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Master Degree in Medical Biotechnologies

Master Thesis

CHARACTERIZATION OF CIRCULATING EXTRACELLULAR VESICLES DERIVED FROM PATIENTS AFFECTED BY ACUTE CORONARY EVENTS: PRELIMINARY RESULTS

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ABSTRACT

Extracellular vesicles (EVs) are membrane-enclosed particles released by cells into the extracellular compartment, playing a crucial role in intercellular communication. Despite the recognition of their existence for over 80 years, only in recent decades significant advancements in their generation mechanism, biological functions, and biomedical applications have been obtained. The aim of this experimental thesis was to set up a protocol to isolate and characterize EVs derived from blood samples of healthy volunteers and patients affected by acute coronary events, as well as to preliminary investigate their biological activity in an *in vitro* endothelial model.

The proposed protocol is based on sequential centrifugations to isolate EVs to be physically and morphological characterized by nanoparticle tracking analysis and transmission electron microscopy. To further support the effectiveness of this approach in allowing EV recovery, the obtained particles were also investigated in terms of specific biomarkers, such as matrix metalloproteinases and specific tetraspanin expression. Furthermore, biological activity was evaluated in terms of their ability to induce interleukin 6 (IL-6) expression and gelatinases (MMP2 and MMP9) secretion by human umbilical vein endothelial cells (HUVECs).

Physical and morphological characterizations showed non-significant differences in concentration and size as well as in morphological appearance between healthy-volunteers- and patients-derived EVs, probably due to inter-individual variability and to the low number of samples. Biochemical evaluation of EVs' gelatinolytic activity revealed a comparable expression of MMP2 in both samples populations and an apparent higher expression of MMP9 in patients-derived samples. Finally, the immunofluorescent staining confirmed the presence of CD63, CD9, and CD81, three tetraspanins recognized as markers able to validate the identity of the isolated vesicles. Considering the preliminary investigation of EVs' biological activity, *in vitro* experiments did not reveal significant differences in their ability to induce IL-6 release by endothelial cells.

In conclusion, the proposed protocol allowed an efficient recovery of a consistent EV fraction from blood samples, allowing their physical and morphological characterization. Despite these promising findings, limitations such as low sample size and inter-individual variability need to be addressed. Future research should involve larger cohorts of patients and further investigation the biological effects of EVs, including their role in cytokine production and immunomodulatory potential, thus expanding our knowledge about the roles of EVs in health and disease.

INTRODUCTION

1.1 Overview of the heart: structure and function

Thanks to a complex network of vessels, blood flows throughout the whole organism, forming the circulatory system. Blood flows through the complex network of blood vessels thanks to the rhythmical contraction of the heart. The heart, thus, represents the central component of this system, acting as a pump that pushes the blood through the vessels assuring oxygen and nutrients supply to the tissues and the constant removal of catabolites.

The heart is located within the thoracic cavity, more precisely in the mediastinum, between the lungs and posterior to the sternal plate. The heart wall is composed of three layers: the endocardium, representing the innermost layer, lining the heart chambers, the myocardium, representing the intermediate layer, corresponding to a strong muscular tissue responsible for the heart's rhythmic contractions, and the epicardium, representing the outermost layer composed by mesothelium overlying some elastin-rich loose connective tissue. Moreover, the heart is enveloped by the pericardium, a protective fibroserous structure composed of two layers: a deeper, thinner serous layer and a thicker superficial fibrous layer made up of irregularly packed connective tissue (Figure 1) [1].

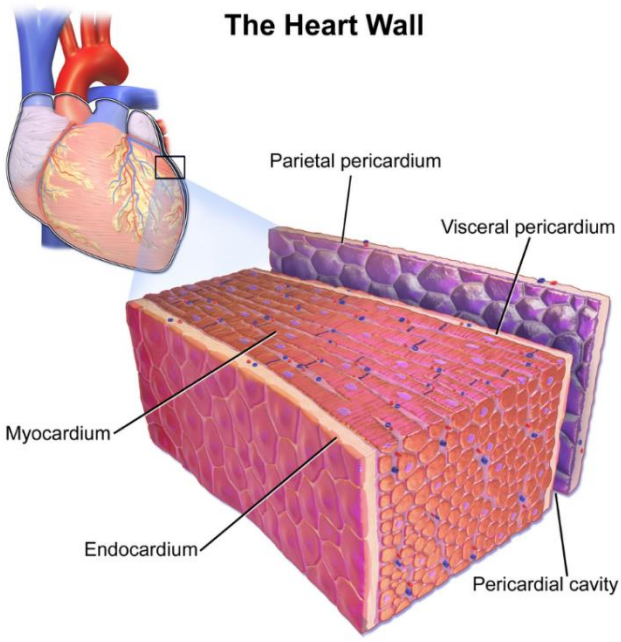

Figure 1. *Cardiac wall section showing the endocardium, the myocardium and the visceral pericardium layers* [2].

The heart's inner structure consists of four chambers: two atria and two ventricles. Atria are two thin-walled chambers located at the heart's upper pole, whose function is to collect the blood from the systemic (right atrium) and pulmonary (left atrium) circulation. The other two chambers, the ventricles, act as muscle pumps to force blood into the arteries allowing its flow throughout the systemic (left ventricle) and pulmonary (right ventricle) circulation. Atria and ventricles, as well as the large vessels originating from them, are separated by valves whose function is to guarantee the unidirectional blood flow. The tricuspid valve on the right side and the bicuspid valve (also known as mitral valve) on the left side control the flow between the atrium and the ventricle. Semilunar valves are located at the site where large arteries stem from the ventricles: the aortic valve controls blood ejection from the left ventricle into the aorta, while the pulmonary valve regulates blood flow from the right ventricle into the pulmonary trunk (Figure 2) [1,3].

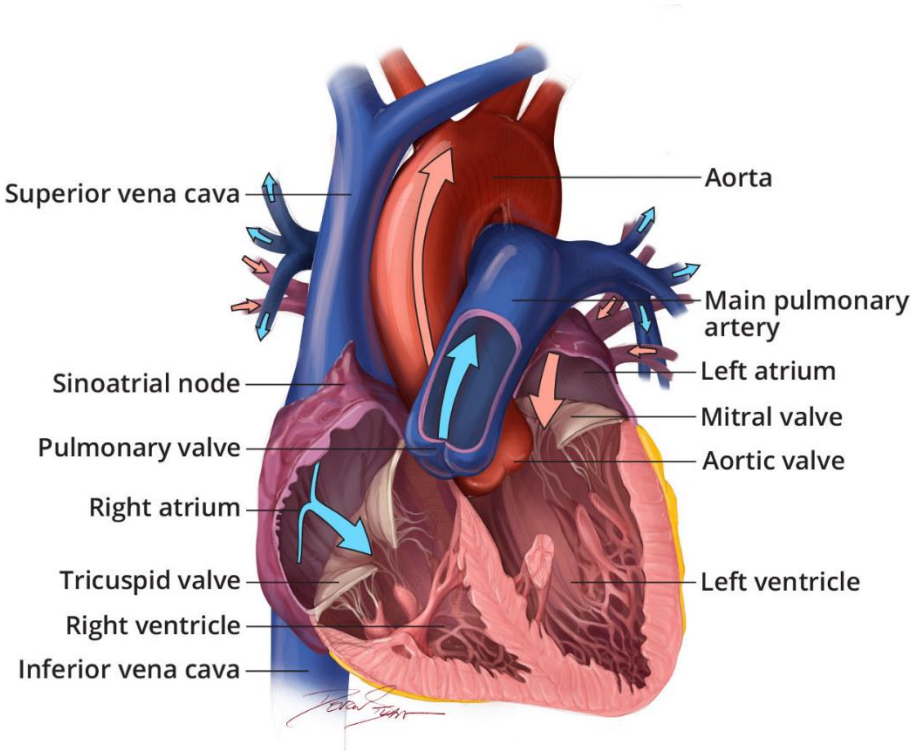

Figure 2. *Anterior view of the hearth (coronal section)* [4].

The whole heart structure is characterized by a specific vascularization, allowing the supply of oxygen and nutrients and the removal of catabolites. This role is covered by coronary vessels: left and right coronary arteries are responsible for oxygenated blood supply, while the coronary sinus is responsible for deoxygenated blood removal.

1.2 Blood vessels: anatomy and function

The vascular system is a complex and dynamic structure playing a critical role in maintaining homeostasis. Its main function is to deliver oxygen and nutrients to tissues and organs throughout the body, while removing catabolites. Deoxygenated blood flows from the periphery to the heart through a sequence of vessels, including capillaries, venules, and veins. Venous blood flows into the right side of the heart before reaching the lungs, where respiratory gasses exchange takes place. Oxygenated blood then returns to the left side of the heart and through the aorta enters the systemic circulation. The aorta, which is the greatest arterial vessel emerging from the left ventricle, then distributes oxygenated blood to the peripheral tissues and organs through arteries, arterioles and capillaries, where the exchange of nutrients and oxygen occurs [1,3].

