

AperTO - Archivio Istituzionale Open Access dell'Università di Torino

Role of Extracellular Vesicles in the Pathogenesis of Vascular Damage

This is the author's manuscript

Original Citation:

Availability:

This version is available <http://hdl.handle.net/2318/1863498> since 2022-06-06T08:04:21Z

Published version:

DOI:10.1161/HYPERTENSIONAHA.121.17957

Terms of use:

Open Access

Anyone can freely access the full text of works made available as "Open Access". Works made available under a Creative Commons license can be used according to the terms and conditions of said license. Use of all other works requires consent of the right holder (author or publisher) if not exempted from copyright protection by the applicable law.

(Article begins on next page)

1 **ROLE OF EXTRACELLULAR VESICLES IN THE PATHOGENESIS OF VASCULAR DAMAGE**

2 Fabrizio Buffolo^{1,3}, Silvia Monticone¹, Giovanni Camussi², Elena Aikawa^{3,4*}

3 ¹Division of Internal Medicine and Hypertension Unit, Department of Medical Sciences,
4 University of Torino, 10126, Torino, Italy (F.B., S.M.)

5 ²Department of Medical Sciences, Molecular Biotechnology Center, University of Torino,
6 10126, Torino, Italy (G.C.).

7 ³Center for Interdisciplinary Cardiovascular Sciences, Department of Cardiovascular
8 Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02115,
9 USA (F.B, E.A.)

10 ⁴Center for Excellence in Vascular Biology, Department of Cardiovascular Medicine,
11 Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02115, USA (E.A.)

12 **Running title:** Vesicles in vascular damage.

13 ***Correspondence to:** Elena Aikawa, MD, PhD

14 Brigham and Women's Hospital, Harvard Medical School

15 3 Blackfan Street, Boston, MA 02115

16 Phone: +1 617-730-7755, Fax: +1 617-730-7791

17 E-mail: eaikawa@bwh.harvard.edu

18

19 **Word count:** 5078 (excluded reference and figure legends)

20 **Key words:** extracellular vesicles, endothelial dysfunction, vascular inflammation,
21 atherosclerosis, vascular calcification

22

1 **Abstract**

2 Extracellular vesicles (EVs) are nano-sized membrane-bound structures released by cells that
3 are able to transfer nucleic acids, protein cargos and metabolites to specific recipient cells,
4 allowing cell-to-cell communications in an endocrine and paracrine manner. Endothelial,
5 leukocyte and platelet-derived EVs have emerged both as biomarkers and key effectors in the
6 development and progression of different stages of vascular damage, from earliest alteration
7 of endothelial function, to advanced atherosclerotic lesions and cardiovascular calcification.
8 Under pathological conditions, circulating EVs, promote endothelial dysfunction by
9 impairing vasorelaxation and instigate vascular inflammation by increasing levels of adhesion
10 molecules, reactive oxygen species and pro-inflammatory cytokines. Platelets, endothelial
11 cells, macrophages and foam cells secrete EVs that regulate macrophage polarization and
12 contribute to atherosclerotic plaque progression. Finally, under pathological stimuli, smooth
13 muscle cells and macrophages secrete EVs that aggregate between collagen fibers and serve
14 as nucleation sites for ectopic mineralization in the vessel wall, leading to formation of
15 micro- and macrocalcification. In this review, we summarize the emerging evidence of the
16 pathological role of EVs in vascular damage, highlighting the major findings from the most
17 recent studies and discussing future perspectives in this research field.

18

1 **Introduction**

2 Cardiovascular disease, causing more than 17.9 million deaths per year and accounting for 31%
3 of global mortality, represents the leading cause of death worldwide¹. Cardiovascular events
4 are the consequence of vascular damage, a continuum of pathological alterations, ranging from
5 early endothelial dysfunction to calcific atherosclerotic lesions, caused by several established
6 risk factors, including arterial hypertension, diabetes, metabolic syndrome, ageing, smoking
7 and physical inactivity².

8 Extracellular vesicles (EVs) are particles loaded with nucleic acids, proteins and metabolites,
9 protected by an outer lipid membrane. According to their biogenesis, EVs can be categorized
10 into two main subgroups: exosomes and microvesicles³. Exosomes are generated by inward
11 budding of the endosomal membrane, forming intraluminal vesicles that are packaged in
12 multivesicular bodies and then released into the extracellular space through fusion with the
13 plasma membrane; microvesicles are generated by outward budding and fission of plasma
14 membrane. EVs are heterogeneous in size ranging from 30-150 nm of exosomes to 100-1000
15 nm of microvesicles³. The latter include vesicles of small size released by normal cells also
16 named ectosomes, and larger apoptotic vesicles released by damaged cells.

17 Since their first description in the late 60s,⁴ extensive knowledge has been gained on the role
18 of EVs in human physiology and disease. Far from being simple biomarkers of cellular injury,
19 EVs released into the extracellular space can mediate cell-to-cell communication and regulate
20 biological processes by means of RNA and protein transfer into recipient cells³.

21 In the cardiovascular field, EVs have been shown to play a dual role: a protective and
22 therapeutic role, with a beneficial effect on vascular function, depending on their cellular origin
23 and cargo⁵ as well as pathological role as mediators contributing to the initiation and
24 progression of vascular damage, from earliest to latest stages⁶.

1 In this review, we will focus on the pathological role of EVs in vascular damage, from the
2 earliest stages of endothelial dysfunction to vascular inflammation, initiation and progression
3 of atherosclerosis, fibrosis and calcification.

4

5 **Extracellular vesicles, endothelial dysfunction and oxidative stress**

6 *Endothelial dysfunction*

7 The endothelium plays a crucial role in maintaining vascular homeostasis and regulating the
8 delicate balance between vasoconstriction and vasorelaxation. Under physiological conditions,
9 the equilibrium is maintained by the release of endothelium-derived relaxing factors (e.g., nitric
10 oxide - NO -, prostaglandins, endothelium-dependent hyperpolarization factors) and
11 endothelium-derived contracting factors⁷. The reduction of endothelium-derived relaxing
12 factors is the main driver of endothelial dysfunction and is considered the initial step of
13 atherosclerosis, the underlying pathology of cardiovascular disease⁸.

14 Increased circulating levels of endothelial-derived EVs have been associated with endothelial
15 dysfunction in different physiological and pathological conditions, including physical
16 inactivity, obesity, diabetes, chronic kidney disease (CKD), pre-eclampsia and coronary artery
17 disease⁹. Several *in vitro* and *in vivo* studies suggest that EVs, beyond their role as biomarkers
18 of impaired endothelial function, can interact directly with the endothelium and play a central
19 role in promoting cellular dysfunction.

20 Despite significant heterogeneity in both cell treatment conditions and isolation protocols, it
21 has been consistently demonstrated through *in vitro* studies that, under pathological conditions,
22 circulating EVs impair vasorelaxation through the reduction of NO bioavailability. The effect
23 is mediated by inhibition of endothelial NO-synthase (eNOS) through activation of
24 ERK1/ERK2 signaling^{10,11} and enhancement of phosphoinositide (PI) 3-kinase pathway¹¹.

1 Similar findings were obtained from studies with animal models, recapitulating hypertensive
2 and hyperglycemic conditions. The results of these studies showed that circulating EVs can
3 harbor and transfer specific molecules, whose concentration is modified by pathological
4 conditions, ultimately altering endothelial and vascular function of recipient vessels, by direct
5 and indirect mechanisms⁹.

6 High blood pressure levels increase the concentration of angiotensin-converting enzyme (ACE)
7 in EVs secreted by adventitial fibroblasts in spontaneously hypertensive rats. Transfer of ACE
8 in smooth muscles cells (SMCs) enhance the ACE activity and angiotensin-II (Ang II)
9 concentration in recipient cells¹². In part by these pathways, adventitial-derived EVs and
10 circulating EVs from hypertensive rats can modulate the vascular function at different levels:
11 by regulating SMCs proliferation, impairing vascular remodeling^{12,13} and affecting
12 endothelium-dependent vasodilatation¹⁴.

13 Studies on animal models that mimic diabetes-induced endothelial dysfunction, showed similar
14 results, although mediated by different mechanisms. Circulating EVs, collected from diabetic
15 mice, display higher levels of arginase 1 (Arg1) than EVs from normoglycemic controls. Arg1
16 converts L-arginine to urea and L-ornithine, reducing L-arginine bioavailability, the substrate
17 for NO production. Hence, transfer of Arg1 to endothelial cells by circulating EVs results in
18 NO reduction and impaired vasorelaxation¹⁵.

19 Translational studies, investigating the effects of human circulating EVs on endothelial cell
20 function, corroborated the hypothesis derived from animal studies, underlying the importance
21 of comorbidities in the determination of EV content and effects. Circulating EVs of patients
22 with metabolic syndrome reduce NO bioavailability via eNOS phosphorylation in endothelial
23 cells¹⁶. In CKD, circulating EVs reduce acetylcholine mediated vasorelaxation, as assessed by
24 reduction of cGMP in recipient endothelial cells¹⁷. Similarly, circulating EVs from patients
25 with myocardial infarction, but not from controls, reduce acetylcholine mediated

1 vasorelaxation^{18,19} by lowering levels of eNOS through ERK1/ERK2 and PI-3 kinase
2 pathways²⁰.

3

4 *Oxidative stress*

5 Oxidative stress plays a critical role in endothelial dysfunction by direct impairment of NO
6 availability²¹. Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase increases
7 oxidative stress, reduces NO availability and weakens endothelium-dependent
8 vasorelaxation²². Similar to the findings on endothelial dysfunction, the evidence of EV role in
9 the regulation of vascular oxidative stress is derived from *in vitro*, animal and translational
10 studies.

11 Under hyperglycemic conditions, cultured endothelial cells release EVs with high levels of
12 reactive oxygen species (ROS) and increased NADPH oxidase activity²³. Similarly, proteomic
13 analysis of endothelial derived EVs exposed to high glucose concentrations, showed a
14 modification of their protein cargo and increased EV-mediated oxidative stress²⁴. The increase
15 in ROS concentration enhances p38 phosphorylation in endothelial recipient cells and activate
16 the endothelial layer²³.

17 Analogous effects of EV-mediated oxidative stress have been shown in ApoE-deficient mice
18 fed a high fat diet. In this animal model, high-glucose-stimulated endothelial EVs demonstrated
19 impaired endothelial function, increased macrophage infiltration and enhanced adhesion
20 protein expression in atherosclerotic lesions by ROS-dependent mechanisms²³.

