Multifaceted role of extracellular vesicles in atherosclerosis

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Multifaceted role of extracellular vesicles in atherosclerosis

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Abstract

Extracellular vesicles (EVs) are small vesicles released by the majority of cells in response to cell activation or death stimuli. They are grouped as small EVs or exosomes, large EVs such as microvesicles (MVs) and apoptotic bodies, resulting from distinct mechanisms of generation. EVs are released into the extracellular space, in most human biological fluids and tissues, including atherosclerotic plaques. They transport complex cargo of bioactive molecules, including proteins, lipids and genetic material and are therefore involved in pathophysiological pathways of cell-cell communication. Indeed, EVs are involved in several processes such as inflammation, coagulation, vascular dysfunction, angiogenesis and senescence, contributing to the initiation and progression of atherothrombotic diseases. Consequently, they behave as a determinant of atherosclerotic plaque vulnerability leading to major cardiovascular disorders. Over the last decade, the field of EVs research has grown, highlighting their involvement in atherosclerosis. However, limitations in both

detection methodologies and standardisation have hindered implementation of EVs in the clinical settings. This review summarizes the effect of EVs in atherosclerosis development, progression and severity, with specific attention devoted to their ambivalent roles in senescence and haemostasis. This review will also highlight the role of MVs as multifaceted messengers, able to promote or to attenuate atherosclerosis progression. Finally, we will discuss the main technical challenges and prerequisites of standardization for driving EVs to the clinics and delineate their relevance as emergent biomarkers and innovative therapeutic approaches in atherosclerosis.

1. Introduction

Cardiovascular diseases (CVD) are the leading cause of death and disability worldwide. Among them, atherosclerosis is the underlying cause of myocardial infarction, stroke, unstable angina, and sudden cardiac death. Atherosclerosis is a progressive, chronic inflammatory disease affecting large blood vessels by triggering the build-up of plaque within the vessel wall. Plaque formation is characterized by an accumulation of lipids and fibrous elements. In the circulation, cholesterol is transported by lipoproteins, primarily as low-density lipoprotein (LDL). An abnormal excess of LDL-cholesterol is retained in the subendothelial space of the artery wall, leading to activation of endothelial cells (EC), and is the initiating event in atherosclerosis. Increased expression of adhesion molecules ICAM-1 on ECs promotes recruitment of inflammatory cells, such as monocytes [1] and neutrophils [2], to the intima. T lymphocytes, although being less abundant than monocytes, also penetrate the intima, and regulate the function of the innate immune cells, as well as the endothelial and smooth muscle cells (SMCs) [3]. Human atherosclerotic lesions

contain T lymphocytes and display markers of adaptive immune activation [4]. Macrophages and SMCs engulf lipids and become foam cells. They release cytokines and reactive oxygen species (ROS) promoting inflammation, senescence, cell death such as apoptosis and necroptosis. The enlargement of the necrotic core and plaque instability lead to plaque rupture and promote thrombosis.

The field of extra vesicles has been growing over the last decade and highlights the involvement of these vesicles in atherosclerosis. Indeed, their concentration increases in patients with CVD, and large amounts of EVs are found within the atherosclerotic plaque [5] [6][7][8]. This review will summarize the latest discoveries on EVs in atherosclerosis, with a specific focus on senescence and hemostasis. Moreover, it will underline how this growing knowledge of EV biology, combined with technological advances on their measurement, accelerates their translation to the clinic.

2. Extracellular vesicles

EVs is a general term of bilipid layer membrane vesicles released from different eukaryotic and prokaryotic cells. Characterization and classification of EVs is a challenge and still debated [9] [10]. Until now, EVs have been classified into three distinct populations: small (<100 nm), medium (100-1000 nm) and large EVs (>1000 nm) also called exosomes, microvesicles (MV) and apoptotic bodies (AB), respectively [9]. Small EVs (sEV) originate from intracellular multivesicular bodies while MVs are released by budding of plasma membranes. ABs are generated during vesiculation of apoptotic cells (Figure 1A). The release of EVs and MVs is an active process involving the endosomal sorting complex required for the transport (ESCRT) system. Additionally, scramblase and flippase activities and the cytoskeleton

participate in MV release [11][12]. EVs are detected in biological fluids in healthy and pathological conditions. In CVD such as atherosclerosis, circulating EV level is increased. They contribute to atherosclerosis development by promoting calcification, plaque progression and thrombus formation after rupture of the plaque [13] [6][14]. EVs are well known for their involvement in intercellular communication [12] by establishing an exchange of bioactive molecules. Indeed, their cargo nucleic acids, proteins, lipids and organelles [15] [16] can be transferred to target cells. Adhesion, internalization of EVs and transfer of their content induce different cellular processes such as inflammation [15] [16], angiogenesis [17], coagulation [18] and senescence [19]. However, the protective role of EVs in atherosclerosis has been reported. EVs released from endothelial cells mediate shuttling of atheroprotective molecules like miRNAs to smooth muscle cells to restore communication with the endothelial cells [20]. Atherosclerotic plasma is rich in EVs and lipoproteins such as high density lipoprotein (HDL) and low density lipoprotein (LDL) (Figure 1B). They share the same range of density and size. The isolation of exosome and MVs from plasma could be contaminated by these lipoproteins [21]. Therefore, it is difficult to separate the effect of lipoproteins and EVs isolated from plasma.

3. Source and role of extracellular vesicles in atherosclerosis

3.1 Involvement of extracellular vesicles in the initiation of atherosclerosis

The notion that EVs are key players in the initiation of atherosclerosis comes from the observation that increased levels of leukocyte and endothelial-derived MVs are associated with carotid remodelling in individuals even before atherosclerosis is detectable [22]. Indeed, there is substantial evidence that MVs are effectors of vessel wall inflammation, notably by regulating leukocytes adhesion to the endothelium and

macrophage polarization to a pro-inflammatory phenotype. Moreover, platelet-derived EVs support monocyte recruitment in systemic inflammation, through the transfer of platelet GP1bα, a platelet adhesion receptor to monocytes, allowing monocytes recruitment in large and small blood vessels [23]. Futhermore, circulating platelet MVs may serve as a transcellular delivery system for RANTES, triggering monocyte arrest to the inflamed and atherosclerotic endothelium [24]. Thus, MVs can act as paracrine messengers that intensify inflammation during atherogenesis by stimulating vascular and inflammatory cells. Moreover, many types of MVs, in particular from leukocytes, endothelial cells and platelets, can induce expression of selectins and cell adhesion molecules involved in leukocyte diapedesis [25]. Neutrophil MVs, by delivering miR-155 [26], inflammatory adipocyte-derived EVs [27] or MVs from HFD-fed rats [28], can induce VCAM-1. Also, ICAM-1 can be induced by oxLDL-derived endothelial MVs, in a model of obesity [1][5]. These findings confirm that diet can influence MV release and subsequent effects on cardio-metabolic health [29]. Indeed, obesity is causally associated with atherosclerosis, and EVs derived from adipocytes might be involved in mediating CVD, specifically by promoting leukocyte attachment to vascular endothelial cells [27]. It has also been described that in a high glucose condition, increased NADPH oxidase activity in endothelial MVs promotes activation of the endothelium and development of diabetes-associated atherosclerosis[30]. Monocyte exosomes also induce adhesion molecules and cytokines via activation of NF-κB in endothelial cells [31]. In addition, mature DC derived-exosomes increase endothelial inflammation and atherosclerosis via TNF-α mediated NF-κB pathway [32] (Figure 2).

