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REVIEW

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Diagnostic and prognostic roles of endothelial- and platelet-derived extracellular vesicles in cardiovascular diseases

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Abstract

Extracellular vesicles (EVs) are membrane-bound structures released by all cell types. They play a critical role in intercellular communication by transferring their cargo, comprising proteins, lipids, metabolites, RNAs, miRNAs, and DNA fragments, to recipient cells. This transfer influences gene expression, signaling pathways, and cellular behavior. Due to their ability to alter the physiology of recipient cells, EVs hold significant therapeutic potential. Additionally, EVs are implicated in various physiological and pathological processes, including immune regulation, cancer progression, and cardiovascular diseases. EVs have been detected in many biological fluids, such as peripheral blood, saliva, urine, cerebrospinal fluid, and breast milk. The cargo of EVs dynamically reflects the physiological and pathological state of their parent cells, making them promising candidates for liquid biopsies in various clinical conditions. Specifically, different EV subtypes in cardiovascular diseases have been studied, with both endothelial and platelet-derived EVs playing significant roles in cardiovascular pathologies. This review focuses on the diagnostic and prognostic potential of endothelial and platelet-derived EVs in cardiovascular diseases, highlighting the role of EV subpopulations.

Keywords Extracellular vesicles, Endothelial-derived extracellular vesicles, Platelet-derived extracellular vesicles, Cardiovascular diseases

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Introduction

Extracellular vesicles (EVs) recently garnered significant interest as natural systemic signaling mediators in intercellular communication [1]. They play crucial roles in both physiological and pathophysiological conditions, such as pregnancy, cancer, sepsis, thrombosis, and tissue regeneration [2, 3]. Due to their ability to cross biological barriers and their presence in biological fluids [1, 4], the analysis of EVs has been proposed as potential biomarkers in many clinical conditions [5–9]. Furthermore, their inherent ability to carry proteins, lipids, metabolites, DNA fragments, mRNA, mi (micro)RNA, and other types of RNAs [10] endows them with remarkable therapeutic potential, being able to influence the physiology of the recipient cells in terms of epigenetic and post-transcriptional modulation [11]. In this review, we aim to investigate the role of endothelial and platelet-derived EVs in cardiovascular diseases.

EV subpopulations

According to the International Society for Extracellular Vesicles (ISEV) guidelines [12, 13], the term "extracellular vesicle" refers to particles naturally released from all types of cells, surrounded by a lipid bilayer and incapable of replication due to the lack of a functional nucleus[12]. EVs are traditionally classified into three main subtypes based on their biogenesis and size: exosomes, microvesicles, and apoptotic bodies [14] (Fig. 1).

Exosomes typically range in size from 30 to 150 nm and are surrounded by a lipid bilayer membrane. They contain a variety of biomolecules, including proteins, lipids, and nucleic acids, which reflect their cellular origin and specific cargo [15]. They are produced intracellularly through the invagination of endosomal membranes, leading to the formation of multivesicular bodies (MVBs) containing intraluminal vesicles (ILVs). The MVBs can either fuse with lysosomes for degradation or the plasma membrane to release ILVs as exosomes into the extracellular space. Exosome biogenesis can be mediated by the endosomal sorting complex required for transport

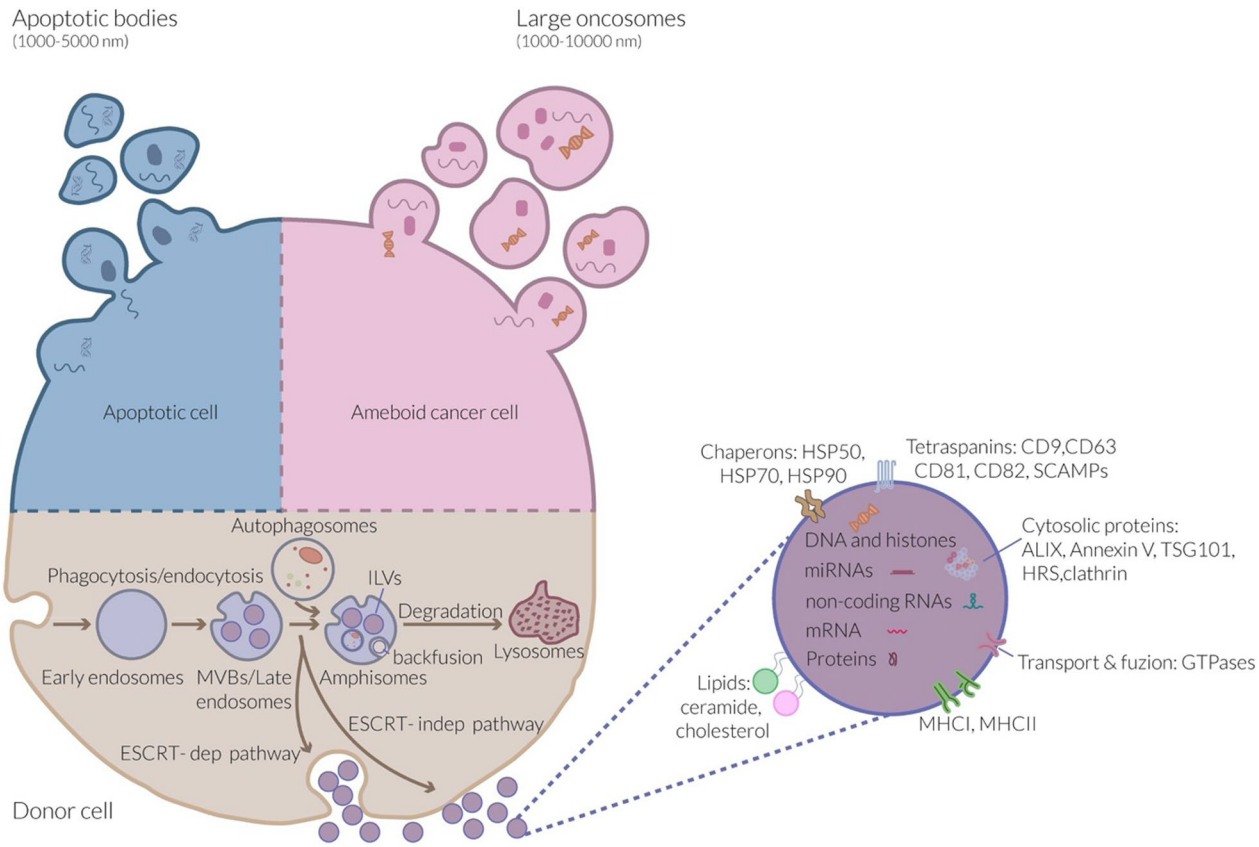


Fig. 1 Extracellular vesicles (EVs) biogenesis. Mechanisms of extracellular vesicle release and EV markers. *ALIX* ALG-2-interacting protein X, *CD* cluster of differentiation, *ESCRT* endosomal sorting complex required for transport, *HRS* hepatocyte growth factor-regulated tyrosine kinase substrate, *HSP* heat shock protein, *ILVs* intraluminal vesicles, *MHC* major histocompatibility complex, *MVB* multivesicular bodies, *sEVs* soluble extracellular vesicles, *TSG101* tumor susceptibility gene 101 protein

(ESCRT) machinery, which is responsible for sorting and loading cargo into ILVs. ESCRT-independent mechanisms involving tetraspanins and ceramides can also contribute to exosome formation [9].

Microvesicles, also known as microparticles, typically range from 100 to 1000 nm and are released by budding directly from the plasma membranes [16]. It has been demonstrated that curcumin stimulates microvesicle release by increasing ceramide synthesis, which enhances microvesicle secretion while reducing intracellular lipid concentrations [17]. Photobiomodulation therapy using laser light has been demonstrated to increase microvesicle release from human keratinocytes via the PI3 kinase-dependent pathway [18]. Additionally, the activation of the P2X7 receptor in glial cells can stimulate microvesicle release, a process involving the ESCRT machinery, particularly the ESCRT-III complex and vacuolar protein sorting 4 (VPS4) [19, 20].

Apoptotic bodies represent the third class of EVs. They have a diameter ranging from 50 to 5000 nm and are released through the blebbing of plasma membranes during apoptosis. Apoptotic bodies express apoptosis markers such as Annexin V and Histone H3 [21, 22].

Several other EV subpopulations have been described, including oncosomes and large oncosomes (LOs), which are particularly relevant in cancer biology [23]. Oncosomes are EVs of 100–400 nm in size, released by tumor cells through blebbing. LOs are significantly larger than oncosomes, ranging from 1 to 10 μm, and are produced by amoeboid tumor cells through specific oncogenic signaling pathways, such as epidermal growth factor receptor (EGFR) and AKT1 [24]. The release of oncosomes and LOs is induced by stimuli that increase intracellular calcium and cytoskeleton remodeling. The shedding process involves the silencing of diaphanous related formin 3 (DIAPH3) and the overexpression of oncoproteins like Caveolin-1 (CAV-1) and heparin-binding epidermal growth factor (HB-EGF) [18]. Both oncosomes and LOs contain oncogenic macromolecules, including metalloproteinases, RNA, CAV-1, and the GTPase, ADP-ribosylation factor 6 (ARF6). These EVs are biologically active and influence tumor cells, endothelial cells, and fibroblasts, promoting tumor progression and metastasis [25]. LOs have been identified in human tumor tissues and the circulation of cancer patients. Their abundance correlates with tumor progression. For these reasons, they have been proposed as potential biomarkers for cancer [26, 27].

