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Review

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Extracellular Vesicles as Mediators in Atherosclerotic Cardiovascular Disease

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ABSTRACT

Atherosclerosis is a chronic inflammatory disease of the arterial intima, characterized by accumulation of lipoproteins and accompanying inflammation, leading to the formation of plaques that eventually trigger occlusive thrombotic events, such as myocardial infarction and ischemic stroke. Although many aspects of plaque development have been elucidated, the role of extracellular vesicles (EVs), which are lipid bilayer-delimited vesicles released by cells as mediators of intercellular communication, has only recently come into focus of atherosclerosis research. EVs comprise several subtypes that may be differentiated by their size, mode of biogenesis, or surface marker expression and cargo. The functional effects of EVs in atherosclerosis depend on their cellular origin and the specific pathophysiological context. EVs have been suggested to play a role in all stages of plaque formation. In this review, we highlight the known mechanisms by which EVs modulate atherogenesis and outline current limitations and challenges in the field.

Keywords: Extracellular vesicles; Atherosclerosis; Inflammation; Plaque, Atherosclerotic; Thrombosis

INTRODUCTION

Ischemic heart disease and stroke, 2 main consequences of atherosclerotic cardiovascular disease (ASCVD), are the leading causes of death worldwide.[1] Atherosclerosis is a disease of the arterial intima, characterized by plaque-forming accumulation of lipoproteins and necrotic debris covered by a fibrous cap. Atherosclerotic lesion formation is a slow progress that remains asymptomatic for decades. Clinical symptoms appear upon significant blood flow reduction due to obstruction or even occlusion of arterial blood vessels, the most well-known examples being myocardial infarction or cerebral stroke. Constitutive risk factors for atherosclerosis are genetics, sex, and age, while the most important modifiable major risk factors include hypercholesterolemia, hypertension, tobacco smoking and diabetes mellitus.

Atherosclerotic lesion formation is initiated by endothelial activation, triggered by the aforementioned risk factors, and by the subendothelial retention of low-density lipoprotein (LDL) as well as other apolipoprotein B-carrying particles.[2] Subsequently, LDL-modification results in the generation of pro-inflammatory mediators that trigger chemokine secretion

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and adhesion molecule expression, which entail leukocyte transmigration into the vascular intima. The pro-inflammatory milieu further promotes the oxidation of accumulated LDL particles, resulting in the generation of oxidized LDL (oxLDL). Likewise, modification of cellular lipids and proteins by lipid peroxidation products results in membrane decoration with oxidation specific epitopes (OSEs), which are recognized as sterile damage-associated molecular patterns (DAMPs) that trigger and propagate the inflammatory process. Concurrently, vascular smooth muscle cells (VSMCs) of the vessel media are stimulated to proliferate and—at later stages—also migrate into the intimal layer. Resident and recruited macrophages, as well as phenotype-switched macrophage-like VSMCs, phagocytose oxLDL and turn into foam cells. At this stage, atherosclerosis is morphologically discernable by visible fatty streaks in the vessel wall. As foam cells accumulate and undergo apoptosis and necrosis, a necrotic core forms. The plaque grows, and remodeling of the arterial wall and extracellular matrix (ECM) are accompanied by plaque calcification. Eventual plaque rupture or erosion can result in thrombus formation.[3,4] Rupture-prone plaques are characterized by large lipid cores and increased inflammation, followed by metalloproteinase-mediated degradation of the fibrous cap. Once ruptured, they release tissue factor (TF) and procoagulant lipids. In plaque erosion, loss of the endothelial monolayer integrity and endothelial desquamation expose procoagulant components of the fibrous cap.[5,6]

Over the past decades, insights on the role of a novel contributor to intercellular communication emerged, namely extracellular vesicles (EVs). EVs comprise various lipid bilayer-delimited vesicles released by a cell, and levels of EVs in circulation have been found to be associated with increased ASCVD risk. Notably, EVs also seem to participate in atherogenesis at all stages. Understanding the precise role and mechanisms of actions of EVs in ASCVD might therefore equip us with novel means to interfere with atherogenesis and prevent its detrimental consequences. The present review aims to summarize the current literature on EVs as pathogenic effectors in atherosclerotic lesion initiation, lesion progression and plaque vulnerability.

1. EVs

In the "minimal information for studies of extracellular vesicles" (MISEV) position paper 2023[7] EVs are defined as "particles that are released from cells, are delimited by a lipid bilayer, and cannot replicate on their own (i.e., do not contain a functional nucleus)." Initially largely dismissed as cell debris, technical advances gradually enabled us to not only more accurately identify various vesicular cell-derived structures, but also determine their diverse sources and functions. The nomenclature of EVs in literature is unfortunately inconsistent and isolation methods should be meticulously compared even if an employed term appears to indicate a specific EV-subset (e.g., exosome – *see below*).[8] Initially, the most popular term was "microparticles," followed by the trend of using the (later on more strictly defined) terms "exosomes" and "microvesicles" to denote 2 subtypes of EVs that may be distinguished by their mode of biogenesis (**Fig. 1**). Exosomes are smaller EVs (<150 nm) of endosomal origin that are initially stored intracellularly in "multivesicular bodies" (MVB) and released upon MVB fusion with the plasma membrane. In contrast, "microvesicles" or "ectosomes" directly derive from the plasma membrane by outward budding and subsequent membrane constriction and separation at the budding site. Their size usually ranges between 150–1,000 nm.[9] Verifying the release mechanism of EVs is tedious and EV isolation methods are limited regarding the purity of the EV subtypes they yield.[10,11] Consequently, the MISEV guidelines encourage the use of more descriptive terms like "small" and "large" EVs together with detailed reports on isolation and characterization methods.[12]

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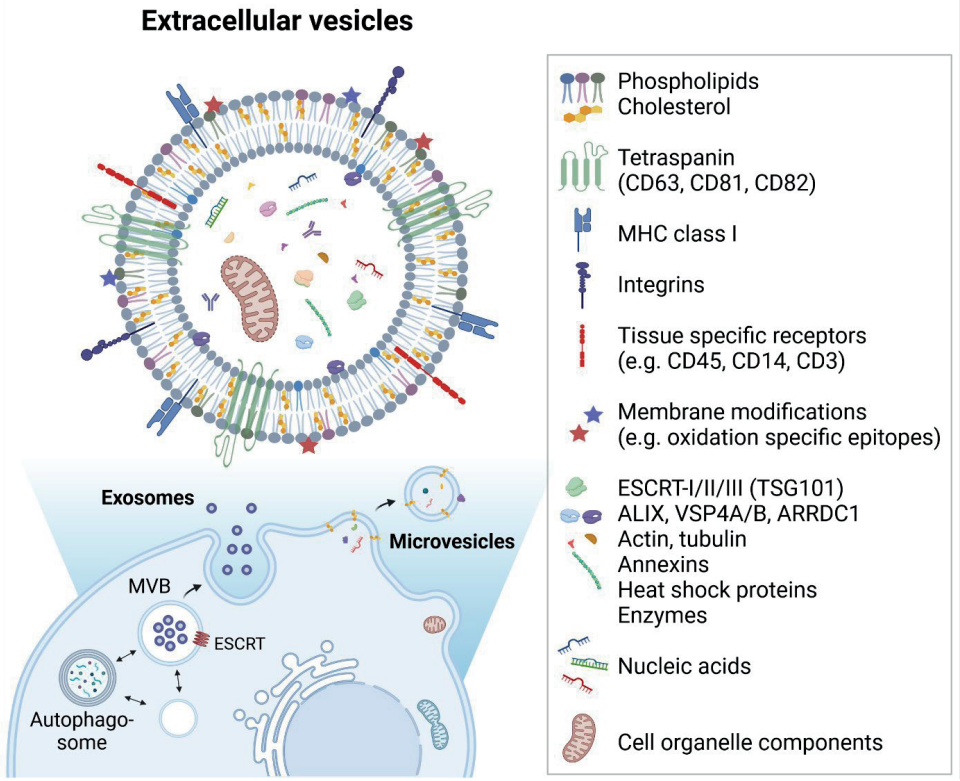

Fig. 1. EV biogenesis and characteristics.
EVs are classically suggested to either be formed intracellularly, assembled in MVBs and be released as "exosomes" or they may form by direct blebbing from the plasma membrane ("microvesicles"). Intersections with the endosomal pathway and autophagy exist. EVs inherit cell-derived (modified) membrane lipids and proteins, and cytosolic cargo, including nucleic acids and cell organelle components.
ALIX, ALG-2 interacting protein X; ARRDC1, arrestin domain containing 1; CD, cluster of differentiation; ESCRT, endosomal sorting complex required for transport; EV, extracellular vesicle; MHC, major histocompatibility complex; MVB, multivesicular bodies; TSG101, tumor susceptibility gene 101 protein; VSP4A/B, vacuolar protein sorting 4 homolog A/B.

In this review, wherever possible, we will indicate whether findings pertain to small (exosomes) and large (microvesicles) extracellular vesicles (sEVs/lEVs). If the main category of EVs remains unclear, we will stick to the term "EVs."