21 Finally, a translational study showed that EVs from patients with acute coronary syndrome are
22 able to increase oxidative stress in recipient endothelial cells, through upregulation of the
23 p22^{phox} NADPH oxidase subunit²⁰, with consequent redox-sensitive activation of mitogen-
24 activated protein (MAP) kinases and ERK1/ERK2 phosphorylation. These effects are partially
25 abolished by inhibition of renin-angiotensin system²⁰.

1 Taken together these data indicate that EVs released under various pathological conditions,
2 may represent endocrine and paracrine mediators able to promote endothelial dysfunction and
3 vascular oxidative stress through various arrays of different pathways. However, although pre-
4 clinical evidence are relatively robust, translational studies are currently limited, involving only
5 few of the many pathological conditions that can alter endothelial function through EV-
6 mediated mechanisms. Moreover, most of the studies evaluated the effects of circulating EV
7 from patients with late-stage disease, such as myocardial infarction and end stage CKD.
8 Whether EVs from patients with the earliest stages of cardiovascular disease harbor the same
9 content and biological properties of EVs from patients with advanced cardiovascular disease
10 remains to be elucidated. The answer to these timely questions is pivotal for future
11 prioritization of potential targets to prevent and treat cardiovascular disease.

12

13 **Extracellular vesicles and vascular inflammation**

14 Accumulating evidence indicates that the inflammatory response, involving both the innate and
15 the adaptative immunity, plays a pivotal role in initiation of atherosclerosis and its
16 complications²⁵. Inflammatory response is now recognized as valuable target for the reduction
17 of cardiovascular events, as demonstrated by the efficacy of colchicine and the IL-1 β inhibitor
18 canakinumab for secondary prevention^{26,27}.

19 Circulating EVs released from different cell types can modulate leukocyte-endothelium
20 interaction and actively participate in the vascular inflammatory response, through diverse and
21 multifaceted mechanisms, including transferring of miRNA, proteins or phospholipids to target
22 cells. Most of the available scientific reports focus on EVs released by monocyte, neutrophils
23 and platelets that seem to act as mediators of vascular inflammation at different levels,
24 including endothelial activation, leukocyte adhesion and diapedesis.

1 *In vitro*, activated monocyte-derived EVs stimulate by autocrine mechanisms the production
2 of pro-inflammatory cytokines, including tumor necrosis factor alpha (TNF- α) and interleukin
3 6 (IL-6), and activation of nuclear factor kappa-light-chain enhancer of activated B cells (NF-
4 κ B)²⁸. Consistently, stimulation of endothelial cells by activated monocyte-derived EVs, leads
5 to NF- κ B activation and expression of several adhesion molecules and pro-inflammatory
6 cytokines, including intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion
7 molecule-1 (VCAM-1), E-selectin, C-C Motif Chemokine Ligand 2 (CCL2) and IL-6^{29,30}. The
8 effects are mediated by mechanisms involving IL-1 and NLR family pyrin domain containing
9 3 (NLRP3) and modulation of mi-RNA cargos. In particular, in monocyte-derived EVs,
10 inflammatory stimuli increase miR-155 and reduce miR-223³⁰ that exhibit opposite effects at
11 vascular level. While mi-R155 stimulates inflammation and atherogenesis, miR-233 displays
12 anti-inflammatory effects by reducing IL-6 and IL-1 β in macrophages and ICAM-1 expression
13 in endothelial cells^{31,32}. Therefore, the combined effect of inflammatory molecules and
14 miRNAs contributes to the pleiotropic effects of monocyte-derived EVs in vasculature.

15 Similar to monocyte EVs, environmental conditions regulate the content and biological
16 properties of neutrophil-derived EVs that have been shown to enhance endothelial
17 inflammation and contribute to atherogenesis. A crucial mechanism is played again by miR-
18 155, transferred from neutrophil-derived EVs to endothelial cells in atheroprone sites³³, where
19 a high ICAM-1 expression promotes EVs adhesion via CD18 binding³³. Under basal
20 conditions, neutrophil-derived EVs display anti-inflammatory effects promoting cells adhesion
21 properties³⁴. On the opposite side, following inflammatory stimuli, neutrophil-derived EVs
22 increase the production of pro-inflammatory cytokines in endothelial cells³⁵. Beyond
23 endothelial activation, neutrophil-derived EVs enhance monocyte adhesion to the endothelial
24 layer and monocyte transmigration by CCL-2 mediated mechanism³³. This is induced by a
25 direct and preferential effect on endothelial cells, rather than on monocyte, suggesting that the

1 EV-mediated cross-talk between neutrophils and endothelium is pivotal for the regulation of
2 leukocyte infiltration in the vascular wall³³.

3 The effects of platelet-derived EVs on the different steps of vascular inflammation have been
4 extensively studied. Platelet-derived EVs regulate endothelial activation and production of
5 inflammatory molecules in both endothelial cells and monocytes^{36,37}. Pathological conditions
6 can alter the cargos of platelet-derived EVs, particularly at miRNA level. MiR-320b, whose
7 transcription is reduced in patients with myocardial infarction, promotes the transcription and
8 production of ICAM-1 in endothelial recipient cells. Therefore, the reduction of miR-320b can
9 contribute to the activation of endothelium with enhanced leukocyte adhesion and diapedesis³⁷.

10 Platelet derived-EVs favor rolling of neutrophils and monocytes on endothelial surface and the
11 interaction between flowing leukocyte and rolling leukocyte, in a P-selectin mediated
12 manner³⁸⁻⁴⁰. This effect is provided by direct transfer of IL-1 β and chemokine (C-C motif)
13 ligand 5 (CCL5) through activated platelet-derived EVs to endothelial cells^{40,41}. Leukocytes
14 then become firmly adhered to endothelial cells through CXC receptor-chemokine interaction,
15 which is enhanced by platelet-derived EVs through activation of both leukocytes and
16 endothelial cells³⁹.

17 Beyond the traditional regulators of endothelial function, other cells can modulate the
18 activation of endothelium by the release of EVs that acts through endocrine mechanisms. In
19 particular, under hypoxic and inflammatory conditions, adipose cells release EVs that increase
20 VCAM-1 and leukocyte adhesiveness of endothelial recipient cells⁴². Moreover, endothelial
21 cell-derived EVs can regulate the activation of endothelial layer by autocrine and paracrine
22 action. Specific environmental conditions (e.g., hypoxia, inflammatory stimuli, hyperglycemia,
23 oxidative stress) alter the proteins, lipids and RNAs carried by endothelial-derived EVs^{43,44}. In
24 particular, under the effect of ROS, endothelial cells release EVs containing pro-inflammatory

1 oxidized phospholipids that stimulate the adhesion of monocyte to endothelial cells with
2 consequent induction of vascular inflammation⁴⁴.

3 In conclusion, several pro-inflammatory stimuli and stressors can modify cargos and biological
4 properties of EVs that can act in an autocrine, paracrine and endocrine fashion, regulating the
5 multiple steps of vascular inflammation. Although the effects of EVs released by various cell
6 types have been investigated in multiple *in vitro* studies, translational research is currently
7 limited. Future studies should explore the effects of specific pathological conditions and
8 diseases in animal models to better understand the diagnostic and therapeutic potential of *in*
9 *vitro* findings.

10 Moreover, most of the studies evaluated the effects of EVs from a single cell type. However,
11 given the variety of cells that contribute to the regulation of vascular inflammation by EV-
12 mediated mechanisms, future efforts should be devoted investigating the pleiotropic and
13 simultaneous effects of EVs from multiple cell types. The synergic effect of EVs from multiple
14 cell sources could unravel novel mechanisms widening the spectrum of EV-mediated effects
15 in vasculature.

16

17 **Extracellular vesicles and atherosclerosis**

18 Infiltration of low-density lipoproteins (LDL) in the subendothelial space is the cornerstone of
19 the initiation of the atherosclerotic process. Exposure of the vessel wall to chronically high
20 circulating LDL via an altered endothelial barrier results in the deposition of LDL in the intima
21 layer⁴⁵. Increase of ROS in the subendothelial space leads to oxidation of LDL with oxidized
22 LDL (ox-LDL) formation. Ox-LDL facilitate the differentiation of monocytes into
23 macrophages that express high levels of scavenger receptors for LDL⁴⁶. Scavenger receptor
24 binds to and uptakes ox-LDL in macrophages by phagocytosis and pinocytosis, leading to a

1 vicious cycle of cholesterol ester accumulation in the form of cytoplasmatic lipid droplets⁴⁵.
2 This process, together with an impairment of the export mechanism of cholesterol mediated by
3 ATP-binding cassette (ABC) transporters (ABCA1 and ABCG1), leads to the formation of
4 lipid-laden foam cells in the atherosclerotic plaque⁴⁶.
5 EVs from different sources, including platelets, endothelial cells, monocytes, macrophages,
6 SMCs and adipose tissue play an important role in the regulation of atherosclerotic plaque
7 development.

8 Platelet-derived EVs can promote the initiation and progression of atherosclerotic lesion in the
9 arterial wall at different steps⁴⁷. Activated platelet-derived EVs increase the internalization of
10 ox-LDL in macrophages, secretion of pro-inflammatory cytokines and formation of foam
11 cells⁴⁸. Moreover, in the latest stage of the atherosclerotic disease, platelet-derived EVs can
12 promote thrombus formation after the rupture or erosion of the atherosclerotic plaque. In fact,
13 platelet-derived EVs harbor negatively charged phosphatidylserine that directly enhances the
14 aggregation of prothrombin complexes and activate the intrinsic and extrinsic coagulation
15 pathways^{47,49,50}. The cross-talk between monocytes and platelets is pivotal for the modulation
16 of EV release by both cell types, and strongly affect their biological properties⁵¹.
17 Monocyte/platelet aggregates are associated with cardiovascular disease and hypertension^{52,53}.
18 Under inflammatory stimuli, their interaction promotes the release of EVs with pro-atherogenic
19 properties and long-lasting effects at vascular levels⁵¹. These effects can be blunted by the
20 inhibition of platelet activation with acetylsalicylic acid, P2Y12 inhibitor and iloprost⁵¹.

