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REVIEW

The Role of Extracellular Vesicle—Derived miRNA in the Atherosclerotic Burden

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In the context of atheroma-related sequelae, the role of extracellular vesicles (EVs) continues to spike interest. Their ability to traffic molecular cargo between cells highlights their role in intercellular communication, and consequently their involvement in mediating molecular events at the basis of physiological and pathologic processes. EVs encapsulate miRNAs within their lumen, shielding them from circulating ribonucleases, which would otherwise catalyze their degradation. However, there is an ongoing debate regarding the implication of miRNA contained within EVs in modulating biological activities on a molecular level. Therefore, the aim of the present review is to discuss the role of EV-derived miRNA, focusing on their implication in molecular mechanisms underlying atheroma formation. EVs of endothelial origin can regulate monocyte activation by transferring miR-10a that targets components of the inflammatory pathway. Tail vein administration of EVs derived from endothelial cells enriched in miR-34c-5p markedly reduces atherosclerosis progression. In patients with stable coronary artery disease, elevated levels of miR-126 and miR-199a in circulating EVs are significantly associated with a reduced incidence of major adverse cardiovascular event rate. These nanoparticles, released by all cells into most biological fluids, hold promise as a liquid biopsy tool as their circulating patterns and cargo can reflect the onset and severity of cardiovascular diseases. *(Am J Pathol 2025, ■: 1—14; https://doi.org/10.1016/j.ajpath.2025.08.006)*

Extracellular vesicles (EVs)—once dismissed as cellular debris[1,2]—are now recognized as key mediators of intercellular communication,[3] with crucial roles in both physiological and pathologic processes. In the context of atheroma, accumulating evidence links EVs to several major risk factors and mechanisms driving plaque initiation, progression, and instability.[4] Atherosclerosis involves the buildup of fatty and/or fibrous material in the innermost layer of the arteries, known as the intima. This condition is marked by the retention of modified lipoproteins in the arterial wall, which triggers the activation of resident macrophages and recruitment of monocyte-derived cells. Key players in the initiation and progression of atherosclerosis include the endothelial monolayer, vascular smooth muscle cells, and inflammatory cells.[5] In this setting, EVs can be at the crossroad of signaling pathways, which are of importance for atherosclerosis development and regression.[6,7]

Although EVs are now recognized as established biomarkers in the context of cardiovascular disease,[8—10] the role of their intraluminal cargo (eg, miRNA), shuttled

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among cells, for instance, endothelial cells (ECs) or vascular smooth muscle cells (VSMCs), that are actively involved in the atheroma formation, needs to be properly clarified.

Therefore, the aim of the present review is to critically discuss the role of EVs, focusing on the molecular mechanisms underlying atheroma formation in which they may be involved in, with a particular focus on miRNA. Indeed, there is an ongoing debate regarding the implication of miRNA contained within EVs in modulating biological activities on a molecular level. In line with the recommendation of the International Society of Extracellular Vesicles to use generic terminology when referring to EV subtypes, without referring to their biogenesis pathways,[11] we consistently use throughout the present review the term EVs instead of exosomes or microvesicles.

The Biology of EVs

EVs are a group of membranous nanoparticles released by all cell types into the extracellular matrix under basal and pathologic conditions.[12] Structurally, EVs are characterized by a phospholipid bilayer enclosing a hydrophilic lumen, within which they can contain a variety of macromolecules. These include proteins, nucleic acids, metabolites, lipids, and even organelles, such as mitochondria[13] (Figure 1). The composition and abundance of EV content are determined by the physiological or pathologic state of the donor cell, as well as by the cell type, the stimuli that influence their release, and the molecular mechanisms at the basis of their biogenesis.[14] This constitutes a promising aspect, as it confers EVs the ability to serve as a proxy indicator of cells, and therefore tissues and even organs. Indeed, not only their molecular content undergoes fluctuation, but also characteristics, such as their size, concentration, phospholipid composition, and derivation, seem to be altered in pathologic conditions.[15]

Traditionally, researchers of this field have categorized EVs into two main groups: exosomes or microvesicles. These two types of EVs are defined by their presumed biosynthesis and size. The term exosome was originally used to describe intraluminal membranous vesicles ranging