Every cell, including plant cells, and even bacteria and mitochondria,[13,14] is capable of releasing EVs. Initially, EVs were perceived as a mean to dispose of obsolete proteins. Nowadays, we know that EVs participate in intercellular communication in an auto-, para- and endocrine manner. EVs inherit content and surface markers from their parental cells.[8,9] Their cargo includes lipids, proteins, metabolites and nucleic acids. Furthermore, the EV-membrane can also display membrane modifications like OSEs, which are a result of lipid peroxidation in conditions of increased oxidative stress, as it can occur during cellular activation and cell death.[15] Altogether, the specific cargo of EVs depends not only on their parental cell, but also on the pathophysiological context.[16] At the recipient cell, signal transmission can include surface (receptor) binding, vesicle uptake and membrane fusion.[9]

The context-specific regulation of EV-biogenesis and cargo-selection indicates their potential as diagnostic and prognostic biomarkers.[16] *In vivo* imaging and quantification of EVs is to some extent possible,[17,18] but measurements in secreted body fluids (e.g., venous blood, saliva, urine, ...) are undoubtedly more feasible and robust. In conformity with our general

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Table 1. Commonly used markers for the determination of EV-origin

Cell of origin	EV marker
Endothelial cell	CD31+, CD41−, CD42−, CD62E+, CD144+
Platelet	CD31+, CD41+, CD42+, CD61+, CD62p+, CD142+
Monocyte	CD11b+, CD14+, CD31+, CD64+
Neutrophil	CD15+, CD66b+, MPO+
Lymphocyte	CD3+, CD45+
Leukocyte	CD45+
Red blood cell	CD235a+

CD, cluster of differentiation; EV, extracellular vesicle; MPO, myeloperoxidase; "+", positive; "-", negative.

perception of blood biomarkers, the concept for circulating EVs is that changes in the levels of bulk EVs or specific subsets of EVs reflect pathological conditions and diseases.

Studies in mice which were intravenously injected with EVs have shown that EVs are rather short-lived (minutes to hours) in the circulation.[19,20] Nevertheless, there is limited understanding of EV-mobility,[21] particularly regarding the speed, quantity and mechanisms of EV-exit and -entry from and to blood circulation.[22] EVs derived from various tissues (e.g., adipose tissue, muscles, lung, liver, ...) can be found in blood circulation, but they only amount to 0.2% of circulating EVs. More than 90% of plasma EVs derive from hematopoietic cells, first and foremost platelets (13%–51%), followed by leukocytes (8%–45%).[23,24] About 5% of the EVs are derived from endothelial cells (ECs).[24] **Table 1** provides an overview of commonly used marker molecules to identify the cellular origin of EVs.[25-29]

Circulating EVs have been shown to be increased in patients with traditional cardiovascular risk factors[30,31] like high blood pressure,[32,33] dyslipidemia,[30,34,35] diabetes,[33,36,37] obesity,[38] metabolic syndrome,[39] smoking,[40,41] unhealthy diet,[42] physical inactivity.[43] Moreover, circulating EV subset levels reflect atherosclerotic changes, such as endothelial dysfunction,[44] carotid intima-media thickness,[44,45] coronary plaque presence and even plaque composition.[46-49] As plaque composition affects plaque vulnerability, circulating EVs might not only indicate disease burden in stable atherosclerosis but also reflect unstable atherosclerotic disease and predict acute occlusive events. Several studies emphasize the independent prognostic value of EVs for future cardiovascular events.[50-53] More detailed information on EVs as biomarkers in ASCVD are provided in already existing reviews.[20,25,54] We here focus on their pathophysiological role in atherogenesis (**Fig. 2**).

Supplementary Table 1 shows mouse studies, which investigate the effect of EV administration on atherosclerosis. These studies demonstrate that the origin and activation status of the cells from which EVs are derived determines their impact on atherosclerotic lesion formation. Notably, if released from unstimulated cells or healthy donors, EVs do not seem to impact or even improve atherosclerosis, whereas EVs derived from cells activated by proatherogenic triggers promote disease.

EVs IN ATHEROSCLEROTIC LESION INITIATION

EVs shed by various cells participate in the initiation of atherosclerosis by affecting and interacting with the endothelium. Thus, they do not only reflect the presence of disease, but also actively participate in pathophysiological processes (**Fig. 3**).

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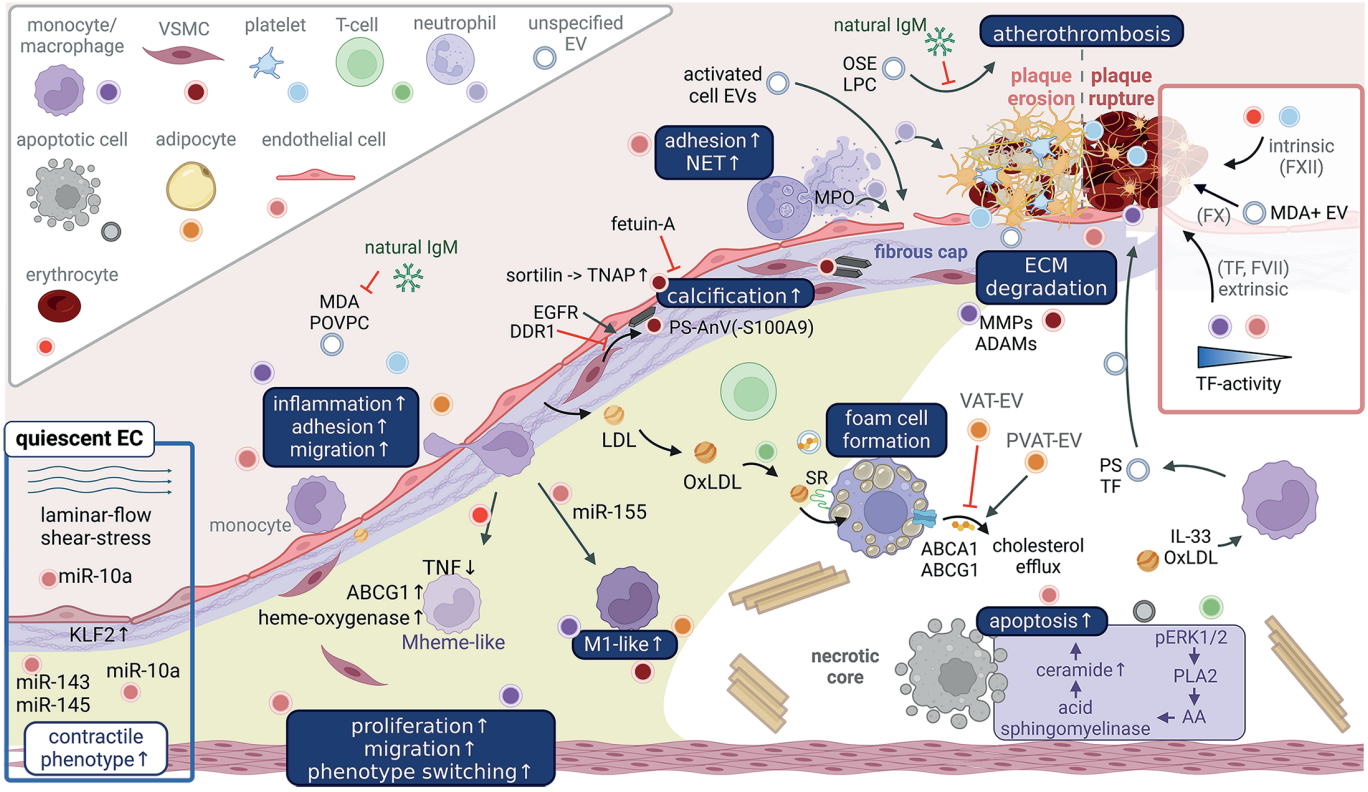

