to contain viral RNA and proteins [8,9], which could explain why some symptoms persist. Still, the exact features and effects of EVs from long COVID patients are not well known. This study is based on the idea that EVs from long COVID patients are different from those from healthy people and that these EVs could harm cell function, possibly contributing to the disease.

1.1 Biogenesis and classifications of EVs

EVs are generally classified into different types based on their cellular origin, size, and biogenesis (**Figure 1**). Exosomes are internally derived from late endosomal membranes and range in size from 30 to 150 nm, with the formation of multivesicular bodies (MVBs) and intraluminal vesicles (ILVs). ILVs are the small vesicles that form within the lumen of MVBs during the inward budding process. When MVBs fuse with plasma membranes, exosomes are formed from ILVs and released outside the cell [10].

This secretion is driven by Rab27 proteins, which are specialized for the trafficking and exocytosis of MVBs under the membrane [11]. Exosome biogenesis is characterized by complex processes, such as exosome sorting mechanisms that include ESCRT (Endosome Sorting Complex Required for Transport), lipids and tetraspanins, all of which are essential to determine the contents of the vesicles [12]. Microvesicles have a diameter between 100 and 1000 nm and their formation takes place with direct budding towards the outside of the plasma membrane [13]. Apoptotic bodies, the largest class of EVs, are formed during apoptosis. Their main function is to promote elimination of dead cells and modulation of the immune response through secretion [14]. They can be recruited and phagocytosed by immune cells, particularly macrophages, contributing to the suppression of various inflammatory processes [15]. The contents of EVs reflect the cell from which they are derived and its pathophysiological state. These vesicles contain an array of biomolecules, including proteins, lipids, and nucleic acids which are capable of influencing the pleiotropic biological functions of recipient cells [16]. Exosomes for example are enriched in tetraspanins such as CD63 and CD81, heat shock proteins such as HSP70, and other bioactive lipids which are important at intercellular communication [17].

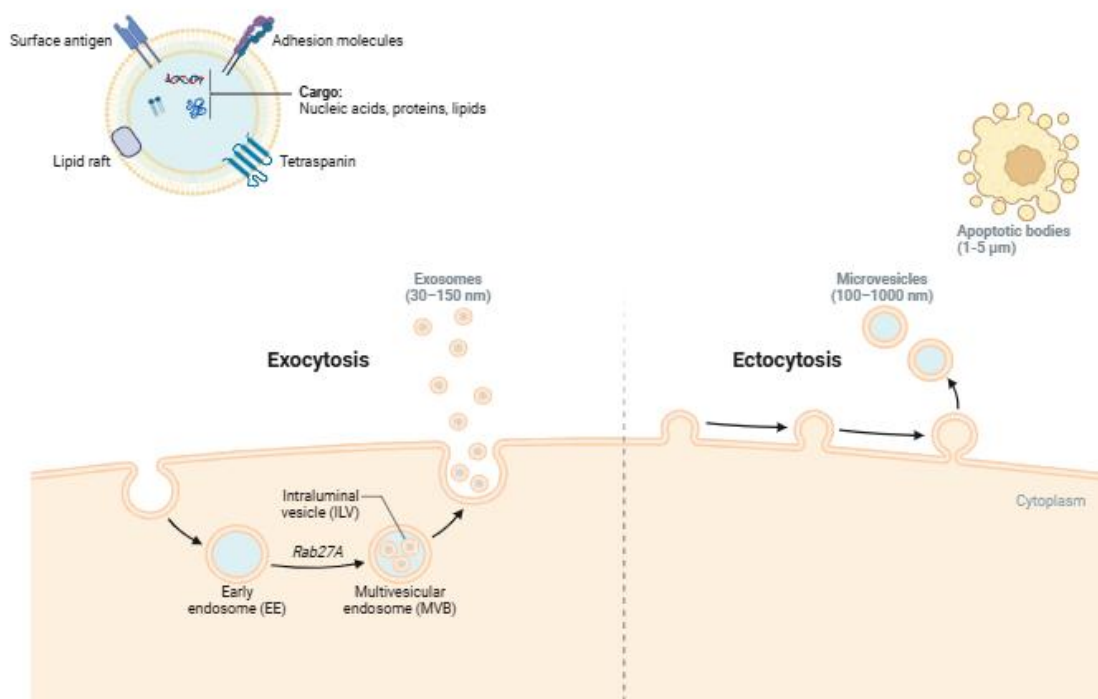


Figure 1: Structure and Biogenesis of EVs. The illustration shows the structure of EVs, with a lipid bilayer containing tetraspanins, adhesion molecules, surface antigens, and cargo (nucleic acids, proteins, lipids) for intercellular communication. Exosomes are formed in multivesicular bodies (MVBs) as intraluminal vesicles (ILVs) and released via exocytosis. Microvesicles arise through ectocytosis, involving plasma membrane budding and fission. Apoptotic bodies are larger vesicles formed during programmed cell death, containing cellular components like organelles and nuclear fragments, and help clear debris and signal during apoptosis.

1.2 Pathophysiological roles of EVs in cardiovascular, neurodegenerative and autoimmune diseases

Since EVs are involved in inter-organ communication, they can be understood more broadly as being involved in systemic disorders, suggesting that they could serve as biomarkers and therapeutic targets [18]. Because they act as specific carriers of bioactive molecules, they could be very useful in terms of therapeutic applications. Indeed, there is a growing body of evidence suggesting their potential role as diagnostic biomarkers and therapeutic agents in multifaceted diseases such as cancer, autoimmune diseases, or neurodegenerative diseases [19].

In heart failure, cardiac EVs from stressed cardiomyocytes, fibroblasts, and immune cells stimulate myocardial fibrosis and dysregulated cardiac remodeling. Cardiomyocyte-derived exosomes enriched in miR-21 promote extracellular matrix deposition in fibroblasts, thus contributing to fibrosis [20]. In addition, EVs from macrophages and neutrophils carry cytokines such as IL-1 β fueling and maintaining a state of inflammation that hinders any repair process in the heart [21]. The NF- κ B signaling pathway is activated by the cargo of EVs, such as miR-146a, which sustains inflammation and contributes to the deterioration of cardiac function. Knowledge of the molecular pathways on which EVs act may open new avenues for the development of interventions aimed at controlling and managing the progression of cardiovascular disease [22].

Exosomes are also involved as mediators of communication between neurons, glial cells, and immune cells in the central nervous system (CNS). EVs exert an influence on neuroinflammatory pathways, synapses, and contribute to the spread of deleterious proteins that contribute to the pathophysiology of neurological diseases [23].

EVs secreted by glial cells also play a role in exacerbating neurodegeneration by altering cellular homeostasis and fueling the pathological process of amyloid beta (A β) protein accumulation in the brain [24]. Microglial EVs carry cytokines and other inflammatory mediators that worsen the neuroinflammatory condition. Activation of signaling pathways, such as NF- κ B and JAK-STAT, regulates the inflammatory response and leads to loss of synaptic structures and neurodegeneration [25,26].

In multiple sclerosis (MS), EVs derived from oligodendrocytes and astrocytes alter the integrity of the blood-brain barrier (BBB) and trigger the activation of immune responses. These vesicles may contain molecular cargoes that can potentiate inflammation by recruiting inflammatory cells and promote demyelination and axonal damage [27].

MiR-146a is an example of an EV-derived miRNA from oligodendrocytes that exerts immune regulation by modifying the activities of microglial cells and T cells. Its absence affects microglial function and proteome, which underscores its importance in maintaining microglial homeostasis and modulating neuroinflammation [28]. EVs carrying α synuclein spread the protein to neighbouring neurons, promoting the progression of Parkinson Disease (PD) [29]. In addition, EVs released by glial cells, particularly microglia, contain pro-inflammatory cytokines and miRNAs such as miR155. This cascade amplifies the inflammatory response, altering neuronal homeostasis and accelerating neurodegeneration, contributing to neuronal loss in PD [30]. These molecular events are linked to activation of the NLRP3 inflammasome, which is a critical mediator of cellular responses to stress and stimulates IL-1 β synthesis and release upon exposure to damage-associated (DAMPs) and pathogen-associated (PAMPs) molecular patterns. Imbalance of this system has been associated with several diseases, including PD, contributing to neurogenic inflammatory cascades that cause damage and neuronal death [31]. EVs are also involved in both direct and indirect antigen presentation processes, as shown in **Figure 2**. In direct presentation, EVs themselves that perform the cross-presentation, without requiring antigen processing by an antigen-presenting cell (APC). This mechanism allows immune modulation without the need for direct cell-cell contact [32]. Like direct presentation, indirect presentation allows for cross-presentation, and antigens are placed on MHC class I molecules however are processed by APCs before being presented. This is critical to the immune system's ability to effectively destroy infected or malignant cells [33].

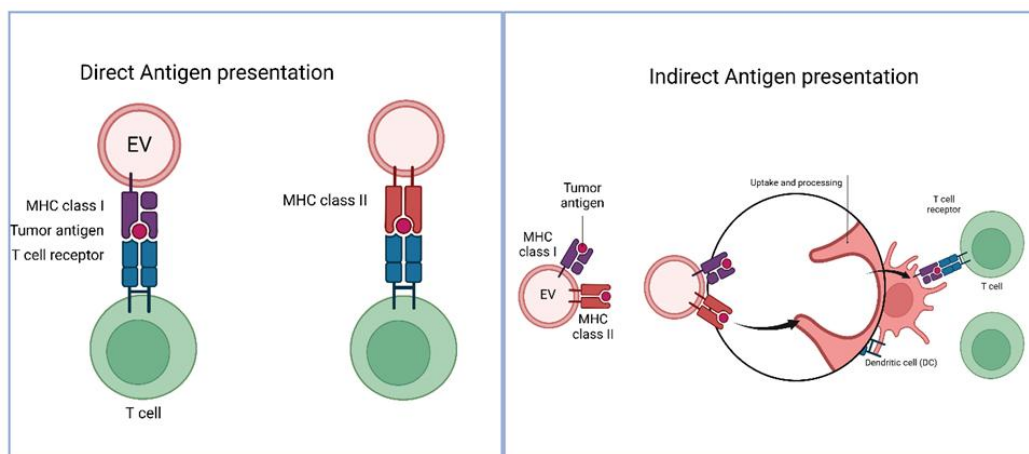


Figure 2. Schematic illustration shows possible direct (left) and indirect (right) antigen presenting processes using EVs. In direct antigen presentation, EVs from antigen presenting cells (APCs) like dendritic cells, initiate T cell activation via the MHC interactions. Also, they can facilitate cross-presentation by showing some exogenous antigens bound onto MHC class I, therefore priming CD8 cytotoxic T cells. In indirect presentation, EVs transfer antigens to APCs, which internalize, process, and present them on MHC class II molecules to activate CD4⁺ T helper cells. In this mechanism, cross-presentation can also take place where some of the processed antigens are presented in MHC class I.

1.3 EVs in tumor progression, osteoporosis, and osteosarcoma

In cancer biology, EVs assume an important factor in tumor growth, development and spread along with the evasion of immune system. Moreover, they can change the landscape of tumor microenvironment and facilitate the escape of immune mechanism thereby enhancing tumor progression [34]. Some evidence claims that EVs derived from tumors can promote angiogenesis. These EVs facilitate the delivery of nutrients and oxygen, hastening the transition to aggressive tumor growth [35]. Tumor cells can express several microRNAs in exosomes that can induce epithelial-mesenchymal transition (EMT) by repressing genes involving cell adhesion, cell movement, and cell invasion including E-cadherin and vimentin [36]. Exosomes also promote cells migration and invasion through contained matrix metalloproteinases (MMPs) which degrade the extracellular matrix, allowing tumor cells to spread to other body organs. Such activities enable the invasion of tissues by tumor cells and alter the microenvironment enhancing conditions for metastatic development [37].

