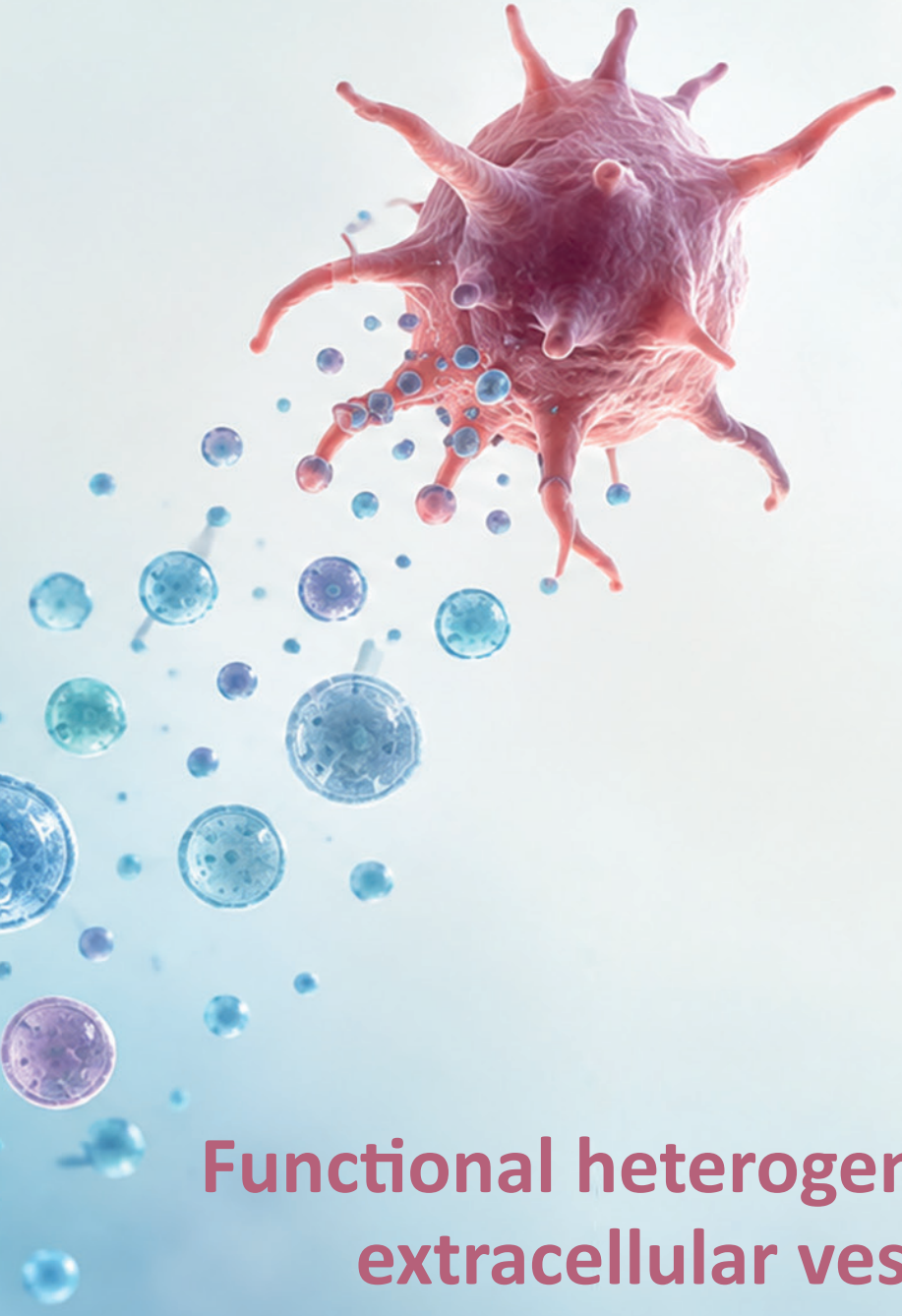


Functional heterogeneity of extracellular vesicles in endothelial inflammation

Xin Xin

Functional heterogeneity of extracellular vesicles in endothelial inflammation

Propositions

1. Extracellular vesicles are functionally heterogeneous, and their effects on endothelial cells depend on their cellular origin and activation state. (This thesis)
2. Lyophilized platelets provide a stable and reproducible source for the generation of platelet-derived extracellular vesicle analogues, facilitating standardized studies of platelet-derived vesicles. (This thesis)
3. Platelet-derived extracellular vesicle analogues are actively internalized by endothelial cells and undergo dose- and time-dependent intracellular trafficking, including trafficking to lysosomal compartments. (This thesis)
4. Extracellular vesicles from different cellular origins can regulate distinct stages of endothelial inflammatory activation, ranging from adhesion molecule expression to leukocyte recruitment and transendothelial migration. (This thesis)
5. Extracellular vesicles derived from different disease-associated conditions can exert distinct effects on endothelial cells, indicating that EV function is shaped not only by cellular origin but also by the pathological environment. (This thesis)