3.2 Involvement of extracellular vesicles in lesion progression

In addition to endothelium activation, aberrant activation of macrophages in the arterial walls by oxLDL is a crucial event in atheroma formation. MVs from activated platelets can increase phagocytosis of oxLDL and production of inflammatory cytokines by macrophages [33]. It has been shown that uptake of distinct types of MVs or spontaneously released vesicles by bone marrow-derived macrophages, coordinated with the activation of multiple TLR ligands, can induce foam cell formation [34], exacerbating inflammation. MVs from activated macrophages carrying TNF-α have been shown to contribute to the propagation of inflammatory signals leading to myocardial infarction [35]. Indeed, MVs could regulate the inflammatory balance in the culprit lesion towards a pro- or anti-inflammatory lesion depending on the nature of the vesicles. Otherwise, the enrichment of unesterified cholesterol in human monocytes or macrophages induce the release of procoagulant MVs. These EVs enriched in cholesterol carry DAMPS and promote the immune response in atherosclerosis [36].

For instance, human plaque MVs carry catalytically active TACE/ADAM17 and significantly enhance the cell surface processing of TACE/ADAM17 substrates TNF, TNFR-1, and endothelial protein C receptor [37]. On the other hand, exosomal MALAT1 derived from oxLDL-treated endothelial cells [38] promotes M2 macrophage polarization, which can reduce plaque inflammation. Macrophage MVs also induce macrophage differentiation through delivery of miR-223 [39] and inhibit cell migration [40]. Similarly, mesenchymal stem cell exosomes polarize macrophage toward an anti-inflammatory phenotype through the miR-let7/HMGA2/NF-κB pathways. [41] Moreover, adipose tissue-derived exosomes may be implicated in the metabolic complications of obesity as they may have a proatherogenic effect by regulating macrophage foam cell formation and polarization [42] (Figure 2). To summarize,

circulating EVs are involved in endothelium activation leading to leucocyte infiltration and inflammation, which are the main initiative steps in atherosclerosis.

3.3 Involvement of extracellular vesicles in the advanced lesion

3.3.1 Relation between extracellular vesicles and the calcification process

Vascular calcification is involved in plaque destabilization. Calcifying EVs have been identified in calcified aortic valves [43], atherosclerotic intimal lesions [44], and medial arterial calcification [43] [45] [46]. These EVs contribute to atherosclerotic calcification by delivering biological signaling [47]. Pathological conditions, such as atherosclerosis characterized by mineral imbalance, can cause SMCs, valvular interstitial cells, and macrophages to release calcifying EVs, which contain specific mineralization-promoting cargos like calcium and phosphate [48]. Moreover, EVs interaction with oxidized and hydroxyl forms of cholesterol may accelerate artery calcification by reprogramming vascular muscle cells to osteogenic differentiation [49].

This mechanism constitutes a continuous source of damaging microcalcifications in atherosclerotic plaques, which becomes unstable and prone to rupture, triggering a local thrombotic event.

3.3.2. Extracellular vesicles in angiogenesis during atherosclerosis

In addition to calcifications, increased vasa vasorum within atheroma could aggravate plaque burden and vulnerability. Thus, the balance between pro- and anti-angiogenic factors is important for the atherosclerotic plaque fate. Therefore, the promotion of angiogenic processes by EVs may have both beneficial and deleterious effects. On

the one hand, exosomes derived from oxidized LDL-stimulated macrophages attenuate the growth and tube formation of endothelial cells [50]. On the other hand, EVs derived from foam cells can promote SMC migration [50] or angiogenesis through a transfer of microRNA-150 [51] [41]. Atherosclerotic plaque CD40 positive MVs also stimulate endothelial proliferation, and angiogenesis and could be involved in intraplaque neovascularization [52]. Altogether, atherosclerotic plaque MVs stimulate endothelial proliferation after CD40 ligation [52]. In diabetic *ApoE*$^{-/-}$ mice, adipocyte-derived exosomes aggravate atherosclerosis by increasing vasa vasorum angiogenesis [53]. The promotion of an angiogenic response may have deleterious effects. These processes occurring in advanced plaques lead to instability and rupture of the plaque, one of the major risks of thrombosis, and to events such as heart attack and stroke (Figure 2).

3.3.3 Connection between extracellular vesicles and cell death

Cholesterol ingestion and the inflammatory context promote cell death within the plaque (early to advanced atherosclerosis). Cell death is generally divided into two category based on regulation (apoptosis) or non-regulation (necrosis). Cells undergo apoptosis through different pathways and generate apoptotic bodies [54] in atherosclerotic plaque. Endothelial cells can deliver miR-126 through their apoptotic bodies and induce CXCL12 in endothelial cells and SMCs [55] (Figure 2). During atherosclerosis development, impairment of the clearance of apoptotic cells, called efferocytosis, leads to the accumulation of dead bodies and the formation of the necrotic core in the advanced plaque. In the last few years, necroptosis, a regulated form of cell death was described in advanced plaque [54]. Necroptosis is a caspase-independent death program regulated by phosphorylation of Receptor Interacting

Protein kinase 1 and 3 that activate the phosphorylation of Mixed Lineage Kinase domain-like (MLKL) involved in the rupture of the plasma membrane. Several stimuli in the plaque, including high levels of oxidized LDL (oxLDL), oxidative stress, tumor necrosis factor-alpha (TNFα), induce necroptosis. Interestingly, the generation of EVs could also be mediated by MLKL and the ESCRT system [56]. Additionally, the recognition of dead bodies is mediated by different receptors such as MerTK and CD47 in atherosclerosis. MerTK can be cleaved by the metalloproteinase ADAM-17 from plaque EVs [37] that could lead to an efferocytosis defect. Moreover, MV from cardiosphere derived-cells induce MerTK expression on macrophage by transferring miR-26a, which inhibits ADAM-17 expression [57]. This process could enhance efferocytosis (Figure 2). During apoptosis, cells release apoptotic bodies and MVs. These MVs are highly pro-coagulant and participate in atherogenicity [58] [58] [59]. However, EVs from apoptotic cells could contribute to survival mechanisms. Indeed, endothelial EV carrying EPCR and activated protein C (APC) could also promote cell survival by induction of cytoprotective and anti-inflammatory effects [60]. Furthermore, endothelial cells in culture could release EV containing caspase-3 and 8 [61]. Statins, a treatment to reduce CVD risks, increase the detachment of apoptotic cells and release of endothelial MVs. Thus, endothelial-derived–MVs contribute to the sorting of several pro-apoptotic factors preventing cell detachment and apoptosis. This mechanism could explain the statins protective effect on the endothelium [61]. EVs from apoptotic platelets induce monocyte adhesion, and polarization into resident phagocyte macrophage [62]. This mechanism could be considered as beneficial. Taken together, EVs from cell in apoptosis or necrosis could have deleterious and protective effects on atherosclerosis progression.