EVs are known to mediate communication among the various cells of the bone environment in the regulation of both bone formation and bone resorption. They should nevertheless, in chronic states, there is an overpopulation of these types that can induce extensive bone loss. This section will more specifically focus on EVs in the chronic disease osteoporosis and the malignant condition osteosarcoma [38]. This broad deviation in EVs signaling aggravates the already existing inappropriate balance in the processes of bone remodelling and, in addition, interferes with the ability of bone to self-regenerate, thus causing the chronic bone tissue loss observed in osteoporosis [39]. Osteoclast-derived EVs, for instance, contain microRNAs such as miR-214, which accelerates the maturation of osteoclasts and suppresses the activity of osteoblasts, creating a vicious cycle of enhanced resorption and undermined formation. This dysregulation contributes to the pathological changes of osteoporosis disease characterized by increased resorption and decreased formation, resulting in weaker bone tissue [40].

It has shown that the delivery of bioactive molecules through mesenchymal stem cells (MSC)-derived EVs enhances the regenerative potential of bone tissue, promoting bone formation and improving bone density. MSC-EVs are therefore particularly useful in the treatment of osteoporosis as they aim to reverse the negative balance between bone resorption and formation [41]. This therapeutic approach appears to be a promising new strategy for the treatment of osteoporosis-related bone loss. In fact, MSCs derived EVs could increase differentiation and function of osteoblasts through osteogenic factors contained in the EVs, increasing bone formation and inhibiting excessive bone resorption [42].

Regarding osteosarcoma, EVs secreted by this condition facilitate osteoclastogenesis, enhance bone resorption, and alter the bone microenvironment. In addition, these vesicles contain matrix metalloproteinases (MMPs) and pro-inflammatory cytokines which destroy the extracellular matrix and assist in the infiltration of tumor cells into adjacent structures, facilitating the aggressive nature of the disease [43]. Even in this case, EVs derived from MSCs appear to have antitumor activity, indicating their potential to counteract bone damage caused by osteosarcoma and inhibiting the aggressiveness of cancer cells. These EVs, as they have been demonstrated to inhibit cancer cell growth, encourage cell apoptosis, limit the spread of cancer, and greatly reduce bone destruction [44].

1.4 EVs in long COVID: from inflammation to biomarker discovery

Cytokines, microRNAs, and viral proteins are contained in vesicles secreted by infected cells and various immune cells, and they promote immune deterioration and prolonged chronic inflammation, conditions associated with long COVID disease [45]. Patients in recovery are quite vulnerable to endothelial damage caused by the still ongoing autoimmune responses, impacting various organs and contributing to the common symptomatology of this serious condition [46]. Patients with long Covid and their relatives may experience a reduction in their standard of living. It is known that exosomes can migrate to the central nervous system and penetrate the blood-brain barrier to increase glial cell activation and lead to a prolonged inflammatory response [47]. Other areas of the brain may be affected by such neurotoxic exosomes contributing to memory loss or cognitive deficits in people with long Covid. Studies have indicated that neurons from individuals with long COVID contain EVs that resemble in content those found in Alzheimer's disease and in fact contain proteins associated with neurodegeneration, strengthening the evidence that exosomes are involved in the development and progression of neurodegenerative disorders associated with this condition [48]. EVs have attracted great interest as potential biomarkers for long COVID, particularly for their ability to reflect the pathophysiological processes. Thanks to the development of bioinformatics, new insights have emerged for the identification and characterization of disease-related biomarkers. The deconvolution of the molecular composition of exosomes has been facilitated by advances in nanomaterials and bioinformatics techniques, which have provided clear insights into their composition. These innovations will improve diagnostic accuracy and may provide personalized approaches to manage the disease [49].

2. Aim of the study

This research aims to clarify on elucidating how EVs might be involved in long COVID pathology. It examines the cellular and molecular features and functions of EVs regarding their interaction with certain cells and tissues, particularly the lungs and blood vessels. Recent literature suggests that EVs from patients with long COVID are postulated to possess molecules that influence cellular processes, providing some rationale as to why certain symptoms, such as fatigue and breathlessness, can be persistent and prolonged. Understanding these processes can aid in elucidating the mechanisms of disease pathogenesis and how long-term consequences evolve.

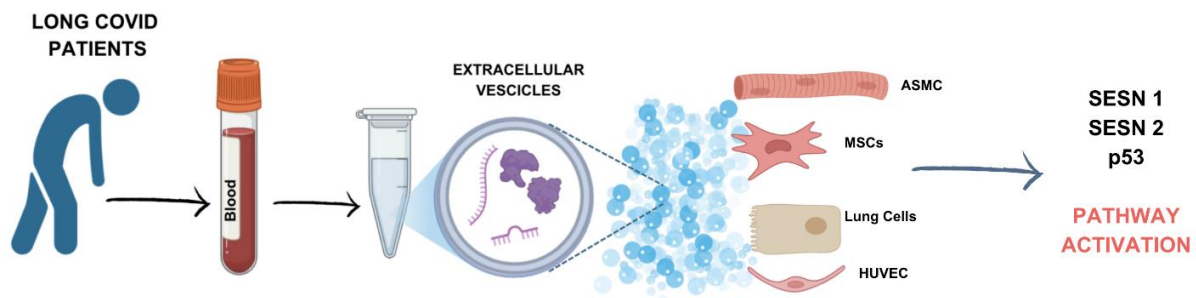
3. Materials and methods

3.1 Design and setting of the study

This study was an in vitro comparative experiment that looked at EVs.

EVs were isolated from the peripheral blood of 32 participants: 16 individuals diagnosed with long COVID and 16 healthy controls, matched for age and sex.

After isolation, the EVs were characterized to understand their features. Several techniques were employed, including nanoparticle tracking analysis (NTA), atomic force microscopy (AFM), and flow cytometry, to evaluate their size, shape, and the surface markers they express.



Functional assays were then performed to evaluate the effects of the EVs. Various human cell lines were exposed, including lung epithelial cells and human umbilical vein endothelial cells (HUVECs), aortic smooth muscle cells (ASMCs), and mesenchymal stem cells (MSCs), to the EVs from the patient group. This allowed to assess the activation of stress-related pathways (such as SESN1/2, p53, and p21), changes in RUNX2 expression, and mitochondrial respiration under metabolic stress.

Finally, it was explored post-transcriptional regulatory mechanism, with a specific focus on miR-204, by carrying out transfection experiments.

3.2 Patients

Blood samples were collected from 16 long COVID patients (8 men and 8 women) and 16 healthy volunteers (8 men and 8 women) to investigate the role of EVs.

Inclusion and exclusion criteria have been described elsewhere (RespiCOVID, Crisafulli [50]); in brief, the study involved outpatients who had previously been hospitalized for SARS-CoV-2 infection. long COVID patients were matched with healthy participants for age and BMI. All participants met the World Health Organization (WHO) criteria for long COVID (<https://www.ncbi.nlm.nih.gov/books/NBK605675/>). Each person provided written informed consent and underwent a full clinical evaluation, including a detailed medical history, to rule out other health conditions. The study was approved by the Ethics Committee of the Azienda Ospedaliera Universitaria Integrata of Verona, Italy (no. 2785CESC), and all procedures were conducted in accordance with the Declaration of Helsinki.

3.3 Six-Minute Walk Test (6MWT)

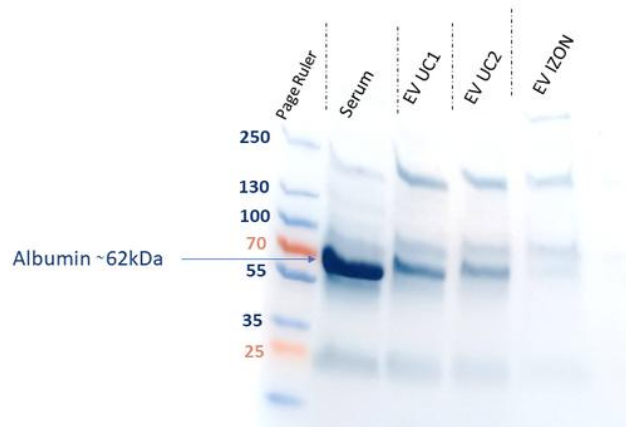
The six-minute walk test (6MWT) was performed on a straight 30-meter course. Start and end points were marked using two cones. Participants with long COVID were instructed to walk as far as possible within six minutes. The procedure followed the American Thoracic Society (ATS) guidelines [51]. The distance covered (in meters) was recorded. Predicted values were calculated according to the equation by Enright et al [52]. Heart rate (beats per minute) and peripheral oxygen saturation (SpO₂) were monitored at rest and at one-minute intervals using a Pulsox-5 device (Konica Minolta Inc.). Blood pressure was measured at baseline, at peak, and during the first minute of recovery.

3.4 Pulmonary function tests

Pulmonary function tests were conducted in accordance with international guidelines [53], with results expressed as percentages of predicted values based on the Official Statement of the European Respiratory Society [54,55]. Measurements were obtained using a flow-sensing spirometer (Jaeger MasterScreen PFT System), which was connected to a computer for data analysis. The parameters recorded included forced vital capacity (FVC), forced expiratory volume in the first second (FEV₁), and total lung capacity (TLC). Diffusing capacity (DLCO) and transfer coefficient (KCO) were assessed using the single-breath method.

3.5 EVs isolation from human blood serum

EVs were isolated using the qEVOriginal-70 nm Gen 2 column (Izon Science Limited ©), following a previously reported method [56]. Subsequent protein analysis using Amido Black staining confirmed the absence of albumin, thereby validating the selected EV isolation technique (**Figure S1**).



Supplemental Fig 1. Detection of proteins evaluated by amido black in serum (lane a), differential ultracentrifugation lanes (b and c) and by Izon columns (d)
Serum = raw serum diluted 1:100

3.6 Nano Tracking analysis (NTA)

Nanoparticle Tracking Analysis (NTA) is founded on the dynamic light scattering (DLS) phenomenon, where the diffusion coefficient of each tracked particle is calculated in an aqueous environment using the Stokes–Einstein equation [57]. NTA was performed using a Malvern NanoSight NS300 instrument (Worcestershire, UK). This analysis was also utilized to determine the concentration of EVs prior to the commencement of subsequent experiments.

3.7 Atomic Force Microscopy (AFM)

AFM was conducted using an NT-MDT Solver Pro atomic force microscope (Moscow, Russia), which was equipped with an NT-MDT NSG01 golden-coated silicon tip. The analysis was performed in semi-contact mode at a scanning frequency of 1 Hz to produce optimized images.

To ensure the accuracy of the measurements, the microscope was calibrated using a TGQ1 calibration grating from NT-MDT, which helped to correct for potential nonlinearity and hysteresis. EVs were prepared for analysis by loading them onto a bracket covered with an inert mica surface. The analysis commenced after allowing 30 minutes for solvent evaporation.

The resulting images were subsequently processed using the Scanning Probe Image Processor (SPIP™) program [58].