Fig. 2. EVs in atherosclerotic lesion progression and atherothrombosis.
Homeostatic ECs under laminar flow are characterized by endothelial KLF2-expression and atheroprotective EC-derived EVs that carry miR-10a and miR-143/145. In contrast, various noxes and EVs derived from activated cells promote monocyte inflammation, adhesion and transmigration. EVs from stimulated cells promote M1-like differentiation or foam cell formation of monocytes and macrophages. Only erythrocyte-derived EVs are known to promote Mheme-like differentiation of monocytes. T-cell derived EVs and cholesterol-bearing EVs promote cholesterol uptake of macrophages, whereas VAT-derived EVs inhibit ABCA1- and ABCG1-mediated cholesterol efflux, and PVAT-EVs promote cholesterol efflux. Monocyte/macrophage and endothelial EVs mostly promote VSMC proliferation, migration and phenotype switching. Furthermore, EVs participate in macrophage apoptosis induction, in part by sustaining ERK1/2 phosphorylation, which ultimately leads to an increase of pro-apoptotic ceramides. Pro-inflammatory agents (oxLDL, IL-33) promote the release of procoagulant PS- and TF-bearing EVs from monocytes. EVs from monocytes and ECs promote coagulation via the extrinsic coagulation pathway (FVII, TF), whereas EVs from erythrocytes and platelets activate the intrinsic coagulation pathway (FXII). MDA-bearing EVs provide a platform for FX aggregation and activation. OSE-EV catalyzed coagulation can be mitigated by natural IgM antibodies. EVs carry metalloproteinases (MMPs, ADAMs) and participate in ECM degradation. They may further promote EC desquamation and neutrophil adhesion and NET-expulsion, which contribute to plaque erosion-related atherothrombosis. Lastly, the PS-Annexin V-S100A9 membrane complex of VSMC-derived EVs as well as their cytosolic TNAP facilitate calcification nucleation. EGFR promotes, whereas DDR1 prevents the release of calcifying EVs from VSMCs.
AA, arachidonic acid; ABCA1/ABCG1, ATP-binding cassette transporter A1/G1; ADAM, a disintegrin and metalloproteinase; AnV, Annexin V; DAMP, damage-associated molecular pattern; DDR1, discoidin domain receptor 1; EC, endothelial cell; ECM, extracellular matrix; EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; EV, extracellular vesicle; FVII/FX/FXII, coagulation factor 7, 10, 12; IgM, immunoglobulin M; IL, interleukin; KLF2, Krüppel-like factor 2; LDL, low-density lipoprotein; LPC, lysophosphatidylcholine; LPS, lipopolysaccharide; MDA, malondialdehyde; miR, microRNA; MMP, matrix metalloproteinase; MPO, myeloperoxidase; NET, neutrophil extracellular trap; OSE, oxidation-specific epitope; oxLDL, oxidized low-density lipoprotein; PAMP, pathogen-associated molecular pattern; pERK1/2, phosphorylated extracellular signal-regulated kinase 1/2; PLA2, phospholipase A2; POVPC, 1-palmitoyl-2-(5-oxovaleroyl)-sn-glycero-3-phosphocholine; PS, phosphatidylserine; PVAT, perivascular adipose tissue; SR, scavenger receptor; TF, tissue factor; TNAP, tissue non-specific alkaline phosphatase; TNF, tumor necrosis factor; VAT, visceral adipose tissue; VSMC, vascular smooth muscle cell.

1. EVs released under the influence of ASCVD risk factors induce endothelial dysfunction

In patients with an ASCVD risk factor profile, elevated levels of circulating EVs have a functional impact on atherosclerosis initiation by activating ECs, promoting endothelial barrier permeability and leukocyte attachment and subsequent transmigration. For example, lEVs isolated from high-fat diet (HFD)-fed rats[55] as well as cholesterol-induced monocyte-derived lEVs[56] induce adhesion molecule expression and reactive oxygen species (ROS) formation in ECs. Moreover, lEVs isolated from patients with metabolic syndrome decrease transendothelial electrical resistance of human aortic ECs and promote monocyte

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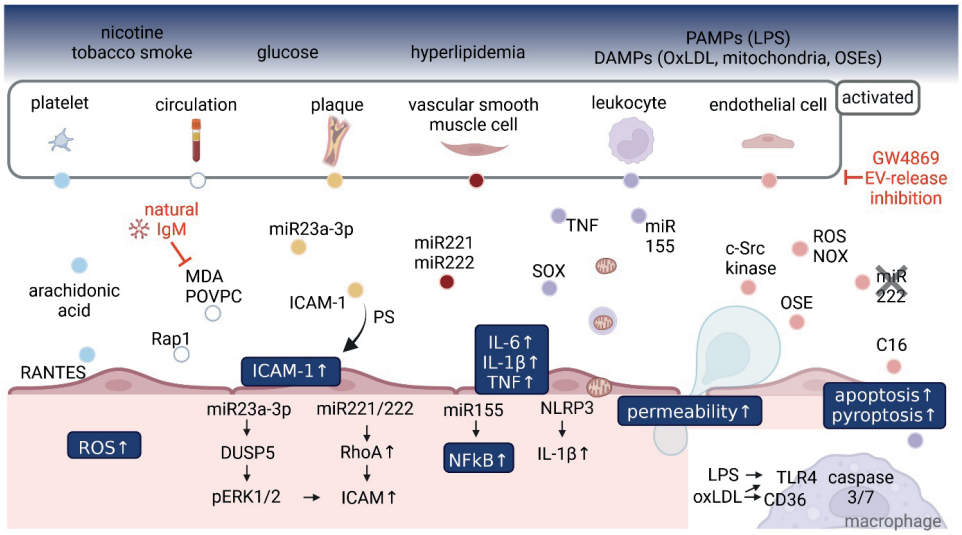

Fig. 3. EV as inducers of endothelial activation and dysfunction.
EVs released under the influence of ASCVD risk factors (nicotine/tobacco smoke, glucose, hyperlipidemia, ...) or by otherwise stimulated cells (PAMPs: LPS; DAMPs: OxLDL, mitochondria, OSEs, ...) activate endothelial cells (ROS increase, NF-κB pathway activation). This induces the release of pro-inflammatory cytokines (IL-6, IL-1β, TNF) and the expression of adhesion molecules (ICAM-1), leading to increased endothelial permeability and leukocyte transmigration and promoting endothelial cell death (apoptosis, pyroptosis). Various microRNAs (miR221/222, miR23a-3p, miR155), phospholipid-related molecules (arachidonic acid, PS, C16), cytokines (TNF, RANTES), oxidative stress-associated molecules (MDA/POVPC, ROS, NOX, SOX) and proteins relevant for signal transduction (Rap1, c-Src kinase) are involved in these processes. Inhibition of the release of EVs by activated cells (e.g., with GW4869) can mitigate EV-mediated pro-inflammatory intercellular communication. Natural IgMs neutralize the effect of OSE-bearing (MDA, POVPC) EVs.
ASCVD, atherosclerotic cardiovascular disease; CD, cluster of differentiation; C16, C16 ceramide; c-Src kinase, proto-oncogene tyrosine-protein kinase Src; DAMP, damage-associated molecular pattern; DUSP5, dual specificity phosphatase 5; EV, extracellular vesicle; ICAM-1, intercellular adhesion molecule 1; IL, interleukin; LPS, lipopolysaccharide; MDA, malondialdehyde; miR, microRNA; NF-κB, nuclear factor kappa-B; NLRP3, NLR family pyrin domain containing 3; NOX, NADPH oxidase; OSE, oxidation-specific epitope; OxLDL, oxidized low-density lipoprotein; PAMP, pathogen-associated molecular pattern; pERK1/2, phosphorylated extracellular signal-regulated kinase 1/2; POVPC, 1-palmitoyl-2-(5-oxovaleroyl)-sn-glycero-3-phosphocholine; PS, phosphatidylserine; RANTES, regulated upon activation, normal T Cell expressed and presumably secreted; Rap1, Ras-associated protein 1; RhoA, Ras homolog family member A; ROS, reactive oxygen species; SOX, superoxide radical; TNF, tumor necrosis factor; TLR4, Toll-like receptor 4.

transcytosis *in vitro*. Both effects are abolished by Rap1 GTPase inhibition,[57] a molecule involved in signal transduction and cytoskeleton rearrangements.[58] Infusion of sEVs isolated from abdominal aortic VSMCs of diabetic mice or isolated from murine bone marrow-derived macrophages, which were cultured under high-glucose conditions, aggravates atherosclerotic plaque formation in apolipoprotein E (ApoE)$^{-/-}$ mice.[59,60] Furthermore, high glucose stimulates human coronary artery ECs to release lEVs with increased pro-inflammatory ROS and NADPH-oxidase (NOX) activity,[61] as well as pro-apoptotic C16 ceramide content enrichment.[62] *In vitro* experiments implicate miR-221 and -222 related increase of RhoA activity in the induction of adhesion molecule expression in ECs, which were incubated with high-glucose treated VSMC-derived sEVs.[60] Thus, EVs that are released under conditions related to hyperlipidemia and diabetes have the capacity to induce endothelial activation.

Not only metabolic dysbalance, but also chemical stimulation elicits cellular release of EVs that induce endothelial dysfunction. Nicotine incites monocytes to release sEVs *in vitro* and *in vivo* in HFD-fed ApoE$^{-/-}$ mice, and injection of nicotine-induced sEVs promotes plaque formation in ApoE$^{-/-}$ mice without influencing the weight or serum cholesterol and triglyceride levels of the animals. Importantly, nicotine-induced plaque formation could be mitigated

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by miR-155 inhibition, or prevented by GW4869 via inhibition of EV formation.[41] GW4869 is one of the most commonly used pharmacological inhibitors of EV release. It inhibits neutral sphingomyelinase-2 (nSMase2), a key enzyme in ceramide generation.[63] Notably, smokers have higher circulating levels of CD14+ sEVs that are enriched in miR-155. *In vitro*, these sEVs increased CCL-2, interleukin (IL)-6, tumor necrosis factor (TNF) secretion and intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) expression of ECs. Interestingly, these effects did not occur if the EVs had been depleted of the monocyte-derived subset.[41] However, as discussed in the following chapters, other studies have shown that EVs from various cell types may induce endothelial dysfunction and promote monocyte adhesion to endothelium.

