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1	ROLE OF EXTRACELLULAR VESICLES IN THE PATHOGENESIS OF VASCULAR DAMAGE
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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, leukocyte and platelet-derived EVs have emerged both as biomarkers and key effectors in the 5 6 development and progression of different stages of vascular damage, from earliest alteration 7 of endothelial function, to advanced atherosclerotic lesions and cardiovascular calcification. Under pathological conditions, circulating EVs, promote endothelial dysfunction by 8 9 impairing vasorelaxation and instigate vascular inflammation by increasing levels of adhesion molecules, reactive oxygen species and pro-inflammatory cytokines. Platelets, endothelial 10 cells, macrophages and foam cells secrete EVs that regulate macrophage polarization and 11 contribute to atherosclerotic plaque progression. Finally, under pathological stimuli, smooth 12 muscle cells and macrophages secrete EVs that aggregate between collagen fibers and serve 13 14 as nucleation sites for ectopic mineralization in the vessel wall, leading to formation of micro- and macrocalcification. In this review, we summarize the emerging evidence of the 15 pathological role of EVs in vascular damage, highlighting the major findings from the most 16 17 recent studies and discussing future perspectives in this research field.

1 Introduction

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

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

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

In the cardiovascular field, EVs have been shown to play a dual role: a protective and therapeutic role, with a beneficial effect on vascular function, depending on their cellular origin and cargo⁵ as well as pathological role as mediators contributing to the initiation and progression of vascular damage, from earliest to latest stages⁶. In this review, we will focus on the pathological role of EVs in vascular damage, from the
 earliest stages of endothelial dysfunction to vascular inflammation, initiation and progression
 of atherosclerosis, fibrosis and calcification.

4

5 Extracellular vesicles, endothelial dysfunction and oxidative stress

6 Endothelial dysfunction

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

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

Despite significant heterogeneity in both cell treatment conditions and isolation protocols, it has been consistently demonstrated through *in vitro* studies that, under pathological conditions, circulating EVs impair vasorelaxation through the reduction of NO bioavailability. The effect is mediated by inhibition of endothelial NO-synthase (eNOS) through activation of ERK1/ERK2 signaling^{10,11} and enhancement of phosphoinositide (PI) 3-kinase pathway¹¹. Similar findings were obtained from studies with animal models, recapitulating hypertensive
 and hyperglycemic conditions. The results of these studies showed that circulating EVs can
 harbor and transfer specific molecules, whose concentration is modified by pathological
 conditions, ultimately altering endothelial and vascular function of recipient vessels, by direct
 and indirect mechanisms⁹.

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

Studies on animal models that mimic diabetes-induced endothelial dysfunction, showed similar results, although mediated by different mechanisms. Circulating EVs, collected from diabetic mice, display higher levels of arginase 1 (Arg1) than EVs from normoglycemic controls. Arg1 converts L-arginine to urea and L-ornithine, reducing L-arginine bioavailability, the substrate for NO production. Hence, transfer of Arg1 to endothelial cells by circulating EVs results in 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 vasorelaxation^{18,19} by lowering levels of eNOS through ERK1/ERK2 and PI-3 kinase
 pathways²⁰.

3

4 *Oxidative stress*

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

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

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

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

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

12

13 Extracellular vesicles and vascular inflammation

Accumulating evidence indicates that the inflammatory response, involving both the innate and the adaptative immunity, plays a pivotal role in initiation of atherosclerosis and its complications²⁵. Inflammatory response is now recognized as valuable target for the reduction of cardiovascular events, as demonstrated by the efficacy of colchicine and the IL-1 β inhibitor 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 of pro-inflammatory cytokines, including tumor necrosis factor alpha (TNF- α) and interleukin 2 3 6 (IL-6), and activation of nuclear factor kappa-light-chain enhancer of activated B cells (NF- $(\kappa B)^{28}$. Consistently, stimulation of endothelial cells by activated monocyte-derived EVs, leads 4 to NF-kB activation and expression of several adhesion molecules and pro-inflammatory 5 cytokines, including intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion 6 7 molecule-1 (VCAM-1), E-selectin, C-C Motif Chemokine Ligand 2 (CCL2) and IL-6^{29,30}. The effects are mediated by mechanisms involving IL-1 and NLR family pyrin domain containing 8 3 (NLRP3) and modulation of mi-RNA cargos. In particular, in monocyte-derived EVs, 9 inflammatory stimuli increase miR-155 and reduce miR-223³⁰ that exhibit opposite effects at 10 vascular level. While mi-R155 stimulates inflammation and atherogenesis, miR-233 displays 11 anti-inflammatory effects by reducing IL-6 and IL1-β in macrophages and ICAM-1 expression 12 in endothelial cells^{31,32}. Therefore, the combined effect of inflammatory molecules and 13 miRNAs contributes to the pleiotropic effects of monocyte-derived EVs in vasculature. 14