With the advent of newer technologies, several additional EV subpopulations with distinct characteristics and functions have been discovered [5]. Among them, exomeres, EVs, and supermeres have been described. Exomeres, isolated using asymmetric-flow field-flow fraction, are non-membranous nanovesicles with a size ≤ 50 nm [28]. They support cell proliferation and promote the production of other exomeres [29]. Supermeres, discovered using optical trapping techniques, are approximately 25 nm in size [30] and exhibit significantly higher in vivo uptake than small EVs and exomeres [31]. Supermeres are highly enriched with cargo involved in various pathological processes, including cancer-glycolytic enzymes, transforming growth factor-β-induced (TGFBI), miR-1246, (MET), glypican 1 (GPC1), argonaute-2 (AGO2)-Alzheimer's disease - amyloid precursor protein (APP) - and cardiovascular diseases - angiotensin converting enzyme 2 (ACE2), angiotensin converting enzyme ACE), proprotein convertase subtilisin/kexin type 9 (PCSK9) [31].

Identifying new EV subpopulations highlights the complexity and diversity of EV subsets, each likely playing unique functional roles. Beyond the classification mentioned above, EV subpopulations share overlapping properties in size and markers.

While certain markers have been proposed as specific to particular EV subtypes (e.g., CD63, CD81, and CD9, tetraspanins abundantly present in exosomes, or Alix and Tsg101, associated with endosome biogenesis), these markers have been found in more than one EV subpopulation [8, 32]. Therefore, further research and efforts to standardize EV isolation protocols are essential for advancing the precise subtyping of EVs [33, 34].

Size alone cannot be used to differentiate between EV subtypes. Due to overlapping sizes and the lack of specific markers, distinguishing between different EV subtypes remains challenging. As a result, the ISEV has simplified the classification of EVs into two main size-based subtypes: small (< 200 nm) and medium-large (> 200 nm) EVs [12].

EVs interact with recipient cells through various mechanisms. They can bind to the surface of target cells and be internalized through fusion with the cellular bilayer or via phagocytosis, macropinocytosis, lipid raft-mediated endocytosis, clathrin-mediated endocytosis, or caveolin-mediated endocytosis [35].

EVs exert their biological effects by releasing their bioactive molecular cargo, composed of proteins, DNA, mRNA, miRNA, small nucleolar RNA, and other non-coding RNAs. These molecules activate different target cell signaling pathways, altering gene expression through translation and post-translational modifications of recipient cell RNA [35, 36].

EV characterization

The nature of EV preparations should be determined through both EV size analysis and the examination of EV surface markers, such as tetraspanins (CD9, CD63,

CD81), cytosolic proteins (e.g., TSG101, ALIX), and the absence of non-EV markers (e.g., cytochrome C) [37]. If the nature of the EVs cannot be confirmed, alternative terms such as extracellular particles (EP) may be more appropriate [12].

Several methods are commonly used to analyze EV size. Among these, Nanoparticle Tracking Analysis (NTA) measures the size distribution and concentration of EVs in liquid suspensions. It is effective for particles ranging from 50 to 300 nm but does not distinguish non-EV structures, such as lipoproteins or protein aggregates [38]. Dynamic Light Scattering (DLS) measures the size of EVs by analyzing light scattering fluctuations induced by their Brownian motion. It is suitable for measuring monodisperse populations of EVs. It provides a single peak for each run. However, it may overestimate diameters due to low concentrations of outliers or particle clustering [39]. Transmission Electron Microscopy (TEM) is used to analyze EV size, morphology, and purity. TEM provides high-resolution images that allow for the differentiation of EV subpopulations with different sizes. Using vitrification and low electron dose imaging conditions, Cryo-TEM retains the native state of EVs, enabling accurate size measurements [40, 41]. Immunolabeling Electron Microscopy (IEM) is used to detect specific proteins on the EV surface through immunogold labeling [42], differently from Atomic Force Microscopy (AFM), which provides high-resolution topographical images and measures the radius of EVs [43].

The most frequently used markers for EV characterization include CD9, CD63, CD81, Flotillin-1 Alix, and TSG101 [8]. These markers are consistently identified across multiple studies and are considered standard for ascertaining the EV nature of analyzed particle samples [44, 45]. Western blotting remains the most used method for detecting EV markers. However, it has limitations, such as being semi-quantitative and requiring a high amount of sample input [46], while mass spectrometry analysis has been used to identify the EV protein cargo [47, 48]. More recently, fluorescent NTA (FNTA) and Nano-Flow Cytometry (nFCM) were developed for high-throughput, multiparametric analyses of EVs, including EV size, concentration, charge, and composition. These techniques use fluorescent reagents to target different EV components, allowing for subtyping and studying EVs as potential biomarkers [49]. Single-Particle Interferometric Reflectance Imaging Sensing (SP-IRIS) combines microfluidics with immunodetection and interferometric imaging to detect unlabeled EVs based on their expression of marker proteins. This method provides detailed information on EV size and protein content [38]. Lab-on-a-chip devices enable rapid purification and multiparametric characterization of EVs without labeling. They use advanced imaging techniques, such as direct stochastical reconstruction microscopy (dSTORM), for high-resolution analyses [50].

Interestingly, flow cytometry showed great potential for studying EV concentration and composition. It also allows for an indirect measurement of EV particle size [7]. Advanced flow cytometers with small particle detectors analyze even small EVs at a single event level. Using membrane-specific probes for staining EV membranes has extended the sensitivity of classical flow cytometers, enabling the detection of EVs as small as 70 nm [51]. A recently optimized protocol for EV identification and enumeration in fresh samples, based on the lipophilic cationic dye staining of EVs, has demonstrated high repeatability [52]. This method has proven to be highly specific, opening new possibilities for studying the potential role of EVs as biomarkers for various diseases, including cardiovascular diseases and cancer [8, 53, 54]. This technique can also be combined with fluorescent labeling to analyze specific EV subpopulations and their cellular origins [55]. Overall, flow cytometry demonstrates the ability to analyze particles at the single-event level and it is a potent tool for distinguishing EVs from non-EV circulating particles [14]. Recent advances in flow cytometry for EV detection have made this technique reproducible, robust, scalable, and quick, being therefore considered particularly suitable for clinical translation of EVs [56, 57].

EV isolation methods

Several methods have been used to isolate EVs, each with advantages and disadvantages. Among these, ultracentrifugation is considered the gold standard method for isolating EVs. This method relies on increasing centrifugal force (10,000–20,000 xg for large EVs, 100,000–120,000 xg for small EVs). While it is simple and low-cost, it has limitations in terms of separation yield and the presence of contaminants in resulting EV preparations, such as non-vesicular protein complexes and apolipoprotein particles [58]. Size-exclusion chromatography (SEC) has also been used for blood EV isolation. It is scalable, preserves EV integrity, and removes soluble proteins and small molecules, but it does not remove lipoparticles [59]. SEC isolates EVs with moderate to high purity and yield but requires additional concentration steps [58, 60, 61]. Polymer-based precipitation is a quick and convenient method that provides a high yield of EVs. However, it results in low purity due to the co-isolation of soluble non-EV material, and the precipitation reagent needs to be removed [58]. Immunoaffinity capture, based on surface marker specificity, is a rapid technique that isolates high-purity EVs without requiring specialized equipment, even if it has a low throughput and yield [62].

Additionally, EVs have been isolated using fluorescence-activated cell sorting (FACS), a powerful technique capable of identifying and isolating EVs at the single-particle level. This method enables EV identification, enumeration, and isolation from any fresh matrix, achieving high repeatability in EV counts while avoiding artifacts generated by other enrichment procedures. It is highly specific, efficiently excluding contaminants such as high-density lipoprotein (HDL), low-density lipoprotein (LDL), and chylomicron proteins. Moreover, due to its high purification efficiency, it can detect EVs in any biological fluid, thereby opening new possibilities for using EVs as biomarkers for several diseases, including cancer, infectious, neurological, and cardiovascular diseases[63]. However, this technique allows for the isolation of a limited amount of EVs in terms of yield, and different instruments may have incomparable sensitivity.

Ultrafiltration is another effective technique for isolating EVs. It utilizes membrane filters with specific pore sizes, concentrating particles based on their molecular weight. This method benefits the processing of large sample volumes [64]. EV separation by ultrafiltration is simple, fast, and does not require expensive equipment. However, it may lead to EV deformation and breakage, potentially altering results. To mitigate this, cellular debris are removed through centrifugation, and columns with a 50–100 kDa molecular weight cut-off are used to eliminate contaminants. This method allows for a high EV recovery percentage with a mean size of 100 nm [65].