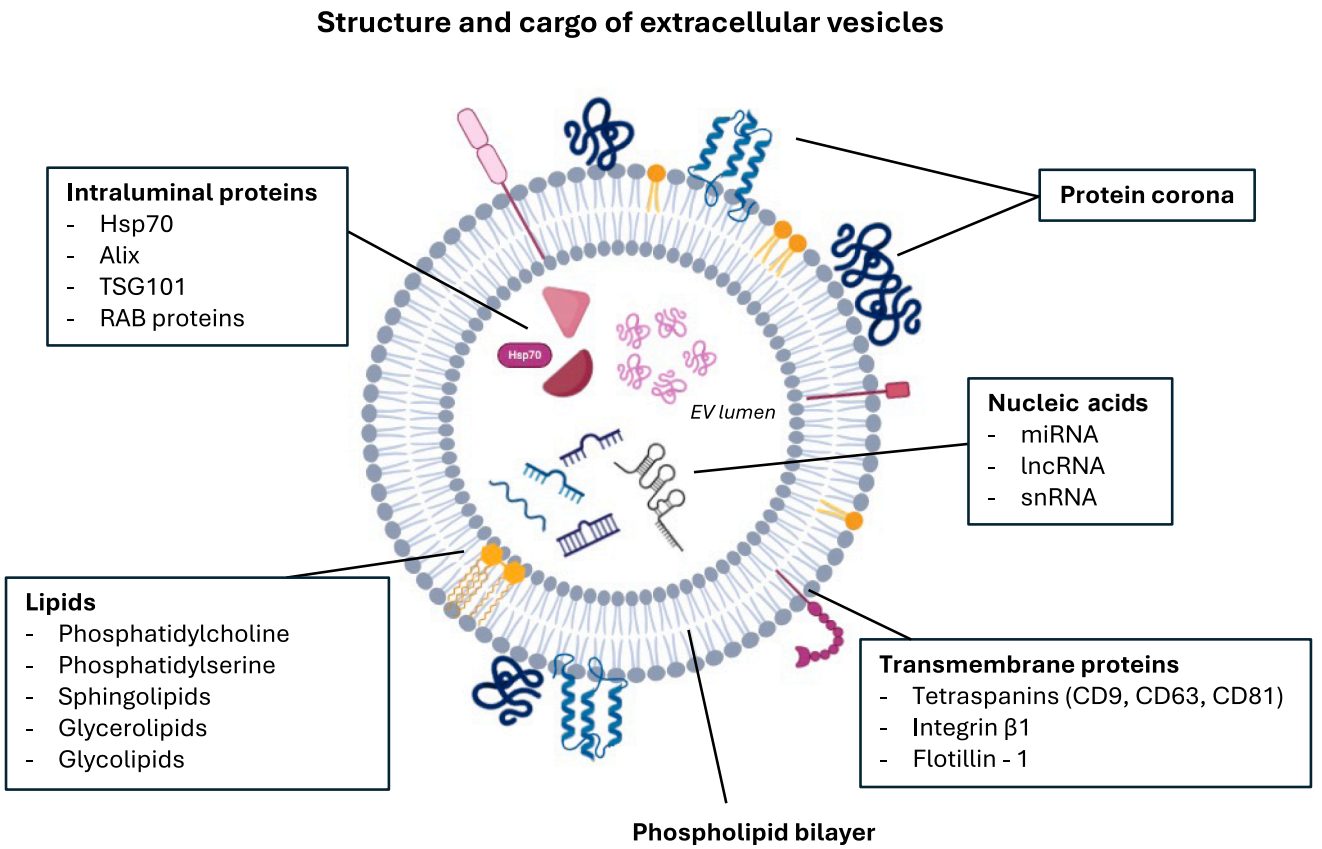

Figure 1 Structure and molecular cargo of EVs. This schematic illustrates the key structural components and biomolecular content of extracellular vesicles. EVs are enclosed by a lipid bilayer rich in phosphatidylcholine, phosphatidylserine, sphingolipids, and glycolipids. Embedded within the membrane are transmembrane proteins, such as tetraspanins (CD9, CD63, and CD81), integrins, and flotillin-1. EVs also carry a protein corona adsorbed to their outer surface. The intraluminal space contains cytosolic proteins, including heat shock protein (Hsp) 70, Alix, tumor susceptibility gene (TSG101), and Ras-related in brain (RAB) proteins, as well as various RNA species, such as miRNA, long noncoding RNA (lncRNA), and small nuclear RNA (snRNA). These molecules contribute to EV-mediated intercellular communication and functional regulation in physiological and pathologic contexts.

between 30 and 100 nm in diameter, thought to originate from the inward budding of the endosomal membrane during the maturation of multivesicular endosomes—intermediates of the endosomal system—which are then secreted once these multivesicular endosomes fuse with the cell membrane.[16] Microvesicles, on the other hand, were known as the larger EVs (50 to 1000 nm), generated by a direct, outward budding from the cell membrane and released into the extracellular space.[17]

To avoid imprecise experimental expectations, it is recommended to use operational terms based on various criteria: size [defining EVs as small (<100 or <200 nm) or medium/large (>200 nm)], density range (low, middle, or high), biochemical composition [such as the presence of specific proteins, like tetraspanins (CD63 or CD81) or annexin A5], and the conditions of the cell of origin (eg, hypoxic EVs or cardiomyocyte-derived EVs).[11] Mechanisms related to the biogenesis and molecular cargo sorting of EVs have been uncovered in recent years. For exosome sorting and formation, subunits of the endosomal sorting complex required for transport (ESCRT) are involved to various extents according to the specific process (molecule loading or EV scission). The ESCRT machinery can be subdivided into functionally distinct subcomplexes known as ESCRT-0, ESCRT-I, ESCRT-II, and ESCRT-III. In mammalian cells, these complexes are sequentially recruited to maturing endosomes. The early ESCRT machinery, consisting of ESCRT-0, ESCRT-I, and ESCRT-II, recruits and sequesters ligand-receptor signaling complexes and leads to their incorporation into intraluminal vesicles within multivesicular bodies. Intraluminal vesicles can be directed toward lysosomal degradation by the multivesicular body−lysosome fusion or toward secretion into the extracellular space through multivesicular body−plasma membrane fusion. The latter, through the action of Ras-related in brain (RAB) GTPases, leads to the budding of exosomes.[18] Mechanisms governing microvesicle formation remain less clear. The release of microvesicles is likely faster, given that their content only needs to accumulate at the plasma membrane, followed by immediate release once they have undergone formation and fission.[19] There is, however, evidence on ESCRT-dependent release of microvesicles, specifically mediated by ESCRT-III proteins.[20]

Structural Composition of EVs

The EV structure consists of a phospholipid bilayer that surrounds a hydrophilic lumen, capable of carrying a variety of lipids, proteins, metabolites, and nucleic acids (coding and noncoding).[20] Lipids constitute basic structural components of EVs, responsible for maintaining their stability within the extracellular environment. EV membrane lipids are organized in a bilayer structure and, like cellular membranes, include a range of glycerophospholipids, including phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine, and phosphatidylinositol, sphingolipids (sphingomyelin, ceramides, glycosphingolipids, and glycosylceramides), glycerolipids (diacylglycerols), cholesterol, and glycolipids.[21] The relevance of phosphatidylserine, a negatively charged phospholipid, has been identified in EV preparations and appears to play a functional role in signaling mechanisms with certain cell types, as EVs can engage cells through phosphatidylserine-mediated attachment and uptake.[22] The externalization of phosphatidylserine to the outer EV membrane leaflet enables its binding by numerous plasma membrane receptors either directly or indirectly, via certain bridging proteins.[23]

Proteins are arguably the most extensively studied components of EVs' biomolecular content. Through the combined effort of numerous studies, some detailed catalogues reporting the protein abundance within EVs have been produced (Table 1). Whether located in the lumen [eg, heat shock protein (Hsp) 70, Hsp90, and tumor susceptibility gene 101 (TSG101)], embedded in the membrane (eg, Flotillin-1),[24] or on the outer surface, EV proteins may play many, crucial functional roles. For instance, fundamental interactions with cells, including EV-mediated signal transduction or EV uptake, are mediated by the binding between EV surface proteins and the cellular plasma membrane. It is widely recognized that various proteins, including tetraspanins (CD9, CD63, CD81, and CD82), integrins (eg, integrin β1), extracellular matrix proteins, immunoglobulin superfamily members, proteoglycans, and lectins, are responsible for EV-cell interactions.[25] However, the precise mechanisms governing these interactions remain unclear. For instance, although CD9 and CD63 have been implicated in regulating EV uptake and delivery,[26] it seems