21 Endothelial EVs are crucial in the development of the atherosclerotic processes, and local pro-
22 atherogenic stimuli can alter endothelial EV release, regulating endothelial and macrophage
23 function locally and at distant sites. *In vitro*, Ox-LDL and IL-6 increase packaging of miR-92a-
24 3p in EVs secreted by endothelial cells, which in turn activates endothelial proliferation and
25 angiogenesis. In humans, miR-92a-3p is increased in endothelial-derived circulating EVs from

1 patients with coronary artery disease, indicating that this pathway is particularly relevant in
2 patients with cardiovascular disease⁵⁴.

3 Endothelial EVs modulate macrophage polarization in opposing directions depending on
4 environmental stimuli. Macrophages polarize towards two different phenotypes: “classic” pro-
5 inflammatory phenotype (M1), associated with atherosclerotic progression and foam cells
6 formation and proliferation, or anti-inflammatory phenotype (M2), associated with anti-
7 atherogenic properties⁵⁵. Ox-LDL-treated endothelial cells release EVs that polarize
8 macrophages toward M1 phenotype. On the other hand, stimulation with Kruppel like factor 2
9 (KLF2), a critical regulator of the anti-inflammatory response in atherosclerotic plaque,
10 induces EV production from endothelial cells that stimulate M2 polarization⁵⁶. Ox-LDL treated
11 endothelial cell derived-EVs contain low levels of Metastasis Associated Lung
12 Adenocarcinoma Transcript 1 (MALAT1)⁵⁷, a long non-coding RNA (lncRNA) that promotes
13 M2 polarization of macrophages, with consequent increase of inflammation and foam cell
14 formation⁵⁸. Moreover, reduction of MALAT1 in endothelial-derived EVs leads to elevated
15 ROS production and dendritic cell maturation through nuclear factor erythroid 2-related factor
16 2 (Nrf2) signaling, further contributing to plaque progression⁵⁷.

17 The effect of macrophage-derived EVs in the atherosclerosis is mainly played at local and
18 paracrine level, regulating the delicate balance between macrophage recruitment and migration
19 and the phenotype switch of SMCs, through paracrine cross-talk between macrophages, foam
20 cells and SMCs. In macrophages, ox-LDL alters mi-RNA content of EVs, increasing miR-146a
21 concentration that in turns inhibits macrophage migratory capacity and promote lipid-laden
22 macrophage engulfment⁵⁹. On the other hand, foam cells of macrophage origin release EVs
23 that enhance SMC migration and intimal adhesion by integrin transfer and activation ERK/Akt
24 pathway⁶⁰. SMCs that migrate from the media into the intima layer undergo a phenotypic
25 switch towards a macrophage-like phenotype and accumulation of oxidized-lipid, contributing

1 to foam cells formation and atherosclerotic plaque progression⁶¹. Ultimately, the progression
2 of atherosclerotic plaque is characterized by the development of a necrotic core, driven by
3 apoptosis of macrophages, SMCs and endothelial cells and by impaired efferocytosis, the
4 process of dead cell clearance⁴⁵. Macrophage-derived EVs enhance macrophage and
5 endothelial cell apoptosis by transferring of lncRNA GAS5, thus contributing to the formation
6 and development of the necrotic core⁶².

7 In metabolic syndrome a relevant role is played by adipose tissue release of circulating EVs,
8 whose content and biological properties are regulated by environmental conditions and food
9 intake. In high fat-induced conditions, adipose tissue-derived EVs favor M1 transition and
10 atherosclerotic plaque progression through NF- κ B activation⁵⁵. Moreover, under a high-fat
11 diet adipose tissue-derived EVs contribute to engorgement of macrophages by down-
12 regulation of ABCA1 and ABCG1, with consequent reduction of cholesterol export and
13 increased foam cell development⁴⁶. Finally, circulating EVs of patients with metabolic
14 syndrome increase SMC proliferation and migration⁶³. This effect seems to be mediated by
15 ras-associated protein-1 (Rap1), a protein that is notably increased in EVs of patients with
16 metabolic syndrome compared with controls, and its levels correlate with body mass index,
17 and waist and hip circumference⁶³.

18 In conclusion, EVs from different sources modulate the atherosclerotic process by a complex
19 interplay of multiple pathways. Given the multifaceted nature of the atherosclerotic plaque
20 that involves several cell type function in a complex and dynamic processes, traditional tools
21 of *in vitro* cultures and assays may be limited. The study of Oggero and colleagues⁵¹
22 demonstrated how the interaction between different cell types may alter EV content and their
23 biological effects. At the same time, EVs from the same source can activate different
24 pathways in different recipient cells. It is hard to define by traditional and simplistic models
25 the final effects of this complex network. *Ex vivo* organ culture approach and three-

1 dimensional (3D) models that better recapitulate the morphological complexity of the
2 atherosclerotic plaque may represent the future directions to address this unmet need.
3 Moreover, the replication of the organ/tissue properties by 3D-bioprinting may expand the
4 applicability of these models for the discoveries of specific therapeutic candidates targeting
5 EVs and cells.

6

7 **Extracellular vesicles and vascular fibrosis and calcification**

8 *Fibrosis*

9 Collagen and elastin are the main components of vascular extracellular matrix (ECM). Type I
10 and type III collagen fibers are the most abundant types of collagens in large and medium
11 arteries, representing together more than 90% of fibrillary collagen. Vascular wall of large
12 arteries is also rich in elastin, and the balance between collagen and elastin plays a pivotal role
13 in the development of arterial stiffening⁶⁴. Turnover of collagen and elastin is slow and mainly
14 regulated by matrix metalloproteinases (MMPs) and elastolytic enzymes (cathepsins), involved
15 in collagen and elastin degradation⁶⁴. Endothelial-derived EVs contain several matrix-
16 degrading proteins, including MMPs⁶⁵. Pro-inflammatory and pro-thrombotic conditions
17 increase MMP-10 in endothelial EVs⁶⁶, and endothelial EVs increase and activate MMP-2,
18 mediating ECM remodeling⁶⁷.

19 Lysyl oxidase (LOX) and lysyl oxidase-like (LOX-L) enzymes are members of a family of
20 proteins directly involved in ECM regulation, modulating the cross-linking of collagen and
21 elastin fibers. LOXL-2 is present in endothelial EVs and localized on the surface membrane of
22 EVs directly interacting with ECM components. Hypoxic conditions increase LOXL-2
23 expression in endothelial EVs and LOX enzymatic activity, explaining how environmental
24 conditions may influence ECM remodeling through EV-mediated pathways⁴³. Patients with

1 atherosclerotic cerebrovascular disease display higher levels of LOXL-2 in circulating
2 endothelial EVs⁶⁸.

3

4 *Calcification*

5 Two main mechanistic initiators trigger and drive vascular calcification in humans:
6 hyperphosphatemia in CKD and chronic inflammation. Although these two pathological
7 conditions are mechanistically different, they can coexist and in some cases exert their action
8 in a synergistic manner⁶⁹.

9 In CKD, hyperphosphatemia leads to the mineralization of the media layer with gross and
10 aligned mineral deposit among elastin fibers (Monckeberg's syndrome), independently of
11 atherosclerotic plaque formation⁷⁰. Hyperphosphatemic calcification is faster than
12 inflammatory-driven calcification, both in humans with CKD and in experimental animal
13 models⁶⁹.

14 Inflammatory-driven vascular calcification is characteristically localized within the intima of
15 atherosclerotic plaque⁷¹. Calcifying EVs (100-300 nm) released from macrophages and SMCs
16 act as nucleating foci of mineralization within the plaque, leading to the formation of spherical
17 or ellipsoidal microcalcifications that later merge, forming large macrocalcifications (≥ 50
18 μm)⁷¹. The role of microcalcification and macrocalcification in the vascular wall and plaque
19 stability is radically different. Macrocalcification is usually localized in a deep portion of the
20 plaque, and while it reduces vascular wall compliance, it may stabilize the plaque⁷². On the
21 other hand, microcalcification is destabilizing, particularly when located in the thin fibrous cap.
22 Microcalcifications increase atherosclerotic plaque vulnerability by accumulation of high local
23 stress around their poles. Particularly, microcalcifications between 5 and 30 μm in diameter
24 are considered to be the most harmful⁷³.

1 EVs aggregate and nucleate mineral and then merge in the plaque forming microcalcification
2 in the gaps between collagen fibers, with an inverse correlation between collagen density and
3 microcalcification size⁷⁴. Mechanisms underlying EV aggregation are only partially
4 understood. Some authors proposed that interactions between negatively charged EVs and
5 extracellular components may be involved, especially in the context of high matrix turnover⁷⁵.
6 EV surface may interact with fibronectin or collagen by integrin binding^{76,77}, interacting with
7 specific collagen sequences⁷⁷. Annexin A1 (ANXA1) can directly contribute to EV-EV
8 tethering and aggregation, and actively promotes nucleation of mineralizing foci⁷⁸. Moreover,
9 in pro-inflammatory conditions with high organic phosphate, ANXA1 facilitate the
10 development of microcalcifications by enhanced tissue nonspecific alkaline phosphatase
11 (TNAP) activity⁷⁸.

12 TNAP is an enzyme that converts inorganic pyrophosphate into free phosphate⁷⁹. It is loaded
13 into EVs secreted by macrophages and SMCs, and its role is critical in inflammatory-driven
14 osteogenic calcification in atherosclerotic plaques⁷⁹. On the contrary, hyperphosphatemic
15 calcification in CKD is largely TNAP-independent and driven by TNAP-negative SMC-
16 derived EVs⁸⁰. In CKD, inorganic phosphate enter the cell by endocytosis and is actively
17 shuttled outside the cells by SMC-derived EVs, in a TNAP-independent process⁸¹.

18 Sortilin is a multiligand sorting receptor that exerts multiple roles, including regulation of
19 inflammation and lipid metabolism⁸². Higher circulating sortilin levels are associated with
20 increased rate of cardio-cerebrovascular events and aortic calcification⁸³. Under osteogenic
21 conditions, sortilin enhances the loading of activated TNAP in SMC-derived EVs in a Rab-11-
22 dependent pathway, enhancing inflammatory-driven calcification⁸⁴. C-terminal serine
23 phosphorylation of sortilin and sortilin dimerization by intermolecular disulfide bonds are two
24 essential steps for sortilin loading in EVs⁸⁵.