3.8. Flow Cytometry and Analysis

EVs samples were subjected to bead-based flow cytometry analysis of surface epitopes using the MACSPlex Exosome kit (Miltenyi Biotec, Bergisch Gladbach, Germany), in accordance with the manufacturer's protocol.

For each condition (Control and long Covid), a total of 4.62×10^7 EVs were diluted in 120 μ l of MACSPlex buffer and incubated overnight with MACSPlex exosome capture beads.

Counterstaining was performed using APC-labeled antibodies against CD6, CD63, and CD81.

Data acquisition of APC-A values was carried out with a BD LSRFortessa X-20 cytometer (BD biosciences, Heidelberg, Germany), and subsequent data analysis was performed with FlowJo software (Ashland, Oregon, United States). For each sample, the median signal intensity of the signals detected for the CD9, CD63, and CD81 capture beads was calculated, and the geometrical mean of these values was utilized as the normalization factor.

3.9 Cell culture

For this research, several different types of cells were used. The A549 cell line (ATCC, Philadelphia, PA, United States) was maintained in DMEM medium (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% FBS (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) and 1% penicillin-streptomycin antibiotics (Lonza, Basel, Switzerland). Human Aortic Smooth Muscle Cells (HAOSMCs) were cultured in Smooth Muscle Cell Growth Medium 2 (PromoCell, GmbH Sickingenstr. 63/65 69126 Heidelberg, Germany. Cat.No. C-22062), enriched with the specific SupplementMix (PromoCell, GmbH Sickingenstr. 63/65 69126 Heidelberg, Germany Cat.No. C-39267) containing growth factors and insulin.

Human bone marrow–derived mesenchymal stem cells (hBM-MSCs) were grown in MesenPRO RS Basal Medium (12747-010, Gibco, Life Technologies Corporation, 3175 Staley Rd., Grand Island, NE, USA) with added supplements and antibiotics. HUVEC cells (PromoCell GmbH Sickingerstr. 63/65 69126 Heidelberg, Germany. Cat.No. C-12200, Heidelberg, Germany) were maintained in M200 medium supplemented with 10% low serum growth (LSGS; Thermo Fisher Scientific, Waltham, MA, USA) and 1% penicillin-streptomycin (Lonza, Basel, Switzerland). All cells were cultured in a humidified CO₂ incubator at 37°C. When the cultures reached approximately 80% confluence, they were treated EVs extracted from the serum of either long-COVID patients or healthy controls. The vesicles were added directly to fresh culture medium to obtain a final concentration of 9.8×10^8 vesicles per milliliter. The treatment was maintained for 18 hours before the cells were collected for further analysis.

3.10 Cellular staining

A549, HUVEC, HAOSMC, and MSCs cells were separately seeded onto chamber slides in their respective basal media. The cells were then stained with a vital fluorescent dye, the Vybrant Cell Labeling Solution (Invitrogen, Waltham, MA, USA), which utilizes 3,3'-diiodo-4',4'-dimethyldiphenylmethane perchlorates (DiO) to emit green fluorescence. The cell nuclei were stained with Hoechst dye.

EVs were stained with 1,1'-diiodo-4,4'-dimethyldiphenylmethane perchlorate (DiI) a red fluorescent dye and subsequently added to the cells for conditioning. Following an 18-hour incubation period, the cells were fixed with 4% paraformaldehyde (PFA) and visualized using the EVOS M5000™ Core Imaging System (Invitrogen, ThermoFisher Scientific, Waltham, MA, USA).

3.11 Immunofluorescence

Immunofluorescence was performed on A549 cells after treatment with EVs, following a protocol already described in the literature [59]. The cells were fixed with 4% paraformaldehyde (PFA), permeabilized with 0.5% Triton X-100, and then processed as reported in previous studies.

To detect the proteins of interest, CK19 (Ref: PA0468, Leica, Wetzlar, Hesse) and Vimentin (Ref: PA0640, Leica, Wetzlar, Hesse) primary antibodies were applied and incubated overnight at 4 °C. The following day, secondary antibodies (Alexa Fluor 488-conjugated anti-goat, 1:300) were added. Cell nuclei were visualized with ProLong™ Gold Antifade Mountant containing DAPI. Negative controls were also included, in which only the secondary antibodies were used (**Figure S2**). Images were acquired with the EVOS M5000™ Core Imaging System (Invitrogen, ThermoFisher Scientific, Waltham, MA, USA).

3.12 Cell transfections

The RUNX2 gene was cloned into the pcDNA3 expression vector as described previously [59]. Briefly, the amplified RUNX2 sequence was first introduced into the pCR™ 2.1 vector, excised by EcoRV/XhoI digestion, and subsequently subcloned into the pcDNA3-Flag-HA construct [59]. For functional studies, A549 cells were cultured to approximately 70% confluence and then transfected with the RUNX2-expressing pcDNA3 plasmid (10 µg/mL) using Lipofectamine 3000 [60]. In parallel, some experiments employed RUNX2 Lenti ORF particles from Origene Technologies, following the manufacturer's instructions.

To examine the effect on mir-204 expression, cells were transfected with a mirVana™ mir-204 mimic at graded concentrations (5, 10, 15, 20, and 25 nM). A scramble sequence served as the control. For these assays, A549 cells were seeded at 3.5×10^5 cells in T25 flasks and maintained in DMEM. Transfection was carried out at 70% confluence with Lipofectamine 3000, and cells were incubated with the complexes for 24 h before collection and storage.

3.13 Western Blotting

Protein concentration was measured using the BCA protein assay kit (Quantum Protein, Bicinchoninic Protein Assay, Euroclone S.p.A. Società a Socio Unico Via Figino, 20/22 20016 Pero, MI), and the readings were taken with a VICTOR microplate reader (PerkinElmer, Waltham, MA, USA). After quantification, all samples were adjusted to the same concentration using Pierce™ RIPA buffer 1X (Cat.Number: 78501 Thermo Fisher Corporation, Waltham, MA, USA) and loading buffer. To prepare them for electrophoresis, the samples were heated at 99 °C for 7 minutes.

Proteins were then separated on precast Mini-PROTEAN® TGX gels (Bio-Rad, 2000 Alfred Nobel Dr, Hercules, CA, United States, California) through SDS-PAGE. The run was carried out first at 125 V for a short time to allow proteins to enter the gel and then continued at 145 V for about an hour. Following separation, the proteins were transferred to PVDF membranes (Cat.Number: 88518 Invitrogen- Thermo Fisher Corporation, Waltham, MA, USA). Before transfer, the membranes were briefly activated with ethanol, rinsed in transfer buffer, and then mounted in the Mini-PROTEAN® Tetra Cell system (Bio-Rad, 2000 Alfred Nobel Dr, Hercules, CA, United States, California). The transfer took place on ice, at 100 V for one hour.

To verify that the proteins had been transferred properly, membranes were stained with Amido Black (Cat. Number: A8181- Sigma-Aldrich-St.Louis, Missouri, USA) and rinsed with MilliQ water. After this check, they were blocked with 5% milk in TBS-Tween for one hour at room temperature. The membranes were then incubated overnight at 4 °C with the following primary antibodies: RUNX2 1:1000 55-62 KDa, (Cat.Number 8486 Cell Signaling-3 Trask Lane Danvers, MA 01923), p53 1:1000 53 KDa (Cat.Number: FNab06083, FineTest), p21 1:1000 21 KDa (Cat. Number: GTX629543, GeneTex), SESN1 1:500 57KDa, (Cat.Number: PA5-98142- Invitrogen Thermo Fisher Corporation, Waltham, MA, USA), SESN2 1:1000 54KDa (Cat Number: ab-178518 Abcam). β -actin 1:10000 48-55 KDa (Cat. Number: MA1-140-Invitrogen Thermo Fisher Corporation, Waltham, MA, USA) was used as a reference protein to confirm equal loading.

3.14 RNA extraction and reverse transcription

Total RNA was extracted from samples using the RNeasy® Protect Mini Kit (Qiagen) according to the manufacturer's instructions. RNA concentration and integrity were measured with the Qubit™ RNA HS Assay Kit (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) and a Qubit 3 Fluorometer (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA, REF Q3321).

Reverse transcription to generate cDNA was performed using a first-strand cDNA synthesis kit (GE, Healthcare Bio-sciences AB, Uppsala), following the provided protocol. All steps were conducted on a GeneExplorer™ Thermal Cycler.

3.15 Gene expression analysis-Real-time PCR

Real-Time PCR was carried out using TaqMan Universal PCR Master Mix (Thermo Fisher Scientific, Waltham, MA, USA) with commercially pre-designed probes (**Table 1**) and iTAQ Universal SybrGreen SuperMix (Bio-Rad, 2000 Alfred Nobel Dr, Hercules, CA, United States, California) with primers listed in **Table 2**.

PCR reactions were prepared in a MicroAmp Optical 96-Well Plate (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA). Each well received 19 μ L of PCR mix and 1 μ L (10 ng) of cDNA. Samples were loaded in triplicate. Plates were briefly centrifuged, covered, and sealed with an Opti-seal membrane (Opti-seal, AB analitica).

Amplification was performed on a LineGene 9620 Real-Time PCR System (Aurogene, Hangzhou Bioer Technology). PCR cycling included 45 cycles using the appropriate mix as previously indicated [61]. Analyses were repeated at least three times, each in triplicate, and Ct values were averaged.

Table 1. List of probes analyzed through TaqMan Real-Time PCR

Gene	Origin	Code
RUNX2	Applied Biosystems	Hs1047973_m1
SESN1	Applied Biosystems	Hs00902782_m1
β-actin	Applied Biosystems	Hs99999903_m1

Table 2. List of primers analyzed through SYBR Green Real-Time PCR

Gene	Origin	Code
β-actin forward	Invitrogen – Thermo Fisher Scientific	5'-GAAGGATTCCTATGTGGGCG-3'
β-actin reverse	Invitrogen – Thermo Fisher Scientific	5'-GGTCTCAAACATGATCTGGGT-3'
SESN2 forward	Invitrogen – Thermo Fisher Scientific	5'-AGATGGAGAGCCGCTTTGAGCT-3'
SESN2 reverse	Invitrogen – Thermo Fisher Scientific	5'-CCGAGTGAAGTCCTCATATCCG-3'

3.16 miRNA 204-5p- EVs

miRNAs were extracted from EVs using the qEV RNA Extraction Kit (Izon Science Limited). Concentration and purity were measured with a Nanodrop ND-1000 (Thermo Scientific).

miR-204-5p was reverse-transcribed into cDNA using the TaqMan™ MicroRNA Reverse Transcription Kit (4366596, Applied Biosystems by Thermo Fisher Scientific, Baltics UAB, Vilnius, Lithuania) with the specific primer. PCR amplification (20 µL total volume, 2 µL cDNA) was performed using TaqMan Universal PCR Master Mix and probes for miR-204-5p (000508, 20X, FAM) and U6 snRNA (001973, 20X, FAM) by Applied Biosystems (Thermo Fisher Scientific, Life Technologies Corporation, Carlsbad, CA, USA).

Amplification consisted of 45 cycles. Data were normalized to U6 snRNA, and relative expression was calculated using the $\Delta\Delta C_t$ method [62].