Arteries are the circulatory system's efferent vessels, meaning they transport blood from the heart to the periphery, while veins are the afferent vessels that transport blood from the periphery to the heart. Capillaries, which connect smaller arteries to smaller veins, are microscopic, thin-walled vessels assuring the interconnection between efferent and afferent vessels. Furthermore, the blood flows in capillaries slowly and with a low and constant pressure, assuring the ideal conditions to sustain metabolic exchanges. Aside from their location and direction of blood flow, these three types of vessels are characterized by their wall histological structure [1].

The wall of arteries and veins is made up of three layers known as *tunicae*. The *tunica intima* lines the interior of the vessel and is directly exposed to the blood flow. It is made up of a simple squamous epithelium called endothelium, which covers a basement membrane and a layer of loose connective tissue. The *tunica media* is generally thicker and mainly consists of smooth muscle cells and elastic fibers. The proportion of these components varies according to the type of vessel and to the characteristics of the blood flow inside them. As a matter of fact, in the arteries the blood flows at high pressure, so the great presence of smooth muscle cells and elastic fibers allows these structures to accumulate the high mechanical energy of the blood ejected from the heart and to use it to sustain blood flow toward the periphery [1].

On the other hand, the lower pressure of blood flowing through the veins accounts for a reduced presence of elastic fibers in the wall of these vessels, where collagen fibers appear to be prevalent. Finally, the *tunica adventitia* corresponds to the outermost layer which is primarily composed of loose connective tissue made up of fibroblasts and associated collagen fibers anchoring the vessels to the surrounding structures and providing structural support (Figure 3) [1].

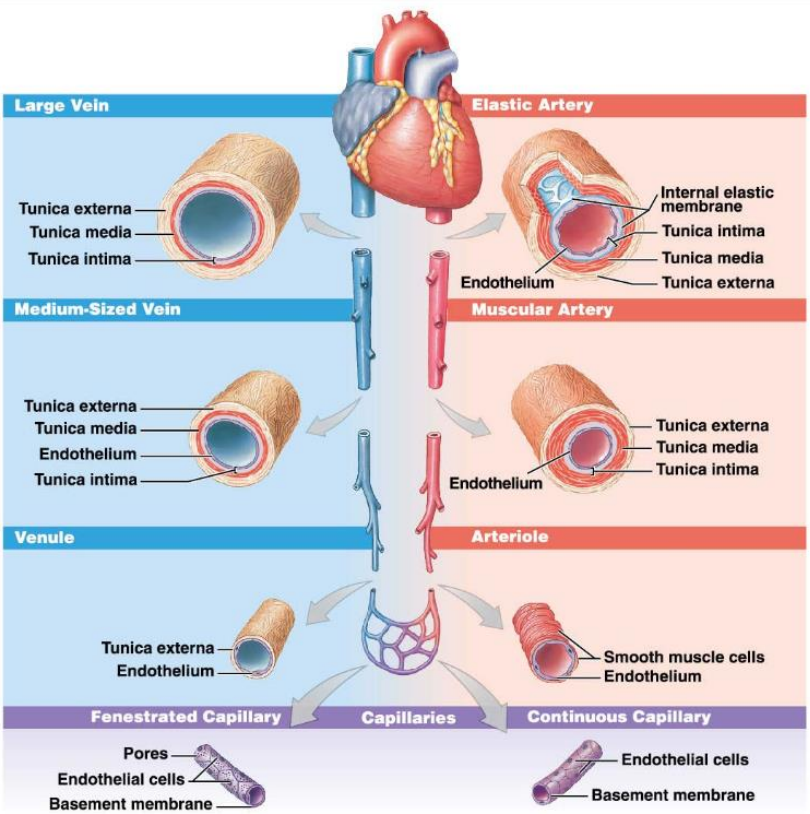

Figure 3. *Structure of the blood vessels in the cardiovascular system, showing the differences between large veins, medium-sized veins, venules, elastic arteries, muscular arteries, arterioles, and capillaries* [5].

1.3 Coronary arteries: the blood vessels of the heart

The heart receives the blood supplying from the coronary arteries. The left and right coronary arteries are the two major arteries arising from the initial part of the aorta, just following the aortic valve. Located along the coronary sulcus, the left coronary artery (LCA) provides blood supply mainly to the left chambers of the heart. It originates the anterior interventricular artery (left anterior descending artery) and the circumflex artery, as well as several other branches mainly originating from the interventricular artery. The right coronary artery (RCA), which runs along the right coronary sulcus, also generates several branches, allowing it to assure blood supply to the posterior two-thirds of the interventricular septum, as well as to the right atrium, ventricle, anterior and diaphragmatic surfaces (Figure 4).

Pathological conditions affecting coronary circulation, such as coronary artery disease (CAD), finally result in an inadequate blood supply to the myocardium due to the constriction of the coronary arteries. The consequent oxygen deprivation causes an initial degeneration of the cardiac muscle, potentially leading to a myocardial infarction (MI) [1,6,7].

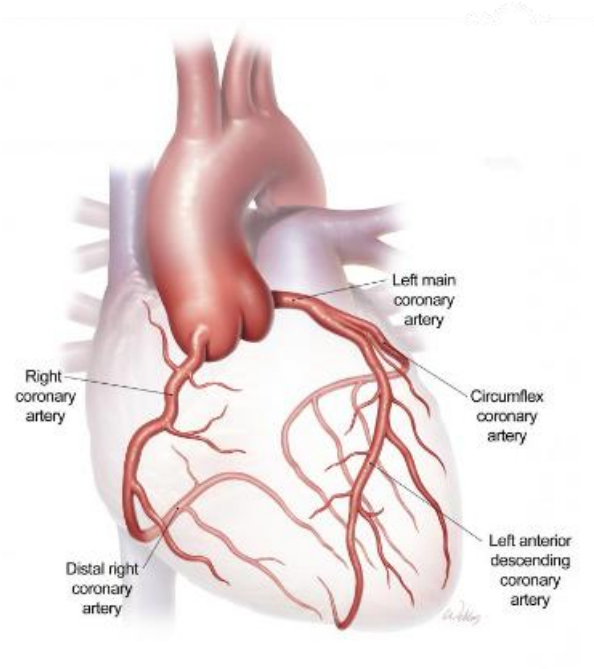

***Figure 4**. Anterior view of the heart, showing the left and right coronary arteries and their major branches [8].*

1.4 Cardiovascular diseases

Cardiovascular diseases account for about one-third of all fatalities at global level. Ischemic heart disease (IHD), also known as atherosclerotic cardiovascular disease (ACD), and coronary artery disease (CAD), are the most common cardiovascular diseases. IHD affects around 126 million people worldwide. The disease, which shows a male prevalence, typically develops during the fourth decade of life and worsens with aging [9].

Considering the global population aging and the increasing prevalence of obesity, diabetes, and metabolic syndrome, the incidence of IHD is predicted to keep rising [9].

Also in Italy, cardiovascular diseases represent one of the most relevant causes of mortality (30.8%): in particular IHD, resulting in an ineffective blood pumping from the heart, accounts for a total of 8.4% mortality, with slight sex-related differences [10].

In coronary artery atherosclerosis, the atherosclerotic plaque is the main cause of the disease. The first step of atherosclerosis development is represented by an endothelial damage resulting in lipoprotein droplets accumulation in the coronary tunica intima. These water-insoluble lipids then bind to some water-soluble lipoproteins, known as apolipoproteins, to move throughout the bloodstream. Circulating low-density lipoproteins (LDL) can undergo oxidation and penetrate the damaged endothelium, where they are internalized by macrophages. These lipid-laden macrophages are known as foam cells, which accumulation results in the development of specific lesions named fatty streaks. These lesions generate mediators able to recruit smooth muscle cells (SMCs) to the fatty streaks where they begin to proliferate and produce extracellular matrix components, among which the most relevant are represented by collagen fibers and proteoglycans. When the amount of extracellular matrix increases, the lesion progresses to a fibrous plaque. The fibrous plaque invades the coronary vessel lumen where it can subsequently calcify. The final lesion is made up of a fibrous cap covering a lipid-rich, necrotic core that may results extremely thrombogenic. The atherosclerotic lesions frequently cause cell death, or apoptosis, which ultimately results in the particulate deposition of tissue factor. Following plaque rupture, fragments can obstruct the vessels, reducing or even blocking blood flow, finally resulting in an imbalance between oxygen demand and supply in the heart, leading to ischemia [11].

The major manifestations of CAD are represented by stable angina, unstable angina and myocardial infarction [11].

Angina consists of chest pain or discomfort occurring because of a lack of blood flow to the heart. From a clinical point of view, angina can be classified as stable and unstable. Stable angina is usually exercise-induced and develops when the heart does not get enough blood supply during physical activity. The pain usually decreases with rest or medications. Instead, unstable angina results from an abrupt, short-term reduction in blood flow to the heart muscle due to plaque accumulation, which narrows vessel lumen, and a subsequent spasm of the coronary arteries. Unstable angina is a more severe manifestation, developing in the absence of physical stimulation. The associated pain worsens with time and is suggestive of a heart attack [12].