Table 1 Catalogues Reporting the Cargo Abundance Within EVs

Database	Website	Main focus
Vesiclepedia	*http://microvesicles.org*	Comprehensive EV data (mRNAs, miRNAs, proteins, and lipids) from >500 studies
ExoCarta	*http://www.exocarta.org*	Curated data on exosomes from various cell types
EVpedia	*https://evpedia.info/evpedia2_xe*	Comprehensive EV proteome, transcriptome, and lipidome for prokaryotes and eukaryotes
exoRBase	*http://www.exorbase.org*	Repository of circRNA, lncRNA, and mRNA from human blood exosome RNA-seq data
EV-TRACK	*https://evtrack.org*	Knowledgebase for EV method and experimental standardization
exRNA Atlas	*https://exrna-atlas.org*	Repository of small RNA and qPCR-derived exRNA profiles from human/mouse biofluids

All websites last accessed August 9, 2025.
circRNA, circular RNA; exRNA, extracellular RNA; lncRNA, long noncoding RNA; qPCR, real-time quantitative PCR; RNA-seq, RNA sequencing.

that tetraspanin depletion is compensated by functionally redundant proteins from the same family,[27] and that other factors, including post-translational modifications (eg, palmitoylation), subcellular localization of EV release, and chemical properties of the cargo, influence EV sorting and delivery.[28]

Although EVs are rich in cytoskeletal, cytosolic, heat shock, and plasma membrane proteins, proteins from intracellular organelles, on the other hand, are less present. The selective inclusion of certain proteins within the EV membrane or lumen depends on a multitude of finely tuned mechanisms and is an aspect that renders them attractive for biomarker studies.[29] Alix, for instance, is an accessory protein of the ESCRT involved in miRNA packaging.[30]

RNA content is also attracting significant interest within the study of EV molecular cargo. Although cellular mRNA ranges in size from 400 to 12,000 nucleotides, RNA detected in EVs predominantly measures approximately 700 nucleotides. Extensive employment of RNA sequencing has demonstrated that EVs can contain multiple RNA biotypes, such as intact mRNA, mRNA fragments, long noncoding RNA, miRNA, rRNA, and fragments of tRNA.[31] EV RNAs in general have been identified as a source of novel disease biomarkers,[32] but miRNAs have received significant attention because of their crucial role in organism development and functions through post-transcriptional gene regulation.

Comprising approximately 40% of all sequencing reads in RNA-sequencing analysis, miRNAs, small, approximately 22-nucleotide single-stranded RNAs, are the most abundant RNA species in human plasma EVs.[33] The question of whether EV-miRNA concentrations within EVs can elicit direct effects is a long-disputed topic.[34] Wei et al[35] estimated that the average abundance of miRNA was of one copy per approximately 100 EVs, implying that, to exert mRNA down-regulation, 11,000 EVs would need to be delivered to the receiving cell, based on the theory of Brown et al[36] stating that approximately 100 miRNAs per cell are necessary as a threshold minimum. However, these stoichiometries may not be so linear for multiple reasons. The miRNA concentrations among different EVs are highly heterogeneous and so a specific quantity may not be ubiquitous to all EVs in general. In addition, in the case that the levels of a given miRNA may already be close to the threshold in a recipient cell, fewer EV-miRNAs would be needed to induce a downstream effect. More recent studies have stated the ability of EV-associated miRNAs to actively participate in cell programming activities.[37] An additional obstacle in studies on EV-derived miRNAs lies in the EV isolation procedures, which can influence the distribution of miRNAs—favoring their association with either the vesicles themselves or the protein fraction.[38]

Thanks to recent advances in live and high-resolution microscopy, as well as innovative EV-labeling techniques, the functional transfer of EV-associated proteins and RNA can now be tracked using novel reporters in both *in vivo* and *in vitro* systems. These developments challenge the traditional model of EV function, which typically involves EVs being released from a donor cell (cell A), traveling to a recipient cell (cell B), and inducing phenotypic changes via endocytosis and cargo delivery. In reality, EVs can also act in an autocrine manner or exert effects independent of cargo delivery—such as modifying the extracellular matrix, interacting with cell surface receptors, or transferring membrane-bound proteins to recipient cells.[39] In addition to transporting luminal molecules, the EV membrane appears to play a central and prominent role in EV functionality, particularly in small EVs. This is because of the distinct arrangement of surface molecules, such as receptors and integrins, that EVs acquire from the biogenesis process.[40,41] Furthermore, their large surface/volume ratio confers EVs a high interfacial energy, making them prone to interact with various biomolecules on their surface.[42] Collectively referred to as the biomolecular corona, these adsorbed biomolecules (proteins, nucleic acids, and metabolites)[43]—but also nanoparticles, such as lipoproteins[44]—significantly influence the molecular, physical, and electrostatic properties of EVs. They also regulate interactions with cells, mobility, and biodistribution.[45,46]

The corona's composition depends on the EV membrane structure, hence reflecting content and state of the parent cells, but also on the composition of the biological fluid in which EVs are dispersed. Furthermore, EVs can act as concentrators for specific molecules on their surface.[47] A recent study using an innovative *in situ* protocol to monitor the dynamics of the EV corona under physiological conditions demonstrated how its formation, evolution, and architecture are influenced by the EV's origin and surface properties, further contributing to the heterogeneity of circulating EVs.[48]

The Atherosclerotic Burden: A Brief Overview on the Pathologic Process

Atherosclerosis is a primary cause of atherosclerotic cardiovascular disease (ASCVD), leading to severe conditions, like heart attack, stroke, and kidney failure.[5] According to estimates from the World Health Organization, ASCVDs accounted for 19.8 million fatalities in 2022 (*https://www.who.int/news-room/fact-sheets/detail/cardiovascular-diseases-(cvds)*, last accessed September 18, 2025). Atherosclerosis is generally the result of ongoing damage of the vascular wall, particularly following EC activation/dysfunction at susceptible sites in major conduit arteries.[49,50] This leads to the up-regulation of adhesion molecules (such as intercellular adhesion molecule 1 and vascular cell adhesion molecule 1) that facilitate monocyte adhesion to ECs and migration into the arterial wall.[51] Within the arterial intima, monocytes transform into macrophages, absorbing lipoprotein-derived cholesterol[52] and

