Exosomes: emerging roles in communication between blood cells and vascular tissues during atherosclerosis

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**Purpose of review**
Microvesicles, in general, and exosomes together with their delivered content in particular, are now being widely recognized as key players in atherosclerosis. We have previously reviewed the role of microvesicles in atherosclerosis pathogenesis, diagnosis and therapy. Here, we focus on the roles of exosomes and discuss their emergent role in mediating activation and response to inflammation, vessel infiltration and induction of coagulation. We will finally give an outlook to discuss novel detection techniques and systems biology-based data analyses to investigate exosome-mediated cell-to-cell communication.

**Recent findings**
Recent research points to a role of exosomes in delivering apoptotic and inflammatory content between blood cells and vascular cells, with a potential contribution of exosomes secreted by adipose tissue. An atheroprotective role of exosomes in response to coagulation that may contrast with the procoagulatory role of platelet-derived larger microvesicles is envisaged. New detection and separation methods and systems biology techniques are emerging.

**Conclusion**
We project that the development of novel detection, separation and analysis mechanism and systems-based analysis methods will further unravel the paracrine and endocrine ‘communication protocol’ between cellular players in atherosclerosis, mediating inflammation, oxidative stress and apoptosis.

**Keywords**
atherosclerosis, exosomes, blood mononuclear cells, endothelial cells, systems biology

**INTRODUCTION**
Atherosclerosis is a chronic condition whereby vessel walls thicken through infiltration of inflammatory cells, fat deposits and cell proliferation. Unstable atherosclerosis, resulting from apoptosis and necrosis, can lead to thrombus formation, causing myocardial infarction and stroke [1]. Several genetic, behavioural, metabolic, environmental and nutritional factors have been linked to its pathogenesis, including familial history, obesity, diabetes mellitus, tobacco smoking, dyslipidemia and ageing. Atherosclerosis is commonly associated with intracellular stress conditions such as systemic inflammation [2], hyperactivation of inflammatory response through release of inflammatory cytokines and deregulated levels of M1/M2 macrophages [3], and oxidative stress [4]. However, to fully appreciate mechanisms contributing to this viscous circle of molecular maladaptation between these conditions [5], it is necessary to understand how the different cellular players communicate between each other during pathogenesis by other ways than by secreting cytokines. Hence, we focus on the role of exosomes that are known to contain proteins and genetic material.
Exosomes in atherosclerosis

KEY POINTS

- Exosomes from vascular and adipose tissues play an important role during atherosclerosis in elevating vascular endothelium apoptosis and dysfunction, while decreasing endothelial cell proliferations and migration.
- Their role is mainly governed by mediating inflammatory and apoptotic cytokines, such as TNF-α, FAS-L and TRAIL, attenuating MAPK and NOTCH signalling.
- Exosomes were further reported to induce ICAM-1 internalization and degradation in endothelial cells, thereby facilitating endothelial cell motility.
- Digital microfluidics techniques will improve exosome separation from other microvesicles and exosome content analysis.
- Systems biology methods are proposed to unravel the highly context-specific communication protocol between vascular, vascular endothelial and adipose tissue as mediated by exosomes.

EXOSOMES AND THEIR ROLE IN HORIZONTAL GENE TRANSFER

Exosomes are bi-lipid membranous vesicles containing protein, lipid and nucleic acid contents that are excreted from cells. They are distinguished from other cell-secreted particles, collectively called microvesicles, such as shedding microvesicles (SMVs) and apoptotic bodies of their smaller size [approximately 40–100 nm of exosomes compared with 100 nm–1 μm for SMV and 1–4 μm for apoptotic bodies] and by their intracellular origin. Several cells of the vascular system can secrete exosomes such as red blood cells, platelets, endothelial cells, monocytes, lymphocytes, dendritic cells, mast cells and cells from the tumour vasculature. Exosomes are found in blood plasma, breast milk, cerebrospinal fluid, saliva and urine, and are extracted using ultracentrifugation [6,7]. They are characterized and identified by integrins and tetraspanins CD63, CD89, CD81, CD9 and CD82, by maturation-related proteins flotillin and annexin, and the heat shock proteins hsp70 and hsp90. Although originally considered as cellular debris, it now becomes clear that exosomes are not a result of casual sampling; instead, they contain selective loads via dedicated packing mechanisms [8*,9*], deliver these loads to targeted cells and contain unique trafficking probabilities. All these mechanisms were found to be dependent on the individual cell, their cellular state and different under physiological and pathological and stress conditions [10].