3.17 High-resolution mitochondrial respirometry

A549 cells were plated in a T75 flask and subsequently detached using 0.05% trypsin. Cell counting was performed via trypan blue exclusion. After centrifugation at 200 g_{max} for 5 minutes at 25 °C, the medium was removed, and cells were resuspended in PBS for aliquoting prior to respirometry. A total of 2×10^6 A549 cells were resuspended directly in the 2 mL chambers of Oroboros Oxygraph-2k chambers (Oroboros Instruments, Corp., Innsbruck, Austria), which were filled with Mir05 medium (0.5 mM EGTA, 3 mM MgCl₂·6 H₂O, 60 mM lactobionic acid, 20 mM taurine, 10 mM KH₂PO₄, 20 mM HEPES, 110 mM sucrose, 1 g/L BSA). The published substrate-uncoupler-inhibitor titration (SUIT)-008 O₂ ce- pce D025 protocol was utilized to measure O₂ flux in pmol·s⁻¹·2 million⁻¹ cells.

The assay was performed at 37 °C. Catalase was added to the medium to create MiR06, with a final concentration of 280 U/mL to induce hyperoxia.

Following the measurement of endogenous whole-cell respiration (ROUTINE state), cells were permeabilized by the addition of digitonin. The optimal concentration for permeabilization was determined by titration experiments, with a final concentration of 50 µg/mL identified as the most effective.

Substrates and inhibitors were added in the following order and final concentrations: pyruvate (5 mM) and malate (2 mM), ADP (2.5 mM), glutamate (10 mM), succinate (10 mM), carbonyl cyanide m-chlorophenylhydrazone (CCCP, multiple 0.5 µM titrations), rotenone

(0.5 μ M), and antimycin A (2.5 μ M). Residual oxygen consumption was subtracted from the oxygen flux as a baseline for all respiratory states. The titration method was completed within 90 minutes.

Data analysis was performed using DatLab 5.2 software (Oroboros, Paar, Graz, Austria), and all chemicals were purchased from Sigma Aldrich/Merck Life Science. This titration method was completed within 90 min.

3.18 Seahorse

To evaluate mitochondrial function, A549 cells were plated in 24-well microplates at a density of either 1.5×10^4 or 2×10^4 cells per well. The cells were simultaneously treated with either control EVs (CTRL) or long COVID EVs (LC).

After approximately 18 hours, the culture medium was replaced with XF-RPMI (Seahorse Bioscience, cat. No. 103576-100) supplemented with 10 mM glucose, 1 mM sodium pyruvate, and 2 mM glutamine, adjusted to pH 7.4. The cells were then incubated for one hour at 37 °C in a non-CO₂ incubator.

Mitochondrial function was assessed using a Seahorse XFe24 Extracellular Flux Analyzer with a Seahorse XF Cell Mito Stress Test Kit. Oxygen consumption rate (OCR) was measured at baseline and after the sequential addition of:

- 1 mM Oligomycin A (Port A),
- 2 mM FCCP (Port B),
- 1 mM Rotenone plus 1 mM antimycin A (Port C).

OCR values were normalized to the DNA content per well, which was determined using the CyQUANT Cell Proliferation Assay Kit. Four biological replicates were included for each condition, and the graphs represent the average of these independent experiments.

3.17 Statistical analysis

The data are presented as mean values accompanied by either standard deviation or standard error of the mean, as specified in each case. When comparing two experimental groups, we employed Student's paired t-test. For analyses involving multiple groups, we utilized one-way or two-way analysis of variance (ANOVA). Subsequently, we conducted specific post hoc tests (Dunnett's or Tukey's) to identify significant differences between individual groups.

To maintain the type I error rate at the predetermined level of $\alpha = 0.05$, we applied appropriate corrections for multiple comparisons. All statistical evaluations were based on two or three independent experiments, each performed in triplicate. Statistical computations were carried out using SPSS for Windows (version 22.0, SPSS Inc., Chicago, IL, USA) or GraphPad Prism (version 9), with p-values ≤ 0.05 considered statistically significant.

4. Results

4.1 Long COVID patients

About four and a half months after their acute infection (4.5 ± 0.6 months), sixteen patients returned for follow-up evaluation. Their main clinical and demographic characteristics are listed in **Table 3**. Each patient had to be hospitalized during the acute COVID-19 phase, with ten requiring oxygen, and two needing an ICU admission. The average length of hospital stay was just under ten days (9.7 ± 6.2 days). By the time of reassessment, the most persistent complaint was fatigue. Patients described this symptom as debilitating and relentless, deeply impacting daily activities and life. Seven individuals identified fatigue as the predominant symptom, one patient experienced rest-related dyspnea, and four developed exertional breathlessness. Additionally, three patients exhibited a chronic cough. **Table 4** summarizes the lung function tests. Patients showed considerable exercise tolerance during the six-minute walk test (6MWT): 15 out of 16 participants covered expected distance targets, with only one male patient achieving under 80% of the predicted value. Patients were asked to evaluate their effort immediately post 6MWT using the modified CR10 Borg Scale [63]. The average score for breathlessness was 2.5 ± 1.86 , while discomfort in the legs was rated slightly higher at 3.25 ± 1.94 . Interestingly, participants tended to perceive leg fatigue as more limiting than shortness of breath ($p < 0.05$).

Oxygen saturation remained stable throughout the test, with mean values of 97.1 ± 1.1 at rest and 96.5 ± 1.6 at peak exercise ($p = 0.120$). However, in two patients, SpO₂ dropped below 95% after completing the walk. A detailed summary of the 6MWT results is presented in **Table 5**.

Table 3: Characteristics of long COVID outpatients.

ID	sex	Age (years)	Height (cm)	Weight (kg)	BMI (kg/m2)	LOS (days)	FUP (months)
1	M	57.5	174	97	32.0	6	4.0
4	M	63.8	170	74	26.0	11	4.0
9	M	65.1	170	81	28.0	9	4.0
11	M	65.7	164	72	26.8	14	4.0
17	M	61.7	182	83	25.1	11	4.0
21	M	62.7	182	81	24.5	2	4.0
29	M	57.4	171	71	24.3	3	4.0
34	M	55.3	174	88	29.1	3	4.0
48	F	55.5	172	62	21.0	6	5.0
49	F	59.6	165	64.5	23.7	9	5.5
50	F	61.0	149	86	38.7	11	4.0
54	F	56.9	160	98	38.3	17	4.0
56	F	55	164	59	21.9	3	4.0
63	F	60.0	155	66.5	27.7	14	4.0
68	F	55	159	67	26.5	11	4.0
74	F	64	150	70	31.1	25	4.0

Characteristics of long COVID outpatients. **LOS:** length of hospital stay for COVID19 infection. **FUP:** follow-up period after hospital discharge; **BMI:** body mass index.

Table 4: Spirometry parameters of subjects.

ID	FVC (l)	FVC%	FEV1 (l)	FEV1%	FEV1/FVC	TLC (l)	TLC%	DLCO_SB (ml/min/mmHg)	DLCO_SB %
1	5.09	121	3.88	116	76.22	6.84	100	26.78	94
4	4.41	115	2.87	95	64.99	4.72	96	17.64	96
9	4.68	125	3.43	117	72.31	6.69	103	26.44	103
11	2.73	80	2.11	79	77.16	4.42	73	18.22	77
17	4.44	97	3.49	98	78.7	6.78	91	26.59	88
21	5.22	115	4.29	121	81.94	7.51	101	30.69	102
29	4.85	120	4.03	126	83.19	6.65	101	23.11	84
34	5.22	123	4.29	126	82.06	7.13	104	24.42	85
48	4.62	140	3.9	138	84.32	5.84	105	21.65	84
49	3.91	135	3.23	132	82.65	5.52	108	21.13	90
50	2,21	104	1,70	97	77,15	3,30	82	20,01	104
54	4,04	147	3,39	146	84,04	5,50	115	19,27	85
56	3,24	109	2,81	111	86,70	5,03	100	15,78	66
63	2,72	112	2,30	112	84,30	4,09	92	16,78	80
68	3,60	132	2,67	116	74,36	4,90	104	20,75	92
74	2,52	119	2,08	119	82,32	3,58	87	14,14	73

FVC: forced vital capacity; **FEV1:** first second forced expiratory volume; **FEV1/FVC:** Tiffenau index; **TLC:** total lung capacity; **DLCO_SB:** single breath diffusing capacity of the lung for carbon monoxide.

Table 5: 6-minute walking test (6MWT) of long COVID outpatients.

ID	Distance (m)	%pred	BorgD rest	BorgL rest	BorgD peak	BorgL peak	SpO ₂ rest	SpO ₂ peak	HRrest (bpm)	HRpeak (bpm)
1	414,00	75,4	0	0	2	5	98	95	79	118
4	500	94,8	0	0	8	8	98	97	59	126
9	495	97,3	0	0	1	1	98	95	94	116
11	472	99,2	0	0	2	4	98	98	60	83
17	548	89,4	0	0	4	4	97	96	80	108
21	669	109,4	0	0	0	1	97	97	70	68
29	699	122,2	0	0	1	1	97	97	80	121
34	709	123,1	0	0	2	3	97	96	89	153
48	600	105,8	0	0	3	4	97	98	54	97
49	505	96,6	0	0	3	3	97	95	83	121
50	687	159,9	0	0	3	1	94	94	66	118
54	448	99,3	0	3	4	5	96	97	64	68
56	600	106,6	0	0	3	4	97	98	70	97
63	447	89,8	0	0	1	1	97	97	86	118
68	563	106,0	0	0	1	3	99	100	76	110
74	420	92,5	0	0	3	4	96	94	75	89

%pred: percentage of the predicted distance value; **BorgDrest** and **BorgDpeak:** points of the Borg scale of perceived dyspnea at rest and at the end of 6MWT; **BorgLrest** and **BorgLpeak:** points of the Borg scale of perceived leg fatigue at rest and at the end of 6MWT; **SpO₂:** O₂ pulse oximetry saturation; **HR:** heart rate.

4.2 EVs analyses from Control and long COVID samples.

EVs from both control subjects and individuals with long COVID (**Figure 3A–H**) were examined using Nanoparticle Tracking Analysis (NTA) and Atomic Force Microscopy (AFM). These complementary techniques made it possible to characterize the size distribution and morphology of the vesicles in detail, enabling a direct comparison between the two groups.