forming foam cells.[53] Specifically, macrophages in atherosclerotic plaques exhibit reduced migration capacity, contributing to unresolved inflammation and the progression of plaques to more advanced stages involving other immune cell subsets and VSMCs.[54] These latter play a unique role by switching from a contractile (differentiated) phenotype in the media to a synthetic (dedifferentiated) phenotype in the intima, as seen in atherosclerotic plaques. VSMCs undergo numerous structural and functional changes in pathologic conditions, potentially losing their original features and acquiring characteristics typical of other cell types, including markers typical of macrophages.[55] Lastly, the role of platelets cannot be overlooked in atherogenesis. These small anucleate cells (2 to 4 μm in diameter) originate from megakaryocytes through thrombopoiesis and circulate for 7 to 10 days. Platelets contribute to the necrotic core of advanced atherosclerotic plaques and produce matrix metalloproteinase-9 and cathepsin G, which degrade the extracellular matrix and promote fibrous cap rupture, releasing necrotic material and forming a thrombus.[56] Currently, classic biomarkers, like total cholesterol, low-density lipoprotein (LDL), and serum triglyceride levels, are insufficient to capture any individual's residual risk, highlighting the need for novel biomarkers that enhance disease detection.[57] Thus, developing biomarkers capable of serving as reliable screening tools could pave the way for personalized interventions and better prevention of clinical events. In this scenario, the dual roles as biomarkers and active participants in disease progression make EVs promising targets for therapeutic interventions aimed at modulating atherosclerotic processes.

EVs in Atherosclerosis: *in Vitro* Evidence

EVs accumulate in human atherosclerotic plaques, where they affect major biological pathways, including inflammation, proliferation, thrombosis, calcification, and vasoactive responses.[58] Indeed, it is well established that most cell types, including ECs,[59] platelets,[10] and lymphocytes,[60] secrete EVs of different size, composition, and subtype. Given that EV characteristics reflect specific signatures of cellular activation and injury, monitoring them allows researchers to assess the onset and progression of certain diseases difficult to detect, such as the case of atherosclerosis[61] (key findings are summarized in Table 2[62–69]). ECs, being one of the first cell types to undergo significant molecular and biochemical changes in individuals with atherosclerosis, offer valuable insights through their EV content and release kinetics, which can serve as initial red flags.

As shown in Figure 2, by mediating intercellular communication, EVs influence endothelial function, macrophage behavior, and inflammatory responses. A healthy, quiescent endothelium releases EVs that suppress proinflammatory responses and steer monocyte activation toward an immunoregulatory state.[70] These anti-inflammatory effects of EC-derived EVs are partly mediated by the transfer of miR-10a to monocytes and macrophages. miR-10a inhibits NF-κB signaling by targeting multiple components of this pathway.[70] In the same study where this was demonstrated, elevated levels of miR-126 and miR-181b were also detected in monocytes exposed to EC-derived EVs; overexpression of these miRNAs similarly attenuated monocyte activation.[70]

Furthermore, EC-derived EVs can reduce monocyte adhesion by down-regulating the expression of adhesion molecules, such as intercellular adhesion molecule 1, a key step in leukocyte diapedesis, in an miRNA-222–dependent manner.[71] ECs exposed to oxidized LDL release EVs enriched with miR-155, which, when transferred to human monocytic THP1 cells, promote a shift in the monocyte/macrophage balance from anti-inflammatory M2 macrophages to proinflammatory M1 macrophages.[62] The potential impact of EC-derived miR-92a on macrophage phenotypic switch cannot be overlooked. EC miR-92a can be transported to macrophages through EVs to regulate Krüppel-like factor 4 levels, thus leading to the atheroprone phenotypes of macrophage and, hence, atherosclerotic lesion formation.[63]

The focal distribution and compositional variation of atherosclerotic plaques along the coronary tree suggest local differences in susceptibility to atherosclerosis. Wall shear stress can modulate endothelial function, smooth muscle cell turnover, and inflammatory adhesion molecule expression, and thus promote atherogenesis.[72] Human umbilical vein endothelial cells exposed to laminar shear stress released EVs enriched in miR-34c-5p that, when transferred to macrophages, promote M1-phenotype repolarization by targeting transforming growth factor beta–induced factor 1 and activating the transforming growth factor-β–Smad3 signaling pathway[73] (Table 3[73–84]).

Given their boundary location, ECs have been proposed to use bidirectional release of distinct EV cargo in quiescent (healthy) and activated (atheroprone) states to communicate with cells within the circulation and blood vessel wall. ECs can release EVs to apical (circulation) and basolateral (vessel wall) compartments. In this context, Raju et al[64] demonstrated that EVs isolated from primary human aortic ECs, previously activated with IL-1β, increased the release of EVs, with miRNA and protein cargo related to atherosclerosis. EC-derived EVs were able to reprogram human monocytes and VSMCs toward an atheroprone signature. On treatment with IL-1β, miRNA-146, miRNA-34c, miRNA-144, and miRNA-374b were increased in apical EC-EVs; miRNA-125b, miRNA-34a, miRNA-21, miRNA-24, and miRNA-126-5p were increased among the basolateral EVs.[64] These results carry significant implications for cardiovascular diseases, as IL-1β–induced activation of EC alters the cargo of EV, a known mediator of inflammation in atherosclerosis. Indeed, circulating EVs in patients with atherosclerosis have been observed to