6. Extracellular vesicle-mediated communication should be interpreted in the context of both the vesicle and the recipient cell rather than by considering EVs as a uniform biological entity.
7. Standardization of EV isolation, characterization, labeling and functional assessment is essential for meaningful comparison between EV studies and for their translation into clinical applications.
8. The biological effects of extracellular vesicles cannot be fully understood without considering the inflammatory context in which EV-mediated communication occurs.
9. Future cardiovascular therapies may benefit from selectively targeting disease-associated EV populations or their interactions with endothelial cells.
10. Science advances not only by discovering new answers, but also by asking better questions.

Xin Xin

19th November 2026

Functional heterogeneity of extracellular vesicles in endothelial inflammation

Xin Xin

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Functional heterogeneity of extracellular vesicles in endothelial inflammation

DISSERTATION

To obtain the degree of Doctor at Maastricht University,
on the authority of the Rector Magnificus, Prof. Dr. Jan Smits,
in accordance with the decision of the Board of Deans,
to be defended in public on

Thursday, 19th of November 2026, at 10:00 hours

By

Xin Xin

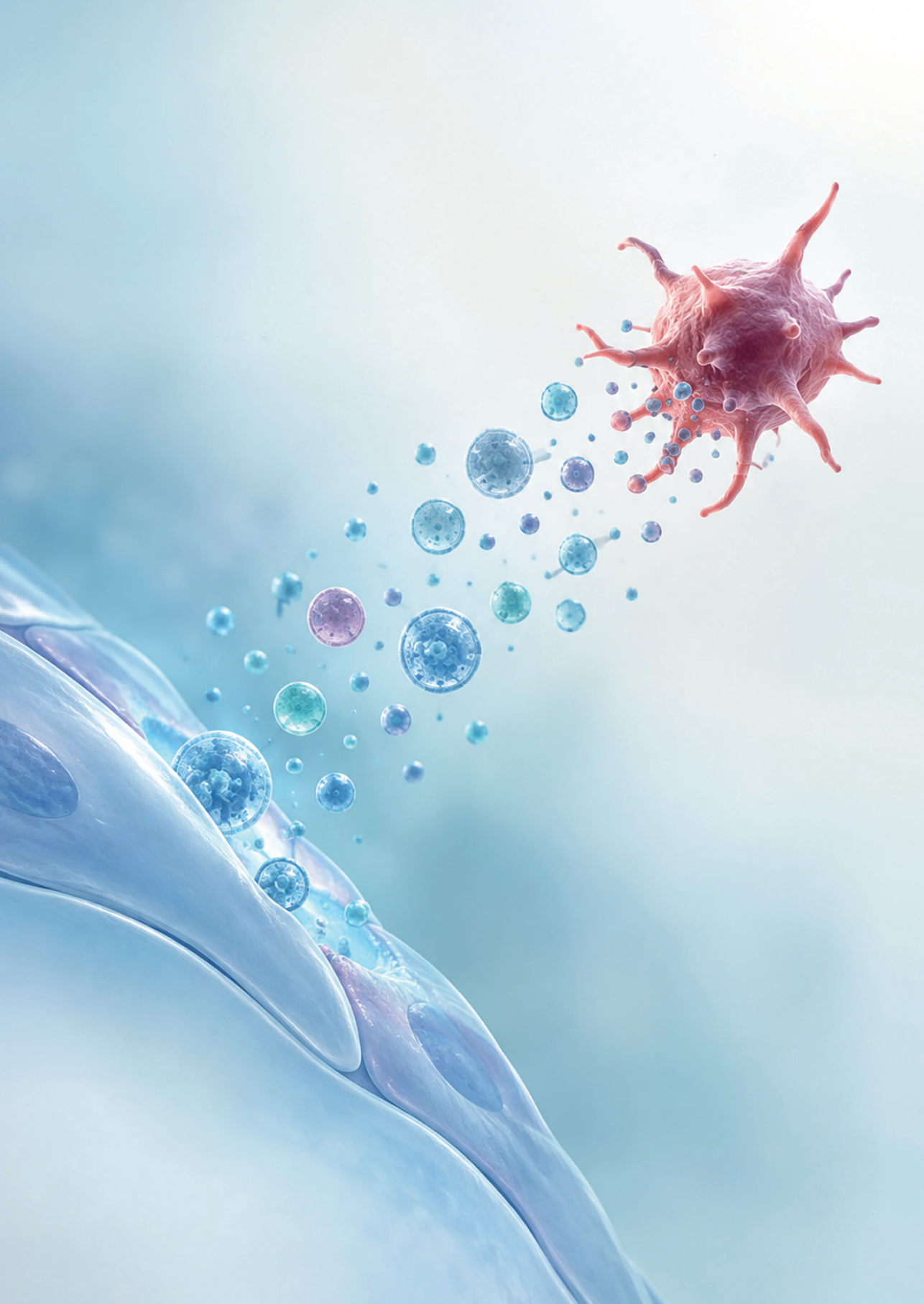
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Chapter 1