2. Atherosclerotic disease related EVs sustain endothelial dysfunction

EVs reflect the status quo of their parental cells. Therefore unsurprisingly, circulating EVs of patients with manifest atherosclerotic disease have been suggested to not only reflect, but also affect atherosclerotic lesion formation by inducing EC apoptosis and inflammation, as suggested by *in vitro* experiments.[64,65] For example, in the study of Zhang et al.,[64] circulating sEVs isolated from patients with coronary artery disease induced expression of the cytokines IL-1β and TNF, as well as expression of ICAM-1, but not of IL-6 and VCAM-1 in ECs. Interestingly, in high-cholesterol fed LDL receptor $(LDLR)^{-/-}$ rats, reduction of EV-release by intraperitoneal injection of GW4869 could attenuate vascular inflammation, including macrophage infiltration, *in vivo.*[66]

EVs that affect the vascular endothelium may not only originate from circulating blood cells, but also from plaque-associated cells. Large and small EVs are present in human atherosclerotic plaques, the majority stemming from immune cells.[67-69] Notably, the concentration of lEVs in human plaques exceeds the concentration in plasma at least 200-fold.[67] The landmark study of Leroyer et al.[67] specified parental cells of human plaque-associated lEVs as 29% macrophages, 27% erythrocytes, 15% lymphocytes, 13% smooth muscle cells, 8% granulocytes, and 8% ECs. Interestingly, platelet-lEVs were not detected.

A comparative study of human circulating and atherosclerotic plaque-derived lEVs showed that *in vitro* only plaque-lEVs transferred ICAM-1 to EC-membranes. Additionally, plaque-lEVs did not affect the release of IL-6, IL-8, monocyte chemoattractant protein-1 (MCP-1) or the expression of VCAM-1 and E-selectin by ECs.[70] Perhaps contradictory, blood and plaque sEVs, but not plaque lEVs, from high cholesterol fed $LDLR^{-/-}$ rats with carotid-ligation increased the surface expression of ICAM-1 as well as VCAM-1 and E-selectin in primary ECs *in vitro* and of the carotid intima *in vivo.*[66] In the study of Rautou et al.,[70] human plaque-lEV mediated ICAM-1 transfer to ECs was inhibited by EV-pre-incubation with Annexin V, a classical binding partner of the membrane component phosphatidylserine (PS), but not by P-selectin glycoprotein ligand-1 (PSGL-1) or P-selectin neutralizing antibodies. The authors therefore concluded that PS-mediated membrane fusion[71,72] is involved in ICAM-1 translocation. Accordingly, Peng et al.[66] found that integrity of the vesicle-structure is important for rat plaque-sEV mediated ICAM-1 increase in ECs. However, they attribute this to EV-mediated transfer of miR-23a-3p and propose that miR-23a-3p targets dual specificity phosphatase 5 (DUSP5) and maintains subsequent ERK1/2 phosphorylation resulting in increased ICAM-1 expression. Notably, EV-induced carotid intimal inflammation, arterial wall thickening and lumen narrowing in standard diet fed $LDLR^{-/-}$ rats could be abrogated by simultaneous injection of miR-23a-3p antagomir.[66]

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Only a few studies evaluate differences of EVs in stable and unstable ASCVD with respect to their influence on endothelial dysfunction. Rautou et al.[70] showed that EVs derived from symptomatic human plaques promoted monocyte adhesion to ECs more strongly than EVs derived from asymptomatic plaques. Since plaque-EV subsets, as defined by cellular origin, do not differ between symptomatic and asymptomatic plaques,[67] the difference in monocyte adhesion might stem from a change in EV-cargo. Accordingly, in the study of Wen et al.,[73] the number of circulating sEVs was lower in patients with unstable atherosclerosis than in those with stable disease, but sEVs from patients with unstable atherosclerosis possessed a greater potential to stimulate EC proliferation and migration.

3. EVs from various vascular cells induce endothelial dysfunction

EC EVs

Endothelial dysfunction can be promoted by autocrine signaling of released pro-inflammatory EVs. Studies *in vitro* allow the dissection of several scenarios of auto-/paracrine signaling of EC-EVs. EVs from quiescent or shortly starved (2-hour) ECs appear to have a moderate pro-inflammatory effect on quiescent ECs, as evidenced by cytokine, adhesion molecule[74] or superoxide[75] induction. However, lEVs derived from (apart from 24-hour starvation) unstimulated ECs suppressed ICAM-1, but not VCAM-1 expression of TNF-stimulated ECs *in vitro* and reduced endothelial ICAM-1 expression of ApoE$^{-/-}$ mice on high-fat, high-cholesterol diet upon injection *in vivo*. The anti-inflammatory effect was attributed to miR-222 and interestingly lEVs derived from high-glucose treated cells were shown to carry lower amounts of miR-222 and possess less anti-inflammatory capacity *in vitro* and *in vivo*.[76] While the effect of EVs derived from stimulated ECs on pre-stimulated tissue remains uninvestigated, several studies confirm that EVs derived from stimulated ECs induce endothelial dysfunction in quiescent ECs in several ways, amongst others,[77] by transferring cytokines and adhesion molecules,[74] by decreasing the phosphorylation of endothelial nitric oxidase synthase in recipient cells,[78] and by impairing adherens junction integrity via transfer of c-Src kinase.[79]

Platelet EVs

Platelet derived EVs represent the largest proportion of circulating EVs.[23,24] Activation of platelets leads to the release of EVs that activate ECs and promote monocyte and neutrophil adhesion *in vitro*.[80–84] The transfer of arachidonic acid and subsequent COX-2 induction in ECs have been suggested to mediate the *in vitro* effects of platelet-derived EVs.[80,81] Furthermore, platelet-derived lEVs were shown to deposit the chemokine RANTES on activated endothelium and murine atherosclerotic arteries *in vitro*.[83]

Leukocyte EVs

Compared to other vascular cells, leukocytes are especially sensitive to inflammatory triggers and naturally efficient mediators of tissue inflammation. Thus, EVs derived from leukocytes, especially monocytes, are particularly important with respect to the induction of endothelial dysfunction and aggravation of atherosclerosis.[41,65,68]

Atherosclerotic lesions accumulate LDL, which is subsequently subjected to lipid oxidation, resulting in the generation of oxLDL that acts as sterile DAMP.[85] OxLDL induces lEV release by primary macrophages, a process mediated by the scavenger receptor CD36 and activation of caspase 3 and 7.[86] In turn, EVs derived from oxLDL-stimulated macrophages activate the nuclear factor (NF)-κB pathway and increase pyroptosis and LDH release of EV-treated ECs *in vitro*.[87]

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OxLDL-induced pro-inflammatory signaling is partly mediated by Toll-like receptor 4 (TLR4).[88,89] A more classical TLR4-ligand is lipopolysaccharide (LPS).[88] LPS-activated monocytes have been shown to release sEVs that trigger NF-κB responses[90] and apoptosis[91] in ECs *in vitro*, and sEVs isolated from LPS-treated murine mature dendritic cells have been shown to increase VCAM-1, ICAM-1 and E-selectin expression of ECs *in vitro* and in the aorta of ApoE$^{-/-}$ mice following infusion *in vivo*.[92] Likewise, neutrophils activated by the chemotactic bacterial peptide N-formylmethionyl-leucyl-phenylalanine release lEVs, which induce endothelial dysfunction *in vitro* and enhance atherosclerotic plaque formation upon infusion in ApoE$^{-/-}$ mice *in vivo*. MiR-155-delivery by lEVs was identified as important mediator of NF-κB activation in recipient ECs.[93] With respect to LPS-treated dendritic cells, membrane-associated TNF of released sEVs was shown to be involved in the induction of NF-κB in ECs *in vitro*. Nevertheless, neither TNF-silencing in dendritic cells nor incubation of sEVs with TNF-neutralizing antibody could fully reduce sEV-induced adhesion molecule expression of ECs, suggesting the presence of additional mediators.[92] In this context, we showed that LPS-treated monocytes release high numbers of naked and lEV-enclosed mitochondria, which incite TNF and type I interferon (IFN) responses in ECs *in vitro*. Depletion of naked mitochondria significantly reduced the pro-inflammatory potential of these EVs. Notably, the potential of the lEVs to induce TNF responses in ECs was strongly dependent on mitochondrial respiratory activity and ROS generation in monocytes. The *in vitro* findings were corroborated by increased levels of EVs that express the circulating mitochondrial marker TOM22 in healthy volunteers following low-dose LPS injection. Moreover, like *in vitro* generated EVs from stimulated monocytes, circulating EVs that were isolated after low-dose LPS injection in healthy volunteers induced TNF and type I IFN responses in ECs *in vitro*.[94] Mitochondria have further been shown to have the ability to activate the NLR family pyrin domain containing 3 (NLRP3) inflammasome, leading to IL-1β secretion[95] and monocyte-derived lEVs were shown to activate ECs *in vitro* in an IL-1β dependent manner.[96]

Apart from DAMPs and pathogen-associated molecular patterns (PAMPs), nutrient changes can also provoke monocytes to shed pro-inflammatory EVs. Small EVs derived from starved monocytes disrupt EC integrity and induce apoptosis of ECs *in vitro*.[91] Infusion of lEVs derived from starved monocytes promoted T-cell infiltration in the vessel wall of ApoE$^{-/-}$ mice, and lEV-uptake by ECs *in vitro* resulted in increased ROS generation as well as CCR2, IL-6, MCP-1, and ICAM-1 expression.[97] Furthermore, EVs released by monocytes, which were incubated with unesterified cholesterol, increased NF-κB-mediated ICAM-1 expression and monocyte adhesion in mouse aortic explants and cultured ECs. Interestingly, the addition of unesterified cholesterol to human monocytes induces mitochondrial complex II-dependent accumulation of superoxide and peroxides in the monocytes as well as their lEVs.[56]

In conclusion, monocytes and macrophages react to DAMPs, PAMPs and nutrient changes with the release of small and large EVs, which induce NF-κB mediated activation of ECs. Mitochondrial activity may be critically involved in the generation of these pro-inflammatory EVs.