Table 2 EVs in Atherosclerosis: *in Vitro* Evidence

In vitro studies		
Study description	Key findings	Snapshot
EVs were isolated from endothelial cells stimulated with ox-LDL.[62]	EVs drive macrophage polarization toward a proinflammatory M1 phenotype via miR-155.	Proatherogenic phenotype
EVs have been isolated from human umbilical venous endothelial cells and co-cultured with human peripheral blood mononuclear cell isolated and differentiated into macrophages.[63]	Intravesicular miR-92a suppressed the expression of target gene Krüppel-like factor 4 (*KLF4*) in macrophages.	Atheroprone phenotype
EVs isolated from primary human aortic endothelial cells, activated with IL-1β.[64]	i) miR-146a, miR-34c, miR-144, and miR-374b were increased in apical EC-EVs ii) miR-125b, miR-34a, miR-21, miR-24, and miR-126-5p were increased among the basolateral EVs.	i) Mediators of inflammation ii) Regulators of inflammation
Human aortic endothelial cells were exposed to EVs isolated from patients with stable coronary artery disease.[65]	EVs from patients with stable coronary artery disease inhibit ZO-1 expression to increase endothelial permeability via transferring highly expressed miR-140-3p into human aortic endothelial cells.	Causative factor in the development of endothelial hyperpermeability during atherosclerosis
EVs were isolated from endothelial cells stimulated with ox-LDL.[66]	EVs derived from endothelial cells treated with ox-LDL are enriched in long noncoding RNA HIF1A-AS2, which suppresses miR-455-5p when transferred in recipient cells.	Increase pyroptosis and vascular inflammation
EVs were isolated from endothelial cells stimulated with ox-LDL and IL-6.[67]	i) ox-LDL and IL-6 increase miR-92a-3p expression in endothelial cell—derived EVs in a dose-dependent manner. Endothelial EVs that incorporated miR-92a-3p foster endothelial angiogenic responses in recipient cells in a STAT3-THBS1—dependent manner. ii) Endothelial-derived EVs transfer miR-92a-3p to VSMCs, but monocytes.	i) Promotion of migration, proliferation, and tube formation in ECs ii) Promotion of VSMC migration and proliferation
EVs isolated from VSMCs overexpressing PCSK9.[68]	These EVs showed changes in a pattern of miRNA involved in both atherosclerosis and inflammation. hsa-miR-34c, hsa-miR-29, hsa-miR-148b, hsa-miR-221, and hsa-miR-125b were down-regulated, whereas hsa-miR-49 was up-regulated.	The genes targeted by these miRNAs were involved in atherosclerosis and inflammation
Thrombin-activated platelet (one unit per milliliter of thrombin reaction at 37°C for 30 minutes).[69]	Platelet-derived EVs enriched in miR-223 inhibit ICAM-1 expression in ECs.	Regulation of thrombosis-inflammation reaction

EC, endothelial cell; HIF1A-AS2, HIF1A antisense RNA 2; ICAM, intercellular adhesion molecule; ox-LDL, oxidized low-density lipoprotein; PCSK9, proprotein convertase subtilisin/kexin type 9; THBS1, thrombospondin 1; VSMC, vascular smooth muscle cell; ZO-1, zonula occludens protein-1.

influence lesion formation by potentially inducing endothelial apoptosis and inflammation. Zhang et al[85] demonstrated that EVs isolated from patients with coronary artery disease stimulated ECs to express IL-1β, tumor necrosis factor, and intercellular adhesion molecule 1, although not IL-6 or vascular cell adhesion molecule 1.

In an *in vitro* study focused on exploring the underlying mechanisms of vascular endothelial hyperpermeability in atherosclerosis, plasma exosomes from patients with stable coronary artery disease were observed to significantly impair vascular endothelial junctions by reducing the expression of critical EC junction components vascular endothelial—cadherin and zonula occludens protein 1 (ZO-1), leading to increased permeability and promoting atherosclerosis. Exosomes from patients with stable coronary artery disease were enriched with miR-140-3p, known to inhibit ZO-1 expression by directly targeting its mRNA, exacerbating endothelial hyperpermeability. This mechanism was further confirmed *in vivo* in atherosclerosis-prone mice, where exosomes from patients with stable coronary artery disease—induced ZO-1 suppression and vascular permeability correlated with larger atherosclerotic lesions. Considering that ZO-1 controls endothelial adherence junctions, cell-cell tension, angiogenesis, and barrier formation, these findings imply the role of circulating EV miRNAs as crucial mediators of endothelial dysfunction in atherosclerosis.[65]

In the context of the atherosclerotic process, pyroptosis and vascular inflammation play a critical role. EVs derived from oxidized LDL—stimulated ECs can trigger EC death