1 Macrophage-derived EVs directly contribute to osteogenic calcification through loading of
2 annexin A5–S100A9 complex in EVs that interacts with phosphatidylserine and acts as
3 nucleation site for mineralization⁸⁶ in CKD environment. S100A9 is also increased in
4 macrophages from patients with type 1 diabetes⁸⁷, and high glucose increases release of
5 calcifying EVs from macrophages through S100A9 signaling⁸⁸, suggesting a crucial role of
6 S100A9 in diabetes- and CKD-induced calcification.

7 Ectopic vascular calcification is the consequence of an impaired balance between calcification
8 promoting factors (e.g., bone morphogenic proteins, NF-κB) and inhibitors (e.g., matrix Gla
9 protein (MGP), fetuin-A, osteopontin, osteoprotegerin)⁸⁹. SMC-derived EVs are
10 physiologically loaded with calcification inhibitors, including MGP and fetuin-A^{80,90}.
11 However, in hyperphosphatemic condition loading of fetuin-A and MGP in EVs is reduced,
12 favoring EV-mediated calcification^{80,90}. Fetuin-A is a glycoprotein produced and secreted by
13 the liver⁹¹, internalized by SMCs and loaded into EVs in a sphingomyelin phosphodiesterase 3
14 (SMPD3)-dependent process⁸¹. Alteration of extracellular calcium and phosphate increases
15 SMPD3 and consequent release of calcifying EVs by SMCs⁸¹.

16 Warfarin, beyond the known anticoagulant effect, promotes and accelerates vascular
17 calcification⁹². The calcification inhibitor MGP is activated by carboxylation of glutamate
18 residues in a vitamin-K dependent manner. Carboxylation and activation of MGP is therefore
19 inhibited by warfarin administration⁹². Similarly to MGP, prothrombin harbors glutamate
20 residues, and it is loaded to SMC-derived EVs via two distinct pathways: multivesicular bodies
21 and membrane budding⁹². Prothrombin loading into EVs inhibits EV-induced calcification,
22 suggesting that warfarin's pro-calcifying effect is partially mediated through inhibition of
23 prothrombin carboxylation⁹². Recent evidence suggests that retinoid acids are able to inhibit
24 vascular calcification by increasing MGP in SMCs. Moreover, all trans-retinoic acid decrease
25 TNAP activity in SMC-derived EVs, further contributing to calcification inhibition⁹³.

1 In summary, the calcification process at vascular levels is driven by activation of multiple
2 pathways, differentially enhanced by various underlying drivers. Several cells have been
3 proved to be crucial in the inflammatory driven process. Macrophages are pivotal in the earliest
4 step (initiation) of the calcification process, releasing calcifying EVs and promoting SMCs
5 osteogenic differentiation. Macrophages and SMCs contribute together to the progression of
6 the calcification process, where inflammation and calcification advance in parallel. Finally, in
7 the late stage of disease, inflammation is substantially diminished and abundant calcification
8 is virtually irreversible⁹⁴. The cross-talk between cells is crucial in each step, but particularly
9 in the initiation and propagation stages. Studies adopting co-culture of macrophages, SMCs
10 and endothelial cells are warranted to understand the complex interplay between cells and
11 regulation of EV release under co-stimulation of several cell types. 3D-bioprinting of cellular
12 hydrogels that mimic the biomechanical properties of aortic valve tissue have been adopted to
13 investigate the biomechanics of calcifying valves⁹⁵. Moreover, acellular 3D models
14 recapitulating calcification in atherosclerotic plaque have been proved to be valuable tools for
15 studying EV-dependent mineralization^{74,96}. The use of these tools should be implemented for
16 the investigation of the complex interaction between cells, EVs and ECM in the atherosclerotic
17 plaque and arterial wall, helping the identification of potential targets to inhibit, decelerate or
18 prevent the complex calcification processes.

19

20 **Conclusions and perspectives**

21 Considerable progress has been made in recent years in understanding the role of EVs in both
22 physiological and pathological regulation of vascular homeostasis and disease, shedding light
23 on EV involvement in various stages of vascular damage. Despite the flourishing research in

1 the field, what is known appears to be only the tip of the iceberg of a much more complex
2 and multifaceted roles played by EVs.

3 Discrepancy in isolation techniques and EVs characterization among studies make it difficult
4 to distinguish between different EVs subpopulations and their relative contribution to the
5 development and progression of vascular damage. Several studies have demonstrated that EVs
6 of different size and/or density display important dissimilarity in content and biological
7 properties. Some isolation methods allow the discrimination of EV subpopulations. Sucrose-
8 based or iodixanol-based density gradient can differentiate EVs according to their relative
9 density, while size-exclusion chromatography discriminates particles and EVs based on their
10 size. Recently, a “single EVs microarray” approach has been proposed for the discrimination
11 of exosome and microvesicles, on the basis of their protein content, at single EV level⁷⁸. The
12 application of these methods to the future basic and translational research could unravel the
13 fine mechanisms driving EV-mediated cardiovascular damage.

14 Translational studies, linking *in vitro* evidence to clinical data are currently limited and
15 should be implemented in the near future. Moreover, the effects of circulating EVs in the
16 different stages of disease have been poorly investigated. The content and biological
17 properties of EVs evolve with worsening and progression of the underlying conditions and
18 risk factors. Understanding the dynamic changes of EV cargos and their effects on
19 pathological processes is crucial for the selection of potential therapeutic targets in timely and
20 tailored fashion.

21 ‘Omics’ data of human-derived EVs from biofluids and tissues could help in this process and
22 guide future pre-clinical studies in a focused and personalized direction. Multi-omics is
23 becoming an appealing strategy for the detection of potential target in cardiovascular
24 disease⁹⁴, and the implementation of this approach at EV level would provide important
25 insights in the comprehension of EV cargos and biological properties in different settings of

1 cardiovascular disease. However, integration of data from transcriptomic, proteomic and
2 metabolomic could be a challenge. Bioinformatic tools and artificial intelligence will play a
3 crucial role in the next years for the management and interpretation of big data derived by
4 multi-omics studies. Pathway and network analysis have been adopted in several studies for
5 the selection of specific drug target^{51,78,97}. However, the final prioritization of the targets is
6 often driven by currently available literature and scientists' research interest. The
7 implementation of machine-learning approaches could overcome these biases by application
8 of unbiased selection guided by integration of pre-clinical and clinical data⁷¹.

9 In conclusion, although slightly decreased in the last decade in high-income countries,
10 mortality for cardiovascular disease is still the leading cause for global deaths worldwide,
11 particularly impacting mid- and low-income countries⁴⁵. Extraordinary efforts are requested
12 to better elucidate the mechanism underlying vascular damage from the earliest to the latest
13 stages of disease; therefore, future research on EV role in this mission will be crucial.

14

15 **Acknowledgments**

16 None.

17

18 **Disclosures.**

19 None.

20

21 **Funding**

22 E.A. laboratory is supported by National Institutes of Health [grant numbers R01HL136431,
23 R01HL147095, and R01HL141917].

24

1 **Figure Legends**

2 **Figure. Pathological role of extracellular vesicles in different stages of vascular damage**

3 Extracellular vesicles (EVs) released by different cells under several pathological conditions
4 mediates initiation and propagation of vascular damage, from endothelial dysfunction to
5 vascular inflammation, atherosclerosis, alteration of extracellular matrix composition and
6 vascular calcification. NO=nitric oxide, NADPH=nicotinamide adenine dinucleotide
7 phosphate, ROS=reactive oxygen species, Rap1=ras-associated protein-1, SMC=smooth
8 muscle cell, LDL=low-density lipoproteins, ERK=extracellular signal-regulated kinases,
9 NFκB=nuclear factor kappa-light-chain-enhancer of activated B cells, TNAP= tissue
10 nonspecific alkaline phosphatase, MGP=matrix Gla protein, ECM=extracellular matrix.
11 Some illustrations of the Figure were prepared using Motifolio drawing toolkit.

12

13

14 **References**

- 15 1. World Health Organization. Cardiovascular diseases (CVDs) Fact Sheet. Published
16 online 2017.
- 17 2. Virani SS, Alonso A, Benjamin EJ, Bittencourt MS, Callaway CW, Carson AP,
18 Chamberlain AM, Chang AR, Cheng S, Delling FN, et al. Heart Disease and Stroke
19 Statistics-2020 Update: A Report From the American Heart Association. *Circulation*.
20 2020;141(9):e139-e596. doi:10.1161/CIR.0000000000000757
- 21 3. van Niel G, D'Angelo G, Raposo G. Shedding light on the cell biology of extracellular
22 vesicles. *Nat Rev Mol Cell Biol*. 2018;19(4):213-228. doi:10.1038/nrm.2017.125
- 23 4. Wolf P. The nature and significance of platelet products in human plasma. *Br J*
24 *Haematol*. 1967;13(3):269-288. doi:10.1111/j.1365-2141.1967.tb08741.x
- 25 5. Jansen F, Li Q, Pfeifer A, Werner N. Endothelial- and Immune Cell-Derived
26 Extracellular Vesicles in the Regulation of Cardiovascular Health and Disease. *JACC:*
27 *Basic to Translational Science*. 2017;2(6):790-807. doi:10.1016/j.jacbts.2017.08.004
- 28 6. Boulanger CM, Loyer X, Rautou P-E, Amabile N. Extracellular vesicles in coronary
29 artery disease. *Nat Rev Cardiol*. 2017;14(5):259-272. doi:10.1038/nrcardio.2017.7