4. The interplay between senescence, extracellular vesicles and atherosclerosis.

Senescence affects several cells involved in the progression of atherosclerosis such as endothelial cells, SMCs, macrophages, foam cells and T cells [63] [64]. Senescence is defined by an arrest of the replicative potential of cells, with telomere shortening and upregulation of cyclin-dependent kinase inhibitor [64] [65] [66] (bullet box 1). However, these cells are still metabolically active, they secrete a panel of pro-inflammatory cytokines and extracellular vesicles as part of the senescence-associated secretory phenotype (SASP) [64] [65] [66] (Key-point box 1). Since the early stage of atherosclerosis, endothelial cells, SMCs and macrophages become senescent [63] [66] and senescent foam cells (SMCs and macrophages) accumulate in the necrotic core [63] [64] due to defective clearance of senescent cells. Indeed, clearance of senescent cells (p16Ink4a positive cells) delays ageing disorders [67]. The vascular endothelium becomes senescent during atherosclerosis [68]. Growing evidence suggests the potential of senescent cells to transfer senescence to other cells through EVs [69] [70] [71] [72]. One recent publication elegantly shows the involvement of senescent EVs in atherosclerosis. [68] MVs from the plasma of elderly patients with acute coronary syndrome induce senescence in endothelial cells *in vitro*, but also under low shear stress conditions. Eendothelial cell MVs from elderly plasma induce senescence of the endothelium and thrombogenicity through Ang II/AT1R/NADPH and the PI3-kinase pathways [68]. Many speculations of the involvement of senescent EVs in atherosclerosis could be done. For example, ADAM-17 expressed on MVs from plaque [37] is associated with senescence [73] and this enzyme cleaves also MerTK [57], a receptor involved in efferocytosis. This could explain the accumulation of senescent cells during atherosclerosis. Moreover,

several miRNAs carried by MVs *in vivo* and *in vitro* target the senescence process and could be involved in atherosclerosis [19]. Indeed, miR-21 and miR-217 carried by plasma small EVs of elderly volunteers, induces endothelial senescence and could be applied to atherosclerosis [19]. Finally, EVs from elderly plasma induce vascular calcification, and this notion may exist in atherosclerosis [47]. Inter-regulatory loops that could interface endothelial senescence and thrombosis have also been described [74]. Interestingly, local production of activated factor X in atherosclerotic plaque was found to induce vascular smooth cell senescence [75] (Figure 3).

5. Involvement of extracellular vesicles in hemostasis during atherosclerosis

Hemostasis involves molecular factors belonging to the coagulation and fibrinolysis systems, which are key actors not only in atherothrombosis but also in the earlier stage of atherosclerosis initiation and progression, in close interaction with the inflammation and angiogenesis processes [76]. EVs represent a biological surface promoting efficiently both procoagulant and fibrinolytic cascades [18] [77] (Figure 4). They are now identified as a key partner contributing to the different steps of atherosclerosis. Most studies have been focused on the procoagulant property of MVs. This explains the considerable number of studies that link the role of EVs to thrombosis formation and plaque rupture in atherosclerosis in the last two decades [59] [78]. Briefly, the exposure of active Tissue Factor (TF) at the EV surface initiates the coagulation process in the presence of high concentrations of anionic phospholipids such as phosphatidylserine (PS) (Key-point box 2 and Figure 3). MVs derived from atherosclerotic plaque display a much higher thrombogenic potential compared to circulating MVs [59]. High expression of TF correlates with high concentration of EVs from leukocytes, SMCs, and erythrocytes within the plaque[59].

In addition to TF bearing MVs that originated from living cells, plaque also contains significant levels of PS and TF positive apoptotic MVs [78]. Tobacco smoke increases the athero-thrombotic effect of epithelial EVs with their pro-coagulant properties[79] and TF-positive MVs from macrophages [80].

As plaque MVs display greater thrombogenicity than plasma EVs, they confer a higher risk for thrombus formation. Indeed, when advanced plaques become unstable and break, they serve as a source of continuous procoagulant EVs released in the bloodstream. This might exacerbate coagulation and lead to ischemic stroke or myocardial infarction. Besides the data regarding MVs in plaque, there are well-documented data providing information on the strong interplay between circulating procoagulant MVs released from different sources and atherosclerosis progression. Activated platelets produce MVs with high-level expression of molecules such as PS, TF, αIIbβ3-integrin (CD41/CD61), glycoprotein IV (CD36), thrombospondin (TSP1), P-selectin (CD62P), P-selectin glycoprotein ligand 1 (PSGL-1), which promote thrombosis. These platelet-derived MVs have been positively correlated with the intima-media thickness of the carotid artery of obese patients and menopausal women [81] [82]. This is also supported by the prognostic value of activated platelets, as it generates TF and thrombospondin-1 positive MVs along with a high number of TF positive monocyte MVs in young patients with high cardiovascular risk [83]. Further studies have demonstrated the critical role of platelet procoagulant MVs in promoting platelet and fibrin deposition to the damaged arterial wall and surfaces with exposed collagen under shear stress, as well as normal flow conditions [84]. Moreover, vessel wall injury can lead to enhanced formation of thrombogenic MVs from endothelial cells, which then amplify the coagulation cascade even further [78].

Several molecules have been shown to modulate the procoagulant potential of MVs in atherosclerosis. For example, atorvastatin treatment in patients with atherosclerosis reduces thrombin generation and expression of tissue factor, P-selectin and GPIIIa on platelet-derived MVs [85]. Conversely, interleukin-33 treatment results in increased production of TF-positive MVs and activity in monocyte subsets, with potential implications for cardiac events during atherosclerosis [86]. As all these studies provide an understanding of how MVs are linked to the coagulation process in atherosclerosis, they might guide the choice of drug target to modulate coagulation and atherosclerotic progress.