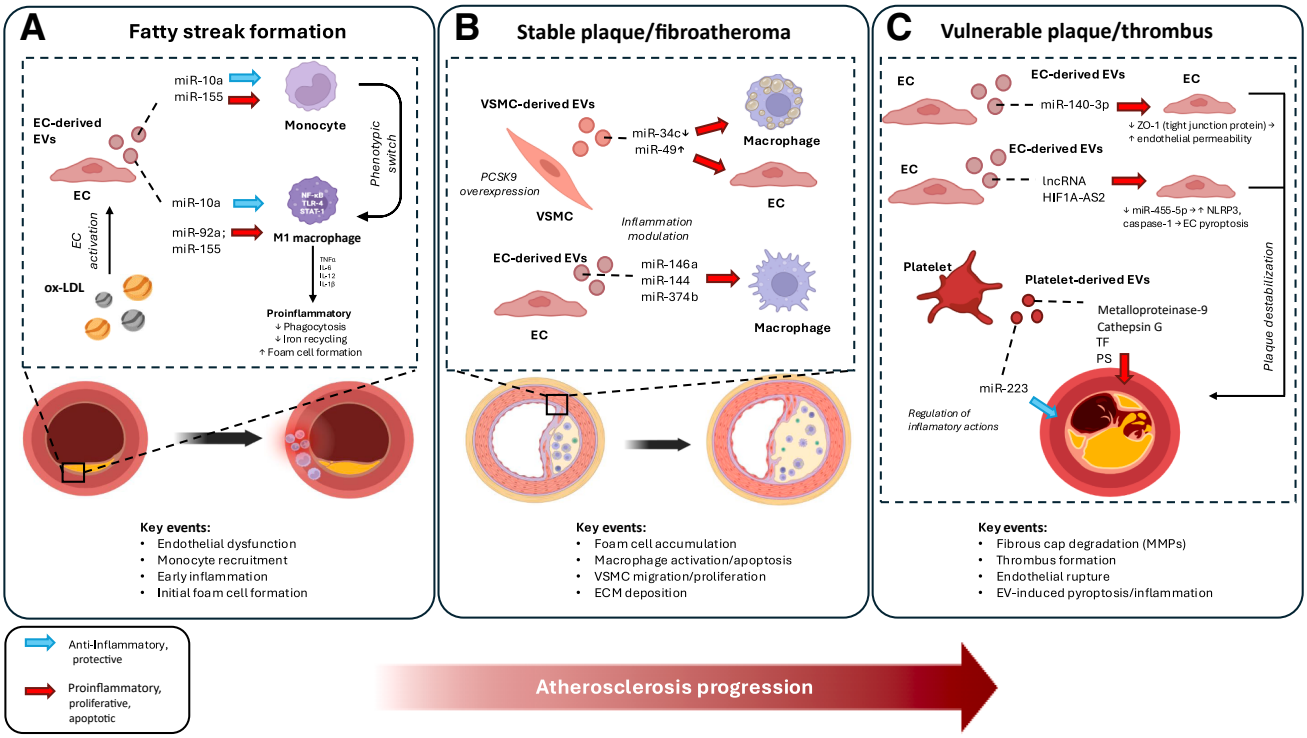

Figure 2 EV-mediated mechanisms in atherosclerosis progression. **A**—**C:** This schematic illustration depicts the contribution of EVs and their molecular cargo to fatty streak formation (**A**), stable plaque/fibroatheroma development (**B**), and plaque destabilization and thrombus formation (**C**). **A:** In early atherogenesis, endothelial cells (ECs) release EVs containing miRNAs, such as miR-10a and miR-155, which modulate monocyte recruitment and macrophage polarization. Exposure to oxidized low-density lipoprotein (ox-LDL) leads to EC-derived EVs to be enriched in miR-92a, that when transported to macrophages favors their phenotypic switch. **B:** During plaque progression, vascular smooth muscle cell (VSMC)—derived EVs exposed to proprotein convertase subtilisin/kexin type 9 (PCSK9) cells carry a pattern of miRNAs (eg, ↓miR-34c, ↑miR-49) that contribute to pro-atherogenic signaling in macrophages and ECs. EC-derived EVs enriched in miR-146a, miR-144, and miR-374b modulate inflammatory responses in macrophages. **C:** In advanced plaques, EC-derived EVs carrying miR-140-3p decrease zonula occludens protein-1 (ZO-1; a tight junction protein), increasing endothelial permeability. EC-derived EVs also contain the long noncoding RNA (lncRNA) HIF1A antisense RNA 2 (HIF1A-AS2), which suppresses miR-455-5p, leading to nucleotide-binding domain, leucine-rich—containing family, pyrin domain—containing-3 (NLRP3) inflammasome activation and endothelial pyroptosis. Platelet-derived EVs contribute anti-inflammatory activities (miR-223) and prothrombotic factors (tissue factor, phosphatidylserine, cathepsin G, and metalloproteinase-9), supporting thrombus formation. Figure was generated using BioRender.com (Toronto, ON, Canada). MMP, matrix metalloproteinase; PS, phosphatidylserine.

or pyroptosis through mechanisms involving the long noncoding RNA HIF1A antisense RNA 2, which suppresses miR-455-5p to activate estrogen-related receptor γ, caspase-1, and inflammasome (nucleotide-binding domain, leucine-rich—containing family, pyrin domain—containing-3).[66]

Treatment with oxidized LDL and IL-6 also increases the expression of miR-92a-3p in ECs and up-regulates the expression level of endothelial EV-incorporated miR-92a-3p, fostering angiogenic responses in recipient ECs in a STAT3—thrombospondin 1—dependent manner.[67] Given the crucial role of EVs in mediating intercellular communication among different cell types, a further step was to investigate the transfer of miR-92a-3p, carried by endothelial EVs, into VSMCs and monocytes, and monitor the resulting phenotypic changes in these recipient cells. Co-culture experiments showed that EV uptake occurred in VSMCs, but not in monocytes, and that the transfer of miR-92a-3p enhanced VSMC migration and proliferation.[67]

During lesion growth, VSMCs switch from a contractile to a proliferative state and migrate into the intima. Phenotypically modified VSMCs can influence neighboring cells in a paracrine manner by secreting a wide range of bioactive molecules,[86] some of which could be packed into EVs to be delivered to recipient cells favoring atherosclerotic processes. In this context, an *in vitro* study investigated EVs derived from VSMCs overexpressing proprotein convertase subtilisin/kexin type 9,[68] one of the primary regulators of LDL cholesterol.[87] These EVs were found to induce a pro-atherogenic phenotype in recipient cells, enhancing adhesion molecule expression in ECs, stimulating proinflammatory cytokine production in monocytes, and promoting oxidized LDL uptake in macrophages.[68] hsa-miR-34c, hsa-miR-29, hsa-miR-148b, hsa-miR-221, and hsa-miR-125b were down-regulated, whereas hsa-miR-49 was up-regulated. These EVs showed changes in miRNAs involved in both atherosclerosis and inflammation.[68]

The contribution of EVs to thrombotic events is because of their procoagulant surface and the expression of highly

Table 3 EVs in Atherosclerosis: Preclinical and Clinical Evidence

Preclinical studies	
Study description	Key findings
Mice fed high-phosphate diet.[74]	EC-derived EV miR-670-3p promotes arterial calcification by targeting IGF-1.
ApoE$^{-/-}$ mice fed high-fat diet for 6 weeks.[75]	EVs derived from ECs expressing the shear-responsive transcription factor Krüppel-like factor 2 reduced atherosclerotic lesion formation by transporting miR-143/miR-145.
ApoE$^{-/-}$ mice fed high-fat diet.[76]	miR-155-5p—enriched EVs promoted the occurrence of carotid atherosclerosis by increasing permeable and angiogenic activity.
ApoE$^{-/-}$ mice fed high-fat diet for 12 weeks.[77]	miR-132/miR-212 contained in adipose tissue—derived EVs exacerbated atherosclerosis progression.
ApoE$^{-/-}$ mice fed high-fat diet for 12 weeks.[73]	Administration of EVs derived from ECs enriched in miR-34c-5p markedly reduced atherosclerosis progression. miR-34c-5p mitigates macrophage infiltration into atherosclerotic lesions.
Human studies	
Study description	Key findings
SAFEHEART cohort.[78]	miR-133a levels in EVs associated to an increased risk of cardiovascular events.
SAFEHEART cohort.[79]	miR-122-5p expression was increased in patients with FH compared with controls. miR-21-5p was higher only in those patients with FH carrying a mutation in LDLR.
Patients with stable coronary artery disease (n = 181). Angiographic coronary artery disease was defined as stenosis of 50% in at least one major epicardial coronary artery.[80]	EV-sorting experiments showed that endothelial cells and platelets were the major cell sources of EVs containing miR-126 and miR-199a. EVs containing miR-126 and miR-199a, but not freely circulating miRNA expression, predict the occurrence of cardiovascular events.
Obese individuals recruited in the cross-sectional SPHERE study.[81–83]	PCSK9 may impact the release of EV-derived miRNAs linked to atherosclerosis (hsa-miR-362, hsa-miR-150, hsa-miR-1244, hsa-miR-520b-3p, and hsa-miR-638).
Obese individuals recruited in the cross-sectional SPHERE study.[82]	BDNF levels have been linked to an increase in 80 EV-derived miRNAs and in a decrease of 59 miRNAs related to atherosclerosis and thrombosis. At least 18 genes were targeted by these miRNAs, 7 of which were involved in depression and cardiovascular risk.
Obese individuals recruited in the cross-sectional SPHERE study.[83]	Nine EV-derived miRNAs (let-7c-5p, miR-106a-5p, miR-143-3p, miR-185-5p, miR-218-5p, miR-331-3p, miR-642-5p, miR-652-3p, and miR-99b-5p) were down-regulated in response to PM_{10} exposure. let-7c, miR-331, miR-185, miR-106a, and miR-652 seemed to mediate the association between PM_{10} exposure and increased fibrinogen levels.
Veterans Affairs Normative Aging Study.[84]	EV-associated miR-223-3p and miR-199a/b positively modified the association between 1-year $PM_{2.5}$ ambient levels and blood pressure.