Reduced or complete lack of blood flow to a portion of the myocardium causes myocardial infarction and it is closely related to coronary artery disease. MI may be “silent”, undetectable, or it could be a catastrophic event leading to hemodynamic deterioration and sudden death [13].

From a clinical point of view, myocardial infarction can be divided, according to the patient's electrocardiogram (ECG) at admission, in two subgroups: ST-segment elevation myocardial infarction (STEMI) and non-ST-segment elevation myocardial infarction (NSTEMI) [14,15].

Typically, a STEMI heart attack occurs due to a complete obstruction of a primary coronary artery. Patients with symptoms of heart attack, but without ST elevation, may be diagnosed with NSTEMI. This last condition often results from a partial blockade of the coronary artery or a blockade in a branch that originates from the primary coronary artery (Figure 5) [15,16].

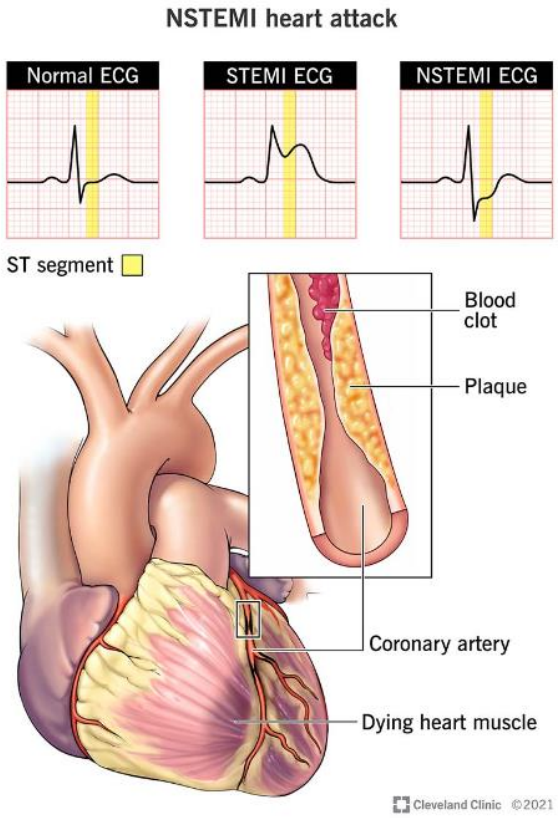

***Figure 5**. Illustration of NSTEMI (Non-ST-Elevation Myocardial Infarction) showing the differences in ECGs between normal conditions, STEMI, and NSTEMI heart attacks [17].*

Coronary artery diseases are pathological conditions characterized by a complex and dynamic interplay between several mediators. Emerging evidence highlighted the significant role of extracellular vesicles (EVs) in the pathogenesis of these conditions. By transporting bioactive molecules (i.e., proteins and RNAs), these vesicles facilitate intercellular communication and contribute to inflammation, thrombosis, and plaque instability.

1.5 Extracellular vesicles overview

Using electron microscopy, Wolf first identified the existence of membrane fragments or "lipid-rich particles" originating from active platelets. He referred to these fragments as "platelet dust", which are actually known as platelet extracellular vesicles (P-EVs) [18].

To date, extracellular vesicles are known as mediators able to provide new ways of communication between cells.

According to the definition of Welsh and colleagues, extracellular vesicles are "particles that are released from cells, are delimited by a lipid bilayer, and cannot replicate on their own (i.e., do not contain a functional nucleus)" [19].

EVs are classified according to their size, biogenesis, and the presence or absence of specific proteins. Moreover, EVs can be categorized into three main subclasses: exosomes, microvesicles, and apoptotic bodies [20].

Exosomes are relatively small extracellular vesicles with a diameter ranging from roughly 50 to 150 nm. Their synthesis relies on the endocytic endosomal pathway, where the cytoplasmic membrane forms inward buds, resulting in the incorporation of membrane molecules and in the creation of early endosomes inside cells. During the later stages of development, early endosomes combine to create late endosomes, causing the endosomal membrane to fold inward and produce intraluminal vesicles (ILVs). These ILVs then fuse together to form multivesicular bodies (MVBs) [21].

Multivesicular bodies may either merge with lysosomes, leading to their breakdown, or merge with the plasma membrane, subsequently releasing exosomes into the extracellular compartment as vesicles carrying cytosolic content [22].

MVBs can be produced by two different mechanisms. The first mechanism is linked to the endosomal sorting complex required for transport (ESCRT), which includes ESCRT-0, -I, -II, -III, and Vps4 complexes. ESCRT-0 plays a crucial role in breaking down ubiquitin-tagged cargo, while ESCRT-I and ESCRT-II contribute to the formation of endosomal membrane buds. ESCRT-III encircles the neck of the created vesicle, while Vps4 breaks the membrane to create the luminal vesicle inside the MVBs [23].

The second mechanism relies on a specific lipid component in the endosomal membrane, rich in sphingolipids, and operates independently of the ESCRT mechanism. Nevertheless, these sphingolipids serve as substrates for neutral sphingomyelinase 2 (nSMase2). nSMase2 catalyzes the conversion of sphingolipids into ceramides on the endosomal membrane. This process causes microdomains to combine and form bigger structures, which then leads to the budding of domains and the creation of intraluminal vesicles. This mechanism involves the transport of several proteins, as well as the activation of several downstream signaling cascades, mainly involving RAS-related proteins. Rab27A and Rab27B are crucial regulatory proteins in this process, and Rab27A has also been shown to be linked to the fusion of the MVBs with the plasma membrane [24].

Microvesicles (MVs) have a size range of 100-1000 nm. Unlike exosomes, they are secreted into the extracellular space by gemming from the plasma membrane via a calcium-dependent process, which induces alterations in the cytoskeleton at the plasma membrane level. External signals induce an elevation in intracellular calcium levels, which disrupts the asymmetry of the double phospholipid layer. This membrane alteration results from the activation of various enzymes, including flippases (inward-directed pumps), floppases (outward-direct pumps), and scramblases (enzymes that facilitate non-specific bidirectional redistribution across the bilayer). These enzymes regulate the movement of phosphatidylserine from the inner to the external side of the membrane. Furthermore, calcium ions participate in the activation of other enzymes, such as gelsolin and calpain, that alter the actin cytoskeleton and enable the breakdown of the actin fibers below the plasma membrane. These structural changes finally alter the curvature and the protrusion of the plasma membrane, allowing microvesicles detaching [24,25].

Lastly, apoptotic bodies are particles with 1000 to 5000 nm size generated by the apoptotic cell membrane bubbling and subsequent disintegration generating protrusions known as apoptotic vesicles during programmed cell death (Figure 6) [26].

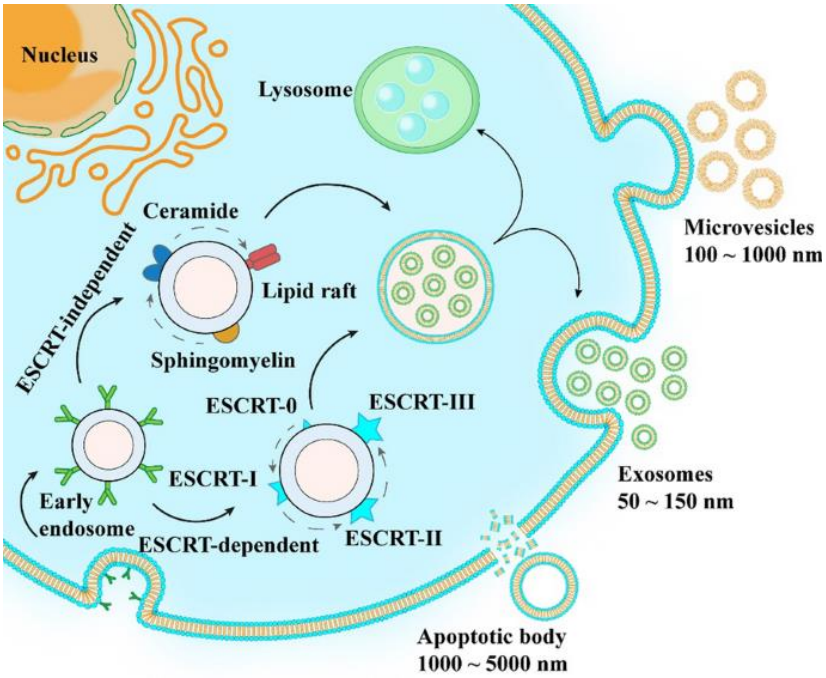

Figure 6. *Biogenesis and release of exosomes, microvesicles, and apoptotic bodies* [24].

It seems that EVs can be generated by all types of cells and that they can be released into many physiological fluids such as blood, saliva, urine, cerebrospinal fluid, breast milk, and semen [27].

