EXTRACELLULAR VESICLE-MEDIATED PROCESSES IN CARDIOVASCULAR DISEASES

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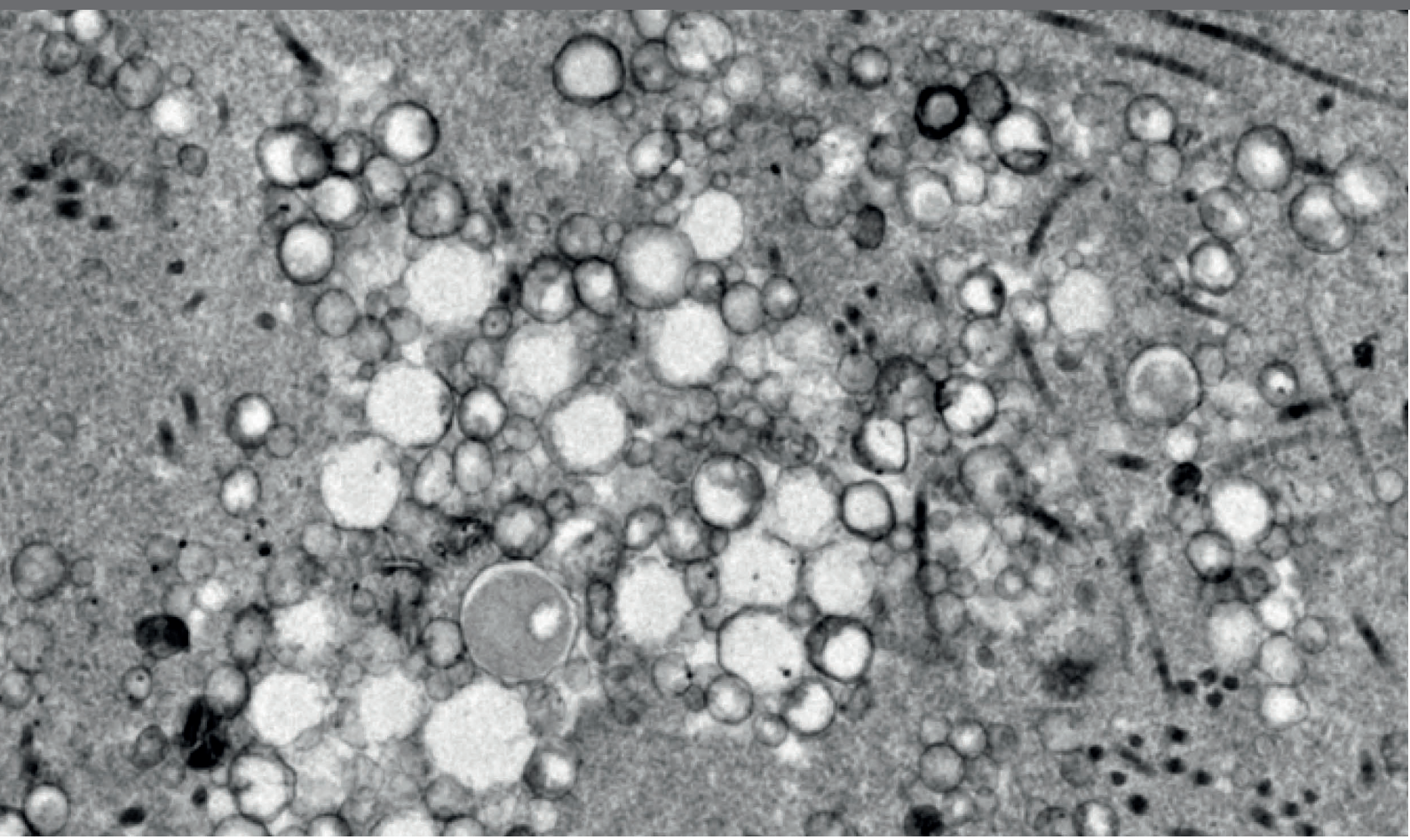

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EXTRACELLULAR VESICLE-MEDIATED PROCESSES IN CARDIOVASCULAR DISEASES

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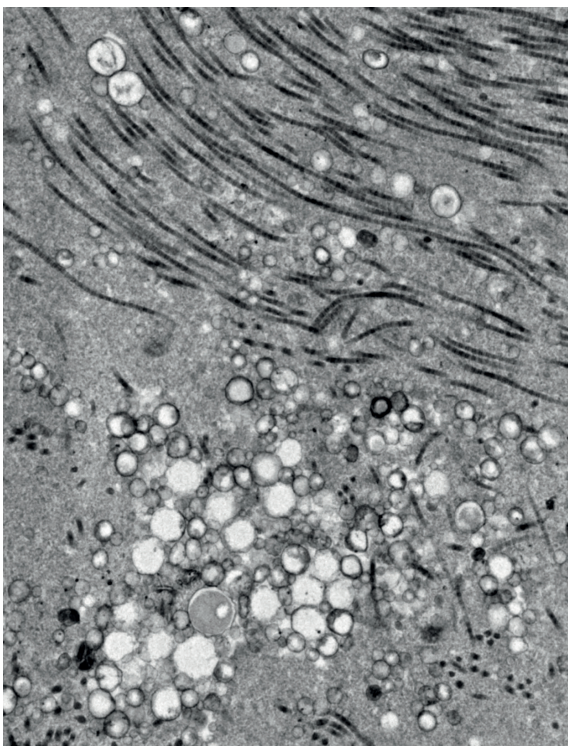

Transmission electron microscopy image of human atherosclerotic plaque shows numerous extracellular vesicles within collagen fibers. Image: Elena Aikawa.

It is long known that many cells can shed extracellular vesicles, small membrane-enclosed cell fragments. Although the existence of extracellular vesicles has been recognized for many years, researchers are only beginning to understand their physiologic significance. Several recent studies have demonstrated that extracellular vesicles released from cells serve as a mode of cellular communication. They can carry diverse molecular payload (e.g. nucleic acids, bioactive lipids and proteins) to distal organs and recipient cells. Extracellular vesicles can be classified into three major groups: exosomes, microvesicles, and apoptotic bodies. All these types of extracellular vesicles can be found in a variety of biologic specimen and their numbers, distribution and composition may serve as biomarkers for various disorders, including cardiovascular disease. Although extracellular vesicle-mediated processes are currently best characterized in the fields of cancer biology and

neurobiology, evidence is accumulating that extracellular vesicles play a key role in the pathophysiology of diabetes, thrombosis, inflammation and cardiovascular calcification.

In this Research Topic, we invited review and methodological articles that advance our understanding of extracellular vesicle-related processes in vascular biology.

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EDITORIAL
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Editorial: Extracellular Vesicle-Mediated Processes in Cardiovascular Diseases

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Keywords: microparticle, atherosclerosis, vascular calcification, diagnostic, biomarker, heart valve

Editorial on the Research Topic

Extracellular Vesicle-Mediated Processes in Cardiovascular Diseases

The review articles provided in this research topic highlight extracellular vesicles (EV) and the processes that they regulate in cardiovascular diseases. Even if landmark achievements have initially been within the field of cancer biology, the importance of EVs in cardiovascular disease is impossible to overlook. To provide the readers with a state-of-the-art knowledge in the field of EV-mediated processes in cardiovascular diseases, we have composed a broad collection of contributions from experts in the field. In this research topic we cover several aspects of EVs, from their (patho-)physiologic functions in cardiac development and regeneration, their role in angiogenesis and atherosclerosis, their involvement in diabetes-related cardiovascular complications, to the exciting implications in cardiovascular calcification. In addition, the optimization of EV measurement methods and their use as biomarkers and potential therapeutics have been highlighted.

Having been disregarded as mere cellular debris for decades, EVs are increasingly being appreciated as integral mediators of cell-to-cell communication. EVs can be classified into three major groups differentiated by size and origin: exosomes (40–100 nm), consisting of cytoplasmic compartments released by exocytotic processes; microvesicles (100–500 nm), generated by budding of the plasma membrane; and apoptotic bodies (~1,000 nm), that are shed by cells undergoing programmed death. As every cell is able to release EVs, their body-wide physiologic and pathologic relevance is self-evident. The proteins and genetic material of EVs may reflect the conditions of the parental cells. To carry message to distal parts of the body, loaded with concentrated cargo miniscule EVs that able to move easily in extracellular space and circulation are likely intendent for a specific function such as interaction via carrying diverse molecular payload (e.g., nucleic acids, bioactive lipids, and proteins) to distal or neighboring recipient cells. This essential biological function of EVs helps to maintain tissue homeostasis in health or contribute to the disease when vesicles acquire pathological properties.

In the contribution by Gross and Zelarayán, the roles of Wnt signaling in cardiac development and during cardiac stress are discussed. The transmission of Wnt signals between cells has been shown to occur through EV. Given the importance of Wnt signaling for general physiology, this likewise highlights EV's physiological relevance, being integral signaling transporters.

Changes in the EV concentrations during cardiovascular diseases have been observed in many studies. Since platelets are among the most abundant blood cells, they represent a major source of circulating EVs. In the article by Zaldivia et al., the functions of EVs derived from platelets are discussed in the pathogenesis of venous thrombosis, atherosclerosis, and myocardial infarction. Yet

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EVs from various cellular origins are also recognized as mediators of cellular crosstalk, particularly in atherosclerosis and angiogenesis. This is discussed in the comprehensive reviews by Badimon et al. and van der Vorst et al., together with the possible exploitation of EVs as prognostic or diagnostic biomarkers, or as therapeutics. The overview by Gustafson et al. expands the discussion by focusing on diabetic complications such as cardiomyopathy and atherosclerosis.

A novel role for EVs was identified in the process of cardiovascular calcification, a complication of atherosclerosis, diabetes, chronic kidney disease, and aortic valve stenosis. Calcifying EVs serve as fundamental building blocks of calcification. In vasculature, calcifying EVs aggregate to form microcalcifications contributing to atherosclerosis plaque rupture and subsequent myocardial infarction. In cardiac valves, calcific aggregates result in increased leaflet stiffness causing aortic stenosis, heart failure, and death. As highlighted in the contribution by Bakhshian Nik et al., EVs derived from smooth muscle cells, valvular interstitial cells and macrophages can modulate cardiovascular calcification. Emerging evidence suggests that platelet-derived EVs may also contribute to vascular calcification, ultimately leading to atherothrombotic complications, as outlined by the article from Schurgers et al..

As defined above, exosomes are a subclass of EVs originating from late endosomal compartments. Although initially thought as a mechanism of cellular waste disposal, exosomes are currently well-established mediators of cell-to-cell communication. The exosomes specialized in communication are termed "signalosomes" in the elegant review by Willis et al., harboring molecular cargo capable of influencing the behavior of recipient cells. Such signalosomes act as immunomodulators in models of lung disease, raising an intriguing possibility for use of purified signalosome-type exosomes for a clinical therapeutic application. The hurdles and perspectives for EV therapy are extensively outlined in this review.

Among such hurdles are the proper purification, potency determination and quantification of EVs, which is largely due to their small size. A novel protocol for the size and count measurement of EV between 50 and 120 nm is presented by Parsons et al.. This promising methodology will improve the accuracy of EV characterization in various biologic fluids from both healthy and diseased individuals.

Accurate categorization of EVs is also crucial for their meaningful implementation as biomarkers. The review by Dickhout and Koenen provides an overview of the prerequisites of the use of EVs as biomarkers, dealing with methods and issues of isolation, determination, and classification. An overview of (pre-)clinical studies using EVs as biomarkers is also provided and the potential of EVs as biomarkers for cardiovascular disease is critically discussed.

With the above reviews, we aim to convince the reader that EVs are integral components of an intricate cellular communication system in health and disease, while simultaneously providing a differentiated overview of the current challenges and obstacles, regarding EV characterization and their implementation as biomarkers or therapeutics. Nevertheless, exciting times are ahead of us as EVs are heading straight to the heart of cardiovascular research.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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MINI REVIEW
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The Mingle-Mangle of Wnt Signaling and Extracellular Vesicles: Functional Implications for Heart Research

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Wnt signaling is an important pathway in health and disease and a key regulator of stem cell maintenance, differentiation, and proliferation. During heart development, Wnt signaling controls specification, proliferation and differentiation of cardiovascular cells. In this regard, the role of activated Wnt signaling in cardiogenesis is well defined. However, the knowledge about signaling transmission has been challenged. Recently, the packaging of hydrophobic Wnt proteins on extracellular vesicles (EVs) has emerged as a mechanism to facilitate their extracellular spreading and their functioning as morphogens. EVs spread systemically and therefore can have pleiotropic effects on very different cell types. They are heavily studied in tumor biology where they affect tumor growth and vascularization and can serve as biomarkers in liquid biopsies. In this review we will highlight recent discoveries of factors involved in the release of Wnts on EVs and its potential implications in the communication between physiological and pathological heart cells.

Keywords: extracellular vesicles, exosomes, Wnt signaling, Wnt secretion, heart remodeling

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WNT SIGNALING PATHWAYS OVERVIEW

Wnts are evolutionarily conserved, secreted glycosylated growth factors, which in humans are encoded by 19 different Wnt genes. There are more than 15 different Wnt receptors and co-receptors, including Frizzled (FZD1-10), LRP5 and 6, and ROR1/2 that are best described. Depending on their binding to receptors and downstream components, Wnt signaling has been classified into canonical (β-catenin-dependent) or non-canonical (β-catenin-independent) pathways. The β-catenin-independent pathways include Planar Cell Polarity (PCP) and Wnt–Ca^{2+} pathway [reviewed in (1)].

The β-catenin-dependent pathway is activated by binding of Wnts with FZDs and LRP5/6, and subsequent GSK3β inhibition, leading to stabilization of cytoplasmic β-catenin. Upon accumulation, β-catenin enters the nucleus binds to TCF (T cell factor)/LEF (lymphoid enhancer-binding factor) transcription factors and regulates the transcription of target genes. Additionally, β-catenin-independent Wnt pathways use different downstream signaling modules. The PCP signaling is activated via FZDs receptors with ROR1/2 and PTK7 as co-receptors, through a cascade of small GTPases RAC1, RHOA and JUN-N-terminal kinase (JNK) activation. This pathway leads to changes in cytoskeleton, cell polarity and activation of JNK-dependent transcription factors and their target genes [reviewed in (2)].

A second β-catenin-independent pathway is the Wnt–Ca^{2+}pathway. Here, Wnts trigger FZD-mediated activation of heterotrimeric G proteins. This activates phospholipase C (PLC), diacyl-glycerol

(DAG) and inositol-1,4,5-trisphosphate [Ins(1,4,5)P3] cascade triggering intracellular Ca^{2+}-release and activation of effectors such as calmodulin-dependent kinase II (CAMKII), calcineurin and protein kinase C (PKC), which activate the transcriptional regulator nuclear factor associated with T cells (NFAT) (3).

In addition to Wnts, several inhibitors, such as Dickkopfs (DKK1-3), secreted Frizzled related proteins (SFRP1-5) and activators, such as R-Spondins (RSPO1-5) are similarly secreted and can regulate the amplitude and specificity of Wnt signaling at the extracellular level (4). Additionally, intracellular cytosolic and nuclear inhibitors fine-tune Wnt signaling.

WNT COMPONENT MOBILIZATION IN EXTRACELLULAR VESICLES

Currently, increasing evidences show the importance of different Wnt signaling branches and their crosstalk among different cell types. Secreted Wnts are lipidated and this hydrophobic moiety hinders them to freely move in the extracellular space. Different experimental proofs showed how their biochemical properties would fit to the idea of "diffusible" morphogens (5). Lipoprotein particles (6, 7), filopodia-surfing (8, 9) and transport on extracellular vesicles (10) were shown to confer biological Wnt activity in different systems [reviewed (11)]. "Extracellular vesicles (EVs)" is a term used for different sub-populations of membrane particles secreted from a plethora of cells into the extracellular space. Based on size and subcellular origin, they are discriminated into exosomes (50–100 nm), microvesicles (100–500 nm) and apoptotic bodies (>1000 nm) (12). Distinct proteins as well as lipid markers allow characterization of different types of EVs (13). CD9 or EMMPRIN are normally found in larger, plasma membrane-derived EVs, while components of the endosomal sorting complexes required for transport (ESCRT) machinery, such as Tsg101 and Alix are markers for the endosome-derived exosomes (14, 15). EVs are purified by differential ultracentrifugation, gel filtration or immunoprecipitation, while their size and composition are investigated by nanoparticles tracking, electron microscopy, immunoblotting and mass spectrometry. Standards for their purification and analysis have been defined and can help to increase reproducibility of EV studies (16). Currently, cell type-specific markers for EVs are missing.

Exosomes carrying Wnts were shown to play key roles under physiological conditions in different systems (17) (**Table 1**). The first evidence that Wnts might be released on membrane-bound structures came from studies of *Drosophila* Wnt, Wingless. In *Drosophila*, exosomes, containing the Wnt secretion factor Evi, transport Wnts across the Drosophila neural-muscular junctions (NMJ) and in the wing imaginal disc (10, 31, 32). Recently, a crosstalk was discovered in tooth development, activating Wnt/β-catenin signaling in mesenchymal cells via exosomal miRNA from epithelial cells (33).

As EVs are detectable in the circulation, it was conceived that their activity spreads systemically. Indeed, under pathological conditions, their functionality has long-range activity influencing metastatic niches far away from their source [reviewed in (34)]. Further examples are: (1) Tethering of autocrine Wnt11 to fibroblast-derived exosomes to influence the migratory phenotype of breast

TABLE 1 | Recent studies about mechanisms and effects of Wnt release in mammalian systems

Mechanism of Wnt release	Genes	Cell/Organism	Pathways/Details	References
Paracrine exosomal Wnts	Wnt3a	Diffuse large B-cell lymphoma	Wnt/β-catenin signaling	(18)
Paracrine exosomal Wnts	Wnt2b Possibly also Wnt10a	Epididymis/mouse	Differentiation/maturation Wnt/STOP	(19)
Paracrine exosomal Wnts	Wnt4	Human umbilical cord MSC in rat skin burn model	Angiogenesis and cell proliferation via Wnt/β-catenin signaling	(20–22)
Paracrine exosomal Wnts	Wnt4	Hypoxic colorectal cancer cells (HCT116, HT29)/endothelial cells (HUVEC)	HIFα-dependent Wnt4 expression Proliferation	(23)
Autocrine exosomal Wnts	Wnt11	Human umbilical cord MSC *in vitro*	Release stimulated by 3,3′-Diindolylmethane	(24)
Polarized exosomal Wnts	Apical/baso lateral Wnt3a, Apical Wnt11	Dog Kidney cells, MDCK	Basolateral Tsg101+ Apical CD63 + apical Wnt secreted in a lipidation-independent manner	(25)
Paracrine exosomal Wnts	Wnt5b	Colon and pancreatic cancer cells Caco-2, Panc-1	Several Wnts found in the supernatant after exosomes purification, such as Wnt3a and Wnt5a from L-cells	(26)
Paracrine exosomal Wnts	Wnt10b	Fibroblasts and breast cancer cells	Proliferation and migration	(27)
Crosstalk of Extravesicular Wnt	Wnt5a	Macrophages and breast cancer cells (SkBr3)	Wnt5a expression and cell invasion	(28)
Paracrine exosomes mobilize autocrine Wnts	Wnt11	Breast cancer cells (MDA-MB-231)	Cancer cell migration	(29)
Paracrine Exosome mobilize autocrine Wnts	Wnt10b	Cortical neurons Rat Optic nerve	Regeneration, mTOR	(30)
Neutral sphingomyelinases dependent trafficking of Wnts onto different EVs	Wnt3a and Wnt5a	Breast cancer cells (SkBr3)	Block of exosomes secretion increases microvesicles release	(13)
Paracrine Exosomal activating Wnt canonical	Wnt/β-catenin	Ischemia/reperfusion rat heart	Enhances cardiomyocyte survival and decreased apoptosis	(20)

cancer cells (29); (2) colorectal tumor cells signal to endothelial cells (EC) by HIFα-induced exosomal Wnt4 secretion, activating Wnt/β-catenin signaling to increase migration and proliferation of ECs (23). Although this work is focused on cancer cells, activation of HIFα upon hypoxia plays a role in cardiac stress (23) and might have similar effects on EC crosstalk in cardiovascular pathologies.

IMPLICATION AND CAVEATS OF THE WNT SIGNALING AND EVS IN THE HEART

Heart function is based on a well-controlled communication system between different cell types. Although, EVs are well appreciated in the process of tumor and infection biology**,** research on cardiac EVs is increasing. So far no direct evidences for secretion of Wnt components on EVs from heart cells exist. Thus, we will integrate evidences from other fields, which may help to advances the knowledge on EVs/Wnt-mediated mechanisms in heart tissue.

In Heart Development and Tissue Regeneration

Wnt signaling is crucial for embryonic development and tissue regeneration (3, 35). Specifically in cardiogenesis, activation of the Wnt/β-catenin signaling induces mesodermal formation, cardiac progenitor cell specification and maintenance, but inhibits further differentiation towards cardiomyocytes (36). Ectopic inactivation of the Wnt/β-catenin signaling in a tissue other than cardiac mesoderm, such as endoderm, is sufficient to trigger differentiation towards cardiac cells, indicating the central role of Wnt in cardiac cell formation (37). Several Wnt ligands are expressed in the early heart including Wnt2, Wnt2b, Wnt11, and Wnt8a, indicating the participation of canonical and non-canonical branches (38). Indeed, initial activation of Wnt/β-catenin signaling is followed by an activation of the Wnt/β-catenin-independent pathway, which represses the canonical signaling and regulates cell processes (39). Moreover, Wnt5a and Wnt11 promote cardiac differentiation in embryonic and adult stem cells through non-canonical pathways and may be necessary to balance β-catenin-dependent proliferation in the outflow tract (38, 40, 41). Hence, Wnt signaling is a network of inter-linked branches engaging different cell populations into intercellular crosstalk. Further details of the role of Wnt signals during cardiogenesis are extensively described elsewhere (36).

Notably, heart regeneration mechanisms vary among species. In contrast to the limited injury-induced regeneration in early stages of life in adult mammal hearts, lower vertebrate like amphibian and teleost fish have sufficient regenerative capacity upon injury mainly by dedifferentiation of cardiomyocytes (35). In mouse, Wnt signaling exerts a similar role on adult cardiac progenitor cell (CPC) homoeostasis as observed during embryogenesis. Wnt/β-catenin activation impairs cardiomyocytes lineage differentiation and enhances endothelial cell (ECs) expansion, whereas its inactivation increases cardiomyocytes and reduced EC lineages (42–44). Accordingly, intra-myocardial injection of Wnt3a post-ischemia reduces CPC differentiation into cardiomyocytes (45). However, the role of Wnt signaling and most importantly the intercellular crosstalk in endogenous regeneration remains unclear. Interestingly, in the regenerative zebrafish hearts Wnt/β-catenin pathway is reactivated upon injury (35). In a recent study, one-day postnatal murine cardiomyocytes, with high regenerative potential, showed enriched Wnt signaling gene networks after ischemic injury (46). Since Wnt signaling becomes inactivated in the postnatal heart during later stages, it was speculated that reactivation of the signaling will confer regenerative capacity to the adult heart, however with impaired cardiac performance. This may imply an initial protective mechanism of the stressed heart to preserve cardiomyocytes function, which eventually fails upon sustained activation of the pathway (**Figure 1**), probably due to a low developmentally permissive transcriptional state of the adult cardiomyocytes (46).

The Stressed Heart: Specific Wnt Component Regulation

Lack of robust regenerative response, upon stress/injury in the adult mammalian heart, results in adaptive tissue remodeling to sustain cardiac output. This finally leads to heart failure development characterized by a switch towards fetal metabolism and re-expression/elevation of developmental genes (60), including genes of the Wnt signaling pathway (13). Therefore, Wnt/β-catenin signaling has been considered a potentially therapeutic target for heart disease (35, 61–64). In the healthy adult heart, Wnt signaling is quiescent but becomes reactivated in different cell types in the ischemic and hypertrophic heart (**Figure 1**) (62–65). Specifically, Wnt/β-catenin activation is found in epicardium, fibroblasts, ECs, smooth muscle cells and CPCs (35) and in cardiomyocytes of the human failing heart (47). Conversely, Wnt inhibition appears to protect the heart from pathological ventricular remodeling (61, 63, 66, 67).

Recent studies indicate that the exosomal content is highly regulated in the heart by various stress conditions and that cardiomyocytes and cardiac fibroblasts release exosomes in *in vitro* studies (56, 68, 69). Moreover, Wnt ligands, FZDs and SFRPs are elevated after ischemic heart injury (48). These observations allow speculating that Wnts traveling on exosomes upon cardiac remodeling may be part of the maladaptive response. After myocardial infarction (MI), cardiac fibroblasts respond to Wnt1 in an autocrine fashion to induce proliferation and fibrogenic genes expression (48). Wnt1, Wnt3a, and Wnt5a regulate proliferation and migration of ECs. Moreover, after MI, β-catenin accumulates in ECs of the rat heart, which suggests activation of canonical Wnt signaling (48). Accordingly, antagonizing Wnt3a/Wnt5a binding to its receptors FZDs prevents heart failure upon ischemia (61). Wnt3a and Wnt5a were already found in exosomes (10, 26, 28). Interestingly, cell-autonomous regulation of Wnt signaling by enhancing β-catenin export on exosomes and reducing its cellular pool was described in tumor cells (70). An exciting idea will be to stimulate exosomal export of β-catenin, thereby reducing signaling activity in heart remodeling and preventing heart failure development.

Upon MI, macrophages are a source of non-canonical Wnts. Genetic ablation of Wnt signaling in mice results in macrophages with anti-inflammatory, reparative, and angiogenic properties and improved left ventricular function and remodeling after MI, possibly by the elimination of non-canonical signaling (51). In the failing myocardium, Wnt5a was most prominently upregulated

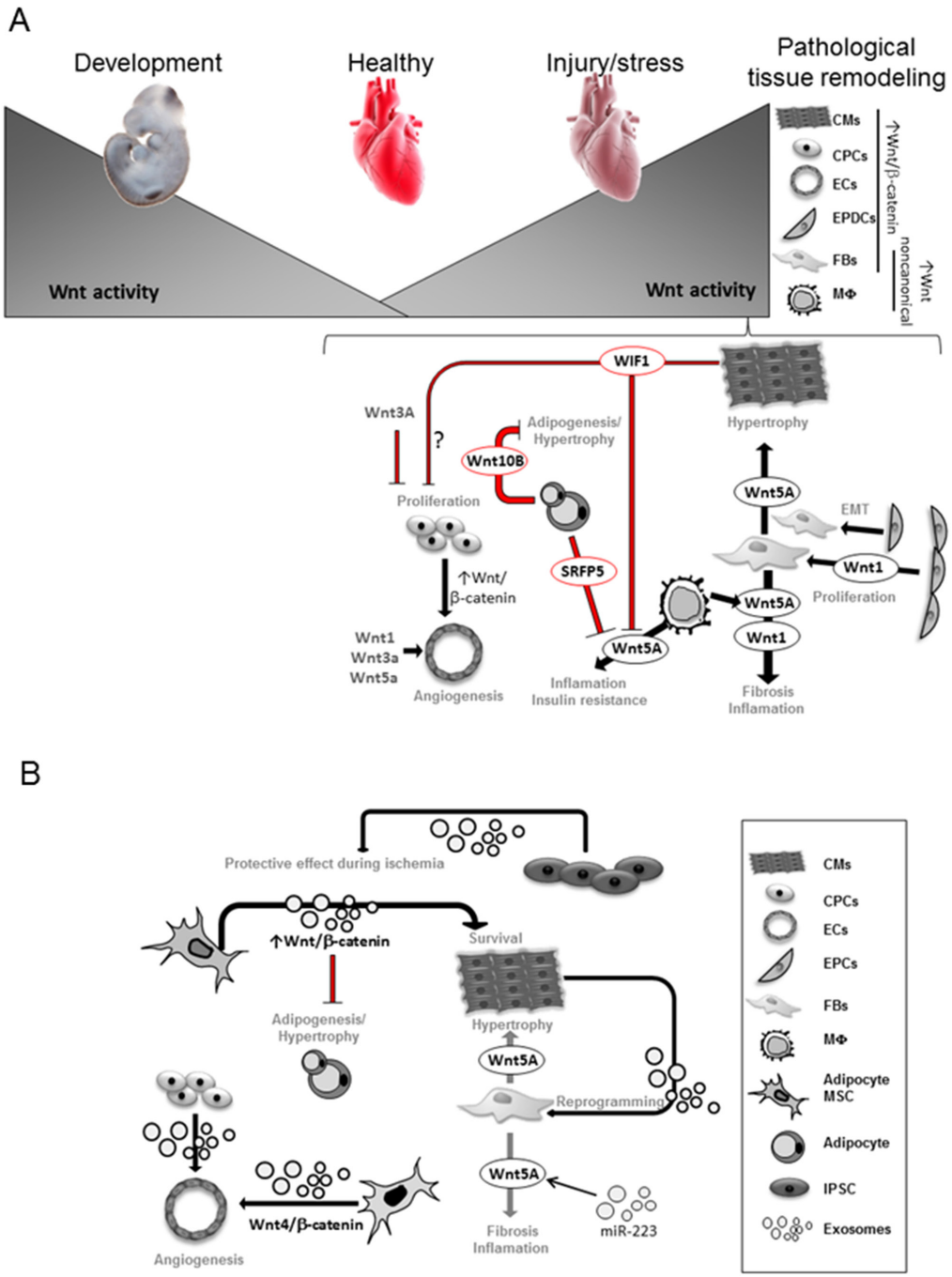

FIGURE 1 | (A) Wnt activity in cardiac tissue and secreted Wnt components described in heart cells. Wnt activity is necessary for heart development and becomes very low in the adult heart. Upon stress, Wnt signaling components are upregulated in different heart cells. Transcriptional dependent-canonical Wnt signaling is known to be activated in CPCs, epicardial-derived cells (EPDCs), fibroblasts (FBs), cardiomyocytes (CMs), endothelial (EC) and smooth muscle cells (35, 47, 48). Non-canonical Wnt components are mainly upregulated in Macrophages (MΦ) and FBs. Wnt1 secretion from EPDCs and FB promotes FB expansion and fibrosis (48); Wnt5a secretion from FB promotes CM-hypertrophy and fibrosis (49, 50); Wnt5a secretion from macrophages (MΦ) induces inflammation and insulin resistance leading to cardiovascular complications (49, 51). Secreted frizzled-related protein 5 (Sfrp5) by healthy adipocytes inhibits Wnt5a function from MΦ. The presence of unhealthy adipocytes with reduced Sfrp5 secretion increased Wnt5a activity (52, 53). Wnt10b from healthy adipocytes balances adipocytes growth (54). CMs-secreted Wnt Inhibitory Factor 1 (WIF1) reduces Wnt5a activity and may affect CPCs proliferation (55). Secreted Wnt1, Wnt3a, Wnt5a as well as activation of Wnt/β-catenin induced by CPCs stimulate angiogenesis (48). **(B)** Potential EVs-mediated signaling crosstalk in heart cells. Exosomes derived from cardiomyocytes (CMs) showed ability to reprogram fibroblasts (FBs) *in vitro* (56, 57). MiR-233 upregulates Wnt5a expression and miR-223 can be found in exosomes (58), thus Wnt5a regulation in cardiac failure may involve exosomal trafficking. Exosomes derived from adipocyte-derived mesenchymal stem cells (MSCs) was shown to activate Wnt/β-catenin signaling pathway, which may affect CM survival and constrains adipogenesis (20). Exosomes derived from umbilical cord MSCs showed a pro-angiogenic effect by delivering Wnt4 and activating Wnt/β-catenin signaling in endothelial cells (ECs) (21). Exosomes secreted from human induced pluripotent cells (iPSCs) showed protective effects on ischemic myocardium (59).

in cardiac fibroblasts and elevated circulating Wnt5a levels were associated with adverse outcomes in patients with dilated cardiomyopathies (49). In mouse and human cardiac fibroblasts, recombinant Wnt5a upregulated the release of Interleukine (IL)-6 and Tissue Inhibitor Of Metalloproteinases 1 (TIMP-1). This might promote myocardial inflammation and fibrosis contributing to heart failure progression (50). Moreover, Wnt5a is known to stimulate hypertrophy in cultured cardiomyocytes (49). Increasing evidence suggests that miR-223 upregulates Wnt5a expression (50) and miR-223 can be found in exosomes (58), hence Wnt5a regulation in cardiac failure may involve exosomal trafficking.

Upon MI, Secreted frizzled-related protein 5 (Sfrp5) functions as an extracellular inhibitor of non-canonical Wnt signaling (52) that antagonizes the pro-inflammatory activity of Wnt5a. Sfrp5 is highly expressed by healthy adipocytes, thus may act as a paracrine cardio-protective adipokine. Obese people with "unhealthy adipocytes" with reduced expression of Sfrp5 and high Wnt5a have an associated insulin resistance with a high risk of cardiovascular complications (53). Moreover, Wnt5a overexpression in myeloid cells augments adipose tissue inflammation; promotes pro-inflammatory cytokines by macrophages and impairs glucose homeostasis (54). Accordingly, Wnt5a ablation in obese mice ameliorates insulin resistance. Thus, Wnt5a crucially mediates cellular crosstalk to finally affect glucose metabolism and cardiac homeostasis. Additionally, Wnt5a induced hypertrophic NFAT activation in cardiomyocytes *in vitro* (49). Another Wnt, Wnt10b constrains mouse white adipose tissue expansion by inhibiting pre-adipocyte differentiation, modifying adipokine secretion and immune-modulatory roles of fat tissue (54). Of note, adipose tissue is an important source of circulating exosomal miRNAs in mice and humans and may regulate whole-body metabolism (71). Exosomes derived from adipocyte-derived mesenchymal stem cells (ADMSCs-ex) significantly ameliorated ischemia/reperfusion-induced myocardial necrosis and apoptosis in rat heart (20). The mechanisms underlying the cardioprotective effects of ADMSCs-ex may be associated with activation of Wnt/β-catenin signaling, a critical regulator of survival and apoptosis of cardiomyocytes (**Figure 1B**).

Hypoxic cardiomyocytes upregulate Wnt Inhibitory Factor 1 (WIF1) (72), which interferes with non-canonical Wnt signaling in monocytes and macrophages and reduces pro-inflammatory activation upon ischemia. In patients with hypoxia-associated disorders such as MI, stroke and pre-eclampsia, an increase of circulating EVs indicates a role of EVs as biomarkers in these pathophysiological states (55). EVs could be regarded as radar signals that confer a population overall fitness and unify their individual regulatory patterns.

Taken together, Wnt signaling activation is key in pathological heart remodeling and EV-mediated signaling may participate in this activation. The contribution of EV-mediated Wnt signaling to block tissue regeneration needs further investigation in order to engineer EV-modifications allowing the recovery of developmental plasticity.

EV-SIGNALING IN CARDIOVASCULAR CELLS

Proper cardiac function relies on communication of cardiomyocytes with other cell types including smooth muscle cells, EC, fibroblasts and immune cells (73). These cells function together by interacting physically or via secreted factors, including lipids, peptide, nucleotides and miRNAs. The adult myocardium secretes exosomes to mediate cell-cell communication (74). Upon cardiac stress, fibroblast-secreted exosomes enriched in miR-21*, which is normally degraded, is taken up by cardiomyocytes to induce cell hypertrophy (69). Moreover, during MI, distinct exosome-contents from border zone and healthy heart cells suggest an adaptive response to injury defined by exosome secretion (75). Primary cardiomyocytes were capable of secreting EVs with the ability to reprogram fibroblasts *in vitro* (56, 57). Thus, cardiomyocytes are able to transfer signals to direct neighboring cell fate (**Figure 1B**). Exosomes are not only potential circulating biomarkers (76) but they are also considered for their potential therapeutic anti-fibrotic and angiogenetic effects as antioxidants protecting cardiomyocytes (77).

Exosome-Mediated Signaling in Endogenous Progenitors

Current data indicate a role for Wnt signaling in the homeostasis of CPCs in the adult heart. However, cardiac functionality may not be directly affected by changing the balance of this small pool but by secreted products (78). In line with this, stem cell injections in the adult heart were suggested to mediate a paracrine regeneration through secreted signals (72) and CPCs are a source of exosomes (79). Under hypoxia conditions, these cells secrete "pro-regenerative" exosomes inducing proliferation of ECs (73). Cardiomyocyte progenitor and mesenchymal stem cell-exosomes have powerful pro-angiogenic effects (80) (**Figure 1B**). Given, the above-discussed action of Wnt activation on stimulating ECs fate, it is tempting to speculate that those "endothelial-pro-regenerative" exosomes may signal through the Wnt pathway.

Since exosomes are carriers of both protective and pathological signals, a better understanding of their content and effect on recipient cell will help to define therapeutic utilities of EVs. And will broaden our understanding of how cells and organs communicate among each other (73).

Regenerative Potential of EVs

Tissue repair requires not only the presence of cells capable to restore damage tissue, but more importantly, requires a microenvironment promoting tissue regeneration. A recent study showed that fibroblast-derived exosomes relocalize Wnt10b into lipid rafts, activating mTOR and promoting axonal regeneration in an inhibitory environment after optic nerve injury (30). It seems that Wnts on different EVs have similar signaling capacities and that loading with specific content is more relevant for their functionality than the EVs used to mobilize (28). This is in agreement with biotechnological approaches where liposomal packaging of Wnts confers a longer stability and high signaling capacity in regeneration models (81, 82).

This microenvironment can be created by exosomes with defined contents, ideally delivering signals affecting cell recruitment, differentiation and immunomodulation. Given the important role of exosomes in tissue regeneration in pre-clinical models, further studies addressing the EVs-mediated signaling are of high interest. Elucidating these mechanisms will offer a great platform for EVs engineering for personalized medicine.

Human pluripotent stem cells (hPSCs) and induced pluripotent cells (iPSCs) have been widely used in translational medicine for their enormous therapeutic potential in tissue repair and regeneration. Isolated exosomes secreted from iPSCs showed protective effects on ischemic myocardium by transferring the endogenous molecules to salvage the injured neighboring cells (59). In this regard, iPSCs-derived exosomes could be used for clinical application as autologous bioactive, cardio-protective exosomes to treat heart diseases and become a clinical tool for personalized medicine (75). Exosomes derived from umbilical cord mesenchymal stem cells showed a pro-angiogenic effect by delivering Wnt4 and activating Wnt/β-catenin signaling in ECs (21). Since activation of Wnt/β-catenin signaling is also pro-angiogenic in the adult heart, it is tempting to speculate that endogenous CPCs may also use EVs for pro-angiogenic signaling. Activation of canonical Wnt signaling was also reported in osteoblast-derived exosomes carrying miRNA to promote osteogenic differentiation. Thus, not only Wnt components may be carried onto EVs but also miRNA regulating Wnt signaling may be involved in cell-cell communication.

Cell therapies can directly support regenerative processes by forming new functional tissues or supporting tissue generation via paracrine mechanisms. Dissecting the precise role of Wnt signaling in cardiac tissue regeneration and the potential use of synthetic EVs may help tailor therapeutic approaches aiming to restore tissue functionality in a non-regenerative environment such as the heart. Moreover, human PSCs provide an excellent tool to address EV-mediated signaling in the context of early cardiogenesis. Developing protocols for exosomes isolation in their *in vivo* environment will allow cell-type and cargo-specific EVs and will enormously advance the field of EV-mediated signaling.

CONCLUSION

Modulation of Wnt signaling is crucial for tissue homeostasis in the developing and postnatal heart. However, the role of Wnt/β-catenin-dependent and -independent pathways in the intercellular crosstalk of heart cells is not fully understood. Activating or inactivating branches of the Wnt-network in specific target cells may be attractive to modulate pathological processes in the cardiovascular system or to enhance regenerative capacities of stem cell therapies. Many of these mechanisms might be mediated by EVs. Hence, understanding Wnt signal transduction via EVs between cell populations and tissues will advance our strategies for therapeutic modulation of these pathways.

AUTHOR CONTRIBUTIONS

Both authors conceived, discussed and wrote the review.

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REVIEW
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Platelet-Derived Microvesicles in Cardiovascular Diseases

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Microvesicles (MVs) circulating in the blood are small vesicles (100–1,000 nm in diameter) derived from membrane blebs of cells such as activated platelets, endothelial cells, and leukocytes. A growing body of evidence now supports the concept that platelet-derived microvesicles (PMVs), the most abundant MVs in the circulation, are important regulators of hemostasis, inflammation, and angiogenesis. Compared with healthy individuals, a large increase of circulating PMVs has been observed, particularly in patients with cardiovascular diseases. As observed in MVs from other parent cells, PMVs exert their biological effects in multiple ways, such as triggering various intercellular signaling cascades and by participating in transcellular communication by the transfer of their "cargo" of cytoplasmic components and surface receptors to other cell types. This review describes our current understanding of the potential role of PMVs in mediating hemostasis, inflammation, and angiogenesis and their consequences on the pathogenesis of cardiovascular diseases, such as atherosclerosis, myocardial infarction, and venous thrombosis. Furthermore, new developments of the therapeutic potential of PMVs for the treatment of cardiovascular diseases will be discussed.

Keywords: microvesicles, platelet-derived microvesicles, cardiovascular disease, therapeutic potential, hemostasis, inflammation, angiogenesis

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INTRODUCTION

Extracellular vesicles (EVs) encompass a broad range of vesicles released from cells (1). EVs can be classified into different subsets according to their size, cellular origin, content or the mechanism leading to their formation (**Table 1**). Microvesicles (MVs)—also referred to as microparticles—are vesicles typically around 100–1,000 nm in size. By contrast, smaller vesicles (30–100 nm) are referred to as exosomes, while larger vesicles containing nuclear materials are referred to as apoptotic bodies. Although there is a general consensus in most studies that apoptotic bodies are particles >1 μm (2, 3), there are several studies that describe apoptotic bodies to have a smaller size range of 0.5 μm (4, 5).

In the context of platelet biology, the plasma membrane fragments shed from activated platelets initially observed to possess procoagulant function were described as "platelet dust" by Wolf (17). Subsequent studies employing electron microscopy demonstrated the budding of vesicles from the platelet plasma membrane (18) thus confirming the cellular origin of the fragments detected by Wolf (17). In fact, 60–90% of EVs have been shown to be derived from platelets as indicated by positive CD41 staining (19). Since then, the role of EVs in the field of cardiovascular research has garnered a huge amount of interest due to their putative role in various pathological conditions.

TABLE 1 | Characteristics of extracellular vesicles.

	Exosome	Microvesicle	Apoptotic body
Size	≈20–100 nm	≈0.1–1 μm	>1 μm
Origin	Multivesicular bodies, internal compartments	Plasma membrane	Cellular fragments
Markers	– Tetraspanins (CD63, CD9, and CD81) – ALG-2-interacting protein X – Tumor susceptibility gene 101 protein – Heat shock 70-kDa proteins – Major histocompatibility complex class I and class II	– Phosphatidylserine (PS) – Integrins – Selectins – CD40 ligands – Other antigens of parental cell	– Histones – Fragmented DNA – PS
Reference	– (6–9)	– (9–14)	– (9, 15, 16)

Elevated levels of platelet-derived microvesicles (PMVs) are observed in diabetes mellitus, sepsis, rheumatoid arthritis, vascular inflammation, and cardiovascular diseases (20–31). Indeed, the pathological events associated with these diseases activate platelets (32–35), which have been demonstrated to increase PMV release, while at the same time, a subpopulation of PMVs coming from agonist-activated platelets have been demonstrated to contribute to pathological events (36). Thus, PMVs may well be both, one of the causes and a consequence of the pathophysiology that drives various diseases.

This review will focus on our current understanding of PMVs in mediating hemostasis, inflammation, and angiogenesis, which are all factors contributing to the pathogenesis of cardiovascular diseases. Furthermore, the clinical relevance of PMVs will be discussed in the context of their therapeutic potential in the treatment of cardiovascular diseases.

FORMATION AND CLEARANCE OF PMVs

Platelet-derived microvesicle formation is complex, and the exact mechanisms involved in the generation of PMVs to date remains to be clearly defined. However, it has been demonstrated that the generation of PMVs can be triggered by various mechanisms: (1) *via* platelet activation by soluble agonists or (2) shear stress or (3) glycoprotein (GP) IIb/IIIa outside-in signaling. In the case of platelet activation in response to soluble agonist stimulation or in response to high shear stress in the vasculature (37–39), sustained elevation of intracellular calcium has been observed, which initiates the loss of lipid asymmetry of the plasma membrane and cytoskeletal reorganization, ultimately leading to PMV generation (**Figure 1**) (40). The exposure of negatively charged phospholipids, such as phosphatidylserine (PS), on the outer leaflet of the plasma membrane is regulated by the calcium-dependent scramblase transmembrane protein (TMEM16F) (41). Likewise, calpain, a calcium-dependent protease, is central to regulating cytoskeletal reorganization thus facilitating PMV shedding (42). By contrast, unstimulated platelets have been demonstrated to generate PMVs *via* GPIIb/IIIa signaling, which destabilizes the actin cytoskeleton, resulting in shedding of PMVs in the absence of soluble agonist stimulation (43). Once generated, PMVs have generally been observed to have a relatively short lifespan with studies demonstrating PMVs to have half-lives of 30 min in mice (44), 10 min in rabbits (45), and approximately 5.5 h in apheresis-derived PMVs (46). Active endocytosis has been demonstrated to be involved in the clearance of MVs (47) including those of PMVs (48). Indeed, several studies demonstrated the involvement of PS-dependent phagocytic processes in the clearance of PMVs in the circulation (44, 49–52). Several opsonins such as complement component C3b, β2-glycoprotein-1, lactadherin, and developmental endothelial locus-1 facilitate this PS-dependent phagocytosis (44, 49–51, 53). Upon engulfment of EVs by phagocytic cells such as macrophages and dendritic cells, liver X receptor (LXR) and peroxisome proliferator-activated receptor pathways are activated, which both are known to be induced by lipid derivatives (54). Indeed, the activation of the cholesterol derivative-sensitive pathway LXR by PMVs has been previously demonstrated in plasmacytoid dendritic cells (pDCs) (52), thereby highlighting the importance of lipid composition of EVs on the target cell responses after engulfment. The internalization of PMVs is not only essential for the clearance of PMVs but also ensures the delivery of the MV content into the target cell and thereby exerting their effector functions. Although studies have shown PS-dependent phagocytosis to be involved in the clearance of PMVs, other mechanisms are also involved in this process, which have previously been reviewed in detail by Mulcahy et al. (55).