Exosomes have been considered as means of horizontal, paracrine gene transfer, delivering microRNA and mRNA between cells of different origin. Transfer processes have been associated to induce gene silencing via modulation of RISC-complex related proteins and microRNA delivery [11], and activate inflammatory response within the target cells [12]. However, the amount as to how much content is delivered remains controversial. On the one hand, it has been reported that the majority of microRNAs that is detectable in serum and saliva is concentrated in exosomes [13] and that small nucleic acid content is enriched [14]. On the other hand, however, a previous study using state-of-the-art sequencing and digital microfluidics techniques reported that only far less than one nucleotide copy of a specific microRNA is present per exosome [15*], suggesting that exosome-mediated gene transfer is dependent on a high amount of exosome trafficking. To this end, databases are emerging that aim to catalogue exosome proteins, RNA and lipids, specific to secreting cell types, organisms and pathophysiological state [16,17]. Apart from their role in paracrine signalling, exosomes also play an endocrine role in communication between adipocytes via vascular tissues, wherein they may act as a carrier of atherogenic content [12,18]. As such, plasma exosomes were found to be modulators of the adiponectin pathway via upregulation of miR-326 and downregulation of let-7a and let-7f in type 2 diabetic patients [19*].

ROLE OF EXOSOMES IN VESSEL WALL INFILTRATION AND ENDOTHELIAL CELL MIGRATION

The role of microvesicles derived from monocytes and their cellular descendants in intercellular communication during inflammation and immune response is well established [6,20,21] and their activation may be in part induced by exosomes from neighbouring cells in a Toll-like receptor-dependent manner [22]. Monocytes are the major progenitor cells of macrophages and dendritic cells and their activation is required for proper immune response, while pathological deregulation leads to inflammation and oxidative stress [5,23,24].

Macrophages and macrophage-derived microvesicles are overrepresented in the vicinity of the arterial endothelium. Monocytes may secrete exosomes to directly induce intima infiltration. As such, Aharon et al. [25] found monocyte-derived exosomes to induce apoptotic effects in endothelial cells and to promote coagulatory pathways by tissue factor release. Leading to further disarrangement of the endothelium, monocyte-derived exosomes can promote endothelial cell migration by miR-150 delivery [26], although exosomes from macrophages
have also been reported by Lee et al. [27] to suppress integrin trafficking mediated endothelial cell migration. Their study demonstrates that exosomes released from human macrophages negatively regulate endothelial cell migration through control of integrin trafficking.

Specifically, macrophage-derived exosomes promote internalization of integrin β1 in primary human umbilical vein endothelial cells (HUVEC) and stimulate trafficking of internalized integrin β1 to lysosomal compartments, resulting in its proteolytic degradation. Exosome-mediated integrin degradation was blocked by bafilomycin A, a lysosomal degradation inhibitor. Apart from mediating integrin surface expression, macrophage-derived exosomes were also shown to effectively suppress collagen-induced activation of the mitogen-activated protein kinase/extracellular signal regulated kinase signalling pathway and HUVEC migration, which are both dependent on integrin β1. These observations provide new insight into the functional significance of exosomes in the regulation of integrin trafficking.

Several studies raise the possibility that exosome-mediated coregulation and common inflammatory activation of macrophages and lymphocytes may allow their wall infiltration. Macrophages are, together with lymphocytes, the first cells that enter the injury site and synergize with them in infiltration of the vessel intima [1]. Macrophage-derived larger microvesicles may promote leukocyte rolling and adherence via elevation of intracellular adhesion molecule (ICAM-1) and NF-κB activation [28]. Direct exosome-mediated communication between lymphocytes and macrophages was also observed [29]. In this study, authors found that exosomes derived from the supernatant of activated, and potentially atherogenic, CD4⁺ T lymphocytes enhanced cholesterol load accumulation in cultured monocytes in a phosphatidyserine receptor dependent manner. Exosomes may also have a coregulatory role of both cells, as they were shown to be able to transfer exogenous siRNA (against mitogen-activated protein kinase-1, which was introduced into exosomes in their setting) to both monocytes and lymphocytes [30] (Fig. 1).