Oxidation specific epitope-bearing EVs

EVs may derive from different parental tissues, but in specific settings their properties are nevertheless alike. Membrane phospholipids are prominent subjects of inflammation-associated oxidative processes[85] and EVs derived from activated cells carry oxidized compounds, such as the OSE malondialdehyde (MDA),[98] or the oxidized phospholipid 1-palmitoyl-2-(5-oxovaleroyl)-*sn*-glycero-3-phosphorylcholine (POVPC).[99] EVs that carry OSEs were shown to activate ECs *in vitro*,[94,99] entailing increased monocyte adhesion.[99] Importantly, natural

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immunoglobulin M (IgM) antibodies that target OSEs can prevent EV-mediated proatherogenic effects,[98100] and several epidemiological data have shown an inverse association of natural IgM levels in plasma with cardiovascular outcomes.[101] Thus, OSE-carrying EVs may represent an important antigenic target, which allows these natural IgM antibodies to mediate protective functions.[85]

EVs IN ATHEROSCLEROTIC LESION PROGRESSION

1. Activated endothelium releases pro-inflammatory EVs

External atherosclerotic stimuli like tobacco smoke[102] and particulate matter[103] as well as internal stress signals like hypoxia,[104] C-reactive protein[105] or IL-1β[106] were reported to activate ECs and stimulate endothelial shedding of EVs *in vitro* and *in vivo*. Compared to quiescent ECs, which shed miR-10a-containing EVs that can suppress inflammation *in vitro* and in peritonitis-induced C57BL/6 mice *in vivo*,[107] activated ECs shed sEVs, whose miRNAomic and proteomic profile is associated with NF-κB-signaling, necroptosis, cytokine-cytokine receptor interaction, and cell cycle regulation. Accordingly, these EVs provoke transcriptional responses associated with monocyte adhesion, migration, inflammation, proliferation, differentiation and apoptosis in recipient monocytes.[106] The precise effect on monocyte differentiation appears to depend on the exact setting. MiR-155-carrying sEVs released by oxLDL-treated ECs may drive untreated monocytes towards anti-inflammatory M2-like polarization[108] and monocytes pre-stimulated with phorbol 12-myristate-13-acetate (PMA) towards pro-inflammatory M1-like polarization.[109] The pro-inflammatory environment of atherosclerotic plaques might favor the latter effect.

The functional impact of EC-EVs on monocyte activation is furthermore influenced by shear-stress and cell morphology. Expression of the transcription factor Krüppel-like factor 2 (KLF2) is a characteristic of ECs under atheroprotective laminar flow,[110,111] and KLF2-transduced EC-EVs suppress monocyte activation *in vitro* and reduce atherosclerosis of HFD-fed ApoE$^{-/-}$ mice upon infusion *in vivo*.[109] Loss of this anti-inflammatory mechanism might contribute to atherogenesis under turbulent flow conditions.[110] Additionally, under laminar flow conditions, ECs maintain an elongated morphology, which is lost at atherosclerosis-prone vessel sites with turbulent blood flow.[110,112] Compared to EC-EVs derived from elongated endothelium, EC-EVs derived from cobblestone-shaped endothelium lack anti-inflammatory properties (e.g., miR-10a) that dampen monocyte activation.[107,113]

It is important to bear in mind, that in each of the abovementioned settings EC-EVs were generated *in vitro*. Adding to the general questionability of *in vivo* validity of *in vitro* findings, the polarized nature of the endothelium further complicates the matter of evaluating the properties of released EVs. Recently, it has been shown that, depending on their budding-site (apical or basolateral), EC-sEVs differ in their miRNA- and protein-cargo. Proteins of sEVs from the apical side are associated with metabolic pathways, whereas proteins from the basolateral side are associated with ECM interactions, cholesterol metabolism and transport, and protein degradation.[106] Thus, functional differences of these EV populations *in vivo* or *in vitro* require further attention in future studies.

2. EVs activate monocytes and facilitate foam cell formation

Monocyte recruitment and foam cell accumulation are hallmarks of the growing atherosclerotic plaque. Newly transmigrated monocytes are faced with a lipid-rich and pro-inflammatory

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milieu, which promotes their differentiation into macrophages with different functional states, the large diversity of which has been revealed by recent single-cell analyses.[114,115] Simplified, the atherosclerotic plaque is dominated by foam-cell macrophages and pro-inflammatory M1-like macrophages, but also contains anti-inflammatory M2-like or Mheme-like macrophages.[116]

In general, EVs derived from vascular or plaque-resident cells, including platelets,[98] macrophages,[117,118] VSMCs,[119] and T-cells,[120] as well as circulating sEVs from patients with acute myocardial infarction,[121] were reported to activate monocytes and induce macrophage foam cell formation *in vitro* and aggravate atherosclerosis in ApoE$^{-/-}$ mice *in vivo*.[121] Apart from suggested roles of miR-223[117] and miR-146a,[118] cholesterol-flux related mechanisms are inevitably central to the involvement of EVs in foam cell formation. Cholesterol accumulation in macrophages is either promoted by increased uptake of cholesterol or its decreased efflux via ATP binding cassette subfamily A member 1 (ABCA1) and subfamily G member 1 and (ABCG1). EVs can have an impact on both. With respect to cholesterol uptake, EVs were shown to either enhance extracellular oxLDL-uptake by macrophages,[121,122] for example by upregulating scavenger receptor expression,[121] or serve as vehicle of cholesterol delivery.[88,120] When compared to their parental cells, EVs may be enriched in cholesterol content, and their ability to induce foam cell formation *in vitro* has been shown to correlate with the amount of cholesterol they carry.[88]

Even though the majority of circulating EVs appear to be pro-inflammatory, one particular subtype, namely erythrocyte-derived EVs, have been reported to have the ability to promote monocyte conversion to an anti-inflammatory Mheme-like[116,123] phenotype. Erythrocyte-derived EVs were shown to upregulate the expression of the cholesterol-efflux transporter ABCG1 as well as the anti-inflammatory enzyme heme-oxygenase-1, resulting in a decreased expression of the pro-inflammatory macrophage surface marker CD86 and concomitant suppression of TNF-secretion. Furthermore, erythrocyte-EVs attenuated oxLDL-induced macrophage foam cell formation *in vitro* and reduced atherosclerosis in ApoE$^{-/-}$ mice upon injection.[124] Even though the actual *in vivo* relevance of the small subset of erythrocyte-EVs could be negligible, their anti-inflammatory properties might be of therapeutic value.

Moreover, the study of Crewe et al.[125] highlights that even "EV-minorities" like adipocyte-derived EVs have the potential to effectively participate in interorgan-communication via the route of circulation. In mice that overexpress mitochondrial ferritin in adipocytes, which is associated with mitochondrial dysfunction in hypertrophic adipocytes in the context of obesity, adipocyte-derived sEVs that carry mitochondrial components were shown to enter the circulation and transfer their cargo to cardiomyocytes. Of note, time was an important factor in this process, since mitochondrial component transfer was only detectable after 11 weeks of observation, but not after 3 weeks. Thus, in chronic conditions like atherosclerosis, the potential importance of proportionally small subsets of EVs should not be dismissed.

Adipocyte-derived EVs were reported to represent the largest category of the small proportion of tissue-derived EVs in circulation.[23] It is likely that EVs from adipose tissue also signal to cells in plaques. It has been shown that sEVs derived from TNF-treated adipocytes increase VCAM-1 expression of ECs and subsequent leukocyte attachment *in vitro*.[126] *In vivo*, administration of visceral adipose tissue (VAT)-derived sEVs from HFD-fed wildtype mice to hyperlipidemic ApoE$^{-/-}$ mice increased plaque burden and leukocyte infiltration in atherosclerotic lesions without changing the weight or plasma-lipid profile of the animals. *In vitro*, adipocyte derived sEVs have been shown to get engulfed by macrophages, but only EVs from visceral as opposed

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to subcutaneous adipose tissue (SAT) facilitated macrophage foam cell generation through ABCA1, ABCG1 mediated cholesterol-efflux decrease as a consequence of downregulated expression of liver X receptor. Moreover, only VAT-sEVs from HFD-fed wildtype mice, as opposed to SAT-sEVs from HFD-fed or VAT-sEVs from chow diet-fed mice, induced TNF and IL-6 in macrophages and promoted a M1-like phenotype.[127] Further adding to the importance of the type of adipose tissue, perivascular adipose tissue (PVAT)-derived sEVs isolated from wildtype mice on standard diet, but not SAT-sEVs, increase ABCA1 and ABCG1 mediated cholesterol efflux and therefore reduce oxLDL-induced macrophage foam cell formation *in vitro*.[128] A follow-up study suggests the involvement of miR-382-5p-mediated induction of peroxisome proliferator-activated receptor gamma (PPARγ),[129] a well-known promoter of cholesterol efflux and suppressor of inflammation in macrophages.[130] In summary, atherogenic diet-fed mouse derived VAT-sEVs promote and PVAT-sEVs inhibit macrophage foam cell formation, emphasizing the importance of interorgan-communication in atherosclerosis.