COMPOSITION OF PMVs

From the humble origin of being just "platelet dust," it is now apparent that PMVs can mediate a diverse range of physiological responses. Their capacity to exert their biological role is attributed to their cell membrane composition and molecular cargo. The phospholipid composition of PMVs is a composite of the platelet plasma and granule membranes with high cholesterol content, also indicating an enrichment of lipid rafts (35). PMVs share many of the antigens as their parental cells that regulate cell adhesion, activation, and coagulation reactions (**Figure 1**) (10–13). These include various GPs, tissue factors (TF), selectins, and coagulation factors V and VIII (10–13). PMVs are packed with numerous biological molecules, which facilitate the transportation and delivery of bioactive mediators that can modulate the function of target cells. PMVs carry cytokines and chemokines such as interleukin (IL)-1β, CXCL4, CXCL7, and CCL5 (11, 56). In addition, a vast amount of proteins, growth factors, and genetic material such as ribonucleic acid (RNA), messenger RNA, and microRNA can be packaged into PMVs (12, 57–60). Moreover,

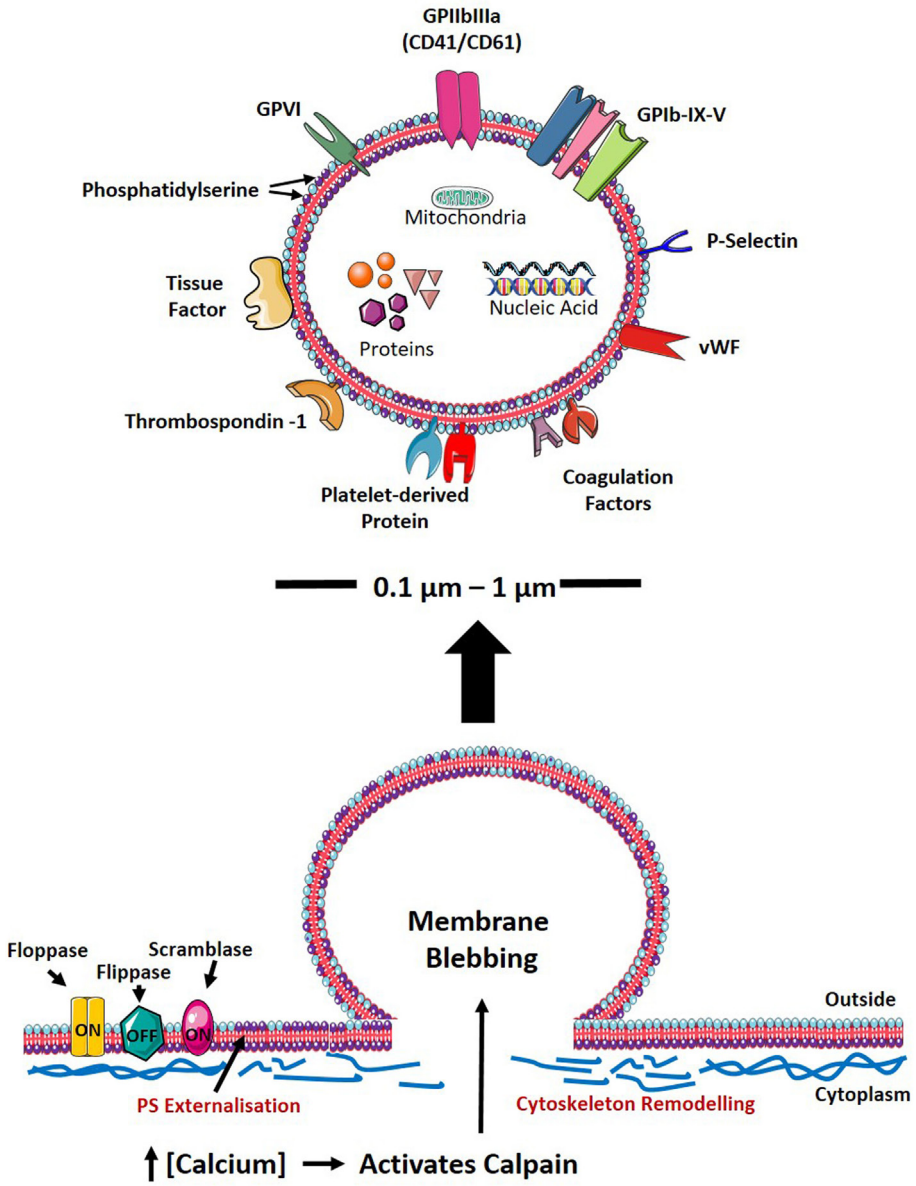

FIGURE 1 | PMV formation and characteristics. Upon cellular activation, the elevation of intracellular calcium inhibits flippase, while activating floppase and scramblase (TMEM16F), mediating the externalization of negatively charged PS (indicated as purple phospholipid). Furthermore, increased intracellular calcium leads to reorganization of the cytoskeleton by activating calpain, thereby cleaving PMVs and releasing them into the circulation. The size, physical characteristics, and cargo of PMVs depend on the environment and agonist(s) causing PMV generation. PMVs share many surface proteins with platelets such as integrins, selectins, adhesion receptors, coagulation factors, and other platelet-derived proteins. PMVs are packed with proteins including growth factors, cytokines/chemokines, and apoptotic proteins. PMVs also carry nucleic acids (mRNA, miRNA, and RNA) and mitochondria. PS, phosphatidylserine; GP, glycoprotein; vWF, von Willebrand factor; RNA, ribonucleic acid; mRNA, messenger RNA; miRNA, microRNA; PMV, platelet-derived microvesicle.

PMVs have recently been described to carry mitochondria, which can influence inflammatory responses (61).

The mechanisms of selective packaging have been demonstrated widely in EVs (62–65). Albeit not fully characterized in PMVs, the difference in lipid composition between PMVs and their parental cell, platelets (35), provide evidence for selective membrane assembly. Interestingly, it has been observed that the physical and biological components of PMVs are influenced by the stimulus used to generate PMVs (66–69). The heterogeneity observed in PMVs may explain why they have a diverse range of biological roles. For instance, larger PMVs may be enriched with more receptors and contents within, and thus can exert more effects, while smaller PMVs can deliver their biological cargo to areas that are otherwise difficult to enter, such as tumors, or to cross, such as the blood–brain barrier. Overall, it is important to understand that various pathological conditions will produce

different types of PMVs carrying unique biological cargos that exert specific effects on targets cells. However, the precise mechanism by which PMVs selectively package and release their biological cargo to influence cellular function needs to be carefully determined in the future.

BIOLOGICAL FUNCTION OF PMVs

Hemostasis

It is perhaps not surprising that PMVs are most widely recognized for their role in mediating hemostasis given the resemblance in lipid composition and biological cargo they share with their parent cell—the platelet. Diseases that affect PMV shedding in the circulation have provided insight into their ability to regulate hemostasis. For example, patients with Castaman's defect, where platelets have an isolated inability to generate MVs display a bleeding phenotype (70). Similarly, Scott syndrome, where platelets cannot externalize PS and generate PMVs, is associated with a marked bleeding diathesis (41, 70–72). These disorders highlight the importance of PMVs in mediating hemostasis.

In accordance with their ability to regulate hemostasis, PMVs display both pro- and anticoagulant properties. The assembly of the tenase and prothrombinase complexes, and thus thrombin generation, is increased in the presence of PMVs due to the PS in the outer leaflet of the cell membrane (73). PMVs also express TF, which can initiate the extrinsic coagulation pathway by activating Factor VII (73, 74). On the contrary, PMVs have anticoagulant activity, which is associated with the binding of the natural anticoagulant, protein S and activation of protein C (75–79). Given the potential pro- and anticoagulant effects of PMVs, the tight regulation of PMV levels is likely an important factor regulating the hemostatic response.

Inflammation

Akin to the wealth of literature demonstrating an important pro-inflammatory role for platelets, there is now a growing body of evidence demonstrating that PMVs can regulate inflammatory responses. The immunoglobulins, antigens, cytokines, and chemokines that PMVs carry can directly regulate immune responses (11, 56, 80). The pro-inflammatory effects of PMVs can be demonstrated through their interactions with monocytes and neutrophils. Mechanistically, PMVs bind to leukocytes and form aggregates and can induce monocytes to release inflammatory mediators including IL-1β, tumor necrosis factor (TNF)-α, monocyte chemoattractant protein-1, and matrix metalloproteinase (MMP)-9, which enhance monocyte migration (81, 82). Lipopolysaccharide-induced PMVs carry IL-1β in its mature form, which can activate endothelial cells and induce leukocyte adhesion thus promoting endothelial inflammation (56). Furthermore, mitochondria that are released *in vivo* in sterile inflammatory diseases, such as rheumatoid arthritis, have been observed to be packaged within PMVs, which can be hydrolyzed by phospholipase A2 IIA to generate bioactive mediators which promote neutrophil pro-inflammatory responses (61).

Intriguingly, there are reports that PMVs can also act as anti-inflammatory mediators. Recently, Dinkla and colleagues (83) have shown that PMVs prevent the differentiation of regulatory T cells into a pro-inflammatory phenotype. PMVs can bind to CCR6-HLA-DR$^+$ regulatory T cell subsets *via* P-selectin and inhibit the production of IL-17 and interferon- γ (83). In accordance, PMVs from platelet concentrates can modify innate immune cells such as macrophages and dendritic cells. Macrophage activation is attenuated in the presence of PMVs as indicated by reduced production of TNF-α and IL-10 (84). PMVs also alter the function of monocyte-derived dendritic cells as demonstrated by their reduced capacity to present antigens, diminished production of pro-inflammatory cytokines and decreased phagocytic activity (84). PMVs can also modify inflammatory effects of the target cell. For instance, PMVs have been demonstrated to regulate the inflammatory responses of mast cells by the transfer of Lipoxygenase 12 (85). This leads to the production of Lipoxin A4, which induces anti-inflammatory and anti-angiogenic responses on endothelial cells by suppressing the generation of pro-inflammatory cytokines (85, 86). Furthermore, pDCs, a subset of dendritic cells that augment inflammatory processes by producing a large amount of pro-atherogenic type 1 interferons were observed to engulf PMVs in a PS-dependent manner (52). PMVs were observed to inhibit pDCs pro-inflammatory response by reducing the secretion of TNF-α and IL-8, signifying an anti-inflammatory mechanism of PMVs (52).

In addition, our group has demonstrated that pentameric protein C-reactive protein (pCRP) binds to different MVs including PMVs (87). These pCRP-MVs, albeit not pro-inflammatory in healthy individuals, can aggravate existing tissue injury by activating the classical complement pathway and enhancing leukocyte recruitment to inflamed tissues (87). MVs not only bind pCRP but also convert pCRP to a highly pro-inflammatory monomer of C-reactive protein (mCRP), which can bind to endothelial cells and generate pro-inflammatory signals (88–91). In addition, autoantigen proteinase 3, an elastin degrading protease, binds to PS expressing MVs, thereby promoting inflammation *via* the generation of reactive oxygen species in neutrophils (92). Thus, these studies further highlight the ability of PMVs to partner with proteins to induce a pro-inflammatory phenotype. Therefore, PMVs may play a dual role in inflammation as they may instigate either pro- or anti-inflammatory responses depending on the cell membrane composition and biological cargo transported by the PMV.

Angiogenesis

In addition to harboring a number of pro-inflammatory cytokines, PMVs may carry growth factors such as vascular endothelial growth factor (VEGF), fibroblast growth factor 2, and lipid growth factors suggesting PMVs may play an important role in regulating angiogenesis (93). In accordance, Kim and colleagues have demonstrated that PMVs can inhibit apoptosis while enhancing cell migration, proliferation, survival, and tube formation in human umbilical vein endothelial cells (93). PMVs may also enhance pro-angiogenic MMP-2 and MMP-9 expression in endothelial cells *in vitro* and *in vivo* (94). Furthermore, PMVs stimulate the growth of endothelial

progenitor cells, thus contributing to the formation of new blood vessels (93).

The role of PMVs in regulating angiogenesis in the context of cardiovascular diseases has been highlighted by Brill and colleagues (95). The authors established that PMVs can induce angiogenesis *in vitro* caused by cytokines, VEGF, basic fibroblast growth factor, and platelet-derived growth factor packaged within PMVs in a process linked to Src, PI-3K, and ERK signaling (95). Moreover, the injection of PMVs in ischemic heart muscle induces the formation of blood vessels in a murine model of myocardial infarction (MI), signifying PMVs capability to induce myocardial angiogenesis in the setting of ischemia (95). PMVs have also been shown to facilitate endothelial repair after arterial injury by enhancing the vasoregenerative capacity of early outgrowth cells (EOCs) (96). PMVs enhance the recruitment, migration, and differentiation of EOCs at the site of injury by enhancing angiogenic growth factors that stimulate resident mature endothelial cells (96). The ability of PMVs to induce angiogenesis has also been demonstrated in the context of neurogenesis following brain injury and tumor progression (97, 98) thus, highlighting the potentially broad role of PMVs in endothelial repair and angiogenesis.

PMVs IN CARDIOVASCULAR DISEASES

Atherosclerosis

The rupturing of an atherosclerotic plaque can lead to MI and stroke, which are leading causes of death and disability globally. Indeed, excessive amounts of PMVs have been observed in patients with atherosclerosis (23–28). The increase in PMV numbers was found to correlate with multiple parameters including carotid artery intima media thickness, lipid-rich atherosclerotic plaques, and plaque burden (23–27). In the setting of atherosclerosis, increased hemodynamic shear stress due to plaque-associated luminal stenosis as well as the accumulation of oxidized low-density lipoprotein can activate platelets and stimulate generation of pro-inflammatory PMVs (33, 99). In atherosclerosis, monocytes adhere to activated endothelial cells, infiltrate the intima, and differentiate to tissue macrophages, which then engulf lipids and form foam cells (100). Smooth muscle cells migrate from the media to the intima and produce extracellular matrix, forming the fibrous cap (100). Macrophages and smooth muscle cells can undergo apoptosis leading to accumulation of extracellular lipid which forms the necrotic core (100). Indeed, PMVs have been implicated with these different stages of atherogenesis. For instance, PMVs encapsulate and transport miR-223 to endothelial cells, which can trigger endothelial apoptosis *via* the insulin-like growth factor-1 receptor (60). PMVs, together with endothelial MVs, increase endothelial permeability thereby influencing vascular endothelial dysfunction, an early step in the development of atherosclerosis (101). The presence of P-selectin expressed by PMVs allows them to interact with leukocytes *via* P-selectin GP ligand-1 thereby facilitating leukocyte accumulation at the site of endothelial injury and enhancing leukocyte infiltration from the blood vessel to the intima (82). Further to this, PMVs can transfer the pro-atherogenic cytokine RANTES to endothelial cells and induce monocytes and endothelial cells to release pro-inflammatory cytokines such as IL-8, IL-1β, TNF-α, and IL-6, further enhancing leukocyte adhesion and infiltration (102–104). PMVs encapsulate active caspase-3 that can induce macrophage apoptosis (105). As a consequence in atherosclerosis, lipids derived from dead macrophages can accumulate and can contribute to the formation of the necrotic core. Furthermore, PMVs have been demonstrated to stimulate smooth muscle cells, leading to the migration of smooth muscle from the media to the intima thereby enhancing lesion progression (106, 107).

However, the main caveat of these studies is the fact that PMVs are only detected in the circulating blood of patients with atherosclerosis but are not found in the atherosclerotic plaque itself (108). This raises the question as to whether PMVs are merely associated with atherogenesis or play an active role in disease pathogenesis. The absence of PMVs in the plaque, while intriguing because of the evidence of PMVs' infiltration in other inflamed tissues, such as arthritic joints (36), could suggest selective removal of PMVs as they engage with other cells and exert their effects at the intraluminal area of the vessel in the setting of atherosclerosis. PMVs are highly subjected to endocytosis/phagocytosis, due to the high expression of adhesion molecules and PS (109). This potentially enhances the clearance of PMVs in comparison with other blood stream-derived MVs, which may possibly be one of the mechanisms contributing to the absence of PMVs in the atherosclerotic plaque. These are important questions for further studies.

Acute Coronary Syndromes (ACS)

High levels of circulatory PMVs have been observed in patients with ACS and are also associated with the degree of elevation of cardiac enzymes, IL-6, and CRP levels (29–31). The elevated levels of PMVs in the plasma of patients with ACS persisted for up to 4 years after MI and is linked with markers of coagulation activation and soluble CD40L (110). In accordance with these findings, PMVs have been demonstrated to correlate with the size of myocardium at risk and microvascular dysfunction after ST elevation MI (STEMI) (111, 112). Also, it appears likely that MVs play an active role in promoting vascular inflammation and cardiac damage in patients after an ACS since MVs, including PMVs containing pro-inflammatory isoforms of CRP, have been demonstrated to be elevated in these patients (87, 88). Furthermore, PMVs independently predict future admission for major bleeding in non-STEMI patients (113). Taken together, these data suggest that PMVs detected in patients with ACS may also act as reporters of vascular inflammation, microvascular obstruction and myocardial damage in cardiovascular diseases (29, 30, 111, 112). While elevated levels of PMVs are often observed in patients with ACS, there are few studies that have reported variances in the levels of PMVs and the lack of association of PMVs with the severity of coronary artery disease (114, 115). The discordance in the levels of PMVs in ACS may be due to the variability of inclusion criteria and medication of the patients enrolled in each study. Also, the lack of consensus in the characteristic and definition of MVs may account for the variations observed in the literature.

Indeed, further studies are required, with establish common protocols and clear MVs nomenclature, to fully elucidate the role of PMVs in ACS.

Thrombotic Disorders

Thrombotic complications are often observed in patients with cardiovascular diseases. Augmented shedding of PMVs is deleterious and may contribute to thrombosis. For instance, in patients with immune thrombocytopenia, there are high levels of PMVs despite low platelet counts, which have been linked to a paradoxical increased risk of thrombotic events (116, 117). Increased PMVs are also detected in other thrombotic diseases such as acute pulmonary embolism and deep vein thrombosis (DVT) (118–121). Likewise, PMVs have been detected to be elevated in the context of thrombophilic states such antithrombin deficiency, protein C deficiency, and the Factor V Leiden mutation—all predispositions to venous thrombosis (122, 123). Increased levels of PMVs are observed to be associated with a heightened risk of venous thromboembolism, and PMVs have been proposed as a biomarker to help diagnose patients with DVT (119, 121). Similar to ACS, variation in plasma levels of PMVs are observed in thrombotic disorders (122, 124–126). Inconsistencies in processing blood samples such as handling, storage and methodology used in isolating PMVs may cause artifactual generation of PMVs and represent one of the underlying reasons for the discrepancies observed in the level of PMVs. Therefore, further studies are needed to fully unravel the role of PMVs in thrombotic diseases.

CURRENT CHALLENGES IN EV RESEARCH

Despite the remarkable progress in the field of EVs, clinical translation of EVs as a diagnostic or prognostic marker of pathological states remains a challenge (127). To fully unravel the potential of EVs in the diagnosis and therapy of cardiovascular diseases, it is imperative to understand the biological roles of PMVs *in vivo*. One of the drawbacks in the field of EVs is that most of the experiments demonstrating the physiological effects of EVs on target cells, such as endothelial cells, have been done *in vitro* in culture. The key weakness of this approach is that endothelial cells under *in vivo* conditions are under constant steady-state exposure to EVs. It is not clear how the response of cells in the *in vitro*, which is typically EV-free, to the sudden exposure of EVs is related to the *in vivo* setting. Furthermore, due to the lack of common practice in sample preparation and analysis, EV counts and phenotypes may vary dramatically between laboratories, making data analyses and clinical translation difficult. Indeed, several studies have highlighted the effects of pre-analytical variables on EV measurements. These include the type of anticoagulant used in collecting blood samples, centrifugation protocol, the storage of samples and staining protocols used for surface membrane antigens for determining the cellular origin of EVs (128–131). In regard to PMVs, preventing platelet activation and ensuring complete removal of platelets during processing of samples are crucial as this may result in inaccurate findings. It is also essential to be mindful of the storage and thawing conditions of samples as this leads to changes in the number of PMVs and Annexin V binding (128–130). In addition to this variability, sample handling, isolation protocol, different antigens used to determine cellular origin, inclusion criteria for patients, and their clinical characteristics may account for the qualitative and quantitative variations observed in the literature regarding the characterization of EVs.

The technological advancements have facilitated new methods to improve the purification and detection of EVs from biological fluids (1, 127, 132–135). These include the following: sensitive single particle detection devices (tunable resistive pulse sensing; nanoparticle tracking analysis; and dynamic light scattering), flow cytometry (conventional; imaging; and impedance based), proteomics, and atomic force microscopy (1, 127, 130, 134, 136–138). However, a combination of multiple methods is still necessary to assess both physical and biological properties of EVs (1, 135). In light of the current limitation of EV studies, the International Society of Extracellular Vesicles endeavors to provide guidelines, harmonizing nomenclatures and practices in an effort to improve reproducibility of EV experiments and to eliminate ambiguity in the field of EVs (139–141). Furthermore, this society has already published several position papers in the Journal of Extracellular Vesicles (139–142) and provides public online databases that catalog EV-associated components, thereby assisting researchers to optimize their practices (EV-TRACK, ExoCarta, Vesiclepedia, and EVpedia).

THERAPEUTIC POTENTIAL OF PMVs

Given the role of PMVs in cardiovascular diseases, this raises the question as to whether MVs can be exploited for therapeutic benefit. Indeed, pharmacological studies that alter the levels of PMVs in the circulation have shown encouraging results (143–146). Also, the physical and biochemical properties of PMVs are advantages that can be utilized for the purpose of developing a therapy for cardiovascular diseases (**Table 2**). For

TABLE 2 | Advantages and disadvantages of extracellular vesicles (EVs) as emerging therapy for cardiovascular diseases.

Advantages	Disadvantages
Natural homing ability and specific transfer of bioactive molecules (152, 153)	Lack of standardization of pre-analytical variables (127, 140)
Highly hydrophobic drugs and hydrophilic drugs can be packaged (152, 154)	No clear nomenclature leading to variable qualitative and quantitative analysis (1, 155)
Good delivery vehicle for drugs based on the ability to cross blood–brain barrier (153, 156)	No recommended isolation protocol for clinical grade production and quality control of EV-based therapeutics (68, 142)
Easy to adapt/optimize content and surface receptors for site-specific delivery (147, 154, 156)	Comprised of heterogeneous components depending on the isolation, handling, and agonist(s) used (67, 69, 128–130)
More stable upon freezing and thawing compared with cells, biocompatible, and non-cytotoxic (142, 156)	The need to establish techniques and methodologies to rigorously quantify and characterized the molecular and physical aspects of EVs (142)

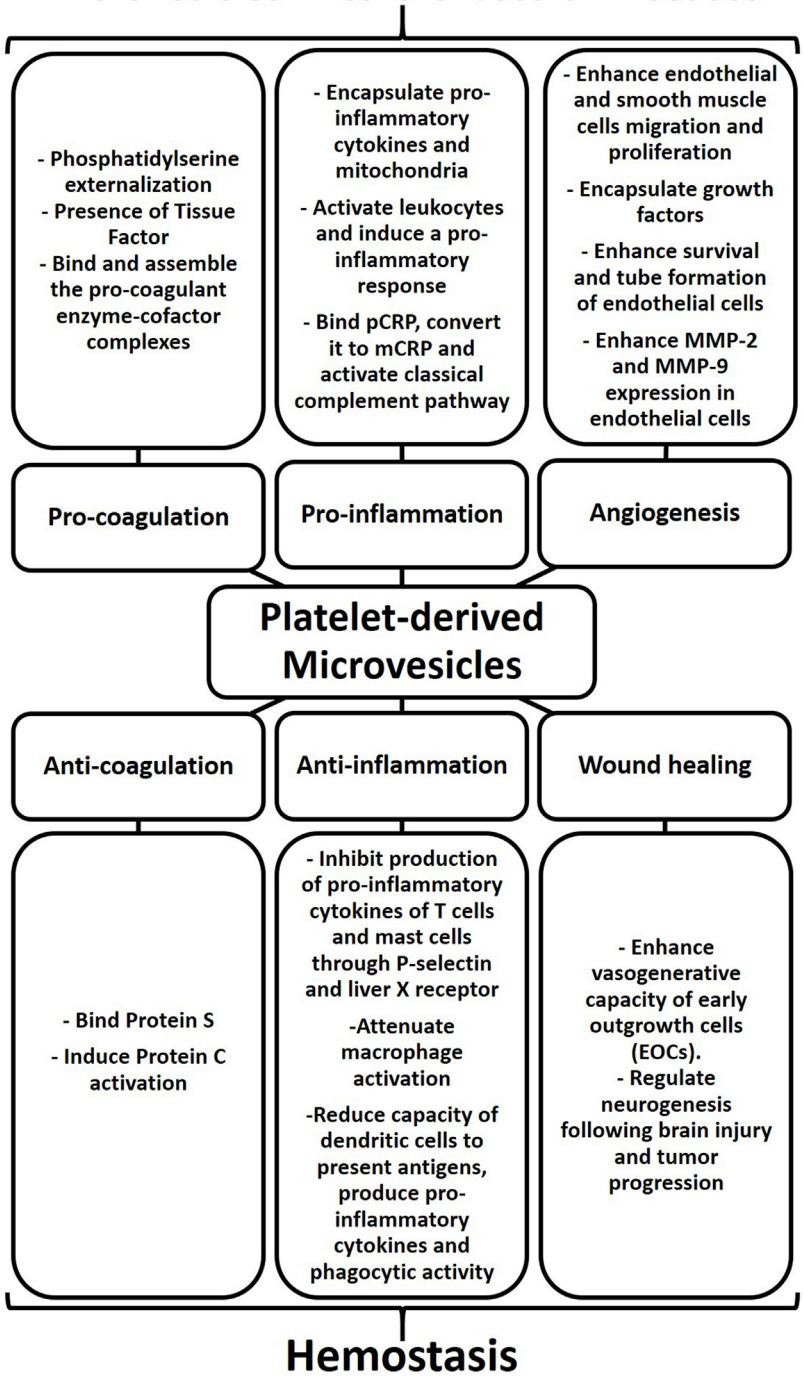

FIGURE 2 | Platelet-derived microvesicles (PMVs) as regulators of hemostasis and contributors to cardiovascular diseases. The physical characteristics of the cell membrane and biological cargo define the biological role of PMVs. PMVs bind natural anticoagulants thereby preventing coagulation. PMVs can also inhibit cytokine production leading to a reduction of vascular inflammation. In addition, PMVs can enhance the vasogenerative capacity of cells, thus highlighting their role in wound healing. While PMVs play a major role in regulating hemostasis, excessive numbers of PMVs can also contribute to cardiovascular diseases. The presence of phosphatidylserine and tissue factor in PMVs can induce procoagulant enzyme–cofactor complexes that favor thrombosis. PMVs can also induce cytokine production, bind protein C-reactive protein (pCRP), and convert it to monomer of C-reactive protein (mCRP), thereby promoting inflammatory responses. The activation of smooth muscle cells, endothelial cells, and leukocytes by PMVs as well as growth factors encapsulated within PMVs can stimulate angiogenesis. Therefore, PMVs may stimulate or dampen coagulation, inflammation, and angiogenesis and may thereby contribute to cardiovascular diseases.

instance, PMV-inspired nanovesicles have been engineered to deliver thrombolysis specifically to sites of clot formation (147). The design of the therapeutic nano vesicle was based on the biological aspects of PMVs that are relevant to thrombus formation. Surface receptors GPIIb/IIIa and P-selectin were used to target the site of clot formation, and the enzyme phospholipase A2 was employed to rupture the vesicle and release the lytic drug, thus allowing the targeting of thrombolysis only at sites of clot formation (147). Thus, this study highlights the feasibility of altering the cargo of EVs and surface receptors for site-specific delivery (**Table 2**). Also, natural PMVs have showed therapeutic potential to treat cardiovascular diseases. Pretreating circulating-angiogenic cells (CACs) with PMVs derived from atherosclerotic patients (PMV-CACs) enhances the re-endothelization capacity of these cells (148). PMV-CACs enhance blood flow and increase capillary density in rats suffering from hind limb ischemia *via* PMV release of RANTES (148). Furthermore, a cardioprotective role of MVs, specifically PMVs, has been demonstrated in a study by Ma and colleagues (149). In this study, the transfusion of PMVs from rats that underwent hind limb ischemia–reperfusion preconditioning resulted in increased levels of PMVs and a reduction of infarct size, indicative of a protective role of PMVs in the context of cardiac ischemia–reperfusion injury. This was further supported by data showing that PMVs transfused into rats with middle cerebral artery occlusion reduced infarct area (150). Similarly, other types of EVs have shown promising therapeutic potential. EVs, mainly MVs and exosomes, displayed a cardioprotective role by enhancing the recovery of cardiac function in a postinfarct heart failure animal model (151). Although the precise mechanisms involved in the protective role of EVs in cardiovascular diseases is still incompletely understood, the capacity of EVs to carry vast biological cargos and their ability to transfer a wide array of bioactive molecules to target cells may explain the beneficial effect of EVs as a therapy for cardiovascular diseases. Furthermore, EVs can be used as a drug delivery system to increase solubility, stability, and bioavailability of hydrophobic drug in the blood circulation (**Table 2**). Sun et al. encapsulated the drug curcumin in exosomes and successfully delivered the drug to activated monocytes, thus inducing an anti-inflammatory response in a preclinical model of septic shock (152). Despite the early promise from these studies involving PMVs, further studies are needed to delineate the potential utility of PMVs in cardiovascular diseases. Further to this, a number of challenges remain in translating therapeutic EVs to the clinic (**Table 2**).

CONCLUSION

Platelet-derived microvesicles have indeed come a long way from their initial descriptions as "platelet dust" to now being considered as major mediators of intercellular communication. Their role in hemostasis, inflammation, angiogenesis, and wound healing may be beneficial or deleterious and can contribute to the pathogenesis of cardiovascular diseases (**Figure 2**). PMVs represent a heterogeneous population of EVs derived from platelets. This heterogenicity is mainly due to the variability of stimuli capable of inducing platelet activation and PMV release. Indeed, the mode of platelet activation seems to define the size, content, and amount of PMVs, which together dictate their fate (cell targeting or not) and their effects and potential involvement in diseases. However, despite the undoubted progress in our understanding of the biological roles PMVs, there remains a pressing need to establish common protocols, analysis, and nomenclature in the field of EV research. Advancing our knowledge of the biological function of EVs holds promise to influence the treatment of cardiovascular diseases. Most interestingly, EV-based therapy represents an emerging and novel biological therapeutic approach for cardiovascular diseases. Therefore, harnessing our knowledge of EV biology may indeed unlock the full potential of EVs for the diagnosis and therapy of cardiovascular diseases and beyond.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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REVIEW
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Microvesicles in Atherosclerosis and Angiogenesis: From Bench to Bedside and Reverse

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Atherosclerosis (AT) is a progressive chronic disease involving lipid accumulation, fibrosis, and inflammation in medium and large-sized arteries, and it is the main cause of cardiovascular disease (CVD). AT is caused by dyslipidemia and mediated by both innate and adaptive immune responses. Despite lipid-lowering drugs have shown to decrease the risk of cardiovascular events (CVEs), there is a significant burden of AT-related morbidity and mortality. Identification of subjects at increased risk for CVE as well as discovery of novel therapeutic targets for improved treatment strategies are still unmet clinical needs in CVD. Microvesicles (MVs), small extracellular plasma membrane particles shed by activated and apoptotic cells have been widely linked to the development of CVD. MVs from vascular and resident cells by facilitating exchange of biological information between neighboring cells serve as cellular effectors in the bloodstream and play a key role in all stages of disease progression. This article reviews the current knowledge on the role of MVs in AT and CVD. Attention is focused on novel aspects of MV-mediated regulatory mechanisms from endothelial dysfunction, vascular wall inflammation, oxidative stress, and apoptosis to coagulation and thrombosis in the progression and development of atherothrombosis. MV contribution to vascular remodeling is also discussed, with a particular emphasis on the effect of MVs on the crosstalk between endothelial cells and smooth muscle cells, and their role regulating the active process of AT-driven angiogenesis and neovascularization. This review also highlights the latest findings and main challenges on the potential prognostic, diagnostic, and therapeutic value of cell-derived MVs in CVD. In summary, MVs have emerged as new regulators of biological functions in atherothrombosis and might be instrumental in cardiovascular precision medicine; however, significant efforts are still needed to translate into clinics the latest findings on MV regulation and function.

Keywords: angiogenesis, atherosclerosis, cardiovascular diseases, cell-derived microvesicles, endothelial dysfunction, inflammation, neovascularization, thrombosis

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Abbreviations: AT, atherosclerosis; CAD, coronary artery disease; cMV, circulating microvesicle; CVD, cardiovascular disease; CVE, cardiovascular event; EC, endothelial cell; eMV, endothelial-derived microvesicle; EPC, endothelial progenitor cell; ErMV, erythrocyte-derived microvesicle; ICAM, intercellular cell adhesion molecule; I/R, ischemia and reperfusion; LDL, low-density lipoprotein; ℓMV, lymphocyte-derived microvesicle; LMV, leukocyte-derived microvesicle; LLT, lipid-lowering treatment; $_{m}$EC, microvascular endothelial cell; $_{m}$eMV, microvascular endothelial-derived microvesicle; MetDiet, Mediterranean diet; miRNA, microRNA; MMP, matrix metalloproteinase; mMV, monocyte-derived microvesicle; MP, microparticle; MSC, mesenchymal stem cell; MV, microvesicle; NO, nitric oxide; pMV, platelet-derived microvesicle; PS, phosphatidylserine; RNA, ribonucleic acid; ROS, reactive oxygen species; SMC, smooth muscle cell; TF, tissue factor; T2D, type 2 diabetes; VCAM, vascular cell adhesion molecule.

INTRODUCTION

Despite significant advances in prevention, diagnosis, and therapeutic intervention focused on strategies for preventing cardiovascular disease (CVD), coronary artery disease (CAD) remains the leading cause of mortality and morbidity worldwide. Since not every individual has the same risk of developing future cardiovascular events (CVEs), an important challenge in cardiovascular medicine is to accurately predict who will develop atherosclerosis (AT)-related complications, such as acute coronary syndromes (ACS). Current tools to predict atherosclerotic vascular complications perform only poor to moderate. This emphasizes that there is an unmet need for novel biomarkers to stratify the risk of atherosclerotic complications in the intermediate and high-risk population on top of classical risk factors.

Atherosclerosis, the hallmark sign of CVD, is a silent hypercholesterolemia-triggered chronic systemic inflammatory process of the artery wall that is characterized by deposition of lipids within the intima of elastic arteries. This leads to structural damage and formation of fatty streaks with subsequent loss of elasticity, which can develop to fiber and atheromatous plaques, resulting in thrombus formation and narrowing of the luminal. Damage to arterial endothelial cells (ECs) is considered the earliest event in atherogenesis. Development of atherosclerotic plaques also includes the continuous crosstalk between EC, smooth muscle cells (SMCs), inflammatory cells, and inflammatory mediators (1) acting through mechanisms that have not yet been completely revealed.

Microvesicles (MVs), with a diameter ranging from 0.10 to 1.00 μm, are small plasma membrane vesicle fragments, also known as microparticles (MPs), that are released by many cell types, such as platelets, leukocytes, ECs, erythrocytes, and SMCs, into different bodily fluids, including plasma, urine, saliva, milk, sweat, semen, and tears, as well as in conditioned media from cell culture experiments (2, 3) (**Figure 1**). Depending on the generation mechanism, there are distinct types of extracellular vesicles, including *MVs*, *apoptotic bodies*, and *exosomes* (4), being MVs the most heterogeneous and studied population so far. The present review particularly focuses on MVs, which are specifically formed by budding of the plasma membrane, a releasing process that is driven by calcium-dependent signaling, activity of several enzymes, cytoskeleton remodeling, and externalization of phosphatidylserine (PS). MVs are shed under basal conditions and their release increases with various stimuli and pathological settings. In contrast to MVs, *apoptotic bodies* are larger permeable membrane vesicles with a diameter >1 μm containing apoptotic nuclear material while *exosomes* constitute the smallest extracellular vesicle type (ranging from 40 to 100 nm in diameter), highly enriched in lipids and tetraspanins, and actively shed from intracellular multivesicular bodies upon fusion with the cell membrane.

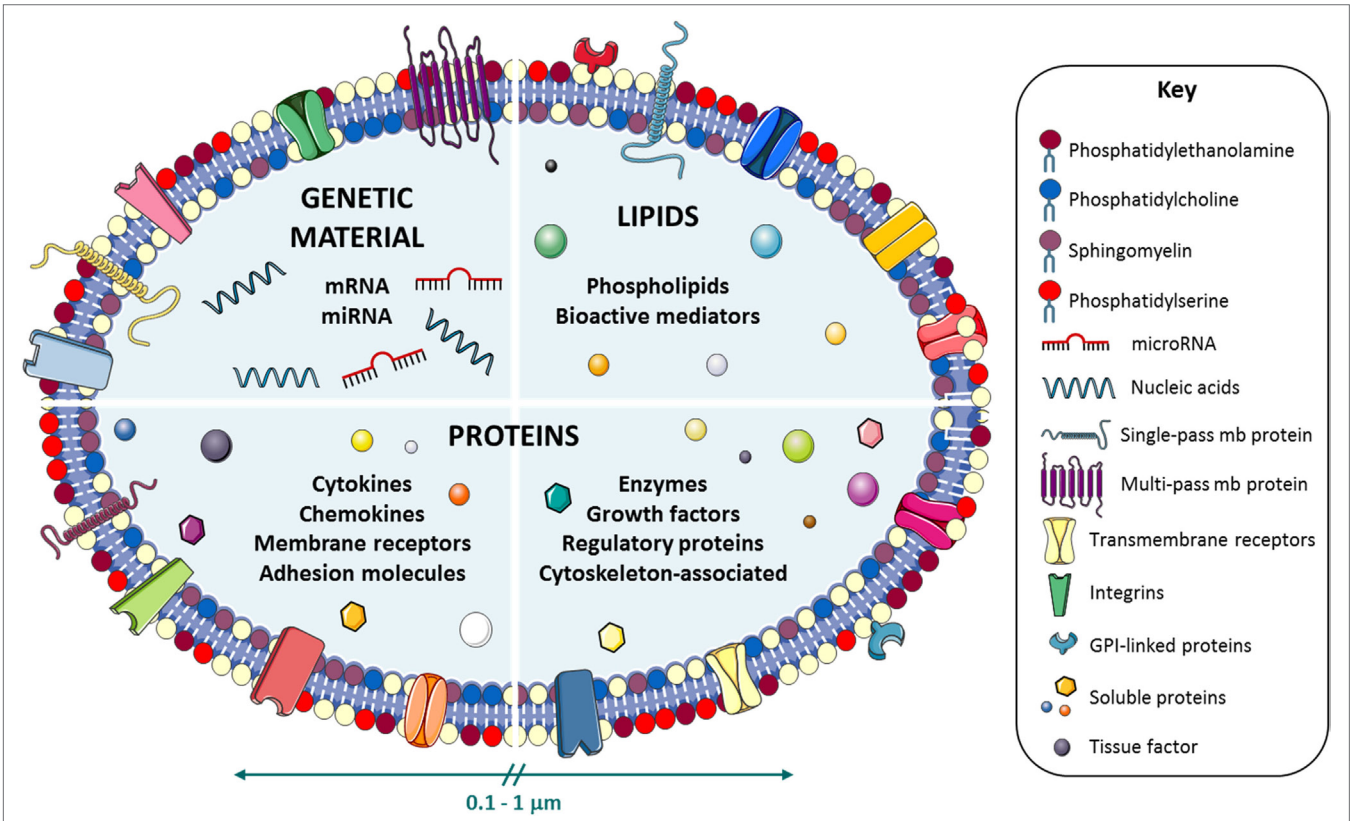

FIGURE 1 | Microvesicle (MV) composition. Schematic representation of the molecular repertoire of the cell-derived MVs. MVs are loaded with distinct components of genetic material [nucleic acids, mRNAs, microRNAs (miRNAs)], lipids (phospholipids and bioactive mediators), and proteins (cytokines, chemokines, membrane receptors, adhesion molecules, enzymes, growth factors, and cytoskeleton-associated and regulatory proteins) to mediate intercellular communication processes.

Microvesicles are specifically composed of lipids, genetic material, such as mRNA, non-coding ribonucleic acids (RNAs) [microRNA (miRNA)], or even small amounts of DNA, and proteins such as transcription factors, cytokines, and growth factors (**Figure 1**). Interestingly, the packaging of distinct biomolecules into MVs seems to occur in a non-randomly fashion. Thus, specific miRNAs were seen to be preferentially sorted into MVs. Blood cells and cultured monocytic THP1 cells actively and selectively secreted MV-loaded miRNAs into the circulation in response to various stimuli (5). Nevertheless, further efforts are needed toward a complete understanding of this regulated sorting mechanism. MVs have been characteristically recognized by the externalization of PS on the outer membrane leaflet. However, this property has recently been a matter of debate. New evidence suggests that some MVs can express cell markers without annexin V binding (6, 7). Interestingly, MVs harbor on their surface transmembrane and receptor proteins from the parental cells from which they derived from. This property, important for specific cell–cell interactions, is also used in MV identification and characterization by high-sensitivity flow cytometry. MVs can deliver their cargo to cells nearby or in remote locations, perpetuating the intercellular communication process. Since their content fluctuates depending on the pathological context, MVs have drawn the attention as a potential source of biomarkers for disease identification (8).

Flow cytometry has been the gold standard methodological choice for MV measurements. Recently, some new methods (9) such as atomic force microscopy have been developed. Today there is still a general need of establishing preanalytical steps for MV isolation and of validating novel techniques. Recent efforts (10–12) are addressed to standardize MV analytical procedures between instruments and laboratories (13).

Microvesicles promote the development and progression of AT, by inducing endothelial dysfunction (ED) and initial lesion formation, influencing cell communication, promoting inflammatory reactions and participation in lipid deposition, neovascularization, calcification and unstable plaque progression, and injured plaque clotting and thrombosis after rupture. Here, we review the current and last data on the role of MVs in AT and CVD, highlighting their relevance for vascular remodeling and neovascularization. In addition, we discuss the emerging interest of MVs as prognostic and diagnostic biomarkers of disease and their potential use as therapeutic agents.

MV-MEDIATED REGULATORY MECHANISMS IN THE DEVELOPMENT AND PROGRESSION OF AT

Several evidences support a direct functional role for circulating MVs in atherothrombosis. They span from the early stages related to the presence of classical cardiovascular risk factors (CVRF) (e.g., hyperlipidemia, diabetes, and hypertension) to acute cardiovascular and cerebrovascular events as result of their effect on intercellular communication processes transferring proteins, non-coding RNAs, and even mRNAs (14) to target cells. Specifically, MVs are able to (1) fuse with plasma membrane of target cells and transfer their cargo into the recipient cells, (2) interact with target cells *via* a receptor-mediated signaling mechanism, and (3) be internalized *via* endocytosis and fused with either endosomes to release their content into the cytosol or lysosomes to be degraded (2). To bridge the gap between atherosclerotic lesion initiation, plaque rupture, and CVEs, multifaceted MV-driven molecular mechanisms of AT progression linked to endothelial homeostasis, inflammation, and thrombosis will be discussed.

Endothelial Dysfunction

Endothelial cells exert natural barrier functions in the vessel wall to prevent pathogen invasion and to maintain vascular integrity, through the balanced release of cell and molecular components acting at paracrine and autocrine level. Vascular ECs change their phenotype and function in response to mechanical injury and systemic factors, such as dyslipidemia, smoking, and other stimuli and/or risk factors.

Recent studies support a direct relationship between the levels of circulating endothelial-derived MVs (eMVs) and the degree of ED in patients with end-stage renal failure (15), congenital heart disease (16), CAD (17, 18), type 2 diabetes (T2D) (19), and obesity and hypertension (20). Indeed, MVs are able to interact with the vascular endothelium and promote cellular dysfunction (**Figure 2**). Thus, MVs from T2D patients have shown to attenuate eNOS expression in cultured EC (21). Furthermore, MVs of endothelial origin have shown to decrease nitric oxide (NO) production *in vitro* (22, 23) and under high glucose conditions through changes in oxidative stress (24) or inducing a temporal cross talk between mitochondria and endoplasmic reticulum (25). These effects have been obtained in *ex vivo* studies (23, 26) and with patient-derived MVs in pathologies such as myocardial infarction (MI) (27), end-stage renal failure (15), metabolic syndrome (28), valvular heart disease and cardiac injury (29), and undergoing percutaneous coronary intervention (30). As a result, vascular tone and endothelial repair capacity are altered. Studies *in vitro* using rat aortic rings provided evidence that eMVs lead to impaired acetylcholine-mediated vasorelaxation (22), and studies *ex vivo* with eMVs from ACS patients induced premature ED, senescence, and thrombogenicity through an angiotensin II-dependent redox activation of phosphoinositide 3-kinase/Akt and mitogen-activated protein kinases pathways (31). In addition, when MVs obtained from plasma of subjects with metabolic syndrome, were injected intravenously in mice, a severe impairment of endothelium-triggered vasorelaxation with decreased NO-synthase expression was detected (28). Horn et al. reported that MVs carry functionally active NO synthase that induce NO production within the MVs, a function downregulated in MVs from patients with ED (32). These results indicate that eMVs have a functional role in the control of vascular homeostasis.

The physiopathological conditions stimulating MVs formation have an impact on MV phenotype and functional activity. Interestingly, Mahmoud and colleagues reported that MVs from healthy individuals or shed under basal conditions had no effects on endothelial function, while *in vitro* generated eMVs exert a protective role on endothelial function in a free-fatty acid-induced model, *via* eNOS/Akt signaling and reduced oxidative stress (33). This recent study reflects on how MVs may behave

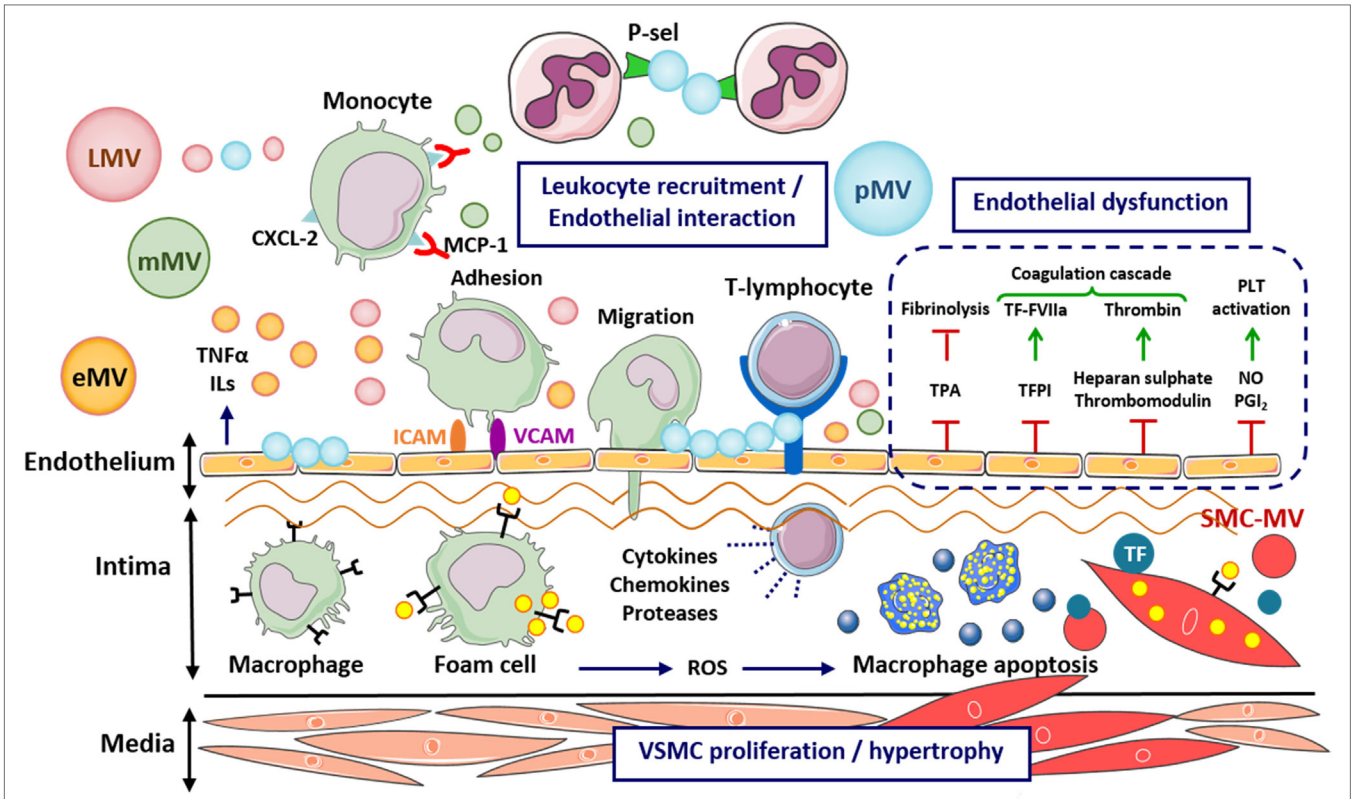

FIGURE 2 | Effects of microvesicles (MVs) on the early stages of atherosclerosis development. Cell-derived MVs are able to interact with the subendothelial matrix, induce endothelial dysfunction, stimulate proinflammatory response, enhance the adhesion and infiltration of leukocytes, as well as oxidative stress, apoptosis, and vascular remodeling, promoting the inflammation and injury of the vessel wall and the progression of atherosclerotic lesions. CXCL-2, C–X–C motif chemokine ligand 2; eMV, endothelial cell-derived microvesicle; FVIIa, coagulation factor VIIa; ICAM, intercellular cell adhesion molecule; LMV, leukocyte-derived microvesicle; mMV, monocyte-derived microvesicle; MCP-1, monocyte chemotactic protein 1; NO, nitric oxide; PGI, prostacyclin; pMV, platelet-derived microvesicle; P-Sel, P-selectin; ROS, reactive oxygen species; SMC-MV, smooth muscle cell-derived microvesicle; VCAM, vascular cell adhesion molecule; TF, tissue factor; TFPI, tissue factor pathway inhibitor; TPA, tissue plasminogen activator; TNF, tumor necrosis factor.

as different biological effectors in health and disease, highlighting the relevance of the environment and pathological stimuli triggering MV release on their phenotype and biological activity.