In addition to their thrombogenic activity, some EVs have fibrinolytic and proteolytic potential. Endothelial and leucocyte MVs carry tPA and uPA respectively, on their surface and can promote plasmin generation *in vitro* [87] [88]. By conveying plasmin, endothelial MVs activate matrix metalloproteases (MMP), which are involved in extracellular matrix degradation and release of growth factors that play a crucial role in tissue remodelling and angiogenesis [77]. Among them, MMP10 is particularly crucial in fibrinolysis as it has been shown to prevent formation of fibrin by proteolytically degrading fibrinogen and may thus prevent thrombus formation [89]. On the other hand, MMP2 and MMP9 accelerate vascular remodelling by degrading matrix components, and high levels of these MMPs have shown to significantly correlate with plaque instability and rupture, resulting in the progression of atherosclerosis [89] [90]. Similar to MMP2 and MMP9, smoke-induced EVs carrying MMP14 may contribute to plaque rupture as it has been shown to exhibit significant gelatinolytic and collagenolytic activity *in vitro* [91]. Interestingly, EVs not only activate pro-MMPs to-active MMPs through plasmin generation, but can also directly vectorize MMP2, MMP9 and MMP10 [77] (Figure 4). Whether fibrinolytic properties carried by

EVs can play a role in atherosclerosis remains to be answered [77] [87]. The fibrinolytic potential of EVs can be modulated by pharmacologic agents, such as rosuvastatin, found to increase the plasminogen level in EVs from the LDL plasma fraction [92]. However, the role of the fibrinolytic potential carried by EVs has not been directly investigated in atherosclerosis and future studies are needed to determine in which extent this may contribute to homeostatic imbalance and provide a potential target for therapeutic manipulation.

6. Translation of extracellular vesicle analysis to the clinic

6.1 Extracellular vesicles as biomarkers of atherosclerosis

Recognition of EVs as potential biomarkers of disease state is illustrated by the growing number of reviews on the topic [93] [94] [95] [96]. However, the measurement of EV remains challenging. Preanalytical variables and technical limitations impact EV determination, therefore impeding sensitivity and repeatability of MV measurements and limiting comparisons between studies. An important step has been the publication of guidelines for sample preparation and analysis [9] [10], resulting from the joint efforts of international scientific societies (The International Society of Extracellular Vesicles (ISEV) and the International Society on Thrombosis and Haemostasis (ISTH)). In practice, standardization of the pre-analytics is crucial since handling, delay and storage of plasma samples are the major sources of artefactual generation or loss of EVs [97]. Regarding the analytical methods available for EVs determination, some methods like electronic microscopy (EM), nanoparticle tracking analysis (NTA) and tunable resistive pulse sensing (TRPS) allow the measurement of particles of small size but they are not adapted to the clinic because they are time-consuming and not available in hospital laboratories. Flow cytometry is

mostly used to enumerate and determine the cellular origin of EVs in clinical samples. However, detection and characterization of exosomes can be challenging, considering their small size, but possible with advanced flow cytometry techniques. Indeed, exosome can be detected after latex bead capture before analysis on flow cytometer [98]. The main disadvantage of these analysis comprises the EV separation with their heterogeneity in size and origin that contrasts with the isolation of EV based on the size.
Since the last years, this method has benefited from considerable improvement, with the development of instruments with increase sensitivity and resolution [10]. Several standardized protocols and calibration tools are now available [9] [10] and pave the way for multicentric studies allowing to delineate the clinical relevance of MVs. Besides flow cytometry, functional assays are available to measure MV dependent procoagulant activity [10].

Several reviews summarized MVs as potential biomarkers for the early identification of subclinical manifestations of atherosclerosis [97] [99] [100] [96]. MVs are clinically relevant biomarkers because they provide some useful and predictive information to stratify patients according to their cardiovascular risks, and to monitor the response to treatment [97] [99] [100] [96]. MVs are detectable in several biological fluids and provide an instant picture of inaccessible tissues, such as the endothelium or atherosclerotic plaque. Moreover, these liquid biopsies may be a signature of the individual underlying atherosclerotic disease. They have the advantage to protect their content from degradation due to their bilayer lipid membrane and allow to perform longitudinal measurements. Levels of EVs in blood are increased in cardiovascular and cardiometabolic diseases, including diabetes mellitus, obesity,

subclinical atherosclerosis, coronary artery diseases and myocardial infarction [101] [93] [97]. The concentration of endothelial and erythrocyte EVs is indeed related to Framingham risk scores for coronary artery disease in healthy patients [97]. Endothelial derived EVs level correlates with the severity of coronary artery disease in patients with diabetes [97]. Previous studies have nicely shown that atherosclerotic plaques contain large amounts of EVs, which could either contribute to plaque progression and be released into the circulation. These EVs, mainly derived from leucocytes, reflecting the local inflammatory environment, could serve as diagnostic and prognostic biomarkers in CVD [93] [94]. Indeed, increased circulating leukocyte MVs positively correlate with high plaque burden in subclinical atherosclerosis [93]. Annexin V positive MVs, released into the circulation by the endothelial cells following vessel wall injury, could be used as a potential biomarker of endothelial damage during atherosclerotic complications [100]. Similarly, elevated endothelial MVs in the circulation of carotid patients may be used to predict plaque instability [102]. Annexin V positive MV originated from apoptotic endothelial cells have been demonstrated to increase significantly in the circulation of type 2 diabetes mellitus patients with asymptomatic atherosclerosis [103]. In HIV infected patients, elevated MVs associated TF activity remarkably correlates with the presence of plaque in the coronary artery [99]. The high circulatory levels of procoagulant MVs from platelet and endothelial cells are indicative of the severity of coronary calcification [95]. On the other hand, lymphocyte CD45 and CD3 derived MVs could be a biomarker of lipid-rich atherosclerotic plaques in familial hypercholesterolemia [96]. MVs are both actors of plaque development and potential promising biomarkers to monitor plaque progression. Altogether, these studies are consistent with the fact that EV detection could predict plaque instability and guide therapeutic options.

Progression of the technical development for measuring MVs, standardization of methods in combination with efforts to define the most interesting subsets of MVs in different clinical contexts are issues that should be addressed in the future. Taken together, the recent development of sensitive and standardized methods will facilitate the introduction of circulating MVs as biomarkers in the clinic.