![FIGURE 1. Role of exosomes in affecting endothelial cell function. Platelet-derived exosomes reduce ICAM-1 expression, reducing monocyte adhesion, while platelet microvesicles under hyperglycaemic conditions induce EC monocyte adhesion via decreased NADPH oxidase activity and increased peroxynitrite. Several blood and vascular cells release exosomes that contain cytokines and apoptosis ligands, promoting inflammation and EC apoptosis. Dendritic cells (DC), T-lymphocytes, NK cells, and some tumour cells, directly release apoptotic ligand FAS-L via exosomes, the first two cell types also in combination with TRAIL. Exosomes from T-cells can also induce FAS-L and TNF-α release from monocytes. Monocytes may also release TNF-α when exposed to exosomes from red blood cells. In turn, endothelial cell apoptosis and dysfunction may exacerbate inflammatory conditions, leading to further T-cell activation or sensitization. Monocyte-derived exosomes can promote endothelial cell migration by miR-150 delivery, while they, however, can also attenuate it via reduced integrin 1β action and collagen expression, suggesting a tightly regulated process. Similarly, exosomes from monocyte-derive macrophages can reduce proliferation by negatively impacting MAPK-signalling. Exosomes from adipose tissue were shown to activate macrophages at the vessel wall, secreting IL-6 and TNF-α in a TLR4/TRIF-dependent way, while plasma-derived exosomes attenuated adiponectin signalling. Endothelial cells secrete exosomes containing the NOTCH ligand DLL-4 that negatively regulates NOTCH signalling and this NOTCH-suppression may be exacerbated by inflammation.](image-url)
EXOSOMES AS A CARRIER AND AN INDUCER OF APOPTOTIC SIGNALS

Exosomes from different vascular cells may induce endothelial cell apoptosis and thereby contribute to vascular dysfunction. As such, platelet-derived exosomes may elevate endothelial cell apoptosis by deregulation of redox metabolism via elevated NADPH activity [31] or by peroxynitrite generation [32]. By similar means, exosomes from red blood cells enhanced tumour necrosis factor-alpha (TNFα) production in monocytes and augmented mitogen-induced CD4+ and CD8+ T-cell proliferation [in an antigen-presenting cell (APC)-dependent manner] [33]. Several lines of evidence exist that elevated exosome-mediated apoptosis occur under inflammatory conditions. As such, exosomes derived from activated T-cells were shown to increase levels of the apoptosis-inducing ligand FAS-L [34]. Likewise, elevated TNF-α levels were found in supernatant of human monocytes exposed to exosomes from the conditioning medium of activated CD4+ lymphocytes [29]. Degranulation of lysosome-derived, multivesicular bodies (as a means of exosome release) in activated T-lymphocytes was observed to increase FAS cell surface death receptor-ligand (FAS-L) and TRAIL (TNF-α related apoptosis ligand) expression in the vicinity of their cell membrane [35], although elevation of FAS-L was observed in T-lymphocytes and natural killer (NK) cells [36]. Likewise, exosomes from dendritic cells express on their surface apoptosis inducing ligands that may further contribute to elevated apoptosis in the endothelium [37]. Apart from the apoptotic role of vascular cell derived exosomes as a response to inflammation, other cells may excrete exosomes that induce an inflammation-mediated apoptotic response. As an example, elevated exosome-mediated FAS-L release (negatively regulated by diacylglycerol kinase alpha) has been observed in tumour cells and attributed as a stress-response to immune attack [38]. Finally, exosome-like vesicles released from adipose tissue were shown to activate macrophages at the vessel wall that can secrete IL-6 and TNF-α in a TLR4/TRIF-dependent way, mediating insulin resistance [39] (Fig. 1).

ROLE OF EXOSOMES IN MEDIATING COAGULATORY PATHWAYS

Platelet-derived microvesicles are responsible for about 70% of the microparticle content in blood serum and affect atherogenesis by activating ECs [40], initiating DC maturation [41] and inducing vascular smooth muscle cell (VSMC) proliferation [42]. Their most prominent role is, however, activation of other platelets and promoting their adherence to the vessel wall, providing a positive feedback that leads to thrombus formation [43]. Heijnen et al. [44] were the first to distinguish platelet-derived exosomes from their larger microvesicles relatives. The studies of Gidlof et al. [45] and Srikanthan et al. [46] suggest that unlike platelet-derived shedding microvesicles, platelet-derived exosomes act rather as anticoagulants or in response to coagulation. Srikanthan et al. [46] provided pre-clinical evidence that exosomes derived from platelet-rich plasma (and after removing nucleated cells by size exclusion filtering) reduced lipid loading and decreased platelet coagulation. This was mediated by decreased CD36 expression, a major mediator of the procoagulative and foam cell-inducing role of oxidized LDL in platelets [46]. Using RNA sequencing, Gidlof et al. [45] found that platelet-derived exosomal miR-320 reduced endothelial ICAM-1 expression, thereby facilitating endothelial cell motility, reducing inflammation and thrombus formation, potentially as a means of protection against further injury. Exosome dependency was validated by the lysosomal inhibitor brefeldin A, which ablated microvesicles expression. They further demonstrate that miR-22, miR-185, miR-320b and miR-4235p were elevated in the supernatant of platelets upon induction of aggregation. Likewise, mast cell exosomes were found to be thrombolytic by stimulating endothelial cells to induce plasminogen activator type I, not only contributing to plaque reduction through fibrinolysis but also to their instability [47].