Extracellular vesicles transport a variety of biologically active substances, including nucleic acids, proteins, lipids, and carbohydrates, both inside and on their surface. These components are transferred from the donor cell to the recipient cell by numerous processes, including direct fusion of membranes, contact between receptors and ligands, endocytosis, and phagocytosis, finally resulting in a modulation of cellular responses [24].

Initially, it was hypothesized that the secretion of EVs had a role in the removal of undesirable substances from cells. Further studies showed that EVs were involved in several intercellular signaling pathways, enabling a wide range of physiological and pathological cellular functions by transporting different biomolecules and allowing the exchange of components between cells [22].

1.6 Role of extracellular vesicles in cardiovascular diseases

Inflammation plays a role in almost all cardiovascular disorders, contributing to the disease's development at various stages. In the context of atherosclerosis, extracellular vesicles are involved in the recruitment of monocytes, in the polarization of macrophages, and in the generation of microcalcifications. These processes together lead to the creation, development, and destabilization of atherosclerotic plaques [28].

Extracellular vesicles isolated from the inner layer of blood vessel lesions have been shown to play a significant role in the advancement of atherosclerotic plaques. Human plaque analysis revealed that the majority of the detected EVs derived from leukocytes, primarily macrophages (29%), followed by lymphocytes (15%) and granulocytes (8%). Also smooth muscle cells, platelets, adipocytes, and endothelial cells are all significant contributors to EVs. Whatever their source, EVs interact with immune cell mediators throughout all stages of disease progression [29].

Thus, EVs contribute to the progression of inflammatory infiltration, leukocyte recruitment, and plaque development. In particular, it has been shown that EVs are involved in macrophage polarization as well as in the conversion of macrophages into foam cells. Furthermore, EVs produced from adipose tissue, induce macrophages to polarize towards the pro-inflammatory M1 phenotype [30].

Untreated atherosclerosis might potentially result in a myocardial infarction, triggering fast immune responses, including both humoral and cell-mediated mechanisms. The release of EVs within the myocardium appears to correspond to the inflammatory response, with a significant increase between 15-24 h following MI. These EVs originate from cardiomyocytes and endothelial cells adjacent to the lesion and stimulate the secretion of chemokines and inflammatory cytokines by invading monocytes [31].

Cardiomyocyte-derived extracellular vesicles have the ability to influence the inflammatory properties of macrophages based on the condition of the cells from which the EVs originate. Endothelial-derived extracellular vesicles are elevated during acute myocardial infarction and stimulate the migration and activation of monocytes from the spleen. Hence, EVs originating from cardiomyocytes and endothelial cells attract peripheral mononuclear cells and modify their characteristics. Moreover, there is a strong correlation between the quantity of EVs and the degree of damage to the heart muscle [32]. This indicates that EVs may have potential diagnostic and prognostic significance in cases of myocardial infarction.

AIM OF THE STUDY

In the past, extracellular vesicles were considered merely as a cellular mechanism intended for the removal of the discarded and catabolic components. Recently, indeed, several studies investigated EVs source and biological role, revealing that these cell-derived particles play a crucial role in maintaining the homeostasis of cells as well as of the whole organism, being involved in intercellular communications, mediating a variety of physiological and pathological processes. It is noteworthy that EVs allow intercellular interactions acting as biological cargos for proteins, lipids, and nucleic acids, thus influencing immune responses, tissue repair, and disease progression. Moreover, EVs composition depends on the cellular origin, allowing them to act as a double-edged sword: in healthy individuals these biological particles play a key role in maintaining homeostasis, while in pathological conditions they are involved in disease progression or defense.

Considering the complex nature of these recently identified intercellular mediators, the aim of this thesis was to set up an experimental protocol to isolate, quantify and characterize circulating EVs from blood samples obtained from both healthy volunteers and patients undergoing programmed coronary catheterization intervention. In addition, a secondary aim of this experimental work was a pilot *in vitro* investigation of the biological effects of the obtained EVs in an endothelial cell model (HUVEC).

MATERIALS AND METHODS

3.1 Blood collection and isolation of extracellular vesicles

Extracellular vesicles were isolated setting up a specific centrifugation protocol, based on the method described by Menck and coworkers [33]. Briefly, blood samples were obtained from human healthy volunteers and patients undergoing planned coronary catheterization intervention using a conventional venipuncture procedure. Whole blood samples were collected using 0.129 M sodium citrate vacutainer tubes (Becton Dickinson, Plymouth, UK) according to standard procedures. Samples were centrifuged at 1500 g for 15 minutes at room temperature in order to separate plasma fraction. For EVs isolation plasma fraction was carefully transferred into a sterile tube and centrifuged at 1500g for 15 minutes at room temperature in order to remove residual platelets and red blood cell debris. Plasma was then transferred into 1.5 ml tubes and centrifuged at 16000 g, at 4°C for 40 minutes. After centrifugation, plasma was carefully removed, while phosphate-buffered saline (PBS) solution was added. This process was repeated 6 times, until all the plasma fraction was completely removed, thus assuring a complete washout of the obtained EVs pellet. Finally, EVs pellet was carefully resuspended in 150 µL of PBS and stored at -80°C for further analysis.

3.2 Nanoparticle tracking analysis

Nanoparticle tracking analysis (NTA) is a high-throughput analytical technique allowing individual EVs analysis. This approach is based on particles' Brownian motion evaluation, allowing the evaluation of their size distribution and concentration (particles/ml). In particular, data are obtained by capturing the scattered light resulting from laser illumination of a suspension of particles (Figure 7) [34,35].

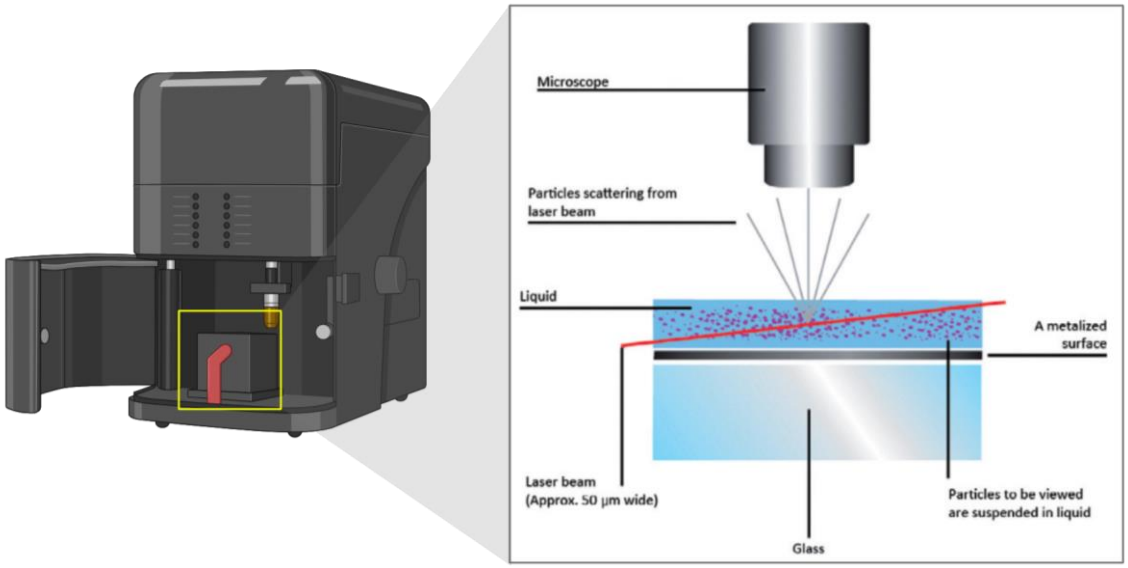

Figure 7. *Schematic illustration of nanoparticle tracking analysis instrument operating principle. NTA technology relies on microfluidics. The solution containing the particles to be analyzed, is irradiated by a laser beam and the resulting scattered light is focused by a microscope while a digital camera is used to record and track the random thermal motion (Brownian motion) of each particle.*

Nanoparticle tracking analysis was performed using a NanoSight NS300 (Malvern Panalytical, Malvern, UK) equipped with 532 nm laser (green) and a scientific sCMOS camera. Before analysis, samples were diluted in ultrapure water, and their concentration was adjusted to reach a particle/frame rate between 30-100 according to the manufacturer's user manual (NanoSight NS300 User Manual, MAN0541-01-EN-00, 2017). For each sample, five consecutive videos of 60 seconds were recorded using the following settings: camera level at 14, temperature at 25°C, and syringe speed at 22 µL/s. The captured videos were elaborated by using the built-in NanoSight Software NTA3.4.003 (Malvern Panalytical, Malvern, UK), fixing the detection threshold at 5.