- 1 7. Godo S, Shimokawa H. Endothelial Functions. *Arterioscler Thromb Vasc Biol.*
2 2017;37(9):e108-e114. doi:10.1161/ATVBAHA.117.309813
- 3 8. Ungvari Z, Tarantini S, Kiss T, Wren JD, Giles CB, Griffin CT, Murfee WL, Pacher P,
4 Csiszar A. Endothelial dysfunction and angiogenesis impairment in the ageing
5 vasculature. *Nat Rev Cardiol.* 2018;15(9):555-565. doi:10.1038/s41569-018-0030-z
- 6 9. Liu M-L, Williams KJ. Microvesicles: potential markers and mediators of endothelial
7 dysfunction. *Curr Opin Endocrinol Diabetes Obes.* 2012;19(2):121-127.
8 doi:10.1097/MED.0b013e32835057e9
- 9 10. Taguchi K, Hida M, Narimatsu H, Matsumoto T, Kobayashi T. Glucose and angiotensin
10 II-derived endothelial extracellular vesicles regulate endothelial dysfunction via ERK1/2
11 activation. *Pflugers Arch.* 2017;469(2):293-302. doi:10.1007/s00424-016-1926-2
- 12 11. Mastronardi ML, Mostefai HA, Soleti R, Agouni A, Martínez MC, Andriantsitohaina R.
13 Microparticles from apoptotic monocytes enhance nitrosative stress in human
14 endothelial cells. *Fundam Clin Pharmacol.* 2011;25(6):653-660. doi:10.1111/j.1472-
15 8206.2010.00898.x
- 16 12. Tong Y, Ye C, Ren X-S, Qiu Y, Zang Y-H, Xiong X-Q, Wang J-J, Chen Q, Li Y-H,
17 Kang Y-M, Zhu G-Q. Exosome-Mediated Transfer of ACE (Angiotensin-Converting
18 Enzyme) From Adventitial Fibroblasts of Spontaneously Hypertensive Rats Promotes
19 Vascular Smooth Muscle Cell Migration. *Hypertension.* 2018;72(4):881-888.
20 doi:10.1161/HYPERTENSIONAHA.118.11375
- 21 13. Ren X-S, Tong Y, Qiu Y, Ye C, Wu N, Xiong X-Q, Wang J-J, Han Y, Zhou Y-B,
22 Zhang F, et al. MiR155-5p in adventitial fibroblasts-derived extracellular vesicles
23 inhibits vascular smooth muscle cell proliferation via suppressing angiotensin-
24 converting enzyme expression. *J Extracell Vesicles.* 2020;9(1):1698795.
25 doi:10.1080/20013078.2019.1698795
- 26 14. Good ME, Musante L, La Salvia S, Howell NL, Carey RM, Le TH, Isakson BE,
27 Erdbrügger U. Circulating Extracellular Vesicles in Normotension Restrain
28 Vasodilation in Resistance Arteries. *Hypertension.* 2020;75(1):218-228.
29 doi:10.1161/HYPERTENSIONAHA.119.13363
- 30 15. Zhang H, Liu J, Qu D, Wang L, Wong CM, Lau C-W, Huang Y, Wang YF, Huang H,
31 Xia Y, et al. Serum exosomes mediate delivery of arginase 1 as a novel mechanism for
32 endothelial dysfunction in diabetes. *Proc Natl Acad Sci USA.* 2018;115(29):E6927-
33 E6936. doi:10.1073/pnas.1721521115
- 34 16. Agouni A, Lagrue-Lak-Hal AH, Ducluzeau PH, Mostefai HA, Draunet-Busson C,
35 Leftheriotis G, Heymes C, Martinez MC, Andriantsitohaina R. Endothelial dysfunction
36 caused by circulating microparticles from patients with metabolic syndrome. *Am J*
37 *Pathol.* 2008;173(4):1210-1219. doi:10.2353/ajpath.2008.080228
- 38 17. Amabile N, Guérin AP, Leroyer A, Mallat Z, Nguyen C, Boddart J, London GM,
39 Tedgui A, Boulanger CM. Circulating endothelial microparticles are associated with
40 vascular dysfunction in patients with end-stage renal failure. *J Am Soc Nephrol.*
41 2005;16(11):3381-3388. doi:10.1681/ASN.2005050535

- 1 18. Boulanger Chantal M., Scoazec Alexandra, Ebrahimian Talin, Henry Patrick, Mathieu
2 Eric, Tedgui Alain, Mallat Ziad. Circulating Microparticles From Patients With
3 Myocardial Infarction Cause Endothelial Dysfunction. *Circulation*. 2001;104(22):2649-
4 2652. doi:10.1161/hc4701.100516
- 5 19. Brodsky SV, Zhang F, Nasjletti A, Goligorsky MS. Endothelium-derived microparticles
6 impair endothelial function in vitro. *Am J Physiol Heart Circ Physiol*.
7 2004;286(5):H1910-1915. doi:10.1152/ajpheart.01172.2003
- 8 20. Abbas Malak, Jesel Laurence, Auger Cyril, Amoura Lamia, Messas Nathan, Manin
9 Guillaume, Rumig Cordula, León-González Antonio J., Ribeiro Thais P., Silva Grazielle
10 C., et al. Endothelial Microparticles From Acute Coronary Syndrome Patients Induce
11 Premature Coronary Artery Endothelial Cell Aging and Thrombogenicity. *Circulation*.
12 2017;135(3):280-296. doi:10.1161/CIRCULATIONAHA.116.017513
- 13 21. Adler A, Messina E, Sherman B, Wang Z, Huang H, Linke A, Hintze TH. NAD(P)H
14 oxidase-generated superoxide anion accounts for reduced control of myocardial O₂
15 consumption by NO in old Fischer 344 rats. *Am J Physiol Heart Circ Physiol*.
16 2003;285(3):H1015-1022. doi:10.1152/ajpheart.01047.2002
- 17 22. Donato AJ, Eskurza I, Silver AE, Levy AS, Pierce GL, Gates PE, Seals DR. Direct
18 evidence of endothelial oxidative stress with aging in humans: relation to impaired
19 endothelium-dependent dilation and upregulation of nuclear factor-kappaB. *Circ Res*.
20 2007;100(11):1659-1666. doi:10.1161/01.RES.0000269183.13937.e8
- 21 23. Jansen F, Yang X, Franklin BS, Hoelscher M, Schmitz T, Bedorf J, Nickenig G, Werner
22 N. High glucose condition increases NADPH oxidase activity in endothelial
23 microparticles that promote vascular inflammation. *Cardiovasc Res*. 2013;98(1):94-106.
24 doi:10.1093/cvr/cvt013
- 25 24. Burger D, Turner M, Xiao F, Munkonda MN, Akbari S, Burns KD. High glucose
26 increases the formation and pro-oxidative activity of endothelial microparticles.
27 *Diabetologia*. 2017;60(9):1791-1800. doi:10.1007/s00125-017-4331-2
- 28 25. Libby P, Ridker PM, Hansson GK, Leducq Transatlantic Network on Atherothrombosis.
29 Inflammation in atherosclerosis: from pathophysiology to practice. *J Am Coll Cardiol*.
30 2009;54(23):2129-2138. doi:10.1016/j.jacc.2009.09.009
- 31 26. Ridker PM, Everett BM, Thuren T, MacFadyen JG, Chang WH, Ballantyne C, Fonseca
32 F, Nicolau J, Koenig W, Anker SD, et al. Antiinflammatory Therapy with Canakinumab
33 for Atherosclerotic Disease. *N Engl J Med*. 2017;377(12):1119-1131.
34 doi:10.1056/NEJMoa1707914
- 35 27. Tardif J-C, Kouz S, Waters DD, Bertrand OF, Diaz R, Maggioni AP, Pinto FJ, Ibrahim
36 R, Gamra H, Kiwan GS, et al. Efficacy and Safety of Low-Dose Colchicine after
37 Myocardial Infarction. *N Engl J Med*. 2019;381(26):2497-2505.
38 doi:10.1056/NEJMoa1912388
- 39 28. Bardelli C, Amoruso A, Federici Canova D, Fresu L, Balbo P, Neri T, Celi A,
40 Brunelleschi S. Autocrine activation of human monocyte/macrophages by monocyte-

- 1 derived microparticles and modulation by PPAR γ ligands. *Br J Pharmacol*.
2 2012;165(3):716-728. doi:10.1111/j.1476-5381.2011.01593.x
- 3 29. Wang J-G, Williams JC, Davis BK, Jacobson K, Doerschuk CM, Ting JP-Y, Mackman
4 N. Monocytic microparticles activate endothelial cells in an IL-1 β -dependent manner.
5 *Blood*. 2011;118(8):2366-2374. doi:10.1182/blood-2011-01-330878
- 6 30. Tang N, Sun B, Gupta A, Rempel H, Pulliam L. Monocyte exosomes induce adhesion
7 molecules and cytokines via activation of NF- κ B in endothelial cells. *FASEB J*.
8 2016;30(9):3097-3106. doi:10.1096/fj.201600368RR
- 9 31. Chen Q, Wang H, Liu Y, Song Y, Lai L, Han Q, Cao X, Wang Q. Inducible microRNA-
10 223 down-regulation promotes TLR-triggered IL-6 and IL-1 β production in
11 macrophages by targeting STAT3. *PLoS ONE*. 2012;7(8):e42971.
12 doi:10.1371/journal.pone.0042971
- 13 32. Tabet F, Vickers KC, Cuesta Torres LF, Wiese CB, Shoucri BM, Lambert G, Catherinet
14 C, Prado-Lourenco L, Levin MG, Thacker S, et al. HDL-transferred microRNA-223
15 regulates ICAM-1 expression in endothelial cells. *Nat Commun*. 2014;5:3292.
16 doi:10.1038/ncomms4292
- 17 33. Gomez I, Ward B, Souilhol C, Recarti C, Ariaans M, Johnston J, Burnett A, Mahmoud
18 M, Luong LA, West L, et al. Neutrophil microvesicles drive atherosclerosis by
19 delivering miR-155 to atheroprone endothelium. *Nat Commun*. 2020;11(1):214.
20 doi:10.1038/s41467-019-14043-y
- 21 34. Dalli J, Norling LV, Renshaw D, Cooper D, Leung K-Y, Perretti M. Annexin 1
22 mediates the rapid anti-inflammatory effects of neutrophil-derived microparticles.
23 *Blood*. 2008;112(6):2512-2519. doi:10.1182/blood-2008-02-140533
- 24 35. Mesri M, Altieri DC. Endothelial cell activation by leukocyte microparticles. *J*
25 *Immunol*. 1998;161(8):4382-4387.
- 26 36. Nomura S, Tandon NN, Nakamura T, Cone J, Fukuhara S, Kambayashi J. High-shear-
27 stress-induced activation of platelets and microparticles enhances expression of cell
28 adhesion molecules in THP-1 and endothelial cells. *Atherosclerosis*. 2001;158(2):277-
29 287. doi:10.1016/s0021-9150(01)00433-6
- 30 37. Gidlöf O, van der Brug M, Ohman J, Gilje P, Olde B, Wahlestedt C, Erlinge D. Platelets
31 activated during myocardial infarction release functional miRNA, which can be taken
32 up by endothelial cells and regulate ICAM1 expression. *Blood*. 2013;121(19):3908-
33 3917, S1-26. doi:10.1182/blood-2012-10-461798
- 34 38. Forlow SB, McEver RP, Nollert MU. Leukocyte-leukocyte interactions mediated by
35 platelet microparticles under flow. *Blood*. 2000;95(4):1317-1323.
- 36 39. Kuravi SJ, Harrison P, Rainger GE, Nash GB. Ability of Platelet-Derived Extracellular
37 Vesicles to Promote Neutrophil-Endothelial Cell Interactions. *Inflammation*.
38 2019;42(1):290-305. doi:10.1007/s10753-018-0893-5