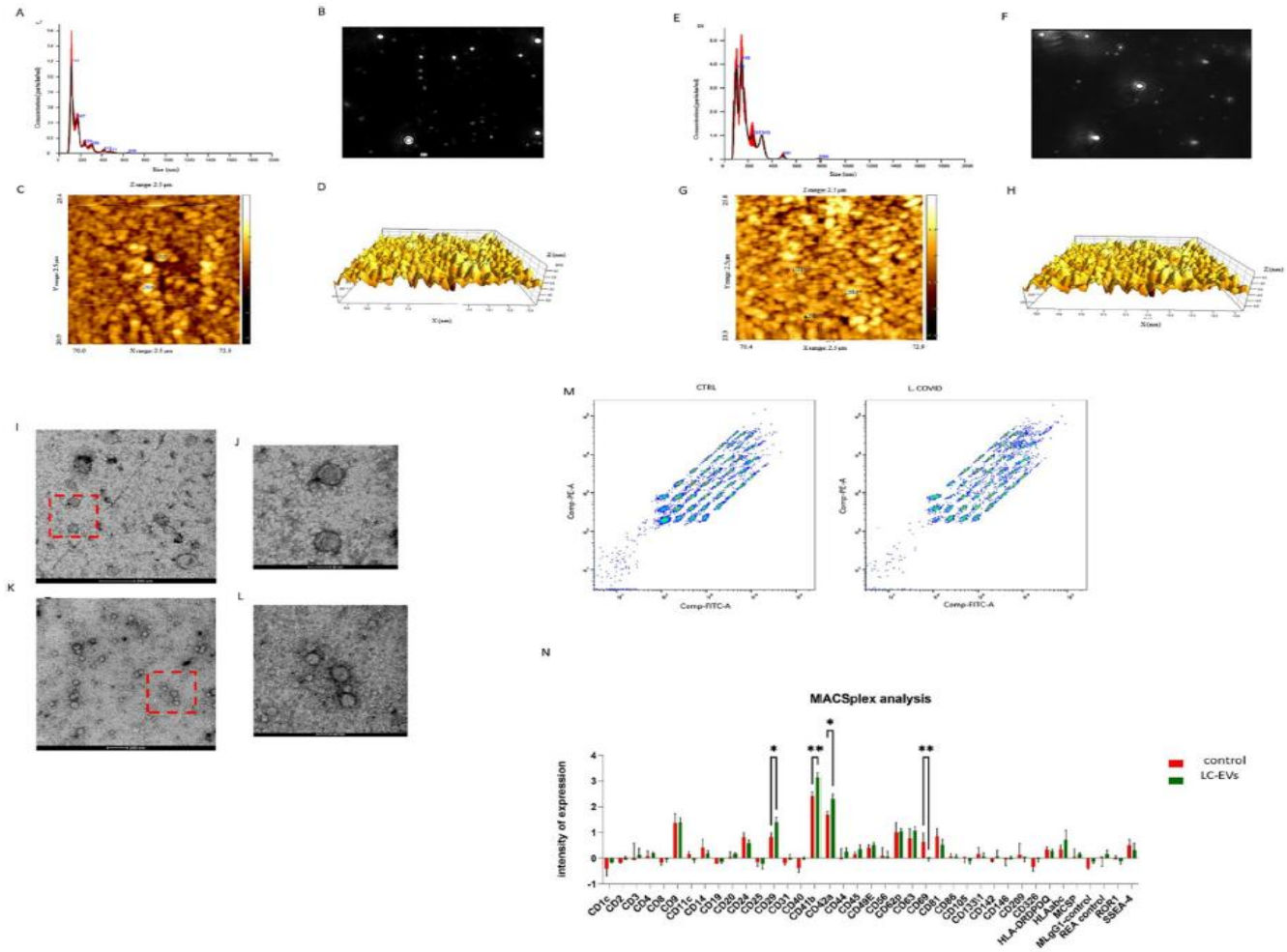


Figure 3. Characterization of EVs. (A) NTA size diagram of EVs from controls, (B) NTA camera capture of EVs light scattering from controls, (C) 2D and (D) 3D AFM analysis of EVs from controls. (E) NTA size diagram of EVs from long Covid subjects, (F) NTA camera capture of EVs light scattering, (G) 2D and (H) 3D AFM analysis. (I) Flow cytometry analyses: The dot plot represents fluorescence intensity data, with APC-A values indicating the expression levels of the analyzed markers. Diagonal clustering suggests a correlation between markers, potentially due to their co-expression on EVs. The applied normalization factor, calculated as the geometric mean of CD9, CD63, and CD81 bead signals, ensures comparability between samples (L). MACSplex analysis of extracellular EVs from Control (CTRL, green) and long Covid (L. COVID, purple) samples. The bar graph represents the intensity of expression for various surface markers detected using bead-based flow cytometry (B). (*p < 0.05; **p < 0.005). (M) Flow cytometry analysis of extracellular vesicles (EVs). Dot plots display fluorescence intensity (APC-A) corresponding to marker expression levels, with diagonal clustering suggesting co-expression of markers on EVs. Data were normalized using the geometric mean of CD9, CD63, and CD81 bead signals to ensure comparability between samples. (N) MACSplex analysis of extracellular vesicles (EVs). Bar graphs show the expression intensity of surface markers in Control (CTRL, green) and Long COVID (purple) samples using bead-based flow cytometry. Experiments were performed in triplicate for NTA, AFM, and flow cytometry. Statistical significance: *p = 0.048; *p = 0.0036.

The differences observed in both the mean and mode values (**Table 6**) fall within the range of the standard deviation.

Sample	NTA		AFM
	Mean	Mode	Diameter
	(nm)	(nm)	(nm)
EVs (C)	171.9 ± 1.3	107.8 ± 3.5	128.5 ± 15.1
EVs (LC)	176.1 ± 2.6	126.9 ± 9.	137.1 ± 30.6

Table 6. A comparison was made between the particle diameters obtained from NTA and those measured by AFM. Each measurement was performed in triplicate, and for AFM analysis, a population of 30 particles was used to calculate the mean and mode values.

Based on this analysis, no major differences were observed in the size distribution between the two groups. The surface of the exosomes was examined using flow cytometry. As illustrated in **Figure 3 (I–L)**, most markers showed similar levels across both populations; however, certain markers displayed notable differences. In particular, EVs from long COVID (LC) subjects exhibited a significant overexpression of markers linked to platelet hyperactivation, including CD29, CD41b, CD42a, and CD69.

4.3 LC-derived EVs drive EMT, fibrotic signaling, and stress response in lung cells

To explore how EVs from long COVID (LC) patients affect lung cells, A549 cells were exposed to EVs isolated from either healthy controls or LC patients for 18 hours. As shown in **Figure 4A**, EVs from both healthy donors and LC subjects were able to cross the lung cell membranes and distribute throughout the cells in a similar manner.

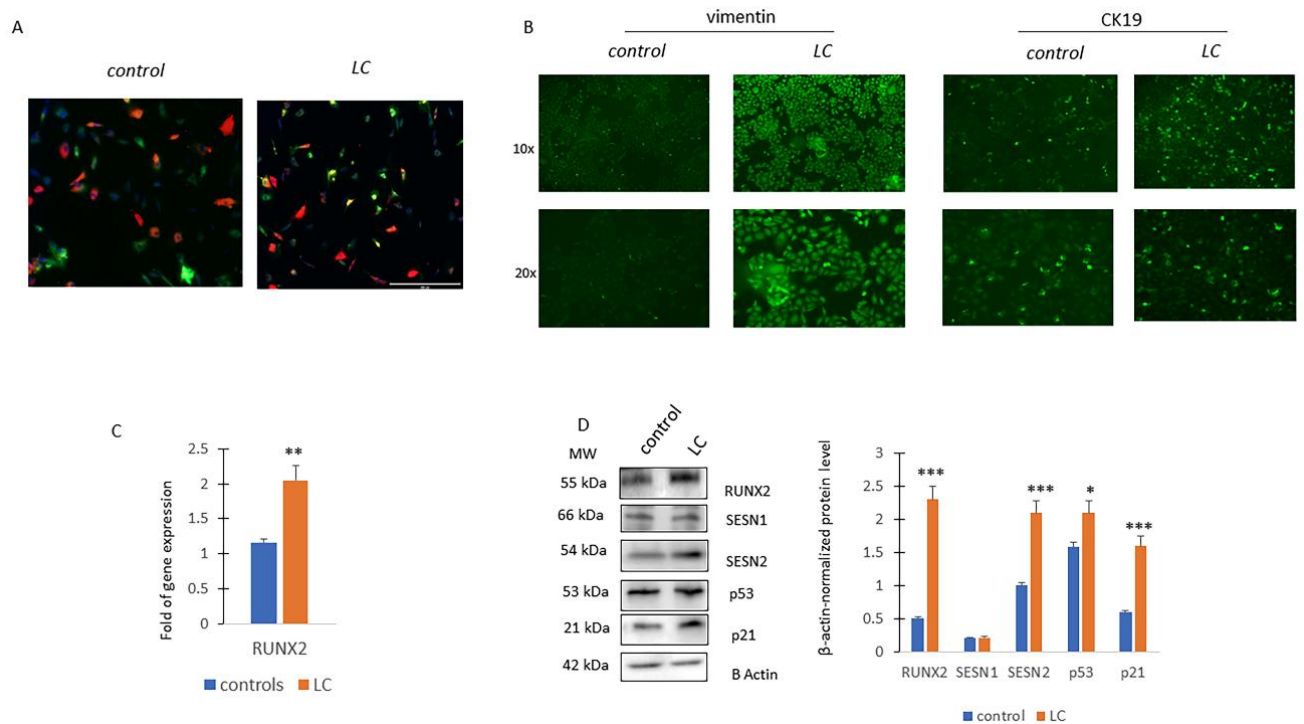


Figure 4. LC-EVs effects on lung cells. (A) The EVs (red) from both the controls and LC crossed the cell (green) membranes and they were completely internalized by the cells after 18 hours. (B) Immunofluorescence staining for vimentin and CK19. (C) Real-Time PCR for RUNX2 in EVs treated lung cells. (D) WB for protein levels (on the left) and optical density of the blots (on the right). (** $p < 0.005$; *** $p < 0.001$ vs controls).

Because vimentin marks the activation of fibroblasts along with cellular migration, and CK19 marks differentiation of cells associated with both neoplastic and regenerative processes [64], their expression in lung cells treated with EVs was examined.

Notably, the expression patterns differed depending on whether the EVs were derived from healthy controls or from long COVID (LC) patients. Vimentin, one of the key drivers of epithelial-mesenchymal transition (EMT) [65], and CK19, which is associated with aggressive tumor metastasis [66,67], both showed significant upregulation in lung cells treated with LC-derived EVs compared to those treated with control EVs (**Figure 4B**). In addition, these findings demonstrated that LC-derived EVs significantly upregulated the expression of RUNX2 in lung cells (**Figure 4C-D**).

RUNX2 is an important factor that regulates epithelial-mesenchymal transition (EMT) processes and fibroblast signaling associated with the development of pulmonary fibrosis [68,69], and it also contributes to fibrotic and inflammatory processes in lung diseases [70]. For this reason, the levels of sestrins, stress-responsive proteins involved in the development of emphysema and known to be overexpressed in patients with chronic obstructive pulmonary disease (COPD), were measured (COPD) [71].

Interestingly, SESN2 expression was elevated in lung cells exposed to LC-derived EVs, whereas SESN1 levels remained comparable to controls (Figure 4D). Since both SESN1 and SESN2 are downstream targets of p53 [72], p21 and p53 were also examined, key mediators of cell cycle arrest in response to DNA damage. The results showed that protein levels of both p53 and p21 were higher in cells treated with LC-EVs compared to control cells (**Figure 4D**).

4.4 LC-derived EVs disrupt mitochondrial function and cellular adaptability under metabolic stress

To determine whether LC-derived EVs influence protein-protein interactions, A bioinformatic analysis was performed using the STRING database (<https://string-db.org/>; accessed 10 September 2024). As illustrated in Figure 5A-B, the predicted interactions within the functional network were statistically significant ($p = 0.00658$), highlighting a robust functional relationship among the proteins involved.