Injured endothelium beyond promoting the recruitment of activated platelets to repair damaged ECs, also facilitates adherence of platelet-derived MVs (pMVs) to the vascular wall (**Figure 2**), inducing in turn the permeability and apoptosis of ECs, the latter by MV transfer of caspase-3 and Rho-kinase enzymes according to the *in vitro* findings reported by Edrissi et al. (34). More specifically, these authors have proved that plasma-derived MVs from rats submitted to chronic cerebral ischemia contain factors involved in the activation of the tumor necrosis factor (TNF)-α pathway that delivered to ECs regulate endothelial permeability *in vitro*.

Vascular Wall Atheroinflammation

The role of MVs on the propagation of endothelial proinflammatory response was initially reported in *in vitro* studies showing that blood-derived MVs (obtained from either plasma and/or cell cultures, and also induced by high-shear stress) raise the release of cytokines and cell adhesion molecules in ECs (35, 36) and in leukocytes (37). In addition, circulating MVs obtained from healthy volunteers after infusion of a chemotactic peptide induced cytokine and chemokine release in *in vitro* EC cultures (38). Similar findings have been reported using MVs from both animal models and patients (39, 40). Thus, MVs obtained from a high-fat diet-fed obese murine model of insulin resistance and T2D induced the expression of vascular cell adhesion molecule-1 and reactive oxygen species (ROS) production in rat cardiac ECs (41). Furthermore, MVs have also shown to increase the monocyte expression of cell surface antigens such as CD11a/CD18 and CD11b/CD18 α_M/β_2 (37) and the endothelial expression of intercellular cell adhesion molecule-1 (ICAM-1) (42, 43). Increasing evidences suggest that regulation of protein expression by MVs is dependent on their miRNA-cargo. Thus, MV-mediated regulation of ICAM-1 expression in ECs is dependent on their content in miRNA-222 and its transfer to the target cells (44). MV-associated inflammatory cascade promotes the binding of monocytes to the endothelium and their infiltration into the atherosclerotic plaque (**Figure 2**) (24, 37, 45–47). Indeed, *in vitro* studies demonstrated that monocyte and leukocyte activation and adhesion is regulated by eMVs transferring miRNA-10 to the monocyte and thereby targeting nuclear factor-κB inflammatory pathway (48) and by MVs that

carry arachidonic acid as well as oxidize phospholipids (45). Another smart study showed that pMVs transfer proatherogenic C–C motif chemokine 5 [regulated on activation, normal T cell expressed and secreted (RANTES)] to either activated ECs or murine atherosclerotic carotid arteries enabling monocyte adherence (46). Thus, MVs might contribute to the early atherosclerotic lesions development by promoting leukocyte adhesion to the endothelium.

Oxidative Stress, Apoptosis, and Vascular Remodeling

The seminal study of Forlow et al. showed that pMVs expressing P-selectin (P-Sel) promoted monocyte infiltration by facilitating leukocyte–leukocyte interactions under detrimental flow conditions (**Figure 2**) (49). Besides, lipid-containing MVs derived from various cell types promote foam cell formation and programmed cell death by inducing lipid and cholesterol accumulation in macrophages through a toll-like receptor induced mechanism *in vitro* and in a human skin model (50). TNF-α-induced eMVs contributed to apoptosis and inflammation of ECs *in vitro* (51). Delivery of the proapoptotic enzymes caspase-1 and caspase-3 to target cells by eMVs, pMVs, monocyte-derived MVs (mMVs), T-lymphocyte-derived MVs (ℓMVs), and erythrocyte-derived MVs trigger macrophage cell apoptosis *in vitro* (52–56), which in turn causes more MV release, amplifying the process and facilitating progression of the atherosclerotic lesion. Thus, human atherosclerotic lesions contain MVs (57, 58) being those of monocyte and macrophage origin the most abundant. Apoptosis cell-derived MVs amplify the initiation and maintenance of inflammation in human monocytes together with interferon γ (59) in contrast to a recent study pointing out that MVs released from apoptotic cells provoke less inflammatory response than MVs from viable cells in monocytes, relating this effect with the specific miRNAs pattern found in these MVs (60). Furthermore, atherogenic low-density lipoprotein (LDL) modified by aggregation induce the release MVs enriched in tissue factor (TF) from SMCs (61) (**Figures 2** and **3**). pMVs increased SMC proliferation

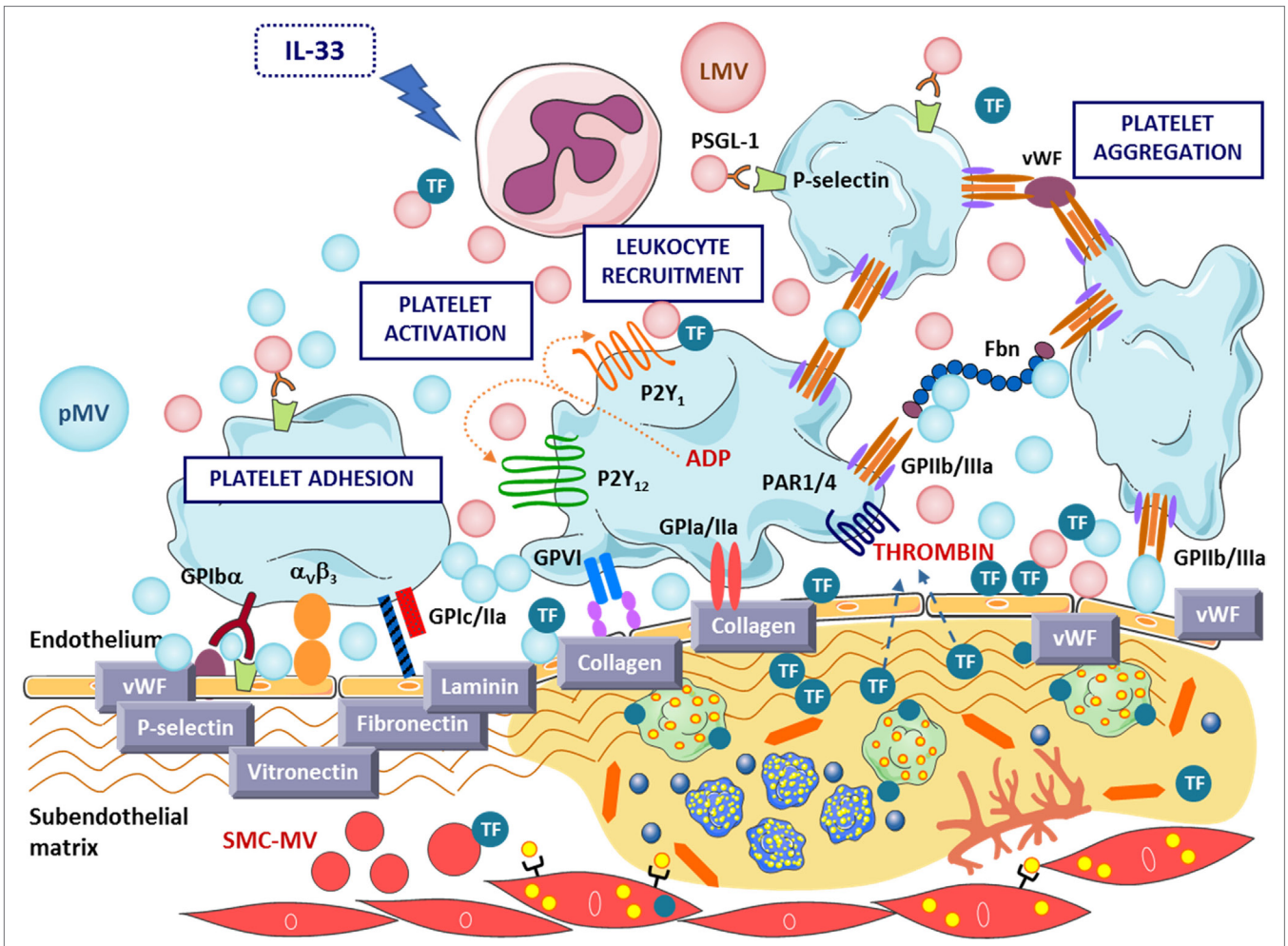

FIGURE 3 | Key prothrombotic mechanisms exerted by microvesicles (MVs) at sites of vascular injury. MVs' adhesion to the endothelial layer enhances platelet adhesion and thrombus formation and contributes to TF release at the injured atherosclerotic plaque. IL-33, interleukin-33; LMV, leukocyte-derived microvesicle; mMV, monocyte-derived microvesicle; pMV, platelet-derived microvesicle; PSGL-1, P-selectin glycoprotein ligand-1; SMC-MV, smooth muscle cell-derived microvesicle; TF, tissue factor; vWF, von Willebrand factor.

(62) by a mechanism independent of platelet-derived growth factor (PDGF) (63) whereas mMVs induced SMC death by caspase-1 (56).

High-risk plaques have a large lipid content covered by a thin fibrous cap penetrated by proinflammatory cells and a diffuse pattern of calcification (64, 65). The level of calcification has associated either with plaque burden (66) and lesion destabilization (67) or with less prone to rupture plaques (68). It seems plausible that the effect of calcification on atherosclerotic lesions evolves from a destabilizing effect in early lesions to a potential stabilizing effect of larger calcium burden in more advanced plaques (69). MVs from ECs, VSMCs, and macrophages seem to be recruited at the site of atherosclerotic plaque calcification and have been associated with the calcification process (70–73). Regarding MV-associated modulation of fibrous cap weakening, several cell-derived MVs can influence the progressive depletion of VSMCs and extracellular matrix degradation through metalloprotease interaction. Specifically, the involved molecular effectors are matrix metalloproteinase 9 (MMP-9), a disintegrin and metalloprotease-10 (ADAM-10) and -17 (ADAM-17) in neutrophil-derived MVs [(74, 75), respectively], MMP-9, matrix metalloproteinase-2 (MMP-2), and MMP-10 in eMVs [(76–78), respectively], and the metalloprotease TNF-α converting enzyme (TACE/ADAM-17) of human atherosclerotic plaques-derived MVs (79). A relevant finding is that the shedding of matrix MVs from arterial intimal SMCs was found higher in athero-prone areas of the human aorta than in athero-resistant areas at the preatherosclerotic disease stage with quantitative electron microscopic analyses (80). Shear stress-induced eMVs-containing miRNA-143 and miRNA-145 prevented VSMC dedifferentiation (81). Likewise, circulating MVs bearing miRNA-223 could penetrate the vascular wall and inhibit VSMC proliferation and migration, resulting in a decreased plaque size (82).

Coagulation and Thrombosis

The potential role of MVs in atherothrombosis is supported by a large number of studies. Procoagulant MVs are located within human advance vulnerable plaques (83). Upon atherosclerotic plaque erosion or rupture, the high-risk vulnerable plaque exposes their vascular contents to the blood flow, the coagulation cascade is activated and, concomitantly, there is recruitment and activation of circulating platelets that may lead to thrombus formation. Thrombosis may compromise arterial blood flow supply leading to the presentation of oxygen deficiency and the presence of MI.

First described as platelet dust (84), MVs are procoagulant biological effectors due to the surface content of negatively charged PS, which confers them high binding capacity for coagulating factors, being prominently higher in MVs than cell surface membrane in the case of platelets (85). The presence of other molecules and receptors including TF (83), factor VIII and Va (86), P-selectin glycoprotein ligand-1 (PSGL-1) (87), glycoprotein IIb/IIIa (88), and protein disulfide isomerase (PDI) (89) on MV surface might further enhance clot formation and thrombosis. TF can be functionally transferred *via* MVs to monocytes and other cells (90, 91). Protease-activated receptor 2 activation favors the shedding of TF-rich MVs through a process involving filamin A. Specifically, the interaction of TF with filamin A translocate cell surface TF to cholesterol-rich lipid rafts, promoting its activity and release into MVs (92). Indeed, procoagulant TF-rich MVs are increased by hyperinsulinemia (93) and found increased in patients with T2D (94). Recently, it has been shown that proinflammatory cytokine IL-33 induces differential TF expression and activity in intermediate monocyte subset as well as the release of procoagulant MVs rich in TF (95), emphasizing the interplay between inflammation and thrombosis (**Figure 3**).

Several key studies have addressed how MVs affect the clotting process (**Figure 3**). Upon endothelial injury, pMVs can bind to subcellular matrix to enhance clotting (96). Noteworthy, we reported that circulating and platelet-derived MVs exert direct effects on atherothrombosis by promoting platelet and fibrin deposition on atherosclerotic arterial wall (97). We performed a proof-of-concept study by perfusing blood with and without exogenously added pMVs to injured atherosclerotic vessel wall and demonstrated that elevated levels of pMVs were able to enhance platelet and fibrin adhesion under conditions of high-shear stress (97, 98). Indeed, a decreased level of pMVs bearing surface epitopes of adhesion and activation was found in blood perfusing prothrombotic surfaces, and also in ST-segment elevation myocardial infarction (STEMI) patients (99), supporting the high tendency to adhere of pMVs (49). High-shear stress-induced pMVs induce enhanced expression of cell adhesion molecules in ECs and monocytes (36) and regulate monocytes involvement in AT and inflammation (37). Interestingly, MVs from healthy subjects support low-grade thrombin generation (100) and are able to activate a stress signaling pathway in ECs leading to increased procoagulant activity (35); and PDI-bearing pMVs promote platelet hyperaggregability and insulin degradation in patients with T2D (89). Furthermore, interactions between platelet P-selection with PSGL-1 of leukocyte-derived MVs (LMVs) are required to concentrate TF activity at the thrombus edge to promote thrombus formation (87). MVs carrying functional TF might enable the growth of unregulated thrombus generation and fibrin formation and thrombus propagation ultimately leading to thrombotic complications (101).

POTENTIAL PROGNOSTIC, DIAGNOSTIC, AND THERAPEUTIC VALUE OF CELL-DERIVED MVs IN AT

CV diseases reflect a continuum of mechanisms underlying the gradually progressing AT. In addition to the pathogenic effects of MVs in AT and thrombosis, circulating levels of MVs (cMVs) of different cellular origin are increased in CVDs and reflect the severity of the different stages of the pathophysiology (102), thus cMVs might serve as potential diagnosis and prognosis biomarkers, which would be valuable tools for cardiovascular risk prediction as well as for evaluating the pharmacological response to therapeutic interventions.

Circulating microvesicles exist in the blood of normal healthy individuals released upon activation and in some cases apoptosis

of vascular cells. Indeed, MV levels show gender-specific differences (103, 104) and changes are observed with age (105), during pregnancy (106), after exercise (107–109) and after a high-fat meal (110). It is important to recognize that differences may arise depending on age, gender, body mass index, lipid, hormone levels, smoking status and other confounding variables in apparently healthy subjects when evaluating cMVs for pathogenic potential (111). Another relevant issue to bear in mind when studying cMVs is their clearance mechanism, which might influence the levels of cMVs and could also be impaired by CVRF.

Cardiovascular Risk Factors

The number and phenotype of cMVs has been associated to major CVRF, such as smoking, diabetes mellitus, obesity, hypertension, dyslipidemia, and metabolic syndrome (**Table 1**). Within hyperlipidemia, it has been shown that in patients with heterozygous familial hypercholesterolemia (FH), pMVs carrying TF identify subclinical atherosclerotic plaque burden (112), and ℓMPs are able to discriminate between lipid-rich and fibrous atherosclerotic plaques in the same patients (113), thus reflecting that chronic exposure to high levels of LDL activates the vascular compartment by distinct mechanisms. Therefore, CVRF correlate with increased numbers of MVs indicating that MVs might be active in triggering thrombosis and further contributing to an increased risk of CVEs.

TABLE 1 | Microvesicle-associated prognostic and diagnostic value in cardiovascular risk stratification and CVDs.

CVRF and atherothrombotic diseases		Reference
CVRF	Smoking	(114–116, 117)
	DM	Insulin resistance (118) DM (119–123) Type 2 DM (124–127) DM with microvascular complications (128)
	Obesity	In children (129) In adults (114, 130, 131) Caloric restriction/bariatric surgery (132)
	Hypertension	(18, 133–135)
	Dyslipidemia	Dyslipidemia (112, 113, 126, 136)
	Metabolic syndrome	Clustering of CVRF (28, 137–139) Cardiometabolic risk (140)
	Uremia	(141)
Atherosclerosis (AT)	FRS	(140, 142)
	ED	(15, 115)
	Calcification	(143)
	PAD	(105, 144)
	Subclinical AT	(19, 112, 113, 145)
	Stable CAD	(17, 146–148)
CVD	ACS	(142, 146, 149–154) STEMI (155, 156)
	Cerebrovascular disease	Ischemic stroke (157–162) Cerebral vasospasm (163) Lacunar infarcts (157) Carotid AT (164)

ACS, acute coronary syndromes; CAD, coronary artery disease; CVD, cardiovascular disease; CVRF, cardiovascular risk factors; DM, diabetes mellitus; ED, endothelial dysfunction; FRS, Framingham Risk Score; PAD, peripheral artery disease; STEMI, ST-segment elevation myocardial infarction.

Atherosclerosis

Several studies have shown an association between cMVs and the Framingham Risk Score (used to predict CVD risk), ED, coronary calcification, peripheral artery disease, and subclinical AT (**Table 1**). Our group has recently reported that subjects at high cardiovascular risk who were about to develop a major CVE have increased levels of blood $CD3^+/CD45^+$ cMV (165). This is in agreement with our previous findings demonstrating that FH patients with subclinical lipid-rich atherosclerotic plaques have significantly higher levels blood-derived circulating ℓMVs than those with fibrous plaques (113). Besides, levels of circulating MVs have been found increased in patients with stable CAD, being MVs of endothelial origin ($CD144^+$, $CD31^+$/Annexin V^+) and bearing miRNA-199a and miRNA-126 the most associated to major adverse CVEs (17, 146–148).

Acute Coronary Syndromes

Cell-derived cMV levels are profoundly increased in patients with ACS (**Table 1**). In a study in patients with STEMI, our group has demonstrated increased levels of pan-LMPs ($CD45^+$), including lymphocytes ($CD3^+$) and monocytes ($CD14^+$) within the first hours of the ACS onset, with partial reductions after 72 h, likely because the inflammatory burst occurred at STEMI onset (155). These data are strongly supported by the fact that platelet- and monocyte-derived cMVs also associate with AMI severity (156) and CVD mortality (166), reflecting the sustained underlying endothelial injury and leukocyte and platelet activation in CVD progression after a CVE.

Cerebrovascular Disease

Circulating microvesicles have been associated with ischemic stroke, cerebral vasospasm, lacunar infarcts and multi-infarct dementia, and carotid AT (**Table 1**). Besides, a recent case-control pilot study suggests that circulating MVs derived from monocytes of M2 phenotype, but not those of M1-phenotype are significantly increased in patients with intracerebral hemorrhage within 12 h of symptom onset (167).

AT-DRIVEN ANGIOGENESIS AND NEOVASCULARIZATION: MVs AS THERAPEUTIC VECTORS

Angiogenesis is an active fine-tuning process of vessel sprouting and growth that depends on a precise interplay between stimulatory and inhibitory signals of ECs, SMCs, and pericytes (168). Notably, recent evidence outlines the importance of EC metabolism for angiogenesis in the context of atherogenesis and AT progression (169). Thus, angiogenic processes have severe consequences on vascular remodeling and plaque stability within

vulnerable atherosclerotic plaques (plaque rupture and intraplaque hemorrhage). The generation of new delicate and frail vessels within the growing atherosclerotic lesion contributes to increase the vulnerability of the plaque to rupture. Intriguingly, it is not fully understood whether an atherosclerotic plaque in the arterial tree will rupture and trigger thrombotic cascade or will stabilize. The highest accumulation of neovessels was found in ACS-related human coronary plaques (170). Paracrine intercellular communication exerted by MVs might play a role in the adaptive response of ischemic tissue to hypoxic stress caused by CVD (171). Interestingly, MVs have been postulated as both pro- and antiangiogenic factors (172–177) (**Table 2**). Indeed, low levels of eMVs-containing β1-integrin and the enzymatically active MMP-2 and MMP-9 showed to promote angiogenesis whereas high levels abolished the angiogenic effects (76). The effect of MVs on angiogenesis is also highly dependent on the quantity, the parental cell type and cell surface content.

Microvesicles from apoptotic ECs (24, 188), endothelial progenitor cells (EPCs) (188), platelets (172, 181, 182, 193, 194), skin wound myofibroblasts (187), and ischemic muscle (183) stimulate endothelial proliferation, survival, migration, repair, and tube formation *in vitro* by activating pro-angiogenic signaling cascades, such as ERK and PI3K/Akt pathways, or through upregulation of MMP-2 and MMP-9 expression in ECs (185) (**Table 2**). Leroyer et al. (186) elegantly demonstrated that carotid plaque MVs stimulate both endothelial proliferation and *in vivo* angiogenesis in a CD40 ligand-dependent manner, which could be modulated by the fibrinolytic activity of eMVs and LMVs (184). Indeed, eMVs play an important role in plasmin formation, which can influence *in vitro* the tube formation of EPCs in a dose-dependent manner (184).

Other cell-specific MVs have also shown proangiogenic potential (**Table 2**). pMVs from atherosclerotic patients increased the neovascularization capacity of circulating angiogenic cells through a RANTES-mediated mechanism (178). pMVs not only were shown to stimulate vascular endothelial growth factor (VEGF)-dependent revascularization after chronic cardiac ischemia (172) but also stem cell repair mechanisms after brain ischemia in rats by increasing angiogenesis and neurogenesis at the infarct zone (179). In addition, the remote conditioning protective effect of pMVs was further proved against cerebral ischemic reperfusion injury (180). Increased levels of MVs from ischemic muscle showed to promote postischemic neovascularization in mouse after hindlimb ischemia (HLI) (183).

TABLE 2 | Proangiogenic potential of distinct cell-derived MVs.

Type of MVs	Source	Function	Mechanism	Reference
Platelet-derived MVs	Patients with atherosclerosis	↑ Neovascularization of CAC	Mediated by RANTES	(178)
	Healthy donors	↑ Angiogenesis *in vitro* and *in vivo*	Dependent on VEGF	(172)
	Healthy donors	↑ Proliferation, angiogenesis and neurogenesis	Differentiation and proliferation of stem cells mediated by MV growth factors (FGF2, VEGF, and PDGF)	(179)
	Healthy rats	Protective effect against cerebral ischemic–reperfusion injury	Mediating remote ischemic preconditioning	(180)
	Healthy donors	↑ Tube formation	*Via* the pertussis toxin-sensitive G protein and the PI3K pathway	(181)
	Healthy donors	↑ Capillary tube formation and reendothelialization	By sensitization of CXCR4 and growth factors	(182)
Endothelial cell (EC)-derived MVs	Mice ischemic hindlimb muscle	↑ Postischemic neovascularization	–	(183)
	Human umbilical vein ECs	↑ Angiogenesis (with low levels of eMVs)	Through β1-integrin and MMP-2 and -9	(76)
	Human ECs	↑ Formation of capillary-like structures	By MV-harboring Sonic Hedgehog	(174)
	Human coronary artery ECs	↑ Vascular endothelial repair	Induced by miR-126-containing MVs	(24)
	Human microvascular EC	↑ *In vitro* tube formation	MV-induced plasmin generation	(184)
	Human umbilical vein ECs	↑ Angiogenesis	By upregulating MMP expression	(185)
Carotid plaque-derived MVs	Endarterectomy specimens	↑ *In vivo* the tube formation	In a CD40 ligand-dependent manner	(186)
T-lymphocyte-derived MVs	Human lymphoid cells	↑ Neoangiogenesis	By NO synthesis pathway	(175)
Myofibroblasts-derived MVs	Skin wound myofibroblasts	↑ Capillary formation	–	(187)
EPC-derived MVs	EPCs from healthy donors	↑ Angiogenesis *in vitro* and *in vivo*	Through eNOS and PI3K/Akt pathway	(188)
MSC-derived MVs	MSC from bone marrow	Promote angiogenesis	–	(189)
	MSC from bone marrow	↑ Postischemic angioneurogenesis	–	(190)
	MSC from umbilical cord	↑ Angiogenesis *in vitro* and *in vivo*	–	(191)
	MSC from umbilical cord	↑ Angiogenesis *in vitro* and *in vivo*	By ↑ VEGF in a HIF-1α independent manner	(192)

CAC, circulating angiogenic cells; CXCR4, C–X–C chemokine receptor type 4; EPC, endothelial progenitor cell; eNOS, endothelial NO synthase; FGF, fibroblast growth factor; HIF, hypoxia-inducible factor; MSC, mesenchymal stem cell; MMP, matrix metalloproteinase; MV, microvesicle; NO, nitric oxide; PDGF, platelet-derived growth factor; PI3K, phosphatidylinositol-3-kinase; RANTES, regulated on activation, normal T cell expressed and secreted; VEGF, vascular endothelial growth factor; MV, microvesicle; MMP-2, matrix metalloproteinase-2.

T-lymphocyte-derived ℓMVs enriched with the morphogen sonic hedgehog increased neoangiogenesis and restored endothelial function after injection in mice by stimulating the NO synthesis pathway (175, 195). Endothelial colony-forming cell-derived MVs were demonstrated to be pro-angiogenic both *in vitro* and *in vivo* through eNOS and the PI3K/AKT pathway (188). Moreover, MVs from bone marrow-derived mesenchymal stem cells (MSCs) showed proangiogenic activities in a rat MI model contributing to cardiac repair (189) and improved postischemic neuroangiogenesis in a stroke model (190) while MSC–derived MVs from umbilical cord enhanced tube-like structure development and further rescued blood flow in a rat model of HLI (191). Indeed, umbilical cord MSC-derived MVs were shown to enhance angiogenesis in a rat model of kidney ischemia (192), suggesting that MSC-derived MVs might exert cardioprotective effects.

In contrast to the findings reported above, placental MVs have been implicated in antiangiogenic processes contributing to impaired perfusion of placenta in patients with preeclampsia (196). In line with these studies, high levels of eMVs inhibited angiogenesis in the cultured segments of hearts by hampering endothelial nitric oxide synthase (eNOS) regulation (176). Oxidative stress is also involved in the antiangiogenic effect of MVs. Lymphocyte-MVs inhibited angiogenesis regulating negatively VEGF pathway (197) whereas eMVs inhibited *in vitro* angiogenesis by impairing acetylcholine-induced endothelial vasorelaxation and NO production in rat aortic rings (22, 173). Recently, eMVs were reported to inhibit capillary-like branch structure formation by microvascular ECs ($_{m}$ECs) *in vitro* and *in vivo* triggered by ROS *via* the expression of CD36 on the target EC (198).

Interestingly, the stimulating environment influencing the generation of MVs is able to switch their angiogenic phenotype. Treatment of MSCs with PDGF generated proangiogenic MVs (199), showing that the cargoes of MVs greatly impact on their effects. In agreement, cavin-2 that is released in eMVs and required for eMV biogenesis acts as a regulator of angiogenesis by producing NO and controlling the stability and activity of eNOS (200). Moreover, we found that C-reactive protein (CRP) is pro-angiogenic (201) and interestingly CRP is carried by circulating MVs in ischemic patients (202). eMVs from patients with chronic thromboembolic pulmonary hypertension increased EC endoglin concentration and stimulated endothelial angiogenesis showing a protective mechanism for ED and vascular occlusion (203). Of note, exercise intensity increases the levels of circulating pMVs with proangiogenic potential, which stimulate EC proliferation, migration, and tubule formation (204). Besides, antihypertensive drugs have shown to regulate both eMV generation and angiogenesis (205). Therefore, MVs might be considered as dual-purpose mediators of cell–cell communication in health and disease, greatly depending on the surrounding environment in which they are formed, their content and the pathophysiological context where they exert their functions.

Similarly, MVs from preconditioned adipose-derived stem cells (ASC) showed proangiogenic potential through the release of miR-31 targeting factor-inhibiting HIF-1 and enhancing ASC therapeutic efficacy (206). Indeed, miRNA delivery is a key mechanism involved in the effects of MV-driven angiogenesis such as EPC-MVs promoting neovascularization and impairing muscle damage after HLI (207) or inducing the survival of human islet transplants (208). Thus, eMVs released upon interleukin-3 activation transferred miRNA-126-3p and pSTAT5 into ECs thereby promoting angiogenesis (209). In this regard, MVs containing miRNA-126 stimulate reendothelialization after vascular injury (24) and administration of vesicles containing miR-126 decreased atherosclerotic plaque formation and favored plaque stability in mice (210). Besides, transfer of miRNA-150 to ECs by mMVs also promoted angiogenesis both *in vitro* and *in vivo* (117).

Tissue factor, the primary cellular initiator of blood coagulation, is also involved in angiogenic processes (211–213). Proangiogenic signaling through TF-dependent MV-mediated activation of PAR-2 has been reported in hypoxic ECs (214). Besides, TF^{+}-MVs were shown to bind to β1-integrin in the surface of ECs to induce proliferation through ERK1/2 (215). In the same line, we have demonstrated that TF-containing eMVs from microvascular ECs ($_{m}$eMVs) interacted *via* paracrine signaling with other $_{m}$ECs and triggered angiogenesis *ex vivo* and postischemic collateral vessel growth *in vivo* (216). The $_{m}$eMVs proangiogenic potential was shown to be regulated by β1-integrin–EC interactions inducing Rac1–ERK1/2–ETS signaling and CCL2 production (**Figure 4**). Taken together, MVs can overwhelm the effects of arterial occlusion and tissue ischemia by stimulating postischemic neovascularization together with tissue reperfusion. Beyond being a promising therapeutic strategy for treating ischemic diseases (217), angiogenesis has also a role in the context of tumor progression and cancer, in which distinct types of MVs by means of cell-cell communication have also a regulatory function (14, 218–228). It is important to join efforts toward the ultimate goal of reaching therapeutical applications of MVs into the clinical arena.

PHARMACOLOGICAL AND NON-PHARMACOLOGICAL INTERVENTION

A deep understanding of the role of MVs in AT might be fundamental for both CVD risk factor control and therapeutic treatment (130). Non-pharmacological interventions lie in overcoming CVRF by lifestyle modifications, such as exercise and diet. In primary prevention, a recent clinical trial showed the benefit of the adherence to the Mediterranean diet in patients at high cardiovascular risk in relation to the incidence of severe CVEs (229). Within the same population, our group has recently demonstrated that decreased levels of cMVs derived from ECs, leukocytes and activated platelets could signal for a reduced rate of major CVEs in high-risk patients under state-of-the-art treatment and receiving a controlled MedDiet supplemented with nuts (230). Dietary modulation of MV release is a relatively new field of study based mainly on short-term studies; further large-scale studies will help to better understand the complex relationship between diet and CVD.

Several drugs have shown to influence cMVs levels (231, 232), their cargoes (233) and even their function (**Table 3**). For instance,

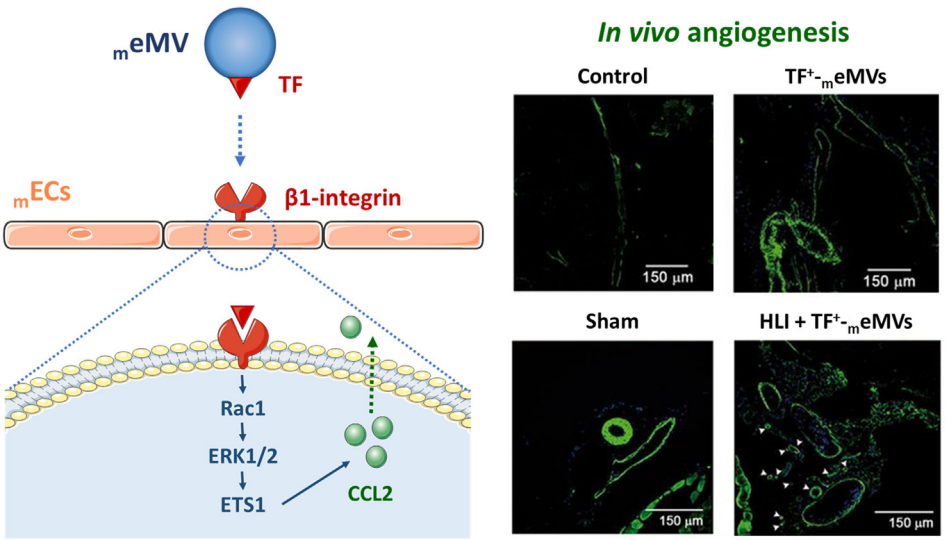

FIGURE 4 | Microvesicle (MV)-mediated neovascularization. TF-positive microvascular endothelial-derived MVs (TF^{+}-$_{m}$eMVs) were shown to interact with endothelial cell surface β1-integrin to induce a Rac1–ERK1/2–ETS signaling cascade that leads to CCL2 production and angiogenesis (216). Representative immunofluorescence images demonstrate that TF^{+}-$_{m}$eMVs enhance collateral capillary formation and angiogenesis *in vivo* after ischemic hindlimbs femoral arteriectomy with antibody against α-actin (green) and nuclear staining (blue). HLI, hindlimb ischemia; $_{m}$eMV, microvascular endothelial-derived microvesicle; TF, tissue factor.

berberine improved endothelial function by reducing $CD31^{+}$/$CD42^{-}$ eMVs and thereby levels of oxidative stress in humans (234, 235) and the adhesion of monocytes to ECs was partially prevented by eMVs after lipid-lowering and antihypertensive treatments (236). Since MV biogenesis and release is not fully understood, distinct therapeutic options are under investigation. Reduced levels of pMVs are associated with the use of distinct anti-platelet drugs such as GPIIb/IIIa inhibitors (237, 238), clopidogrel (239, 240), ticlopidine (121, 122), and acetylsalicylic acid (ASA) (241). In patients under antithrombotic therapy, P-Sel and TF-containing pMVs remain high 6 months after treatment initiation (242), likely due to the fact that low doses of ASA might not be potent enough to prevent the release of pMVs into the microcirculation (243). Our group has reported that ASA intake in patients with diabetes in primary prevention has no effect on pMVs (244). Similarly, antihypertensive drugs like angiotensin II receptor antagonists (245) and calcium channel blockers (246), antioxidants (247), peroxisome proliferator-activator receptor activators (248, 249), hydroxyurea in sickle cell disease (250), and eculizumab in paroxysmal nocturnal hemoglobinuria (251) have also shown influence on MV shedding. Up to now the effect of statins, the cornerstone drugs for lipid-lowering treatment (LLT) in CVD prevention, has been highly debated. While some authors demonstrated that statins may enhance the shedding of MVs (252, 253) many studies have found that statin treatment promote MV inhibition (88, 135, 254–256). Thus, pravastatin and simvastatin decreased pMVs in hypertensive (257) and type-2 diabetic (88, 120) patients. Similarly, atorvastatin diminished the formation of thrombin and the expression of P-Sel, TF, and GPIIIa on pMVs in patients with peripheral artery disease (233) and with dyslipidemia and type-1 diabetes (258). Indeed, in a study focused on the effects of LLT on levels of cMV in atherosclerotic patients in primary prevention, we have reported that the plasma of LLT-treated patients presented lower quantity of MVs and lower content of cell surface activation markers than the plasma from untreated patients with the same blood cholesterol levels (255), indicating a direct benefit of LLT with statins in reducing cell membrane shedding, which may have effects in the beneficial protection against AT characteristic of statins by inhibiting MV generation and the triggering of MV-dependent mechanisms. These data are in agreement to results pointing out the broader use of statins decreasing inflammation and suppressing MV release, an effect that is not shown neither with ezetimibe alone (259) nor with ezetimibe combined with statins (260). Furthermore, several inhibitors of MV shedding such as ROCK inhibitors or calpain, among others, are currently under study. Nevertheless, whether the clinical benefit of these pharmacological strategies is directly related to MV decrease deserves further research.

Therapeutic Potential

In addition to pharmacological modulation, the advantageous characteristics of cell-derived MVs, which are a naturally produced therapeutic agents with potential to be used as delivery drugs to specific cell types (261), open up their potential therapeutic application, especially in cardiac cell therapy. Preclinical studies demonstrated that treatment with the vesicular fraction of the conditioned media of hypoxic MSCs decreased infarct size and improved cardiac function by decreasing oxidative stress, enhancing myocardial viability, and preventing damage to the heart after MI in mouse and pig models (262–264). The mechanism of action might involve the transfer of specific RNAs through embryonic stem cell MVs (265). MSC-derived MVs were able to face the detrimental effects of ischemia and reperfusion (I/R) injury in the kidney (266). Similarly, MVs derived from myocardial ischemia

TABLE 3 | Main studies evaluating the effects of pharmacological therapies on circulating microvesicles.

Type of drug	Therapy dose	Subjects (N)	MV change	Reference
Anti-platelet				
GPIIb/IIIa inhibitors	Abciximab: 250 μg/kg bolus + 12 h 0.125 μg/kg/min Eptifibatide: 180 μg/kg bolus + 18 h 2 μg/kg/min	50 ST-segment elevation patients undergoing percutaneous coronary intervention	↓ GPIV^{+}-pMVs ↓ CD11a^{+}-LMVs	(238)
Clopidogrel	4 weeks, 75 mg/day	26 patients with stable coronary artery disease (CAD)	= CD51^{+}-eMVs ↓ CD42^{+}/CD31^{+}-pMV	(240)
Probucol and ticlopidine	6 months Probucol: 500 mg/day Ticlopidine: 200 mg/day	23 normolipidemic controls and 53 hyperlipidemic patients	↓ CD62P^{+}-pMVs ↓ CD63^{+}-pMVs ↓ Annexin V^{+}-MVs ↓ CD14^{+}-mMVs	(121)
Ticlopidine	1 month, 200 mg/day	21 type-2 diabetic patients	↓ CD62P^{+}-pMVs ↓ CD14^{+}-mMVs	(122)
Acetylsalicylic acid	8 weeks, 100 mg/day	15 patients with CAD	↓ eMVs ↓ pMVs	(241)
	6 months, bolus of 500 mg and 75 mg/day	51 patients with acute coronary syndromes	↑ CD62P^{+}-pMVs ↑ CD142^{+}-pMVs	(242)
	10 days, 100 mg/day	43 patients with diabetes	= pMVs	(244)
Cilostazol	1 month, 150 mg/day	30 controls and 43 non-insulin dependent diabetes mellitus	↓ CD62P^{+}-pMVs ↓ CD63^{+}-pMVs ↓ Annexin V^{+}-MVs	(231)
Antihypertensive				
Angiotensin II receptor antagonists	Eprosartan: 2 months, 600 mg/day	31 hypertensive and 31 normotensive patients	↓ CD42b^{+}-pMVs	(245)
Losartan and simvastatin	24 weeks Losartan: 50 mg/day Simvastatin: 10 mg/day	41 hypertensive patients with hyperlipidemia and/or type-2 diabetes	↓ KMP9^{+}-pMVs ↓ CD51^{+}-eMVs	(135)
Anti-diabetic				
Miglitol	1 month, 150 mg/day	72 non-diabetic patients (37 with hypertension, 35 with hyperlipidemia) and 38 diabetic patients	↓ CD42a/b^{+}-pMVs	(232)
Berberine	1 month, 1.2 g/day	14 vs. 11 healthy subjects	↓ CD31^{+}/CD42^{-} eMVs	(234)
	1 month, 1.2 g/day	12 vs. 11 healthy subjects	↓ CD31^{+}/CD42^{-} eMVs	(235)
Lipid lowering				
Statins	Pravastatin: 8 weeks, 40 mg/day	50 patients with type-2 diabetes	↓ CD61^{+}-pMVs	(88)
	Atorvastatin: 8 weeks, 80 mg/day	19 patients with peripheral arterial occlusive disease and hypercholesterolemia	↓ CD62P^{+}-pMVs ↓ CD142^{+}-pMVs ↓ CD41^{+}-pMVs	(233)
	Atorvastatin: 80 mg/day	19 patients with peripheral arterial occlusive disease	↓ CD62P^{+}-pMVs ↓ CD142^{+}-pMVs ↑ CD144^{+}-eMVs	(253)
	Simvastatin: 80 mg/day; pravastatin: 40 mg/day; lovastatin: 80 mg/day; fluvastatin: 80 mg/day; atorvastatin: 80 mg/day; rosuvastatin: 20–40 mg/day	37 hypercholesterolaemic patients and 37 normocholesterolaemic controls	↓ CD41^{+}/61^{+}-pMVs ↓ CD146^{+}/31^{+}-eMVs ↓ CD45^{+}-LMVs ↓ CD14^{+}-mMVs ↓ CD142^{+}-MVs	(255)
	Simvastatin: 6 months, 20 mg/day	21 hyperlipidemic patients	↓ CD61^{+}-pMVs	(256)
	Simvastatin: 24 weeks, 10 mg/day	41 hypertensive patients	↓ KMP9^{+}-pMVs	(257)
	Atorvastatin: 2 months, 20 mg/day	20 patients with type 1 diabetes and dyslipidemia	↓ pMVs	(258)

(*Continued*)

TABLE 3 | Continued

Type of drug	Therapy dose	Subjects (N)	MV change	Reference
Ezetimibe	10 mg/day	63 patients with coronary heart disease	= MVs	(259)
Ezetimibe with statins	Atorvastatin 80 mg/day vs. atorvastatin 20 mg/day plus ezetimibe 10 mg/day	75 high-risk subjects	↓ pMV with high-dose statin monotherapy	(260)

MV, microvesicle; eMVs, endothelial-derived microvesicles; LMVs, leukocyte-derived microvesicles; mMVs, monocyte-derived microvesicles; MVs, microvesicles; pMVs, platelet-derived microvesicles.

could protect hearts from I/R injury in rats through calcium regulatory proteins to alleviate intrinsic myocardial mitochondrial and endoplasmic reticulum apoptotic pathways (267). Interestingly, the effect of cardiosphere-derived cells (CDC) on the therapeutic regeneration of the infarcted myocardium shown in a clinical trial (268) seems to be mediated by CDC-derived extracellular vesicles (269). ECs have also been shown to be atheroprotective by transferring miRNAs *via* MVs to SMCs (81). Specifically, MVs transported functional miR-143/145 into SMCs after activation by shear stress and they reduced atherosclerotic lesion formation in the aorta. Furthermore, a promising therapeutic application of MVs is the use of synthetic MVs mimicking the natural ones. MV delivery could be clinically useful in several conditions such as MI and inflammatory pathologies. Indeed, a recent work reported that infusion of artificially produced MVs can improve inflammation and ameliorate symptoms in different mouse models of MI, multiple sclerosis, and kidney injury (270). Therapeutic innovation of MVs is still in its infancy, and its applicability is hampered by many shortcomings, such as the *in vivo* biodistribution of MVs that depends on their cellular origin, their half-life and the route of administration (271). Further understanding of the biological mechanisms, efficiency and feasibility of these MV-based therapies is warranted.

CONCLUSION AND FUTURE PERSPECTIVES

Microvesicles actively contribute to AT progression and complication due to their implicit role in cell-to-cell communication. cMVs not only play a direct biological role in AT and neoangiogenesis but also might be susceptible targets for pharmacological modulation and emerge as potential prognostic and diagnostic biomarkers of atherothrombotic CVD. However, limitations and technological constraints have precluded the complete understanding of mechanisms of MV formation and pathophysiological relevance. Most functional effects of MVs have been evidenced in *in vitro* studies with a predetermined MV concentration that is not always comparable to the pathophysiological situation while their exact role in the clinical setting is dismissed. Despite cMVs emerge as promising biomarker candidates, clinical studies in larger cohorts are required to clearly delineate their role as diagnostic and prognostic markers of disease. Until now, the lack of standardization in preanalytical phase guidelines, the difficult implementation as routine in the clinical laboratory as well as the high cost of cMV measurements have curbed their full clinical characterization. However, recent and advanced sensitive technologies together with consensus within the scientific community will undoubtedly shed light on the cMV potential as biomarkers. Besides, exploring the cMV specific targeting to a selective tissue, how they are produced *in vivo* and their specific genomic, transcriptomic, metabolomic and proteomic content is essential to design efficient therapeutically strategies involving MVs. In the next coming years, we will witness advances and breakthroughs in the area of MVs that will translate into their use in diagnostic and therapeutic innovation.

AUTHOR CONTRIBUTIONS

LB, RS, and TP conceived and coordinated the design of the review and wrote the paper. RS made figures. LB and RS edited the paper. All the authors wrote part of the manuscript, provided critical comments, revised the manuscript, and approved the final version of the manuscript.

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Message in a Microbottle: Modulation of Vascular Inflammation and Atherosclerosis by Extracellular Vesicles

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Extracellular vesicles (EVs) have emerged as a novel intercellular communication system. By carrying bioactive lipids, miRNAs and proteins they can modulate target cell functions and phenotype. Circulating levels of EVs are increased in inflammatory conditions, e.g., cardiovascular disease patients, and their functional contribution to atherosclerotic disease development is currently heavily studied. This review will describe how EVs can modulate vascular cell functions relevant to vascular inflammation and atherosclerosis, particularly highlighting the role of EV-associated proteolytic activity and effector proteins involved. Furthermore, we will discuss key questions and challenges, especially for EV-based therapeutics.

Keywords: extracellular vesicles, vascular inflammation, atherosclerosis, proteolytic activity, challenges

INTRODUCTION

Extracellular vesicles (EVs) play a crucial physiological and pathophysiological role, as they have been identified as regulators of cell-to-cell communication (1).