EVs can also be separated by density gradient ultracentrifugation, which combines ultracentrifugation with a sucrose density gradient to separate EVs from particles of different densities. It is widely recognized for providing superior quality EV preparations for reliable functional and structural analyses [66]. However, isolating EVs using density gradient methods can be challenging due to the overlapping densities of EVs and other particles like lipoproteins. Prolonged centrifugation times and optimized gradient compositions can improve separation efficiency [67]. Typical protocols use a discontinuous gradient with varying concentrations of iodixanol (e.g., 5%, 10%, 20%, 40%) and layering the EV-containing sample on the top. The gradient is then centrifuged at high speeds to separate EVs based on their buoyant density. The iso-osmotic properties of iodixanol preserve the integrity of EVs and prevent contamination from protein aggregates [68]. Immunoaffinity techniques, such as using antibody-coated magnetic beads or immunoaffinity chips, are highly effective for isolating specific subpopulations of EVs based on the binding of EV markers. This approach is more efficient than ultracentrifugation and gradient density separation methods for isolating EVs from different sources [69]. Immunoaffinity-based isolation methods have demonstrated higher specificity and efficiency in isolating tumor-derived exosomes than conventional methods, significantly improving the detection of clinically relevant biomarkers in diseases like prostate cancer [70]. Polymer-based EV precipitation techniques use polyethylene glycol to alter the solubility and dispersibility of EVs, allowing for their isolation through low-speed centrifugation or filtration [71].

EVs in biofluids and their implication in cardiovascular diseases

EVs have been identified in many biofluids, such as blood, urine, cerebrospinal fluid, saliva, and breast milk [47]. Their cargo, primarily consisting of proteins, nucleic acids, and metabolites, dynamically reflects the parental cells' physiological and pathological state [72]. For these reasons, EVs have been pointed out as promising candidates for liquid biopsy purposes in many clinical conditions, including cardiovascular diseases [9, 54].

EVs in peripheral blood

EVs are abundantly present in peripheral blood, playing a key role in intercellular communication. They are secreted by blood cells, including platelets and endothelial cells [47]. Platelet-derived EVs (pEVs) are the most abundant in human blood, accounting for 50–90% of all circulating large EVs in healthy individuals [73]. Blood EVs are involved in various physiological and pathological processes, such as immune regulation, coagulation, and intercellular communication, by transferring proteins, lipids, and RNA to target cells [74], as well as promoting the activation of immune cells [74, 75]. Due to their abundance and distinctive characteristics, EVs are promising biomarkers in many pathophysiological conditions. They dynamically reflect clinical changes and are being explored for potential applications in drug delivery and gene therapy[76]. EVs encapsulated with angiostatin have been used to evaluate endothelial damage during chemotherapy [77].

Additionally, small EVs containing proteins like hERG1 and Hsp47 have been linked to myocardial ischemia and heart failure, suggesting their potential as biomarkers for cardiovascular diseases [78]. pEVs have also been associated with major adverse cardiovascular events (MACE) and major adverse limb events (MALE) in patients undergoing femoral endarterectomy. EVs containing Serpin G1 and CD14 are significant indicators of adverse outcomes [79]. Elevated levels of EVs have been linked to conditions such as hypertension, atherosclerosis, and vascular organ damage, suggesting their role as a biomarker of cardiovascular disease progression [80]. pEVs are associated with blood pressure fluctuations and vascular damage, indicating their role as integrative biomarkers for

vascular health. This highlights the relevance of EVs in monitoring and managing hypertension and related vascular conditions [80].

Furthermore, blood EVs promote angiogenesis, cell repair, and the maintenance of cell homeostasis, making them potential tools in regenerative therapies for cardiovascular diseases [81, 82]. It is important to consider that pre-analytical factors, such as blood collection methods, anticoagulants, and sample handling, can significantly affect the quality and quantity of isolated EVs. The optimization and use of appropriate protocols are essential to minimize ex vivo vesiculation and ensure accurate analysis [83]. The isolation and characterization of EVs from peripheral blood samples are complex due to the presence of other particles with similar size, i.e., very low-density lipoprotein (VLDL) [84].

Urinary EVs

Urinary EVs, traditionally studied as biomarkers for genitourinary diseases, are also a rich source of biomarkers for cardiovascular diseases. These EVs are linked to renal cellular senescence and injury in hypertensive patients. Elevated levels of specific markers in urinary EVs, such as p16, have been associated with hypertension and ischemic kidney injury [85]. miRNAs contained in urinary EVs play a crucial role in cardiovascular biology. They influence the development of cardiovascular diseases by altering protein expression and the phenotypes of recipient cells, thus contributing to conditions such as atherogenesis, heart failure, and diabetes [86]. Moreover, EVs derived from human urine-derived stem cells (USCs) show promise in promoting ischemia repair. In a mouse model of hind-limb ischemia, USCs-EVs have been shown to significantly improve perfusion, angiogenesis, and muscle regeneration, facilitating in vitro proliferation of human microvascular endothelial cells (HMEC-1) and mouse myoblast cells (C2 C12) in a dose-dependent manner. This highlights the potential of USCs-EVs in therapeutic applications for ischemic cardiovascular diseases [87]. Urinary EVs have been isolated using various methods, including differential centrifugation and SEC, which are particularly effective for separating EVs from urinary cellular debris [88].

EVs in saliva

Salivary EVs, which are known as biomarkers in head and neck diseases [89], also play a significant role in the pathogenesis and progression of cardiovascular diseases. These EVs are involved in vascular integrity, atherogenesis, and heart failure processes by transferring miRNAs and other molecules that alter protein expression and phenotypes of recipient cells [86]. Their molecular content, including miRNAs and proteins, heavily depends on the tissue or cell type they originate, making them valuable tools for liquid biopsy biomarkers in various cardiovascular conditions [90, 91].

EVs in breast milk

Human breast milk EVs have been shown to mitigate endothelial dysfunction, a key factor in cardiovascular diseases. Milk-derived EVs exhibit anti-inflammatory properties and reduce oxidative stress, which plays a crucial role in maintaining vascular health and preventing the progression of cardiovascular disease [92]. These properties contribute to inflammation reduction, a common underlying factor in many cardiovascular diseases [92, 93]. Growth factors carried by milk EVs, such as vascular endothelial growth factor (VEGF) and epidermal growth factor (EGF), play a crucial role in promoting angiogenesis and improving blood flow in ischemic tissues [92]. Specific miRNAs delivered via small EVs from milk, such as miR-146a, have demonstrated cardioprotective effects by inhibiting the nuclear factor kappa B (NF-κB) signaling pathway, reducing myocardial tissue apoptosis, and enhancing cardiac function following myocardial ischemia–reperfusion injury [94]. EVs are typically isolated from breast milk using ultracentrifugation, SEC, and precipitation-based methods suitable for small sample volumes [95].

EV subtypes involved in cardiovascular diseases

EVs are released by nearly all cell types, but those related to the cardiovascular system primarily come from platelets, erythrocytes, leukocytes, and endothelial cells. Platelet and endothelial-derived EVs have been extensively studied in the cardiovascular system and have been shown to play key roles in hemostasis, inflammation, and vascular integrity [96].

In particular, pEVs are involved in maintaining endothelial and coagulation homeostasis. They support endothelial cell barrier function under normal conditions and in response to inflammation [97]. pEVs have also been shown to promote vascular neoangiogenesis following chronic ischemia, balancing procoagulant and anticoagulant activity in physiological conditions. They induce proinflammatory and procoagulant polarization in diabetic and obese patients, events that may represent a substrate for cardiovascular complications [98]. Endothelial-derived EVs (eEVS) regulate endothelial cell homeostasis by protecting endothelial cells through their antioxidant cargo in an autocrine manner while also inducing endothelial inflammation, activation, neoangiogenesis, and a prothrombotic state [99]. Additionally, eEVs promote vasoconstriction and vascular remodeling in pathological states commonly observed in patients with cardiovascular diseases [100].

Platelet-derived EVs

pEVs are released by activated platelets triggered by the proteinase-activated receptor (PAR) agonist, thrombin receptor agonist peptide SFLLRN (TRAP), or α-thrombin [101]. They are usually isolated by differential centrifugation. According to the analysis by TEM, two types of particles are released: medium-large pEVs, which are described as particles with a diameter of 100 to 1000 nm, expressing proteins like phosphatidylserine (binding Annexin-V), αIIB-β3 and β1, GP1bα, and P-selectin and small pEV, defined as 40–100 nm EVs [101].

It is crucial to properly isolate pEVs from platelets and distinguish them from lipoproteins [102] and megakaryocyte–derived EVs. Both pEVs and platelets are CD41 positive, but pEVs, as their parental cells, express P-selectin (CD62P) [103].

The concentration of pEVs has been shown to increase in the plasma of patients with chronic inflammation and platelet activation, conditions such as cardiovascular diseases, infections, neurological diseases, cancer, and autoimmune diseases (rheumatoid arthritis and systemic lupus erythematosus), with changes in pEV content occurring in different pathological settings [104–106]. The main cargos and roles of pEVs are summarized in Table 1.

Role of pEVs in coagulation and thrombosis

Multiple studies underline the different roles of pEVs in physiological coagulation processes. pEVs demonstrate both procoagulant [107, 108] and anticoagulant [103] functions. A recent study showed that when pEVs concentrations are increased in the blood of healthy donors, they promote fibrinolysis by supporting plasmin generation [103]. pEVs are key players in thrombosis, and their procoagulant activity in blood is 50–100 times higher than that of activated platelets [109]. These EVs mediate several biological processes since they contain platelet membrane proteins and bioactive lipids, such as integrin glycoprotein (GP) IIb/IIIa (CD41/61), GPIX (CD42a), GPIb (CD42b), P-selectin (CD62P), P-selectin glycoprotein ligand 1 (PSGL-1, CD162) and CD40 ligand (CD154) [110]. The physiological basis for these differences in activity may be related to the presence of different pEV subtypes. Genetic studies focused on platelets provide insight into the physiological role of pEVs in coagulation. In Scott syndrome, a rare genetic bleeding disorder, the externalization of phosphatidylserine on activated platelets and pEVs is severely impaired, resulting in a hemorrhagic phenotype [111].