ApoE, apolipoprotein E; BDNF, brain-derived neurotrophic factor; EC, endothelial cell; FH, familial hypercholesterolemia; IGF-1, insulin-like growth factor 1; LDLR, low-density lipoprotein receptor; PCSK9, proprotein convertase subtilisin/kexin type 9; PM, particulate matter; SAFEHEART, Spanish Familial Hypercholesterolemia Cohort Study; SPHERE, Susceptibility to Particle Health Effects, miRNAs and Exosomes.

procoagulant proteins, such as tissue factor, and the externalization of anionic phospholipids, mainly phosphatidylserine, that significantly contribute to EV-associated procoagulant activity[88]; conversely, platelet-derived miRNAs seem to be atheroprotective. The most highly expressed miRNAs in platelet-derived EVs are miR-126-3p, miR-21, miR-223, miR-339, miR-328, miR-22, miR-185, and miR-320b.[89] Li et al[69] reported that thrombin activated platelet-released EVs enriched in miR-223 that when transferred into tumor necrosis factor-α—stimulated ECs (human umbilical vein endothelial cells) inhibited intercellular adhesion molecule 1 expression. Overall, it is known that miR-223 suppresses inflammation largely via controlling translation of its target genes, rather than levels of target gene transcripts.[90] Conversely, EVs isolated from platelet of patients with acute coronary syndrome, which are enriched in miR-126, seem to promote human umbilical vein endothelial cell proliferation and migration.[91]

EVs in Atherosclerosis: Preclinical Evidence

EVs derived from ECs have been described as active participants of the atherosclerotic process. These EVs, besides serving as indicators for endothelial dysfunction, appear to directly interact with the endothelium, therefore further aggravating the process.[92,93] In mice fed high-phosphate diet, EC-derived exosomes enriched with miR-670-3p

were found to significantly enhance VSMC calcification by down-regulating insulin-like growth factor 1.[74] This suppression of insulin-like growth factor 1 promotes osteogenic differentiation, a pivotal process driving the progression of arterial calcification. Considering that the presence and burden of coronary calcification provide direct evidence of the presence and extent of coronary artery disease,[94] EVs act as key mediators of crosstalk between ECs and VSMCs, exacerbating arterial calcification in high-phosphate conditions.[74]

In apolipoprotein $E^{-/-}$ mice, EVs derived from ECs (human umbilical vein endothelial cells) expressing the shear-responsive transcription factor Krüppel-like factor 2 reduced atherosclerotic lesion formation by transporting miR-143/miR-145, which, when transferred to VSMCs, aided in preventing VSMC dedifferentiation.[75] In the same mouse model, miR-155-5p–enriched exosomes promoted the occurrence of carotid atherosclerosis by increasing permeable and angiogenic activity.[76] When apolipoprotein $E^{-/-}$ mice were fed a high-fat diet, mi-132/miR-212 contained in adipose tissue–derived EVs exacerbated atherosclerosis progression by promoting endothelial apoptosis, proliferation, and migration of VSMCs within the plaque.[77] Tail vein administration of EVs derived from ECs enriched in miR-34c-5p, on exposure to laminar shear stress, markedly reduced atherosclerosis progression compared with mice treated with phosphate-buffered saline. This effect could have been partly attributed to the ability of miR-34c-5p to mitigate macrophage infiltration into atherosclerotic lesions.[73]

A summary of the preclinical animal models, implicated miRNAs, and their corresponding effects is given in Table 3.

EVs in Atherosclerosis: Human Evidence

Characterizing EVs from various cell types and their cargo may provide valuable diagnostic and prognostic insight. Demonstrating the prognostic utility of EVs, a study conducted on the Spanish Familial Hypercholesterolemia Cohort Study cohort investigated the EV signature for predicting cardiovascular events in patients with familial hypercholesterolemia (FH), without clinical disease at baseline. Patients who experienced cardiovascular events within an average of 3.3 years had significantly higher levels of EVs, derived from lymphocytes, neutrophils, and activated platelets, compared with age/cardiovascular risk factor/treatment-matched patients with FH who did not experience an event within the same time period. Baseline numbers of these EVs were positively associated with mortality at follow-up. Overall, the characterization of EVs did improve the predictive accuracy of the Spanish Familial Hypercholesterolemia Cohort Study risk model, significantly increasing its area under the curve.[8] An additional analysis of the Spanish Familial Hypercholesterolemia Cohort Study cohort identified a signature of 10 miRNAs among patients with FH experiencing a cardiovascular event and non-FH hypercholesterolaemic relatives without cardiovascular events. Indeed, high plasma miR-133a levels associated to an increased risk of cardiovascular events. miR-133a targets genes involved in regulation of the cell-membrane lipid-receptor LRP6 and inflammatory cytokines (CXCL8, IL-6, and tumor necrosis factor).[78] A small RNA-sequencing analysis of EV plasma from 54 patients with FH and 38 normolipidemic individuals showed that among approximately 2000 miRNAs, miR-122-5p expression was increased in all patients with FH compared with controls, whereas miR-21-5p was higher only in those patients with FH carrying a mutation in LDL receptor (*LDLR*).[79]

Given that atherosclerotic plaques are commonly found in most patients with acute coronary syndrome, it is noteworthy that miR-208a expression was up-regulated in the serum EVs of these patients. Furthermore, among the affected patients, those exhibiting higher miRNA-208a expression tended to be older and presented with more severe clinical indicators (eg, Killip class, creatine kinase–myocardial band, cardiac troponin T, and LDL cholesterol) compared with those with lower miR-208a expression. Additionally, patients with higher miR-208a expression showed a decreased survival rate.[95] Healed plaques, which indicate prior plaque destabilization, are frequently found in coronary arteries. Now that these plaques can be identified in living individuals, it is noteworthy that, in patients with stable coronary artery disease, elevated levels of miR-126 and miR-199a in circulating EVs were significantly associated with a reduced incidence of major adverse cardiovascular event rate. Endothelial cells and platelets were the major cell sources of these EVs.[80]