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

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

3 The effects of platelet-derived EVs on the different steps of vascular inflammation have been extensively studied. Platelet-derived EVs regulate endothelial activation and production of 4 inflammatory molecules in both endothelial cells and monocytes^{36,37}. Pathological conditions 5 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 production of ICAM-1 in endothelial recipient cells. Therefore, the reduction of miR-320b can 8 9 contribute to the activation of endothelium with enhanced leukocyte adhesion and diapedesis³⁷. Platelet derived-EVs favor rolling of neutrophils and monocytes on endothelial surface and the 10 interaction between flowing leukocyte and rolling leukocyte, in a P-selectin mediated 11 manner³⁸⁻⁴⁰. This effect is provided by direct transfer of IL-1β and chemokine (C-C motif) 12 ligand 5 (CCL5) through activated platelet-derived EVs to endothelial cells^{40,41}. Leukocytes 13 14 then become firmly adhered to endothelial cells through CXC receptor-chemokine interaction, which is enhanced by platelet-derived EVs through activation of both leukocytes and 15 endothelial cells³⁹. 16

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

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

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

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

16

17 Extracellular vesicles and atherosclerosis

Infiltration of low-density lipoproteins (LDL) in the subendothelial space is the cornerstone of the initiation of the atherosclerotic process. Exposure of the vessel wall to chronically high circulating LDL via an altered endothelial barrier results in the deposition of LDL in the intima layer⁴⁵. Increase of ROS in the subendothelial space leads to oxidation of LDL with oxidized LDL (ox-LDL) formation. Ox-LDL facilitate the differentiation of monocytes into macrophages that express high levels of scavenger receptors for LDL⁴⁶. Scavenger receptor binds to and uptakes ox-LDL in macrophages by phagocytosis and pinocytosis, leading to a vicious cycle of cholesterol ester accumulation in the form of cytoplasmatic lipid droplets⁴⁵.
This process, together with an impairment of the export mechanism of cholesterol mediated by
ATP-binding cassette (ABC) transporters (ABCA1 and ABCG1), leads to the formation of
lipid-laden foam cells in the atherosclerotic plaque⁴⁶.

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

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

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

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

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

to foam cells formation and atherosclerotic plaque progression⁶¹. Ultimately, the progression of atherosclerotic plaque is characterized by the development of a necrotic core, driven by apoptosis of macrophages, SMCs and endothelial cells and by impaired efferocytosis, the process of dead cell clearance⁴⁵. Macrophage-derived EVs enhance macrophage and endothelial cell apoptosis by transferring of lncRNA GAS5, thus contributing to the formation 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 atherosclerotic plaque progression through NF-KB activation⁵⁵. Moreover, under a high-fat 10 diet adipose tissue-derived EVs contribute to engorgement of macrophages by down-11 regulation of ABCA1 and ABCG1, with consequent reduction of cholesterol export and 12 increased foam cell development⁴⁶. Finally, circulating EVs of patients with metabolic 13 syndrome increase SMC proliferation and migration⁶³. This effect seems to be mediated by 14 ras-associated protein-1 (Rap1), a protein that is notably increased in EVs of patients with 15 metabolic syndrome compared with controls, and its levels correlate with body mass index, 16 and waist and hip circumference ⁶³. 17

In conclusion, EVs from different sources modulate the atherosclerotic process by a complex 18 interplay of multiple pathways. Given the multifaceted nature of the atherosclerotic plaque 19 that involves several cell type function in a complex and dynamic processes, traditional tools 20 of *in vitro* cultures and assays may be limited. The study of Oggero and colleagues ⁵¹ 21 22 demonstrated how the interaction between different cell types may alter EV content and their biological effects. At the same time, EVs from the same source can activate different 23 pathways in different recipient cells. It is hard to define by traditional and simplistic models 24 25 the final effects of this complex network. Ex vivo organ culture approach and three-

dimensional (3D) models that better recapitulate the morphological complexity of the
 atherosclerotic plaque may represent the future directions to address this unmet need.
 Moreover, the replication of the organ/tissue properties by 3D-bioprinting may expand the
 applicability of these models for the discoveries of specific therapeutic candidates targeting
 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 and type III collagen fibers are the most abundant types of collagens in large and medium 10 arteries, representing together more than 90% of fibrillary collagen. Vascular wall of large 11 arteries is also rich in elastin, and the balance between collagen and elastin plays a pivotal role 12 in the development of arterial stiffening⁶⁴. Turnover of collagen and elastin is slow and mainly 13 regulated by matrix metalloproteinases (MMPs) and elastolytic enzymes (cathepsins), involved 14 in collagen and elastin degradation⁶⁴. Endothelial-derived EVs contain several matrix-15 degrading proteins, including MMPs⁶⁵. Pro-inflammatory and pro-thrombotic conditions 16 increase MMP-10 in endothelial EVs⁶⁶, and endothelial EVs increase and activate MMP-2, 17 mediating ECM remodeling⁶⁷. 18

Lysyl oxidase (LOX) and lysil oxidase-like (LOX-L) enzymes are members of a family of proteins directly involved in ECM regulation, modulating the cross-linking of collagen and elastin fibers. LOXL-2 is present in endothelial EVs and localized on the surface membrane of EVs directly interacting with ECM components. Hypoxic conditions increase LOXL-2 expression in endothelial EVs and LOX enzymatic activity, explaining how environmental conditions may influence ECM remodeling through EV-mediated pathways⁴³. Patients with atherosclerotic cerebrovascular disease display higher levels of LOXL-2 in circulating
 endothelial EVs ⁶⁸.