3. EVs promote VSMC migration and phenotype switching

During the formation of an atherosclerotic plaque, VSMCs switch from their classical contractile phenotype to a synthetic state. Synthetic VSMCs show an increase in proliferation and migration. Further differentiation into osteogenic, mesenchymal, fibroblast-like, adipocyte-like, or macrophage-like VSMCs is possible and phenotype-switched VSMCs make up for about 30% of cells in atherosclerotic plaques.[131-133]

Several studies report that sEVs derived from oxLDL-stimulated monocytes or macrophages[134-139] promote the proliferative and migratory ability of VSMCs *in vitro* and *in vivo*.[136] The same has been shown for sEVs released by nicotine-induced macrophages,[140] macrophage foam cells,[141] plasma-derived sEVs from atherosclerotic patients,[141] lEVs from patients with metabolic syndrome and from HFD-fed but not standard diet-fed ApoE$^{-/-}$ mice,[57] and EVs from TNF-stimulated VSMCs.[77] The various microRNAs identified to be enriched in these EVs and transferred to VSMCs, including their proposed targets, are summarized in **Table 2.** Liu et al.[138] further described that sEVs derived from oxLDL-stimulated monocytes reduced apoptosis of VSMCs *in vitro*, which they attributed to miR-106a-3p and its binding to caspase 9. In contrast, sEVs released by LPS stimulated monocytes were reported to deliver caspase 1 to VSMCs, resulting in induction of VSMC death.[142]

With respect to EC-derived EVs, sEVs released by IL-1β -stimulated ECs carry miRNAs and proteins that activate pro-inflammatory and atherogenic pathways in monocytes and VSMCs *in vitro*.[106] Furthermore, sEVs released by LPS- or oxLDL-treated ECs promote VSMC proliferation, migration[143,144] and phenotype switching *in vitro*.[143] In contrast, EVs released by untreated[144] or starved and apoptotic ECs[145] still promote VSMC dedifferentiation,[146] but reduce VSMC proliferation and migration. Injection of lEVs released by starved ECs in wildtype mice reduces neointima formation after wire-inflicted carotid artery injury. MiR-126-3p was identified as the predominant microRNA in these protective EC-EVs, and in a cohort of 176 patients with coronary artery disease, patients with higher levels of miR-126 were less likely to require percutaneous coronary intervention.[145] Finally, miR-143 and 145-enriched lEVs released by ECs under shear-stress or lEVs released by ECs overexpressing KLF2 were found to support maintenance of a contractile phenotype of VSMCs *in vitro*.[147]

In conclusion, EVs derived from activated cells contribute to VSMC proliferation, migration and phenotype switching, promoting their ability to contribute to fibrous cap formation and populate the plaque core as phenotype-switched cells.

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Table 2. Overview of studies on EV-effects on VSMCs

Parental cell type	Treatment	EV type	EV cargo	VSMC target	VSMC effect	Reference
PBMC	LPS	sEV	Caspase-1		Viability↓	Sarkar et al. (2009)[142]
THP-1	oxLDL	sEV	LIPCAR	CDK2↑, PCNA↑	Proliferation↑	Hu et al. (2021)[139]
THP-1 monocyte	oxLDL	sEV	miR-106a-3p	Caspase 9↓	Proliferation↑, viability↑	Liu et al. (2020)[138]
THP-1 monocyte	oxLDL	sEV	circ_100696	miR-503-5p↓ →PAPPA↑	Proliferation↑, migration↑	Liu et al. (2023)[137]
RAW 264.7	oxLDL	sEV	miR-19b-3p	JAZF1↓	Proliferation↑, migration↑	Wang et al. (2022)[136]
THP-1 macrophage	oxLDL	sEV	miR-186-5p	SHIP2↓ →PI3K/AKT/mTOR pathway↑	Viability↑, invasion↑	Ren et al. (2022)[135]
RAW 264.7 macrophage	oxLDL	sEV	miR-503-5p	smad7, smurf1/smurf2↓ TGF-β1↑	Proliferation↑, migration↑	Wang et al. (2021)[134]
RAW 264.7 macrophage	Nicotine	sEV	miR-21-3p	PTEN↓	Proliferation↑, migration↑	Zhu et al. (2019)[140]
J774a.1 foam cell	oxLDL	s+lEV		pErk, pAkt↑ actin cytoskeleton and focal adhesion pathway	Adhesion↑, migration↑	Niu et al. (2016)[141]
Athero patient blood*		sEV	CD45 ↑		Adhesion↑, migration↑	Niu et al. (2016)[141]
MetS patient blood		lEVs	Rap1	ERK5/p38 pathway↑	Proliferation↑, migration↑, inflammation↑	Perdomo et al. (2020)[57]
VSMC	TNF	lEV		MAPK, PCNA↑	Proliferation↑	Paudel und Kim (2020)[77]
HAEC	IL-1β	sEV	miRNAome proteome		Inflammation↑	Raju et al. (2024)[106]
HUVEC	oxLDL	sEV	LINC01005	miR-128-3p↓, KLF4↑	Proliferation↑, migration↑, phenotype switching↑	Zhang et al. (2020)[143]
HUVEC	LPS	sEV			Proliferation↑	Xiang et al. (2021)[144]
	None	sEV			Proliferation↓, migration↓, lipid accumulation↓	
EC	Starved, apoptotic	lEV	miR-126-3p	LRP6↓	Proliferation↓, migration↓	Jansen et al. (2017)[145]
HUVEC		sEV	miR-26a		Dedifferentiation↑	Lin et al. (2016)[146]
HUVEC	Shear-stress, KLF2 → miR-143/145↑	lEV	miR-143/145		Dedifferentiation↓	Hergenreider et al. (2012)[147]

circ, circualar; CD, cluster of differentiation; CDK2, cyclin-dependent kinase2; EC, endothelial cell; ERK, extracellular signal-regulated kinase; EV, extracellular vesicle; HAEC, human aortic EC; HUVEC, human umbilical vein EC; JAZF1, juxtaposed with another zinc finger protein 1; J774a.1, murine macrophage cell line; KLF, Krüppel-like factor; lEV, large extracellular vesicle; LINC, long intergenic non-coding RNA; LIPCAR, long intergenic noncoding RNA predicting cardiac remodeling; LPS, lipopolysaccharide; LRP, LDL receptor-related protein; MAPK, mitogen-activated protein kinase; MetS, metabolic syndrome; miR, microRNA; mTOR, mammalian target of rapamycin; OxLDL, oxidized low-density lipoprotein; PAPPA, pregnancy-associated plasma protein-A; PBMC, peripheral blood mononuclear cell; PCNA, proliferating cell nuclear antigen; pErk, phosphorylated ERK; PI3K, phosphoinositide 3-kinases; PTEN, phosphatase and tensin homolog; Rap1, Ras-associated protein 1; RAW264.7, murine macrophage cell line; sEV, small extracellular vesicle; SHIP2, Src homology 2 domain-containing inositol phosphate phosphatase 2; smad, small mothers against decapentaplegic, smurf, smad ubiquitination regulatory factor; TGF-β1, transforming growth factor-beta1; THP-1, human leukemia monocytic cell line; TNF, tumor necrosis factor; VSMC, vascular smooth muscle cell; "↑", increase; "↓", decrease; "→", leads to.
*Patient with atherosclerotic cardiovascular disease.

4. EVs and necrotic core formation

A dysbalance of cell death and efferocytosis leads to accumulation of dead cell material in the plaque, morphologically defined as the necrotic core, which is a hallmark of unstable, rupture-prone plaques. As indicated above, the inflammatory milieu provokes the release of yet again pro-inflammatory and often pro-apoptotic EVs. Distler et al.[148] explored this concept and showed that EVs derived from T-cells, which have been exposed to various apoptotic stimuli, are phagocytosed by murine macrophages and trigger apoptosis *in vitro*. Huber et al.[149] showed similar effects in human macrophages and identified acid sphingomyelinase activation and subsequent generation of pro-apoptotic ceramides as essential steps in EV-induced apoptosis. More specifically, the authors propose that EVs promote ERK1/2 phosphorylation, and subsequent phospholipase A2 activation entails arachidonic acid generation, which in turn activates acid sphingomyelinase. Simulating plaque conditions, sEVs from TNF-treated ECs were shown to induce macrophage apoptosis *in vitro*,[150] and sEVs from endotoxin-stimulated monocytes were shown to induce VSMC death *in vitro*.[142] Thus, EVs have the capacity to contribute to plaque destabilization by promoting necrotic core formation.