- 1 40. Mause SF, von Hundelshausen P, Zerneck A, Koenen RR, Weber C. Platelet
2 microparticles: a transcellular delivery system for RANTES promoting monocyte
3 recruitment on endothelium. *Arterioscler Thromb Vasc Biol.* 2005;25(7):1512-1518.
4 doi:10.1161/01.ATV.0000170133.43608.37
- 5 41. Lindemann S, Tolley ND, Dixon DA, McIntyre TM, Prescott SM, Zimmerman GA,
6 Weyrich AS. Activated platelets mediate inflammatory signaling by regulated
7 interleukin 1beta synthesis. *J Cell Biol.* 2001;154(3):485-490.
8 doi:10.1083/jcb.200105058
- 9 42. Wadey RM, Connolly KD, Mathew D, Walters G, Rees DA, James PE. Inflammatory
10 adipocyte-derived extracellular vesicles promote leukocyte attachment to vascular
11 endothelial cells. *Atherosclerosis.* 2019;283:19-27.
12 doi:10.1016/j.atherosclerosis.2019.01.013
- 13 43. de Jong OG, van Balkom BWM, Gremmels H, Verhaar MC. Exosomes from hypoxic
14 endothelial cells have increased collagen crosslinking activity through up-regulation of
15 lysyl oxidase-like 2. *J Cell Mol Med.* 2016;20(2):342-350. doi:10.1111/jcmm.12730
- 16 44. Huber J, Vales A, Mitulovic G, Blumer M, Schmid R, Witztum JL, Binder BR,
17 Leitinger N. Oxidized membrane vesicles and blebs from apoptotic cells contain
18 biologically active oxidized phospholipids that induce monocyte-endothelial
19 interactions. *Arterioscler Thromb Vasc Biol.* 2002;22(1):101-107.
20 doi:10.1161/hq0102.101525
- 21 45. Libby P, Buring JE, Badimon L, Hansson GK, Deanfield J, Bittencourt MS, Tokgözoğlu
22 L, Lewis EF. Atherosclerosis. *Nat Rev Dis Primers.* 2019;5(1):56. doi:10.1038/s41572-
23 019-0106-z
- 24 46. Yu X-H, Fu Y-C, Zhang D-W, Yin K, Tang C-K. Foam cells in atherosclerosis. *Clin*
25 *Chim Acta.* 2013;424:245-252. doi:10.1016/j.cca.2013.06.006
- 26 47. Wang Z-T, Wang Z, Hu Y-W. Possible roles of platelet-derived microparticles in
27 atherosclerosis. *Atherosclerosis.* 2016;248:10-16.
28 doi:10.1016/j.atherosclerosis.2016.03.004
- 29 48. Feng C, Chen Q, Fan M, Guo J, Liu Y, Ji T, Zhu J, Zhao X. Platelet-derived
30 microparticles promote phagocytosis of oxidized low-density lipoprotein by
31 macrophages, potentially enhancing foam cell formation. *Ann Transl Med.*
32 2019;7(18):477. doi:10.21037/atm.2019.08.06
- 33 49. Sinauridze EI, Kireev DA, Popenko NY, Pichugin AV, Panteleev MA, Krymskaya OV,
34 Ataulkhanov FI. Platelet microparticle membranes have 50- to 100-fold higher
35 specific procoagulant activity than activated platelets. *Thromb Haemost.*
36 2007;97(3):425-434.
- 37 50. Van Der Meijden PEJ, Van Schilfgaarde M, Van Oerle R, Renné T, ten Cate H, Spronk
38 HMH. Platelet- and erythrocyte-derived microparticles trigger thrombin generation via
39 factor XIIa. *J Thromb Haemost.* 2012;10(7):1355-1362. doi:10.1111/j.1538-
40 7836.2012.04758.x

- 1 51. Oggero S, de Gaetano M, Marcone S, Fitzsimons S, Pinto AL, Ikramova D, Barry M,
2 Burke D, Montero-Melendez T, Cooper D, et al. Extracellular vesicles from
3 monocyte/platelet aggregates modulate human atherosclerotic plaque reactivity. *J*
4 *Extracell Vesicles*. 2021;10(6):12084. doi:10.1002/jev2.12084
- 5 52. Czepluch FS, Kuschicke H, Dellas C, Riggert J, Hasenfuss G, Schäfer K. Increased
6 proatherogenic monocyte-platelet cross-talk in monocyte subpopulations of patients
7 with stable coronary artery disease. *J Intern Med*. 2014;275(2):144-154.
8 doi:10.1111/joim.12145
- 9 53. Gkaliagkousi E, Gavriilaki E, Triantafyllou A, Douma S. Clinical Significance of
10 Endothelial Dysfunction in Essential Hypertension. *Curr Hypertens Rep*.
11 2015;17(11):85. doi:10.1007/s11906-015-0596-3
- 12 54. Liu Y, Li Q, Hosen MR, Zietzer A, Flender A, Levermann P, Schmitz T, Frühwald D,
13 Goody P, Nickenig G, Werner N, Jansen F. Atherosclerotic Conditions Promote the
14 Packaging of Functional MicroRNA-92a-3p Into Endothelial Microvesicles. *Circ Res*.
15 2019;124(4):575-587. doi:10.1161/CIRCRESAHA.118.314010
- 16 55. Xie Z, Wang X, Liu X, Du H, Sun C, Shao X, Tian J, Gu X, Wang H, Tian J, Yu B.
17 Adipose-Derived Exosomes Exert Proatherogenic Effects by Regulating Macrophage
18 Foam Cell Formation and Polarization. *J Am Heart Assoc*. 2018;7(5).
19 doi:10.1161/JAHA.117.007442
- 20 56. He S, Wu C, Xiao J, Li D, Sun Z, Li M. Endothelial extracellular vesicles modulate the
21 macrophage phenotype: Potential implications in atherosclerosis. *Scand J Immunol*.
22 2018;87(4):e12648. doi:10.1111/sji.12648
- 23 57. Li H, Zhu X, Hu L, Li Q, Ma J, Yan J. Loss of exosomal MALAT1 from ox-LDL-
24 treated vascular endothelial cells induces maturation of dendritic cells in atherosclerosis
25 development. *Cell Cycle*. 2019;18(18):2255-2267. doi:10.1080/15384101.2019.1642068
- 26 58. Huang C, Han J, Wu Y, Li S, Wang Q, Lin W, Zhu J. Exosomal MALAT1 derived from
27 oxidized low-density lipoprotein-treated endothelial cells promotes M2 macrophage
28 polarization. *Mol Med Rep*. 2018;18(1):509-515. doi:10.3892/mmr.2018.8982
- 29 59. Nguyen M-A, Karunakaran D, Geoffrion M, Cheng HS, Tandoc K, Perisic Matic L,
30 Hedin U, Maegdefessel L, Fish JE, Rayner KJ. Extracellular Vesicles Secreted by
31 Atherogenic Macrophages Transfer MicroRNA to Inhibit Cell Migration. *Arterioscler*
32 *Thromb Vasc Biol*. 2018;38(1):49-63. doi:10.1161/ATVBAHA.117.309795
- 33 60. Niu C, Wang X, Zhao M, Cai T, Liu P, Li J, Willard B, Zu L, Zhou E, Li Y, Pan B,
34 Yang F, Zheng L. Macrophage Foam Cell-Derived Extracellular Vesicles Promote
35 Vascular Smooth Muscle Cell Migration and Adhesion. *J Am Heart Assoc*. 2016;5(10).
36 doi:10.1161/JAHA.116.004099
- 37 61. Bennett MR, Sinha S, Owens GK. Vascular Smooth Muscle Cells in Atherosclerosis.
38 *Circ Res*. 2016;118(4):692-702. doi:10.1161/CIRCRESAHA.115.306361

- 1 62. Chen L, Yang W, Guo Y, Chen W, Zheng P, Zeng J, Tong W. Exosomal lncRNA
2 GAS5 regulates the apoptosis of macrophages and vascular endothelial cells in
3 atherosclerosis. *PLoS ONE*. 2017;12(9):e0185406. doi:10.1371/journal.pone.0185406
- 4 63. Perdomo L, Vidal-Gómez X, Soletti R, Vergori L, Duluc L, Chwastyniak M, Bisselier
5 M, Le Lay S, Villard A, Simard G, et al. Large Extracellular Vesicle-Associated Rap1
6 Accumulates in Atherosclerotic Plaques, Correlates With Vascular Risks and Is
7 Involved in Atherosclerosis. *Circ Res*. 2020;127(6):747-760.
8 doi:10.1161/CIRCRESAHA.120.317086
- 9 64. Jacob MP. Extracellular matrix remodeling and matrix metalloproteinases in the
10 vascular wall during aging and in pathological conditions. *Biomed Pharmacother*.
11 2003;57(5-6):195-202. doi:10.1016/s0753-3322(03)00065-9
- 12 65. Taraboletti G, D'Ascenzo S, Borsotti P, Giavazzi R, Pavan A, Dolo V. Shedding of the
13 matrix metalloproteinases MMP-2, MMP-9, and MT1-MMP as membrane vesicle-
14 associated components by endothelial cells. *Am J Pathol*. 2002;160(2):673-680.
15 doi:10.1016/S0002-9440(10)64887-0
- 16 66. Martínez de Lizarrondo S, Roncal C, Calvayrac O, Rodríguez C, Varo N, Purroy A,
17 Lorente L, Rodríguez JA, Doevre L, Hervás-Stubbs S, et al. Synergistic effect of
18 thrombin and CD40 ligand on endothelial matrix metalloproteinase-10 expression and
19 microparticle generation in vitro and in vivo. *Arterioscler Thromb Vasc Biol*.
20 2012;32(6):1477-1487. doi:10.1161/ATVBAHA.112.248773
- 21 67. Lozito TP, Tuan RS. Endothelial cell microparticles act as centers of matrix
22 metalloproteinase-2 (MMP-2) activation and vascular matrix remodeling. *J Cell*
23 *Physiol*. 2012;227(2):534-549. doi:10.1002/jcp.22744
- 24 68. Goetzl EJ, Schwartz JB, Mustapic M, Lobach IV, Daneman R, Abner EL, Jicha GA.
25 Altered cargo proteins of human plasma endothelial cell-derived exosomes in
26 atherosclerotic cerebrovascular disease. *FASEB J*. 2017;31(8):3689-3694.
27 doi:10.1096/fj.201700149
- 28 69. Hutcheson JD, Blaser MC, Aikawa E. Giving Calcification Its Due: Recognition of a
29 Diverse Disease: A First Attempt to Standardize the Field. *Circ Res*. 2017;120(2):270-
30 273. doi:10.1161/CIRCRESAHA.116.310060
- 31 70. Ix JH, Katz R, Kestenbaum B, Fried LF, Kramer H, Stehman-Breen C, Shlipak MG.
32 Association of mild to moderate kidney dysfunction and coronary calcification. *J Am*
33 *Soc Nephrol*. 2008;19(3):579-585. doi:10.1681/ASN.2007070765
- 34 71. Rogers MA, Aikawa E. Cardiovascular calcification: artificial intelligence and big data
35 accelerate mechanistic discovery. *Nat Rev Cardiol*. 2019;16(5):261-274.
36 doi:10.1038/s41569-018-0123-8
- 37 72. Imoto K, Hiro T, Fujii T, Murashige A, Fukumoto Y, Hashimoto G, Okamura T,
38 Yamada J, Mori K, Matsuzaki M. Longitudinal structural determinants of
39 atherosclerotic plaque vulnerability: a computational analysis of stress distribution using
40 vessel models and three-dimensional intravascular ultrasound imaging. *J Am Coll*
41 *Cardiol*. 2005;46(8):1507-1515. doi:10.1016/j.jacc.2005.06.069