3

4 *Calcification*

5 Two main mechanistic initiators trigger and drive vascular calcification in humans: 6 hyperphosphatemia in CKD and chronic inflammation. Although this 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.

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

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

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

Sortilin is a multiligand sorting receptor that exerts multiple roles, including regulation of inflammation and lipid metabolism⁸². Higher circulating sortilin levels are associated with increased rate of cardio-cerebrovascular events and aortic calcification⁸³. Under osteogenic conditions, sortilin enhances the loading of activated TNAP in SMC-derived EVs in a Rab-11dependent pathway, enhancing inflammatory-driven calcification⁸⁴. C-terminal serine phosphorylation of sortilin and sortilin dimerization by intermolecular disulfide bonds are two essential steps for sortilin loading in EVs⁸⁵. Macrophage-derived EVs directly contribute to osteogenic calcification through loading of annexin A5–S100A9 complex in EVs that interacts with phosphatidylserine and acts as nucleation site for mineralization⁸⁶ in CKD environment. S100A9 is also increased in macrophages from patients with type 1 diabetes⁸⁷, and high glucose increases release of calcifying EVs from macrophages through S100A9 signaling⁸⁸, suggesting a crucial role of S100A9 in diabetes- and CKD-induced calcification.

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

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

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

19

20 Conclusions and perspectives

Considerable progress has been made in recent years in understanding the role of EVs in both physiological and pathological regulation of vascular homeostasis and disease, shedding light on EV involvement in various stages of vascular damage. Despite the flourishing research in the field, what is known appears to be only the tip of the iceberg of a much more complex
 and multifaceted roles played by EVs.

3 Discrepancy in isolation techniques and EVs characterization among studies make it difficult to distinguish between different EVs subpopulations and their relative contribution to the 4 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 size. Recently, a "single EVs microarray" approach has been proposed for the discrimination 10 of exosome and microvesicles, on the basis of their protein content, at single EV level⁷⁸. The 11 application of these methods to the future basic and translational research could unravel the 12 fine mechanisms driving EV-mediated cardiovascular damage. 13

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

'Omics' data of human-derived EVs from biofluids and tissues could help in this process and guide future pre-clinical studies in a focused and personalized direction. Multi-omics is becoming an appealing strategy for the detection of potential target in cardiovascular disease⁹⁴, and the implementation of this approach at EV level would provide important 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 data ⁷¹ .
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 countries ⁴⁵ . 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	
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17	
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24	

1 Figure Legends

2 Figure. Pathological role of extracellular vesicles in different stages of vascular damage Extracellular vesicles (EVs) released by different cells under several pathological conditions 3 4 mediates initiation and propagation of vascular damage, from endothelial dysfunction to vascular inflammation, atherosclerosis, alteration of extracellular matrix composition and 5 6 vascular calcification. NO=nitric oxide, NADPH=nicotinamide adenine dinucleotide 7 phosphate, ROS=reactive oxygen species, Rap1=ras-associated protein-1, SMC=smooth muscle cell, LDL=low-density lipoproteins, ERK=extracellular signal-regulated kinases, 8 9 $NF\kappa B$ =nuclear factor kappa-light-chain-enhancer of activated B cells, TNAP= tissue nonspecific alkaline phosphatase, MGP=matrix Gla protein, ECM=extracellular matrix. 10 Some illustrations of the Figure were prepared using Motifolio drawing toolkit. 11 12 13 14 References World Health Organization. Cardiovascular diseases (CVDs) Fact Sheet. Published 15 1. 16 online 2017. Virani SS, Alonso A, Benjamin EJ, Bittencourt MS, Callaway CW, Carson AP, 17 2. Chamberlain AM, Chang AR, Cheng S, Delling FN, et al. Heart Disease and Stroke 18 Statistics-2020 Update: A Report From the American Heart Association. Circulation. 19 2020;141(9):e139-e596. doi:10.1161/CIR.000000000000757 20 van Niel G, D'Angelo G, Raposo G. Shedding light on the cell biology of extracellular 21 3. vesicles. Nat Rev Mol Cell Biol. 2018;19(4):213-228. doi:10.1038/nrm.2017.125 22 Wolf P. The nature and significance of platelet products in human plasma. Br J 23 4. Haematol. 1967;13(3):269-288. doi:10.1111/j.1365-2141.1967.tb08741.x 24 25 5. Jansen F, Li Q, Pfeifer A, Werner N. Endothelial- and Immune Cell-Derived Extracellular Vesicles in the Regulation of Cardiovascular Health and Disease. JACC: 26 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 artery disease. Nat Rev Cardiol. 2017;14(5):259-272. doi:10.1038/nrcardio.2017.7 29

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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; NFkB, 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.