These findings support a procoagulant role for pEVs. However, it remains challenging to determine whether the increased bleeding risk is due to the altered release of procoagulant pEVs or impaired cellular exposure to phosphatidylserine on platelets and other cells.

Role of pEVs in obesity

Understanding the role of EVs in cellular crosstalk in impaired metabolic states could provide new therapeutic targets and biomarkers for obese and diabetic patients. In obese patients, decreased level of pEVs $CD41^{+}$ and increased phosphatidylserine and Factor V expression are observed, features linked to a prothrombotic state. These changes may contribute to the increased cardiovascular and oncological risk in obese patients [112]. The PCSK9 pathway is currently being studied as a new therapeutic target for hypercholesterolemia treatment. In obese adults, PCSK9 may influence the release of EVs from cells involved in atherosclerosis (platelets, endothelium, neutrophils, macrophages) [113]. Since PCSK9 enhances platelet activation [114] and the release of proinflammatory cytokine from macrophages, it may regulate EV release and contribute to induce a proinflammatory state. This could be mediated by the release of interleukin 6 (IL-6) and interleukin 8 (IL-8) induced by endothelial cells,

Table 1 Platelet-derived EVs main cargo and roles

Platelet-derived EVs	
Cargo	**Role**
Caspase 3 and Rho-kinase	Proapoptotic
Tissue factor, phosphatidylserine	Procoagulant, oxidative stress
NADPH oxidase, disulfide isomerase, NOS	Downregulate cardioprotective miR223
miR-1915-3p, miR-4,507, miR-3,656	Reduced in early-stage AMI
miR-122-5p	Correlates with ACS severity
miR-126-3p, miR-199-5p	Inverse correlation with MACE
miR-223, miR-339 and miR-21	Inhibit PDGFRβ (biomarker of atherosclerosis)
CXCL7 and CXCL10	More expressed in T2DM

NAPDH nicotinamide adenine dinucleotide phosphate oxidase, *NOS* nitric oxide synthase, *AMI* acute myocardial infarction, *ACS* acute coronary syndrome, *MACE* major adverse cardiovascular events, *PDGFRβ* Platelet-derived growth factor receptor beta, *T2DM* type 2 diabetes mellitus

activated macrophages, and proinflammatory pEVs [115]. Moreover, IL-8 levels are directly related to an increased cardiovascular risk in healthy individuals [116]. Therefore, one of the therapeutic effects of PCSK9 inhibition could be linked to the decrease of inflammation, atherogenic process, and cardiovascular risk [105] mediated by the modulation of proinflammatory EVs (Fig. 2).

Role of pEVs in diabetes

Platelets are considered one of the most important mediators of inflammation in type 2 diabetes mellitus (T2DM) and are involved in many cellular crosstalk pathways [117]. Compared to euglycemic controls, higher levels of of circulating EVs, pEVs, erythrocytes-, leukocytes-, monocytes-, and endothelial-derived EVs have been found in T2DM patients [118]. These data support the use of EVs as promising biomarkers for early T2DM diagnosis. pEVs released in a diabetic context show high levels of CXCL7 and CXCL10. These pEVs target endothelial cells in the aorta, kidneys, and retina, inducing increased production of reactive oxygen species (ROS) [119] and raising the expression of adhesion molecules, consequently increasing the risk of endothelial injury. This could promote diabetic complications, such as diabetic retinopathy, diabetic nephropathy, and atherosclerosis [120]. In a recent study, Souzu and colleagues showed that cilostazol and sarpogrelate hydrochloride (both antiplatelets drugs) significantly reduced the concentration of circulating pEVs in diabetic patients, exploring a possible link between antiplatelet therapies and the reduction of EV-mediated vascular inflammation in diabetes [121].

Role of pEVs in cardiovascular diseases

Impaired concentrations of pEVs have been associated with an increased cardiovascular risk. High levels of pEV have been observed in patients with cardiovascular risk factors and after cardiovascular events [122]. pEVs inhibit atherothrombosis by preventing oxidized-LDL binding and cholesterol loading into macrophages, affecting the class B scavenger receptor CD36, inhibiting platelet thrombosis, and reducing the expression of CD36 and cholesterol accumulation in cultured murine macrophages [123]. Therefore, pEVs are involved in preventing lipid overload and occurrence of atherosclerosis [124]. Furthermore, pEVs are key players in maintaining endothelial barrier function and stabilizing vasculature [125] (Fig. 3).

Endothelial and platelet-derived EVs increase endothelial permeability by locally inducing apoptosis through the delivery of caspase 3 and Rho-kinase enzymes to target cells [126]. pEVs adhere to the subendothelium and activate endothelial cells, further recruiting activated platelets to the endothelium-damaged areas. pEVs also activate pro-inflammatory cytokines such as IL-1, IL-6, and IL-8, and induce the production of intercellular adhesion molecule 1 (ICAM-1). This effect is possibly related to miRNAs carried by pEVs [127]. Additionally, pEVs increase vascular smooth muscle cell (SMC) proliferation

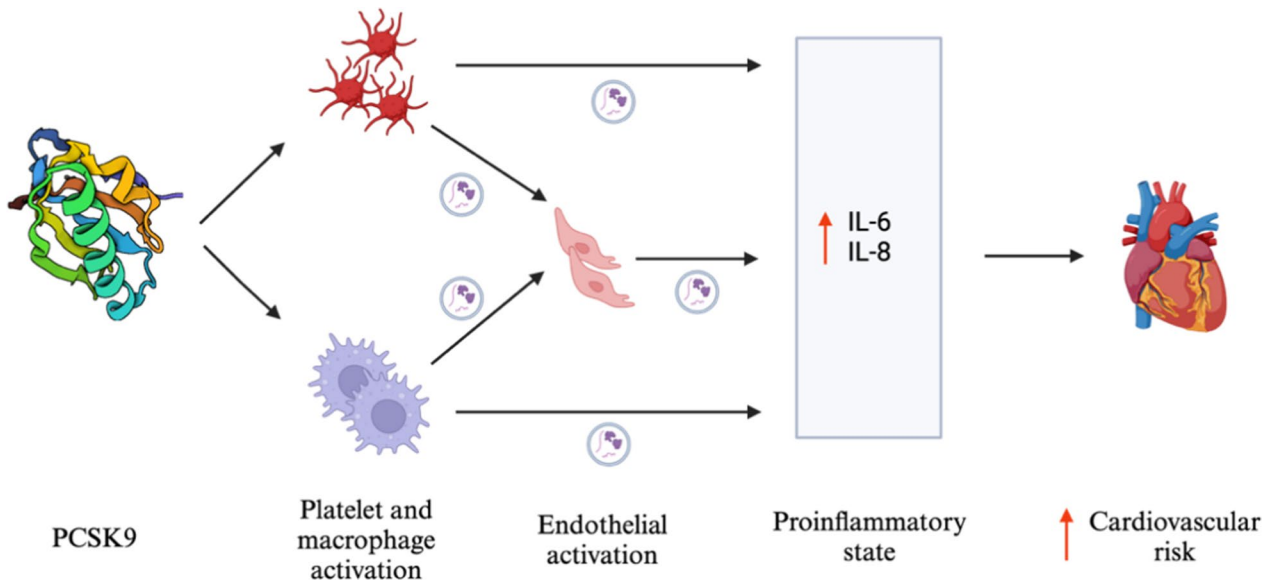
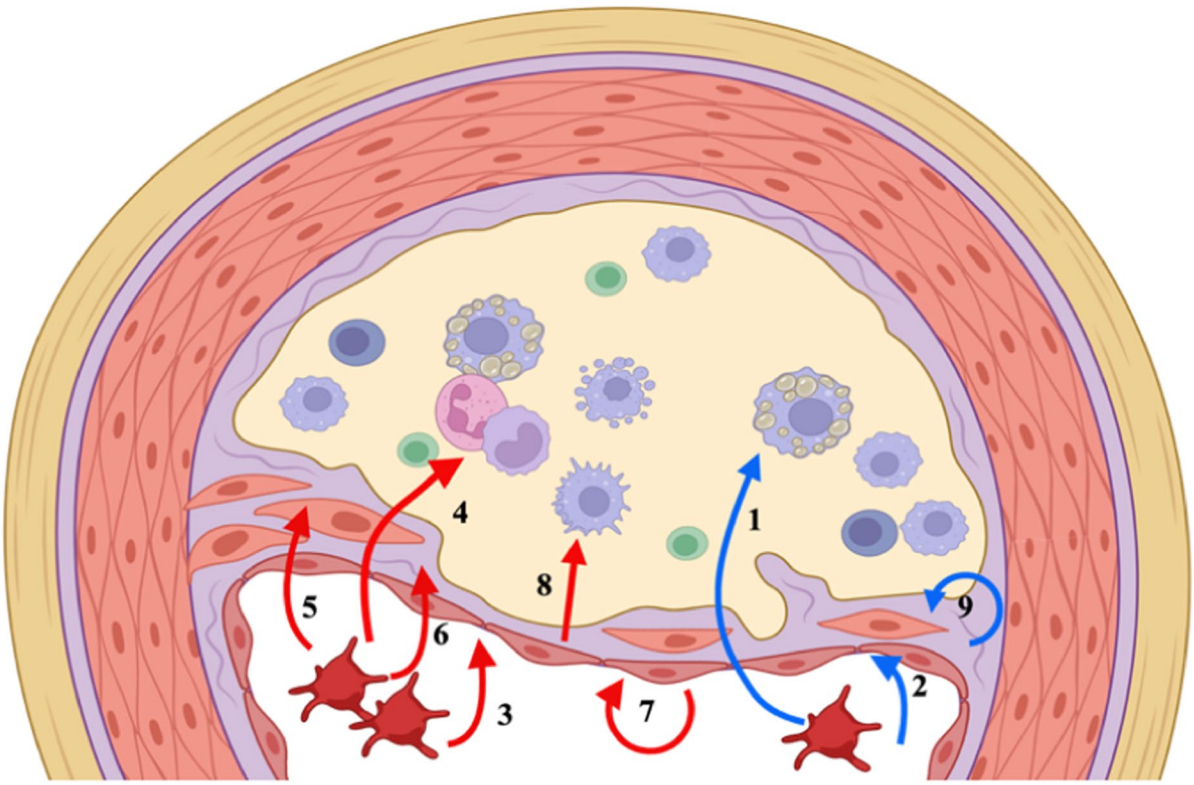