3.3 Transmission electron microscopy

Due to the dimensions, EVs can not be resolved with standard optical microscopy approaches but require the use of electrons instead of photons to obtain images with a resolution down to the nanometer [35]. For this reason, the morphology of the isolated EVs was observed using a transmission electron microscope (TEM). Briefly, 20 µL of each sample (starting concentration: 4.5 µg/mL of protein) was let to adsorb onto a formvar/carbon copper grid (150 mesh, Electron Microscopy Sciences, Hatfield, PA, USA) for 10 minutes at room temperature. Then the grid was washed twice in water and then incubated with 1% uranyl acetate (1+30 minutes at room temperature, in the dark) to negatively stain the biological membranes. The excess of uranyl acetate was removed using Whatman filter paper and then the grid was air-dried overnight. Images were analyzed using a ZEISS EM-109 transmission electron microscope with an Olympus Mega View G2 TEM CCD Camera System with integrated TEM imaging Platform iTEM (Olympus, Hamburg, Germany). The identification of extracellular vesicles was carried out at high magnification (≈140.000X).

3.4 Gelatin zymography

The zymographic technique is a variant of the classic electrophoretic analysis method that allows for the visualization of MMPs' enzymatic activity on gels due to the presence of a suitable substrate copolymerized within it [36]. According to EVs-associated gelatinases (MMP2 and MMP9) expression [37,38,39], gelatin zymography was performed on equal amounts of pure EVs (5×10^8 particles/ml). This biochemical approach was used also to investigate EVs biological activity, by analyzing the conditioned cell culture medium resulting from endothelial cell stimulation with the isolated EVs.

The gel used for zymography (zymogram) consisted of a 7.5% acrylamide running gel and a 4% stacking gel. The running gel was prepared by mixing 950 µL of 40% acrylamide (ITW Reagents, Monza, Italy), 1.25 mL of Tris-HCl 1.5 M pH 8.8, 2.7 mL of 0.2% gelatin, 50 µL of 10% ammonium persulfate (APS), 50 µL of 10% sodium dodecyl sulfate (SDS) and 10 µL of N, N, N', N' - tetramethyl-ethylenediamine (TEMED). The stacking gel was prepared by mixing 250 µL of 40% acrylamide, 625 µL of Tris-HCl 0.5 M pH 6.8, 1.575 mL of deionized water, 25 µL of 10% APS, 25 µL of 10% SDS and 5 µL of TEMED.

Samples were mixed with non-reducing loading buffer (Tris-HCl 50 mM pH 6.8, 2% SDS, 0.1% bromophenol blue, 10% glycerol) to preserve enzymatic activity.

In addition to the samples of interest, a molecular weight marker (Euroclone, Milan, Italy) and a purified MMP-9 marker (Abcam, Cambridge, UK) were loaded.

The electrophoretic separation was performed on a Mini-PROTEAN IV® electrophoresis module (Bio-Rad, Hercules, CA, USA) at 100 mV in Tris-glycine-SDS buffer, pH 8.3.

After electrophoretic separation, gels were rinsed for 2 hours in 2.5% Triton X-100 (Sigma-Aldrich, Milan, Italy) aqueous solution to remove SDS and allow enzyme renaturation. Gels were then incubated overnight at 37°C in digestion buffer (Tris-HCl 50 mM pH 7.5, 10 mM CaCl2, 0.15 mM NaCl), to guarantee the optimal conditions for enzymatic activity. After digestion, the gels were fixed in 50% methanol, 10% acetic acid solution for 2 hours at room temperature, then stained with Coomassie Blue G250 solution (Sigma-Aldrich, Milan, Italy) for 1 hour and finally destained in 5% methanol, 7% acetic acid solution.

Enzymatic activity can be detected in the form of clear bands against a uniform blue background, corresponding to the undigested gelatin. Experimental conditions adopted during zymographic assay allow gelatin digestion also by gelatinases pro-enzymatic forms (pro-MMP2 and pro-MMP9), and this issue should be considered when analyzing the obtained results [38]. Appropriately destained gels were digitally acquired using a ChemiDoc transilluminator (Bio-Rad, Hercules, CA, USA) and gelatinolytic activity was quantified by densitometric analysis using ImageJ software.

3.5 Immunofluorescent tetraspanin staining

Immunofluorescent tetraspanin staining is an advanced analytical approach designed for the characterization of extracellular vesicles, enabling the measurement of the size, concentration and expression of EVs surface markers through interferometric detection. This technology allows high-resolution imaging and precise identification of EVs subpopulations by capturing them on surfaces functionalized with specific antibodies [40].

Microarray chips (Lunis, #251-1045, Unchained Labs, Pleasanton, CA, USA) coated with antibodies against CD9 (HI9a), CD63 (H5C6) and CD81 (JS-81) were pre-scanned according to the manufacturer's instructions on a Leprechaun instrument (Unchained Labs, Pleasanton, CA, USA) to generate baseline measurements of pre-adhered particles before sample incubation.

The EV samples were diluted 1:10 and 1:20 in incubation buffer in order to avoid oversaturation of the chips. After the pre-scanning step, 50 µL of the diluted samples were carefully loaded onto the chips and incubated overnight, at room temperature in a sealed 24-well Luni plate.

At the end of the incubation, chips were rinsed using the automated Luni Washer (Unchained Labs, Pleasanton, CA, USA) and incubated for 1 hour at room temperature with the fluorescently labeled antibodies anti-CD9 (CF®-488-labeled), anti-CD63 (CF®-647-labeled) and anti-CD81 (CF®-555-labeled) (Unchained Labs, Pleasanton, CA, USA) diluted 1:500 in blocking buffer (Figure 8). At the end of the incubation, chips were washed and transferred into a Leprechaun instrument (Unchained Labs, Pleasanton, CA, USA) for fluorescent signal scanning.

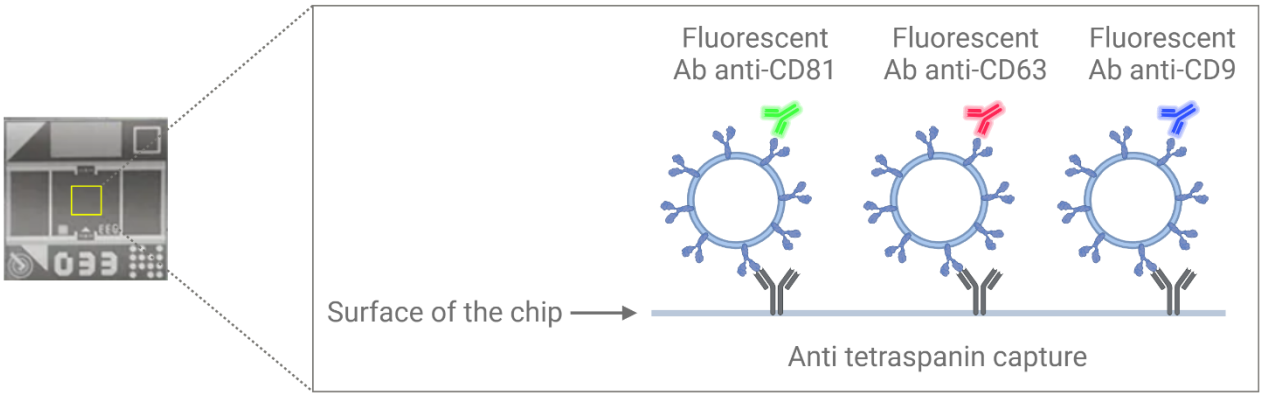

***Figure 8**. Schematic illustration of the immunofluorescent tetraspanin staining approach for the characterization of extracellular vesicles (EVs). The experimental design relies on EVs capture on a surface coated with antibodies specific for tetraspanins and subsequent stain with a fluorescent antibody cocktail (anti-CD63, anti-CD81, and anti-CD9 antibodies).*

3.6 Cell Culture

Primary human umbilical vein endothelial cells (HUVEC CRL-1730, ATCC®, Manassas, VA, USA) were used to investigate the biological effects of EVs. As HUVEC are primary cells, experiments were performed using only cells that underwent less than 6 passages in culture.

HUVEC were grown at 37°C and 5% of CO_2, in Kaighn's modification of Ham's F-12 medium (F12K) (ATCC®, Manassas, VA, USA) supplemented with penicillin 0.02 units/ml, streptomycin 0.02 µg/ml, amphotericin B 0.05 ng/ml (PSF solution, Euroclone, Milan, Italy), endothelial cell

growth supplement (EGCS) (Corning, Berlin, Germany) 0.06 mg/mL, heparin (Sigma-Aldrich, Milan, Italy) 0.1 mg/mL and 10% fetal bovine serum (FBS) (Gibco, Milan, Italy). To avoid interferences from FBS intrinsic extracellular vesicles, before EVs stimulation HUVEC were starved overnight in cell culture medium without FBS, and the same conditions were maintained during stimulation. To test EV biological effects, HUVEC were stimulated for 24 hours with 2×10^9 EVs, 100 ng/ml of TNF-α (#H8916, Sigma-Aldrich, Milan, Italy) (positive control) or left untreated (control condition).

3.7 Enzyme-linked immunosorbent assay

Interleukin (IL)-6 secretion by HUVEC in response to EV stimulation was evaluated through enzyme-linked immunosorbent assay (ELISA), a widely used biochemical assay allowing the detection of an analyte in a liquid sample by adsorbing it to a solid phase which is physically immobilized on the assay support [41].