ROLES OF ENDOTHELIAL CELLS AND ENDOTHELIAL-DERIVED EXOSOMES AND MICROVESICLES

Endothelial-derived exosomes and other microvesicles present only a sparse fraction of the circulating microvesicles. Yet, they have been ascribed a multifaceted role including atherosclerotic effects such as increasing arterial stiffness, inflammation and thrombosis, or atheroprotective roles such as promoting angiogenesis, endothelial cell differentiation and cell survival [48]. Endothelial-derived microvesicles may be activated under hyperglycemic conditions to induce monocyte-adhesion in other endothelial cells vis a via increased NADPH oxidase activity [49]. They may further act as diagnostic markers in atherosclerosis and endothelial cell functions [50,51]. As such, the amount of endothelium-derived microparticles, considered as markers of endothelial cell (dys-)function, was positively correlated with incidence of atherosclerosis in hypercholesterolemia [52] and with incidence of chronic kidney disease in children who also show increased arterial stiffness and atherosclerosis [53].
Endothelial-derived exosomes may contain RNA and proteins that are reflective of the underlying specific stress conditions [12] and have further implicated in vascular development through attenuation of NOTCH signalling [54]. Specifically, authors found that endothelial and tumour cells express the NOTCH ligand Delta-like 4 (Dll4), which negatively regulates NOTCH. It thereby changes endothelial cells to a tip-cell phenotype, supporting vessel branching. Likewise, as NOTCH signalling in endothelial cells is necessary for protection of EC exposed to stress to prevent endothelial senescence and inflammation, exosomes containing Dll4 and inflammation-induced NOTCH depression may contribute to EC dysfunction and apoptosis [55, 56].

**FURTHER ROLE EXOSOMES UNDER ATEROGENIC STRESS CONDITIONS**

Exosomes and other microparticles have been shown to play an atheroprotective or atherogenic role in several conditions accompanying atherosclerosis [57]. Exosomes from mature dendritic cells may contribute to immune system hyperactivation during atherosclerosis, as they transfer the ability to induce naive T-cell priming in an ICAM-1 dependent way to B lymphocytes [58], potentially reducing their reactivity to ox-LDL. Maturation of plasmacytoid dendritic cells can, in turn, occur by endothelial microvesicles, leading to T-cell stimulation and the secretion of inflammatory cytokines [59]. Furthermore, an exosome-mediated link between systemic calcium deregulation, smooth muscle cells calcification and increased vessel stiffness has also been made by analysing data in chronic kidney disease patients [60]. In contrast, exosome-mediated communication between endothelial and smooth muscle cells may be atheroprotective under endothelial shear stress conditions due to turbulent blood flow near bends and bifurcations by containing expression of the miR-143/145 cluster and dependent on the expression of the shear stress responsive Krüppel like factor 2 (KLF2) [61].

**OUTLOOK: NOVEL DETECTION TECHNIQUES**

Currently, only a few studies focus specifically on exosomes. We therefore expect that improvement and standardization of separation procedures and co-culturing protocols will further increase inter-study comparability and exosome role clarification. Exosome-specific labels or, likewise, of their nucleic acid content that may trace them after in-vivo re-injection may further shed light into their pathophysiological role during atherogenesis [62*]. Novel detection methods going beyond ultracentrifugation or size exclusion techniques will increase sensitivity of exosome and microparticle detection and speed up analyses cycles [62*]. These techniques will include microfluidics devices for on-chip isolation, quantification and characterization of circulating microparticles [63]. They will further include droplet-based segmented-flow microfluidics for extraction of nucleic acids [64], microchip-based RNA extraction, droplet digital and analogue RT-PCR for mRNA and miR amplification [65], and plasmonics for detecting their biomarker-relevant content [66].