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EVs AND PLAQUE VULNERABILITY

1. EVs in plaque calcification

After initial plaque formation and VSMC proliferation and migration, calcium can start to deposit especially in the fibrous cap covering the plaque. The role of calcification with respect to plaque vulnerability is still under discussion and reviewed elsewhere.[151] Briefly, it is commonly distinguished between micro- and macrocalcification in the fibrous cap of the plaque. The current mainstream belief is that initial microcalcification in the fibrous cap of the plaque enhances its chance to rupture, whereas later-developing macrocalcifications stabilize the cap.

EVs at the center of calcification nucleation

The process of calcification per se is understood to occur in a pro-calcific environment, which might ensue in the course of inflammation and cell death. "Matrix vesicles" have long been suggested to be the source of nucleation for calcium-phosphate crystals and hydroxyapatite formation.[152] The term "matrix vesicles" emerged independently but evidently describes yet another subset of EVs, nowadays also referred to as "calcifying EVs." Calcifying EVs are reported to be in the size range of 30–500 nm[153-155] and are released from membranous protrusions.[155,156] Initially, they were described to derive from chondrocytes,[157] but in the atherosclerotic plaque they are believed to derive from osteogenic VSMCs[154] or macrophages.[155] *In vitro*, osteogenic VSMCs release an increased number of EVs[158-160] and proteomic analysis revealed that VSMC-derived sEVs share components with osteoblast-derived EVs, in particular calcium-binding proteins (Annexins) and ECM proteins.[158]

In the extracellular space, calcifying EVs bind to negatively charged surfaces like collagen fibrils and proteoglycans of the ECM. Direct interaction of VSMC-sEVs and type I collagen *in vitro* is suggested to involve interactions with integrins.[161] The vesicle membrane and lumen serve as a platform, where pro-calcifying factors can be focally accumulated in high concentrations.[152,155,157] At the vesicle membrane, Annexins may form pores that allow calcium influx or they participate in PS-Ca2+ complexes as starting points of calcification.[152,157] Compared to apoptotic bodies and VSMC lysates, VSMC-EVs are selectively enriched in Annexin II, V and VI, of which, Annexin VI was confirmed to be of particular importance for vascular calcification.[154] In macrophage-derived calcifying EVs, S100A9 content was found to correlate with their degree of calcification, and the authors concluded that a PS-Annexin V-S100A9 membrane complex facilitates hydroxyapatite nucleation.[155] In the vesicle lumen, tissue non-specific alkaline phosphatase (TNAP) converts the mineralization inhibitor inorganic pyrophosphate to pro-mineralizing free inorganic phosphate.[152,157] Sortilin was shown to regulate the loading of TNAP into EVs, and sort-1 deficiency reduces arterial calcification but not bone mineralization of LDLR$^{-/-}$ mice on high-fat, high-cholesterol diet.[162] The release of sortilin-enriched calcifying EVs from mouse immortalized aortic VSMCs (MOVAS) can be promoted by Nε-Carboxymethyl-Lysine, a key active component of advanced glycation products in serum.[163] In contrast, VSMCs can take up fetuin-A, a circulating plasma glycoprotein, and the presence of fetuin-A in VSMC-derived vesicles prevents calcification *in vitro*.[164]

Of course, vesicle-mediated calcification can also be regulated on the level of EV-biogenesis. Epidermal growth factor receptor (EGFR) influences caveolin-1 trafficking and promotes the biogenesis of calcifying EVs from VSMCs *in vitro* and in aortas of mice with chronic kidney disease *in vivo*,[165] whereas DDR1 prevents the release of calcifying EVs from VSMCs *in vitro* and in aortas of LDLR$^{-/-}$ mice *in vivo*.[166]

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Calcifying EVs and plaque vulnerability

Regarding the role of calcifying EVs in plaque vulnerability, carotid tissue specimens from symptomatic atherosclerotic patients revealed a higher number of vesicles in vulnerable (cap thickness <100 μM) than stable fibrous caps. Additionally, vulnerable fibrous caps contained more vesicles with signs of calcification.[153] In 3D-collagen hydrogels, the sEVs of human VSMCs calcified in a way that reminds of microcalcifications.[167] In a similar 3D-model of an atherosclerotic fibrous cap, Hutcheson et al.[168] observed that VSMC-derived calcifying EVs aggregate between collagen fibers and initially calcify in the pattern of microcalcification, but ultimately form macrocalcifications.

The importance of pro-calcifying stimuli including EVs

Importantly, even though EVs are present in the ECM of vessels from young and healthy individuals, in the physiological state, they do not necessarily possess calcifying potential.[154] However, calcification or calcifying potential might be tipped off by several factors, including a rise in extracellular and intracellular calcium or phosphate concentrations[152,158,159,169] or TNF-[158] and nicotine-stimulation of the parental cell.[160] Furthermore, lipoprotein (a) induces sEV release of human VSMCs and promotes calcification, possibly by supplying oxidized phospholipids,[167] which induce pro-calcifying oxidant stress.[170]

Thus, one may speculate that OSE-carrying EVs contribute to VSMC calcification. Similarly, other pro-inflammatory EVs may also contribute to VSMC calcification. Indeed, atherosclerosis-associated EVs such as macrophage-derived lEVs released under high-glucose conditions,[171] EVs derived from VSMCs of rats with chronic kidney disease,[172,173] sEVs from LPS-stimulated macrophages,[174] and lastly lEVs from ECs stimulated with TNF,[175] have all been shown to promote osteogenic VSMC-switching *in vitro* and *in vivo*[171] and induce associated calcium-dependent NOX activity and ROS generation.[159,160,172,173] MiR-32 might play a role in this process.[171,176] Importantly, EVs that promote VSMC switching towards an osteogenic phenotype are sometimes also termed “calcifying EVs.”

2. EVs in extracellular matrix degradation

A common mediator of both plaque rupture and erosion are metalloproteinases, which are able to break down the ECM components of the fibrous cap or the endothelial basal membrane and thus critically regulate plaque stability.

Metalloproteinases are represented by three groups, the “a disintegrin and metalloproteinase” (ADAMs), ADAMs with thrombosponding motif (ADAMTs) and matrix metalloproteinases (MMPs). The role of metalloproteinases in ASCVD is reviewed elsewehere.[6,177] Of note, even though some targets of metalloproteinases have been specified (e.g., MMP 1, 13 – fibrillary collagens I, II, III; MMP 2, 9 – denatured collagen, collagen IV and laminin), MMPs usually degrade multiple matrix components with variable efficiency. Furthermore, MMPs may activate other MMPs, the most prominent example being MMP-14, which cleaves pro-MMP2.[6]

Clinically, various studies highlight a relationship between metalloproteinases and atherothrombotic events. Rizza et al.[178] showed that consideration of circulating ADAM17 substrate-levels improves the prediction accuracy of the Framingham Recurring-Coronary-Heart-Disease-Score, and ADAMs or MMPs were shown to regulate endothelial permeability[179] and correlate with[180,181] or influence[182] plaque instability. It is possible that, although not investigated, EV-bound metalloproteinases are implicated in these associations.

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First of all, immune cell-derived EVs have been shown to incite the release of metalloproteinases by VSMCs and fibroblasts, *in vitro*. For instance, macrophage-derived sEVs elevate MMP-2 expression in VSMCs,[183] and EVs derived from apoptotic and activated T-cells and monocytes induce the synthesis of MMP-1, MMP-3, MMP-9, and MMP-13 in various fibroblasts.[148]

Secondly, the secretion and function of metalloproteinases has been described to be regulated by the endosomal pathway[184-186] as well as tetraspanins[187] and PS,[188] revealing an intersection of MMP-trafficking with EV-release mechanisms. Indeed, circulating EVs isolated from wildtype mice have been shown to carry surface-bound MMP-10[189] and further studies suggest the presence of MMP-1, MMP-2, MMP-7, MMP-13, MMP-14, MMP-15, MMP-16[190] or MMP-2, MMP-9, and MMP-14[191] on the surface of fibronectin-degrading EC-EVs, whereas the concurrent presence of tissue inhibitor of metalloproteinases-1 (TIMP-1) and TIMP-2 is controversial. *In vitro* generated leukocyte-derived EVs were reported to carry MMP-9 and -14,[192,193] ADAM10, ADAM15 and ADAM17,[194-196] and proteinase-3 and elastase.[192,197] Importantly, Canault et al.[198] reported the presence of ADAM17 on human plaque EVs as opposed to EVs from healthy human internal mammary arteries. Mechanistically, some studies suggest the involvement of p38 mitogen-activated protein kinases (MAPK) and c-Jun N-terminal kinase (JNK) pathway in the EV-related release of metalloproteinases.[183,189,193]

While Lozito and Tuan[190] reported that cytokine-enriched (IL-1β, TNF) and hypoxic conditions did not affect MMP localization to EVs, several other studies describe increased EV-associated metalloproteinase release by various cell types in response to specific stimuli,[154,189,192-197] for instance, LPS- or thrombin-stimulated ECs,[189] calcium-stimulated VSMCs,[154] or tobacco-stimulated leukocytes.[193,195]

3. EVs in atherothrombosis

Ultimately, plaque progression can culminate in atherothrombotic events. This is either triggered by plaque rupture, and subsequent exposure of thrombogenic plaque content (e.g., tissue factor), or plaque erosion, where the subendothelial matrix and neutrophil extracellular traps (NETs) provide a hotspot for coagulation factor activation.[199] Apart from plaque-derived coagulation activators, a thrombogenic state also needs to be considered.