6.2 Extracellular vesicles as therapeutics in atherosclerosis

EVs might be used as therapeutics [55] [20] [104] [105] [106] [107]. In the last 10 years, several clinical studies have suggested the potential use of EVs as therapeutic vectors by either modifying their cytosolic content or loading them with molecules that could be transferred to target cells through specific interactions. Several reviews related the clinical trials using EV to therapies in different diseases, especially CVD [108] [6]. Two preclinical studies performed on murine models of atherosclerosis have demonstrated a decrease of atherosclerotic plaque size thanks to the injection of vesicles derived from human endothelial cells [20] [55]. Apoptotic bodies from endothelial cells from atherosclerotic patients or *ApoE*$^{-/-}$ mice contain miR-126, that mediate the atheroprotective action through increased CXCL12 expression, decrease of V-CAM1, and mobilization and incorporation of Sca-1 positive progenitor cells to the plaque [55]. Endothelial cells could also release exosomes rich in miR-143/146 but not in miR-126. The uptake of these exosomes by SMC in the plaque reduces atherosclerotic lesions in the *ApoE*$^{-/-}$ model [20]. Other studies related to the treatment of myocardial infarction used EVs. Indeed, injection of EVs from iPSC-derived cardiomyocyte could exert a protective effect by regulating inflammation and

angiogenesis [107]. On the same line, exosomes from stem cells could be used to promote cardiac function in the pathological heart [109].

Since a decade, several studies have used the properties of EVs as a cargo of specific bioactive molecules to deliver their content and treat cells, tissue and diseases. Nanostructures of melittin, a known pore-forming peptide that encapsulates anti-JNK2 siRNA, decreased plaque size and thrombotic risk in *ApoE*$^{-/-}$ [106]. Moreover, injection of lipidoid nanoparticles of *CCR2* siRNA, in *ApoE*$^{-/-}$ mice affected by myocardial infarction reduces the recruitment of inflammatory monocytes (Ly6C high) at the infarct site and attenuates the inflammation [105]. Recently, chitosan nanoparticles of 200 nm were optimized to deliver a specific miRNA that promotes the efflux of excess cholesterol from foam cells to the liver. These chitosan nanovesicles can transfer miR-33 to macrophages, *in vitro* and *in vivo*, reducing cholesterol efflux or reversing cholesterol transport, suggesting a therapeutic use [104].

Clinical translation is still a challenge but remains possible, as evidenced by ongoing clinical trials of non-engineering EV therapies that are safe, well-tolerated in cancerous diseases and very promising in chronic kidney diseases [110] [111].

7. Conclusions

EVs are potent vehicles of bioactive molecules, which influence various processes in atherosclerosis. Their involvement in vascular pathophysiological responses is more complex than initially thought and it demonstrates not only EVs diversity but also the complexity of the mechanisms they are involved in. Indeed, they play detrimental

roles by promoting inflammation, thrombosis, vascular dysfunction and senescence but can also be protective, through their involvement in fibrinolysis and cell survival.

The notion that MPs are conveyors of biological information with the potential deleterious or beneficial role is an exciting prospect, suggesting that they could control the stability/instability of atherosclerotic plaques. Detectable in the bloodstream, they provide a signature of tissue injuries and behave as an attractive source of biomarkers for translation to the clinics. Therefore, they open new research areas as emergent diagnostic and therapeutic targets. However, proof of these attractive concepts remains to be fully established *in vivo* and faces numerous challenges:

- to develop animal models defective for MV production, to better understand the role of EVs, the cellular and molecular determinant triggering their ambivalent involvement in the pathophysiological process. Since they can either promote or inhibit coagulation, fibrinolysis, angiogenesis and survival, they may consequently guide the evolution of the atherosclerotic diseases

- to identify the cargo components based on the heterogeneity of EVs and their subpopulation, thanks to the -omic technologies. This cargo of bioactive molecules, such as proteins, lipids, nucleic acid, could contribute to increase or resolve disease severity. Defining the molecular profiling of EVs will help identify atherosclerotic related specific signatures and exploit the full biological potential of MVs as emergent biomarkers

- to better understand mechanisms controlling the spatiotemporal release of EVs and their clearance. Indeed EVs, as professional in cell-cell communication, could be associated with, internalized or cleared by cells. In cardiovascular diseases such as

atherosclerosis, the generation of EVs and their origin in circulation or tissues should be established, since they could originate from circulating cells or the vessel structure with endothelial cells, fibroblasts and smooth muscle cells. Thus, the EV clearance process or association with circulating cells should be investigated since EVs in the circulation could be attributed to the increase or defect of EV generation and clearance

- to overcome the remaining challenges to drive MV in clinical practice, in particular (1) resolve technological limitations and develop standardization, two prerequisites to validate their clinical interest at a multicenter level and (2) validate the benefit of therapeutic modulation of MV in the prevention of future cardiovascular complications

- to establish whether EVs could be used as therapeutic agents or therapeutic vectors. Using the property of EVs (small vesicle, biomolecular cargo, great internalization) holds a promising avenue of therapeutics in cardiovascular disease with engineered vesicles. Moreover, EVs isolated from patients could also be transferred to another patient and resolve diseases. The bio-engineering vesicle field will expand in the future, employing new material, thus a new way of delivery, and different ways to produce them.

Key-point box 1. Senescence

- Senescence is a cellular program involved in different processes such as embryonic development, cancer-protection, ageing and CVD [65] [64] [66].

- Senescence is beneficial when it is transient (acute senescence) whereas aberrant accumulation of senescent cells (chronic senescence) is detrimental and leads to inflammation and decline of tissue functions.
- Therapies that target senescent cells (senotherapies) enhance CVD.
- Triggers of cellular senescence are diverse such as telomere shortening, DNA damage, proteotoxic stress, ROS, RAAS, hypertension, hyperglycaemia, secreted molecules, cytotoxic compounds, radiation, oncogene activation, mitotic stress, tumour-suppressor inhibition, secreted molecules [65].
- Cellular senescence is characterized by a permanent cell-cycle arrest and by a specific secretome named senescence-associated secretory phenotype (SASP).
- Intracellular markers of senescence used are senescence-associated β-galactosidase (SA-β-gal) activity at pH 6.0, proliferative marker Ki67, cyclin-dependent kinase inhibitors such as p21CIP1, p16INK4a and p19ARF, p14ARF, p27KIP1 or p15Ink4b; DNA damage response is detected with γ-histone H2AX or tumour suppressor p53-binding protein 1; senescence-associated heterochromatic foci is evaluated with chromobox protein homologue 3 (HP1-γ), histone H3K9me3 or core histone macro-H2A.
- SASP is mainly composed of cytokines ((IL)-1α, IL- 1β, IL-6, IL-8, IL-18, TNF-α), chemokines (CCL2,), proteins (iNOS, PAI-1, MMP-1, -2, -3, -7, -8, -9, -10, -12, -13, and -14) and EVs (exosomes and microvesicles) [72] [70].

Key-point box 2. Coagulation and fibrinolysis

- Blood clot is the result of coagulation.
- Coagulation cascade involves a series of proteolytic reactions. The activation of the coagulation process begins with the tissue factor VII pathway and results in thrombin

generation. Thrombin converts fibrinogen to fibrin. Crosslinking of fibrin is mediated by Factor XIIIa (Figure 4). Many actors play a key role in coagulation such as platelet, neutrophils with their neutrophil extracellular traps and endothelium [2].