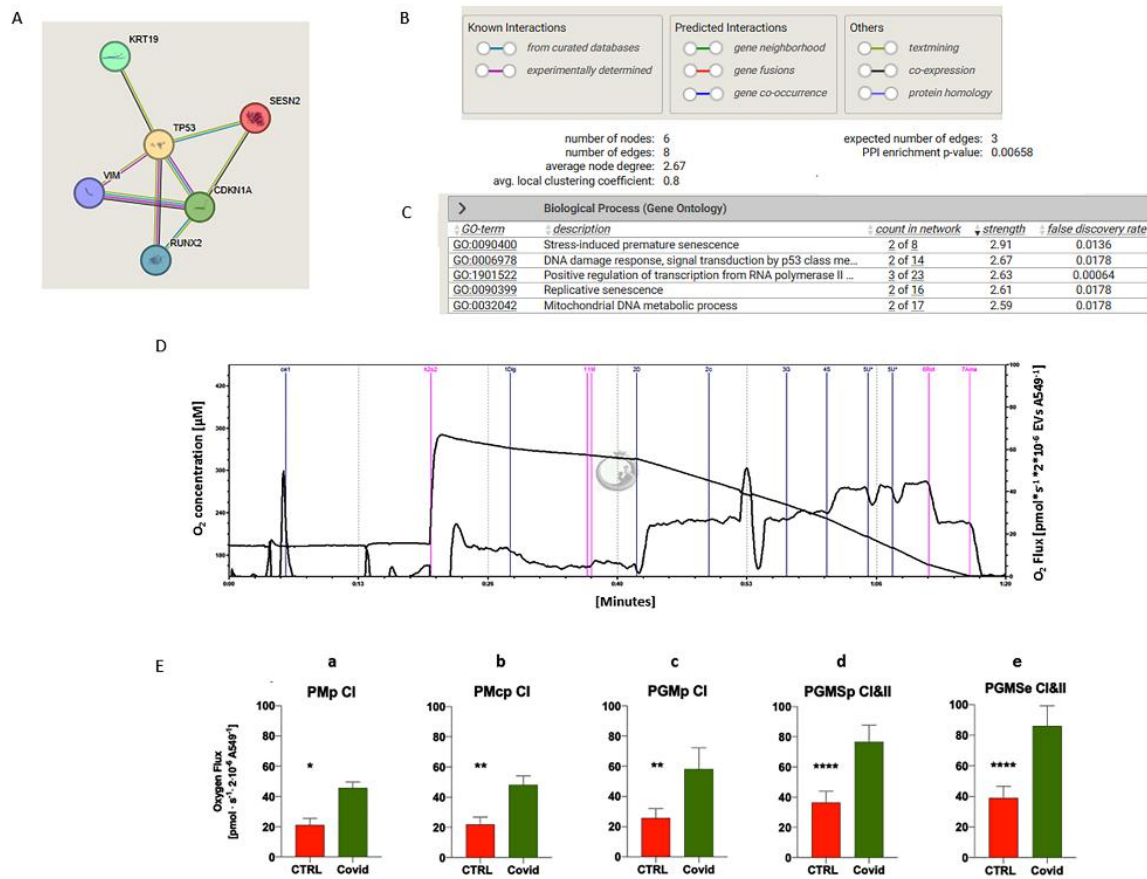


Figure 5. Protein interactions and respiratory capacities. (A) Functional protein interactions identified by STRING analyses (B) and enrichment analysis (PPI enrichment p-value: 0.00658). (C) Biological Process identified by string analysis. (D) Representative O2k trace. (E) CI uses saturating concentrations of pyruvate, malate, and glutamate as substrates (a, b, c). Max ETS is achieved using saturating concentrations of pyruvate, malate, ADP, glutamate, succinate (for complex II), and then titrated with CCCP to uncouple respiration to achieve maximal respiration. Complex I (d) and MaxETS (e) respiration for control and longCovid EVs-treated A549 cells. P, pyruvate; M, malate, p, OXPHOS state, c, cytochrome c; G, glutamate; S, succinate; e, electron-transfer state; CI, complex I; CII, complex II. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.0001$.

Furthermore, functional enrichment analysis of protein-protein interactions highlighted that the modulated proteins are involved in multiple biological processes (Figure 5C). Given that mitochondrial structural abnormalities have been reported in long COVID patients [73], the focus has been on the “mitochondrial DNA metabolic process” (GO:0032042). To further explore mitochondrial function, high-resolution respirometry using the Oroboros O2k under hyperoxic conditions to maximize cellular respiratory capacity was performed (Figure 5D). Following an 18-hour treatment with EVs from control and long COVID (LC) subjects, cells exposed to LC-derived EVs exhibited significantly higher oxygen consumption compared to controls (Figure 5E).

To explore the mechanisms behind the increased oxygen consumption, a MitoStress test was performed using the Seahorse system. Under conditions of metabolic stress induced by higher cell density, where cells compete for limited nutrients and oxygen, LC-EV-treated cells showed a decline in metabolic capacity. Notably, Maximal Respiration decreased significantly as cell density increased in these cells, whereas cells exposed to control EVs maintained stable Maximal Respiration (**Figure 6A**). These findings indicate that LC-EVs compromise the cells' capacity to adapt under conditions of limited resources. Consistently, both ATP production (**Figure 6B**) and basal respiration (**Figure 6C**) were reduced at higher cell densities in the presence of LC-EVs, further supporting the notion that these vesicles negatively affect mitochondrial adaptability under metabolic stress.

By expanding the number of nodes in the protein interaction analysis, further biological processes have been discovered, including apoptotic cell death induced by DNA damage (**Figure S2**).

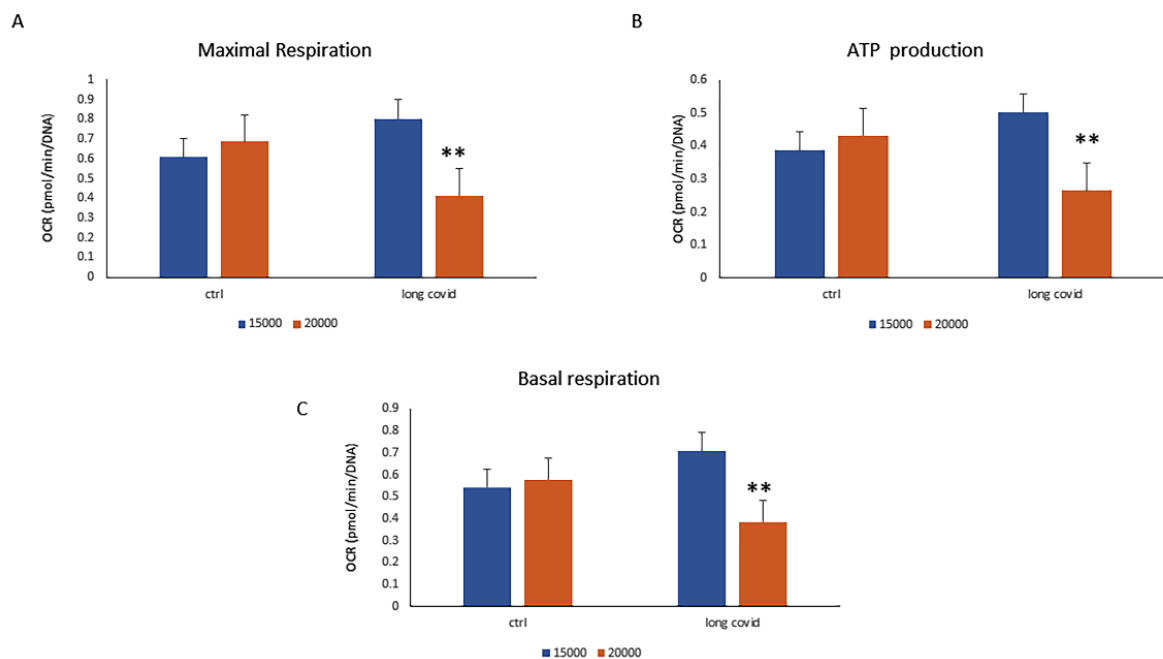
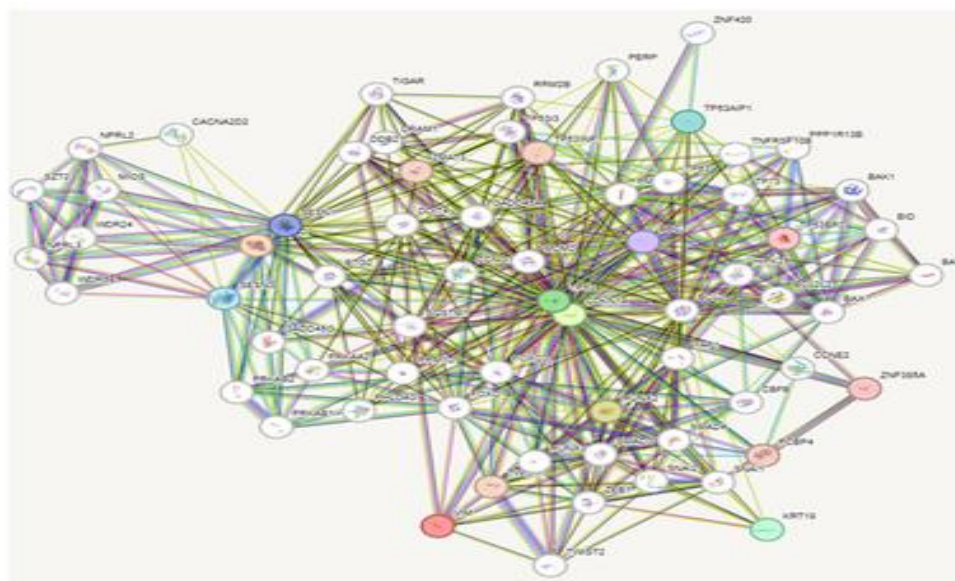


Figure 6. Metabolic stress response in cells treated with EVs. (A) Maximal respiration measured using the Seahorse MitoStress test decreases significantly in LC-EV-treated cells as cell density increases, whereas control vesicle-treated cells maintain stable respiration. (B) ATP production is reduced in LC-EV-treated cells at higher cell densities, indicating impaired energy generation. (C) Basal respiration also declines in LC-EV-treated cells under resource-limited conditions. OCR: Oxidative consumption rate $p < 0.005$; *** $p < 0.001$).



Biological Process (Gene Ontology)					
GO term	description	count in network	strength	signal	false discovery rate
GO:0072331	Signal transduction by p53 class mediator	17 of 93	1.74	4.87	6.96e-21
GO:0072332	Intrinsic apoptotic signaling pathway by p53 class mediator	13 of 56	1.84	4.36	5.99e-17
GO:0008630	Intrinsic apoptotic signaling pathway in response to DNA damage	13 of 75	1.71	3.84	1.09e-15
GO:0097193	Intrinsic apoptotic signaling pathway	18 of 166	1.51	3.83	7.18e-19
GO:0032007	Negative regulation of TOR signaling	11 of 52	1.8	3.63	5.95e-14

Figure S2. Expansion of the protein–protein interaction network revealed additional biological processes, notably apoptotic cell death pathways activated in response to DNA damage.

4.5 RUNX2 overexpression and miR-204 modulation drive p53/p21 pathway and SESN2 activation in lung cells

RUNX2 mRNA levels were analyzed in the cargo of EVs derived from long COVID (LC) patients and control subjects. RUNX2 expression was found to be elevated in LC-derived EVs compared to controls (**Figure 7A**). To determine whether RUNX2 contributes to the increase in SESN2 and the activation of the p53/p21 pathway, RUNX2 was overexpressed in A549 cells. Forced expression of RUNX2 resulted in activation of the p53/p21 pathway and increased SESN2 levels (**Figure 7B**), consistent with the effects observed in cells treated with LC-derived EVs. The involvement of molecules regulating RUNX2 post-transcriptionally was subsequently investigated. An in-silico analysis using TargetScan identified a putative miR-204 binding site within the 3'-UTR of RUNX2 mRNA, located in an evolutionarily conserved region (**Figure 7C**). To assess the effect of miR-204 on RUNX2 expression, A549 cells were transfected with a miR-204 mimic using Lipofectamine at concentrations ranging from 5 to 25 nM. RUNX2 protein levels decreased in a dose-dependent manner in response to miR-204 mimic (**Figure 7D**). Analysis of miR-204 content in EV cargo revealed higher levels in LC-derived EVs compared to controls (**Figure 7E**).