Extracellular vesicles are small spherical vesicles, consisting of a lipid bilayer membrane encasing a small organelle-free cytosol, that are released by cells into the extracellular environment (2). It has been shown that most cell types can release EVs, originating from various subcellular membrane compartments (3). Nowadays, EVs are generally classified into three main classes, i.e., exosomes, microvesicles (MVs), and apoptotic bodies (3). Exosomes arise from intracellular compartments called multivesicular bodies (MVBs) and are released by an active process, leading to fusion of these MVBs with the plasma membrane (4). Exosomes typically have a size of 30–100 nm, i.e., representing the smallest subgroup of EVs, and are enriched for tetraspanins (CD9, CD63, and CD81) or other markers, such as flotillin and tumor susceptibility gene 101, which are often used to distinguish them from other populations of EVs (5). The second class of EVs is MVs, which are typically larger in size (ranging from 100 to 1,000 nm) and are produced by budding off directly from the plasma membrane in a process called microvesiculation (5). Microvesiculation involves the externalization of phosphatidylserine (PS) followed by cytoskeleton rearrangement and the formation of membrane curvatures (6, 7). Therefore, MVs membranes are also enriched in PS (detectable by Annexin A5) and the membrane composition resembles that of the parental cell (8). The third type of EVs is apoptotic bodies with a size of >1 μm. These vesicles are released from apoptotic cells through membrane blebbing and therefore contain apoptotic nuclear material (9).

However, although the field is rapidly evolving, it is still quite challenging to specifically isolate, characterize, and classify the different populations of vesicles as discussed below.

Extracellular vesicles can cargo a large variety of biomolecules, such as various DNA, RNA, and microRNA species, bioactive lipids, and proteins. The latter include receptor ligands, by which EVs can interact with target cells (2), and proteolytically active enzymes, by which these vesicles can influence many cellular functions (10). This review will give a brief overview on how EVs can modulate vascular cell functions relevant to vascular inflammation and atherosclerosis, particularly highlighting the role of EV-associated proteolytic activity and effector proteins involved. Furthermore, we will discuss key questions and challenges, especially for EV-based therapeutics.

EVs IN VASCULAR INFLAMMATION AND ATHEROSCLEROSIS

Recent years, great efforts have already been made to elucidate the role of EVs in cardiovascular diseases (CVDs), which is still the major cause of mortality worldwide. CVDs are mainly caused by atherosclerosis, a chronic inflammatory disease initiated by a continuous damage of the vascular endothelium leading to endothelial dysfunction (11). It has already been clearly shown that inflammation and endothelial injury augment the release of EVs (12, 13), generally reflecting the pro-inflammatory state of the parental cell. In addition, EVs influence thrombus formation which can occur after plaque rupture (3). Indeed, atherosclerotic lesions contain and release EVs, derived from leukocytes, platelets, smooth muscle cells (SMCs), and endothelial cells, during all stages of atherosclerosis development (14, 15) (**Figure 1**). As a consequence, patients with CVD mediated by endothelial damage show significantly elevated levels of circulating cell-derived EVs (16). This observation has therefore also been the starting point to investigate EVs as potential prognostic and diagnostic biomarkers. While most research has focused on the presence and function of MVs, also exosomes have been observed in human atherosclerotic lesions (17), although their functional roles remain largely unexplored.

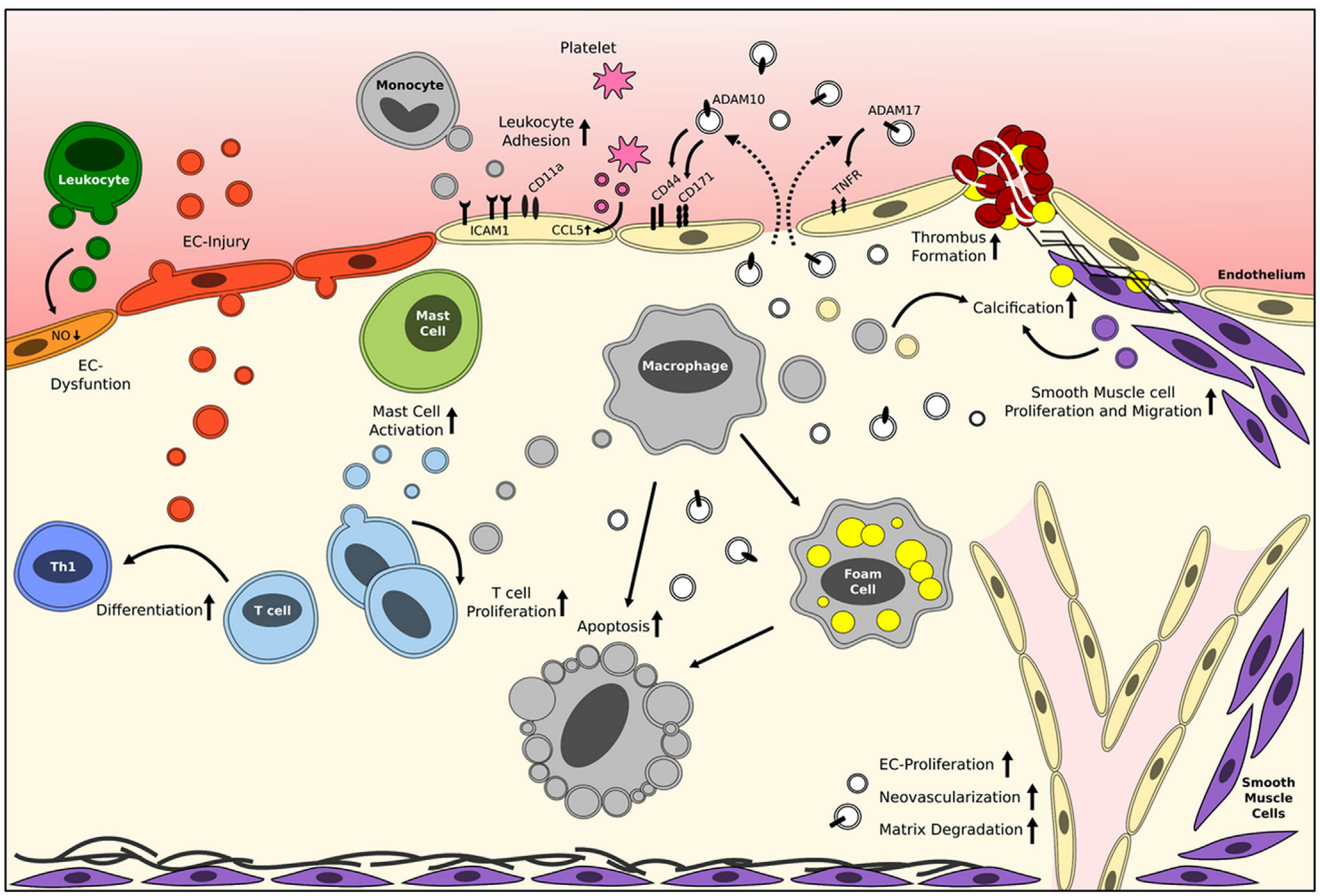

FIGURE 1 | Reported roles of EVs in vascular inflammation and atherosclerosis. Brief schematic representation of the reported effects of circulating cell-derived and plaque-derived EVs on different processes in atherosclerosis development. The mentioned effector molecules are merely examples, and it should be noted that many more exist. White vesicles are of unknown origin/parental cell. EC, endothelial cell; EV, extracellular vesicle. Please refer to **Table 1** for more detailed information.

Several *in vitro* studies clearly show that platelet and leukocyte-derived MVs from unstimulated cells increase the release of pro-inflammatory cytokines from endothelial cells and leukocytes, especially interleukin (IL)-6 and IL-8 (18, 19). Release of these cytokines will inherently promote monocyte adhesion to the endothelium and migration into the atherosclerotic lesions. MVs released from human atherosclerotic plaques were shown to increase the expression of endothelial adhesion molecules such as intercellular adhesion molecule 1 and monocyte adhesion molecule receptors, like CD11a, thereby further augmenting monocyte adhesion (18, 20). Furthermore, CCL5 (RANTES) is transferred from healthy platelet MVs to activated endothelial cells and can thereby enhance leukocyte adhesion (21). Endothelial and leukocyte MVs have also been shown to induce endothelial cell dysfunction, by decreasing the production of nitric oxide (NO) (22, 23). This is the result of an inhibition of the endothelial NO synthase and/or an increase in caveolin-1, increasing local oxidative stress (24, 25). Besides this mediator, MVs can act as potential markers of endothelial dysfunction, as nicely reviewed in Ref. (26). Together, these data clearly show that MVs, derived from various (vascular) cell types, can greatly influence the initiation of atherosclerosis development.

Microvesicles derived from macrophages and fibroblasts have also been implicated in later stages of lesion development, as they can stimulate foam cell formation by lipid/cholesterol uptake in macrophages (27). Furthermore, several reports have indicated that T cell-derived MVs can contribute to monocyte and macrophage apoptosis, *via* two proposed mechanisms (28, 29). The first mechanism involves the phagocytosis of MVs by monocytes and macrophages, leading to an increased cellular content of membrane phospholipids. These phospholipids are likely cleaved by phospholipase A2 into arachidonic acid, which will subsequently result in an increased amount of proapoptotic ceramides inside the cells (28, 29). The second mechanism involves MVs containing caspase-1 or caspase-3, which can induce target cell apoptosis (30, 31). Several studies have shown that MVs also play an important role in lymphocytes, as both human atherosclerotic plaque and *in vitro* generated dendritic cell MVs can stimulate T cell proliferation (32, 33). Most likely this influence is mediated by the presence of major histocompatibility complex class II presence on the MVs secreted from macrophages and dendritic cells (32). Furthermore, endothelial cell-derived MVs can promote lymphocyte differentiation toward a more proatherogenic T helper-1 phenotype as shown by priming of naive T cells with dendritic cells which were matured with endothelial MVs (34). On their turn, activated T cells release MVs that can induce mast cell activation, degranulation, and cytokine release (35). Mast cells are also present in the arterial wall, where they can contribute to atherosclerosis development (36).

Furthermore, MVs have significant effects on plaque stability as they can influence SMC proliferation and migration, *via* protease-activated receptor interaction or various microRNAs (37, 38). In addition, plaque MVs can contribute to matrix degradation as they contain several active proteases (39), which will be discussed in more detail later. This influence on matrix degradation is also one of the mechanisms by which MVs could potentially contribute to intraplaque neovascularization. It has also been shown that human plaque MVs can increase endothelial proliferation, a crucial step in neovascularization, *in vitro* as well as *in vivo* in matrigel plugs (40). During human atherosclerosis development, intimal calcification occurs at different stages of lesion development (41). Moreover, endothelial, SMC, and macrophage-derived EVs are present at the sites of calcification (3), nicely reviewed in Ref. (42). EVs released from SMCs have the potential to stimulate calcification by these same SMCs, mediated by sortilin-dependent regulation of alkaline phosphatase trafficking (43). In addition, EVs enriched in bone morphogenetic protein 2 released from endothelial cells can promote calcification in vascular SMCs (44).

In the latest stages of atherosclerosis, i.e., plaque rupture and thrombosis, MVs can also play an important role. MVs/EVs carry various proteolytic factors that likely contribute to matrix degradation, as shown in cancer (45) and could thereby potentially also influence plaque destabilization. In addition, human plaque MVs have been shown to be particularly prothrombogenic (15). Plaque MVs can contribute to the coagulation pathway *via* two different pathways: the presence of tissue factor on the surface of MVs and the exposure of PS on the outer membrane layer (3, 46). In contrast to MVs, exosomes seem to have antithrombotic effects. Platelet aggregation was suppressed by platelet-derived exosomes by inhibiting platelet CD36 (47). The procoagulant role of MVs is more elaborately reviewed in Ref. (48).

Besides communication between different cells within an atherosclerotic plaque, it is generally assumed that EVs, as they are relatively stable, mediate cross talk with cells at relatively large distances. This is particularly relevant for CVDs, which is widely acknowledged to be a systemic disease, and the basis for the "vulnerable patient concept" (49, 50). Indeed, it has already been long recognized that clinical symptoms in CVD patients (e.g., myocardial infarction or stroke) are often followed by secondary CVD events. Moreover, CVDs are often associated with several comorbidities, e.g., diabetes, chronic kidney disease, non-alcoholic steatohepatitis, small cerebral vessel disease, and heart failure. It is likely, yet it remains to be determined, that EVs play a crucial role in this systemic intercellular communication.

PROTEOLYTIC ENZYMES/EFFECTOR MOLECULES IN EVs

Extracellular vesicles are known to carry a large amount of bioactive molecules, including proteins/enzymes. Still, relatively little is known on the influence of various (atherogenic) stimuli on EV composition and thus EV function. Proteomic analysis recently identified several proteolytical enzymes in EVs, such as the cell surface-bound sheddases a disintegrin and metalloproteinases (ADAMs), soluble ADAMs with thrombospondin motifs (ADAMTSs), as well as cell surface-bound and soluble matrix metalloproteinases (MMPs) (51).

A disintegrin and metalloproteinases are involved in ectodomain shedding of various transmembrane proteins, thereby regulating cell adhesion, migration, and cell–cell communication (52).

ADAM10 and ADAM17 are the best studied members of this family. ADAM17 is considered the primary enzyme for shedding of tumor necrosis factor (TNF), and its receptors (TNFR1 and 2), and the epidermal growth factor receptor ligands (53). On the other hand, ADAM10 is physiologically critical for Notch signaling *via* receptor cleavage (54). ADAMs have been reported to mediate various exosome/MV functions, e.g., by cleavage of EV surface molecules, releasing them as soluble factors in the target cell microenvironment. Indeed, ADAM17 is present on MVs released from atherosclerotic lesions and shown to cleave pro-TNF from these vesicles, which could provide a means to locally release pro-inflammatory mediators at large distances from the cell/site from which the MVs are released (39). In addition, plaque MVs have been shown to increase the shedding of TNF and its receptor (TNFR) from the surface of endothelial cells in an ADAM17-dependent manner (39), further supporting a role for ADAM17$^+$ MVs in the regulation of (systemic) vascular inflammation.

Little is known on the role of other EV-associated ADAMs in relation to atherosclerosis. In exosomes, secreted from ovarian carcinoma cells, especially ADAM10 has been shown to be crucially involved in the cleavage of CD171 (L1) and CD44 (55), two important cell adhesion molecules. Cleavage did not only occur in the released exosomes but also already in the earlier phases of vesicle formation in the endosomal compartment. ADAM17 is also able to cleave CD171, although this occurs only at the cell surface demonstrating that different ADAMs are involved in distinct cellular compartments (55), and thus potentially in different EV populations. Other ADAMs such as ADAM15 (56), have also been identified in exosomes. Tumor cell-derived exosomes, enriched in ADAM15, display a high binding affinity for integrin αvβ3 and suppress cell adhesion, migration and growth (56). Exosomes derived from macrophages have also been shown to express ADAM15 and demonstrate described tumor inhibitory effects (56). The functional contribution of ADAM proteases in EVs to CVD disease progression, however, remains to be determined.

ADAMs with thrombospondin motifs are relatively comparable to ADAMs, but have thrombospondin-like motifs instead of transmembrane and cytoplasmic domains and are therefore generally secreted as soluble proteins (45). A large subgroup of ADAMTSs is known as aggrecanases, because they can proteolytically cleave proteoglycans and are involved in cartilage degradation (57). This degradation of cartilage by aggrecanases has been associated with the progression of arthritis (58). Recently, it has been shown that rheumatoid synovial fibroblasts secrete MVs containing aggrecanase activity, most likely mediated by ADAMTS1, ADAMTS4, or ADAMTS5 (59). Synovial fluids in rheumatoid arthritis also contain T cell- and monocyte-derived MVs, which can induce the synthesis of several MMPs in fibroblasts (60). Considering the role of various ADAMTS proteases in inflammation and vascular biology (61, 62), it is likely that EV-associated ADAMTSs are implicated in CVD. However, there are no clear indications for such a role of ADAMTSs in EVs in other pathologies, such as CVDs, yet.

Matrix metalloproteinases are a family of zinc-dependent endopeptidases, which are also crucial to extracellular matrix degradation and cleavage of surface proteins. It has already been shown that EVs released from mouse melanoma cells and human colorectal carcinoma cells have gelatinolytic and collagenolytic activity, indicating the presence of active MMPs (63, 64). Indeed, more recently several MMPs have been detected in EVs derived from tumor cells (45). Interestingly, there is also a positive correlation between the quantity of shed vesicles, the amount of vesicle bound lytic enzymes and the *in vitro* invasive capability of different human cancer cell lines (65). Since MMPs also play a role in CVD (66), a role of MMPs in EVs in CVD can be expected but has surprisingly not been evaluated so far.

CLINICAL POTENTIAL AND CHALLENGES

Targeting EVs seems like a promising novel therapeutic option, where EVs containing RNA, DNA, or proteins involved in disease pathogenesis can be blocked. Blockage of EVs and especially the delivery of their cargo to the target cell can be achieved in various ways, e.g., by inhibiting the vesicle release, uptake or formation [reviewed by El Andaloussi et al. (67)]. Vesicle formation can be suppressed by inhibiting crucial cellular compartments, for instance by ceramide or syndecan proteoglycans blockage. Furthermore, the release of vesicles can be blocked by inhibiting GTPases, which are needed for the fusion of MVBs with the plasma membrane. In addition, EVs could be used as therapeutic delivery tools. For this purpose, both endogenously produced EVs and EVs, which are deliberately packaged with specific components can be used (68). For example, a recent proof of concept study has shown that EVs could deliver specific siRNA to mouse brains (69). In the context of CVD, a recent study has shown that *in vitro* generated endothelial EVs could reduce atherosclerosis formation by the transfer of miRNAs (38). The first clinical trials using EVs have also already been started in the field of antitumor immunotherapy. Two separate phase I trials used Good Manufacturing Practice compatible protocols to isolate EVs from dendritic cells and could show a good feasibility and safety of EV administration in patients (70). The phase II trial that followed unfortunately did not give the expected positive outcomes, but combined these results clearly show the therapeutic potential of EVs.

In addition to their therapeutic use, EVs could also be used as biomarkers as they are also found in several body fluids, such as blood (71) and urine (72), making them easily accessible for prognostic or diagnostic purposes. Emphasizing the prognostic potential, it has already been shown that Cystatin C, Serpin F2, and CD14 MV levels correlate with an increased risk for cardiovascular event and mortality (73). In addition, miRNA content of EVs has already been clearly linked with disease outcome (74). More details about the clinical potential of EVs and their use as biomarkers are recently elaborately reviewed in Ref. (75).

The field of EV research is rapidly progressing, although the EV research complexity and challenges are still considerable (76). EVs represent a very heterogeneous population, both in size and composition. This has led to some confusing and variable nomenclature, although as described before some consensus has already been achieved. Another major difficulty is the presence of non-EV components in preparations of EVs, which have

TABLE 1 | Summarizing described studies supporting the role of EVs in vascular inflammation and atherosclerosis.

Cell origin	Species origin	Study type	Activation stimuli	Main findings	Reference
ECs	Mouse/ human	*Ex vivo/in vitro*	n.a.	MVs attenuate EC-mediated vasodilation *ex vivo* and reduced NO release *in vitro*	(24)
ECs	Human	*In vitro/in vivo*	Hydrogen peroxide	Elevated levels of $CD144^+$ EVs reflect EC injury *in vitro* and correlate with CVD risk *in vivo*	(16)
ECs	Human	*In vitro/in vivo*	n.a.	MVs correlate with decreased arterial function *in vivo* and decreased NO release *in vitro*	(22)
ECs	Human	*Ex vivo*	n.a.	MVs impaired vasorelaxation and NO production by rat aortic rings	(23)
ECs	Human	*In vivo/in vitro*	High glucose	MVs derived from high-glucose ECs impaired endothelial function and increased macrophage infiltration after injection into mice and increased NADPH oxidase activity and ROS levels *in vitro* (compared with MVs from untreated ECs)	(25)
ECs	Human	*In vitro*	Various apoptosis inducer	MVs from apoptotic ECs contain caspase-3	(30)
ECs	Human	*In vitro*	TNF	DCs matured with MVs resulted in priming of naïve T cells toward more proatherogenic T helper-1 phenotype	(34)
ECs	Human	*In vitro/in vivo*	KLF2 or shear stress	EVs are enriched in miR-143/145 and control SMC gene expression and phenotype *in vitro* and reduce atherosclerotic lesion formation in mice	(38)
ECs	Human	*In vitro*	TNF	EVs enriched in bone morphogenetic protein 2 promote calcification in SMCs	(46)
SMCs	Human	*In vitro*	n.a.	EVs stimulate calcification of SMCs in a sortilin-dependent manner	(45)
PMNs	Human	*In vitro*	Formyl peptide and phorbol ester	MVs stimulate EC activation and cytokine release	(19)
Monocytes	Human	*In vitro*	Endotoxin	Monocyte-derived MVs contain caspase-1 and induce cell death of SMCs	(31)
Fibroblasts	Mouse	*In vitro*	n.a.	MVs stimulate macrophage foam cell formation, which is enhanced by TLR stimulation	(27)
DCs	Human	*In vitro*	LPS	Released MVs from activated DCs can fuse with resting DCs and activate T cells	(33)
T cells	Human	*In vitro*	Apoptosis inducers	MVs increase macrophage apoptosis and stimulated macrophage MV release	(28)
T cells	Human	*In vitro*	IL-2	MVs perturb lipid homeostasis of macrophages and thereby induce apoptosis	(29)
T cells	Human	*In vitro*	PMA	T cells release MVs that induce mast cell activation, degranulation and cytokine release	(35)
Platelets	Human	*In vitro*	n.a.	MVs increased monocyte adhesion to ECs and chemotaxis	(18)
Platelets	Human	*In vitro*	n.a.	MVs enhance monocyte rolling/arrest by depositing RANTES on ECs	(21)
Platelets	Human	*In vitro*	Thrombin	Exosomes inhibit atherothrombotic processes by reducing CD36-dependent lipid loading of macrophages and by suppressing platelet thrombosis	(49)
Plaques	Human	*Ex vivo*	n.a.	MV are more abundant and thrombogenic in plaques compared with plasma	(15)
Plaques	Human	*Ex vivo*	n.a.	First ultrastructural evidence of plaque exosomes	(17)
Plaques	Human	*Ex vivo*	n.a.	MVs stimulate intercellular adhesion molecule 1-dependent monocyte adhesion	(20)
Plaques	Human	*In vitro*	n.a.	MVs express MHC-I and MHC-II and induce T cell proliferation	(32)
Plaques	Human	*In vitro*	n.a.	ADAM17, present on plaque MVs cleaves pro-TNF from these vesicles Plaque MVs increase TNF shedding and its receptor (TNFR) from ECs	(40)
Plaques	Human	*In vitro/in vivo*	n.a.	MVs increased EC proliferation *in vitro* and stimulated *in vivo* angiogenesis in matrigel assays in mice	(41)
Plaques	Human	*Ex vivo*	n.a.	Plaque MVs contribute to the coagulation pathway *via* two different pathways: the presence of tissue factor on the surface of MVs and the exposure of PS	(48)
Plasma	Human	*Ex vivo*	n.a.	Cystatin C, Serpin F2, and CD14 MV levels correlate with an increased risk for cardiovascular event and mortality	(75)
Plasma	Human	*Ex vivo*	n.a.	MVs containing miR-126 and miR-199a predict the occurrence of cardiovascular events	(76)

DC, dendritic cell; EC, endothelial cell; KLF2, Krüppel-like factor 2; LPS, lipopolysaccharide; MHC, major histocompatibility complex; MV, microvesicle; NO, nitric oxide; PMA, 4-beta-phorbol 12-myristate 13-acetate; PMN, polymorphonuclear leukocytes; PS, phosphatidylserine; SMC, smooth muscle cell; TLR, toll-like receptor; TNF, tumor necrosis factor; EV, extracellular vesicle; IL, interleukin; n.a., not applicable.

comparable features (77). Currently, various isolation methods are used to isolate EV subtypes, such as differential (ultra)centrifugation, density gradient centrifugation, size exclusion chromatography, and immunocapture. All of these methods result in EV preparations of different composition and especially purity. For example, ultracentrifugation not only pellets EVs but also protein aggregates, while lipoproteins have similar size and density as EVs and are therefore often co-isolated (78). Recently, more confounding factors of ultrafiltration and protein analysis have been identified (79). Since, these different methods have not yet been tested side by side on a single EV sample, reliable quantitative comparisons regarding recovery and purity are difficult. Another interesting point that needs consideration is the influence of medication on EVs. For example, several antiplatelets agents, such as aspirin, can inhibit platelet activation and the related release of MVs (3). Antihypertensive agents have also been shown to reduce circulating platelet- and monocyte-derived MVs (3). In addition, statin therapy influences the composition of endothelial MVs (80). Besides the variety in contaminating factors in the different isolation methods, another major limitation is the unstandardized and often inadequate reporting on the specific methods used. Previously, the International Society for Extracellular Vesicles already introduced the minimal information for studies on EVs guidelines (81). More recently, to further improve the reliability of EV-related data/publications an international consortium developed the EV-TRACK (transparent reporting and centralizing knowledge in EV research) platform (82). This platform urges researchers to report more specific and detailed parameters which are necessary to fully interpret the obtained data and compare different studies. In addition, a recent review gives some methodological guidelines to study EVs (83). All these efforts clearly show the intention to standardize EV procedures, which will also be necessary to advance this research field toward comparable/supportive studies, crucial to pave the way toward clinical trials.

CONCLUDING REMARKS

In the context of CVD and in particular atherosclerosis, a large variety of risk factors and contributing factors have already been identified and are currently targeted to treat this pathology, such as inflammatory molecules and lipids/lipoproteins. Although EVs have already been shown to be of crucial importance in the modulation of vascular inflammation and atherosclerosis (**Table 1**), at least *in vitro*, little is known on their therapeutic potential for CVD. Moreover, there are still several major limitations that should be overcome, such as detailed characterization and isolation procedures. Therefore, more preclinical studies are necessary before attempting to translate this research field to human medicine. In conclusion, EVs are promising targets for vascular inflammation and atherosclerosis and future research will further elucidate the full potential of such vesicles in disease prognosis, diagnosis and therapy.

AUTHOR CONTRIBUTIONS

EV and RJ: drafting the manuscript. MD: concept and design; drafting the manuscript.

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REVIEW
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Extracellular Vesicles as Protagonists of Diabetic Cardiovascular Pathology

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Extracellular vesicles (EVs) represent an emerging mechanism of cell–cell communication in the cardiovascular system. Recent data suggest that EVs are produced and taken up by multiple cardiovascular cell types, influencing target cells through signaling or transfer of cargo (including proteins, lipids, messenger RNA, and non-coding RNA). The concentration and contents of circulating EVs are altered in several diseases and represent explicit signatures of cellular activation, making them of particular interest as circulating biomarkers. EVs also actively contribute to the progression of various cardiovascular diseases, including diabetes-related vascular disease. Understanding the relationships between circulating EVs, diabetes, and cardiovascular disease is of importance as diabetic patients are at elevated risk for developing several debilitating cardiovascular pathologies, including diabetic cardiomyopathy (DCM), a disease that remains an enigma at the molecular level. Enhancing and exploiting our understanding of EV biology could facilitate the development of effective non-invasive diagnostics, prognostics, and therapeutics. This review will focus on EV biology in diabetic cardiovascular diseases, including atherosclerosis and DCM. We will review EV biogenesis and functional properties, as well as provide insight into their emerging role in cell–cell communication. Finally, we will address the utility of EVs as clinical biomarkers and outline their impact as a biomedical tool in the development of therapeutics.

Keywords: extracellular vesicles, diabetes, cardiovascular, atherosclerosis, cardiomyopathy, miRNAs

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INTRODUCTION

The prevalence of diabetes mellitus (DM), especially type 2 DM (T2DM), is steadily increasing and is predicted to rise substantially over the next decade (1, 2). Mortality rates of individuals with T2DM are consistently elevated, with an overall excess risk of death from any cause of ~27% (3). There is abundant epidemiological and mechanistic evidence underscoring the role of T2DM as an independent risk factor for accelerated cardiovascular disease (4, 5). Individuals with T2DM are at high risk for developing several cardiovascular disorders, including coronary heart disease, stroke, peripheral arterial disease, and diabetic cardiomyopathy (DCM) (6). Much of the vascular burden associated with T2DM is caused by the chronic, injurious effects of hyperglycemia on the micro- and

Abbreviations: CM, cardiomyocytes; DCM, diabetic cardiomyopathy; DM, diabetes mellitus; ECs, endothelial cells; EVs, extracellular vesicles; mRNA, messenger ribonucleic acid; MVs, microvesicles; MVBs, multivesicular bodies; T2DM, type 2 diabetes mellitus.

macro-vasculature [see Ref. (7) for a comprehensive review]. Indeed, many of the earliest pathological responses to hyperglycemia are manifested in the vascular endothelial cells (ECs) that interface with elevated blood glucose levels. Traditionally, the activation of pathological inflammatory processes through both paracrine and endocrine cellular communication has served as the centerpiece for the purported development of diabetic cardiovascular pathologies (8). However, a third mechanism of intercellular communication, involving the intercellular transfer of extracellular vesicles (EVs), is emerging as an important mediator. Much remains to be explored regarding the contribution of these EV pathways to cardiovascular complications in T2DM patients.

EXTRACELLULAR VESICLES

Nomenclature and Biogenesis

Extracellular vesicles are a heterogeneous population of small cell-secreted phospholipid bilayer-bound structures naturally released into the extracellular space. Secretion of EVs appears to be conserved across species, as they have been identified in fundamentally all eukaryotes and many prokaryotes (9). Using current conventions, EVs are classified into three major subtypes based on biogenic, morphological, and biochemical properties: exosomes, microvesicles (MVs), and apoptotic bodies (**Table 1**). Characterization and classification of this heterogeneous population of membrane vesicles has been challenging and the source of heated debate, but based on current evidence, a working basis for a consensus has recently been reached (10). Garnering focused attention have been exosomes, which are the smallest subgroup of EVs at approximately 30–100 nm in diameter. Exosomes are generated within the endosomal system, initially forming as intraluminal vesicles inside multivesicular bodies (MVBs) in the endosomal compartment during the maturation of early into late endosomes (**Figure 1**) (11). The formation of MVBs has been shown to be mediated by the endosomal sorting complex required for transport (ESCRT) machinery, which sequesters ubiquitinated transmembrane proteins and drives intraluminal membrane budding (12, 13). However, ESCRT-independent exosome biogenesis pathways have been suggested, primarily *via* tetraspanin-dependent mechanisms (14, 15). MVBs have a bipartite fate; either degradation through fusion with lysosomes or exocytosis as exosomes after fusion with the plasma membrane. The release of exosomes into the extracellular milieu appears to be facilitated, in part, by SNARES and Rab proteins (16).

Microvesicles (also known as microparticles or ectosomes) tend to be larger in size, approximately 100–1,000 nm in diameter, and arise in a biogenically distinct fashion. They are formed by the outward budding and scission of extracellular membrane (**Figure 1**) (17). The release of vesicles is preceded by the budding of cytoplasmic protrusions, which detach through the fission of their stalk. It is thought that dynamic interactions between cholesterol-rich microdomains regulated by animophospholipid translocases initiates formation, followed closely by vesicle budding induced by translocation of phosphatidylserine to the outer-membrane leaflet and contraction of cytoskeletal structures by actin-myosin interactions (18, 19).

Apoptotic bodies are the largest subtype of EVs, encompassing a wide size range of approximately 1–5 μm in diameter. Unlike exosomes and MVs, which are generated in both physiological and pathological conditions, apoptotic bodies are only generated by plasma membrane blebbing of apoptotic cells (20). While commonly regarded as purely cellular debris, in the emerging context of EV cellular communication, apoptotic bodies represent a potentially untapped source of biologically useful information; having various cargoes, including organelles, packed tightly within their structures (21).

Function

Extracellular vesicles are secreted from most cell types and are able to elicit diverse responses in recipient cell types. This is accomplished by engagement of EV surface proteins with receptors on recipient cells or through internalization of EVs into recipient cells, thereby transporting EV cargo into the cell. Uptake mechanisms include endocytosis, fusion with the recipient cell's membrane or uptake *via* binding of EV surface proteins such as tetraspanins to the target cell's membrane (22, 23). The notion that exosomes and MVs act as effectors of cellular communication is founded on ample data showing that they can transport bioactive molecules to target cells—either locally, or systemically by entering biological fluids—and transfer select cargo to affect molecular pathways and the behavior of

TABLE 1 | Common classifications of extracellular vesicles (EVs).

Characteristics	EVs		
	Exosomes	**Microvesicles**	**Apoptotic bodies**
Biogenesis	Sorted as intraluminal vesicles in multivesicular endosomes and secreted after the fusion of multivesicular bodies with the plasma membrane	Fission and outward budding from the plasma membrane directly into the extracellular environment	Generated through apoptotic fragmentation and blebbing
Size	30–100 nm	100–1,000 nm	1–5 μm
Markers	Tetraspanins (CD9, CD63, CD81), heat shock proteins (HSPA8, HSP70, HSP90), Annexin A2, Enolase 1, Flotilin-1, and TSG101		TSP, 3Cb
Cargo	DNA, RNA (messenger RNA, miRNA, lncRNA), Proteins (cytokines), Lipids		

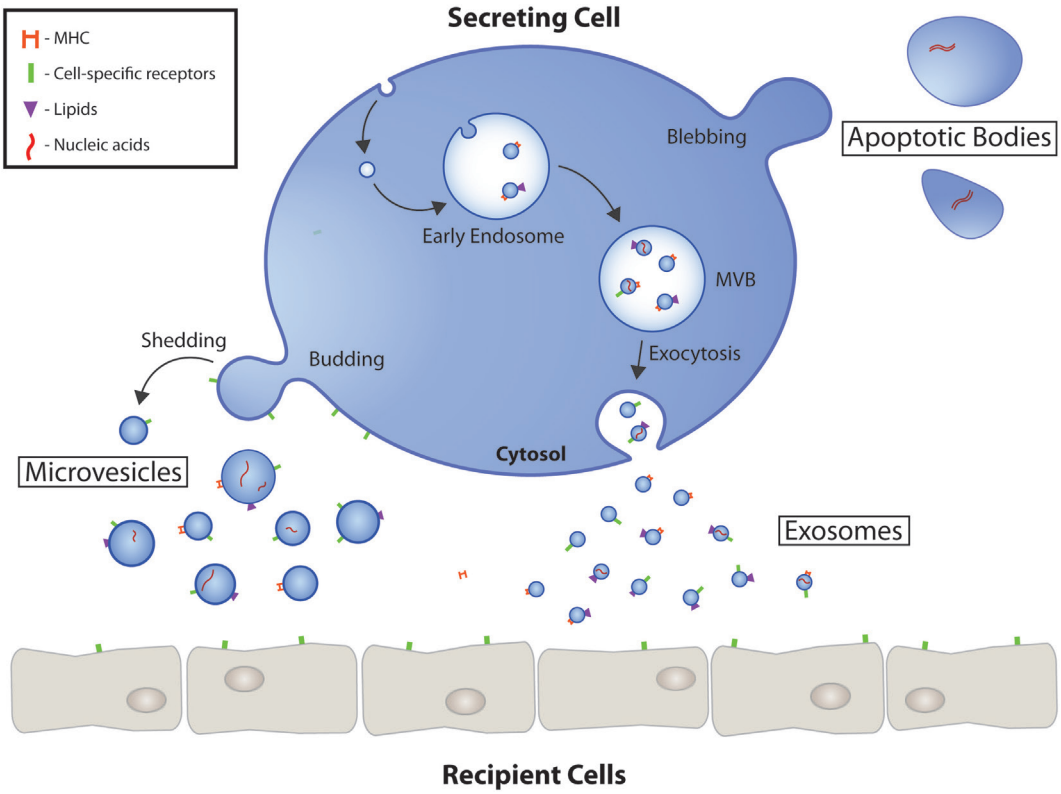

FIGURE 1 | Extracellular vesicle (EV) biogenesis and secretion. Schematic representation of the origin and release of EVs by eukaryotic cells. Exosomes are formed as intraluminal vesicles by budding into early endosomes. MVBs typically have two fates; fusion with lysosomes or fusion with the plasma membrane, which allows the release of their content into the extracellular milieu. Microvesicles arise as a result of outward budding and fission of the plasma membrane mediated by phospholipid redistribution and cytoskeletal protein contraction. The largest EVs, apoptotic bodies, are formed during programmed cell death mediated in part by actin-myosin mediated membrane blebbing. EVs have numerous markers ranging from proteins, to lipids, to nucleic acids. MVB, multivesicular body.

recipient cells (24–26). Cargo can include genetic material such as DNA, messenger RNA, non-coding RNA (e.g., miRNA), as well as proteins, carbohydrates, lipids, and in unique circumstances, organelles such as mitochondria. Regarding cell–cell communication, while many avenues of effector action have been described, EV-associated miRNAs have received the most thorough examination. They serve as potent biomolecules that direct multiple cellular processes *via* negative regulation of target genes at the posttranscriptional level (27). Distinctive surface markers including cellular receptors and transmembrane proteins on both exosomes and MPs appear to provide a means of increasing cellular interaction specificity. *In vitro* findings have also shown distinct cargo, including genetic material, proteins, and other molecules, in exosomes and MPs, and correspondingly discrete functions (28, 29). Finally, although less well studied than exosomes and MPs, apoptotic bodies have been suggested to harbor functional capabilities, in particular, carrying miRNAs known to direct vascular protection (30).

EV Enrichment

Although there is an intense focus on the biogenesis, cargo, and subsequent function of EVs and their heterogeneous subpopulations, many efforts are stifled by limitations imposed by current isolation and characterization methodologies. There are many strategies available for the enrichment of EVs, the most popular being ultracentrifugation, size exclusion chromatography, and commercially available EV precipitation kits. Ultracentrifugation is the gold standard for EV isolation, being used in more than 50% of reports (31, 32). Differential ultracentrifugation employs a series of centrifugation cycles with varying centrifugal force and duration, escalating from 400 to 100,000 *g*, leading to the preferential isolation of EV subtypes that have unique densities; apoptotic bodies (2,000 *g*), MVs (10,000–20,000 *g*), and exosomes (≥100,000 *g*) (33–36). Although standard ultracentrifugation offers a relatively pure sample, it can also precipitate large proteins not associated with EVs. High centrifugal forces can also be potentially damaging to EVs. These protocols are also particularly time intensive, require expensive equipment, and are difficult to implement with small amounts of starting material (33). More recently, contamination by protein and particle aggregates has been partially addressed through the adoption of density gradient centrifugation, which utilizes density gradients to separate specific EV populations (32).

Other techniques have been developed to better meet time, sample quantity, and equipment sensitive situations. In particular, many commercial kits offer comparatively rapid precipitation of EVs through the incorporation of polymers such as polyethylene glycol (37). However, preparations from commercial kits have been shown to have low purity and potentially impaired functionally due to co-precipitation of non-vesicular contaminants such as lipoproteins and polymer material (38). As a result, commercial kits may be more suited for high-throughput EV cargo characterization, such as miRNA profiling. Size-based EV isolation techniques, such as filtration and size exclusion chromatography, are also available (39, 40). Such methodologies offer several advantages, including moderately rapid isolation, ease of use, reduced contaminant concentrations, and maintenance of functionality. Size-exclusion chromatography is particularly advantageous as it uses gravity, resulting in the preservation of EV structure, integrity, and biological activity. However, the extended isolation time represents a significant drawback for clinical studies, especially if high throughput sample processing is required (37).

Immunoaffinity (IA) purification is the least prevalent method of EV isolation, typically involving magnetic microbeads coated with an antibody that recognizes surface markers on the EV surface. While in principle IA represents a specific means for the identification and isolation of specific EVs, the lack of established and well-characterized EV markers limits its validity and utility. This method is additionally limited by the physical surface area of EVs available for binding. While potentially resulting in lower yields with higher purity, it may, however, result in concentration underestimations and false negative results (32, 37). While all the aforementioned techniques can be utilized to isolate EVs from culture media as well as biofluids, considerable care and caution should be exercised during optimization to ensure efficient enrichment. The lack of standardized isolation and characterization techniques has hindered advancement of the field. This is particularly evident when technique-to-technique comparisons are conducted, during which significantly different particle concentrations, characteristics, and functions can arise from biologically similar samples. Indeed, recent studies have suggested caution in the interpretation of EV investigations due to the likelihood of confounding factors, including co-purification of protein and lipid complexes (41, 42).

EVs IN DIABETIC AND ATHEROSCLEROTIC PATHOLOGIES

Obstructive atherosclerotic diseases—disorders leading to the narrowing of the arterial lumen through the formation of atherosclerotic plaques—are thought to be central to the development of diabetic macrovascular complications (43). Metabolic dysfunction in individuals with T2DM has been shown to exacerbate and accelerate the pathological mechanisms underlying the development of atherosclerotic disease (44) (**Figure 2**). This is rooted in non-resolving proinflammatory pathogenic activation of the vascular endothelium; leading to platelet activation and adhesion, as well as the recruitment and trans-endothelial migration of circulating monocytes and neutrophils, which drive plaque expansion (45).

Endothelial cell (EC)-derived EVs have been described as important markers and mediators of vascular dysfunction. While patients with various types of vascular diseases have increases in levels of circulating EC-derived EVs, this is particularly evident in patients with both atherosclerosis and T2DM, who display markedly increased levels of circulating EC-derived EVs (46, 47). EVs appear to actively participate in the pathological progression of atherogenesis; from atherosclerotic lesion initiation to progression (48). For example, increased circulating levels of EC-derived EVs in T2DM appear to be associated with increased vascular dysfunction and are an independent risk factor for decreased arterial elasticity; a known change during atherogenesis (49). The decrease in arterial elasticity and successive development of high shear-stresses within the vasculature may further modulate EV function. In particular, platelet-derived EVs under high shear-stress conditions were shown to induce IL-8, IL-1β, and IL-6 production in ECs, which could indicate participation in vascular damage and atherosclerosis (50). Traditionally, the inflammatory response is mediated by the activation of the vascular endothelium and subsequent attraction of inflammatory cells, stimulation of the coagulation and complement systems, and increases in vascular permeability (51). Monocyte recruitment from the bloodstream represents one of the earliest processes of atherosclerotic plaque formation. While EVs isolated from healthy mouse plasma or endothelium can suppress monocyte activation, several experiments have shown that EVs isolated from activated ECs, platelets, or from atherosclerotic plaque, can promote the adhesion of monocytes to the endothelium by increasing the expression of adhesion molecules on both ECs and monocytes (52–54). Of note, Rautou et al. demonstrated that EVs isolated from symptomatic atherosclerotic plaques were more potent at promoting endothelial intercellular adhesion molecule 1-dependent monocyte adhesion and transendothelial migration than EVs from asymptomatic plaques (53). In addition, long-term feeding of high-fat diet to rats resulted in increased numbers of circulating EVs that were associated with an increased potential to induce pro-inflammatory reactive oxygen species and vascular cell adhesion molecule 1 expression in rat ECs *in vitro* (55). Similar observations have been made in the setting of hyperglycemia, where high-glucose conditions increased NADPH oxidase activity in endothelium-derived EVs, subsequently amplifying endothelial activation (56).

It is well established that phenotypic switching of smooth muscle cells has an important role in the progression of vascular diseases such as atherosclerosis (57). In the early stages of atherogenesis, smooth muscle cells acquire a synthetic phenotype and migrate from the media to the intima, subsequently proliferating and contributing to plaque development. Interestingly, platelet-derived EVs have been shown to actively induce vascular smooth muscle mitogenesis (58), while transfer of EVs from ECs to smooth muscle cells has been shown to either inhibit (59) or promote smooth muscle cell proliferation (60). In later stages of atherogenesis, microcalcification of vulnerable plaques can contribute to plaque destabilization and rupture (61). It is now evident that EVs play an active role in both the initiation and progression of calcification (62, 63). For example, New et al.

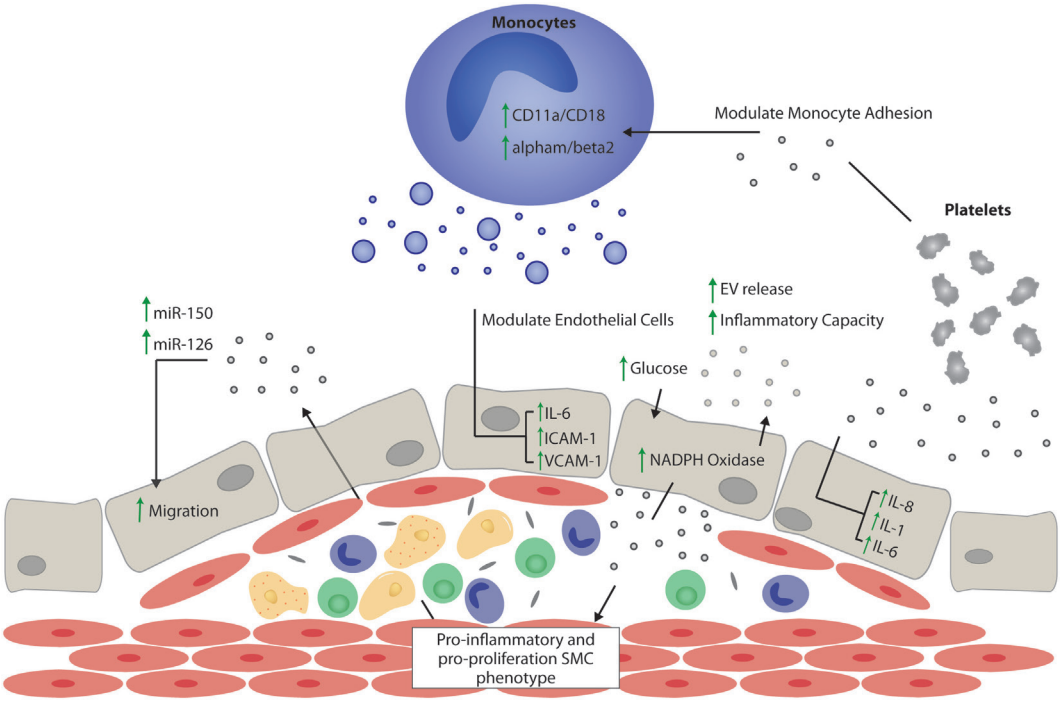

FIGURE 2 | Extracellular vesicle (EV) effects on atherogenesis. Schematic representation of the potential proatherogenic and antiatherogenic effects of EVs, focusing mainly on the role of EVs in inflammation, thrombosis, and endothelial function. Vesicles of endothelial origin in the presence of hyperglycemia might stimulate pro-inflammatory and pro-proliferative smooth muscle cell phenotype switching. EVs stimulated by atherosclerotic plaque niche might stimulate or decrease vascular inflammation depending on their cargo proteins and noncoding RNAs. The presence of the miRNAs, miR-150, and miR-126, in endothelial vesicles is important in autoregulation of migration, while miR-150 is important in maintaining vascular smooth muscle cell differentiation. Vesicles of platelet origin promote endothelial and monocyte inflammation *via* interleukin (IL)-dependent mechanisms, and together with monocyte-derived vesicles, promote thrombosis by upregulating adhesion molecules. EVs released by monocytes contribute to endothelial inflammation by increasing leukocyte adhesion and activating the IL-6 pathway in endothelial cells. SMC, smooth muscle cell; interleukin-6, IL-6; interleukin-8, IL-8; interleukin-1, IL-1; intercellular adhesion molecule 1, ICAM-1; vascular cell adhesion molecule 1, VCAM-1.

provide clear support for the role of macrophage-derived EVs in the nucleation of microcalcifications (62).