Fig. 2 Impact of PCSK9 on cardiovascular risk via EV release. PCSK9 expression induces a pro-inflammatory state through the activation of platelets, macrophages, and endothelial cells mediated by the release of EVs. *PCSK9* proprotein convertase subtilisin/kexin type 9, *IL* interleukin. Created with Biorender.com



1. Platelet EVs inhibit foam cell formation
2. Platelet EVs stabilize endothelial barrier
3. Platelet EVs activate endothelium and induce ICAM-1
4. Platelet EVs induce IL-1, IL-6, IL-8
5. Platelet EVs stimulate inflammatory vascular remodeling
6. Platelet EVs promote coagulation and platelet adhesion in sub-endothelium
7. Endothelial EVs activate endothelium and increase permeability
8. Endothelial EVs activate macrophages
9. Endothelial EVs reduce vascular inflammation

Fig. 3 Role of platelet and endothelial-derived EVs in arterial inflammation and atherosclerosis. Summary of proinflammatory and anti-inflammatory effects mediated by EVs on vascular, endothelial, and immune cells in atherosclerosis. *ICAM-1* intercellular ddhesion molecule 1, *IL* interleukin, *EVs* extracellular vesicles. Created with Biorender.com

and migration through interactions with CD40 and P-selectin on the tissue. Furthermore, pEVs induce a pro-inflammatory phenotype of SMCs and stimulate vascular remodeling [128].

pEVs also promote coagulation by binding to the subcellular matrix following endothelial injury [129]. Increased levels of pEVs enhance platelet and fibrin adhesion to damaged atherosclerotic vessel walls under high shear stress [130]. High levels of pEVs expressing procoagulant tissue factor (TF) have been observed in hyperinsulinemic patients and in T2DM patients [131]. These findings explain the relationship between pEV levels in blood samples, insulin resistance and cardiovascular risk.

pEV levels in the blood have been proposed as a new biomarker of cardiovascular risk due to their link to vascular damage. In patients with acute coronary syndrome, increased levels of circulating pEVs, containing polygenic immunoglobulin receptor, (pIgR), cystatin C, and complement factor C5a, were observed [132]. It is also known that EVs enhance platelet aggregation in patients who were non-responders to the antiplatelet drug clopidogrel, as well as in diabetic patients. Blocking pEVs formation may reduce platelet hyperreactivity [133]. The inhibition of purinergic receptor 2Y1 (P2Y1) and P2Y12 receptors reduces platelet aggregation and affects the release of distinct subpopulations of pEVs.

Specifically, the use of ticagrelor (a potent P2Y12 receptor inhibitor used in acute coronary syndromes) reduces the release of procoagulant pEVs from activated platelets, potentially contributing to the observed clinical benefits in patients treated with this drug [134].

Moreover, many miRNAs present in EV cargoes have been studied as diagnostic and prognostic biomarkers in stable coronary artery disease and acute myocardial infarction (AMI).

pEVs enriched with NADPH oxidase, disulfide isomerase and nitric oxide synthase impair myocardial anti-inflammatory activity by downregulating miR-223, which plays a cardioprotective role in cardiac tissues [135, 136].

Small pEV miR-1915-3p, miR-4,507, and miR-3,656 were significantly less expressed in AMI compared to stable coronary artery disease patients, suggesting that these miRNAs might be predictive for AMI at an early stage [137]. Ling H. and co-workers showed that the expression of miR-122-5p in small EVs isolated from the serum of patients affected by unstable angina and AMI was significantly higher than that in the control group, and expression levels differed between unstable angina and AMI patients. Additionally, increased levels of miR-122-5p in blood-derived small EVs have been correlated to the severity of coronary stenosis [138]. Patients with low levels of miRNA-126-3p and miRNA-199-5p showed a higher risk of further cardiovascular complications [139]. Recently, it has been shown that EVs derived from activated platelets carrying miR-223, miR-339 and miR-21 were transferred to SMCs, inhibiting the expression of platelet-derived growth factor receptor beta (PDGFRβ), potentially enhancing unfavorable remodeling and atherosclerotic plaque thrombosis in animal models. These results suggest a possible role of EVs as an atherosclerosis thrombosis marker [140].

Remote ischemic preconditioning (RIPC), a brief non-harmful ischemia of myocardial tissue that protects cell viability, decreases circulating levels of pEVs in patients with coronary diseases undergoing conventional antiplatelet therapies. This may consequently induce increased EV clearance/uptake or alter EV release. Clinical variables such as statin use, diabetes, and hypertension may affect the effectiveness of remote ischemic preconditioning [98].

High levels of CD31 +/Annexin V+ pEVs indicate a higher risk for coronary revascularization and cardiovascular death. Their levels increase in patients with impaired coronary artery function and cardiovascular risk factors, and they can be used as an independent factor to predict the occurrence of cardiovascular events in patients with stable coronary artery disease [141]. Moreover, it has been shown that CD3 +/CD45 + and SMA-α + circulating EVs are increased in subjects with high cardiovascular risk [142].

In acute stroke, the levels of eEVs and pEVs correlate with lesion size and clinical prognosis. Consequently, investigating them could provide benefits for stroke diagnosis and prognosis [143, 144].

Recently, a correlation has been demonstrated between patent foramen ovale and migraine with aura. This correlation depends on a prothrombotic state sustained by high levels of pEVs expressing functionally active TF and phosphatidylserine, which can promote thrombin generation, increase oxidative stress through elevated ROS production, and impair the blood oxidized/reduced (GSH/GSSG) glutathione ratio. Following patent foramen ovale closure, 100% migraine remission and a reversion of prothrombotic status were observed [145].

In conclusion, genomic and proteomic analyses of pEVs may provide valuable diagnostic and prognostic information in the early stages of atherosclerosis and offer a new therapeutic target for personalized therapies [124].

Endothelial-derived EVs

eEVs are particles released by endothelial cells under cellular stress conditions, such as endothelial activation or damage [146]. eEVs consist of various subtypes of EVs, including small and medium-large EVs, and express several parental cell-specific markers, such as E-selectin (CD62E) and VE-cadherin (CD144). Other common markers used to identify eEVs include platelet endothelial cell adhesion molecule-1 (CD31), ICAM-1 (CD54), endoglin (CD105), vascular cell adhesion molecule-1 (VCAM-1) (CD106), melanoma cell adhesion molecule (CD146), integrin aV (CD51) and von Willebrand factor (vWF) [147, 148]. Since eEVs specifically express CD62E and CD144, they can be identified using specific antibodies [149]. Additionally, eEVs carry several RNAs, primarily involved in angiogenesis, proliferation, and differentiation (i.e., miR-10b, LET71, miR-126, miR-100, miR-27b, miR-30a, miR-25, miR-221) [150]. It has also been shown that the lipid cargo of IL-1α-stimulated eEVs contains higher levels of phosphatidylserine and phosphatidylethanolamine, which could promote thrombin formation through a TF-dependent mechanism leading to a prothrombotic state [151]. The main cargo and roles of eEVs are summarized in Table 2.