IL-6 presence in the conditioned cell culture supernatants was analyzed using an ELISA kit (#EH2IL6, Invitrogen, Waltham, USA), following manufacturer's instructions. Briefly, 50 µL of biotinylated antibody reagent as well as 50 µL of IL-6 standard and conditioned cell culture supernatants, were added to each well and incubated for 2 hours at room temperature. At the end of the incubation, the plate was washed 3 times and 100 µL of streptavidin-HRP solution were added to each well and incubated for 30 minutes at room temperature. At the end of the incubation step, the plate was washed 3 times and 100 µL of TMB substrate were added to each well and incubated for 30 minutes, at room temperature in the dark to allow the color reaction. The reaction was stopped by adding 100 µL of stop solution to each well and the absorbance at 450 nm was read using a Victor4X Multilabel Plate Reader (Perkin Elmer, Milan, Italy).

RESULTS

The protocol set up in our laboratory allowed the isolation of the particles of interest. The pellet obtained at the end of the 6 centrifugation rounds was composed of extracellular vesicles, as confirmed through different characterization approaches (NTA, immunofluorescent tetraspanin staining and TEM analyses).

4.1 Quantification and physical characterization of extracellular vesicles

After the isolation, the obtained EVs were quantified using nanoparticle tracking analysis. As shown in figure 9a, the mean size of EVs isolated from healthy volunteers ranged from 110 to 150 nm, while the mean size of EVs isolated from patients ranged from 100 to 140 nm, highlighting a non-significant difference between the two set of samples. Furthermore, NTA approach allows the estimation of the number of particles in solution, after estimating the sample volume. As shown in figure 9b EVs concentration in healthy-volunteers- and patients-derived samples was similar ($1.27 \times 10^{11} \pm 1.9$ particles/ml in healthy volunteers derived samples and $1.14 \times 10^{11} \pm 1.8$ particles/ml in patients derived samples).

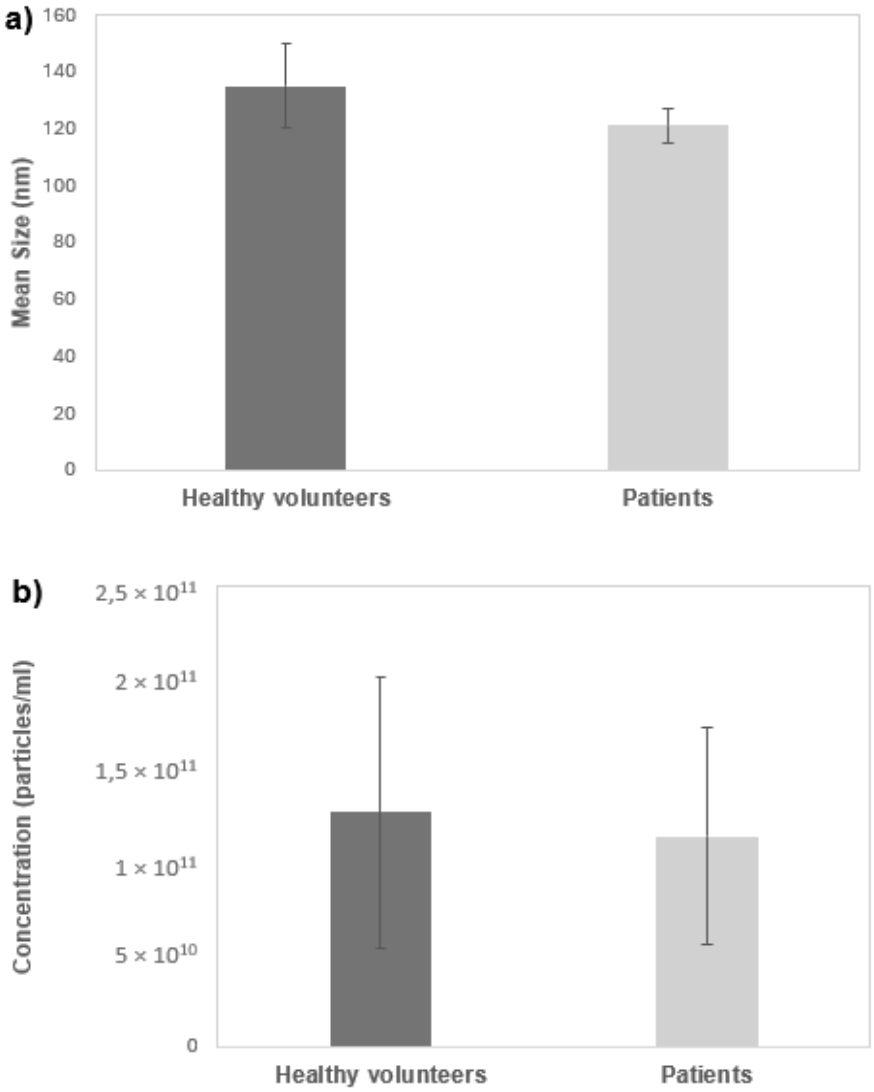

Figure 9. *Healthy-volunteers- and patients-derived EVs mean size (a) and (b) concentration distribution.*

4.2 Morphological characterization of extracellular vesicles

The morphology of the extracellular vesicles isolated from blood samples was evaluated by TEM analysis, as their size is below the diffraction limit of visible light. Furthermore, in order to increase the final resolution of the image, EVs were negatively stained with uranyl acetate, a compound commonly used to enhance the contrast of biological membranes. TEM analysis highlighted the presence of EVs, showing heterogeneity both in size and morphology. As shown in figure 10, some EVs derived from both a healthy volunteer and a patient appear larger in size and display the typical cup-shaped morphology (figure 10 section a and c) while others appear smaller and with a well-defined membrane surrounding a clear and granular inner material (figure 10 section b and d). Moreover, TEM analysis confirmed the structural integrity of the isolated extracellular vesicles.

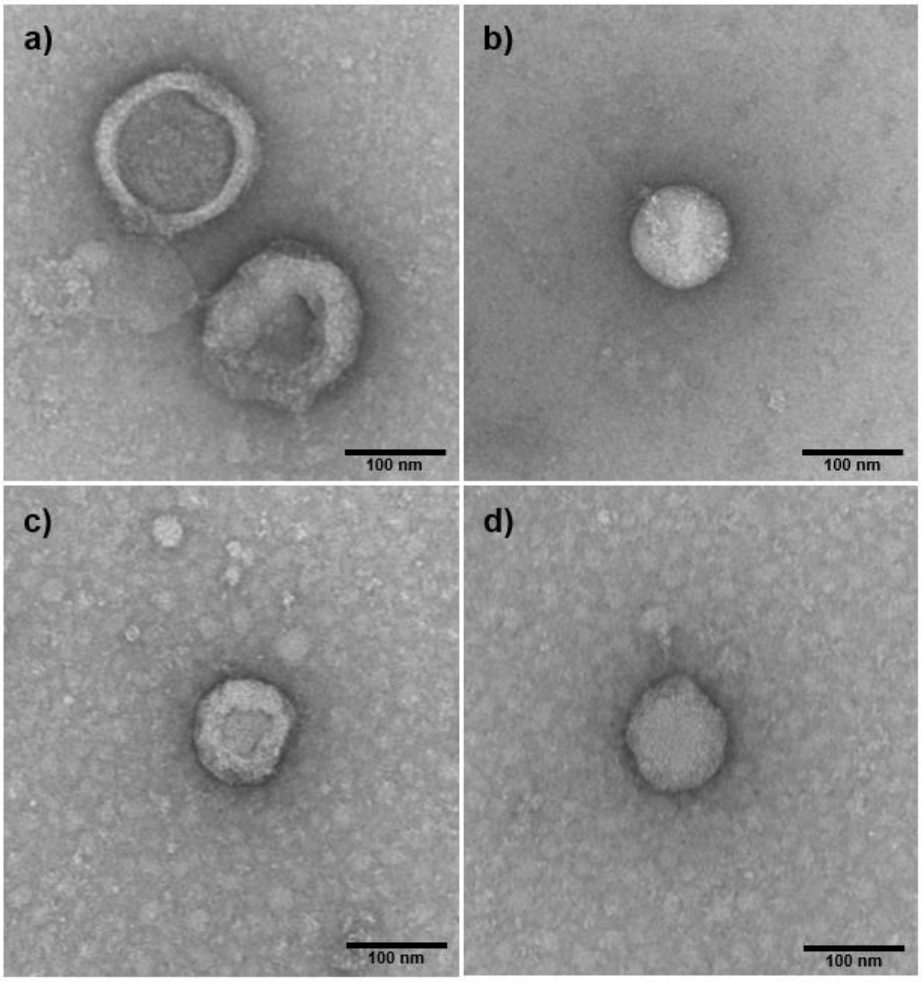

***Figure 10**. Representative TEM images of EVs, showing a cup-shaped morphology (a,c) or a simple membrane surrounding a clear, granular material (b,d). Scale bar 100 nm. Magnification 140000X.*

4.3 Detection of extracellular vesicles-associated proteolytic activity

The proteolytic activity of the obtained EVs was evaluated by gelatin zymography, an experimental approach allowing to visualize MMP2 and MMP9 enzymatic activity. Since gelatinases are known to be associated with EVs, an equal amount of extracellular vesicles isolated from 3 healthy volunteers and 3 patients was analyzed. As shown in figure 11, in both types of samples it is possible to identify a clear band corresponding to active MMP2 (~62 kDa). Even if a slight inter-individual difference in gelatinolytic activity could be appreciated, a non-significant difference exists between the two populations. According to zymogram results, also a ~92 kDa clear band could be observed, corresponding to the MMP9 zymogen (pro-MMP9). In healthy volunteers derived samples, pro-MMP9 expression appears homogeneous between the different donors, while in patients derived samples MMP9 zymogen shows a clear inter-individual variability, finally resulting in an apparently higher activity associated with patients derived EVs.