Taken together, these results strongly suggest that EVs produced during the pathogenesis of diabetes and atherosclerosis not only promote the development of pro-inflammatory vascular conditions but also encourage the development of early atherosclerotic lesion development by promoting monocyte adhesion and infiltration to the sub-endothelial space, as well as through their ability to stimulate smooth muscle cell migration and proliferation and their role in instigating calcification. These findings provide unique insights into the pathogenesis and perhaps accelerated presentation of diabetes-associated atherogenesis.

Although there appears to be a strong relationship between EVs and atherogenesis, the exact functional interplay has yet to be fully explored. EV-associated miRNAs have received particular attention as they can be efficiently isolated from liquid biopsies and have substantial functional implications (64). Collectively, miRNAs have been shown to modulate vascular inflammatory, calcification, and thrombus formation pathways related to diabetes and atherosclerosis (65, 66). Jansen et al. described the differential regulation and selective packaging of miRNAs during T2DM pathology when compared to non-diabetic controls (67). Additionally, large-scale miRNA profiling of plasma EVs from patients with T2DM has revealed significant dysregulation of miRNAs, independent of body mass index, age, or sex (67). In-depth mechanistic studies have validated some functional roles of EV-associated miRNA dysregulation. Wu et al., in particular, found that the miRNA-126/VEGFR2 pathway was downregulated in untreated T2DM, potentially governing vascular integrity (68). Additionally, EC-EV transfer of miRNA-126 has been shown to be abrogated in high glucose settings, highlighting the importance of EV cargo maintenance in physiology (69). Karolina et al. has highlighted the promise of utilizing specific dysregulations in EV-associated miRNA cargo clinically by assessing the circulating EV-associated miRNA profiles of 219 participants with either metabolic syndrome, T2D, hypercholesterolemia, or hypertension, showing that each disorder had its own specific EV miRNA profile (70). While results of many studies, including the aforementioned one, have highlighted the unique dysregulation of EV-associated miRNA during the development of vascular disorders such as atherosclerosis, their utility as clinical biomarkers remain unfulfilled. This is in

part a result of the complex and often multifactorial function of EVs, limiting our ability to efficiently delineate miRNAs directly associated with early disease processes.

EVs IN DIABETIC CARDIAC PATHOLOGY

Prolonged asymptomatic, yet progressive, phases of DCM make diagnosis of this condition particularly challenging (71). DCM is a complex condition and is defined as the presence of left ventricular (LV) dysfunction in individuals with T2DM in the absence of arterial hypertension, coronary artery disease, or evidence of other structural cardiac disease (71). While T2DM is a well-known risk factor for atherosclerotic disease, its role in development of DCM is less established. Epidemiological evidence suggests a high prevalence (30–40%) of cardiomyopathy in individuals with T2DM (72–75). Perhaps unsurprisingly, several signaling pathways (including inflammation, oxidative stress, and endothelial dysfunction) that are dysregulated under diabetic conditions and contribute to atherosclerotic disease also appear to enhance myocardial dysfunction (i.e., DCM) and accelerate heart failure (76–78). Clinically, DCM begins by presenting itself as early stage DCM, characterized by an abnormal myocardial energy metabolism, systolic, or diastolic dysfunction (i.e., impairment of the contraction of relaxation of the heart, respectively) and reduced LV strain (defined as regional deformation, or lengthening, shortening and thickening of the LV) (79). Over time, the progression of DCM can lead to overt heart failure, associated with cardiomyocyte hypertrophy, myocardial fibrosis, and ultimately cardiomyocyte death (80). This vulnerability to DCM may in part be due to the convergence of multiple risk factors, such as chronic hyperglycemia, resulting in detrimental effects on various cell types within the heart (81).

Recent attention has been focused on understanding the mechanisms of communication between the diverse cell-types in the heart, particularly, as it relates to disease pathogenesis (**Table 2**). These cells include cardiomyocytes (CMs), accounting for 25–35% of all cells in the heart (82), ECs (comprising 60% of the non-myocyte cells cardiac tissue cells), smooth muscle cells, hematopoietic-derived cells, and fibroblast cells (83). Each of these cell types play an important role in healthy and diseased cardiac function as they can contribute to the processes of ventricular hypertrophy, steatosis, fibrosis, and impaired angiogenesis, all of which can lead to diabetic cardiac complications, including cardiomyopathy (81). Extensive cross-talk occurs among these cells, and emerging evidence has implicated EVs in this communication (84). That being said, the link between EVs produced under T2DM conditions and increases in cardiac oxidative stress, cardiac inflammation, myocardial fibrosis, and other aspects of the pathogenesis of cardiomyopathy, has not been extensively studied to date, and remains an area ripe for future studies.

TABLE 2 | Extracellular vesicle-derived miRNA regulation of the diabetic heart promotes the development of diabetic cardiomyopathies.

miRNA	Source/recipient	Target/process
miRNA-1/ miRNA-133A	Cardiomyocytes (CMs)	Independent predictors of myocardial steatosis (85)
miRNA-320	CMs/endothelial cells (ECs)	Impairs angiogenesis by targeting IGF1, Hsp20, and Ets2 (86)
miRNA-503	ECs/pericytes	Impairs migration and proliferation following its transfer to vascular pericytes (87)
miRNA-126	Endothelial progenitor cells	Alters EC repair processes; reduces VEGFR-2 expression (68)
miRNA-21*	Cardiac fibroblasts/CMs	Promotes cardiac hypertrophy by targeting Sorbin and SH3-domain-containing protein 2 and PDLIM5 (88)

The main function of CMs is to generate contractile force in the heart, and although not considered to be a secretory cell, they can secrete cytokines, chemokines, and various factors such as ANP, and BNP as well as EVs (84). CM-derived EVs have been implicated in diabetic cardiomyocyte steatosis (85). Accumulation of lipids in the myocardium has been associated with non-ischemic cardiomyopathy (including DCM) and LV hypertrophy. Elevated levels of miR-1 and miR-133a were observed in EVs derived from lipid-loaded HL-1 CMs; levels were also increased in the serum of mice fed a high fat diet, and in the circulation of diabetic patients with myocardial steatosis (85). Unfortunately, no mechanism for miR-1/miR-133a function in steatosis was described, but being identified as independent predictors makes them important biomarkers. Recently, CM-derived EVs were shown to communicate with the endothelium, and it was demonstrated that this cross-talk is altered in the setting of diabetes; contributing to dysfunctional angiogenesis in DCM (86). While cardiomyocyte-derived EVs isolated from wild-type mice promoted angiogenesis, EVs isolated from diabetic rats exerted antiangiogenic effects; this was attributed to higher levels of antiangiogenic miR-320, and lower levels of angiogenic miR-126 (86).

Endothelial cells play a critical role in facilitating myocardial contraction and CM survival (89). Microvascular rarefaction is a major manifestation of diabetes-mediated ischemic cardiovascular disease, resulting from endothelial cell death and insufficient myocardial angiogenesis (90). Early in diabetes, high blood glucose leads to endothelial dysfunction, which can promote microvascular rarefaction over time (91). Several miRNA-based mechanisms have been proposed to explain vascular dysfunction in diabetes. For example, when exposed to elevated glucose concentrations, the levels of miR-503 in the endothelium increase, inhibiting EC proliferation and angiogenesis by targeting CCNE1 and Cdc25A (92). Moreover, transfer of miRNA-503 from EC-derived EVs impaired pericyte migration and proliferation, thereby decreasing angiogenesis and modulating vessel permeability by interfering with the production of VEGFA and EFNB2 (87). In healthy conditions, ECs release EVs that contain miR-10a, which can be transferred to monocytes, where it represses several components of the NF-κB signaling pathway to dampen their inflammatory activation (54). These EVs additionally contain high levels of miR-126, which can promote vascular endothelial repair through the targeting of SPRED-1 (69), a negative regulator of the VEGF signaling pathway (93, 94). However, in pathological hyperglycemic conditions, miR-126 expression is reduced in EC-EVs, impairing EC repair due to a lack of SPRED-1 targeting (69). Another group revealed reduced miR-126 expression in circulating EVs and endothelial progenitor cell-derived-EVs from

patients with uncontrolled diabetes (68). Furthermore, exposing endothelial progenitor cells to these EVs downregulated VEGFR2 decreased migration ability, and increased apoptosis and ROS production (68).

Cardiac fibroblasts are involved in the fibrotic response that accompanies DCM. The differentiation of fibroblasts to myofibroblasts, together with their proliferation and production of extracellular matrix, contributes to the increased stiffness of the myocardium that promotes diastolic dysfunction (95). In neonatal rat cell culture, paracrine factors from cardiac fibroblasts elicit detrimental changes in CM electrophysiology that resemble those seen in cardiac pathologies (96); however, the role of EVs was not assessed. Hyperglycemia contributes to diabetic cardiac fibrosis as it can promote proliferation, myofibroblast differentiation, and collagen synthesis by cardiac fibroblasts (97–99). A potential culprit for the observed effects is miR-21, which targets DUSP5, a negative regulator of p38 and JNK signaling (100). Cardiac fibroblasts also secrete EVs that target CMs and appear to be enriched in miRNA passenger strands, which are typically eliminated during miRNA biogenesis. Transfer of miR-21* from cardiac fibroblasts to CMs induced cardiac hypertrophy by downregulating Sorbin and SH3-domain-containing protein 2 and PDZ and LIM domain 5 (PDLIM5) (88). Inhibition of miRNA-21* in mice with angiotensin II-induced heart hypertrophy suppressed the observed cardiac pathology (88).

Secretion of cardiac EVs appears to be an intricately regulated process that can mediate both local and systemic effects. *In vitro* cellular stretch and *in vivo* pressure overload in a mouse model induced the release of EVs from CMs that were enriched with angiotensin type I receptor (AT1R) (101). This was associated with the transfer of active AT1R to various tissues including the mesenteric artery and skeletal muscle, which upon injection into AT1 knockout mice, affected peripheral vascular resistance and blood pressure (101). The full extent of EV-mediated cell–cell communication among the cells locally in the heart or distally in systemic circulation has not yet been explored, and whether circulating EVs can be taken up by CMs, pericytes, or fibroblasts in the heart is not known. Additionally, the impact of diabetes on this form of communication is just coming into view. From initial studies, it appears that EVs are major protagonists in eliciting cardiovascular dysfunction in diabetics. Further elucidation of these pathways and mechanisms may reveal novel biomarkers and potential therapeutic strategies.

DIABETIC CEREBROVASCULAR CROSS TALK

Cardiovascular dysfunction, especially overt heart failure, has been proposed as a major cause of cognitive dysfunction in the elderly; commonly referred to as "vascular dementia" (102). An increasing body of evidence suggests that even the relatively mild effects on cardiac output that are observed in DCM are independently associated with impairment in various cognitive domains (103). Diabetes is associated with a breakdown in the blood brain barrier, a unique structure that protects the brain from detrimental systemic circulating factors (104). It is currently unclear whether cardiac output directly impacts cognitive function or whether both of these phenomena are driven by an independent factor. Based on the current body of evidence highlighting deleterious effects of inflammatory EVs on vascular ECs, it would seem conceivable to hypothesize that these effects on the brain and heart vasculature may be mediated by circulating EVs. Indeed, while still emerging, there is a body of evidence suggesting that EVs in diabetic microvascular settings may increase blood–brain barrier permeability (105, 106). A recent study found that the anti-inflammatory miRNA, miR-146a is decreased in the brains of diabetic mice, and that this is associated with accumulation of cellular prion protein (107). Interestingly, delivery of EC-derived exosomes loaded with miR-146a could decrease levels of cellular prion protein and could restore short-term memory (107). Additional research in this emerging area is clearly warranted and may shed light on the pathobiology of vascular dementia and its link to cardiac disease.

EVs AS BIOMARKERS OF DIABETIC CARDIOVASCULAR PATHOLOGIES

The paucity of effective diagnostic modalities and pharmacological interventions for DCM has fueled the search for novel circulating biomarkers that may be more reflective of disease status (108). Given their abundance in multiple bodily fluids and the modulation of the abundance, source, and contents (e.g., miRNAs) of EVs in response to pathological stimuli, EVs are attractive candidates as biomarkers (109). While numerous studies are underway to examine the utility of EVs as biomarkers (110–112), there has been a particularly intensive focus in oncological and neurological diseases. Understanding changes in EV contents will generate insight into potential disease mechanisms mediated by cell–cell communication that can be targeted therapeutically.

The complexity and chronic nature of cardiovascular pathologies appear to have impeded the field's ability to correlate disease states with unique EV changes, slowing their adoption into biomarker studies. Nonetheless, there is considerable excitement in utilizing EVs as a novel diagnostic tool due to their inherent ability to transport miRNAs. miR-146a, in particular, may play an important role in the pathogenesis of both atherosclerosis (54, 113, 114) as well as the development of cardiomyopathies (115, 116) through the regulation of inflammatory pathways. Interestingly, it appears that transfer of miR-146a between cells may play an important role. For example, transfer between ECs and CMs plays a role in peripartum cardiomyopathy, and blocking this communication reverses pathology (115). In addition, the demonstration that miRNA-containing EVs are released into circulation from cardiac cells highlights the need for additional investigation (117). Identification of the cellular source of these EV-derived miRNA, understanding the mechanisms of packaging and secretion, and characterizing their functional roles remain a matter of active investigation. Nonetheless, a detailed characterization of EVs released into the circulation by CMs has lagged and appears prime for fruitful investigation.

THERAPEUTIC POTENTIAL OF EVs

The involvement of EVs in the pathology of diabetic cardiovascular pathologies serves as a strong impetus to develop EV-based therapeutics. The combination of innate biocompatibility, low toxicity and immunogenicity, stability, and selective uptake make them an ideal delivery vehicle for therapeutics (118). Current therapeutic approaches aim to use EVs to deliver small RNAs in an attempt to reverse pathological miRNA-based communication with anti-miRNA oligonucleotides or to stimulate protective communication with synthetic miRNA mimics (119, 120). More specific delivery of anti-miRNAs or miRNA mimics to target cells is being achieved by engineering vesicles with cell-selective surface proteins (121), which should reduce off-target effects.

Many hurdles remain to be overcome before EV-based therapeutics might be used in the clinic to treat cardiovascular diseases. Nevertheless, the proven utility of using small RNAs in a cardioprotective manner in mouse and large animal models to prevent pathological changes such as fibrosis, cardiac hypertrophy and inflammation (122–124) highlights their potential as efficacious therapeutic targets. The ability to load EVs with particular cargo such as miRNAs, suggests the possibility of using EVs to deliver miRNA-based cardiovascular therapeutics. The field of miRNA-based therapeutics is advancing rapidly and over the last 10 years, research focused on circulating EVs, and the miRNA they contain, has revealed diverse and important roles (24). That being said, much still remains to be revealed regarding the role of EVs in cell–cell communication in health and diabetic cardiovascular disorders. Specifically, it may be advantageous to understand the effects of the chronic inflammatory environment in diabetes on the packaging and release of endothelial EVs and their subsequent interactions with CMs. Better understanding the role of endothelial-derived EVs may allow for in-depth probing of currently employed diabetes therapeutics such as sodium-glucose cotransporter-2 inhibitors, which are believed to have cardioprotective benefits (125). Advancing our understanding of the role of EVs in cardiovascular disease will help identify the cellular source and destination of EVs, subsequently allowing for the exploration of specific cellular interactions. Furthermore, improving our understanding of EV organ-tropism will aid in the targeting of specific tissues, improving the efficiency of miRNA-based therapies.

CONCLUSION

Extracellular vesicles in liquid biopsies, such as blood, urine, or saliva, as well as localized tissue EV content remain a relatively untapped source of detailed information for both basic researchers and clinicians alike. The innate ability of EVs to shield biologically complex information from degradation and sensitivity to minute changes in physiology highlight their potential as sensitive and specific biomarkers. Early studies into their biology suggest that they may be critical mediators of cardiovascular diseases such as atherosclerosis and DCM. There are a number of unexplored avenues particularly regarding the interactions between elevated glucose levels, endothelial EVs, and dysfunction in cardiac tissues. Understanding the potential roles of EVs in diabetes associated cardiac dysfunction will be critical in understanding the mechanisms of currently employed therapeutics and for the development of more efficacious agents. The largest roadblock in illuminating the roles of EVs in the cardiovascular field remains a thorough understanding of the vesicle population, which in suit relies heavily upon our ability to apply accurate vesicle isolation techniques. The development of a harmonized nomenclature for EVs will be essential for both meaningful dialog between researchers and ensuring reproducibility of results across laboratories (109). To better understand the role of EVs in multifactorial conditions such as diabetic pathologies, a number of gaps in fundamental knowledge should be addressed. The most pressing is to better understand the mechanisms of EV biogenesis, delivery, and degradation upon which a more normalized nomenclature can be developed. Building upon this, the development of accurate *in vivo* vesicle tracking models will be essential in validating much of what is currently known for translation into the clinic. Finally, utilizing large clinical cohorts for the examination of vesicle concentrations, populations, and cargo should be performed to examine vesicle heterogeneity in multiple patient populations. Although the precise physiological and pathological functions of EVs remain at a nascent stage of understanding, their obvious potential as biomarkers and vehicles for therapeutic intervention could transform our approach to understanding and treating diabetic cardiovascular pathologies.

AUTHOR CONTRIBUTIONS

DG and SV researched the data for the article, substantially contributed to discussion of the article and wrote the article. DG, SV, and JF contributed to conceptualizing the article as well as reviewing and editing of the manuscript before submission.

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REVIEW
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Extracellular Vesicles As Mediators of Cardiovascular Calcification

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Involvement of cell-derived extracellular particles, coined as matrix vesicles (MVs), in biological bone formation was introduced by Bonucci and Anderson in mid-1960s. In 1983, Anderson et al. observed similar structures in atherosclerotic lesion calcification using electron microscopy. Recent studies employing new technologies and high- resolution microscopy have shown that although they exhibit characteristics similar to MVs, calcifying extracellular vesicles (EVs) in cardiovascular tissues are phenotypically distinct from their bone counterparts. EVs released from cells within cardiovascular tissues may contain either inhibitors of calcification in normal physiological conditions or promoters in pathological environments. Pathological conditions characterized by mineral imbalance (e.g., atherosclerosis, chronic kidney disease, diabetes) can cause smooth muscle cells, valvular interstitial cells, and macrophages to release calcifying EVs, which contain specific mineralization-promoting cargo. These EVs can arise from either direct budding of the cell plasma membrane or through the release of exosomes from multivesicular bodies. In contrast, MVs are germinated from specific sites on osteoblast, chondrocyte, or odontoblast membranes. Much like MVs, calcifying EVs in the fibrillar collagen extracellular matrix of cardiovascular tissues serve as calcification foci, but the mineral that forms appears different between the tissues. This review highlights recent studies on mechanisms of calcifying EV formation, release, and mineralization in cardiovascular calcification. Furthermore, we address the MV–EV relationship, and offer insight into therapeutic implications to consider for potential targets for each type of mineralization.

Keywords: calcification, atherosclerosis, aortic stenosis, hyperphosphatemia, extracellular vesicles, matrix vesicles

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BACKGROUND

Bonucci reported the appearance of "roundish bodies" in initiation of the calcification process in guinea pig and rat tibial–femoral epiphyseal plates in 1967 (1). One year later, Anderson used electron microscopy on tissue sections to demonstrate vesicular structures in the mouse cartilage epiphysis (2). Subsequent studies have suggested that mineralization depends on secretion of *matrix vesicles (MVs)*, with diameter of 30–400 nm (3, 4), from chondrocytes, osteoblasts, odontoblasts, tenocytes, and cementoblasts (5). MVs released from specific sites on cell membranes [apical microvilli (3)] exhibit similar cytosolic and plasma membrane profiles apparent in their cell of origin (6). Compared to their parent cells, MVs carry augmented levels of acidic

lipids such as phosphatidylserine (PS) and sphingomyelin, but diminished levels of neutral phospholipids of phosphatidylcholine and lysophospholipids (5). Chondrocytes residing in the epiphyseal plate experience hypoxic conditions due to presence of collagen fibrils and proteoglycans, which restrict oxygen and nutrients delivery (7). Development of blood microvessels into this zone creates oxidative stress caused by the sudden elevation in nutrients, oxygen, calcium ions (Ca^{2+}), and phosphate ions (Pi) (8). This process leads to enrichment of mitochondria with Ca^{2+}, which results in secretion of Ca^{2+}-loaded vesicles into the cytosol. It has been proposed that the formation of complexes between PS and Ca^{2+} either with Pi or annexins in these vesicles, diminishes the Ca^{2+} level within the cytosol, expedites actin depolymerization, and consequently leads MVs to pinch-off from the cell and release into the extracellular environment (8, 9). Of note, apoptotic bodies originating from apoptotic cell membrane rearrangement during terminal stage of mineralization in epiphyseal plate differs from active formation and release of MVs (10). Released MVs interact with glycosaminoglycans and initiate extracellular mineral deposition (11).

Chondrocyte differentiating factors such as thyroxine (T3), bone morphogenetic protein 6, retinoic acid, and Indian hedgehog may give rise to MV generation by inducing changes in cell phenotype (12). Osteogenic cell types abundantly express annexin I, II, IV, V, VI, and VII. Annexins function as voltage-gated channels or Ca^{2+}-binding agents, mediate inflammation responses, and regulate structural properties of both cells and MVs membranes (4, 11, 13). The most abundant proteins in MVs are annexins II, V, and VI, which can accelerate the calcification process by either providing required Ca^{2+} for mineralization or partaking in PS–Ca^{2+}-annexin complexes (14). Additionally, other membrane proteins such as calbindin D9k can provide Ca^{2+} for MVs (15).

Pyrophosphate (PPi), which originates from nucleotide pyrophosphatase phosphodiesterase (NPP1) hydrolysis of nucleoside triphosphates, inhibits mineralization. Progressive ankylosis (ANK) carries PPi into extracellular milieu. Tissue non-specific alkaline phosphatase (TNAP, on the outer leaflet of MV membranes) hydrolyzes PPi into free phosphate (Pi) and provides free Pi for complexing with Ca^{2+} and mineral formation (5, 7, 16). Type III Na/Pi transporters (PiT-1) on the MV membrane facilitate Pi internalization. Bone morphogenetic protein 2 and parathyroid hormone upregulate expression of these transporters (7). Additionally, MVs contain phosphatases and membrane phosphohydrolases, such as TNAP, AMPase, ATPase, nucleoside triphosphate pyrophosphohydrolase (NTPPase, NPP1, and PC-1), and PHOSPHO-1 that elevate the intravesicular Pi concentration within the MVs (5, 17). This highly ions concentrated environment, adjacent to the MV membrane, where Ca^{2+} and Pi meet, provokes calcium phosphate precipitation followed by an increase in pH and mineral stabilization. Membrane–mineral associations are mediated by PS located on the luminal side of the MV lipid bilayer. PS is an anionic phospholipid with tendency to bind Ca^{2+}. The complexes of PS–Ca^{2+}–Pi may serve as initial nuclei for hydroxyapatite precipitation (7, 13, 18). Studies have also suggested that the mineralization process can begin intracellularly with contribution of pre-nucleation clusters within endosomes, which initiate mineralization in order to maintain Ca^{2+} and Pi in homeostatic concentration and fulfill energy trade-off (5).

Matrix vesicles are equipped with matrix metalloproteases (MMPs) that degrade and remodel the ECM (3, 19). Particularly, MMP-2, MMP-3, MMP-9, and MMP-13 are located in MV membranes and play a key role in matrix remodeling and propagation of mineralization (5, 19, 20). In ECM, collagen fibrils and proteoglycans provide charged regions, which are favorable sites for accumulation of the calcium phosphate nanoparticle clusters that form in MVs. The amorphous apatite resulting from this accumulation transforms into structured and crystalline mineral following ECM associations (5). MVs interact with ECM proteins *via* their integrin receptors and surface motifs, such as CD9, CD63, and Hsp70 (5) (**Figure 1**). In addition to their remodeling potential and ECM binding, MVs in growth plate ECM can affect the proliferation and fate of resident cells, due to the activation of parathyroid hormones and gene-related peptide through their loaded proteins (5). This paracrine signaling property is similar to other subtypes of extracellular vesicles (EVs) ubiquitous to many cells and tissues.

It is worth mentioning that skepticism exists on the existence and role of MVs in bone growth and formation. Studies have suggested that calcification originates from cell death and debris, which nucleate mineral, and observations of MVs are attributed to sample preparation artifacts (21). In recent studies, however, isolation of MVs from chicken tibias using multiple centrifugations and application of microscopy techniques such as transmission electron microscopy on growth plate during bone formation have provided support for MVs as mediators of mineralization (22, 23). Furthermore, knockout of Stx4a, a regulatory factor for secretion of MVs, decreases number of MVs and leads to reduced bone mineral density (24). Recent interest in non-calcifying EVs (e.g., exosomes) and the related development of assay techniques may build on the observational studies of the past and help clarify the derivation and function of MVs in the mineralization of hard tissues.

EVs WITH NON-OSTEOGENIC ORIGINS

Matrix vesicles represent one specific subtype of EVs. Generally, EVs function to maintain both intracellular and extracellular homeostasis. Two major pathways mediate EV release into the ECM: (i) *via* multivesicular bodies (MVB), containing several EVs wrapped by plasma membrane and (ii) through direct budding of a single EV from cellular plasma membrane (4). Depending on the release mechanism, EVs are generally referred to as exosomes and microparticles, respectively. Both EV types carry a subset of cargos representing their parental cell (11, 25). MVB trafficking and fusion/fission from the plasma membrane requires activity of Rab GTPases, specifically Rab27a, Rab27b, Rab35, and Rab11 (26, 27). EV structure consists of a metabolically active membrane with transmembrane proteins and an inner core, which typically carries RNAs, soluble proteins, and lipids (11, 25, 28). EVs often contain sets of small RNAs, such as miRNA, tRNA, mRNA, piRNA, snRNA, Y-RNA, and vault RNA, which can be protected from RNase degradation and encoded at target cells

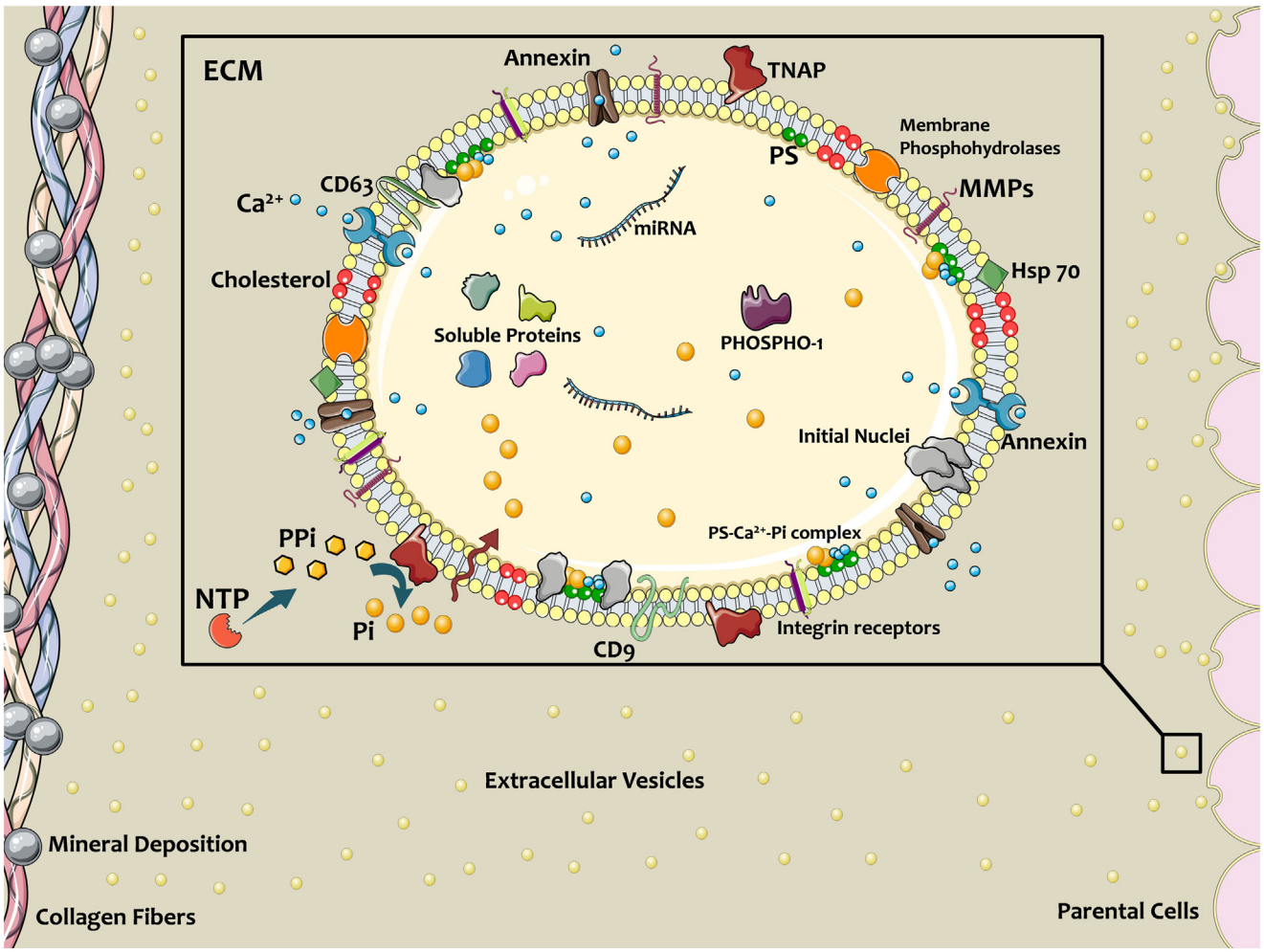

FIGURE 1 | Schematic of extracellular vesicles (EVs) contributing in calcific mineral formation. Osteogenic cells release EVs into ECM to nucleate bone or cardiovascular mineral. EV membranes contain a specific lipid profile that differs from the parent cell. Annexins on EVs facilitate Ca^{2+} entrance, and tissue non-specific alkaline phosphatase (TNAP) activity converts PPi to phosphate ions (Pi), thereby providing the necessary ionic components for mineralization. PiT-1 transporters transfer Pi into the EV lumen. Coincidence of these ions and formation Ca^{2+}–PS–Pi within the EV lead to mineral initiation. Membrane enzymes and proteins interact with and attach to the ECM, directing the localization of calcification. Figure created using Servier Medical Art images (http://smart.servier.com).

(27, 29). The difference in RNA ingredients of parental cells and EV cargo demonstrates the selective mechanism of RNA loading. EV RNA cargo can serve as biomarker that indicate the phenotypic state of parental cells, as well as messengers that can interact with other cells (27). EVs play a key role in cell–cell interaction and data trafficking in both normal and pathological conditions. For instance, miRNA-enriched EVs from endothelial cells can regulate gene expression and resultant phenotypic transitions in smooth muscle cells (SMCs) (30). Divergence from normal physiological conditions toward pathological ones induces release of dysfunctional EVs with pathologic cargo and may affect tissue homeostasis and cellular phenotypes (4). This section serves as a short primer on the complex mechanisms associated with EV release and cargo loading. These processes are reviewed in greater detail elsewhere (31–34). The current review focuses on EVs that play a direct role in depositing mineral in cardiovascular tissues. Current evidence, discussed in the following sections, suggests that these EVs share commonalities with both MVs and other EV populations, such as exosomes.

CALCIFYING EVs IN CARDIOVASCULAR CALCIFICATION

Calcification contributes to pathological remodeling in different locations throughout the cardiovascular system, such as the arterial intima and media and the aortic valve (35, 36). Electron microscopy demonstrated the presence of needle-like hydroxyapatite nanocrystals in EVs extracted from atherosclerotic lesions of apolipoprotein E-deficient mice (37). EVs released into the atherosclerotic lesion have a Ca/P ratio of 0.66, indicating incomplete calcification [compared to the ratio in normal adult murine bones of 1.71 (38)]. High resolution microcomputed tomography imaging revealed microcalcification in the fibrous cap of atherosclerotic plaques composed of calcified EV

aggregates (39, 40). These observations indicate a role for EVs in the formation and progression of cardiovascular calcification, but mechanistic studies demonstrating causality are difficult. One major challenge in EV research is to distinguish between different EV populations, such as calcifying EVs, MVs, exosomes, apoptotic bodies, and microparticles, due to their shared size and shape characteristics (41). Application of multiple and consecutive centrifugations followed by size-based filtration and sucrose gradient-based ultracentrifugation to isolate EVs of known density have been used to separate different cellular-derived particles and EVs (25, 42). EVs with various sizes and densities pellet based on the centrifugation speed, i.e., large, medium, and small EVs precipitate at low, moderate, and high speeds, respectively (25). However, these techniques are often unable to separate the various EV populations. Calcifying EVs secreted by SMCs cultured under osteogenic conditions exhibit increased mass density compared to other EVs, likely due to mineral formation. Therefore, calcifying EVs precipitate more quickly under the application of ultracentrifugation (~100,000*g*) (41), permitting enrichment of these EVs and subsequent proteomic profiling (25, 41). Once proteomic fingerprints are established, membrane proteins (tetraspanins), ER-related proteins, mitochondrial proteins, exosomal markers, endosomal markers, and ECM factors (such as surface molecules and integrins) specific to various EV populations help map and separate EVs into subsets based on their origins (25).

In certain pathological conditions (such as chronic kidney disease), extracellular Ca^{2+}/Pi concentrations increase, thereby cells release EVs with high levels of these ions (43). This may be in contrast to inflammation-driven osteogenic differentiation (e.g., in atherosclerosis). Release of EVs from cardiovascular cells with osteogenic phenotypes promotes mineralization *via* generation of free phosphate from sources such as ATP and pyrophosphate (44). Further, dystrophic mineral deposition resulting from cell death may also contribute to a significant portion of cardiovascular calcification (45). The diverse contributors have led to confusion surrounding the mechanisms of cardiovascular calcification (46); however, numerous studies have demonstrated similarities between cardiovascular calcification and bone metabolism (47–51). Annexins present on calcifying EVs play a dual role of Ca^{2+} uptake and counteracting the calcification inhibitory activity of fetuin-A during ectopic mineralization (4, 11). High phosphate imbalance present in chronic kidney disease may accelerate calcification nucleation in EVs (46). In this condition, TNAP activity may accelerate the calcification process in both non-osteogenic and osteogenic EVs through removing PPi, a competitive inhibitor to Ca^{2+} associations with Pi (39). EVs with calcifying potential may also contain imbalanced and dysfunctional miRNAs, which induce the gene expression and protein synthesis of osteogenic markers, such as RUNX2, Smad1, osterix, TNAP, chaperones, and pro-inflammatory factors (4, 11). Unlike the physiological process of MV-mediated mineral deposition, however, calcification of EVs may inhibit their ability to reach their intended target cells, further promoting a loss in tissue homeostasis and pathological remodeling (52) (**Figure 1**).

Future works are needed to improve upon EV isolation techniques in order to reduce noise from non-calcifying EVs. Advancements in EV characterization tools may allow careful comparative studies to understand the similarities and differences between MVs and calcifying EVs liberated from the three reported cellular contributors to calcification in cardiovascular tissues: vascular SMCs, macrophages, and valvular interstitial cells (VICs). While these cell populations do not represent all cells involved in cardiovascular calcification, direct EV-related contributions to mineral nucleation from other cells remain unreported. Improvement of EV isolation and assay techniques may also allow for determination of the relative roles of the various cell populations in the mineralization process.

Smooth Muscle Cell-Derived Vesicles

Smooth muscle cells and osteoblasts share similar mesenchymal origins and under pathological stresses SMCs can exhibit an osteoblast-like phenotype (48). In a hyperphosphatemic environment (e.g., chronic kidney disease) or inflammation-driven atherosclerosis, vascular SMCs upregulate expression of osteogenic differentiation genes (53) and release EVs enriched with pro-calcific biomarkers (44). Hyperphosphatemic and osteogenic conditions decrease circulating factors, such as fetuin-A (51) and matrix Gla protein (50), which inhibit extracellular mineralization, but increase TNAP and annexins II and VI in SMC-derived EVs, which promote extracellular mineralization. Similar to its function in bone, TNAP activity leads to increased available Pi and reduction of mineralization inhibitors such as PPi (49). The formation of calcifying EVs begins with a series of intracellular trafficking processes that produce the EVs with calcification-promoting factors. A specific trafficking protein, sortilin, is a key player in the formation of calcifying EVs secreted by vascular SMCs. Sortilin transports TNAP into SMC-derived EVs, thereby increasing EVs calcifying potential (49).

Materials science imaging techniques revealed that, once released into the ECM, SMC-derived calcifying EVs tend to aggregate and form microcalcifications in areas with sparse collagen, whereas, large calcifications (larger than 200 μm) are bordered by dense collagen fibers (36). Large calcifications (macrocalcifications), shaped by accumulation of small calcifications (microcalcifications), gradually form mature mineral. Microcalcifications formed within the fibrous cap of vulnerable atherosclerotic plaques further potentiate plaque rupture, whereas larger macrocalcifications beneath stable fibrous caps may promote plaque stability. EVs collected from SMCs cultured in pro-calcific conditions and incubated within an *in vitro* collagen hydrogel system, mimicking aspects of atherosclerotic lesions, showed the progression of calcification from single calcifying EVs to EVs aggregation and fusion to the formation of microcalcifications to growth into large calcifications (36). Collagen acts as a scaffold to direct the shape and size of the calcifications generated from this growth process (36). Collagen fibrils, specifically type I and III in arteries, bind to EV membranes and mediate calcification propagation in the ECM (48). **Figure 2** illustrates the mechanism by which EVs associate to form mineral within the tissue, beginning with EV accumulation and aggregation to fusion and mineralization. In addition to binding and directing the calcification of EVs, collagen may also play a role in phenotypic changes and EV formation within SMCs (54). Discoidin domain receptor-1

FIGURE 2 | Schematic of extracellular vesicle (EV)–collagen interaction to form microcalcifications in the atherosclerotic fibrous cap. Collagen degradation in vulnerable fibrous caps allows calcifying EVs to accumulate. The initial mineral nuclei forms within individual EVs and EV aggregation and fusion drives mineral maturation and growth (36).

(DDR-1), a collagen receptor, regulates SMC phenotype by sensing extracellular collagen. DDR-1-depleted SMCs exhibit elevated collagen production, EV release, and mineral deposition. DDR-1 functions as a regulator of TGF-β signaling pathways, acting as a switch between pro-fibrocalcific and anti-fibrocalcific TGF-β signaling (54). Therapeutic strategies to control these pathways and prevent or reverse SMC-driven calcification will require a better understanding of the mechanisms that lead to their formation within the cell, nucleation of mineral outside the cell, and the role of the ECM in calcification propagation. SMC-derived EVs are the most studied type of calcifying EVs in cardiovascular tissues and the mechanisms identified in these cells may help inform research into other cellular drivers of cardiovascular calcification.

VIC-Derived Vesicles

Valvular interstitial cell phenotypic changes play a vital role in ECM remodeling and mineral deposition that lead to calcific aortic valve disease (55). VICs have a high phenotypic plasticity and can transform from a fibroblastic phenotype to myofibroblast- or osteoblast-like cells in response to pathological conditions, such as hyperphosphatemia and pro-inflammatory cytokines. VICs exhibit high sensitivity to their mechanical environment and undergo phenotypic changes *in vitro* in response to changes in substrate stiffness and mechanical stretch (55). VICs may also influence valvular endothelial cells (VECs) through EV secretion. Valve homeostasis depends on appropriate interactions between VECs and VICs (55, 56). This intercellular interaction occurs when VECs take up VICs-derived EVs containing perinuclear proteins (35). In calcifying milieu, VICs express osteogenic mRNAs of PiT-1, RUNX2, Msx2, and TNAP (57), and pro-calcific VIC-derived EVs resemble MVs from chondrocytes and SMCs, demonstrating elevated annexins II, V, and VI (35). Similarly, immortalized rat VICs cultured in high calcium and phosphate media release EVs with elevated calcium and annexin VI, and electron microscopy revealed co-localization of annexin VI with EVs in calcified human aortic valves (57). Though aortic valve calcification constitutes a major unmet clinical problem, investigations into the extracellular mechanisms through which mineral nucleation and growth occurs remain scant. More studies are needed to understand the role of VIC EVs in this process and the similarities and differences between these EVs and the more well-studied SMC-derived EVs.

Macrophage-Derived Vesicles

Atherosclerosis creates moderate hypoxia (2% oxygen) for local cells and leads to activation of pro-inflammatory responses such as Akt and β-catenin pathways in macrophages (58). Additionally, oxidized lipids, IL-6, and TNF-α (pro-inflammatory cytokines), and mechanical stimuli contribute to both increased inflammation and subsequent ectopic calcification (59). Pro-inflammatory macrophages secrete elastolytic cathepsins and collagen-degrading MMPs (e.g., MMP-2 and -9), and the resultant proteolytic ECM degradation and remodeling causes atherosclerotic plaque instability and rupture, the leading cause of cardiovascular morbidity (60, 61). Inflammation precedes and may serve as a requisite step for the onset of both atherosclerotic and aortic valve calcification (61). Monocytes internalize forming minerals and secrete more inflammatory cytokines and intensify pathologic condition (62). Cytokines secreted by pro-inflammatory macrophages exacerbate calcification by activating apoptosis or osteogenic pathways activation in SMCs and VICs (46). In addition to an indirect role in pro-calcific remodeling, macrophages can directly contribute to cardiovascular calcification through release of calcifying EVs in hyperphosphatemic milieu (46). Macrophage-derived calcifying EVs contain the tetraspanin exosomal markers of CD9, CD63, CD81, and TSG101 (28, 63). These EVs also exhibit immunopositivity for CD68 (63) (**Figure 3A**). In EVs released by macrophages, calcium mineral nucleates on complexes containing S100A9 [a pro-inflammatory and pro-thrombotic factor (11)], PS, and annexin V on the EV membrane (4). Accumulation and aggregation of these EVs results in mineral growth within atherosclerotic plaques (**Figure 3B**). Of note, macrophages contribute to both vascular and valvular calcification; therefore, macrophage-derived EV calcification could provide a link between mineral depositions within these tissues.

CONCLUSION

Calcifying EVs play an important role in the initiation and development of cardiovascular calcification. Though calcifying EVs in cardiovascular tissues appear to share commonalities with MVs, they seem to be derived from different origins within cells. The overlapping characteristics between EVs and MVs underscore the fact that research in cardiovascular calcification

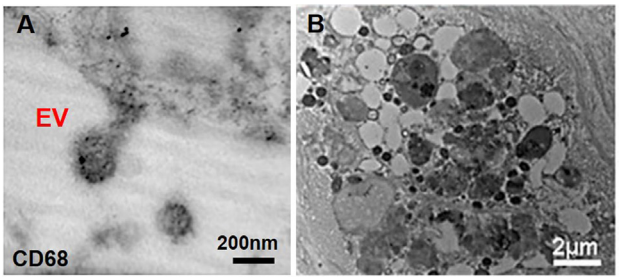

FIGURE 3 | Macrophage-derived extracellular vesicles (EVs) within atherosclerotic cap. **(A)** Macrophage-derived EVs contain the CD68 glycoprotein (immunogold staining); **(B)** released EVs have various size and morphology and aggregate to build calcific mineral within the atherosclerotic fibrous cap (63).

has been informed by pioneering research in bone physiology. However, the noted differences between cardiovascular calcifying EVs and bone MVs warrant further investigation. The type and quality of mineral that forms appear different in the two tissues. Further, the appearance of calcific mineral in cardiovascular tissues associates strongly with a decrease in bone mineral density—a phenomenon known as the calcification paradox (59). Studies into the differences between the fundamental building blocks of calcification—calcifying EVs in cardiovascular tissues and MVs in bone—may provide new insight into the observed divergence in mineral within these tissues and present therapeutic options that avoid unwanted off-target effects.

AUTHOR CONTRIBUTIONS

AN researched the topic and drafted the manuscript. JH helped draft the original manuscript and edited and revised the manuscript text. EA provided intellectual contributions to the original manuscript draft and substantially edited and revised the manuscript text.

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REVIEW
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Initiation and Propagation of Vascular Calcification Is Regulated by a Concert of Platelet- and Smooth Muscle Cell-Derived Extracellular Vesicles

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The ageing population continues to suffer from its primary killer, cardiovascular disease (CVD). Despite recent advances in interventional medicinal and surgical therapies towards the end of the 20th century, the epidemic of cardiovascular disease has not been halted. Yet, rather than receding globally, the burden of CVD has risen to become a top cause of morbidity and mortality worldwide. Most CVD arises from thrombotic rupture of an atherosclerotic plaque, the pathologic thickening of coronary and carotid artery segments and subsequent distal ischemia in heart or brain. In fact, one-fifth of deaths are directly attributable to thrombotic rupture of a vulnerable plaque. Atherosclerotic lesion formation is caused by a concert of interactions between circulating leukocytes and platelets, interacting with the endothelial barrier, signalling into the arterial wall by the release of cytokines and extracellular vesicles (EVs). Both platelet- and cell-derived EVs represent a novel mechanism of cellular communication, particularly by the transport and transfer of cargo and by reprogramming of the recipient cell. These interactions result in phenotypic switching of vascular smooth muscle cells (VSMCs) causing migration and proliferation, and subsequent secretion of EVs. Loss of VSMCs attracts perivascular Mesenchymal Stem Cells (MSCs) from the adventitia, which are a source of VSMCs and contribute to repair after vascular injury. However, continuous stress stimuli eventually switch phenotype of cells into osteochondrogenic VSMCs facilitating vascular calcification. Although Virchow's triad is over 100 years old, it is a reality that is accurate today. It can be briefly summarised as changes in the composition of blood (platelet EVs), alterations in the vessel wall (VSMC phenotypic switching, MSC infiltration and EV release) and disruption of blood flow (atherothrombosis). In this paper, we review the latest relevant advances in the identification of extracellular vesicle pathways as well as VSMCs and pericyte/MSC phenotypic switching, underlying vascular calcification.

Keywords: extracellular vesicles, vascular smooth muscle cells, perivascular mesenchymal stem cells, vascular calcification, platelets, phenotypic switching

EXTRACELLULAR VESICLES

Extracellular vesicles (EVs) are currently considered as important physiological players. Their secretion represents a universally active cellular function in all living organisms from bacteria to humans (1). EVs are highly heterogeneous structures that differ in size, biochemical content and mode of secretion. Current nomenclature distinguishes three populations of EVs: (1) exosomes (30–100 nm), which originate when multivesicular bodies fuse with the plasma membrane, (2) microvesicles/ectosomes (100–1,000 nm), which are generated by budding of the plasma membrane and (3) apoptotic bodies (>1,000 nm), which are formed in the process of programmed cell death (2–5). Each family is composed of small phospholipid membrane-enclosed entities released spontaneously, or, in response to cell activation or apoptosis (6). EV release is stimulated via multiple physiological and pathological conditions, making them potential diagnostic biomarkers for monitoring various diseases (7). Their presence has been detected in a number of bodily fluids from healthy individuals, such as peripheral blood, urine, saliva and synovial fluid to name a few (1, 8, 9). In terms of pathological states, first breakthroughs in the field of EV research were made in oncology and immunology, yet today cardiovascular disease represents one of the most intensively studied and rapidly developing areas of the extracellular vesicle field (4). The number of circulating EV levels has been shown to be associated with various cardiovascular and metabolic disorders, including atherosclerosis and diabetes mellitus (8). In the vasculature, EVs are released from platelets, endothelial cells, smooth muscle cells, erythrocytes and leukocytes (6, 7).