Role of eEVs in coagulation and thrombosis

eEVs play different roles in coagulation [146]. It has been demonstrated that activated protein C (APC)-stimulated human umbilical vein endothelial cells (HUVEC) release eEVs with anticoagulant properties [152]. Furthermore, EVs released by the complement activation exhibit prothrombinase activity, expressing

Table 2 Endothelial-derived EVs main cargo and roles

Endothelial-derived EVs	
Cargo	**Role**
miR-10a, 19a, 23b, 101, 143, and 145	Suppress NF-kB, limit atherosclerotic plaque
miR-126 and miR-181b	Reduce monocyte activation
miR-146a	Reduces cardiac contractile function
$A_{2A}R$	Hyperhomocysteinemia in CAD
miR-126-5p	Reduces endothelial dysfunction, improves cardiac function in HF
miR-1, miR-30, miR-133a, miR-208, miR-499	More sensible than hs-TnI in AMI
miR-499-5p	Superior to hs-cTnT in NSTEMI vs acute HF
miR-208a	Correlates with severity and mortality in AMI
miR-192, miR-194 and miR-34a	Correlate with LVEDD in AMI
miR-423-3p	Reduces cell apoptosis in IRI
miR-214	Prevents calcium overload
miR- 155	Inflammatory macrophage polarization
miR-29b and miR-455	Antifibrotic
miR-712 and miR-205	Enhance endothelial inflammation
HSP70, Arginase-1	Endothelial dysfunction
miR-122 and miR-192	Obesity and insulin resistance
miR-29a	Obesity, correlates with NT-proBNP
miR-410-5p	Cardiac fibrosis
miR-132 and miR-17-5p	Inverse correlation with BMI
miR-423-5p, miR-520c-3p and miR-15a	Morbid obesity and metabolic syndrome
miR-483	Insulin resistance, dyslipidemia, obesity, risk of developing T2DM
miR-21	Reduced in T2DM and metabolic syndrome
IgG	Retinal damage in T2DM
TGF-β1	Apoptosis in renal glomerular cells

$A_{2A}R$ adenosine A2 A receptor, *HSP70* heat shock protein 70, *IgG* immunoglobulin G, *TGF-β1* trasforming growth factor beta 1, *NF-kB* nuclear factor kB, *CAD* coronary artery disease, *HF* heart failure, *AMI* acute myocardial infarction, *NSTEMI* non-ST elevation myocardial infarction, *LVEDD* left ventricle end-diastolic diameter, *IRI* ischemia–reperfusion injury, *NT-proBNP* N-terminal pro-B natriuretic peptide, *BMI* body mass index, *T2DM* type 2 diabetes mellitus

binding sites for factor Va [153]. Furthermore, eEVs released under C-reactive protein (CRP) stimulation may cause vascular injury by impairing tetrahydro-L-biopterin (BH4)-dependent nitric oxide (NO) production [154]. Angiotensin II receptor type 1 antibodies, identified in patients with hypertension, stimulate a dose- and time-dependent release of eEVs, which increases ROS production and reduces NO synthesis in endothelial cells[155]. Hyperglycemia-induced eEVs enhance ROS release via the xanthine/xanthine oxidase system and NADPH oxidase, both of which are essential for the expression of vWF by HUVEC, leading to the formation of platelet strings on their surface [156]. Hypoxia/reoxygenation-induced eEVs exert pro-apoptotic and oxidative effects on cardiomyocytes via the p38 and JNK1/2 signaling pathways, suggesting that reducing eEVs levels could prevent myocardial cell death [157]. eEVs released under plasminogen activator inhibitor-1 (PAI-1) stimulation enhance thrombin generation [158] and induce neutrophil recruitment to the lungs, potentially priming lung injury from other pathogens [159]. eEVs released by ionizing radiation and tumor necrosis factor alpha (TNF-α) stimulation induce a procoagulant status, mediated by TF expression and ROS formation. On the other hand, antioxidative treatments inhibit eEVs activity, thereby reducing thrombogenicity. This evidence supports the use of N-acetylcysteine to prevent late thrombosis in antiproliferative treatments when combined with anticoagulants [160]. eEVs spontaneously released by quiescent endothelial cells exhibit anti-inflammatory activity. Such an activity has been attributed to the transfer of miR-10a from eEVs to monocytes/macrophages, suppressing NF-κB activity, and miR-126 and miR-181b, suppressing monocyte activation [161]. These experimental models highlight the ambivalent role of eEVs in coagulation, supporting the hypothesis that eEV activity depends on the specific trigger causing their release. Additionally, high levels of blood eEVs in endothelial dysfunctions may be linked to the pathophysiology of the increased cardiovascular risk in this context.

Role of eEVs in obesity

EVs can transfer miRNAs and affect organism insulin sensitivity [162]. In particular, miR-192 and miR-122 have been shown to be associated with insulin resistance, and the levels of these miRNAs are increased in obese patients. Furthermore, EV concentrations in blood correlate with increased waist circumference, visceral fat quantity, and increased triglyceride/HDL ratio in humans [163]. It has been observed that the inhibition of miR-122 reduces hepatic cholesterol and fatty acid synthesis in mice and primates [164]. Circulating levels of EVs containing miR-29a and miR-194 are increased in obese mice and are associated with impaired cardiac function, demonstrated by reduced left ventricle ejection fraction (LVEF) and elevated N-terminal pro-brain natriuretic peptide (NT-proBNP). Whereas treatment with miR-29a or miR-194 inhibitors improves cardiac function [171, 172]. One proposed mechanism of reduced cardiac function is myocyte mitochondrial damage. This hypothesis is confirmed by reduced ATP production, basal oxygen consumption, and impaired mitochondrial complex I activity, suggesting a new therapeutic target to restore cardiac function in obese patients [165, 166].

EVs containing miR-410-5p were elevated in high-fat diet rats and were associated with cardiac fibrosis via SMAD7 pathway [167].

Other miRNAs have been suggested as candidates for obesity biomarkers (Fig. 4). Obese patients exhibit lower levels of EVs containing miR-132 and miR-17-5p compared to non-obese controls, and miR-17-5p shows a negative correlation with body mass index (BMI) in obese individuals [173]. The increased circulating levels of EVs carrying miR-423-5p, miR-520c-3p, and miR-15a predict morbid obesity in men with an accuracy of 93.5%. Specifically, miR-520c-3p levels are inversely related to BMI, fat mass, waist circumference, blood lymphocyte counts, as well as levels of fasting glucose, glycated hemoglobin, and lipopolysaccharide-binding protein [168, 174]. Additionally, levels of EVs that transport miR-483-5p positively correlate with insulin resistance, dyslipidemia, and obesity [169, 175].

Fig. 4 miRNA in endothelial-derived EVs circulating in obese patients. EVs show a specific miRNA signature in the obesity state. *Mir* miRNA. Created with Biorender.com

Role of eEVs in diabetes

miRNAs transported through EVs could have specific signatures suitable for predicting T2DM, cardiovascular risk and vascular complications (Fig. 5). For example, EVs carrying miR-21 were significantly lower in patients with T2DM and inversely associated with waist circumference, BMI, insulin levels and insulin resistance [170]. The previously mentioned miR-483-5p, display a statistically significant correlation with the risk of developing T2DM and cardiovascular disease over time [169].

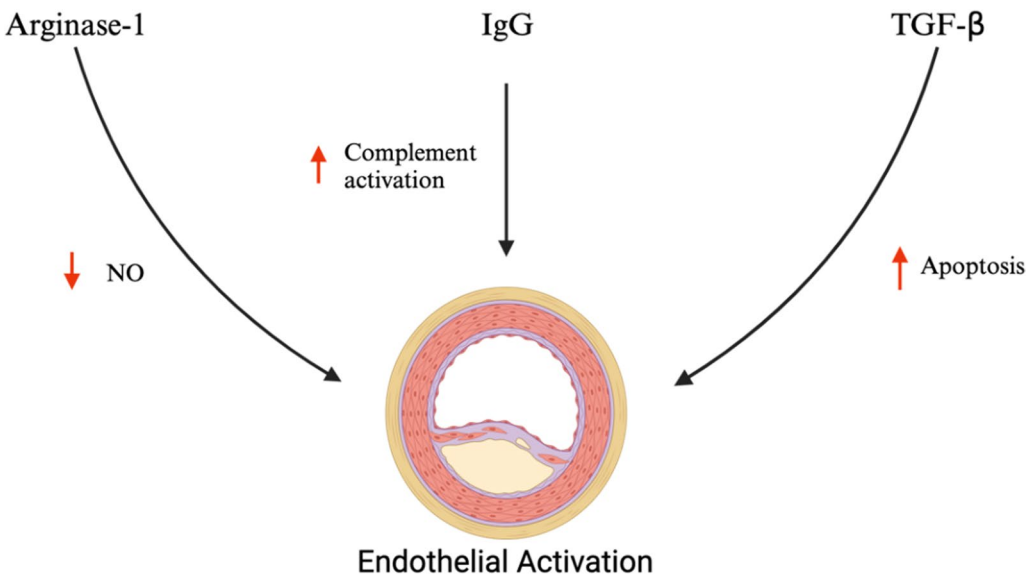


Fig. 5 Endothelial-derived EVs and endothelial activation in type 2 diabetes mellitus. Mechanisms of endothelial dysfunction and inflammation promoted by EVs in diabetic patients. *IgG* immunoglobulin G, *NO* nitric oxide, *TGF-β* trasforming growth factor beta. Created with Biorender.com

Serum-derived EVs play a crucial role in vascular dysfunction in diabetes by delivering arginase-1 to endothelial cells in homozygous mutated diabetic mice, which reduces NO production and contributes to endothelial dysfunction and vascular complications. This effect was also observed in heterozygous mutated nondiabetic mice, highlighting the broad impact of these EVs on vascular health [171]. Additionally, in diabetic patients, EVs carrying IgG are linked to retinal damage through activation of the classical complement pathway. Elevated concentrations of IgG-enriched EVs were found in these patients, and their reduction correlated with decreased retinal vascular damage [172]. Similarly, EVs released by mesangial cells and podocytes under high-glucose conditions contribute to diabetic nephropathy by inducing podocyte injury. Berberine treatment was shown to protect podocytes by inhibiting the transfer of TGF-β1, reducing apoptosis in renal glomerular cells, thus offering a potential therapeutic approach for mitigating diabetic vascular complications [173].