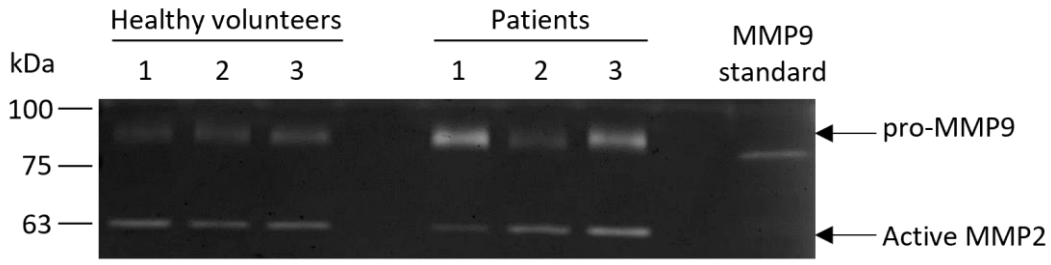

***Figure 11**. Representative zymogram of pure EVs isolated from 3 healthy volunteers and 3 patients.*

4.4 Characterization of extracellular vesicles surface biomarkers

Surface characterization of the extracellular vesicles isolated from blood samples was obtained by immunofluorescent tetraspanin staining, an experimental approach allowing the discrimination of EVs from other blood components showing similar density and size such as lipoproteins.

Figure 12 schematically summarizes EVs detection on the chip: each dot represents an individual EV, while the color indicates the presence of specific tetraspanin markers, based on antibody binding. Briefly, in the first step of the experiment EVs were captured on the chip through antibody binding, then the captured EVs were stained with a combination of fluorescent antibodies against CD63, CD81, and CD9, allowing the visualization of surface markers positivity.

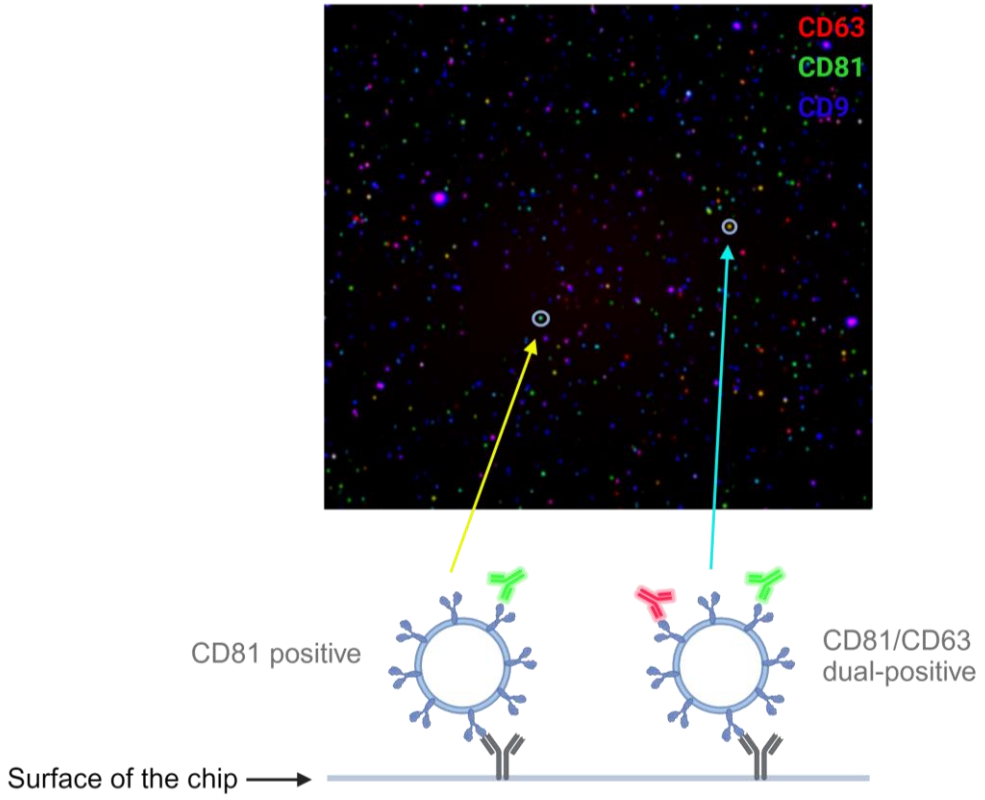

***Figure 12**. Representative image showing EVs detection mechanism. Capture antibodies are blocked on chip surface allowing individual EV capture. After EVs capture, a mix of fluorescently labeled antibodies is added allowing surface marker positivity detection. Each dot corresponds to an individual EV, while different colors indicate marker positivity (blue = CD9 positive EVs, green = CD81 positive EVs, red = CD63 positive EVs, merge = multiple positivity). The yellow arrow indicates a representative CD81 positive EV, while the cyan arrow indicates a representative CD81/CD63 dual positive EV.*

Table 1 summarizes the estimated concentration of EVs positive for each specific biomarker (CD63, CD81, and CD9). Immunofluorescent tetraspanin staining highlighted different surface biomarkers prevalence in the two populations, with a strong prevalence of CD63 and CD9 expression in EVs derived from the healthy volunteer sample compared to those derived from the patient sample.

***Table 1**. Surface biomarker positive extracellular vesicle concentration.*

	Biomarkers positive EVs concentration (particles/ml)		
	CD63	**CD81**	**CD9**
Healthy volunteer	$3.13 \times 10^9 \pm 1.79 \times 10^8$	$3.9 \times 10^8 \pm 1.9 \times 10^7$	$> 1 \times 10^{10}$
Patient	$6.82 \times 10^8 \pm 4.6 \times 10^7$	$1.47 \times 10^8 \pm 2.3 \times 10^7$	$1.67 \times 10^9 \pm 2.3 \times 10^8$

4.5 Preliminary evaluation of extracellular vesicles biological activity

Biological activity of the obtained EVs was evaluated in an *in vitro* endothelial cell model in terms of IL-6 expression and gelatinolytic activity.

EVs ability to induce IL-6 expression by endothelial cells after 24 h stimulation was evaluated by means of ELISA assay. As shown in figure 13, HUVEC stimulation with EVs derived from healthy volunteers or patients did not result in a significant difference in IL-6 expression.

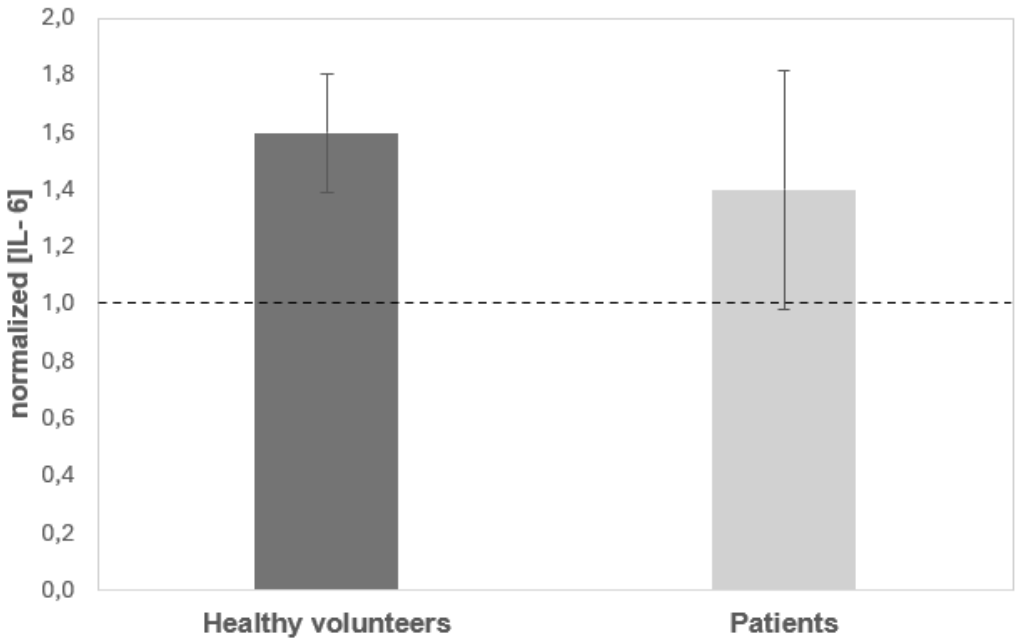

***Figure 13**. Quantification of IL-6 expression by HUVEC stimulated with EVs derived from healthy volunteers and patients. Data were normalized on control condition (cells without stimulation) cytokine expression.*

Conditioned medium from cells stimulated with EVs was also used to evaluate their ability in promoting cellular gelatinolytic activity. As shown in figure 14a, both types of EVs induced a comparable cellular release of active MMP2, while they were not able to induce MMP9 release. As shown in figure 14 section b and c, it is worth nothing that all the observed MMP2 activity derived from cell stimulation, as the corresponding gelatinolytic activity was not present in the media used for cellular stimulation.