- 1 73. Kelly-Arnold A, Maldonado N, Laudier D, Aikawa E, Cardoso L, Weinbaum S.
2 Revised microcalcification hypothesis for fibrous cap rupture in human coronary
3 arteries. *Proc Natl Acad Sci USA*. 2013;110(26):10741-10746.
4 doi:10.1073/pnas.1308814110
- 5 74. Hutcheson JD, Goettsch C, Bertazzo S, Maldonado N, Ruiz JL, Goh W, Yabusaki K,
6 Faits T, Bouten C, Franck G, et al. Genesis and growth of extracellular-vesicle-derived
7 microcalcification in atherosclerotic plaques. *Nat Mater*. 2016;15(3):335-343.
8 doi:10.1038/nmat4519
- 9 75. Hsu HHT, Tawfik O, Sun F. Mechanism of dystrophic calcification in rabbit aortas:
10 temporal and spatial distributions of calcifying vesicles and calcification-related
11 structural proteins. *Cardiovasc Pathol*. 2004;13(1):3-10. doi:10.1016/S1054-
12 8807(03)00093-0
- 13 76. Sung BH, Ketova T, Hoshino D, Zijlstra A, Weaver AM. Directional cell movement
14 through tissues is controlled by exosome secretion. *Nat Commun*. 2015;6:7164.
15 doi:10.1038/ncomms8164
- 16 77. Hodroge A, Trécherel E, Cornu M, Darwiche W, Mansour A, Ait-Mohand K, Verissimo
17 T, Gomila C, Schembri C, Da Nascimento S, et al. Oligogalacturonic Acid Inhibits
18 Vascular Calcification by Two Mechanisms: Inhibition of Vascular Smooth Muscle Cell
19 Osteogenic Conversion and Interaction With Collagen. *Arterioscler Thromb Vasc Biol*.
20 2017;37(7):1391-1401. doi:10.1161/ATVBAHA.117.309513
- 21 78. Rogers MA, Buffolo F, Schlotter F, Atkins SK, Lee LH, Halu A, Blaser MC, Tsolaki E,
22 Higashi H, Luther K, et al. Annexin A1-dependent tethering promotes extracellular
23 vesicle aggregation revealed with single-extracellular vesicle analysis. *Sci Adv*.
24 2020;6(38). doi:10.1126/sciadv.abb1244
- 25 79. Blaser MC, Aikawa E. Roles and Regulation of Extracellular Vesicles in Cardiovascular
26 Mineral Metabolism. *Front Cardiovasc Med*. 2018;5:187.
27 doi:10.3389/fcvm.2018.00187
- 28 80. Kapustin AN, Davies JD, Reynolds JL, McNair R, Jones GT, Sidibe A, Schurgers LJ,
29 Skepper JN, Proudfoot D, Mayr M, Shanahan CM. Calcium regulates key components
30 of vascular smooth muscle cell-derived matrix vesicles to enhance mineralization. *Circ*
31 *Res*. 2011;109(1):e1-12. doi:10.1161/CIRCRESAHA.110.238808
- 32 81. Kapustin AN, Chatrou MLL, Drozdov I, Zheng Y, Davidson SM, Soong D, Furmanik
33 M, Sanchis P, De Rosales RTM, Alvarez-Hernandez D, et al. Vascular smooth muscle
34 cell calcification is mediated by regulated exosome secretion. *Circ Res*.
35 2015;116(8):1312-1323. doi:10.1161/CIRCRESAHA.116.305012
- 36 82. Goettsch C, Kjolby M, Aikawa E. Sortilin and Its Multiple Roles in Cardiovascular and
37 Metabolic Diseases. *Arterioscler Thromb Vasc Biol*. 2018;38(1):19-25.
38 doi:10.1161/ATVBAHA.117.310292
- 39 83. Goettsch C, Iwata H, Hutcheson JD, O'Donnell CJ, Chapurlat R, Cook NR, Aikawa M,
40 Szulc P, Aikawa E. Serum Sortilin Associates With Aortic Calcification and

- 1 Cardiovascular Risk in Men. *Arterioscler Thromb Vasc Biol.* 2017;37(5):1005-1011.
2 doi:10.1161/ATVBAHA.116.308932
- 3 84. Goettsch C, Hutcheson JD, Aikawa M, Iwata H, Pham T, Nykjaer A, Kjolby M, Rogers
4 M, Michel T, Shibasaki M, et al. Sortilin mediates vascular calcification via its
5 recruitment into extracellular vesicles. *J Clin Invest.* 2016;126(4):1323-1336.
6 doi:10.1172/JCI80851
- 7 85. Itoh S, Mizuno K, Aikawa M, Aikawa E. Dimerization of sortilin regulates its
8 trafficking to extracellular vesicles. *J Biol Chem.* 2018;293(12):4532-4544.
9 doi:10.1074/jbc.RA117.000732
- 10 86. New SEP, Goettsch C, Aikawa M, Marchini JF, Shibasaki M, Yabusaki K, Libby P,
11 Shanahan CM, Croce K, Aikawa E. Macrophage-derived matrix vesicles: an alternative
12 novel mechanism for microcalcification in atherosclerotic plaques. *Circ Res.*
13 2013;113(1):72-77. doi:10.1161/CIRCRESAHA.113.301036
- 14 87. New SEP, Aikawa E. Molecular imaging insights into early inflammatory stages of
15 arterial and aortic valve calcification. *Circ Res.* 2011;108(11):1381-1391.
16 doi:10.1161/CIRCRESAHA.110.234146
- 17 88. Kawakami R, Katsuki S, Travers R, Romero DC, Becker-Greene D, Passos LSA,
18 Higashi H, Blaser MC, Sukhova GK, Buttigieg J, et al. S100A9-RAGE Axis
19 Accelerates Formation of Macrophage-Mediated Extracellular Vesicle
20 Microcalcification in Diabetes Mellitus. *Arterioscler Thromb Vasc Biol.*
21 2020;40(8):1838-1853. doi:10.1161/ATVBAHA.118.314087
- 22 89. Wang Z, Zhang L, Sun Z, Shao C, Li Y, Bao Z, Jing L, Geng Y, Gu W, Pang Q, Li L,
23 Yan J. Mechanisms of Matrix Vesicles Mediating Calcification Transition in Diabetic
24 Plaque. *Heart Lung Circ.* 2020;29(1):112-117. doi:10.1016/j.hlc.2019.04.022
- 25 90. Reynolds JL, Skepper JN, McNair R, Kasama T, Gupta K, Weissberg PL, Jahnen-
26 Dechent W, Shanahan CM. Multifunctional roles for serum protein fetuin-a in inhibition
27 of human vascular smooth muscle cell calcification. *J Am Soc Nephrol.*
28 2005;16(10):2920-2930. doi:10.1681/ASN.2004100895
- 29 91. Triffitt JT, Gebauer U, Ashton BA, Owen ME, Reynolds JJ. Origin of plasma
30 alpha2HS-glycoprotein and its accumulation in bone. *Nature.* 1976;262(5565):226-227.
31 doi:10.1038/262226a0
- 32 92. Kapustin AN, Schoppet M, Schurgers LJ, Reynolds JL, McNair R, Heiss A, Jahnen-
33 Dechent W, Hackeng TM, Schlieper G, Harrison P, Shanahan CM. Prothrombin
34 Loading of Vascular Smooth Muscle Cell-Derived Exosomes Regulates Coagulation
35 and Calcification. *Arterioscler Thromb Vasc Biol.* 2017;37(3):e22-e32.
36 doi:10.1161/ATVBAHA.116.308886
- 37 93. Rogers MA, Chen J, Nallamshetty S, Pham T, Goto S, Muehlschlegel JD, Libby P,
38 Aikawa M, Aikawa E, Plutzky J. Retinoids Repress Human Cardiovascular Cell
39 Calcification With Evidence for Distinct Selective Retinoid Modulator Effects.
40 *Arterioscler Thromb Vasc Biol.* 2020;40(3):656-669.
41 doi:10.1161/ATVBAHA.119.313366