- EVs are involved in coagulation thanks to the exposure of anionic phospholipids and tissue factor that mediate the assembly of coagulation factors and the formation of fibrin [87] [77].
- Fibrinolysis is the result of clot lysis by degradation of fibrin [77] [88].
- Tissue plasminogen activator (tPA) from endothelial cell and urokinase plasminogen activator (uPA) convert plasminogen to plasmin that degrades fibrin in thrombus.
- A subset of EVs carry fibrinolytic molecules like uPA and tPA, which convert plasminogen to plasmin and promote clot lysis by degrading fibrin in the thrombus [77] [88].
- Proteolysis occurs to clot lysis through the action of matrix metalloproteinase (MMP-1,9,10)

Figure legend

Figure 1. Heterogeneity of extracellular vesicles.

(A) Extracellular vesicles are heterogeneous in size and content. The cargo of EVs is dependent on cell origin and stimuli (pathologic or physiologic). (B) EVs get along with lipoproteins in blood. High density lipoprotein (HDL); low density lipoprotein (LDL); very low density lipoprotein (VLDL).

Figure 2. Impact of extracellular vesicles during atherosclerosis.

Figure 3. Interplay between senescence, extracellular vesicles and atherosclerosis.

Figure 4. Involvement of extracellular vesicles in hemostasis during atherosclerosis.

Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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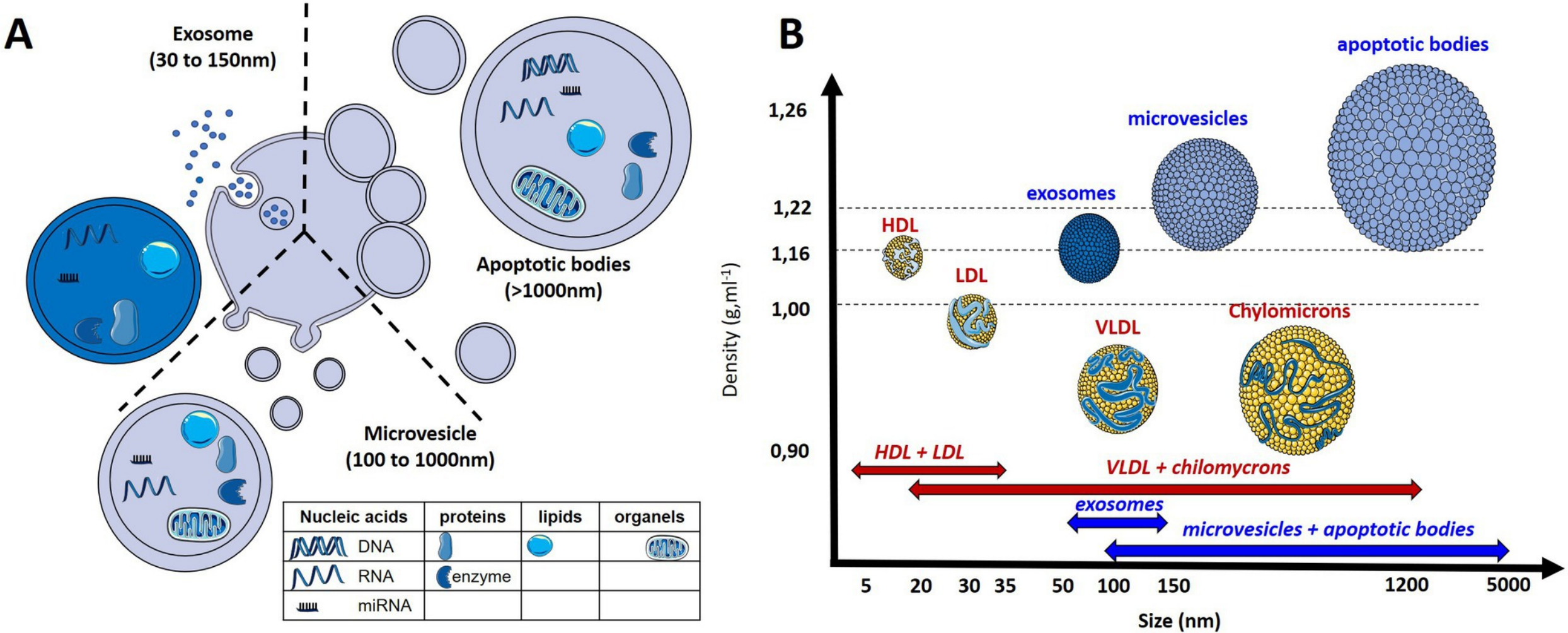
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Density (g,ml-1)
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Apoptosis
TF expression
Inflammation
IL-1α
miR-10a
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thrombosis
coagulation
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Cell death
inflammation
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Migration
OxLDL engulfment
M2 polarization
ADAM 17
efferocytosis
Angiogenesis
miR-150
Angiogenesis
miR-126
miR-222
angiogenesis
Adhesion molecules
ICAM-1, VCAM-1, CCL2
Monocyte attraction & attachment
Adhesion molecules
ICAM-1
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CXCL12
CCL5
GP1bα
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Necrotic core

Monocyte | Macrophage | Neutrophil | Dendritic cell | Lymphocyte | Erythrocyte | Platelet | Endothelial cell | Smooth muscle cell | Foam cell