Obesity remains a significant independent risk factor for atherosclerosis, even when accounting for other established contributors, such as hypertension, dyslipidemia, and smoking.[96] In obese subjects, proprotein convertase subtilisin/kexin type 9 may impact the release of EVs derived from atherosclerotic components (ie, platelets, endothelium, monocytes/macrophages, and neutrophils) as well as EV-derived miRNAs linked to atherosclerosis (hsa-miR-362, hsa-miR-150, hsa-miR-1244, hsa-miR-520b-3p, and hsa-miR-638) and their related targeted genes [eg, *LDLR*, toll-like receptor 4 (*TLR4*), and estrogen receptor 1 (*ERS1*)].[81]

Obesity is also known to be associated to complex social and psychological facets, including depression. In this scenario, genome-wide association studies have identified pleiotropic genes that are in common for mood disorders and cardiometabolic diseases. Among these genes, brain-derived neurotrophic factor has been described to be involved in the etiology of both obesity and depression.[97] Brain-derived neurotrophic factor levels have been linked to an increase in 80 EV-derived miRNAs and in a decrease of 59 miRNAs related to atherosclerosis and thrombosis. At

least 18 genes were targeted by these miRNAs, 7 of which were involved in depression and cardiovascular risk.[82] According to the knowledge that metabolic syndrome is associated with an approximately twofold increased risk of developing ASCVD, EVs isolated from these patients showed increased levels of Ras-associated protein 1 (Rap1)−EVs, a feature correlating with cardiovascular risk, including stenosis. Rap-1 is a small GTPase that belongs to the Ras family of GTPases, and it is a key player in metabolic diseases.[98]

Although numerous experimental and clinical studies now support a positive association between the atherosclerotic process and exposure to various air pollutants, the precise mechanisms behind this relationship remain unclear.[99] Air pollutants impact the selective loading of EV cargo, including miRNA and proteins, which can modify the functionality of the recipient cells.[100] Thus, although clinical outcomes have not been described, it is worth highlighting the liaison between particulate matter (PM), EV-miRNA content, and cardiovascular risk factors that contribute to the progression of atherosclerosis. In participants randomly selected from the Veterans Affairs Normative Aging Study, EV-associated miR-223-3p and miR-199a/b positively modified the association between 1-year $PM_{2.5}$ ambient levels and blood pressure, suggesting that the magnitude of the effect of $PM_{2.5}$ levels on blood pressure differs depending on the levels of these EV-derived miRNAs. *In silico* analysis showed that EV-associated miR-223-3p and miR-199a/b could potentially target several proteins implicated in important cardiovascular functions.[84] Short-term exposure (day before blood drawing) to PM_{10} has been described to associate with an increased release of EVs. This effect was mainly due to EVs derived from monocyte/macrophage components ($CD14^+$) and from platelets ($CD61^+$). Nine miRNAs were down-regulated in response to PM_{10} exposure, five of which (let-7c, miR-331, miR-185, miR-106a, and miR-652) seemed to mediate the association between PM exposure and increased fibrinogen levels.[83]

Finally, although not in the remit of this review, it is important to highlight the recent development of an EV-based index of aging, termed EVaging, by stratifying EV profiles by age decade. With the aim to investigate the ability of this index to predict chronological age and assess its capacity to reflect aging-associated processes and cardiovascular risk profiles, Burrello et al[101] demonstrated the association between an EVaging index and age in healthy individuals, which was capable of distinguishing cardiovascular risk profiles in patients, correlating with cardiovascular outcomes and likelihood of fatal cardiovascular events according to the European Society of Cardiology Systematic Coronary Risk Evaluation, and reflecting age-associated comorbidities.

A summary of the various human-based studies and the implicated miRNAs is reported in Table 3.

Future Directions

EVs possess peculiar characteristics that render them promising in the context of drug delivery, offering a potential solution to stability issues and avoiding off-target tissue adverse effects. The amphipathic nature of EV membranes provides a versatile chaperone for transporting hydrophobic and hydrophilic molecules. Indeed, besides safeguarding their luminal cargo even in biofluids, the EV surface is loaded with proteins conferring them a natural affinity for specific tissues at distant sites, rendering them versatile signaling mediators in the context of new therapeutic tools.[102] EVs have targeting properties that allow them to release the drugs directly in the target tissue or cell, enhancing therapeutic properties and efficacy of the drugs.[103] However, for EVs to be successfully translated into clinically applicable therapeutic tools, substantial advancements are necessary. Challenges include methods for consistent and safe large-scale production of EVs,[104] separation and characterization methods, definition of biomarkers for EV safety, and studies of EV pharmacokinetics, biodistribution, and mechanism of action. In this context, it will be also crucial to understand the interactions between EVs and lipoproteins *in vivo*, which could positively or negatively affect EV biodistribution.[105]

Although interest in the clinical application of EVs is rapidly increasing, most EV-related clinical trials are centered on diagnostic and companion diagnostic purposes, with relatively few exploring their therapeutic potential.[106] The clinical translation of EV-based therapies faces several key challenges, including an incomplete understanding of their mechanism of action, the need to ensure long-term safety, and the establishment of appropriate dosing regimens and routes of administration. Furthermore, logistical barriers, such as limited scalability, high production costs, and time constraints, represent significant obstacles to their broader clinical adoption.[102]

Conclusions

With the introduction of advanced separation and characterization technologies, it has become evident that not all EVs are identical. Some originate from endosomal compartments, others from plasma membrane blebbing, and some from cytokinetic bridges. In the context of the atherosclerotic burden, characterized by a reciprocal relationship between different cell types (eg, endothelial cells, fibroblasts, smooth muscle cells, and inflammatory cells), EVs occupy a unique niche, offering valuable insights for developing new EV-centered strategies for treatment of ASCVD. Machine learning approaches, which are adept at

capturing nonlinear associations and integrating vast amounts of medical information, including multi-omics data, can be used to extract crucial features from EV content and build diagnostic models, achieving superior specificity and sensitivity for ASCVD prognosis.[107]

Author Contributions

A.S.R. wrote the original draft and performed the literature review; I.F. performed the literature review; S.C. critically revised the manuscript; A.R. critically reviewed the sections pertaining to the biology of extracellular vesicles; and C.M. and M.R. conceived the topic of the manuscript and wrote the manuscript.

Disclosure Statement

None declared.

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