Role of eEVs in cardiovascular diseases

eEVs have been implicated in many different cardiovascular pathologies [174]. In post-partum cardiomyopathy, the 16-kDa N-terminal prolactin fragment induced the release of miR-146a-enriched eEVs, further internalized by cardiomyocytes. This resulted in the downregulation of miR-146a target genes, and the depression of contractile function [175]. It has been shown inhibiting miR-146a attenuated a post-partum cardiomyopathy-like phenotype characterized by increased miR-146a expression in cardiomyocyte-restricted Stat3 knockout mice [176].

Moreover, blood-derived eEV were increased in many vascular pathologies (obesity, hypertension, thrombosis, inflammation) [177], and endothelial dysfunction is an independent predictor of vascular diseases. Therefore, measuring CD31 +/Annexin V+ eEVs could have a diagnostic value [146]. It has been, in fact, demonstrated that eEVs release is increased in patients with endothelial dysfunction [178]. Notably, high levels of blood CD31 +/Annexin V+ EVs were associated with several pathological conditions like arterial and venous thrombosis [179]. A recent study suggested that TF-positive eEVs could serve as a prognostic biomarker for COVID-19, as TF levels correlated with disease severity and mortality [180]. eEVs from coronary artery disease patients with hyperhomocysteinemia contained high levels of ubiquitinated A_{2A} receptors for adenosine ($A_{2A}R$), underscoring the potential of eEVs as diagnostic markers and therapeutic targets in this context [181].

The prognostic role of eEVs in patients with abdominal aortic aneurysm undergoing Endo Vascular Aortic Repair (EVAR) has recently been highlighted [9]. A 5-year follow-up of EVAR implantation showed that eEV levels were higher in patients with endoleak complications compared to those that did not develop any endoleak, suggesting that eEVs are valuable prognostic biomarkers for endoleak detection [55]. Additionally, eEV levels in the blood have been shown to be independent predictors of future cardiovascular events in patients with high risk for coronary heart disease. EV levels positively correlated with the risk of developing cardiovascular disease, increasing from low-risk patients to those with AMI. The analysis of eEV in the blood could help to predict future cardiovascular events and support a multi-biomarker strategy to enhance primary prevention and to better stratify the patients [188]. Lipoxin A_4 exerts anti-inflammatory and endothelial protective activities by upregulating miR126–5p in HUVEC, inducing the release of HUVEC-derived EVs. HUVEC-derived eEVs, carrying miR126-5p, promote endothelial cell proliferation and maintain vascular integrity, as well as reduce endothelial dysfunction in cardiovascular pathologies [99].

In AMI patients, circulating miRNAs, like miR-1, miR-30, miR-133a, miR-208, and miR-499, are increased and their peak concentrations occurred before the rise in troponin T (cTnT) [182]. Notably, miR-499-5p effectively discriminated between non-ST-elevation myocardial infarction (NSTEMI) and acute congestive heart failure (CHF) in elderly patients with modest cTnT elevation [183]. MiR-208a has been proposed as a prognostic marker in AMI, as high levels of miR-208a in blood were correlated with a high 1-year mortality rate, aging, and greater AMI severity, creatine kinase (CK)-MB, cTnT peak and elevated LDL concentration at clinical presentation [184]. Additionally, its sensitivity as a diagnostic biomarker is higher than Troponin I during the first 4 h following an AMI [185]. MiR-192, miR-194, and miR-34a levels correlated with left ventricular end-diastolic dimension one year after AMI, making them potential markers for predicting the risk of ischemic heart failure (HF) after AMI[186]. Reduced miR-126 levels reduced the angiogenic capacity of early outgrowth cells and circulating CD34 +cells, hindering their ability to improve cardiac neovascularization and function in chronic HF, suggesting a new therapeutic target for HF treatment [187]. Some microRNAs have demonstrated protective effects on cardiomyocytes during chronic myocardial ischemia. Ischemic post-conditioning can enhance this cardioprotective effect by upregulating the release of miR-423-3p-carrying EVs from cardiac fibroblasts, targeting RAP2 C pathway and consequently reducing cardiomyocyte cell apoptosis in ischemic conditions [188]. MiR-214 was upregulated during ischemic injury and plays a cardioprotective role in HF by preventing calcium

overload in ischemic cardiomyocytes [189]. MiR-155 induced macrophage polarization into an inflammatory phenotype that interacts with myocardial fibroblasts, increasing inflammation and reducing cardiac function in acute coronary syndrome (ACS) and heart failure. Conversely, exercise-induced upregulation of miR-29b and miR-455 reduces matrix metalloproteinase-9 (MMP9) activity and, thus, fibrosis in cardiac tissue [136]. In recent work, Yao et al. developed an exosome spray based on mesenchymal stem cell-derived small EVs, administrated via a minimally invasive thoracoscopy approach. This EV-based therapy reduced cell apoptosis, fibrosis, and infarct size, enhanced cardiac function, increased left ventricular wall thickness and promoted proliferation in an experimental neonatal rat cardiomyocyte model [190]. These results suggest a potential clinical application of EVs in regenerative medicine. EVs also modulate vascular endothelial dysfunction and plaque formation. It has been demonstrated that EVs containing miR-712 and miR-205, released by endothelial cells subjected to turbulent blood flow, reduce the activity of metalloproteinase-3 tissue inhibitor and consequentely they enhance metalloproteinase activity, contributing to endothelial inflammation [100]. Moreover, LDL and homocysteine induce the release of HSP70-containing eEVs, enhancing monocytes adhesion to endothelial cells, and impairing endothelial integrity. Moreovew, they attact lymphocytes and platelets to the endothelium, accelerate inflammation, and induce differentiation of monocytes to macrophages [191]. In contrast, miR-126-containing eEVs reduce VCAM-1 expression on endothelial cells, limiting inflammatory cell recruitment, activating chemokine ligand CCL12, and inhibiting apoptosis in vascular endothelial cells. These events produce plaque stabilization, inflammation reduction, and vascular re-endothelialization [192]. Furthermore, miR-143/145-enriched eEVs prevent smooth muscle cell de-differentiation and limit atherosclerotic plaque formation [193].

Molecular pathways and pathogenesis associated with EVs in cardiovascular disease

EVs play a crucial role in intercellular signaling in various pathological contexts. In cardiovascular diseases, endothelial and platelet-derived EVs contribute to homeostasis in physiologic conditions, and their impairment due to dyslipidemia, hyperglycemia, and inflammation could provide insights into the physiological basis of cardiovascular diseases. Many molecular pathways activated by endothelial and platelet-derived EVs in cardiovascular diseases are related to inflammation, endothelial activation, apoptosis, and fibrosis [136].

Platelet activation via ligand-receptor interactions, such as GPIIb/IIIa, P-selectin, factor V, integrin, tissue factor, and phosphatidylserine, promotes platelet aggregation, inflammation, and leucocyte activation [114]. The NF-kB/interleukin pathway is triggered by proinflammatory pEVs releasing specific miRNAs (e.g. hsa-miR-362, hsa-miR-150, hsa-miR-1244, hsa-miR-520b-3p, and hsa-miR-638) that target inflammation- and atherogenesis- related genes (LDLR, TLR4, ESR1). This induces the release of IL-6 and IL-8 and an increased expression of ICAM-1, in which PCSK9 activity is involved by facilitating proinflammatory cytokines and adhesion molecule release [117–119]. This process contributes to endothelial injury, heightened atherogenesis, and increased cardiovascular risk. The release of pEVs induced by CXCL7 and CXCL10 enhances inflammation and ROS production through CXCR2, subsequently activating NF-κB, promoting diabetic target organ damage, and increasing cardiovascular risk.

pEV blood levels are elevated in HF patients, suggesting their potential as biomarkers of HF [194]. Caspases and Rho-kinase enzymes transferred by pEVs to endothelial cells have been associated with endothelial activation and inflammation [131]. pEVs carrying miR-223, miR-339 and miR-21 enhances adverse vascular remodeling [132]. Additionally, eEVs released under CRP stimuli and hyperglycemia conditions impaired vascular integrity, leading to vascular damage mediated by ROS production [159]. eEVs released under hypoxia conditions activate p38 and JNK1/2 pathways, causing a pro-apoptotic and inflammatory state [162]. A circular RNA (CircUbe3a) delivered by EVs promotes cardiac fibrosis by sponging miR-138-5p, further repressing Rho-kinase pathway and promoting proliferation and migration of recipient cardiac fibroblast in post-AMI murine models [195].

Future perspectives

Future applications of EVs in cardiovascular diseases require a deeper understanding of disease onset and the EV contributions to pathophysiological progression [196, 197]. In secondary prevention, EVs could serve as valuable tools for assessing individual patient risk for cardiovascular disease and predicting the incidence of major adverse cardiovascular events. By analyzing EV levels and their cargo (e.g., miRNAs and proteins), it may be possible to define a specific"epigenetic signature". This approach would enable more stringent management of risk factors in patients identified as having a higher risk based on their EV signature [198].

Additionally, circulating EVs could be targeted to achieve systemic modulation of the pathophysiological mechanisms involved in cardiovascular diseases.