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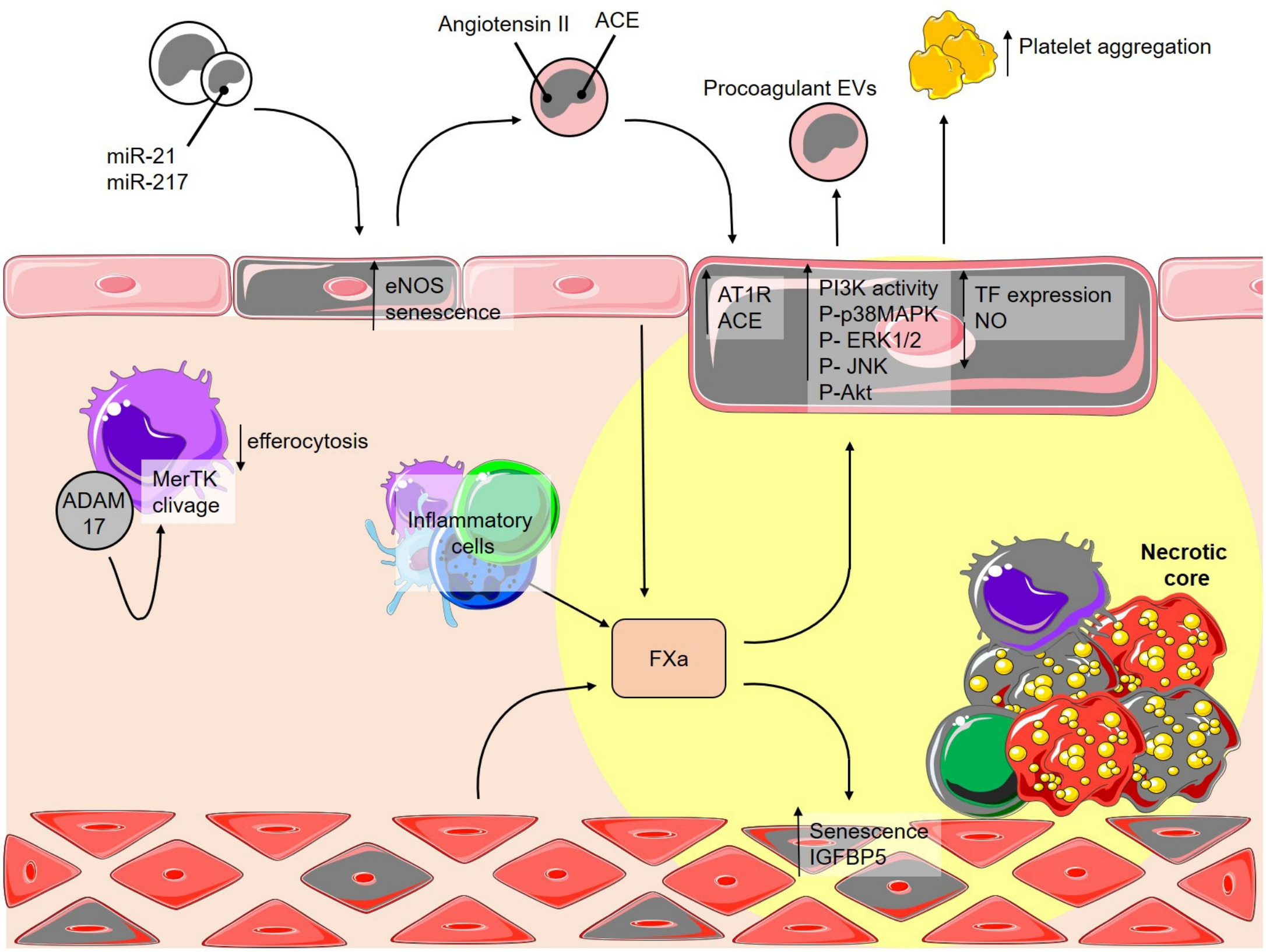
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P- JNK
P-Akt
TF expression
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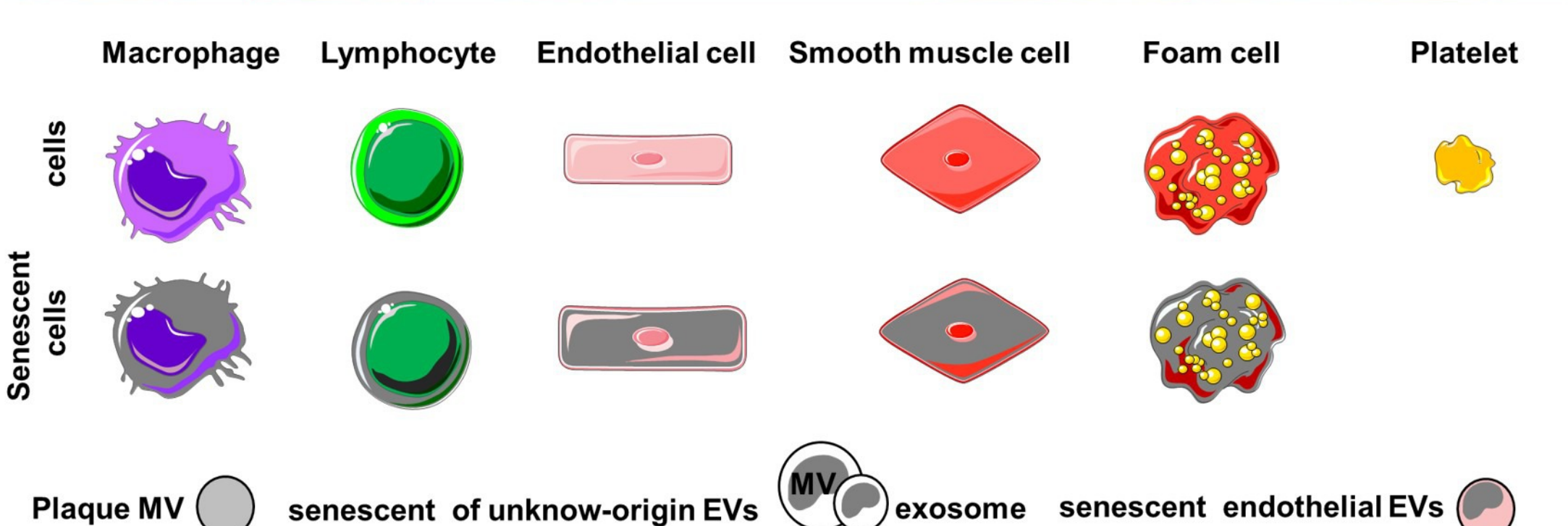
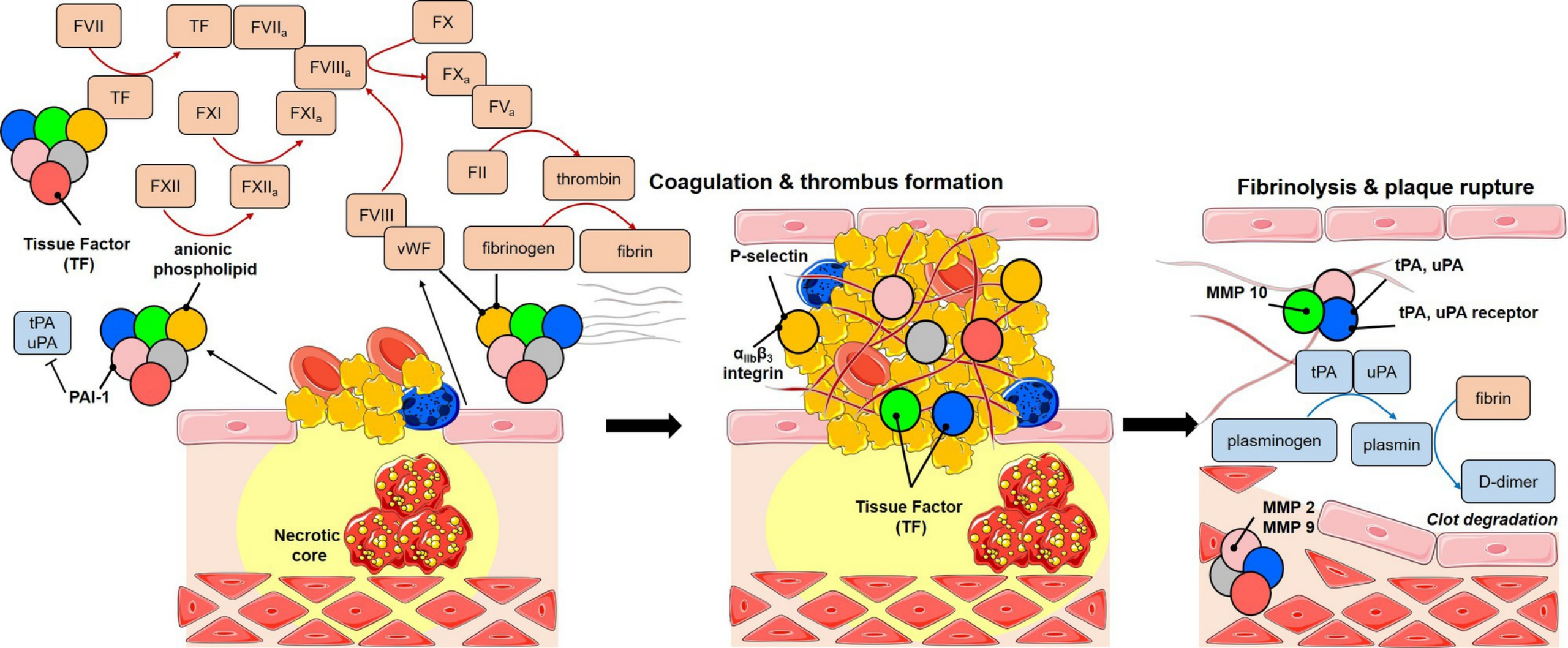
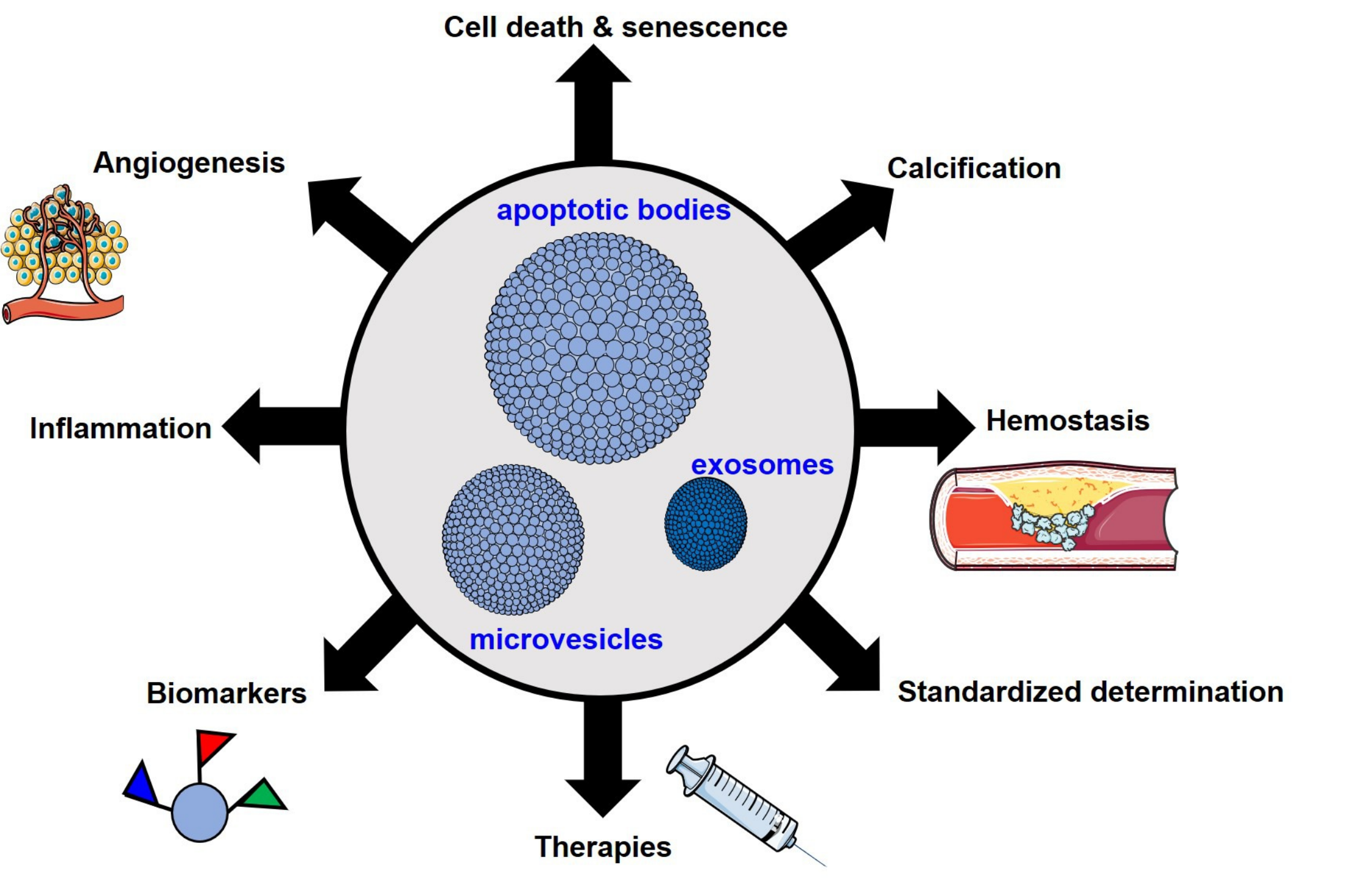
cells
FXa
Necrotic
core
Senescence
IGFBP5


Macrophage
Lymphocyte
Endothelial cell
Smooth muscle cell
Foam cell
Platelet
cells
Senescent
cells
Plaque MV
senescent of unknow-origin EVs
MV
exosome
senescent endothelial EVs


FVII
TF
FVII$_a$
FX
FVIII$_a$
FX$_a$
TF
FV$_a$
FXI
FXI$_a$
FII
thrombin
FXII
FXII$_a$
FVIII
vWF
fibrinogen
fibrin
Tissue Factor (TF)
anionic phospholipid
tPA
uPA
PAI-1
Necrotic core
Coagulation & thrombus formation
P-selectin
$\alpha_{IIb}\beta_3$ integrin
Tissue Factor (TF)
Fibrinolysis & plaque rupture
MMP 10
tPA, uPA
tPA, uPA receptor
tPA
uPA
plasminogen
plasmin
fibrin
D-dimer
MMP 2
MMP 9
Clot degradation


Cell death & senescence
Angiogenesis
Calcification
apoptotic bodies
Inflammation
Hemostasis
exosomes
microvesicles
Biomarkers
Standardized determination
Therapies