Platelet-Derived Extracellular Vesicles

EVs from platelets were first described in 1967 by Peter Wolf from human blood samples, where they were originally referred to as "platelet dust" (10). Further studies demonstrated that EVs are released when platelets attach to the vessel wall (11). Later it was reported that platelet-EVs are composed of two different types: exosomes and microvesicles (12). Since then platelet EVs have been shown to be involved in several processes in the human body, such as coagulation and atherosclerosis (13). Given that EVs express phospholipids on their surface, they are capable of binding (activated) coagulation factors. Interestingly, their coagulation activity is 50–100 times higher compared to activated platelets (14). In fact, a genetic disorder that is associated with deficient EV formation by platelets leads to bleeding (15). This suggests that promotion of coagulation by EVs is an important physiological mechanism (6).

On the other hand, platelet EVs are known to accumulate in platelet concentrates (16). Transfusion of platelet concentrates is associated with adverse reactions in the recipient, more often than any other blood-derived product (17). This might be explained by the fact that platelet EVs are rich in inflammatory molecules (e.g., CD40L), so they can adhere to leukocytes by CD62P – PSGL-1 interactions and transport pro-inflammatory signals. Several studies have demonstrated that platelet EVs isolated from platelet concentrates can modulate the phenotype and activities of leukocytes and vascular cells (see below). Platelet EVs retain many properties of their parent cells, such as the presence of surface specific antigens (**Table 1**), the ability to deposit chemokines to the vessel wall and to confer inflammatory signals to distal sites (28). In human blood, platelet-derived EVs are the most abundant population of EVs, despite the fact that erythrocytes are about 30 times more numerous than platelets (29). It has been estimated that in circulation 70–90% of all EVs are derived from platelets, 10% originate from granulocytes and only 5% come from endothelial cells, red blood cells and monocytes (1). The number of circulating platelet EVs is also influenced by cardiovascular medication. Antiplatelet agents, antihypertensive agents and cholesterol-lowering drugs inhibit EV release from platelets (30, 31). However, in studies on patients treated with statins, consensus on the number of circulating platelet EVs has not been established (30, 32). Of note, it has been proposed that the majority of the circulating CD41-positive EVs actually originate from megakaryocytes, rather than from platelets (33). In addition, platelet-derived EVs might be distinguished from their megakaryocyte-derived counterparts through the detection of surface molecules such as CD62P, LAMP-1, CLEC-2 and GPVI.

Vascular Inflammatory Functions of Platelet-Derived EVs

Besides the involvement of platelet-derived EVs in the coagulation process, evidence also points towards a role in immune- and inflammation-related processes. For example, platelet-derived EVs have been shown to influence vascular cells (endothelial cells and smooth muscle cells) and leukocytes, thereby changing their phenotype and function. EVs are considered to play an important role in cell-cell communication, their membrane-enclosed content, small size and repertoire of surface receptors facilitate long distance transport within bodily fluids (5, 8, 34). EVs can influence target cells by providing ligands which augment the secretion of growth factors or cytokines, transfer of cell adhesion molecules or reprogram target cells through their genetic make up (1, 28, 29). When isolated platelet EVs are incubated with

TABLE 1 | Surface markers found on platelet EV.

Cluster of differentiation (CD) or abbreviation	Trivial or full name	Reference
CD9	Tetraspanin-29	(18)
CD29	Integrin β1	(19)
CD31	PECAM-1	(19)
CD36	Platelet GPIV	(20)
CD42a	Platelet GPIX	(21)
CD42b	Platelet GPIbα	(19, 22)
CD63	Tetraspanin-30	(19)
CD59	Membrane attack complex inhibition factor	(20)
CD61	Integrin beta 3	(22)
CD154	CD40 Ligand	(23, 24)
CD184	CXCR4	(23)
PAR-1	Protease-activated receptor-1	(23)
CD321	Junctional adhesionmolecule-A	(25)
TSP-1	Thrombospondin-1	(21)
VN	Vitronectin	(26)
VWF	Von Willebrand Factor	(27)

TABLE 2 | Cardiovascular/metabolic diseases associated with increased platelet-EV levels.

Disorder	Reference
Hypercholesterolemia and subclinical atherosclerosis	(37)
Coronary calcification	(38)
Carotid atherosclerosis	(39)
Coronary heart disease	(40)
Acute coronary syndrome	(41–44)
Peripheral arterial disease	(45–48)
Hypertension	(49, 50)
Venous Thrombo-embolism	(51, 52)
Stroke	(53–55)
Diabetes mellitus	(56–59)
Metabolic syndrome and obesity	(60–63)

Table dapted from Aatonen et al. (64) and Ridger et al. (65)

monocytes, platelet EVs readily bind to monocytes and phagocytic uptake of platelet EVs can be observed over time (35). In chemotaxis assays, monocytic cells are actively attracted by platelet EVs, an effect that can be blocked by antibodies against CCL5. Prolonged incubation of monocytes with platelet EVs results in a notable change of surface marker expression, indicating a polarisation of the monocytes to M2-type macrophages (35). Finally, platelet EVs were found to induce the secretion of TNFα from monocytic cells more strongly than platelets, whereas incubation with platelets led to a robust release of GM-CSF (35). Similar studies have demonstrated that platelet EVs induce the differentiation of macrophages into dendritic cells (20) and that platelet EVs are even able to "reprogram" the gene expression profile and function of macrophages (36). Although platelet EVs are present both in diseased patients and healthy subjects, increased levels have been associated with various pathological disorders, such as atherosclerosis and diabetes mellitus (**Table 2**).

Pathological remodelling of the vasculature involves an intricate and dynamic interaction between blood cells (platelets, leukocytes), vascular cells (endothelial cells, smooth muscle cells and adventitial cells) and their direct microenvironment (66). EV-mediated signalling between hematological cells and vascular cells is also of importance in this process. Elevation of platelet EVs in cardiovascular disease appears to be a common process, their interaction with the vascular endothelium has been an area of high interest. However, current knowledge in this field is still very limited and our understanding relies mainly on *in vitro* experiments. The importance of leukocyte-endothelium signalling in pathophysiological conditions of the vasculature is already well known. More recently the role of platelet EVs in these processes has been demonstrated. *In vitro* experimentation has shown that shear stress-activated platelet EVs facilitate the interaction between monocytes and endothelial cells. This is facilitated by increases in inflammatory cytokine levels and cell adhesion molecules on both cell types (67). Another study indicated that platelet EVs contain bioactive lipids (e.g., arachidonic acid), that stimulate ICAM-1 expression in HUVECs, leading to enhanced monocyte-endothelial interactions (68). Platelet EVs may also activate endothelial cells and leukocytes, more specifically, neutrophils by surface molecules CD41 and CD62P, further demonstrating their importance in modulating inflammation (69). It has been also shown that platelet EVs can deposit inflammatory molecules such as CCL5 (RANTES) during rolling interactions over endothelial cell monolayers, facilitating the subsequent recruitment of monocytes (25). In this study, rolling interactions depended on P-selectin and GPIb, while transfer of CCL5 was dependent on integrin αIIbβ3 and junctional adhesion molecule A (25).

Interestingly, more than 700 miRNAs have been found to be stored in platelets (70). Platelets also can contain the functional miRNA processing machinery required for the processing of miRNA precursors (71). Moreover, evidence is accumulating that platelets and platelet-EVs can horizontally transfer nucleic acids to endothelial cells (72, 73). Platelet EVs isolated from activated platelets contain significant amounts of miRNA, further to this, functional transfer of miRNA from platelets to endothelial cells was found to occur through vesicle formation (74, 75). Uptake of platelet EV-associated miRNA results in modulation of endothelial target gene expression demonstrated by a downregulation of ICAM-1 (72).

Besides the endothelial lining of the vessel wall, VSMCs can also be influenced by platelet EVs. Platelets can adhere directly to VSMCs, facilitated by the interaction of CX_3CR1 on platelets with CX_3CL1 on VSMCs (76). Although platelets do not make contact with VSMCs under healthy conditions, such encounters might occur after vascular damage, increased endothelial permeability or through intraplaque haemorrhage. Upon endothelial denudation, platelets in a thrombus might release EVs that then come in contact with VSMCs. Increased permeability of the endothelial lining might permit the passage of circulating EVs and during intraplaque haemorrhage, EVs might be formed due to platelet contact with the highly thrombogenic plaque interior. On the other hand, a study investigating EVs isolated from atherosclerotic plaques did not observe a significant fraction of platelet-EVs within plaque (77). It is possible that platelet-EVs lose their surface markers by enzymatic shedding after activation (78), that platelet-EVs are rapidly phagocytosed by macrophages (35, 36), or that platelet-EVs indeed constitute only a minor fraction of the total EV content within a plaque. Nevertheless, a potential influence of platelet EVs on the behaviour and phenotype of VSMCs was investigated in our recent work. Binding studies using CFSE-labelled platelet EVs and VSMCs revealed that the platelet EV–VSMC interaction is metal ion-dependent and that αIIbβ3 on platelet EV is the primary receptor that mediates interactions with VSMCs (79). Platelet EVs induce migration and proliferation of VSMCs in a CXCL4-dependent manner. Prolonged incubation of VSMCs with platelet EVs results in an increased adhesiveness for THP1 monocytic cells under flow conditions and an increase in interleukin 6 production, indicating that platelet EVs have pro-inflammatory effects on VSMCs. Interestingly, the incubation of cultured VSMCs with platelet EVs led to a phenotypic switch towards a synthetic phenotype, as evidenced by morphological changes and a reduced expression of the contractile marker calponin (79). Although direct contact of VSMCs with platelet EVs leads to changes in proliferation, migration, marker expression and phenotype of VSMCs, the possibility exists that platelet EVs might alter the behavior of surrounding cells e.g., the endothelium, thereby indirectly affecting VSMCs by released factors or the transfer of

endothelial EVs, analogous to what has been observed during abnormal shear stress (80).

Taken together, platelet EVs influence both phenotype and behaviour of leukocytes and vascular cells, thus are important initiators and propagators in vascular remodelling and downstream processes, such as calcification.

Initiation and Propagation of Vascular Calcification Is Regulated by Vascular Smooth Muscle Cell Function

Vascular smooth muscle cells (VSMCs) are the most abundant cell source of the vasculature. Unlike most cells, VSMCs arise from several lineages (81). They are critical to maintaining structure and function of the vascular system (82). Their role is central to vessel dilation and constriction as well as vessel remodelling. VSMCs produce components of the vascular extracellular matrix (ECM), therefore altering the composition of connective tissue and can increase the number of VSMCs present in the vasculature by proliferating. VSMCs are commonly considered to be heterogeneous, having either contractile or non-contractile (synthetic) properties (83). This heterogeneity is present in both developing and adult vasculature and is the most defining feature of VSMCs. It has been hypothesised that the different characteristics and functions of VSMCs originate from early developmental cues, as well as spatiotemporal gene regulation of differentiation markers (84).

While in a contractile state, VSMCs contract and relax to enable blood flow around the body. In this contractile state, they express highly VSMC-specific markers for contractility such as SM-αA, calponin and SM22α. These cells have low motility, hence decreased cellular migration is observed, as well as decreased levels of proliferation and a reduced production of extracellular matrices. This enables the blood vessels to maintain elasticity allowing proper function and delivery of blood supply to the anatomy. When synthetic, VSMCs exhibit a marked decrease in expression for VSMC-specific contractility markers, but express more highly markers for matrix metalloproteinase, collagenase, osteopontin and an increase in production of EVs. Phenotype switching enables VSMCs to maintain blood flow as well as support the vascular niche. During vessel repair, migration and proliferation of VSMCs is necessary. Additionally, increases in expression of growth factors such as PDGF, TGF and VEGF, as well as an increased production of ECM is required to reconstruct vasculature following injury.

Vascular Smooth Muscle Cell Phenotypic Switching

Terminal differentiation of VSMCs is not a definitive end and it is possible to switch between phenotypes depending on the demand of the vascular niche. Contractile VSMCs are generally referred to as quiescent differentiated cells, whereas the synthetic state is associated with plasticity and appropriately referred to by some as dedifferentiated VSMCs. Several pathologies of the vasculature are associated with VSMC phenotype switching such as atherosclerosis, restenosis, aneurysm and calcification (85–87). Vascular calcification, the deposition of hydroxyapatite crystals along the vessel, decreases vessel flexibility, impairs proper blood flow and is associated with cardiovascular disease mortality (88).

While culture conditions for *in vitro* maintenance of human VSMCs in either synthetic or contractile phenotypes have been identified, the precise mechanisms that enable VSMC phenotype switching remains unknown (89). Literature has sparse number of papers implicating miRNAs and proteins in either maintenance or inducing VSMC phenotype switch (90). Furthermore, several identified miRNA and proteins have been eluded to modulation of vascular pathologies associated with VSMC phenotype switching or a dysregulation in phenotype switching (90). During pathological phenotypic switching, VSMCs can adapt an osteogenic, chondrogenic or inflammatory phenotype (91–93).

Adding further interference to the quandary of VSMC phenotype switching, wall resident adventitial progenitor cells have been recently implicated as vessel wall regulators (94, 95). Adventitial progenitors have been found to differentiate into osteoblasts, chondrocytes, adipocytes, macrophage as well as VSMCs. This has led to the hypothesis that adventitial progenitor cells are master regulators of the vascular niche. When progenitor dysfunction occurs, differentiation of VSMCs to an osteoblastic, chondrogenic or macrophage-like capacity are formed. This is yet to be fully appreciated.

VSMC-Derived Extracellular Vesicles

In 1967, Anderson first used the name "matrix vesicles" in cartilage development and calcification (96). Tanimura and co-workers were the first to report an association between small membrane encapsulated particles, matrix vesicles, and vascular calcification (97). Vesicular structures have been found in both intimal and medial layers and are likely derived from VSMCs. The release of EVs from VSMCs was first described as a rescue mechanism against calcium overloading, in an attempt to prevent apoptosis (98).

Today, it is appreciated by many that the role of EVs released by VSMCs is significant in VSMC phenotype switching and calcification. VSMCs have been found to release a variety of EVs when in either synthetic or osteogenic phenotype. A group of VSMC EVs have been identified and somewhat characterised by tetraspanin markers CD9, CD63 and CD81 (99). Furthermore, EVs share similarity to osteoblast EVs, having calcium binding capacity and osteoblast-like ECM production. Interestingly, calcific conditions *in vitro* increase expression of SMPD3 and subsequent EV genesis. Inhibition of SMPD3 completely ablates generation of EVs and calcification. Additionally, *in vivo* identification of CD63 with calcification of vessels of chronic kidney disease (CKD) patients implicates that SMPD3 is a potential novel therapeutic target to inhibit EV genesis and thus vascular calcification.

Chen and associates recently identified a novel role between VSMCs and EVs in calcification (100). Initially they characterised EVs from four sources, two from cellular-derived EVs the others from matrix vesicles. Curiously, they identified that both populations of cellular-derived EVs possessed the capacity to enhance calcification, however matrix vesicles did not possess this ability, even though all four EV types were uniformly endocytosed by VSMCs. They eluded to novel increases in expression for MEK1, Erk1/2, Nox1 and SOD2 alongside increases in intracellular calcium ion content, EV biogenesis and calcification. All EVs were found to express tetraspanins CD9, CD63 and CD81, however, the proportion of expression differed significantly between the media and cellular EVs. Cellular-derived

EVs expressed more significantly for CD63 that strikingly coincides with Kapustin et al. *in vivo* observation of CD63 co-localisation with calcification in calcified CKD vessels (99).

Differences between mineralising and non-mineralising EVs were first revealed by Kirsch et al. within chondrocytes, where they identified high expression of annexin A5 within calcifying EVs (101). The precise function of annexins in calcification has not been fully unravelled (102). Regarding *in vitro* VSMC calcification, annexin A2 has been highlighted in calcium regulation resulting in VSMC EV generation (103). Annexin A2 may bind to fetuin-A on the cell membrane of VSMCs, this is in turn internalised by endocytosis preventing fetuin-A from blocking mineral formation. Increases of annexin A2, annexin A5 and alkaline phosphatase co-localisation are proportional to decreases in fetuin-A expression within *in vitro* VSMC calcification models. Macrophage-derived EV calcification is induced via binding of annexins A2 and A5 to phosphatidylserine (PS) (104). Annexin A5 with S100A9 binding to PS is critical for osteoblast-derived ECM production, interestingly this mechanism also occurs during macrophage production of calcifying EVs. Further interrogation of annexins A2, A5 and A6 in VSMC calcification, phenotype modulation and EV genesis will add to our understanding of the roles of annexins in the cellular context of VSMCs.

Propagation of calcification via vitamin K-antagonist (VKA) treatment led to the identification of matrix Gla-protein (MGP) as a potent calcification inhibitor (105). Twenty years have now passed since MGP inhibitory action was identified, ten years since the mechanism of MGP activation was eluded to by means of serine phosphorylation and gamma-glutamyl carboxylation (91, 106, 107). More recently it has been demonstrated that VSMC-derived EVs contain copious amounts of Gla-proteins from circulation (108). Prothrombin (PT), like MGP, contains a Gla domain. It has been recently demonstrated that production of calcifying VSMC-derived EVs can be inhibited by PT interaction. The Gla domain of PT interacts with the surface of EVs, preventing nucleation sites for calcification (108). This finding has been substantiated by the localisation of PT in calcified regions of the iliac/femoral arteries from a cohort of patients with CKD. Furthermore, circulating levels of PT are reduced in patients with vascular calcification. Accordingly, in absence of MGP, VSMC-derived EVs act to induce calcification, suggesting a potential novel role for inhibition of calcification via PT-EV interactions.

Recent findings have implicated a key role for Sortilin in VSMC calcifying EV biogenesis and release. Originally, Sortilin 1 was identified as driving factor in EV-facilitated calcification through

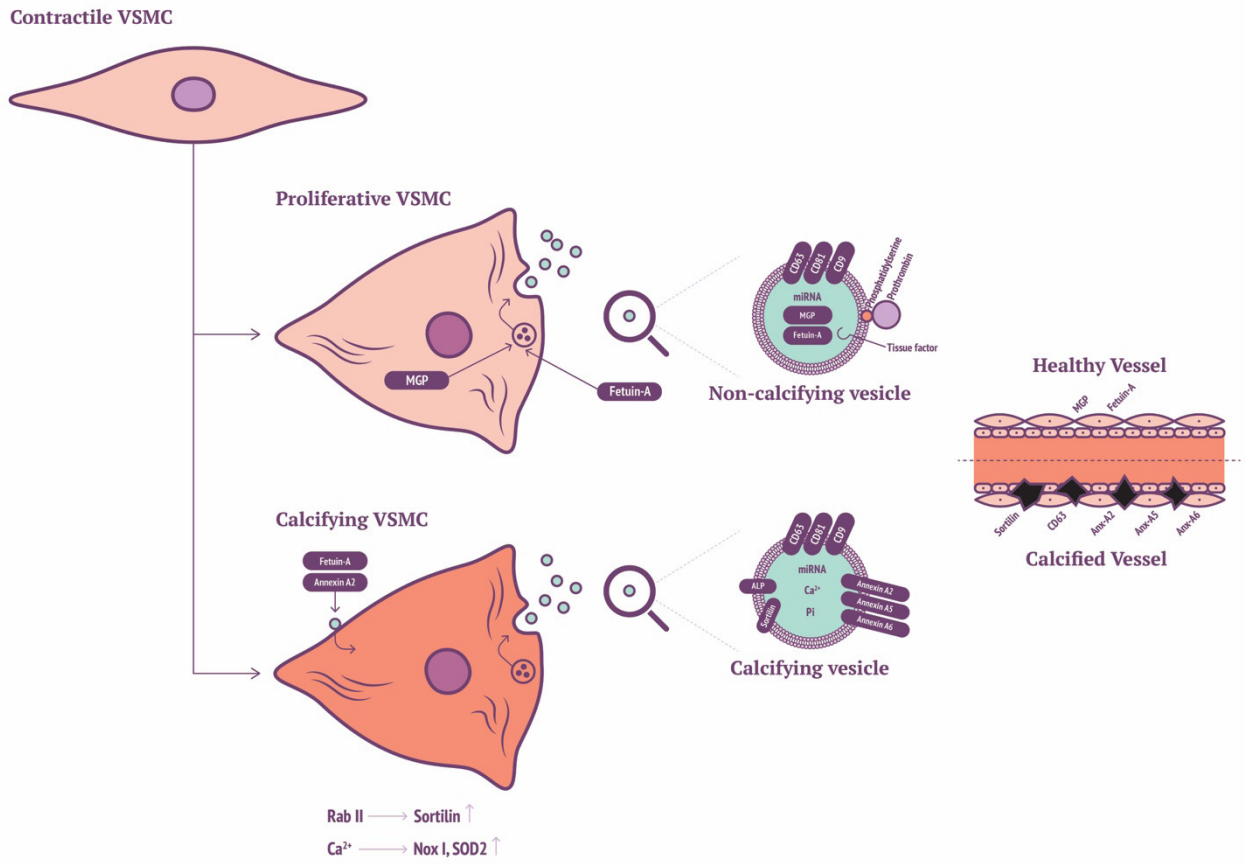

FIGURE 1 | VSMC with Sortilin, MGP and annexin production of calcifying EVs.

Rab11 interaction (109). More recently, it was shown that Sortilin 1 localises in human calcifying vessels (110). In addition, Sortilin 1 plays a role in VSMC calcification *in vitro*. Sortilin 1 contributes to vascular calcification via tissue nonspecific alkaline phosphatase (TNAP) although regulation of TNAP expression within VSMC calcification remains poorly understood. Notably, Sortilin 1 has been identified as a driver in generation of osteoblasts, while TNAP expression was unaffected. This eludes to a potential novel mechanism in the production of VSMC-derived calcifying EVs, which might be a unique mechanism in vascular calcification (**Figure 1**).

Characterisation of EV cargo has been standardised to falling into either protein, RNA or lipid (111). The composition of lipid content in circulating EVs might provide novel insights into mechanisms for VSMC calcification. RNA content of VSMC-derived EVs may be mRNA, miRNA, lncRNA or circRNA, with miRNA being the most characterised in VSMC phenotype modulation and calcification (112). RNA lie within the unique position of being both genotype and phenotype which gives them a role in cellular regulation and pathology (113). Potently a wide array of miRNAs have been implicated by a variety of mechanisms contributing to, or inhibiting the development of vascular calcification (**Figure 2** and **Table 3**). MiRNAs which either induce VSMC to an osteoblast-like pro-calcific phenotype or inhibit associated phenotype and calcification. The specific mechanism of which has been reviewed elsewhere (133, 134). Although the role of miRNAs within vascular calcification is being unravelled, many miRNAs have been identified via RNA screening between normal and calcified VSMCs. The origin of miRNA source in a pathological context remains elusive and requires further investigation.

Whether there is cross talk of EVs from different facets of the vascular niche or if there is a dysregulation in VSMC maintenance remains an important question. Understanding the cross talk of EVs between vascular cells might be significant to appreciating the precise mechanisms for vascular calcification. MiR-206, ARF6 and NCX1 have been identified as endothelial cell-released EV content capable of regulating VSMC contractile phenotype (135). Furthermore, given the observed increase of endothelial EV release following injury, it has been noted that endothelial cell EVs from human pulmonary artery not only interacted with pulmonary VSMCs, but interaction induced proliferation and had a seemingly anti-apoptotic effect on VSMCs (136). This suggests a direct pathological consequence of endothelial EV to VSMC crosstalk during and following intimal vessel injury.

MESENCHYMAL STEM CELLS AS PERIVASCULAR PROGENITORS

As described above, VSMCs are known to exhibit elaborate phenotypical plasticity and diversity during normal development, disease, and repair of vascular injury. The current theory of vascular calcification is that upon injury VSMCs dedifferentiate. VSMCs become synthetically active and under stress factors, like elevated

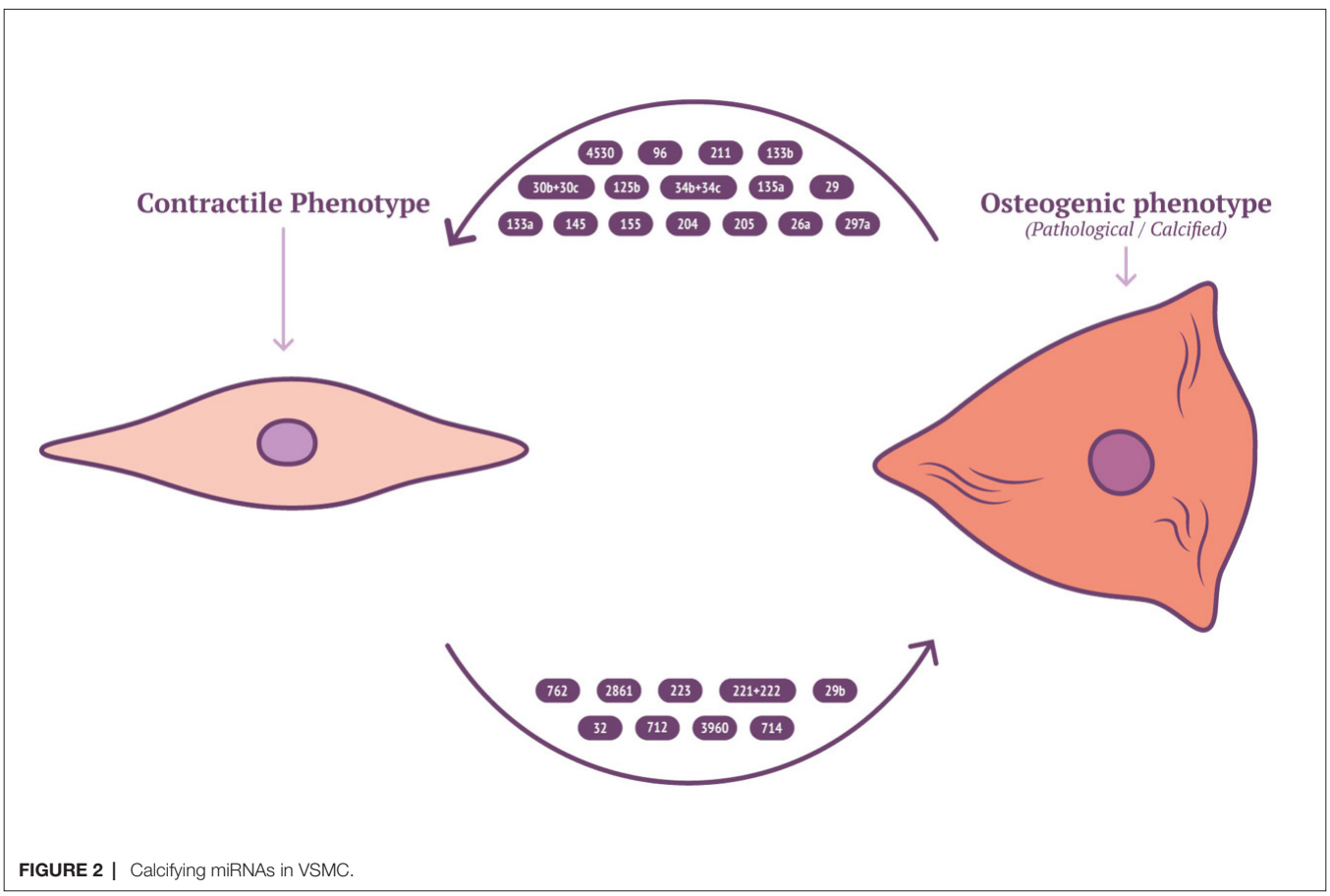

FIGURE 2 | Calcifying miRNAs in VSMC.

TABLE 3 | MiRNA associated with phenotypic switching of VSMC.

Reference	Calcifying miRNA	MiRNA Inhibiting Calcification	Paper
(114)	29b	133b, 211	MicroRNAs 29b, 133b and 211 regulate vascular smooth muscle calcification mediated by high phosphorous
(115)	32		MicroRNA-32 promotes calcification in vascular smooth muscle cells: implications as a novel marker for coronary artery calcification
(116)	3960, 2861		Runx2/miR-3960/miR-2861 positive feedback loop is responsible for osteogenic transdifferentiation of vascular smooth muscle cells
(117)		29	MiR-29-mediated elastin down-regulation contributes to inorganic phosphorous-induced osteoblastic differentiation in vascular smooth muscle cells
(118)		125b	MiR-125b regulates calcification of vascular smooth muscle cells
(119)		135a	MiR-135a suppresses calcification in sensence VSMCs by regulating KLF4/STAT3 pathway
(120)		204	MicroRNA-204 regulates vascular smooth muscle cell calcification *in vitro* and *in vivo*
(121)	221 + 222		MiRNA-221 and miRNA-222 synergistically function to promote vascular calcification
(122)		125b, 145, 155	Decreased microRNA is involved in the vascular remodelling abnormalities in chronic kidney disease (CKD)
(123)	762, 714, 712		Micro RNAs that target Ca(2 + transporters are involved in vascular smooth muscle cell calcification
(124)		30b, 30c	Bone morphogenetic protein-2 decreases microRNA-30b and microRNA-30c to promote vascular smooth muscle cell calcification
(125)		96	Down-regulation of miR-96 by bone morphogenetic protein signalling is critical for vascular smooth muscle cell phenotype modulation
(126)		205	MicroRNA-205 regulates the calcification and osteoblastic differentiation of vascular smooth muscle cells
(127)		133a	MiR-133a modulates osteogenic differentiation of vascular smooth muscle cells
(128)		4530, 133b	Differential expression of microRNAs in severely calcified carotid plaques
(129)		26a	MiR-26a regulates vascular smooth muscle cell calcification *in vitro* through targeting CTGF
(130)		297a	MicroRNA-297a regulates vascular calcification by targeting fibroblast growth factor 23
(131)	223		Inorganic phosphate accelerates the migration of vascular smooth muscle cells: Evidence for the involvement of miR-223
(132)		34b + 34c	MicroRNA-34b/c inhibits aldosterone-induced vascular smooth muscle cell calcification via a SATB2/Runx2 pathway

calcium and phosphate levels or uremic toxins (e.g., during CKD), switch to a dedifferentiated synthetic phenotype and later become osteoblast like cells (99, 137). Genetic fate tracing strongly indicated mature VSMCs to be a major contributor to the development of atherosclerotic plaque remodeling (138). However, not all elements of plaque development and progression may be indicative as a result of VSMC phenotype switching. The fact, that atherosclerotic human vessels can contain complete trabecular bone with fully formed bone marrow sinusoids even containing hematopoietic cells is difficult to explain without new concepts, such as vascular stem cells. Indeed, the presence of a specialised progenitor population of VSMCs localised in the adventitia of muscular arteries has been suggested by several groups.

The perivascular niche houses pericytes, which are present at intervals along microcapilaries and pericyte-like cells are also located in the adventitia of large arteries. Peault and coworkers were the first to show that many pericytes are MSCs (139, 140). However, it remains elusive whether all pericytes are MSCs. A pericyte is defined as a cell that is completely or partially embedded in the endothelial basement membrane (141). The fact that MSCs are also located in the adventitia of large arteries distant from vasa vasorum and in the endosteal niche of the bone marrow indicates that not all MSCs are pericytes (94, 142). The recent evidence that the perivasculature represents the MSC niche explains why MSCs can be isolated from virtually all organs and tissues.

It was first described over 25 years ago that pericytes have an osteogenic potential (143) and subsequently pericyte-like cells with osteogenic capacity were isolated from VSMCs nodules of human aorta (144). A decade ago it has been reported that $Sca1^+/CD34^+/PDGFR\beta^+$ cells that reside in the adventitia of arteries possess a differentiation capacity towards smooth muscle cells and osteoblasts *in vitro* (145). Interestingly, these progenitors express the Hedgehog receptor Ptc1 as well as most other hedgehog pathway members including Gli1-3 (145). Other groups have reported that perivascular MSCs were also found to be progenitors of white adipocytes (146) follicular dendritic cells (147) and skeletal muscle (148), while also playing a major role in fibrotic response (149–151). Thus, various studies suggest the presence of adventitial MSC-like cells with osteogenic and myogenic potential. However, until recently the involvement of these MSC-like pericytes in cardiovascular disease development remained unclear.

Role of Perivascular MSC-Like Cells in Vascular Calcification

Using genetic labeling and *in vivo* fate tracing experiments it was recently revealed that the Hedgehog transcriptional activator Gli1 specifically labels perivascular MSC-like cells (94, 142, 151, 152). $Gli1^+$ cells reside in the pericyte niche with direct contact to endothelial cells of the microvasculature and in the adventitia of large arteries (94, 151). $Gli1^+$ cells possess all criteria that have been used to define a MSCs including surface marker expression, tri-lineage differentiation and plastic adherence (94, 151). Inducible genetic fate tracing, the gold standard technique to dissect cellular hierarchies, indicated that $Gli1^+$ cells are a major cellular source of myofibroblast in fibrosis of all major organs such as lung, kidney, liver, heart and bone marrow (142, 151).

The question is however, whether adventitial $Gli1^+$ MSC-like cells are involved in acute injury repair and chronic vascular disease progression. Using *in vivo* genetic fate tracing, it was demonstrated

that after wire injury of the femoral artery about 50% of newly formed VSMCs were derived from adventitial Gli1$^+$ cells (94). Furthermore, FACS isolated adventitial Gli1$^+$ MSC could be differentiated into calponin$^+$, αSMA$^+$, smoothelin$^+$ VSMCs in vitro (94). This data indicates that adventitial Gli1$^+$ MSC are indeed progenitors of VSMCs.

The role of adventitial Gli1$^+$ MSC in vascular calcification was studied in triple transgenic Gli1CreER; tdTomato, ApoEKO mice. Mice were pulsed with tamoxifen in order to genetically tag Gli1$^+$ cells by expression of the bright red fluorochrome tdTomato. Mice were subjected to either subtotal nephrectomy and western diet or sham surgery with standard chow. Interestingly, a continuous low frequency migration of Gli1$^+$ cells into the media during aging in the sham group was observed. Gli1$^+$ cells acquired markers of VSMCs such as α-SMA and calponin suggesting that progenitor cells continuously replace lost VSMCs during aging (94). Importantly, these data indicate a great migration of Gli1$^+$ cells into the media and neointima during chronic injury and atherosclerosis. Multiple co-staining experiments indicated that adventitial Gli1$^+$ cells first differentiated into contractile VSMCs (α-SMA$^+$, calponin$^+$) and then underwent a phenotypic switching with loss of contractile VSMC markers and acquisition of synthetic VSMC markers such as Tropomyosin alpha 4 (TPM4) and non-muscle myosin heave chain 2b (nmMHC2b) (94). Importantly, during vascular calcification a high percentage of Gli1$^+$-derived cells acquired nuclear expression of the transcription factor Runx2 indicating differentiation into osteoblast-like cells. Imaging experiments after injection of a fluorochrome conjugated bisphosphonate (Osteosense) indicated that calcified areas showed intense accumulation of tdTomato$^+$ cells. Importantly, genetic ablation of Gli1$^+$ cells in triple transgenic Gli1CreER; iDTR; ApoEKO mice before onset of CKD completely abolished vascular calcification in both the intima and media. Thus, clearly demonstrating that adventitial Gli1$^+$ cells are important progenitors of synthetic VSMCs and osteoblast-like cells in the vascular wall. Adventitial Gli1$^+$ cells can be considered an important therapeutic target in vascular calcification. Interestingly, we observed a Shh domain in the adventitia of human arteries where endothelial cells of vasa vasorum stained positive for Shh, whereas surrounding cells showed Gli1 expression. Further staining experiments in calcified human arteries showed intense Gli1 expression around calcified intima and media areas.

While these data clearly demonstrate an important role of adventitial MSC-like cells in vascular calcification there are still various open questions that need to be answered (**Figure 3**). It is unclear whether the Gli1$^+$ population is a homogenous progenitor population or whether several subpopulations exist. Single-cell qPCR analysis of sorted Gli1$^+$ cells for reported markers of adventitial progenitors indicates heterogeneity with three distinct subpopulations (unpublished data). Thus, it will be important to differentiate between these subpopulations. Next, it remains elusive as to whether Gli1-expressing cells of human arteries may also secrete EVs and thereby contribute to vascular calcification. Finally, while our data clearly indicates migration and differentiation of Gli1$^+$ cells during acute injury repair and chronic disease progression, the underlying molecular pathways that activate migration and differentiation of the progenitor population remain obscure. It is currently not known whether EVs are involved in migration and differentiation of MSCs. Further, we must await as to whether adventitial Gli1$^+$ cells produce EVs or interact with EVs from other cells such as platelets, immune

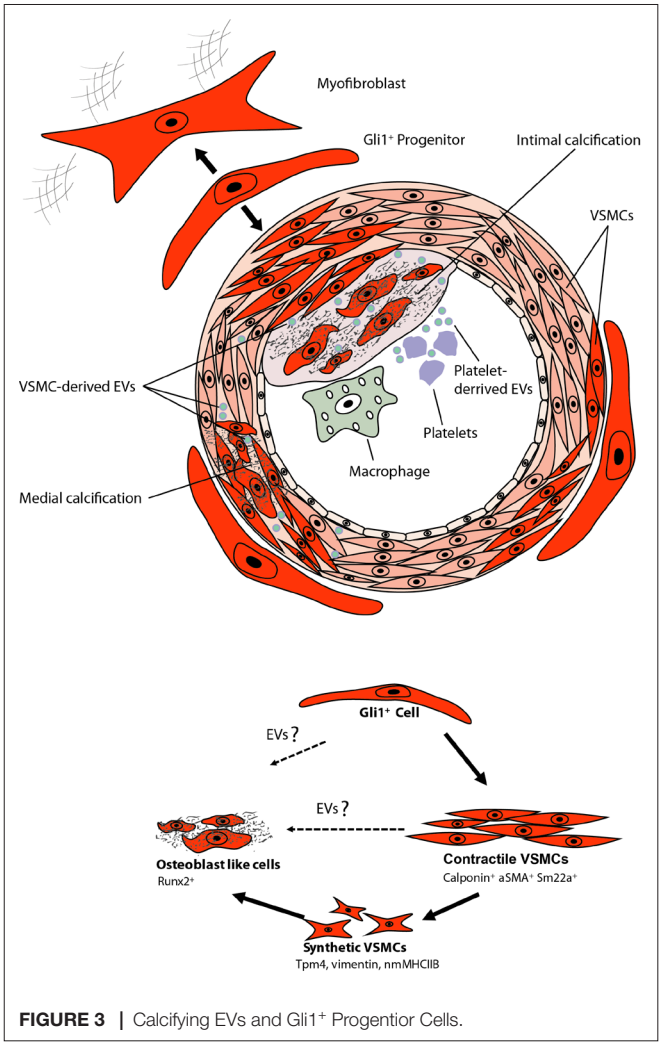

FIGURE 3 | Calcifying EVs and Gli1$^+$ Progentior Cells.

cells and VSMCs. Experiments in myelofibrosis demonstrated that a malignant hematopoietic clone in particular megakaryocytes can activate Gli1$^+$ cells to leave their niche partly by CXCL4 release (142). Thus, involvement of platelet and cellular EVs might be an explanation for activation and migration of Gli1$^+$ cells. Further studies are needed to answer whether EVs are involved in Gli1$^+$ cell recruitment and differentiation.

CONCLUSION

The biology of vascular disease and calcification is complex and still poorly understood with respect to cause and consequence of players and pathways, and thus complexity increases continuously by ongoing elucidation of novel players and pathways. A subset of EVs act as mediators of cell-induced extracellular matrix calcification in the pathogenesis of cardiovascular disease. On the other hand, it has become evident that platelet and cellular vesicles play an important role in cellular communication. Whether such communication from platelet EVs can transpire through endothelial cells to the media

and adventitia remains unknown, and so a role for EV crosstalk in calcification remains open with promise.

AUTHOR CONTRIBUTIONS

LS wrote the manuscript, supervised writing process and was responsible for the final version; AA wrote the manuscript; DK wrote the manuscript; MH wrote the manuscript; RRK wrote the manuscript; RK wrote the manuscript and was responsible for the final version.

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Toward Exosome-Based Therapeutics: Isolation, Heterogeneity, and Fit-for-Purpose Potency

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Exosomes are defined as submicron (30–150 nm), lipid bilayer-enclosed extracellular vesicles (EVs), specifically generated by the late endosomal compartment through fusion of multivesicular bodies with the plasma membrane. Produced by almost all cells, exosomes were originally considered to represent just a mechanism for jettisoning unwanted cellular moieties. Although this may be a major function in most cells, evolution has recruited the endosomal membrane-sorting pathway to duties beyond mere garbage disposal, one of the most notable examples being its cooption by retroviruses for the generation of Trojan virions. It is, therefore, tempting to speculate that certain cell types have evolved an exosome subclass active in intracellular communication. We term this EV subclass "signalosomes" and define them as exosomes that are produced by the "signaling" cells upon specific physiological or environmental cues and harbor cargo capable of modulating the programming of recipient cells. Our recent studies have established that signalosomes released by mesenchymal stem/stromal cells (MSCs) represent the main vector of MSC immunomodulation and therapeutic action in animal models of lung disease. The efficacy of MSC-exosome treatments in a number of preclinical models of cardiovascular and pulmonary disease supports the promise of application of exosome-based therapeutics across a wide range of pathologies within the near future. However, the full realization of exosome therapeutic potential has been hampered by the absence of standardization in EV isolation, and procedures for purification of signalosomes from the main exosome population. This is mainly due to immature methodologies for exosome isolation and characterization and our incomplete understanding of the specific characteristics and molecular composition of signalosomes. In addition, difficulties in defining metrics for potency of exosome preparations and the challenges of industrial scale-up and good manufacturing practice compliance have complicated smooth and timely transition to clinical development. In this manuscript, we focus on cell culture conditions, exosome harvesting, dosage, and exosome potency, providing some empirical guidance and perspectives on the challenges in bringing exosome-based therapies to clinic.

Keywords: exosomes, extracellular vesicles, exosome-based therapeutics, mesenchymal stem cells, preclinical

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INTRODUCTION

The intracellular transfer of diverse moieties *via* extracellular vesicles (EVs) has been proposed to be a widespread process. Cells release diverse EVs that include exosomes, microvesicles (MVs) and apoptotic bodies (1, 2). The classification of such EV subtypes is mainly based on their biogenesis and resultant biophysical properties, such as size, density, and predominant protein markers. Originally, the class of EVs generated through the endosomal pathway (exosomes) was assumed to represent a mere mechanism for the cell to jettison unwanted moieties (3, 4). We now understand that exosome biogenesis is a process governed by the endosomal-sorting complex machinery and involves the formation of intraluminal vesicles within multivesicular bodies (MVBs). Mature MVBs fuse with the plasma membrane and subsequently secrete the enclosed exosomes into the extracellular environment. During their biogenesis, exosomes associate with an array of bioactive cargo from their parental cell. Such cargo has been reported to include genetic information in the form of small noncoding RNAs, free fatty acids, surface receptors, and proteins (**Figure 1**) (5, 6). It is considered that the biophysical properties of EVs, including their cargo, reflect the stimulus triggering their formation (7), implying specific packaging of "*message*" prior to export from the parent cell. In turn, the secretion of these biologically loaded signaling vectors to the extracellular environment represents an important method of cell-to-cell communication, dubbed the "new endocrinology".

Exosomes have been shown to play important roles in a broad range of pathological conditions, such as cancer (9), liver and kidney disease (10), neurodegenerative disorders (11), and numerous cardiopulmonary disorders (12, 13). More recently, in addition to their prognostic and diagnostic value, exosomes have also been reported to represent novel therapeutic reagents across multiple disciplines.

EXOSOME-BASED THERAPEUTICS

The therapeutic capacity of exosomes generated by mesenchymal stem/stromal cells (MSCs) that have been derived from different organs, such as bone marrow, umbilical cord, adipose tissue, or placenta has been tested in various disease models. In the cases where cells and their respective exosomes were studied in parallel, exosome treatment has demonstrated a similar or even superior therapeutic capacity to MSC treatment (14). MSC-exosomes have provided beneficial effects in numerous disease models promoting functional recovery and neurovascular plasticity following traumatic brain injury (15), reducing myocardial infarction size (16, 17), ameliorating hypoxia-induced pulmonary hypertension (18), aiding repair of kidney injury (19, 20), and orchestrating neurological protection by the transfer of microRNA (21, 22). MSC-exosome-based approaches for the treatment of different disease models are highlighted in **Table 1**.

While the functional roles of exosomes have been extensively reported [reviewed in Ref. (43–46)], few reviews have addressed the challenges underlying the transition of exosome-based therapies

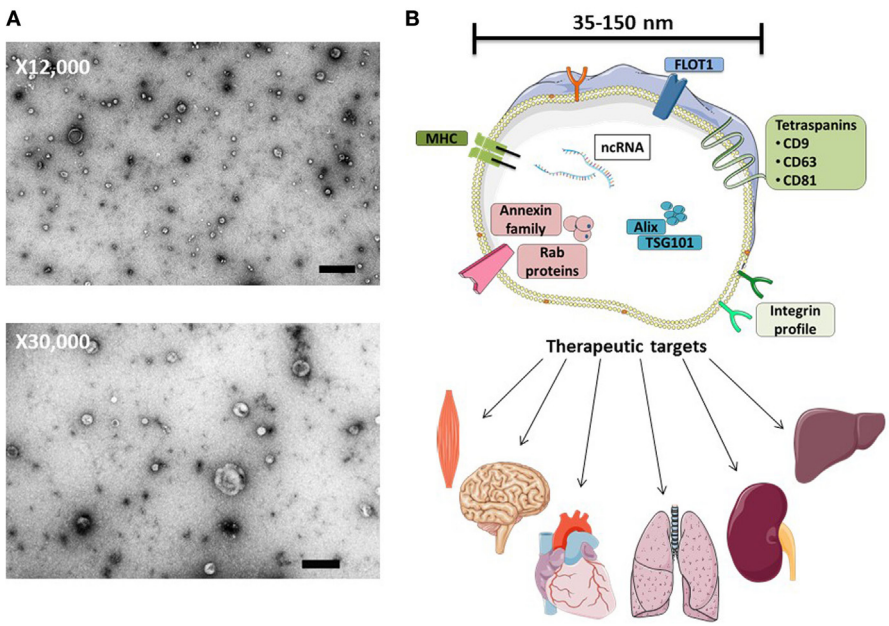

FIGURE 1 | MSC-exosome morphology and composition. **(A)** Transmission electron microscopy (TEM) images of human bone marrow-derived MSC-exosomes (low magnification, 12,000×, scale bar = 500 nm, and high magnification, 30,000×, scale bar = 100 nm) representative TEM images adapted from Ref. (8). **(B)** MSC-exosomes are surrounded by a phospholipid bilayer and may contain proteins, such as annexins (these are important for transport); tetraspanins such as CD9, CD81, and CD63; and other proteins, such as Alix and TSG101, that are involved in exosomal biogenesis from endosomes. MSC-exosome therapy has shown beneficial effects in numerous preclinical models, demonstrating histological and functional benefits in multiple organs. Abbreviations: FLOT1, flotillin-1; MHC, major histocompatibility complex; TSG101, tumor susceptibility gene 101.

TABLE 1 | Summary of MSC-exosome-based approaches for the treatment of different disease models.