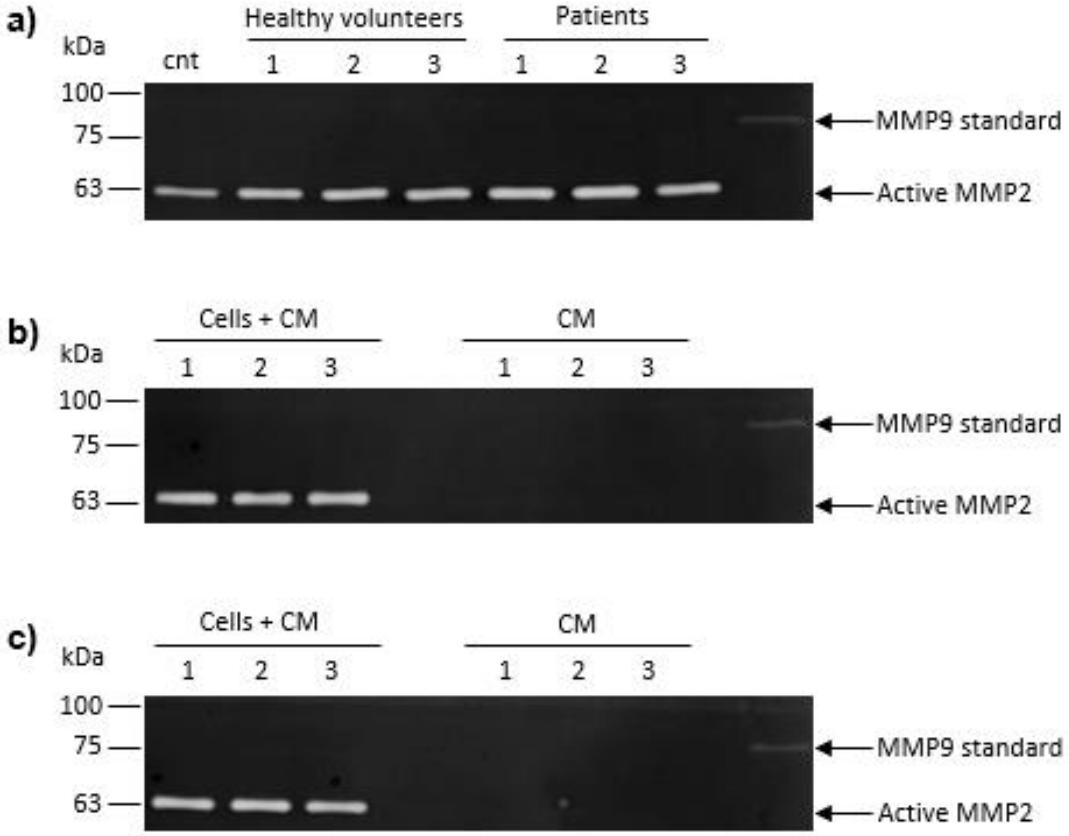

***Figure 14**. Representative zymograms of conditioned media derived from HUVEC stimulation with EVs isolated from healthy volunteers and patients. cnt = negative control (F12K without FBS), CM = conditioned medium used for cell stimulation (F12K without FBS + 2 × 10^9 EVs).*

DISCUSSION

Extracellular vesicles are membrane-enclosed particles released by cells into the extracellular compartment. Although evidence of their existence has been documented for over 80 years, it is only in recent decades that the mechanism involved in their generation [22], the signaling pathways underlying their biological functions [42] as well as their biomedical applications [43] gained scientific interest. In recent years, the number of available publications focused on EVs has increased exponentially, allowing a great improvement of the existing knowledge related to EVs biology. To date, it is known that EVs are involved in several biological functions as well as in the development and progression of various diseases through their key role in mediating intercellular communications [44]. However, although there are many areas of consensus concerning the mechanisms underlying EVs-mediated cell-cell communications, several challenges and shadowed areas remain, thus fostering new studies to unveil in detail EVs biological role.

The protocol that has been set up in this experimental thesis, based on sequential centrifugations, allowed the isolation of small extracellular particles from venous blood samples. In order to confirm that the isolated particles were EVs, the first part of the study has been dedicated to their characterization.

After isolation, EVs have been analyzed using NTA technology: data obtained with this approach supported the successful isolation of a relevant number of particles with a size range that was consistent with the available literature on EVs. In order to further characterize the isolated particles, representative samples were observed with a transmission electron microscope. TEM analysis not only confirmed the NTA estimation of the obtained particles size range, allowing their clear distinction from lipoproteins, but also provided detailed morphological insights. As a matter of fact, microscopical observation revealed the presence of two primary morphologies: vesicles with well-defined membranes and granular inner material as well as cup-shaped ones [45], another result in line with the available literature on EVs, thus further supporting the effectiveness of the proposed protocol in successfully recover circulating extracellular vesicles.

Furthermore, it is worth noting that the observed heterogeneity in morphology and size could derive from the intrinsic complexity of the source tissue. Despite being liquid, the blood is a connective tissue, composed of different cell types, each of which could contribute to EV generation, as well as the endothelium lining the vessel lumen.

After a preliminary physical and morphological characterization, the obtained particles were evaluated also in terms of specific biomarkers [37,38,39,50]. Gelatinases are known to be a specific class of enzymes associated with EVs [37,38,39]: zymographic analysis of pure samples highlighted a comparable MMP2 activity in both the evaluated populations associated to a probable higher presence of pro-MMP9 in patient-derived samples. The observed increase of the MMP9 zymogen in pathological samples could be related to the well-recognized involvement of MMPs and especially MMP9 in cardiovascular disease progression [46,47,48,49]. Finally, in order to validate the assumption that the isolated particles mainly represented the circulating EV fraction, a high-throughput immunofluorescent staining analysis has been performed. Considering that CD63, CD9 and CD81 tetraspanins are well established surface markers for EVs [50], their immunofluorescent analysis confirmed that the isolated particles obtained with the proposed protocol were essentially EVs.

After confirming EVs successful isolation, considering the critical role of these mediators in intercellular communications, the second part of this experimental study was dedicated to a preliminary investigation of their biological activities in an *in vitro* endothelial model. Since literature evidence supports EVs involvement in cardiovascular diseases, onset and progression [28], the obtained particles were assessed for their ability to induce a pro-inflammatory response by endothelial cells. Considering that IL-6 is one of the most common pro-inflammatory cytokines released in response to tissue injuries [51], its release by HUVEC stimulated for 24 h with EVs derived from healthy volunteers and patients affected by acute coronary events has been evaluated. The obtained results highlighted non-significant differences in IL-6 expression, a result that could be explained by the different pathological condition affecting the studied patients as well as by the selected time point for cytokine response evaluation.

The biological activity of the isolated EVs has been also evaluated in terms of gelatinolytic activity: although HUVEC stimulation with extracellular vesicles derived from both healthy volunteers and patients for 24 h induced a comparable MMP2 secretion, it was not able to induce MMP9 secretion. Also in this case, the observed lack of MMP9 expression could be due to the time point selected for gelatinolytic activity assessment.

The obtained results support the effectiveness of the proposed protocol in allowing a successful isolation of the circulating extracellular vesicle fraction. Nevertheless, the preliminary *in vitro* evaluation of the EVs' biological activity highlights the need to further improve the selected protocol.

In particular, the low numerosity of the analyzed samples could be the main responsible of the lack of difference both in physical parameters and biological activity observed in EVs derived from healthy volunteers and patients, as inter-individual variability (i.e., age, sex, diet, as well as medications and comorbidities in patients) strongly affected the overall results.

In conclusion, the results obtained in this experimental thesis are promising in terms of methodological approach but need to be strengthen in terms of EVs biological activity evaluation. In particular, the evaluation of released cytokine profile and the immunomodulatory potential of the isolated extracellular vesicles as well as studies focused on their effects on endothelial glycocalyx composition would be of great interest in expanding the current knowledge on EVs involvement in cardiovascular diseases.

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