EVs engineering, providing a highly selective biological delivery system, could be used to transport bioactive

molecules to specific cells involved in cardiovascular pathophysiology.

EVs are released during both acute and chronic phases of cardiovascular disease, affecting cardiomyocyte homeostasis and cardiac function [199]. Blocking these signaling pathways using selective inhibitors of EV cargo (e.g., siRNA, proteins) could modulate the mechanisms involved in cardiovascular disease development. Similarly, therapies based on cardioprotective EVs containing miRNAs and proteins associated with anti-inflammatory activity and scar remodeling could improve patient outcomes by promoting cardiomyocyte healing, atherosclerotic plaque remodeling, and favorable microenvironment polarization.

EVs as biomarkers of cardiovascular disease

EVs are increasingly discussed as potential biomarkers for various pathological conditions [7, 9, 200–202]. In cardiovascular disorders, specific expression patterns of eEVs and pEVs that contain certain proteins and miRNAs could reflect systemic inflammatory dysregulation, a proatherogenic environment, and neurohormonal dysfunction, all linked to an increased cardiovascular risk and higher mortality in heart failure [203, 204]. The use of EVs as biomarkers could provide a reliable tool for better stratifying patients and identifying those with a higher probability of developing cardiovascular diseases. EVs could serve as diagnostic markers in acute conditions such as ACS or acute decompensated HF and predict patient prognosis following cardiovascular events [205, 206].

For example, detecting elevated levels of procoagulant pEVs in blood could guide anti-aggregating strategies for post-percutaneous coronary intervention in AMI patients, allowing for more aggressive or lenient protocols based on platelet activation. Furthermore, identifying specific cardiotoxic eEV subsets could predict a more severe phenotype in heart failure patients. We have previously discussed how eEVs containing certain cardio-specific miRNAs increase during myocardial infarction. The EV analysis showed to be more sensitive than troponin assays if performed during the initial hours. The EV levels can also distinguish between ACS and CHF in patients with modest cTnT increase at the disease onset. Moreover, the EVs levels correlate with worse clinical outcomes in AMI patients and act as a prognostic marker for 1-year mortality and cardiac function in AMI patients [207].

It has also been observed that eEV levels are significantly increased in chronic heart failure, reflecting endothelial stress [208]. Additionally, increased eEV levels have been detected in conditions like psoriasis, indicating the EV role in endothelial dysfunction and atherogenesis [209]. Moreover, eEVs are implicated in the pathogenesis of vascular damage, contributing to endothelial dysfunction and inflammation [210]. It has also been demonstrated that pEVs play a crucial role in cardiovascular diseases, including atherosclerosis and ischemic stroke. They contribute to disease mechanisms and serve as biomarkers for disease severity and outcomes [211, 212]. pEVs have been found to be elevated in conditions like thrombotic antiphospholipid syndrome, indicating ongoing platelet activation [213]. In rheumatoid arthritis, increased pEV levels are linked to a procoagulant state, emphasizing their involvement in systemic inflammation and thrombosis [214]. In the context of ischemic stroke, pEV levels at hospital admission correlate with stroke severity and outcomes, positioning them as useful markers for risk stratification [212]. In diabetic microvascular complications, the analysis of circulating eEVs and pEVs, along with their miRNA profiles, offers a non-invasive method for early disease detection [215]. Likewise, the analysis of EV biomarkers, such as CD41 +/CD61 + EVs in coronary artery disease, enhances diagnostic accuracy [216]. Although further studies involving more patients are needed, EV analysis could represent a promising advance in traditional diagnostic protocols and risk assessment for cardiovascular patients.

Therapeutic use of EVs in cardiovascular diseases

One of the most interesting therapeutic uses of EVs is drug delivery. EVs can be loaded with specific cargo, such as proteins, RNAs, or miRNAs. Because EVs can cross biological barriers, including the blood–brain barrier, they represent a natural and more efficient alternative to synthetic drug delivery systems[1]. The EV cargo could be used to prevent endothelial and platelet activation, cellular apoptosis, and inflammation by targeting the key molecular pathways in cardiovascular diseases [217]. However, no regulatory EV products have been approved yet, and further research are therefore mandatory.

Another key area of research is the potential use of heart-derived EVs as cardioprotective agents to enhance cardiac remodeling in post-AMI patients [217]. EVs from cardiac stromal/progenitor cells have been shown to decrease cardiomyocyte apoptosis by activating pro-survival pathways, such as Akt and ERK1/2 [217]. Conversely, the transfer of miR-181b to macrophages through EVs led to a cardioprotective effect due to the induction of favorable macrophagic polarization and downregulation of protein kinase C δ (PKCδ; prkcd) gene transcription, leading to a reduced proinflammatory gene expression [218, 219]. It has been demonstrated that intramyocardial and intracoronary injections of EVs in AMI preclinical models resulted in reduced microvascular obstruction, improved LVEF, increased LV stroke volume, diminished infarct size, and enhanced blood vessel

density after a 3-month follow-up [217]. Systemic intravenous infusion of EVs from clonal immortalized human embryonic mesenchymal stem cells induced a significant decrease in infarct size (30–40%) and lesion transmurality [220–222]. These findings were confirmed by cardiac magnetic resonance (CMR)-based preclinical trials aimed at delivering EVs to the infarct region [221, 223, 224]. This approach resulted in better right heart function, decreased ventricular volumes, and infarct size, as well as less fibrosis and an anti-inflammatory macrophage polarization compared to controls [223]. Another study using intracardiac EVs overexpressing miR-486-5p in primate models showed improved LVEF, reduced end-systolic volume, decreased infarct size, and greater vascular density in the scar region [224].

These benefits were confirmed in post-AMI animal models via intracardiac or intrapericardial EV administration. The EV injection resulted in improved CMR systolic function parameters and cardiac volumes, decreased scar size and mass, and it created a conducive anti-inflammatory macrophagic microenvironment [221, 225]. Ongoing trials are examining the safety and efficacy of EVs in human patients following percutaneous coronary intervention (PCI) in AMI (NCT04327635), coronary artery bypass grafting (NCT05669144), and non-ischemic cardiomyopathies (NCT05774509).

Limitations

The use of EVs as diagnostic and therapeutic options is limited by several factors [226]. Technical challenges include the need for specialized laboratory equipment for cell culturing, EV harvesting, isolation, cargo loading, and formulation. The cell lines used for EV production must be carefully selected and cultivated under strict standard conditions. Due to their nature and origin, EVs are particularly prone to contamination, represented by proteins, phospholipid fragments, lipoparticles, or viruses [226]. A rigorous sterile environment must be maintained, and contamination clearance must be ensured. Different approaches to cargo loading, achieved through either passive or active methods, have been developed, with the latter showing better performance [227]. Storage conditions pose a logistical challenge as EVs should be stored at −80 °C, although biological activity alterations have been observed, making this process expensive and difficult to manage [228]. Lyophilization has been studied as a potential alternative for long-term storage [228].

Safety remains a major concern in EV therapy. The lack of regulation regarding safety, potency, and monitoring assays represents the primary obstacle to standardizing EV therapy from animal studies to human trials, leading to low reproducibility in batch-to-batch clinical studies. Furthermore, data on dose–response, biodistribution, pharmacokinetics, and in vivo pharmacodynamics are scarce and non-standardized. Additionally, definitive data on the long-term side effects of EV therapy, such as immunologic or oncologic events, are lacking, which raises concerns about the safety of human EV therapy. EVs exhibit low immunogenicity and long-term stability, with homologous EVs proving to be safer than heterologous EVs [229]. However, more evidence, particularly regarding human use, is still needed. In AMI patients, EVs have demonstrated immunomodulatory effects, including increased circulating levels of IL−1α, which blocks the inflammatory effects of IL-1α/β, and reduces levels of TNF-α and granulocyte–macrophage colony-stimulating factor (GM-CSF) 30 days post-AMI [223]. Inflammatory reactions noted in xenogenic models, often discussed in the context of histologic rejection at cardiac biopsy, are more likely related to factors such as needle injury [221]. A proarrhythmic effect of EVs was documented in some studies, likely related to the proarrhythmic effect of engraftment and the coupling of survival cells in cardiac tissue. Otherwise, more recent trials showed that EVs inhibited the induction of ventricular arrhythmias, according to the favorable scar healing observed in previous studies [217].

To avoid unexpected systemic reactions, some researchers proposed localized myocardial somministration of EVs using polymeric or pericardial scaffolds as well as EV-based sprays [223, 230]. However, these methods are invasive and require thoracotomy.

Conclusions

EVs provide deeper insights into the mechanisms involved in systemic cellular crosstalk under pathological conditions, linking cardiovascular disease, inflammation, endothelial and platelet activation, and metabolic dysfunction. They represent an innovative diagnostic tool, enabling the use of risk stratification and prognostic methods in cardiovascular diseases. Platelet-derived and endothelial-derived EV cargoes influence cellular homeostasis and play crucial roles in various cardiovascular pathologies. Future studies focusing on EVs may confirm their potential as additional diagnostic and prognostic biomarkers across different diseases. Additionally, EVs have considerable promise for therapeutic uses in drug delivery and regenerative medicine, providing customized treatments for patients by targeting specific pathways involved in their disease and reversing the cellular changes associated with cardiovascular disease progression.

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Competing interests
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