Disease model	MSC-product "nomenclature"	Isolation method	Dose assessment	Dose	Reference
Respiratory					
Bronchopulmonary dysplasia	Exosomes	Density Cushion	Cell equivalent	0.5×10^6	Willis et al. (8)
Pulmonary hypertension	Exosomes	SEC	Protein	0.1–10 µg	Lee et al. (18)
Pulmonary hypertension	Exosomes	UC (100K × *g*)	Protein	25 µg	Aliotta et al. (23)
Acute lung injury	Microvesicles (MVs)	UC (100K × *g*)	Cell equivalent	1.5×10^6	Zhu et al. (24)
Silicosis	Exosomes	Sucrose gradient	Protein	40 µg	Phinney et al. (25)
Pneumonia	MVs	UC (100K × *g*)	Cell equivalent	9×10^6	Monsel et al. (26)
Cardiovascular					
Myocardial infarction	Exosomes	ExoQuick	Cell equivalent	4×10^6	Yu et al. (27)
Myocardial infarction	Extracellular vesicles (EVs)	UC (100K × *g*)	Protein	80 µg	Bian et al. (28)
Myocardial infarction	Exosomes	ExoQuick	Protein	80 µg	Teng et al. (29)
Ischemia/reperfusion	Exosomes	HPLC	Protein	0.4 µg	Lai et al. (16)
Ischemia/reperfusion	Exosomes	HPLC	Protein	0.4–0.8 µg	Arslan et al. (17)
Neurological					
Traumatic brain injury	EVs	Anion exchange chromatograph	Protein	30 µg	Kim et al. (30)
Laser-induced retinal injury	Exosomes	UC (110K × *g*)	Protein	10 µg	Yu et al. (31)
Optical nerve crush	Exosomes	UC (100K × *g*)	ExoELISA	3×10^9	Mead and Tomarev (32)
Stroke	EVs	UC (110K × *g*)	Cell equivalent	2×10^6	Doeppner et al. (33)
Stroke	Exosomes	UC (100K × *g*)	Protein	100 µg	Xin et al. (34)
Musculoskeletal					
Cardiotoxin injury	EVs	UC (100K × *g*)	Protein	5 µg	Lo Sicco et al. (35)
Hepatic					
Drug-induced liver injury	Exosomes	UC (100K × *g*)	Protein	0.4 µg	Tan et al. (36)
Liver fibrosis	Exosomes	UC (100K × *g*)	Protein	250 µg	Li et al. (37)
Gastrointestinal					
Colitis	EVs	UC (100K × *g*)	Protein	50–200 µg	Yang et al. (38); Fang et al. (39)
Dermatological					
Wound healing	Exosomes	UC (100K × *g*)	Protein	160 µg	Zhang et al. (40)
Wound healing	Exosomes	UC (120K × *g*)	Protein	100 µg	Fang et al. (39)
Renal					
Ischemia/reperfusion	MVs	UC (100K × *g*)	Protein	100 µg	Zou et al. (41)
Acute kidney injury	MVs	UC (100K × *g*)	Protein	100 µg	Bruno et al. (42)

Ultracentrifugation (UC): 100,000–120,000 × g (100K–120K × g). Size-exclusion chromatography (SEC). ExoQuick and ExoELISA refer to a commercially available exosome isolation kit and CD63 capture (exosome) ELISA, respectively (Systems Biosciences, CA, USA).

from animal models to clinical development. Furthermore, the full realization of their therapeutic potential has been hampered by a lack of standardization in exosome isolation and characterization. Herein, we will focus on the therapeutic application of MSC-exosomes and outline topics relevant to the facilitation of their development as a pharmaceutical preparation, focusing on exosome harvesting, dosing and potency, and providing guidance on the current challenges in bringing exosome-based therapies to clinic.

MESENCHYMAL STEM/STROMAL CELL ORIGIN: WHERE ARE THE EXOSOMES COMING FROM?

A comprehensive characterization of the tissue/cellular source of exosomes is imperative for exosome-based therapeutics. Detailed methods for obtaining human MSCs from several tissues, including bone marrow (BMSCs), Wharton's jelly (WJMSCs), umbilical cord blood, and adipose tissue are well reported (47, 48). By definition, MSCs must adhere to plastic, demonstrate a baseline differentiation potential to osteocytes, chondrocytes, and adipocytes *in vitro*, and express the presence of widely accepted surface markers (**Table 2**) (49). However, donor-to-donor variability remains a prominent challenge. Studies have found that BMSCs obtained from older donors have slower proliferation and reduced differentiation potential *in vitro*. Furthermore, discrepancies in the

TABLE 2 | Minimal criteria for defining MSCs, as put forth by The International Society for Cellular Therapy.

Characterization of mesenchymal stem/stromal cells (MSCs)
1. Adherence to plastic in standard culture conditions
2. Phenotype: Positive (≥95%) CD105 CD45 CD73 Negative (≤2%) CD34 CD90 CD14 or CD11b CD79a or CD19 HLA-DR
3. *In vitro* differentiation: osteocytes, adipocytes, and chondrocytes.

Human leukocyte antigen–antigen D related (HLA-DR). Adapted from Dominici et al. (49).

differentiation capacity and transcriptome profiles are reported to be tissue and species dependent (50–52). To what extent do these uncertainties affect the therapeutic capacity of MSCs and their resultant exosomes remains unclear. Thus, in addition to validated MSC isolation procedures, investigators should adhere to carefully selected donor eligibility criteria in accordance with the appropriate ethical and regulatory approval, employing strict control measures to prevent risk of relevant communicable disease agents or diseases (RCDADs), such as human immunodeficiency virus (HIV), hepatitis C virus, and cytomegalovirus. Moreover, donor screening should include a comprehensive medical record review, physical assessment, and medical history interview, with records documented in compliance with appropriate regulatory frame. The International Society of Extracellular Vesicles (ISEV), the Food and drug Administration (FDA), the International Council for Harmonization (ICH) of Technical Requirements for Pharmaceuticals for Human Use, and the European Medicines Agency (EMA) provide extensive guidance for the development and generation of novel biological medicines with regard to donor/patient care, product safety, and quality (53–56). The demonstration that exosomes generated by MSCs isolated from WJMSCs are as effective as BMSC-exosomes in treating rodent disease models (8) may facilitate standardization and consistency of MSC lines for exosome harvesting. Moreover, the umbilical cord may possess several advantages over bone marrow. First, the umbilical cord represents a more readily available source than bone marrow. Second, it is often viewed as discarded medical waste that does not require any invasive procedures or cadaver procurement.

"PHYSIOXIA" CONSIDERATIONS IN ESTABLISHING MSC CULTURE CONDITIONS FOR EXOSOME PRODUCTION

Previous studies have reported that the protein and RNA profile of exosomes reflect the cell culture conditions and microenvironmental stimuli that triggered their release. With this in mind, it begs to question can "stimulating" and/or preconditioning cells be used as a means to generate a more homogenous or efficacious exosome population? Interestingly, in an experimental model of hyperoxia-induced bronchopulmonary dysplasia (BPD), Waszak and coworkers found that the conditioned media (CM) derived from hyperoxia-preconditioned rat BMSCs (95% O_2, for 24 h) provided greater protection *in vivo* compared to CM collected from cells grown under control conditions (57). Clearly, in the absence of further characterization, one can only speculate where the observed augmentation of activity resides. Other studies have found that MSCs cultured in hypoxia conditions (<5% O_2) exhibited an altered protein expression pattern compared to MSCs cultured in the so-called "normoxia" (58). Furthermore, in a murine hind limb ischemia model, they showed that intra-arterial injection of MSCs cultured in both "normoxic" or hypoxic conditions enhanced revascularization compared with saline controls; however, the functional recovery of mice that received hypoxia preconditioned MSCs was faster (59). These reports suggest that preconditioning MSCs in different oxygen environments may improve their tissue regenerative potential.

Indeed, "hypoxia" (1% O_2) has been shown to increase exosome production in numerous cell types *in vitro*. Previous studies have shown that the hypoxia-induced elevation in exosome secretion is chiefly governed by hypoxia-inducible factor-1 alpha (HIF-1α) and is independent of apoptosis (60). Often, laboratory cell culture conditions are at atmospheric oxygen levels (21% O_2, corresponding to a PO2 of ~159 mmHg); following adjustment to 5% CO_2, this equates to ~19.95% O_2 (~150 mmHg). Although, it is challenging to accurately measure tissue oxygen concentrations *in vivo*, it is well recognized that most of the human body tissue is normally exposed to much lower O_2 levels. This can range from 160 to 100 mmHg in the alveoli, >35 mmHg in the brain, and ~25 mmHg in skeletal muscle (61–63). Notably, in the bone marrow, arguably the most common origin of MSCs, the PO_2 is reported to be ~40 mmHg, while the umbilical cord vasculature PO2 is reported to be between 10 and 30 mmHg (63). Organ oxygen levels have been extensively reviewed (63). However, it is important to remember that the different techniques used to measure oxygen concentration *in vivo* are subject to their own advantages and limitations.

In vivo, the oxygen concentration of an organ is an indication of its physiological state and reflects the balance between oxygen delivery and its metabolic consumption. Consequently, in a physiological condition, organs are subject their own unique "physioxia" status. On balance, the routine laboratory cell culture conditions expose MSCs to oxygen levels higher than those in their physiologic niches, and this departure from "physioxia" may precipitate a "perceived hyperoxia" response. Thus, it is important to recognize this factor when interpreting results of experiments performed in atmospheric "normoxia," and also to realize that the impact of this factor may vary, depending on the particular study and the metrics assessed.

In turn, several questions remain unanswered. The optimal oxygen concentration for *in vitro* MSC culture and the effect that it may have on subsequent exosome production remains undefined at this point. Existing reports indicate that optimization is likely to be both MSC origin and disease model specific. Thus, additional studies assessing the effect of oxygen levels on MSCs and their resultant exosomes are much needed.

EXOSOME HETEROGENEITY

Cells generate three major EV classes: apoptotic bodies, MVs, and exosomes. Arguably, it is often assumed each subtype represents a homogenous vesicle population that can be distinguished based on biophysical properties such as size or density. However, it has become obvious that even within such subtypes, there is heterogeneity (2, 64). Although, the field lacks tools to distinguish vesicles from different routes of biogenesis, recent evidence has demonstrated that MSCs release distinct EV subpopulations that differ in biophysical, proteomic, and RNA repertoires. Specifically, Kowal and coworkers found that large-, medium-, and small-sized EVs can be isolated by sequential low-, intermediate-, and high-speed centrifugation, respectively. Among the small-EVs (exosomes), four subcategories were defined by their

degree of enrichment in CD63, CD9, and/or CD81 tetraspanins (64). In accordance, Lai et al. found that MSCs secrete many distinct subtypes of vesicles, which differ in RNA and protein composition (65). It is relevant to note that the study involved an immortalized, iPS-derived MSC cell line that will likely secrete a more restricted range of exosome subtypes than those generated by primary cells.

In our hands, ongoing studies aim to address the relationships between MSC-exosome subtypes and therapeutic efficacy and to explore the hypothesis that a discrete subtype is responsible for the therapeutic activity in our established experimental models of BPD, a chronic lung disorder of infants (8). Here, we isolated exosomes from either human BMSCs or WJMSCs by differential centrifugation, followed by tangential flow filtration and iodixanol density floatation before separating exosome subtypes by size-exclusion chromatography (SEC) (described in **Figure 2**). This approach separates MSC-exosome subtypes based on their size, and in accordance with previous

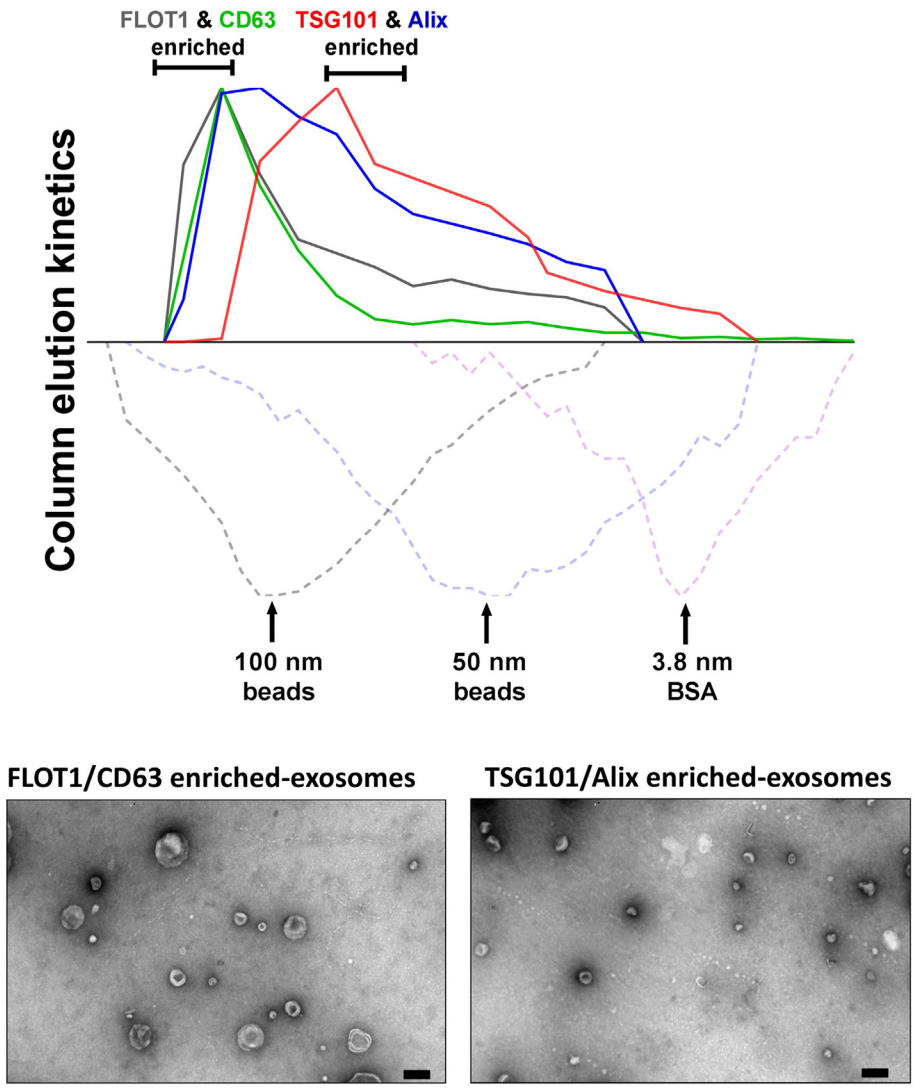

FIGURE 2 | Isolation of MSC-exosomes subtypes by size-exclusion chromatography (SEC). Briefly, exosomes were isolated directly from cell culture supernatants following a 36 h harvest period in serum-free-media. Cell culture media were subjected to differential centrifugation, 300 × *g* for 10 min, followed by 3,000 × *g* for 10 min, and 13,000 × *g* for 30 min to remove any cells, cell debris, and large apoptotic bodies in suspension, respectively. Conditioned media (CM) was concentrated 50-fold by tangential flow filtration (TFF) and the exosomes were purified using OptiPrep™ (iodixanol) cushion density flotation (3.5 h at 100,000 × *g*, 4°C), as previously described (8). Heterogeneous exosomes were further purified by size using size-exclusion chromatography (SEC). Here, sepharose CL-2B (80 mL) was washed with 1 × SSPE buffer [containing 1 mM EDTA and 149 mM NaCl in 0.20 mM phosphate buffer (pH 7.4)]. The column was packed with washed sepharose CL-2B to create a column with an internal diameter of 1.6 cm and height of 40 cm. Exosomes (1 ml, corresponding to 60 × 10^6 MSC equivalents) were added to column with a flow rate of 1 ml/min. Fractions (1 ml) were collected and assessed by dot plots and electron microscopy. The elution kinetics of 100 nm, 50 nm, and bovine serum albumin (BSA) were used to estimate exosome elution kinetics. TSG101, Alix, CD63, and FLOT1 levels were assessed by dot plots and are reported as relative intensity. Here, we identify two distinct MSC-exosome subtypes. The larger exosomes (>80 nm) have a greater flotillin-1 (FLOT1) and CD63 enrichment, while smaller exosomes (<80 nm) have a greater TSG101 and Alix ratio.

reports, we demonstrate a shift in protein markers associated with different exosome subtypes. Specifically, we found that CD63 and flotillin-1 (FLOT1) is associated with "large"-exosomes (>80 nm), while Alix and TSG101 is enriched in "small"-exosomes (<80 nm). Few studies assess the ratio of such markers; however, further investigation is warranted as it may provide a tool for distinguishing exosome subtypes. Ongoing *in vivo* studies are testing whether the different MSC-exosome subtypes exhibit differential therapeutic efficacy in a number of animal models currently utilized by our group and collaborators.

Regulatory frameworks often require a mechanism-of-action that details the identity, quantification, and characterization of such bioactive substances responsible for the therapeutic effect(s). Moreover, disclosure of non-active components ("excipients") in drug preparations should be acknowledged (56, 66). Knowing exosome subtypes harbor different protein and genetic cargo, it is fair to speculate that they likely mediate different effects on targets cells. Thus, improved separation techniques that distinguish between "non-active" and therapeutic exosome subtypes may help focus the search for the bioactive substance(s) responsible for such beneficial effects. The "one size fits all" hypothesis may not work for exosome-based therapeutics. Although a specific exosome subset may induce beneficial therapeutic effects in a specific disease model, it is important to recognize that a different exosome subpopulation may afford the beneficial effects in a different disease model.

EMERGING THEMES IN EXOSOME ISOLATION

Isolation methods impact exosome integrity, *in vivo* biodistribution and metabolic fate (56). Exosome isolation techniques from various biological fluids and cell culture medium have been extensively reviewed (67–69). It is well established that widely applied exosome isolation techniques, such as differential ultracentrifugation (UC), promote vesicle aggregation and often co-isolate soluble factors and protein (70). Thus, a consensus in the field has shifted toward more "*gentle*" isolation techniques to ultimately reduce contaminants (non-EV material), maintain integrity, and isolate "bioactive" vesicles from heterogeneous EV populations. To date, popular avenues of investigation include gradient density isolation and SEC, with the latter being more suited to enclosed tissues culture systems. Variations of such approaches have been shown to effectively separate exosomes from proteins and soluble factors in different biological fluids (71, 72). However, layered density-based procedures may achieve enrichment rather than true exosome isolation, where the influence of UC parameters coupled with high-sucrose concentrations may change the osmotic environment (69). Furthermore, UC methods are impracticable for large-scale bioprocessing.

Several emerging technology platforms have shown promise in isolating exosomes from various sample matrices, with each method exploiting a particular biophysical trait of exosomes such as their size, density, shape, or surface receptors (**Figure 3**) (72).

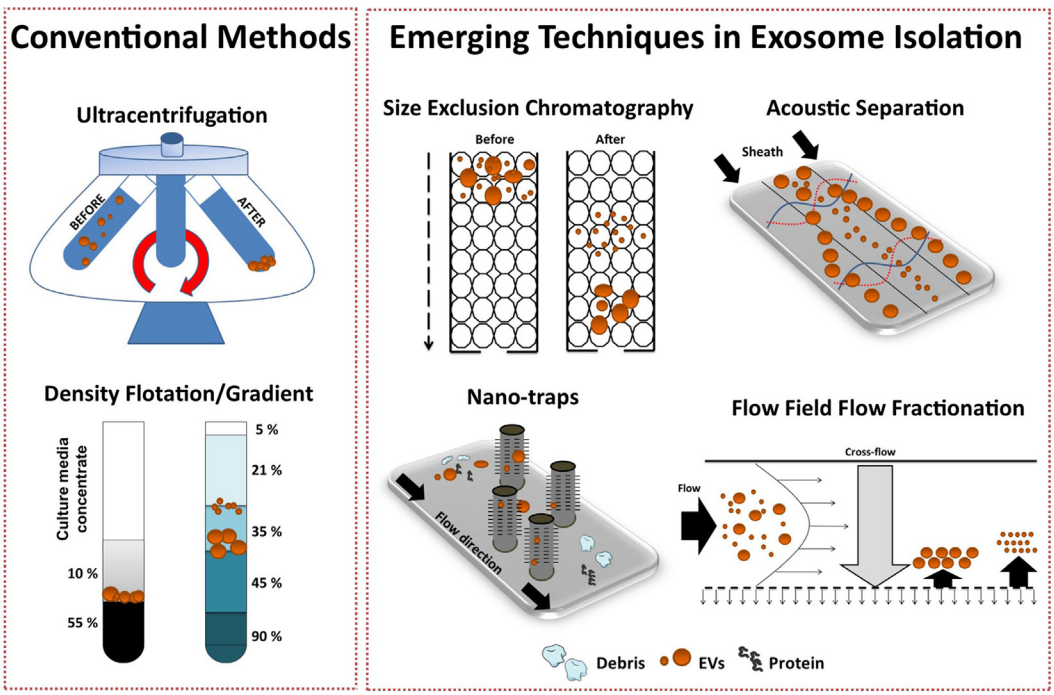

FIGURE 3 | Conventional and emerging exosome isolation techniques. Ultracentrifugation (UC) is the most common exosome isolation method. Here, sedimentation of solutes including vesicles is governed by their size/density. Variations of UC such as layered or cushion based-density gradient UC are also widely employed. New methods are required to facilitate large-scale, high-yield production of exosomes for clinical applications. Several emerging technology platforms have shown promise in isolating exosomes from various sample matrices. Techniques, such as size-exclusion chromatography, ciliated micropillars nano-traps, acoustic wave separation technology, and flow field-flow fraction (F4), exploit unique biophysical traits of exosomes.

The final goal is an isolation method that is label free, distinguishes between exosome subtypes and interfering components, and can facilitate a large-scale production of exosomes.

Recently, Lee and colleagues employed a label-free acoustic nano-filter system to isolate exosomes based on their size (73). Specifically, using ultrasound standing waves exert differential acoustic force, they isolated exosomes from both cell culture media and stored red blood cell products. They effectively separated exosomes (149 nm) and MVs (410 nm). Although, its application for high-throughput exosome preparations is yet to be established, previous studies have used label-free acoustic wave systems to isolate circulating tumor cells (74).

Exosomes can also be separated by size with 10 nm accuracy using variations of flow field-flow fractionation (F4) (75). Asymmetrical F4 (AF4) is a one-phase chromatography method that uses parabolic-flow to drive exosomes across a flow channel. A crossflow intercepts the parabolic-flow perpendicular to the channel and distributes particulate components against the flow chamber wall. Subsequently, exosomes are separated based on differences in diffusivity. Smaller particles diffuse further from the accumulation wall and are eluted earlier than larger ones. Successful attempts have been made using AF4 to isolate exosomes from human neural stem cell culture (76) and melanoma cell lines (77). AF4 approaches provide promise for "label-free" isolation of large-scale exosome production.

To effectively utilize the size difference between exosomes, other EV subtypes and cellular debris, Wang and colleagues fabricated a porous silicon nanowire-on-micropillar "nano-trap" made from ciliated micropillars (78). This fabricated microfluidic device preferentially traps exosomes with a diameter of 40–100 nm, while filtering out proteins, larger EVs, and cellular debris. Moreover, trapped exosomes can be recovered by dissolving the porous silicon nanowires in PBS buffer. However, in this proof-of-concept study, the authors noted poor vesicle retention (~60%) and only assessed small sample volume (30 μl), thus scalability is yet to be demonstrated.

Overall, to support the development of exosome-based therapeutics, research efforts should focus on the development of "label-free" exosome isolation techniques that can support high-throughput systems/scale-up requirements and are capable of distinguishing exosome subtypes. Although a number of highly sophisticated technologies for EV isolation have emerged recently, their application is mainly in the biomarker field, as tools for exosome-based diagnostics. Although such emerging technologies hold great promise, the large-scale preparation of isolated exosome subtypes to be used as the basis of exosome-based pharmaceutical products will probably depend, at least in the near future, on modifications of classic industrial processes such as SEC.

DOSE EVALUATION

Currently, investigators use several different methods to quantify exosome dosage, making inter-study comparison troublesome. Common quantitative practices include reporting cell equivalents, protein concentration, and/or specialized quantitative analytical measurements by instruments, such as tunable resistive pulse sensing (TRPS) and nanoparticle tracking analysis (NTA), with each method harboring its own advantages and limitations [for recent reviews (79–82)]. The need to standardize exosome dosing is imperative. Of interest, methods such as TRPS are currently used to verify particle size and characterization for liposome-encapsulated forms of doxorubicin and are accepted within the definition of bioequivalence as set forth by the FDA and EMA. Although enumeration of analytical criteria is beyond the scope of this review, we acknowledge that the field is limited by current technology and lacks the ability to accurately assess exosomes at a single vesicle level. Thus, to aid inter-study comparison, we recommend that in addition to providing extensive detail of standardized cell culture conditions and pre-analytical protocols, investigators should measure exosome concentration using multiple quantification tools, where possible. A summary of the advantages and limitations of common methods used to determine exosome dose are highlighted in **Table 3**. Establishing an exosome potency assay is a novel approach which holds great promise in standardizing exosome dosing.

DEVELOPING AN EXOSOME POTENCY ASSAY

The definition of the bioactive substance(s) will remain a crucial question in the preclinical development of exosome-based therapeutics. With an orchestra of bioactive cargo and diverse physiological effects (**Figure 1B**), identification of "*one*" bioactive substance or a singular mechanism-of-action appears improbable. By FDA standards, potency is defined as the products specific ability or capacity to affect a given result (66). With no "gold-standard" technique for their quantification, assessment of exosome potency would be a valuable tool in overcoming the inconsistencies in preparations and batch-to-batch variation. For example, exosomes obtained from two separate donors may be normalized *via* a given quantitative method; however, the "*bioactive*" load may differ, subsequently the potency and degree of efficacy will not be the same. Thus, investigators should consider employing a unique exosome potency unit (EPU) to standardize practices and minimize variation between different samples. Presently, attempts to define an exosome potency metric utilized the immunomodulatory properties of MSC-exosomes. For example, Jiao et al. described an *in vitro* potency assay for MSC-exosomes based on the release of IL-10 from mononuclear cells following incubation with exosome preparations, and other studies have shown that T-cell proliferation assays may be modified to provide the basis for assays on exosome immunomodulatory potency (29, 83). Growing evidence also suggests that MSC-exosomes can modulate macrophage phenotypes (8, 35). Macrophages play a pivotal part in regulating immune responses. They assume both phagocytic "defensive" roles and exhibit regulatory "anti-inflammatory" actions, facilitating both the initiation and the resolution of inflammation (84). With this consideration in mind, our ongoing studies are exploring an *in vitro* macrophage polarization assay as a means of assessing MSC-exosome potency. Briefly, the potency assay involves adding MSC-exosomes to murine bone marrow-derived macrophages (BMDMs) that are polarized to the classically activated

TABLE 3 | A summary of the advantages and limitations of common methods used to determine exosome dose.

Exosome dosing method	Information acquired	Advantages	Limitations
Protein	Total protein amount	Fast Low cost	May not reflect bioactive ingredient. May measure non-exosomal-associated protein. Does not reflect particle concentration/size/distribution
Nanoparticle tracking analysis (NTA)	Particle concentration, size, and distribution (range 10 nm–2 μm)	Fluorescent-NTA available Provides absolute EV concentration, size and distribution	Difficulties in determining vesicle aggregates and size heterogeneity in biological samples
Tunable resistive pulse sensing (TRPS)	Particle concentration, size and distribution (size range <40 nm–10 μm)	Provides absolute EV concentration, size and distribution. Can also measure particle surface charge	Different pore sizes are needed to assess biological samples that contain both exosomes (for example, <150 nm) and larger EVs (>150 nm) Can detect non-exosome material within size range
ELISA	Specific concentration of EV marker (for example, CD9 or CD63)	Specific to "exosome" protein	May not reflect bioactive exosome population. Time consuming. Provides non-specific information about exosome size/distribution
Dynamic light scattering (DLS)	Particle concentration and size (range <1 nm–10 μm)	Fast Small volume required	Difficulties in measuring heterogeneous samples
Flow cytometry	Particles concentration and size (size range ~>150 nm)	Non-specialized (typical) laboratory equipment Fluorescently labeled EVs	Detection limit (~<150 nm) *(cytometer dependent)*
Cell equivalents	Cell number	Low cost Fast	Requires standardized tissue culture procedures. Does not reflect particle concentration/size/distribution
Emerging tools for estimating exosome dose	**Description**		
"Fingerprinting assays"	Quantifies surrogate markers (for example levels/ratios of exosome markers such as CD63, CD9, and CD81) as an indication of potency and/or dose		
Exosome potency assays	Quantifies the ability of an exosome preparation to elicit the desired biologic/therapeutic action or surrogate activity *in vitro* and/or *in vivo*		

NTA and DLS size ranges were obtained from http://malvern.com. TRPS detection range was obtained from http://izon.com.

(proinflammatory) M1-phenotype. The functional endpoint is the capacity of MSC-exosomes to suppress the mRNA induction of TNFα. The half maximal effective concentration (EC_{50} value, 50% inhibition in TNFα mRNA levels, relative to M1 control) is transformed to an arbitrary EPU (described in **Figure 4**) (8). In turn, an EPU could potentially be applied to standardize dosing between different exosome preparations. In all cases, potency assays need to be disease specific, fit-for-purpose, and employ relevant functional end-points.

MANUFACTURING AND SCALE-UP

In 2002, Lamparski et al. described a method for the production and characterization of clinical grade exosomes derived from dendritic cells for their application in cancer vaccine clinical trials (85). Using ultrafiltration coupled with a sucrose/deuterium oxide UC cushion, they isolated vesicles (50–90 nm in diameter) containing major histocompatibility complex (MHC) class-I, -II, and CD1, and tetraspanin molecules (CD9, CD63, and CD81) In addition, in 2005, Navabi and coworkers detailed the development of a method for the preparation and characterization of good manufacturing practice (GMP)-grade exosomes from the ascites fluid of ovarian cancer patients (86). Since then our understanding of exosome biology has improved and the development of specialized isolation and characterization methods has allowed investigators to more accurately isolate and characterize exosome populations.

Considering the development of exosome-based therapeutics, lessons could be learned from cell therapy. The *in vitro* expansion of cells (such as MSCs) is required to deliver an effective therapeutic dose, with the absence of having a detrimental impact on the quality of the cell. Upon scaling-up, process analytical technology (PAT), a system proposed by the FDA, may be implemented to monitor the manufacturing process through continuous measurement of cell parameters (87, 88). Monitoring bioprocess parameters, such as population doubling time, temperature, metabolite concentrations, pH, pO_2 and pCO_2, may help ensure optimal exosome quality and quantity, as previous reports have shown that subtle acidic pH shifts may impair exosome aggregation inhibiting forces and, in turn, promote aggregation and reduce functionality (56).

Recently, the inevitable shift to using tissue culture bioreactors has been used to generate large-scale EV preparations. Indeed, Watson and colleagues demonstrated that hollow-fiber bioreactors promotes enhanced exosome production (~40-fold greater EVs/ml of CM) when compared to conventional 2D tissue culture preparations (89). However, it remains unclear if such methods enhance generalized EV production or simply reflect a reduction

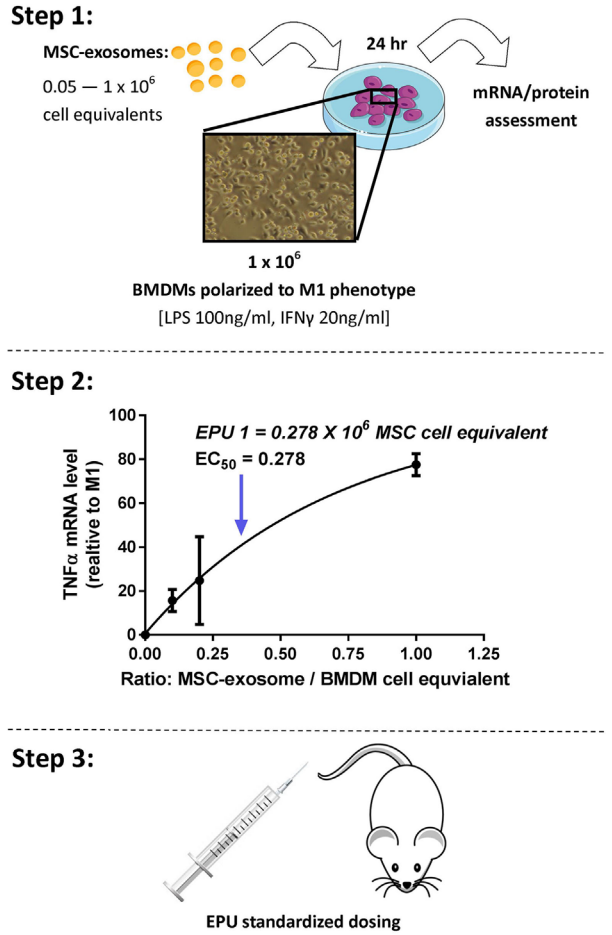

FIGURE 4 | Stepwise approach to developing an MSC-exosome potency assay. Considering limitations in current exosome quantification techniques, assessment of exosome potency would be a valuable tool to standardize exosome dosing. Step 1: primary murine bone marrow-derived macrophages (BMDMs) were obtained by flushing the femur and tibia of 6- to 8-week-old FVB mice. M1 polarization was initiated by lipopolysaccharide (LPS) 100 ng/ml and interferon-γ (IFNγ) 20 ng/ml stimulation. Macrophage polarization (1×10^6 BMDMs) was initiated with/without the presence of MSC-exosome preparations (0.05–1×10^6 cell equivalents), providing several ratios of MSC-exosome cell equivalents-to-BMDMs. After 24 h, total RNA was isolated and TNFα mRNA levels were assessed by RT-qPCR. The functional endpoint is the capacity of MSC-exosomes to suppress the mRNA induction of TNFα. Step 2: an EC_{50} value (50% reduction in TNFα mRNA, relative to M1 control) is transformed to an arbitrary exosome potency unit (EPU). Data were adapted from our recent work (8), where we demonstrated purified human umbilical cord MSC-exosomes, dose dependently suppressed mRNA levels of TNFα in alveolar macrophages *in vitro*. Step 3: an EPU can be applied to standardize dosing between different exosome preparations or as a means of correlating potency to exosome quantity.

in exosome re-uptake. Under the considerations discussed above, relating to EV diversity and the possibility that only specific exosome subtypes may represent the therapeutic agent, it is premature to assume that production of higher EV numbers will necessarily yield a higher efficacy final product. Optimization of MSC culture conditions will, therefore, require the parallel development of a dependable and easily adoptable potency assay.

"OFF-THE-SHELF" EXOSOMES

With evidence to suggest that exosomes can be stored at −20°C for up to 6 months with no loss to their biochemical activity, "off-the-shelf" exosome-based products represent an attractive pharmaceutical formulation (56, 66, 90). Although standardized storage procedures remain to be defined, current storage protocols use isotonic buffers to prevent pH shifts during storage, avoid freeze–thaw cycles and are absent of dimethyl sulfoxide (DMSO) and glycerol as previous reports have shown these agents may impact exosome integrity (91). With a lack of data addressing the impact of storage time and excipients on exosome structural stability and functional efficacy, more studies are warranted to help define a provisional "shelf-life" for exosome-based products and facilitate the manufacturing and distribution process.

EV-BASED THERAPY IN CLINICAL TRIALS

Promising preclinical data that demonstrated dendritic cell-derived EVs containing MHC–peptide complexes could alter tumor growth in immune competent mice led to a phase I anti-melanoma clinical trial conducted in France (92) and a phase I anti-non-small cell lung cancer clinical trial in the United States (93) (clinical trial applications highlighted in **Table 4**). Both clinical trials administered autologous dendritic cell-derived EVs that met their respective current GMP standards. Such clinical trials to date are important for their demonstration of both the feasibility and the short-term safety of autologous EV administration, but safety considerations for therapies based on exogenous exosome-based products will arguably be more stringent. Nevertheless, it is very encouraging to note that preclinical studies have established immunomodulation as the main therapeutic mechanism of MSC-exosomes action. Immunomodulation is clearly involved in the autologous exosome clinical trials mentioned above, and this may provide guidelines and precedent for clinical trials using exogenous exosomes. In this context, a recent clinical case involving treatment of a steroid-refractory graft-vs-host disease patient with MSC-EVs derived from unrelated bone marrow donors produced encouraging results (94).

Ultimately, issues raised in this review aim to provide a basic guidance for investigators on key issues to consider for the smooth transition of exosome-based therapies from the preclinical model into clinical development (**Figure 5**). Among them, determining the optimal dose, the appropriate time window for exosome administration, the number of doses, and route of administration that achieves maximal efficacy without adverse effects are the most important issues to resolve. Such issues will be disease/model specific and clearly beyond the scope of this work.

SUMMARY

Exosome-based therapeutics represent a most promising next generation approach for treating a diverse number of diseases, particularly diseases the pathogenesis of which involves a primary (or major) inflammatory component. The efficacy of MSC-exosome treatments has been robustly established in numerous preclinical models, but development of large-scale GMP-grade

TABLE 4 | Exosome-based therapy: clinical trials.

Disease	Phase	Vesicle cellular source	Route of administration	Isolation method	Modified (Y/N)	Status	Reference
Melanoma	I—open label	Autologous monocyte-derived dendritic cells	SC	UF/UC sucrose cushion	Y	Complete	Escudier et al. (92)
Non-small cell lung cancer	I—open label	Autologous monocyte-derived dendritic cells	SC and intradermal	UF/UC sucrose cushion	Y	Complete	Morse et al. (93)
Colon cancer	I—open label	Autologous ascites	SC	UC sucrose cushion	N	Complete	Dai et al. (95)
Colon cancer	I—open label	Plant based	–	Not declared	Y	Ongoing	NCT01294072
Type I diabetes	I—open label	Umbilical cord blood (allogeneic) MSC	–	Not declared	N	Ongoing	NCT02138331
Non-small cell lung cancer	II—open label	Tumor cell	Pleural or peritoneal cavity	Not declared	Y	Complete	Besse et al. (96)
Wound healing (Ulcer)	I—open label	Plasma (autologous)	–	Not declared	N	Enrolling	NCT02565264

MSC, mesenchymal stem/stromal cells; SC, subcutaneous; UF, ultrafiltration; UC, ultracentrifugation.
Adapted from Lener et al. (56).

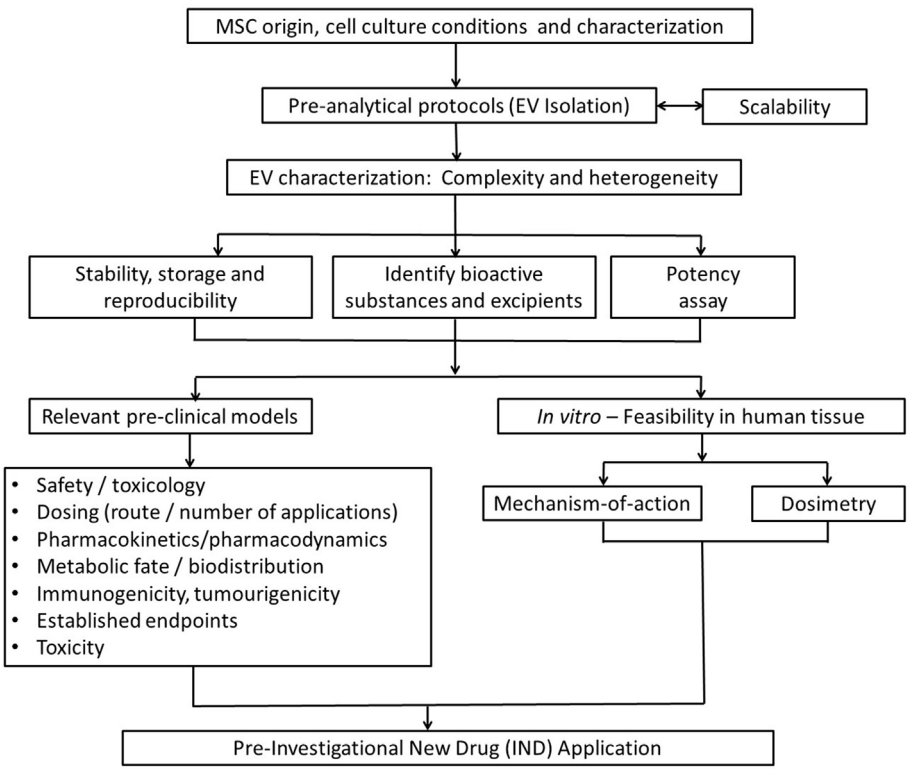

FIGURE 5 | Strategic flowchart for the preclinical testing of exosome-based therapeutics. EV, extracellular vesicle. Adapted from Ref. (97).

exosome-based pharmaceuticals and subsequent clinical trials demand the resolution of several technological and mechanistic issues, reflecting the cautious navigation in unknown seas for this relatively novel field. Among the major issues to be resolved are the definition of an EPU, the standardization of MSC culture conditions and protocols for exosome harvest and storage. Although safety considerations need also to be addressed, it is expected that safety concerns for cell-free, exosome-based clinical trials will be arguably milder than those relevant to live cell MSC trials currently in progress, as mutagenicity and oncogenicity concerns will be null.

AUTHOR CONTRIBUTIONS

GW participated in data collection, analysis, and manuscript writing. SK and SAM contributed to final article editing and approval.

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A Protocol for Improved Precision and Increased Confidence in Nanoparticle Tracking Analysis Concentration Measurements between 50 and 120 nm in Biological Fluids

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Nanoparticle tracking analysis (NTA) can be used to quantitate extracellular vesicles (EVs) in biological samples and is widely considered a useful diagnostic tool to detect disease. However, accurately profiling EVs can be challenging due to their small size and heterogeneity. Here, we aimed to provide a protocol to facilitate high-precision particle quantitation by NTA in plasma, the supernatant of activated purified platelets [the platelet releasate (PR)] and in serum, to increase confidence in NTA particle enumeration. The overall variance and the precision of NTA measurements were quantified by root mean square error and relative standard error. Using a bootstrapping approach, we found that increasing video replicates from 5 s × 60 s to 25 s × 60 s captures led to a reduction in overall variance and a reproducible increase in the precision of NTA particle-concentration quantitation for all three biofluids. We then validated our approach in an extended cohort of 32 healthy donors. Our results indicate that for vesicles sized between 50 and 120 nm, the precision of routine NTA measurements in serum, plasma, and PR can be significantly improved by increasing the number of video replicates captured. Our protocol provides a common platform to statistical compare particle size distribution profiles in the exosomal-vesicle size range across a variety of biofluids and in both healthy donor and patient groups.

Keywords: nanoparticle tracking analysis, extracellular vesicles, plasma, serum, platelets, platelet releasate, particle enumeration

Abbreviations: EVs, extracellular vesicles; FTLA, finite track length adjustment; NTA, nanoparticle tracking analysis; PR, platelet releasate; RMSE, root mean square error; RSE, relative standard error.

INTRODUCTION

Extracellular vesicles (EVs) are released by nearly all eukaryotic cells and are found in a diverse range of human biofluids. They regulate a diverse range of biologic and inflammatory pathologic processes and have been implicated in myriad of diseases (1–3). As such, they have emerged as a promising source of future biomarkers in biofluids with both diagnostic and prognostic value (4, 5). However, accurately profiling EVs can be challenging due to their small size and heterogeneity (6). While important advances have been made (5), optimization of procedures for EV quantification across laboratories is of great significance to the EV field with implications for both basic and clinical research.

Nanoparticle tracking analysis (NTA) is widely used to determine the particle size distribution of a sample (6–9). Particle size distribution describes the concentration of particles as a function of size (10). In brief, particle size is determined by focusing a laser beam through a suspension of particles. The light scattered by individual particles in solution allows visualization of particles and each individual particles' displacement is recorded over disjointed time intervals (8, 11, 12). The mean square displacement for each particle is then used, alongside liquid temperature and viscosity, to calculate individual particle sizes using the Stokes-Einstein equation. The concentration of particles is determined by counting total particles and representing the concentration as a binned histogram (13).

The validity of particle size distributions for a sample depends on accurate sizing of particles as well as precise concentration measurements. Accuracy is generally within 5% of the expected particle size once correct hardware and software setting have been applied (7, 14, 15). However, NTA concentration measurements have been shown to have low precision, due to variation in the number of particles detected between video replicate measurements of the same sample (12). For low particle counts, it has been suggested that increasing video replicates could lead to improved concentration measurements (7).

Here, we sought to determine the effect of increasing video replicates on the precision of particle concentration quantitation using NTA in the biofluids of plasma, serum, and the supernatant of activated purified platelets [the platelet releasate (PR)]. Although plasma and serum are widely used in clinical diagnoses (2, 16, 17), the PR is an important biofluid to also include as contains a variety of EVs (18–20) and its contents play a fundamental role in hemostasis, wound healing, and the inflammatory response (21–25). In our analysis, we initially used a bootstrapping approach to investigate the precision of particle concentration measurements in all three biofluids. We found that increasing video replicates led to a reproducible increase in the precision of NTA particle concentration quantitation in these biofluids and we validated our findings in an extended clinical cohort.

MATERIALS AND METHODS

Plasma, PR, and Serum Isolation

Human plasma, serum, and platelets were obtained from healthy adult volunteers in accordance with approved guidelines from the UCD research, and with ethical approval from St. Vincent's University Hospital and the Rotunda Hospital Research Ethics committees. All subjects gave their informed written consent according to the declaration of Helsinki.

Isolation of platelet-free plasma and PR was as described (19). Briefly, 44 ml of blood was drawn into acid citrate dextrose blood collection tubes (BD, Franklin Lakes, NJ, USA) and the first 4 ml was discarded. Blood was centrifuged at 150 × *g* for 10 min at room temperature and platelet-rich plasma was aspirated. Platelets were pelleted from platelet rich plasma by centrifugation at 720 × *g* for 10 min at room temperature and platelet poor plasma was aspirated. Platelets were suspended in a modified Tyrode's buffer (130 mM NaCl, 10 mM trisodium citrate, 9 mM $NaHCO_3$, 6 mM dextrose, 0.9 mM $MgCl_2$, 0.81 mM KH_2PO_4, 10 mM Tris pH 7.4). Platelet count was adjusted to 1×10^9/ml and platelets were stimulated with 1 U thrombin/ml at 37°C in a PAP-4 aggregometer under constant stirring. The supernatant from the thrombin activated platelets was centrifuged three times at 10,000 × *g* for 10 min at 4°C to remove the aggregated platelets and cell debris, leaving the activated platelet supernatant, or PR.

Serum was prepared by drawing 4 ml whole blood into serum blood collection tubes (BD, Franklin Lakes, NJ, USA) and processed according to manufacturer's instructions. In brief, samples were rested upright for 60 min to allow RBCs to clot. The RBC clot was subsequently pelleted by centrifugation at 1,300 × *g* for 10 min and serum was aspirated. All plasma, serum, and PR samples were stored at −80°C prior to NTA analysis.

NTA of Biological Samples

Particle size distribution in PR, plasma, and serum samples was determined by NTA using a NanoSight NS300 system (Malvern Technologies, Malvern, UK) configured with a 488 nm laser and a high sensitivity scientific CMOS camera. Samples were diluted (PR 1:20–1:50, plasma 1:100–1:2,000, serum 1:500) in particle-free PBS (Gibco, Waltham, MA, USA) to an acceptable concentration, according to the manufacturers recommendations. Samples were analyzed under constant flow conditions (flow rate = 50) at 25°C according to Ref. (7, 26). For bootstrapped samples, 30 s × 60 s successive videos were captured with a camera level of 16. Data were analyzed using NTA 3.1.54 software with a

TABLE 1 | Overall variance of nanoparticle tracking analysis measurements shows an exponential decay in variance as *n* number bootstrapped video replicates increase.