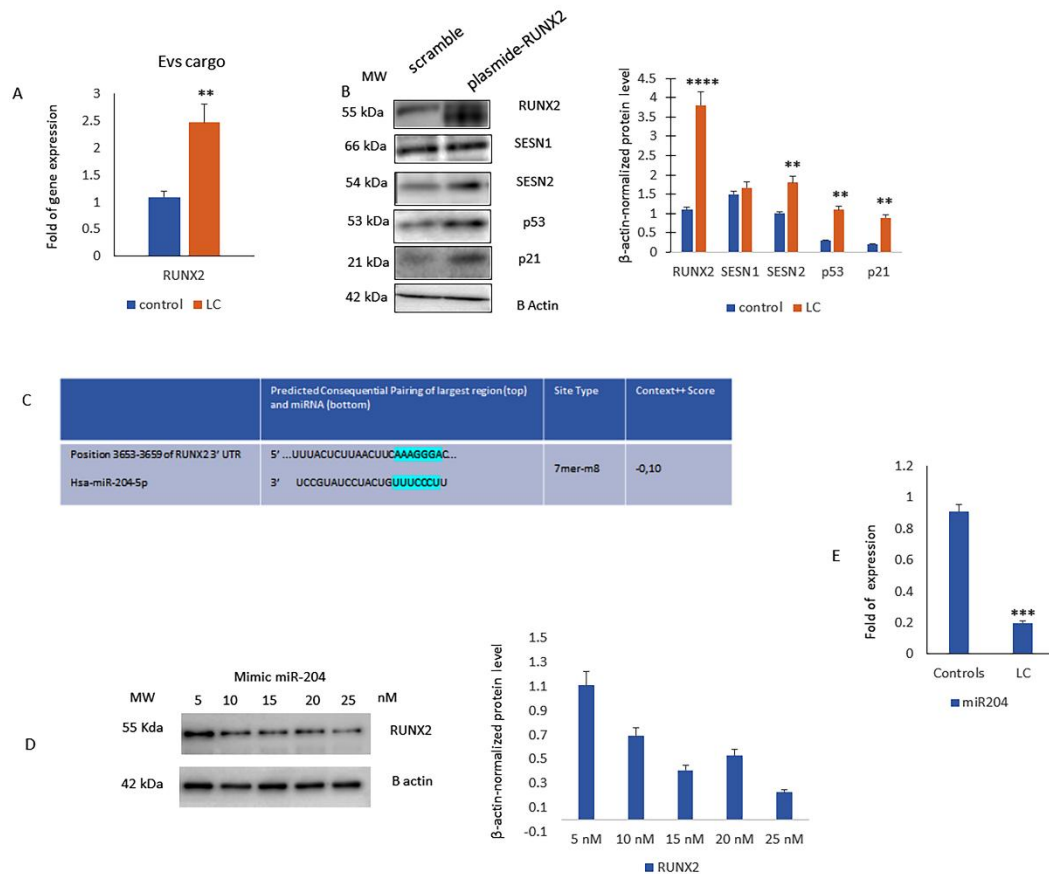


Figure 7. MiR 204/RUNX2 EVs. (A-B) Increased RUNX2 gene expression in LC EVs compared to controls WB for proteins levels (on the left) and optical density of the blots (on the right) in control or forced RUNX2 expression on A549 cells. (C) Putative miR-204 binding region in RUNX2 (Prediction score in Target Scan database: 0.10). (D) WB (on the left) and optical density of the blots (on the right) for RUNX2 protein levels on A549 cells transfected with mimic miR 204 at concentrations ranging from 5 to 25 nM. (E) Real-Time PCR for cargo miR 204 in controls and LC subject. (* $p < 0.05$; ** $p < 0.005$; *** $p < 0.001$; **** $p < 0.0001$ vs controls).

4.6 RUNX2 overexpression and stress response activation in endothelial and aortic smooth muscle cells exposed to LC-derived EVs

It was also observed a significant increase in RUNX2 expression in both endothelial cells (EC) and aortic smooth muscle cells (ASMC). As shown in **Figure 8A**, EVs from both control and long COVID patients were able to enter endothelial cells, and after 18 hours, they were fully localized within the cytoplasm.

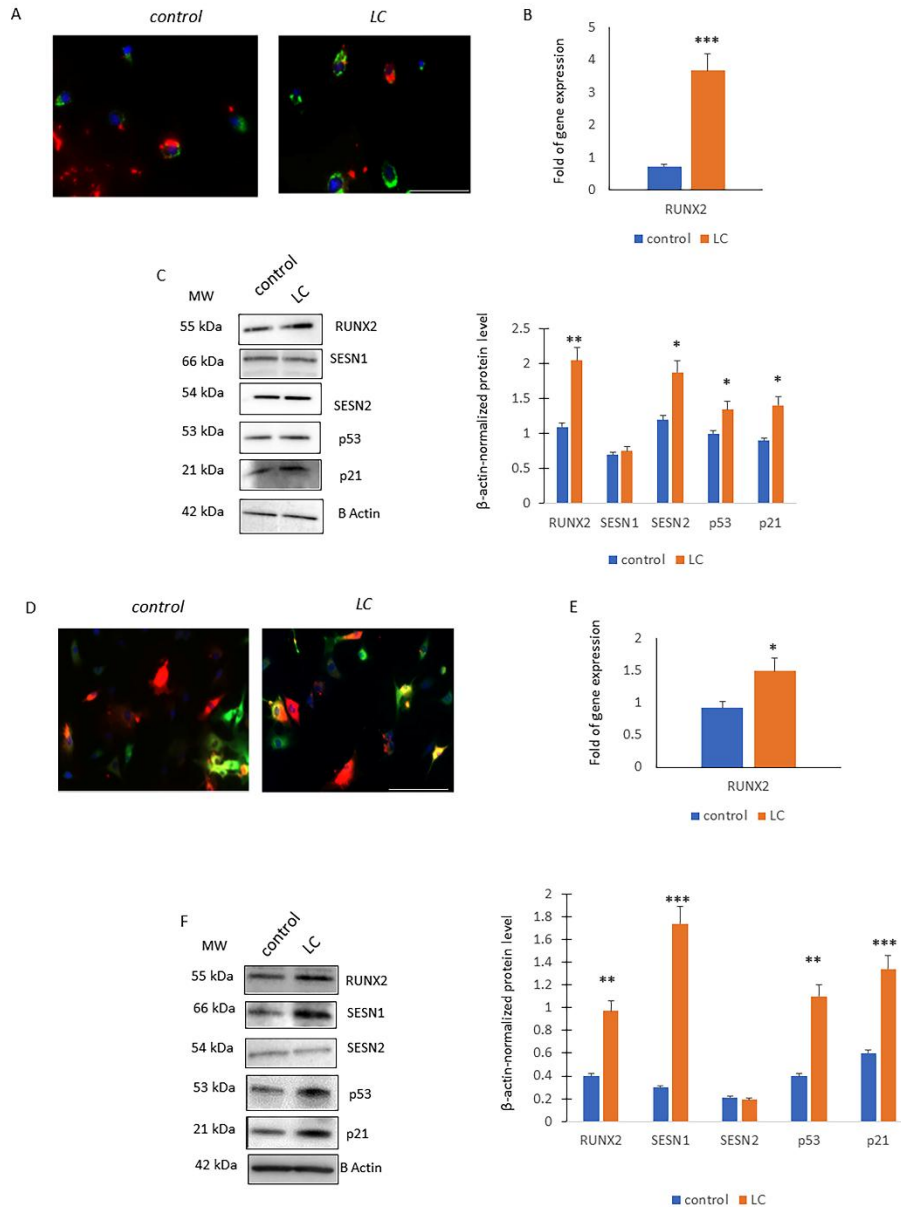


Figure 8. Effects of EVs on endothelial cells (EC) and ASMCs. **(A)** The EVs (red) from both the controls and LC crossed the cell (green) membranes and were completely internalized into the EC cells. **(B)** Real-Time PCR for RUNX2 and **(C)** WB for protein levels (on the left) and optical density of the blots (on the right) of EVs treated ECs. **(D)** The EVs (red) from both the controls and LC crossed the cell (green) membranes and were completely internalized into the ASMCs. **(E)** Real-Time PCR for RUNX2 and **(F)** WB for protein levels (on the left) and optical density of the blots (on the right) of EVs treated ASMCs. (* $p < 0.05$; ** $p < 0.005$; *** $p < 0.001$ vs controls).

Both gene expression (**Figure 8B**) and RUNX2 protein levels (**Figure 8C**) were higher in cells treated with long COVID-derived EVs compared to controls. Additionally, SESN1 levels were similar, while p53, p21, and SESN2 levels were higher in endothelial cells exposed to long COVID-derived EVs compared to controls. Similarly, EVs from both control and long COVID subjects efficiently entered ASMCs, with complete internalization observed within 18 hours (**Figure 8D**).

In ASMC has been seen an upregulation of the RUNX2 gene (**Figure 8E**) and related protein levels (**Figure 8F**) following treatment with long COVID EVs. Higher levels of SESN1 were observed in ASMC treated with EVs-derived LC compared to controls, while SESN2 levels were similar in both groups (**Figure 8F**). Higher levels of p53 and p21 were detected in lung and endothelial cells treated with long COVID-derived EVs compared to control EVs (**Figure 8F**).

4.7 RUNX2 and stress pathway activation in mesenchymal stem cells exposed to long COVID-derived EVs

Since RUNX2 is a key transcription factor for osteogenic commitment and differentiation of Mesenchymal Stem Cells (MSCs), the impact of long COVID-derived EVs on a human MSC line was investigated. The data demonstrated that similarly to the above-investigated cells, EVs from both control and long COVID sources entered the mesenchymal stem cells, with complete internalization observed within 18 hours (**Figure 9A**).

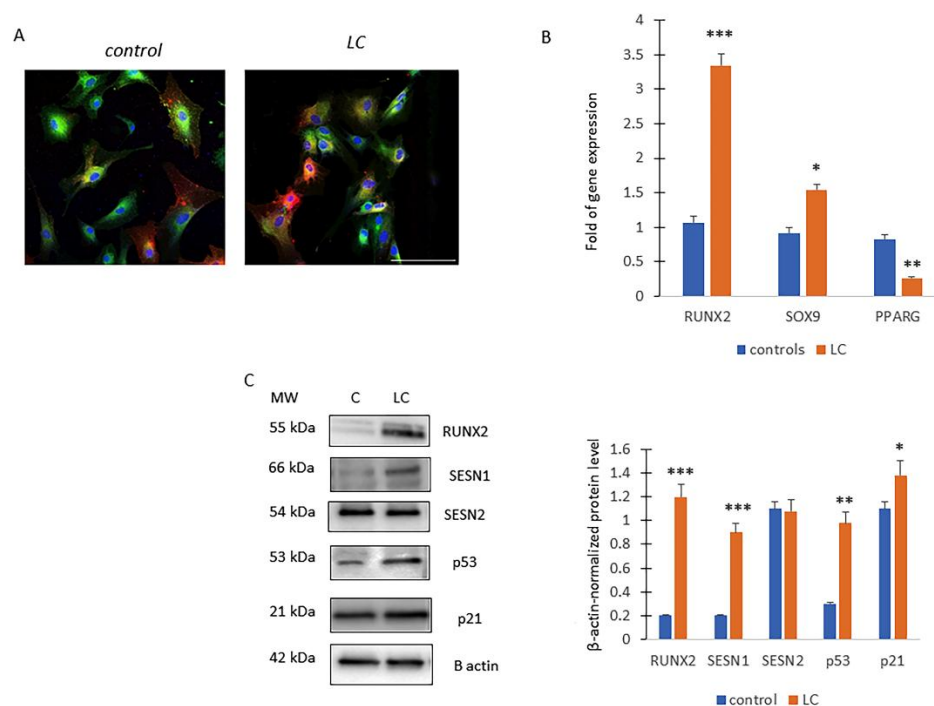


Figure 9. Effects of LC-EVs on hMSCs. (A) The EVs (red) from both the controls and LC crossed the cell (green) membranes and were completely internalized by the cells after 18 hours. (B) Real-Time PCR for RUNX2, SOX9 and PPARG2. (C) WB for protein analyses (on the left) and optical density of the blots (on the right). (* $p < 0.05$; ** $p < 0.005$; *** $p < 0.001$ vs controls).