An elevated number of circulating EVs in patients with acute coronary syndrome already hints at a role of EVs in the atherothrombotic event.[53] Moreover, their implication in immunothrombosis is emphasized by the specific elevation of platelet- and monocyte-derived EVs and EV-aggregates in patients with coronary artery disease, who required percutaneous coronary intervention.[200] The elevation of circulating EV numbers could simply be the consequence of a systemic reaction to the acute occlusive event. However, evidence of elevated procoagulant[201] and malondialdehyde-bearing (MDA+)[98] EVs specifically at the culprit lesion-site implicate EVs as mediators in the atherothrombotic process.

EVs in coagulation

Physiological hemostasis requires a balance of pro- and anti-coagulant factors. Endothelial- and leukocyte-derived EVs can serve as a platform for fibrinolytic plasmin generation.[202,203] Furthermore, platelet-derived sEVs were reported to inhibit platelet adhesion to collagen and platelet aggregation *in vitro* and occlusive thrombosis in damaged murine carotid arteries upon injection *in vivo*.[204] Nevertheless, even in healthy individuals, EVs mainly have procoagulatory potential.[205,206] They enhance platelet deposition on human atherosclerotic

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arteries, thrombus formation,[205] and accelerate fibrin polymerization, leading to higher plaque density and impeding fibrinolysis.[206] The negative surface of exposed PS on the EV-surface *per se* serves as a platform for coagulation activation. Furthermore, procoagulant lysophosphatidylcholine (LPC) is enriched in EV-membranes.[207] Moreover, a subset of circulating EVs carries OSEs,[98] which, depending on individual levels of neutralizing natural IgM associated with them, promote coagulation to varying extent.[100] Additionally, 10% of circulating lEVs express TF.[208] It has been shown that platelet-associated TF and lEV-associated TF contribute additively to overall blood-TF activity.[209] TF-transfected EVs show increased adherence to collagen-IV and fibronectin as well as enhanced TF-activity due to its greater concentration on the EV-surface.[210] Interestingly, neutrophil adhesion molecules and secretion products further amplify TF-activity,[209] implicating TF-bearing EVs in the process of NET-mediated thrombosis during plaque erosion.

In general, all blood EV subsets are primarily considered procoagulant, but they differ in their procoagulatory potential and TF expression. When stimulated,[211] monocytes generate the most procoagulant EVs, followed by ECs, granulocytes and then platelets.[212] Of note, the platelet-lEV surface is proposed to have 50–100-fold higher procoagulant activity than the activated platelet membrane surface.[213]

Extrinsic/intrinsic coagulation pathway activation by EVs

EV-associated TF activity follows the same order as overall procoagulant activity of EV-subsets. It is highest in monocyte EVs and lower in EC-EVs. Platelet, granulocyte and erythrocyte EVs show no TF activity.[212,214-216] Complementary to their lack of extrinsic pathway activation, platelet-derived and red blood cell-derived EVs were shown to induce thrombin generation via factor XII of the intrinsic coagulation pathway[215,217,218] and blocking the extrinsic pathway by FVII-inhibition did not influence this.[215] EV-associated PS was shown to contribute to the activation of the intrinsic coagulation pathway.[211,217,218] In contrast, monocyte-derived EVs did not induce FXII-dependent thrombin generation,[217,218] neither did purified phospholipids with similar PS-activity as the EVs. However, FVII-inhibition hampered the procoagulatory potential of monocyte-derived EVs,[215] most likely by impeding TF-induced thrombin generation.[219,220] FVIIa also mediates procoagulant TF+-EV release by ECs *in vitro*[221] and *in vivo* in wild type mice.[222,223]

Interestingly, the OSE-targeting natural IgM antibody LR04 inhibits EV-mediated propagation of coagulation irrespective of the extrinsic and intrinsic coagulation pathway by preventing factor Xa assembly on MDA+ EVs.[100]

Factors mediating the procoagulatory potential EVs

PS-bearing EV-release and TF-expression both have been suggested to relate to cholesterol availability and subsequent PS and TF localization to lipid rafts.[220,224,225] Monocyte-derived EVs have much higher concentration of TF and PSGL-1 than their parental cells, but no enrichment of CD45. TF and PSGL-1, but not CD45, accumulate in lipid rafts and depletion of membrane cholesterol hinders their release.[220] The cytoskeletal protein filamin-A could be essential for TF localization to lipid rafts.[224,226]

Another regulatory mechanism of cellular and hence lEV-associated TF and PS surface-expression is proposed to relate to intergrin-arf6 mediated TF and PS internalization, which is inhibited by ATP.[227] Likewise, various pro-inflammatory stimuli like IL-33, TNF, oxLDL and also EVs released by stimulated cells promote the release of procoagulant, TF-enriched EVs from vascular or plaque related cells.[86,228-231] Interestingly, TF can also be transferred between

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different cell types via EVs and enhance the procoagulant state of the recipient cell, albeit this phenomenon is usually reported for EVs derived from activated cells.[91,220,232-234]

Altogether, the data suggest that EV-associated promotion of coagulation occurs especially in a pro-inflammatory setting, as it would be the case in the plaque. Indeed, when compared to arterial wall lEVs[235] or plasma lEVs,[67] plaque derived lEVs prove to have much higher thrombogenicity. Curiously, there was no significant difference in TF-expression between EVs isolated from plasma or plaque material, or between EVs extracted from asymptomatic and symptomatic plaques. Furthermore, there was no difference in thrombin-generating activity of EVs from asymptomatic and symptomatic plaques.[67]

EVs in plaque erosion

In the context of plaque erosion, EVs released by activated cells enhance TF exposure and thrombogenicity of ECs.[91,234] Large EVs released by stimulated ECs promote neutrophil adhesion and their release of NETs.[79] Furthermore, myeloperoxidase-bearing neutrophil-derived EVs can cause endothelial injury *in vitro*,[236] and EVs from activated monocytes, platelets and ECs can further contribute to EC apoptosis and pyroptosis[87,91,237,238] or ward it off.[239] Once EC integrity has been lost, the subendothelial matrix provides a new platform for thrombus formation. Platelet-lEVs may bind to fibrinogen, fibronectin and collagen-coated surfaces, but not to monolayers of neither unstimulated nor stimulated ECs.[240] Collagen,[80,81] as well as thrombin,[80,241] activate platelets and lead to their shedding of platelet aggregation-promoting EVs, further propagating the pro-thrombotic process.

CONCLUSION

EVs participate in intercellular signaling at all stages of ASCVD. They relay parental cell and environmental information by exposing and transferring membrane-associated components (e.g., integrins, modified phospholipids, tissue factor), and by sharing their cytosolic cargo (e.g., cytokines, miRNAs). Furthermore, they serve as a catalyzing platform for extracellular processes like calcification, coagulation and fibrinolysis. Although most evidence illustrates EVs as amplifiers of ASCVD, atheroprotective functions have been demonstrated in selective experimental settings, in particular, regarding EVs from ECs. Current insights are mostly derived from *in vitro* experiments, while existing *in vivo* studies rely on artificial EV-isolation and injection of concentrated amounts of EVs and may obscure or skew relevant biological effects. In this context, the diversity of current isolation methods, and the consequent heterogeneity in purity and subtype composition of EV isolates, still pose obstacles in uncovering the exact pathophysiological role of EVs in ASCVD. Although comprehensive and extensive EV-research is being facilitated by emerging "-omics" technologies and advances in single-EV analysis, the crucial task of distinguishing biologically relevant effects from experimentally introduced alterations should not be dismissed. Compliance with the standardized MISEV guidelines and detailed reporting of experimental methods is paramount to overcoming these potential challenges. In specific settings, however, if analyses of EVs (e.g., derived from tissue or body fluids) reproducibly convey diagnostic or prognostic information, artificial effects from isolation procedures may be less important. Potential EV-related biomarkers of ASCVD have already evolved, but need to be validated in large multicentric clinical trials to demonstrate their methodological reproducibility and clinical benefit. Lastly, natural and bioengineered EVs have emerged as potential therapies, for instance, by conferring protective effects of their cells of origin (e.g., stem or progenitor

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cells), or serving as vehicles for drug delivery. Clearly, research on EVs in ASCVD is rapidly evolving and bears many challenges, but with the promise of great rewards within reach.

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SUPPLEMENTARY MATERIAL

Supplementary Table 1

Overview of mouse studies investigating the effect of EV administration on atherosclerosis

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