	Root mean square error (RMSE) represented as % of *n* = 5 video replicates (±SD)		
***n* number**	**Plasma**	**Platelet releasate**	**Serum**
5	100	100	100
10	70.4 ± 4.27	70.4 ± 1.88	70.2 ± 5.33
15	57.6 ± 3.81	58.0 ± 3.58	58.0 ± 4.93
20	50.5 ± 4.24	49.9 ± 3.26	51.4 ± 3.98
25	44.5 ± 3.32	45.2 ± 2.28	45.4 ± 2.82

Average RMSE (±SD) for each plasma, PR and serum is represented as a percentage of RMSE at 5 min. A consistent % reduction in RMSE observed for each sample with increased n bootstrapped samples.

detection threshold of 5. For the validation cohort, 15 s × 60 s videos were captured with a camera level of 16 and a detection threshold of 10.

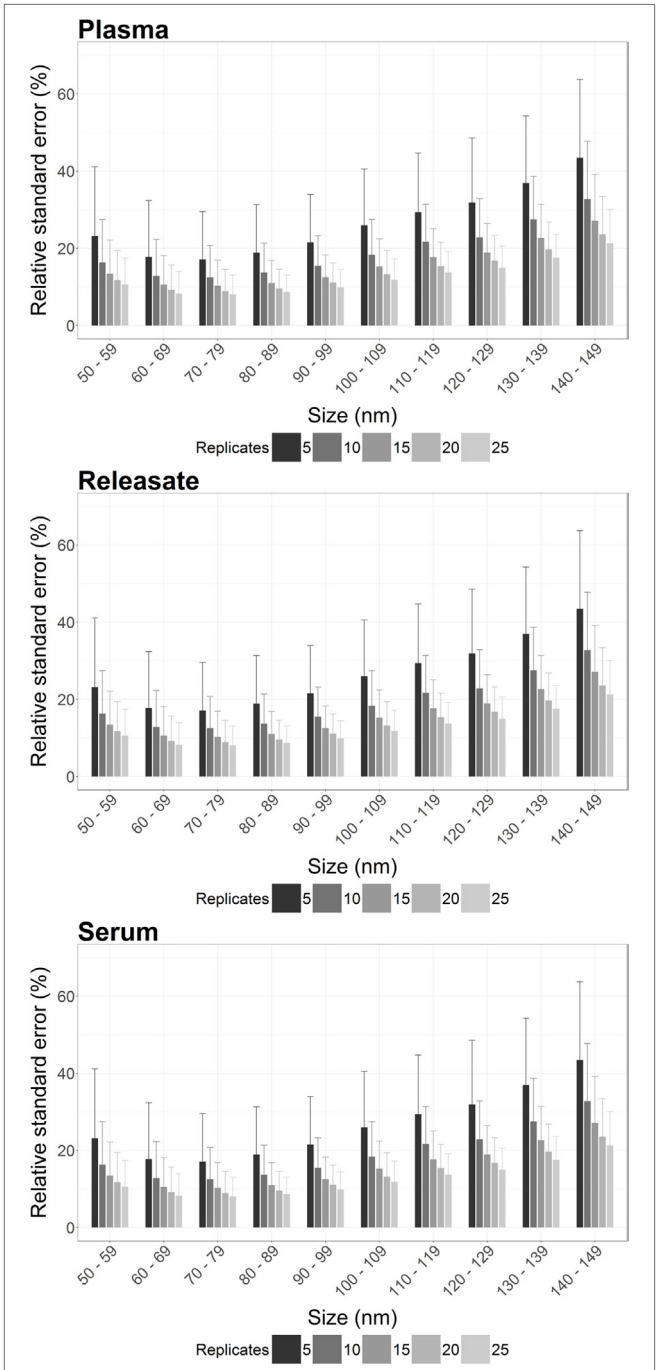

FIGURE 1 | The precision of nanoparticle tracking analysis (NTA) concentration measurements is enhanced by increasing the number of video replicates in all bin widths. For plasma, platelet releasate and serum the average relative standard error (RSE) per bin width from a total of 900 bootstrapped samples was reproducibly decreased by increasing *n* video replicates. As RSE is a measure of the statistical precision of a sample measurement, the reduction in RSE with increased video replicates indicated that more video replicates led to increased precision of NTA measurements.

Statistical Analysis of Nanoparticle Analysis Tracking Data

Particle size distributions were binned into 10 nm bin widths using NTA 3.1.54 software for all video replicates to determine concentration measurements. To understand the variability in the estimates across the complete range of bin widths, we quantified the root mean square error (RMSE) of 100 bootstrap samples of $n = 5$, 10, 15, 20, and 25 NTA recordings, which were sampled from the available 30 concentration measurements. The RMSE is the error sum of squares scaled to the data from which it was derived and was calculated as follows:

$$\mathrm{RSME}_m = \sqrt{\frac{\sum_{i=1}^{N_{\mathrm{bins}}} \sum_{j=1}^{N_{\mathrm{boot}}} \left(x_{ij} - \overline{x}_i\right)^2}{N_{\mathrm{bin}}\left(N_{\mathrm{boot}} - 1\right)}}$$

where N_{bins} is the number of bins considered, N_{boot} is the number of bootstrap samples taken for each bin, x_{ij} is the j^{th} bootstrap mean for bin i and $\overline{x}_i$ is the mean of the bootstrap means for bin i.

Next, to determine the precision of the concentration estimates, we quantified the relative standard error (RSE) as follows:

$$\mathrm{RSE}_n = \frac{\frac{s}{\sqrt{n}}}{\overline{c}_n} \times 100 = \frac{s}{\overline{c}_n \sqrt{n}} \times 100$$

where s is the SD of measurements, $\overline{c}_n$ is the sample mean concentration estimate, and n is the number of samples. Precision of our clinical cohort was quantified by RSE where $n = 5$ or 15 video replicates. Initially, 100 bootstrap samples were sampled from the available 30 concentration measurements and the RSE was calculated for each bin width for n = 5, 10, 15, 20, and 25 video replicates. In our validation cohort, RSE was calculated from $n = 5$ and $n = 15$ video replicates for each bin width.

Statistical Analysis

All bootstrapping, RMSE, and RSE calculations were performed using the open source programming language for statistical computing R 3.3.1 (27) and the integrated development environment RStudio 1.0.136 (28).

TABLE 2 | The precision of nanoparticle tracking analysis concentration measurement at 100–110 nm is increased by increasing the number of video replicates.

	Plasma	Platelet releasate	Serum
***n* number**	**Maximum relative standard error (RSE) (%)**	**Maximum RSE (%)**	**Maximum RSE (%)**
5	95.3	49.2	63.1
10	52.8	25.8	26.6
15	41.0	21.2	20.2
20	31.7	15.5	17.4
25	27.6	14.7	14.2

Focusing on particles from 100 to 110 nm, the maximum RSE is significantly decreased by increasing video replicates.

RESULTS

Reduction in the Overall Variance of NTA Measurement by Increasing Video Replicates

Using a bootstrapping approach, we examined the effect of increasing video replicates on the total variance of NTA measurement of particles in plasma, PR, and serum. RMSE, a measure of total variance, was calculated to show the variability of measurement at each time point. Plasma, PR, and serum were isolated from three healthy donors and subjected to analysis by NTA. 30 s × 60s consecutive videos were captured for each sample. All experiments were performed in triplicate. RMSE was calculated for 100 bootstrap samples of $n = 5$, 10, 15, 20, and 25 video replicates. RMSE was normalized to $n = 5$ video replicates with $n = 5$ representing 100% variance. For plasma, PR, and serum increasing video replicates led to a decrease in total variance across all samples (**Table 1**). On average, doubling the number of video replicates from $n = 5$ to $n = 10$ led to a ~ 30% reduction in the

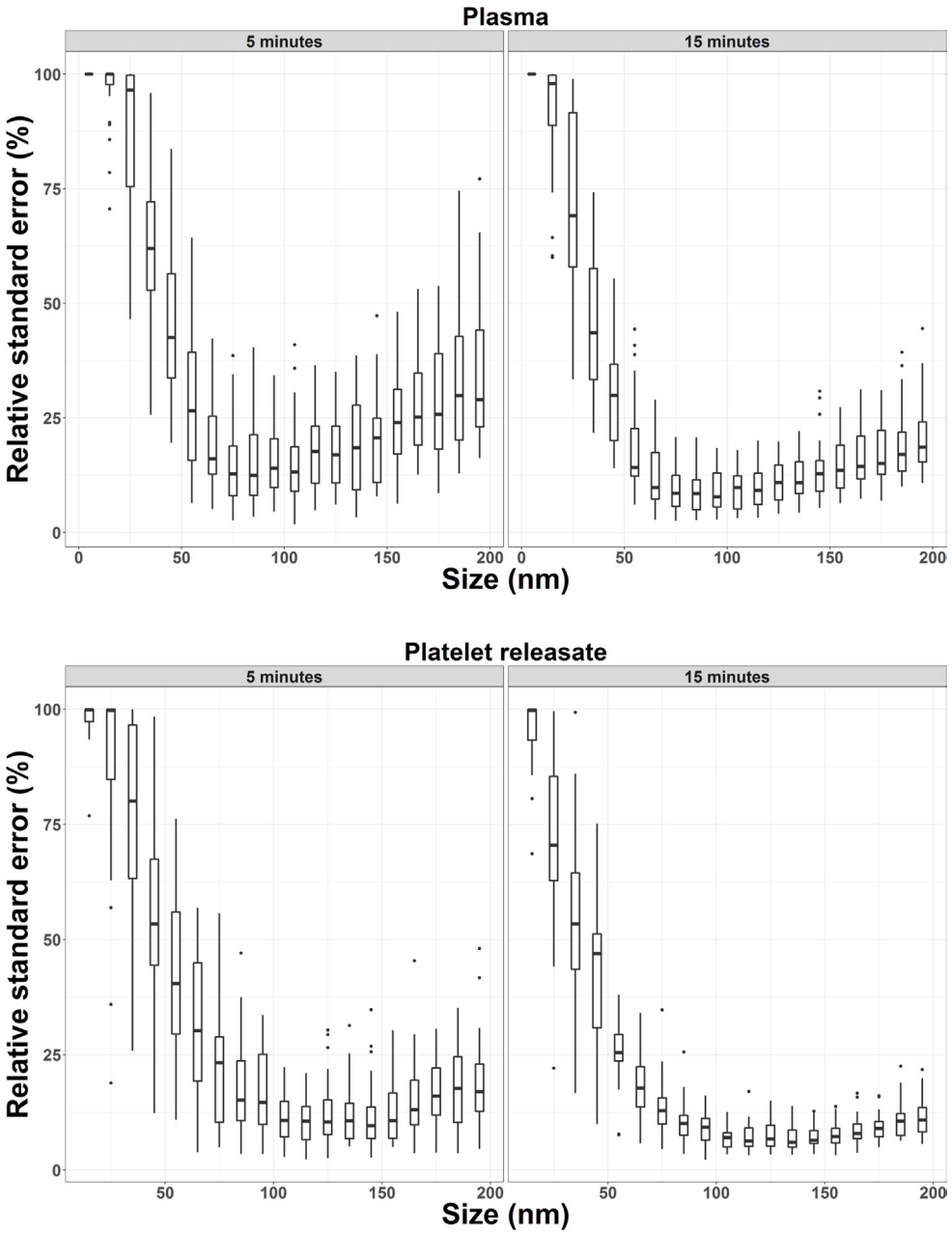

FIGURE 2 | Precision of nanoparticle tracking analysis concentration measurements from 32 healthy donors is increased by increasing the number of video replicates in all bin widths. For plasma and platelet releasate, the average relative standard error per bin width from 32 healthy donors was reproducibly reduced by increasing video replicates from $n = 5$ to $n = 15$.

initial variance, with further reductions in total variance reproducibly replicated in all samples with increasing video captures.

Precision of NTA Concentration Measurements in Individual Bin Widths Is Improved with Increasing the Number of Video Captures

Individual RSE was calculated per 10 nm bin width for 100 bootstrap samples from plasma, PR, and serum, for $n = 5$, 10, 15, 20, and 25 video replicates. A reduction in RSE was reproducibly observed across all bin widths for plasma, PR, and serum with increasing video replicates (**Figure 1**; Table S1 in Supplementary Material).

However, also of interest is the maximum RSE across all bootstrapped samples. Focusing on particles from 100 to 110 nm, the maximum RSE was found to be excessively high for $n = 5$ replicates only, with a maximum RSE for plasma of 95.3, 49.2% for PR, and 63.1% for serum (**Table 2**). However, increasing n video replicates had a reproducible significant reduction in maximum RSE. For example, for $n = 10$ video replicates, maximum RSE for plasma was 52.8, 25.8% for PR, and 26.6% for serum. Further reductions in maximum RSE were evident with increased n video replicates (**Table 2**). This pattern was reproduced for all bin widths. The maximal RSE for all bin widths and samples can be found in Table S1 in Supplementary Material. As RSE is a measure of the precision of concentration measurements by NTA, an overall reduction in maximal RSE indicates that increasing video replicates leads to a reproducible increase in the precision of particle concentration measurements by NTA.

Validation of Increased Precision of NTA Concentration Measurements in a Larger Cohort of 32 Donors

Next, we wished to determine if the increase in precision of concentration quantitation observed in our bootstrapped samples could be replicated in a larger cohort ($n = 32$). 15 s × 60s NTA video replicates were captured for PR and plasma from 32 healthy donors. The first five videos were analyzed to determine the RSE of $n = 5$ video replicates for every 10 nm bin width for each donor. These five, plus an additional 10 videos were then analyzed to determine the RSE of $n = 15$ video replicates. By increasing the video replicates from 5 to 15, the precision of NTA concentration quantitation was improved (**Figure 2**; **Table 3**). The maximum RSE in plasma was reduced from 64.37 to 44.34%, while the maximum RSE in PR was reduced from 76.23 to 38.01%. In accordance with the findings from our bootstrapped samples, the precision was most improved in the bin centers corresponding to vesicles of 50–120 nm in size. For example, taking the bin center from 100 to 110 nm for illustrative purposes, the maximum RSE was found to be high with $n = 5$ replicates with maximum RSE for plasma of 41.0%, and 22.3% for PR (**Table 4**). Increasing n video replicates had a reproducible significant effect on maximum RSE. When video replicates were to $n = 15$, maximum RSE was 18.0 and 12.6% for plasma and PR, respectively, indicating a greatly improved precision of NTA concentration measurements (**Table 4**). In a similar manner, the maximum error was also calculated for each bin width across all samples (Table S2 in Supplementary Material).

TABLE 3 | Increased precision of nanoparticle tracking analysis concentration measurements in a larger cohort of 32 healthy donors of particles from 50 to 120 nm.

	Plasma	Platelet releasate
***n* number**	**Maximum relative standard error (RSE) (%)**	**Maximum RSE (%)**
5	64.37	76.23
15	44.34	38.01

TABLE 4 | Precision of nanoparticle tracking analysis concentration measurements is increased in particles from 100 to 110 nm by increasing the number of video replicates in a cohort of 32 healthy donors.

		Plasma		Platelet releasate	
***n* number**	**Size (nm)**	**Average relative standard error (RSE) (%)**	**Maximum RSE (%)**	**Average RSE (%)**	**Maximum RSE (%)**
5	100–110	15.6 ± 15.6	41.0	11.5 ± 11.5	22.3
15	100–110	9.3 ± 9.3	18.0	6.8 ± 6.8	12.6

For particles from 100 to 110 nm, average RSE (±SD) is decreased by increasing the number of video replicates. Maximum RSE is also significantly decreased by increasing the number video replicates.

DISCUSSION

Here, we sought to determine the effect of increasing video replicates on the precision of EV concentration quantitation using NTA. Initially, we used a bootstrapping approach to investigate the precision of particle concentration measurements in plasma, the PR, and in serum. Our results indicated that the precision of routine NTA measurements can be significantly improved in the particle size range of 50–120 nm for all biofluids analyzed and this held true even as we extended our analysis to plasma and PR in a larger cohort of 32 donors.

As the NTA method is the current gold standard to measure the size and concentration of small particles in biological samples and has the potential to be a useful diagnostic tool to detect disease (8, 29), our protocol has much relevance to the field. In fact, with NTA-acquisition settings constant between analyses, our approach will enable the mean, mode, and median particle size together with EV concentration to be more precisely compared between differential samples. In this way, we conclude that we now provide a common platform to statistically compare particle size distribution profiles from the plasma and PR of patients with a variety of pathologies.

ETHICS STATEMENT

Human plasma, serum and platelets were obtained from healthy adult volunteers in accordance with approved guidelines from the UCD research ethics committee, and with ethical approval

from St Vincent's University Hospital and the Rotunda Hospital Research Ethics committees. All subjects gave their informed written consent according to the declaration of Helsinki.

AUTHOR CONTRIBUTIONS

MP, DM, PS, MG, AM, and PM designed research; MP, DM, PS, MG, KO, HO, CM, FÁ, AM, and PM performed experiments; MP, DM, PS, MG, AM, and PM analyzed data; MP, DM, PS, MG, KO, HO, CM, FÁ, AM, and PM wrote the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/article/10.3389/fcvm.2017.00068/full#supplementary-material.

TABLE S1 | Relative standard error per bin width from bootstrap samples. 30 x 60 second consecutive videos were captured for each sample, plasma, platelet releasate (PR), and serum from 3 donors in triplicate. RSE was calculated for 100 bootstrap samples of n = 5, 10, 15, 20, and 25 video replicates. Average RSE per n video replicates for each bin size was calculated for all 900 bootstrapped samples. A reduction in RSE was reproducibly observed across all bin widths for plasma, platelet releasate (PR) and serum with increasing video replicates.

TABLE S2 | Relative standard error per bin with from 32 donor validation. 15 x 60 second consecutive videos were captured for each sample, plasma and platelet releasate (PR) for 32 healthy donors. The first five videos were analysed to determine the RSE of n = 5 video replicates for every 10 nm bin width for each donor. These five, plus an additional 10 videos were then analysed to determine the RSE of n = 15 video replicates. The average RSE for each bin size for 5 and 15 minutes shows a reduction for each bin size. The maximum RSE for was reduced for each bin size for both biological samples.

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REVIEW
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Extracellular Vesicles as Biomarkers in Cardiovascular Disease; Chances and Risks

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The field of extracellular vesicles (EV) is rapidly expanding, also within cardiovascular diseases. Besides their exciting roles in cell-to-cell communication, EV have the potential to serve as excellent biomarkers, since their counts, content, and origin might provide useful information about the pathophysiology of cardiovascular disorders. Various studies have already indicated associations of EV counts and content with cardiovascular diseases. However, EV research is complicated by several factors, most notably the small size of EV. In this review, the advantages and drawbacks of EV-related methods and applications as biomarkers are highlighted.

Keywords: microparticle, atherosclerosis, vascular remodeling, diagnostic, platelet, endothelial cell, monocyte

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INTRODUCTION

After having been disregarded for decades, extracellular vesicles (EV) are now in sharp focus as mediators of cell-to-cell communication and their importance is currently being investigated for many diseases. Progress is quick, particularly in the field of tumor biology, but also in other areas e.g., cardiovascular diseases, EV-related findings are gathering strong interest (1–3). Being identified as mere cellular "dust" in the 1960s, it has become clear that EV are much more than that. Extracellular vesicles are derived from parent cells and tissues and can be classified into roughly 3 classes: (I) microvesicles that originate from budding of the cell membrane, (II) exosomes, that have endosomal/intracellular organelle origin and (III) apoptotic bodies, that are generated during programmed cell death. However, there appears to be quite some overlap between those classifications, in a sense that microvesicles can be in the size range of exosomes or *vice versa* and/or carry supposed endosomal markers (4). All body fluids have been found to contain EV, hinting toward their abundance and their possible physiologic roles. In addition, given the specific cellular origin of the EV, they may contain interesting information, reflecting cellular functions or health status and thus ultimately revealing physiologic or pathophysiologic disease states. This would make EV excellent biomarkers. Whereas most biomolecular biomarkers (e.g., circulating proteins) do not contain information about the original cellular and tissue context, such information is often contained in EV, in the form of a palette of cell-specific surface markers and corresponding membrane-enclosed EV content. A sole determination of the cellular origin of EV e.g., in plasma could provide information about the nature, severity and prognosis of a particular disorder. A further perspective is offered by the analysis of the content of EV from patient specimen, since proteins or nucleic acids within EV can yield clues about the pathophysiologic mechanisms underlying the disease. The range of cardiovascular pathologies in which EV are suspected to play a role is very wide and still expanding. However, as the field of EV is still developing, the methods for optimal analysis of EV determination, purification, and analysis are heavily debated. As established

biomarkers such as circulating proteins have validated methods (e.g., ELISA) and clearly defined pre-analytical variables (e.g., concerning sample preparation), these are much less clearly defined in the field of EV. In this overview, the current chances and risks of the use of EV as biomarkers will be discussed.

ISOLATION AND MEASUREMENT OF EV—ARE WE GETTING CLOSER TO A GOLD STANDARD?

Isolation of EV

At first sight, the isolation of EV from biologic fluids appears rather straightforward. In theory, EV can be isolated to purity solely based on their physicochemical properties, because they are larger in size than the protein fraction yet smaller than whole cells, more dense than the lipid fraction, with a rather defined density range and quite robust due to their membrane encapsulation. In addition to these physical properties, EV possess a palette of surface markers specific for the parent cell type. Thus, there are many possible approaches for EV isolation (summarized in **Table 1**). The most widely used method remains centrifugation in its variants density gradient, differential and ultracentrifugation. The small size of EV is exploited in a number of sequential centrifugation steps, starting at low speed (300 g) to remove any intact cells and continuing at higher speeds to obtain fractions enriched in microvesicles (20,000 g) and exosomes (100,000 g). The sedimentation behavior of EV can be modified by using density gradients, allowing separation from proteins and other components. Although processing times can be quite long, a clear advantage of centrifugation is the possibility to process larger volumes, such as collected cell culture supernatants. The use of centrifugation is somewhat losing its popularity since the report of (lipo)protein and/or RNA-protein complex contaminations and loss of EV integrity after pelleting (5, 6).

Gaining ground is size-exclusion chromatography (SEC), which is a quick and straightforward method for removing proteins and other contaminations from EV-containing fluids. Columns for SEC can be ordered commercially or easily cast in the lab, using common 10–50 mL syringes, SEC-medium and fine gauze to retain the gel in the column. Depending on the column length (and diameter), they can handle volumes in the mL range, although the use of a large column inherently leads to dilution of the EV-containing eluate. There is still some debate whether SEC-purified EV are free of (lipo)protein contaminations (7, 8) and it is recommended to perform appropriate controls. Conventional and ultrafiltration has also been used successfully to isolate EV (9). The principle is similar to SEC, as EV are separated based on their size properties. In a recent study, the use of a set of sequential filters with different pore sizes resulted in preparations of pure and size-defined EV (10). Although the filter system described in this study is custom made and thus not commercially available (as many published experimental setups), the approach appears to have potential to become a standard method for EV isolation.

TABLE 1 | Overview of current isolation methods of EV from plasma.

Isolation method	Principle	Advantages (A)/Drawbacks (D)
Differential centrifugation	Sedimentation and/or density	A: Large volumes can be processed A: Widely used and facile method D: Risk of contaminations with plasma proteins D: Risk of EV aggregation/loss of integrity D: Quality depends on rotor type
Size exclusion chromatography	Size (largest elute first)	A: Well-accepted and facile method A: Good separation/recovery of EV A: Preserves integrity of EV D: Sample volumes small to medium D: Does not discriminate between EV origins D. results in sample dilution
Filters	Size	A: Processing of large volumes possible A: Allows size fractionation of EV D: Does not discriminate between EV origins D. risk of EV fragmentation
Microfluidics	Physical behavior of EV (size)	A: High sample recovery A: Suitable for small sample volumes A: Maintains EV integrity and properties D: Low sample throughput D: Not appropriate for large volumes D: Need of equipment for flow cell construction
ExoQuick™	Precipitation using polyethylene glycol	A: Quick and straightforward sample handling A: Can be scaled up for larger samples A: Amenable to larger sample numbers D: Risk for contaminations with plasma proteins
Magnetic beads/affinity chromatography	Immuno-affinity by surface markers	A: Discriminates between EV origins A: Less contamination with plasma proteins A: Amenable to larger sample numbers D: Does not discriminate between EV sizes D: Often needs highly specific antibodies
Fluorescence-activated cell sorting (FACS)	Light scattering, fluorescence	A: Size- and surface marker-based sorting D: Long processing times D: Costly equipment

Considering the increasing interest in microfluidics, it is not surprising that this principle is implemented in the design of novel EV isolation techniques [reviewed in Gholizadeh et al. (11)]. An elegant study made use of microfluidic mixing cells in combination with visco-elastic sheath fluids, in which the smaller exosomes were driven to the walls of the flow cells, while larger particles (microvesicles) and the sample fluid remained in the center of the flow path. The 3 fluid streams (from the 2 walls and the center) were collected separately and the 2 sample streams

originating from the walls contained the isolated exosomes (12). The recovery of EV was found to be very high using this method, yet the actual setup is still designed for small sample volumes and low throughputs. Another widespread method for EV, and particularly exosome isolation is precipitation. Here, polymers (e.g., polyethylene glycol) and proprietary chemicals are used to cause specific precipitation of EV by disturbing the solvation layer around the membranes. Although this method is quickly and easily performed on low as well as higher sample volumes, there is a considerable risk of co-precipitation of contaminants.

All techniques mentioned above are based on the physicochemical characteristics of EV (e.g., size, surface potential, density) and inherently do not distinguish between different cellular origins. Yet since EV also share many cellular (surface) markers with their parent cells, the opportunity is created to specifically isolate EV using these markers (see below). Thus, the use of affinity chromatography or labeled magnetic beads is an attractive alternative or a complement to physical techniques such as centrifugation and SEC. One study took advantage of the binding affinity of EV for heparin and could enrich EV from biologic samples using heparin coupled to agarose, which is a commonly used reagent in protein purifications (13). Although this method is straightforward and resulted in highly enriched EV preparations, there are many abundant plasma and serum proteins with high affinity for sulfated glycosaminoglycans, e.g., antithrombin, CXCL4, and apolipoproteins. A higher level of specificity can be achieved by using antibodies against specific surface markers to isolate EV. Needless to say, this requires markers unique for a particular EV subset and corresponding antibodies with high specificity. The potential to separate exosomes from extracellular vesicles depends on the cellular origin of the EV preparations (e.g., the exosome marker CD9 is present both on exosomes and microvesicles from platelets). However, physicochemical separation principles can be used to enrich or deplete the EV preparations in/from exosomes prior to implementing an affinity-based isolation method. Alternatively, EV have been sorted using sorting by FACS, combining scattering and marker-based detection methods (14). However, apart from the limitations described below, a modern FACS sorter might not be accessible due to high costs and the processing times per sample might be quite long.

Taken together, there is as yet no golden standard for EV isolation. The optimal method of EV purification depends, as often, on the characteristics of the starting material and the demands of the downstream applications. The good news in this respect is that many, often multidisciplinary research groups are in the course of developing innovative methods to achieve high quality EV preparations.

TABLE 2 | Overview of current measurement principles of EV.

Detection method	Principle	Advantages (A)/Drawbacks (D)
Flow cytometry	Light scattering, fluorescence	A: Fast recording and high throughput A: Combined size and surface marker analysis D: Limited possibilities using older machines D: Artifacts possible due to swarm detection
Tunable resistive pulse sensing	Coulter effect (electrical resistance changes)	A: Feasible and accurate size determination D: Membranes may clog D: Accurate measurements are slow
Nanoparticle tracking analysis	Light scattering, (fluorescence)	A: Feasible and accurate size determination D: Requires careful calibration D: Accurate measurements are slow
Dynamic light scattering and Raman spectroscopy	(In-)elastic light scattering	A: Feasible and accurate size determination A: Raman yields information about composition D: Low throughput D: Requires high technical proficiency
Transmission electron microscopy	Transmission of accelerated electrons	A: Size and structure determination A: Yields impressive images D: Low throughput D: Requires high technical proficiency
Atomic force microscopy	Power exerted to cantilever	A: Size and structure determination A: Gives information about surface markers D: Low throughput D: Requires high technical proficiency
Enzyme-linked immunosorbent assay	Antigen binding to antibodies, fluori-/colorimetric	A: Highly specific and facile method A: High throughput D: Limited information about counts and size
Western blotting	Antigen binding to antibodies, chemiluminescence	A: Facile method for composition analysis A: Medium throughput D: Limited information about counts and size
Surface plasmon resonance	Proteins binding to ligands,	A: Highly specific and facile method A: High throughput D: Limited information about counts and size D: Rather expensive and specialized equipment

Measurement and Characterization of EV

A similar story can be told for the measurement and isolation of EV. The distinct properties of EV can likewise be used to measure and characterize EV. A summary of the current methods is summarized in **Table 2**. In general, their small dimensions and rather heterogeneous size distribution rather hampers accurate measurement of EV (15). This particularly applies for flow cytometry, as older generation machines (that are still commonly used) generally lack the capability to (accurately) measure particles sized below 300 nm. Another complication is the low refractive index of EV and thus the rather low capability to scatter light in aqueous solutions. In addition, common cytometer optics poorly distinguish single EV from EV swarms, which complicates the exact determination of EV counts in samples (16). On the other hand, modern flow cytometers have optics that allow the measurement of single particles as small as 100 nm and combined with careful apparatus setup and parameter adjustment (the authors refer to www.exometry.com), reliable characterization of

EV using flow cytometry is possible. This, combined with the use of fluorescently-labeled antibodies and the high throughput of the method, makes flow cytometry still a method of choice for EV determinations.

Two other commonly used techniques are tunable resistive pulse sensing (TRPS) and nanoparticle tracking analysis (NTA). The former is based on the current of ions over a permeable membrane with pores that have tunable size. Particles that block the pores result in increased membrane resistance, from which a distribution of size and numbers can be derived. The method is quite reliable over a range of EV sizes and concentrations, yet care should be taken not to clog the pores of the membrane or to apply excessive pressure (15). The detection of EV by NTA is based on light scattering combined with recording of their Brownian motion paths using a microscope-camera setup. Knowing the viscosity and temperature of the sample medium, the EV size can be derived from their diffusion coefficients calculated using the Stokes-Einstein equation. A prerequisite is that viscosity and temperature are exactly defined and that the machine is calibrated using particles that have similar size and scatter characteristics as the EV analyzed (15, 17). Nevertheless, the inaccuracies in EV counts obtained by NTA can be quite high, but protocols have been developed to minimize sample-to-sample variations (18, 19). A similar technique is dynamic light scattering, which like NTA is based on elastic Rayleigh scattering and makes use of the Stokes-Einstein equation for calculating the diffusion coefficients. A further with potential method is Raman spectroscopy, which is based on inelastic light scattering making this method attractive also for analyzing EV, since inelastic light scattering contains information about the molecular composition of the EV (17, 20). Drawbacks are the high technical and mathematical complexity of the method and the long processing times of the analyses.

The same may apply for electron microscopy. Although electron micrographs of EV preparations are recommended to be included in EV-focused publications, the proper sample preparation and recording of (transmission) electron micrographs of EV requires a high level of technical proficiency. The images do reveal useful information about EV ultrastructures and if combined with immunologic detection methods (immunogold labeling), even information about molecular composition may be obtained. An interesting evolution of electron microscopy is cryo-electron tomography, in which a series of electron micrographs is recorded at various angles and then composed to a 3D image (21). A recent study has proven cryo-electron tomography to be feasible for EV, at least those derived from platelets, revealing a surface covered with platelet receptors linked to the actin cytoskeleton (22). A final non-optical microscopy method worth discussing is atomic force microscopy [reviewed in Sharma et al. (23)]. The technique works by a microscopic tip at the end of a cantilever that scans (surface immobilized) biologic specimen, somewhat like a microscopic turntable. The force exerted on the tip and cantilever is used to reconstruct an image of the specimen. The microscope can be set in various modes, each with their specific properties for obtaining information. Major advantages of this technique are that it is optimally suited for nanoscale specimen and that both structural features and information about surface molecules can be recorded, as the tips can be functionalized with specific antibodies.

Besides physical (optical) techniques, EV can also be determined using biochemical (immunologic and enzymatic) techniques. By making use of the presentation of negatively charged phospholipids (notably phosphatidyl serine) on their surface, EV can be captured e.g., using immobilized annexin A5 and subsequent detection using the prothrombinase reaction, which is highly dependent on negatively charged phospholipids (24). However, since not all EV express phosphatidyl serine, a significant fraction might be missed using this method. In addition to enzymatic assays, immunologic methods or "bulk immunologic assays" (BIA) might also be applied (25). The many surface molecules presented on EV and their small size can be exploited to design specific ELISAs for EV subgroups. In addition, EV content can be resolved on SDS PAGE gels followed by western blotting. A more sophisticated method is surface plasmon resonance spectroscopy, which measures refractory index changes as a result of mass bound to a golden surface under flow conditions. When the surfaces are functionalized with specific antibodies against EV surface markers, EV can be measured specifically within biologic samples, as was recently demonstrated for endothelial EV (26). Although the above assays have the advantage that they allow the qualitative and specific analysis of EV, the results of BIA may be difficult, if not impossible, to translate to absolute counts and to particle sizes.

Similar to their isolation, the measurement of EV is accompanied by restrictions that lie in their small size and their physicochemical properties. Also here, a gold standard of EV determination is not clearly defined. Chances are offered by the exploitation of the palette of surface markers carried by EV combined with a technique that specifically scans particles in the correct size range. As technology stands now, flow cytometry still has the highest potential to become a standard method.

DIFFERENTIATION OF THE CELLULAR ORIGIN OF EV

As mentioned in the introduction, EV can originate from virtually every cell or tissue. In the vasculature, the primary cell types that would release EV eligible as biomarkers are red blood cells, platelets, leukocytes, and vascular cells. Among those cell types, red cells and platelets greatly outnumber the others. Thus, EV derived from red cells and platelets are common in plasma. Some studies provide an estimation of the relative numbers of EV from different cell types in blood, yet due to the uncertainties and variations (as outlined above), the authors refrain from listing numbers in this overview. For example, it is still debated whether the platelet-derived EV found in plasma actually originate from platelets or from megakaryocytes (27).

Crucial for a meaningful exploitation of circulating EV as biomarkers is the differentiation of cellular origin. Many studies use surface molecules that function as indicators of the parental cells of the EV investigated. Although many cells are successfully being typed using surface markers, the use of such markers might be accompanied by difficulties. First, EV and in particular

exosomes, are much smaller, making their measurement by e.g., flow cytometry technically more challenging (see below). Second, while aggregates of whole cells can readily be excluded from a flow cytometric analysis, the exclusion of EV aggregates is hampered by the large variation of EV size, meaning that a pair of 2 smaller EV can have a size similar to another larger EV. This might give rise to seemingly double-positive EV, which are in fact aggregates composed of EV from different cellular origins. Third, cellular activation is often required for EV formation, yet is also accompanied by activation of proteases (e.g., of the ADAM family), as is reported for platelets and endothelial cells (EC) (28, 29). Residual protease activity on EV might lead to loss of surface markers in time, which is relevant as a physiologic factor, but also as a pre-analytical variable to be taken into account. The latter even impacts characterization of EV from isolated cells, as surface molecules may be lost during storage, giving rise to day-to-day variations. A fourth difficulty is the availability of cell-specific markers (and corresponding specific antibodies). Platelets and EC for example, share quite a number of surface markers (e.g., P-selectin, CD31, β_3 integrins, thrombospondin, von Willebrand factor, and many more), which complicates a clear differentiation, particularly in samples where platelet-derived and EC-derived EV outnumber those from other sources. Platelet- and EC-derived EV are often distinguished by the use of a common (e.g., CD31) and a platelet-specific marker (CD41 or CD42), bearing the risk that platelet-derived EV with poor antibody binding (e.g., by loss of a marker) can falsely be counted as EC-derived EV. This also applies in cases that cell-specific EV need to be isolated from biologic fluids. Since the common techniques of differential centrifugation or size exclusion chromatography do not distinguish between EV from different cell types, the correct use of markers for the enrichment of cell-specific EV from platelet- or erythrocyte-derived contaminants or protein aggregates is crucial.

POTENTIAL OF EV AS BIOMARKERS—RECENT EXAMPLES

Despite the above reservations, surface markers are widely used in studies exploring levels of particular EV in health and disease. An accurately adjusted flow cytometer with size calibration and optics suitable for small particle analysis considerably facilitates EV determination. The number of flow cytometers that are capable of measuring EV available on the market is increasing. By combining small particle measurement with specific surface markers, information about EV content in biologic fluids can be obtained with a satisfactory level of accuracy. Several reported markers used for the determination of EV from particular cell types are summarized in **Table 3**. Although a large variety of techniques for EV analysis is available (**Table 2**), almost every patient sample-based study has been performed using flow cytometry. A recent multicenter collaborative workshop was organized by the International Society on Thrombosis and Haemostasis (ISTH) Vascular Biology Standardization Subcommittee to evaluate a new, universal standardization protocol to measure platelet EV counts using flow cytometry (38). This new standardization protocol was based on side scatter (SSC) and forward scatter (FSC) of pre-defined beads rather than only FSC before, dependent on which parameter is used as the best resolving size parameter in specific flow cytometers present in the laboratories. The study showed that this bead-based assay has potential for standardization of measurement of platelet EV numbers, however this procedure is not suitable to measure particle size.

TABLE 3 | Examples of markers used for the determination of the cellular origin of EV.

Cell type	Markers	References
Monocyte	AnxA5, CD11b, CD14, CD31, CD64, CD142	(30–32)
Lymphocyte	CD3, CD45	(31)
Neutrophil	AnxA5, CD35, CD66b, MPO	(32–34)
Platelet	AnxA5, CD31, CD41, CD42, CD61	(31)
Megakaryocyte	CD62P-, LAMP-1, full-length filamin A	(27)
Endothelial cell	VCAM-1, CD62E, CD144, CD31, CD41-, CD42-	(32, 35)
Red blood cell	AnxA5, CD235a	(36, 37)

AnxA5, annexin A5; MPO, myeloperoxidase; LAMP, lysosome-associated membrane protein; VCAM, vascular cell adhesion molecule.

Altered levels of EV were found in cohorts of patients with variety of cardiovascular diseases. Several recent reviews provide an excellent and comprehensive overview of the relevant studies (3, 7, 39–41) and highlights of original work are listed in **Table 4**. In general, the origin of the EV investigated in most studies is derived from 3 main cell types: endothelial cells, leukocytes, and platelets. This is not surprising, since all 3 cell types are in direct contact with the blood. In addition, the endothelial lining of the vessel wall constitutes a huge surface (approx. 7,000 m^2) and in the case of (systemic) inflammation, cytokines may increase the activation state of the endothelium, giving rise to the release of numerous EV. The same counts for platelets, since their sheer numbers combined with their capability to release EV upon activation can result in steep increases in EV numbers during pathologic conditions such as arterial and venous thrombosis. When surface markers become more defined and the analysis techniques more refined, also EV from rare cell types that are underrepresented in biologic samples may be detected.

Most of the studies show a positive correlation between EV counts and the cardiovascular disorder investigated, regardless of the cell type of origin. This may reflect the common observation that cells show an increase release of EV after activation. EVs have shown to be upregulated in patients with endothelial dysfunction or atherosclerosis (42, 59), in patients with deep vein thrombosis or pulmonary embolism (60–62), in patients with cerebrovascular diseases(63–66) or in patients that show cardiovascular risk factors like type-2 diabetes mellitus (67), severe hypertension (68) or obesity (69). Determining the EV's parent cells harbors the possibility to obtain additional information about the pathophysiology of a particular disorder. For example, in a small case-control study of one of the study arms of the PREDIMED trial with participants following a Mediterranean Diet, the EV levels of different cell types were measured. Participants suffering a cardiovascular event (CVE)

TABLE 4 | Cardiovascular disorders (CVD) with involvement of EV.

Pathologic setting	EV from cell types involved	References
EV as risk factor for CVD	Endothelial cells	(14, 36, 42–48)
	Platelets	(14, 36, 42, 46–48)
	Leukocytes (unspecified)	(42, 46, 47)
	Monocytes	(46, 48)
	Lymphocytes	(43)
	Hematopoietic cells	(43)
	Smooth muscle cells	(43)
	Erythrocytes	(36)
Vascular calcification	Smooth muscle cells	(49–52)
	Macrophages	(53)
	Endothelial cells	(54)
	Platelets	(54)
	Leukocytes (unspecified)	(54)
Coronary artery disease and acute coronary syndrome	Endothelial cells	(37, 44, 55–58)
	Platelets	(44, 55, 57, 58)
	Erythrocytes	(37)
	Leukocytes (unspecified)	(55, 58)
	Monocytes	(31)

within 1 year of intervention, showed increased EV release from lymphocytes and smooth muscle cells. Participants that did not have a future CVE within the follow-up time showed reduced EV release from these cells (43). In a case-control study at the NIH Stroke program, even different subtypes of EVs from endothelial parent cells could be distinguished. This study compared endothelial cell-derived EV levels in 20 patients with a mild stroke (NIHSS score < 5) to the EV levels of 21 patients with moderate to severe stroke (NIHSS score ≥ 5), and to the levels of 23 age-matched healthy volunteers. Using flow cytometry, they observed significantly higher phosphatidyl serine$^+$ EV counts in patients compared to the controls, and all endothelial derived EV counts were elevated in the moderate to severe stroke group compared to controls. In patients with acute ischemic stroke, three endothelial cell microparticle (EMP) phenotypes (Endoglin$^+$ EMP, phosphatidyl serine$^+$ EMP, and ICAM-1$^+$ EMP) correlated significantly with brain lesion volume, with ICAM-1$^+$ EMP ($P = 0.002$) showing the strongest correlation. These data combined suggest a possible role of endothelial-derived EV numbers as a biomarker for severity and brain lesion size in patients with ischemic stroke (64).

Endothelial dysfunction is an independent predictor of vascular disease. Therefore, quantitative measurements of CD31$^+$/Annexin A5$^+$ EVs were assessed by Sinning and colleagues in patients with stable coronary artery disease (CAD). EV levels were higher in patients that later developed a major adverse cardiovascular and cerebral event (44). This study also finds that the presence of diabetes and male gender are significantly positively correlated to the number of EVs, a factor that has to be taken into account during risk stratification.

In addition, since EVs can be purified, their numbers and contents can be enriched manifold, which opens the possibility for the identification and determination of biomarkers that were previously too dilute to be measured in biologic fluids. Some studies have exploited this to identify miRNAs with prognostic value for cardiovascular diseases (14, 36). EVs of thrombin-stimulated platelets have elevated levels of miR-223 in complex with Argonaute 2, are taken up by HUVEC cells *in vitro*, and regulate gene expression levels through regulatory elements in the 3′UTR region of two specific mRNAs (70). This is only one of the examples in which platelets can alter specific gene regulation in HUVEC cells.

Moreover, the proteins cystatin C, serpin G1 and F2, and CD14 found in EVs have been identified as potential biomarkers by Kanhai et al. in 2013, using the Athero-Express discovery cohort (71). This was the first large, single-center cohort of 1,060 patients, to describe the protein content of EVs is related to increased risk of secondary cardiovascular events. Increased levels of cystatin C, serpin F2, and CD14 were correlated to an increased risk of myocardial infarction, vascular events and all-cause mortality, whereas increased levels of CD14 was also correlated to an increased risk of the occurrence of an ischemic stroke. This study only takes total EV protein levels in account, and not the number of EV.

Another prospective single-center cohort study showed that the EV protein levels polygenic immunoglobulin receptor, cystatin C and complement C5a were independently associated with acute coronary syndrome (72). This study also indicates an important discrepancy between male and female patients, where male patients show a strong correlation between the aforementioned proteins and ACS, whereas female patients did not.

Patients at risk for CVD with high LDL levels are often treated with statins. Statins prevent cardiovascular events, possibly not only by reducing plasma LDL levels. The METEOR trial aims to determine the effect of rosuvastatin on subclinical atherosclerosis. Patient serum samples and LDL-EVs were analyzed for their protein content of von Willebrand factor (vWF), Serpin C1, and plasminogen. Rosuvastatin-treated patients have higher levels of plasminogen and vWF in LDL-associated EVs, serum plasminogen levels were also increased but to a lesser extent, and serum vWF levels were not increased (73). This study concludes that this could be a possible new intermediate between statin therapy and coagulation.

Possibly the most studied potential biomarker is the coagulation potential of EVs exposing tissue factor (TF$^+$ EV) in cancer patients, to evaluate the relative risk on developing venous thromboembolism (VTE) which is a complication in many cancer patients. In a multinational, prospective cohort study, the procoagulant activity of TF$^+$ EV was evaluated using an in-house TF$^+$ EV activity assay based on fibrin generation. The TF$^+$ EV activity was measured in patients with various types of advanced cancer, and correlated with the development of venous thromboembolism (VTE). A high fibrin generation test outcome was associated with a two-fold increased risk for VTE, with the strongest association in patients with pancreatic cancer (four-fold increase) compared to patients with other tumor types

(1.5 fold increase). The activity of TF^+ EV measured using the fibrin generation test correlated poorly with the more commonly used TF-dependent Xa assay (74). However, there are also studies that do not find correlation of EVs with risk for VTE (75), or only a correlation with mortality but not with thrombosis (76). Therefore the role of TF^+ EV as biomarker for VTE in cancer patients remains a matter of debate. Not only EVs derived from cancer cells but also EVs from monocytes expose TF. Although platelets were found to express TF (77), EVs from platelets and erythrocytes lack TF but did induce thrombin generation in a FXII-dependent matter (78).

Despite these examples of protein content in or on EVs, most of the current studies are limited to absolute counts of EV from particular cell types. The number of studies showing correlations or associations of EV numbers with disease prognosis, severity or occurrence is steadily increasing. It must be noted that the majority of studies have a relatively low number of subjects included in the investigation. The largest study to date on EV numbers in CVD is the study of Amabile et al. (2014) where 844 individuals in the Framingham Offspring cohort were studied (45). In this cohort, endothelial-derived EVs were associated with the presence of several cardiometabolic risk factors, including higher triglyceride levels, hypertension, and metabolic syndrome. The highest correlation was found with elevated triglycerides. However, this study only focused on large vesicles $\geq$ 500 nm, thereby risking that a substantial amount of the sample consists of apoptotic bodies.

Another complication is the lack of standardization of sample processing and measurement, making it difficult to compare studies from different laboratories. The same counts for the selection of surface markers analyzed and the corresponding allocation to the EV's parent cell types. A final issue is the heterogeneity of the sample populations investigated, meaning that there are quite some indications that EV are associated with a particular cardiovascular disease, yet hard evidence for such association in large cohorts of defined subjects is still largely lacking. It has to be noted that the above complications largely apply for biomarker research in general.

CONCLUDING REMARKS

Without doubt, the potential of EV as biomarkers is considerable. They may contain information about the original tissues, the pathophysiologic context and the severity of disease. On the other hand, the field is still relatively young and the progress in technologic development for accurate analysis is somewhat lagging behind the desires and ambitions of the investigators working in the area. Still, there is increasing consensus about the standardization of sample preparation and analysis of EV, meaning that studies are becoming more and more reliable and comparison between study locations becomes increasingly feasible. Taken together, interesting and exciting times are awaiting us, as EV do seem to be a big step toward the highly anticipated liquid biopsies.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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