In hMSCs, gene expression levels of RUNX2 were increased following treatment with long COVID-derived EVs compared to controls (**Figure 9B**). Additionally, SOX9 gene expression was increased, while PPARG expression was decreased (**Figure 9B**). Higher levels of RUNX2, SESN1, p53, and p21 proteins were detected in MSCs treated with LC-derived EVs. (**Figure 9C**).

5. Discussion

The data indicate a pathogenic mechanism in long COVID mediated by EVs. Flow cytometric analysis revealed a marked overexpression of markers associated with platelet hyperactivation in LC-derived EVs. In particular, CD29, CD41b, CD42a, and CD69 were elevated, all of which are known to play key roles in platelet aggregation [74,75]. The downregulation of these markers has been associated with hemorrhagic shock, underscoring their relevance in hemostasis [76]. Altered platelet activity has previously been reported in individuals with long COVID (LC) [77]. Damage to vascular endothelial cells is known to increase the expression of platelet-binding adhesion molecules, including von Willebrand factor (VWF), selectins, and integrins, while simultaneously downregulating anti-thrombogenic mediators. This endothelial impairment facilitates platelet activation and the thrombus formation. Accordingly, sustained elevation of VWF antigen and its propeptide has been observed in patients recovering from COVID-19, persisting for up to six weeks following symptom resolution [78].

Increased serum levels of plasminogen activator inhibitor type 1 have been reported in COVID-19 patients extending up to four months post-infection. These observations provide evidence for a sustained hypercoagulable condition in individuals experiencing ongoing symptoms after SARS-CoV-2 infection [79,80].

When lung cells were exposed to EVs from both healthy donors and LC patients, the vesicles crossed the cell membrane and spread throughout the cytoplasm. This occurred with EVs from both groups, showing that they can enter cells regardless of their origin. However, differences became apparent upon examining the expression of particular molecular markers across different cell lines, where LC-derived EVs exhibited unique expression profiles.

Vimentin and CK19, both associated with epithelial-mesenchymal transition (EMT) and neoplastic invasion [81], were markedly increased in cells treated with LC-derived EVs. This aligns with previous evidence, as vimentin plays a key role in EMT, while CK19 is associated with tumor progression and metastasis [81]. Increased RUNX2 expression in LC-EV-treated cells further supports its involvement in profibrotic signaling and pulmonary fibrosis [69,82]. Its connection to cellular movement, tissue penetration, and inflammatory respiratory disorders emphasizes its importance as a molecular target for comprehending the pulmonary manifestations present in LC patients. Sestrins constitute a group of evolutionarily conserved proteins that mediate cellular responses to stress conditions, encompassing oxidative harm, nutritional deficiency, and other adverse stimuli [83].

Operating as stress-detecting elements, they influence crucial molecular networks, such as mTOR and AMPK signalling, thus contributing to energy equilibrium maintenance and cellular viability promotion during challenging environments [84]. Dysregulation of these proteins has been linked to several diseases, including cancer and metabolic disorders, making them potential therapeutic targets in stress-related conditions [84]. In lung cells, SESN2 levels increased after exposure to LC-derived EVs, indicating a response to stress-response program. SESN2 is known to protect against different forms of stress, including genotoxic, energetic, and oxidative damage [85]. Since sestrins are activated by p53 [86], the p53-p21 pathway was examined, revealing increased levels of both p53 and p21 in cells treated with LC-derived EVs. This points to activation of the p53 pathway, which is commonly involved in cellular responses to DNA damage, oxidative stress, and other stress conditions [87].

Enhanced expression of p21, a gene regulated by p53, typically results in halting of the cell division cycle, providing cellular machinery with sufficient time for damage restoration or, when restoration proves unsuccessful, progression toward senescence or programmed cell death [87]. Sestrins may additionally participate in these mechanisms by facilitating oxidative stress management and modulating signalling networks associated with cellular viability and metabolic processes. Computational analysis of protein interaction networks provides additional support for these observations, demonstrating meaningful functional connections among the dysregulated proteins, especially within mitochondrial genomic metabolic pathways. EVs derived from long COVID patients (LC-EVs) demonstrated a bifunctional impact on mitochondrial performance. Cellular populations exposed to LC-derived EVs and examined under oxygen-rich conditions exhibited elevated oxygen utilization, as determined through Oroboros O₂k oxygraph measurements. This observation suggests amplified mitochondrial respiratory activity, potentially indicating heightened metabolic processes or an adaptive cellular response to stress conditions triggered by LC-EVs. Under conditions of increased metabolic stress, such as higher cell density, LC-EV-treated cells displayed a significant decline in maximal respiration (Seahorse MitoStress test). This indicates that although LC-EVs may initially enhance mitochondrial activity, they ultimately reduce the cells' metabolic adaptability, increasing vulnerability when resources like oxygen and nutrients are limited. A consequence of this reduced adaptability is a decrease in ATP production and basal respiration at higher cell densities. The reduction in peak respiratory capacity observed in cells treated with LC-EVs indicates that mitochondrial effectiveness in ATP synthesis during stressful conditions becomes impaired.

Conversely, cellular populations exposed to control vesicles demonstrated consistent respiratory patterns and ATP generation, reflecting maintained mitochondrial integrity. Collectively, these findings indicate that LC-EVs interfere with metabolic equilibrium in target cells, particularly during periods of elevated metabolic requirements. These discoveries may hold significant relevance for comprehending how long COVID affects cellular energy production, potentially explaining the exhaustion and other enduring manifestations experienced by affected individuals. Research has documented that patients with long COVID encounter persistent fatigue and activity-related lower extremity discomfort [88], symptoms that can manifest even during minimal exertion such as standardized six-minute ambulatory assessments.

To explore a potential mechanism by which long COVID EVs (LC) may affect the RUNX2/p53/sestrins pathway, the levels of RUNX2 mRNA carried by the EVs were analyzed. An increase in RUNX2 mRNA was observed in EVs from long COVID patients. Considering that previous studies have reported variable gene expression levels for miRNAs within EV cargo [89,90], the levels of microRNA-204 were also investigated. MicroRNA-204 was selected based on in silico analysis using TargetScan, along with previous evidence supporting its role in regulating RUNX2 [91].

Lower levels of miR-204 were observed in the cargo of EVs from long COVID subjects, consistent with the elevated RUNX2 levels detected in both the EVs and the cells treated with LC-derived EVs. The increase in RUNX2 levels in these cells also triggered activation of the p53/p21/Sestrins pathway, as confirmed by forced upregulation of RUNX2 through transfection with a RUNX2-overexpressing plasmid. However, the involvement of other miRNAs or regulatory factors in the observed signaling effect cannot be excluded. In addition to lung cells, the study included endothelial cells, aortic smooth muscle cells (ASMC), and hMSCs, where increased RUNX2 expression was also observed following treatment with long COVID-derived EVs.

In ASMC and mesenchymal cells, increased SESN1 levels were observed following treatment with LC EVs. This effect may reflect cell type-specific mechanisms, with each cell selectively upregulating a particular Sestrin to prevent redundant stress responses [83].

Both SESN1 and SESN2 modulate mTOR pathways and cellular defense against oxidative damage, enabling cells to selectively regulate specific Sestrin isoforms according to the stressors they encounter. This selective regulation allows for more precise cellular adaptation strategies.

The concurrent elevation of RUNX2, p53, p21, and either SESN1 or SESN2 demonstrates that long COVID-derived EVs exert broad-spectrum effects across diverse cell populations that are critical for cardiovascular and respiratory physiology. Examination of human mesenchymal stem cells revealed enhanced RUNX2 expression accompanied by corresponding modifications in SOX9 and PPARG transcript levels.

Previous studies have also linked simultaneous increases in RUNX2 and SOX9 to hypoxia-induced mineralization [92].

The findings indicate that EVs from long COVID patients may create hypoxic conditions, resulting in elevated levels of these transcription factors and potentially contributing to abnormal tissue mineralization and fibrosis. These observations are particularly relevant given that mesenchymal stem cells (MSCs) have been proposed as a promising therapeutic approach for COVID-19 [93–95].

However, because EVs from long COVID patients can affect MSC function, care is needed. Giving MSCs when abnormal EVs are present could reduce their effectiveness. In some cases, MSCs might become fibrotic, which could make the profibrotic environment caused by long COVID EVs worse. This shows why caution is important when using cell therapies in these conditions.

6. Conclusions

The research reveals that the EVs extracted from long COVID (LC) patients exhibit a unique molecular signature that has the potential to invoke profound cellular changes. These EVs have an increased composition of platelet activation markers and enhance the prothrombotic state, in accordance with the vascular dysfunction observed in long COVID. EVs from long COVID patients (LC-EVs) boost the expression of RUNX2 and initiate stress-related signaling pathways that involve p53, p21, and Sestrins in the target cells, indicating a response to oxidative and metabolic stress. Additional functional research shows that mitochondria exhibit less metabolic adaptability in LC patients, which could explain the fatigue and exercise intolerance. A specific mechanism was observed involving reduced miR-204 and increased RUNX2 mRNA in LC-EVs, suggesting an influence in the modulation of gene expression in target cells. These phenomena affected various types of cells such as lung, endothelial, smooth muscle, and mesenchymal stem cells, which signifies a systemic effect. The investigation shows that LC-EVs modulate mesenchymal stem cell (MSC) function, indicating that MSC-based treatments for long COVID patients should be approached with caution, as they could potentially lead to profibrotic changes. To conclude, EVs secreted from LC patients are crucial in the pathogenesis of long COVID, as they disrupt cellular equilibrium and drive systemic dysfunction.

Author's contribution

The research presented was carried out by the candidate under the supervision of the Principal Investigator and in collaboration with the research group. The candidate was responsible for the key experimental phases, the strategic management of clinical samples (blood collection, serum separation, and storage) and the isolation of EVs according to the specialized protocols described herein. The candidate personally performed the NTA and AFM analysis and the molecular characterization via RT-PCR and Western Blotting across multiple cell lines. The candidate led the interpretation of the results and the overall drafting of this manuscript. This work has been published in the international peer-reviewed journal *Cell Communication and Signaling* (2025), confirming the candidate's role in the study's conceptualization and technical execution.

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