- 1 94. Aikawa E, Blaser MC. 2020 Jeffrey M. Hoeg Award Lecture: Calcifying Extracellular
2 Vesicles as Building Blocks of Microcalcifications in Cardiovascular Disorders.
3 *Arterioscler Thromb Vasc Biol.* 2021;41(1):117-127.
4 doi:10.1161/ATVBAHA.120.314704
- 5 95. van der Valk D, van der Ven C, Blaser M, Grolman J, Wu P-J, Fenton O, Lee L, Tibbitt
6 M, Andresen J, Wen J et al. Engineering a 3D-bioprinted model of human heart valve
7 disease using nanoindentation-based biomechanics. *Nanomaterials.* 2018;8(5):296.
- 8 96. Ruiz JL, Hutcheson JD, Cardoso L, Bakhshian Nik A, Condado de Abreu A, Pham T,
9 Buffolo F, Busatto S, Federici S, Ridolfi A, et al. Nanoanalytical analysis of
10 bisphosphonate-driven alterations of microcalcifications using a 3D hydrogel system
11 and in vivo mouse model. *Proc Natl Acad Sci U S A.* 2021;118(14):e1811725118.
12 doi:10.1073/pnas.1811725118
- 13 97. Schlotter F, Halu A, Goto S, Blaser MC, Body SC, Lee LH, Higashi H, DeLaughter
14 DM, Hutcheson JD, Vyas P, et al. Spatiotemporal Multi-Omics Mapping Generates a
15 Molecular Atlas of the Aortic Valve and Reveals Networks Driving Disease.
16 *Circulation.* 2018;138(4):377-393. doi:10.1161/CIRCULATIONAHA.117.032291
- 17

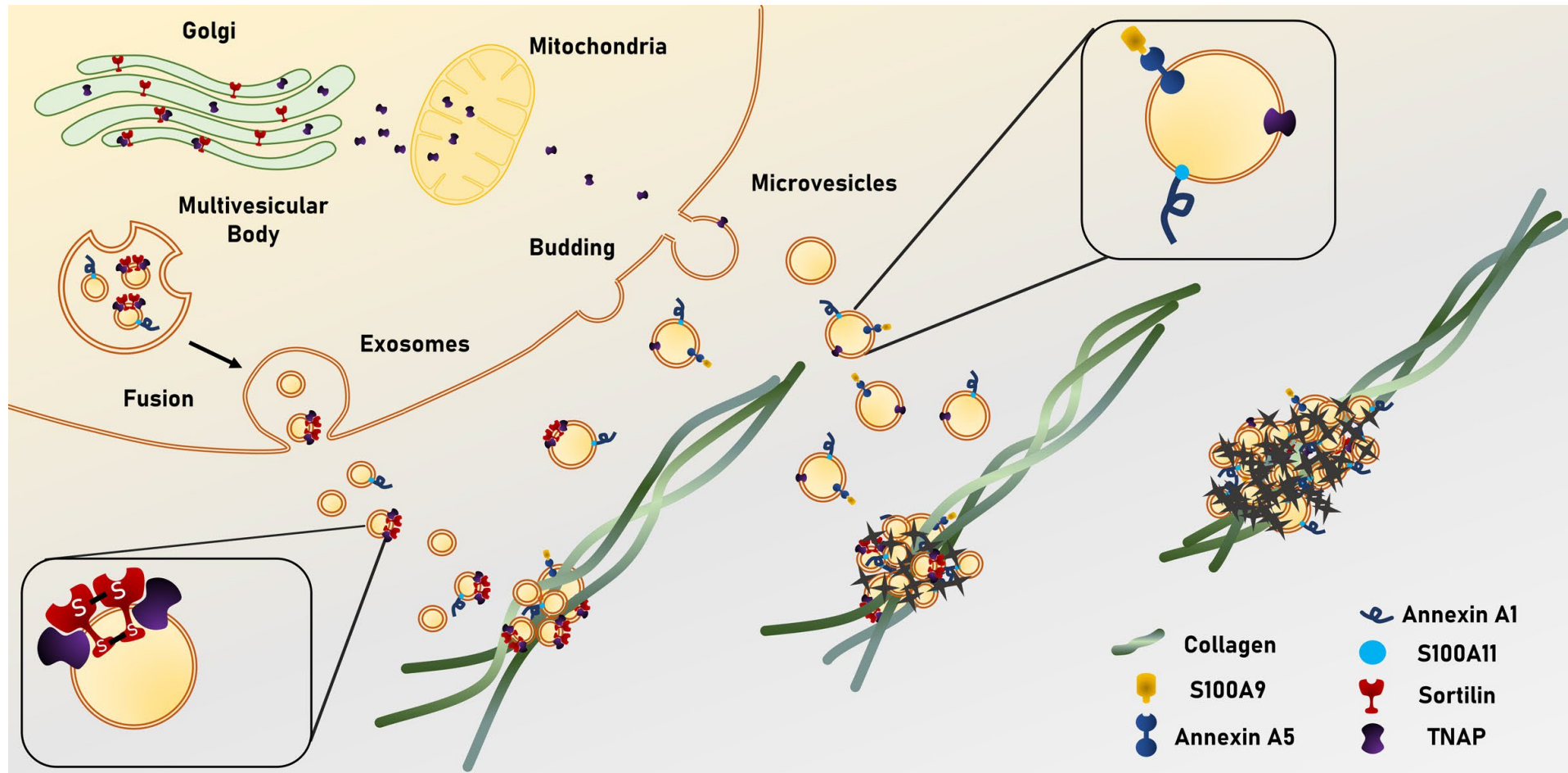


Figure 1. Extracellular vesicles (EVs) biogenesis and role in the development of cardiovascular calcification. EVs are released by cells by 2 mechanisms: direct budding or fission of the plasma membrane, generating microvesicles; multivesicular bodies generation of intracellular vesicles and their release in the extracellular space through fusion with the plasma membrane, generating exosomes. EVs and microvesicles drive calcification process in the arterial wall through multiple pathways, including TNAP (tissue nonspecific alkaline phosphatase) generation of free phosphate, sortilin-mediated loading of TNAP into EVs, annexin A1 tethering of EVs and S100A9 enhancement of EV-mediated ectopic calcification process. Some illustrations of the Figure were prepared using Motifolio drawing toolkit.

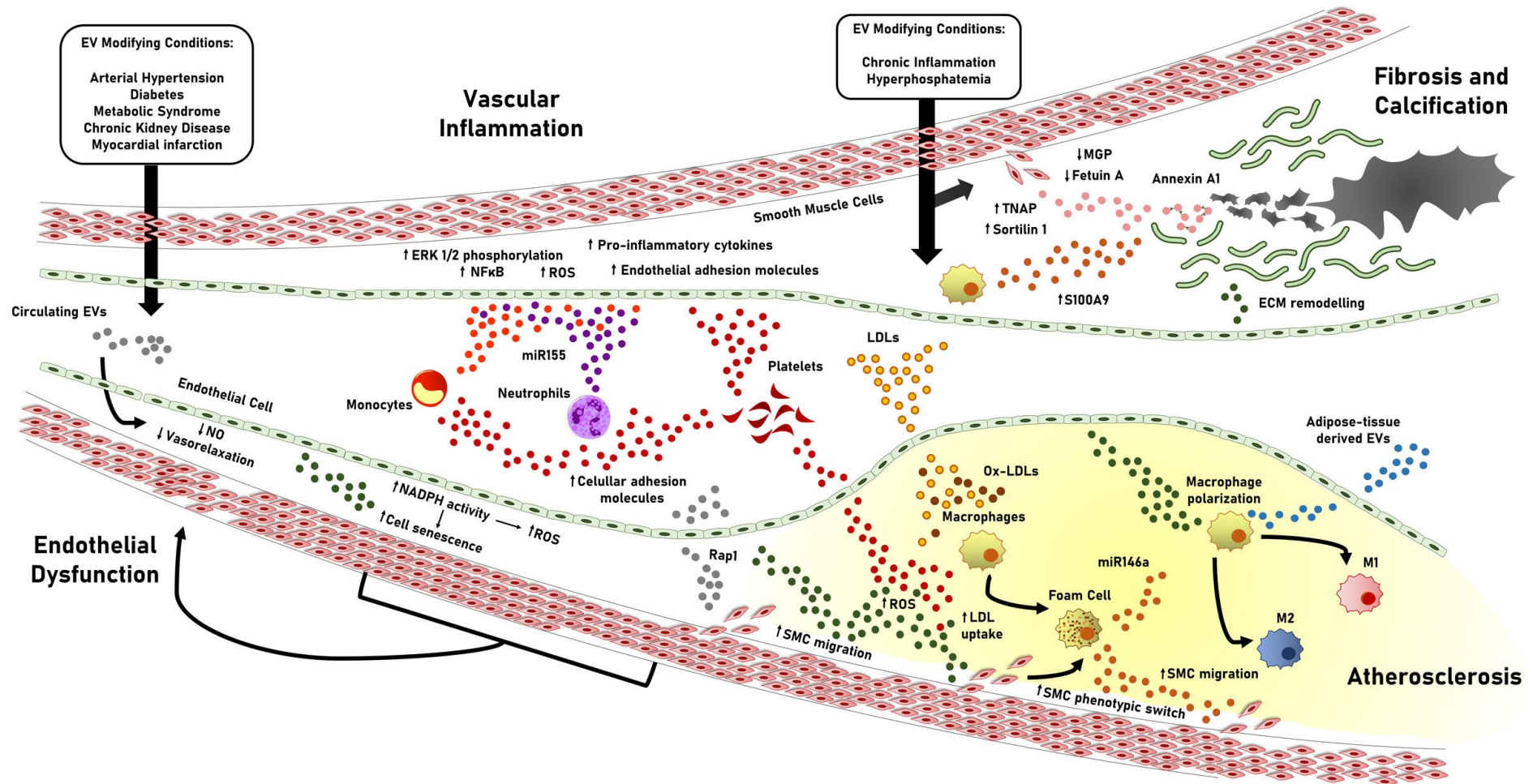


Figure 2. Pathological role of extracellular vesicles (EVs) in different stages of vascular damage. EVs released by different cells under several pathological conditions mediates initiation and propagation of vascular damage, from endothelial dysfunction to vascular inflammation, atherosclerosis, alteration of extracellular matrix composition, and vascular calcification. Some illustrations of the Figure were prepared using Motifolio drawing toolkit. ERK indicates extracellular signal-regulated kinase; LDL, low-density lipoproteins; MGP, matrix Gla protein; NFκB, nuclear factor kappa-light-chain-enhancer of activated B cells; NO, nitric oxide; Rap1, ras-associated protein-1; ROS, reactive oxygen species; SMC, smooth muscle cell; and TNAP, tissue nonspecific alkaline phosphatase.