**OUTLOOK: SYSTEMS BIOLOGY BASED DATA ANALYSIS**

As intracellular communication is complex and highly dependent on cellular context, systems biology techniques will help to generate context-specific hypotheses of the role of specific microvesicles dependent on state of the disease, the expression status of the cell, the secretion frequency and trafficking properties of microparticles, and the type and amount of content delivered. Promising methods therefore are cue-signal-response studies using partial least square regression (PLSR) methods which is a statistical procedure combining multivariate regression with parameter reduction. PLSR allows us to study how signals (exosome content) translate cues from the secreting cell (its gene/protein expression state) to elicit a specific response in the recipient cells. To this end, Gray et al. [67*] demonstrated that depending on the preconditioning of cardiac progenitor cells (hypoxia vs. normoxia at different time points), different clusters of miRs were packed into exosomes derived from these cells. They then identified that these specific clusters gave rise to differential physiological responses in recipient endothelial cells such as assessing their capability for tube formation, thereby suggesting a way how changes of miR composition could be used to direct outcome in target cells.

We propose here to use this method to study how hypoxic and reperfusion-induced atherogenic stress in monocytes would translate into specific endothelial cell responses mediated by specific exosome loading (Fig. 2a). As such, we propose to determine how a physiological stress condition 1 consisting mainly of a mixture of acute and chronic hypoxia induced macrophages to release an exosome content E1 (e.g. containing a certain combination of miR#3 and miR#4), which translates to a specific response in endothelial cells such as reactive oxygen species (ROS) production, while a
Acute hypoxia  
Chronic hypoxia  
Oxidative stress  
Inflammation  
Hyperlipidemia

**FIGURE 2.** Systems biology approaches to study exosome-mediated signalling during atherosclerotic conditions. (a) Proposed cue-signal-response analysis to investigate how different stress conditions in macrophages translate into changes in endothelial cell phenotype using partial least square regression (PLSR) similar to Gray *et al.* [67]. The method seeks for canonical types of exosome loadings (E1, E2, E3; each containing a certain combination of miRs) that are characteristic for in-vivo conditions wherein different combinations of stressors simultaneously apply. Thereby the method aims to separate effective exosome mRNA from bystander content. (b) Once most important exosome content and most affected pathways in endothelial cells have been identified from [a], computational signal transduction analyses may help to assess the effect of exosome content on a detailed level. Content released from exosomes may trigger specific pathways relevant to EC function (apoptosis, dysfunction, proliferation), while further exosome content may also exacerbate or attenuate the signalling response. Computational models can assess the net effect of exosome content on signalling by including quantitative expression levels from pathway proteins and exosome content.

stress condition 2 that is mainly determined by inflammation and hyperlipidemia would lead to an exosome content E2 and induces an endothelial phenotype consisting of deregulated ICAM-1 expression and EC migration.

In contrast to such large-scale systems biology approaches, targeted system models that focus on specific pathways may help to study how specific exosome content may change signal transduction processes in the target cells. Such analysis would allow further segregation of exosome bystander content from such relevant to intercellular signalling. An example therefore would be to study how exosome-delivered interleukin (IL)-6, TNF-α or FAS-L from monocytes/macrophages would affect pathways of endothelial cell apoptosis, dysfunction and proliferation (Fig. 2b). How system models can help to improve clinical diagnostics and treatment in the field of cancer therapy and apoptosis was recently reviewed by us [68]. We thereby collected studies from translational systems biology, whereby theoretical predictions helped to design molecular
interventions to re-establish impaired cancer cell apoptosis and personalized dosages of chemotherapy based on molecular profiling of apoptosis proteins. It is therefore expected that similar personalized medicine approaches may assess grade of inflammation and susceptibility to coagulation from quantifying exosome content, or allowing the design of optimal exosome-content for therapeutically attenuating these processes.

CONCLUSION
Exosome-mediated intercellular signalling during atherosclerosis is a multifacet set of processes whose context-dependent roles are slowly being deciphered. Still, exosome isolation from serum and from their sister microparticles is tedious, and only a few studies are specifically focussed to study exosomes and their role in preclinical and clinical research. Nevertheless, the recent progress in the field of exosome separation and detection, the application of systems biology based data analysis to unravel the ‘communication protocol’ between the involved cells and tissues, and the potential exploitation of exosome delivery for therapeutic purposes make exosome research an attractive field for complex, systemic diseases such as atherosclerosis.

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Conflicts of interest
There are no conflicts of interest.

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