General introduction and outline of this thesis

Cardiovascular Disease and Endothelial Dysfunction

Cardiovascular disease (CVD) remains a leading cause of mortality worldwide, affecting approximately 523 million people and resulting 18.6 million deaths globally in 2019, imposing a significant socioeconomic burden[1]. Despite ongoing advancements in prevention, lifestyle interventions, pharmacological treatments, and interventional therapies that have improved the control of traditional risk factors such as dyslipidemia, hypertension, and diabetes, considerable cardiovascular risk persists in a large population[2,3]. This suggests that, in addition to these classic risk factors, there are still complex and not fully understood pathological mechanisms involved in the occurrence and progression of cardiovascular disease.

Mounting evidence indicates that vascular inflammation and endothelial dysfunction are key drivers in the development of cardiovascular disease[4,5]. Vascular endothelium, a dynamic monolayer of cells that lines the circulatory system, plays a crucial role in maintaining vascular homeostasis, including regulating vascular tone, coagulation, vascular permeability, and immune responses[6]. Under the influence of inflammatory stimulation and pathological microenvironment, endothelial cells can transition from a resting state that maintains vascular homeostasis to an activated state, exhibiting pro-inflammatory, pro-adhesion, and pro-thrombotic phenotypes[7,8]. Persistent endothelial activation further disrupts vascular homeostasis, leading to endothelial dysfunction characterized by increased oxidative stress, decreased nitric oxide bioavailability, and persistently enhanced inflammatory signaling, accompanied by upregulation of cell adhesion molecules, such as intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion protein 1 (VCAM-1), and increased secretion of inflammatory factors[9,10]. Therefore, endothelial dysfunction is not only an important pathological feature of cardiovascular disease but is also increasingly becoming a potential therapeutic target.

Extracellular vesicles as mediators of intercellular communication

Extracellular vesicles (EVs), nanosized particles encapsulated by membranes and released from almost all cell types, are increasingly recognized as important mediators of intercellular communication[11]. EVs carry a variety of bioactive molecules, including proteins, lipids, and nucleic acids, which can be taken up by recipient cells, resulting in biological effects that directly alter the phenotype and functional state of the recipient cells, thereby regulating cell function both locally and over long distances[12].

In the context of cardiovascular diseases, EVs are widely considered to participate in various pathological processes such as coagulation regulation and vascular inflammation[13]. Increasing research indicates that EVs can induce endothelial activation, promote platelet activation,

and leukocyte recruitment, thereby exacerbating vascular inflammation and endothelial dysfunction[14,15]. Among the various populations of EVs, platelet-derived EVs (P-EVs) have emerged as one of the most extensively studied subtypes, because platelets are a major source of circulating EVs and are critically involved in both haemostasis and vascular inflammation[13].

Platelet-derived extracellular vesicles

During activation, apoptosis, or storage, platelets release a significant number of EVs[16]. P-EVs are the primary source of circulating EVs, accounting for over 70% of the total population[17]. P-EVs retain some surface features of their originating platelets and carry a variety of bioactive cargoes[18]. Their molecular composition can change depending on the platelet activation state and stimulation mode, leading to functional diversity[19]. Numerous studies have shown that P-EVs play a role in processes such as vascular inflammation, angiogenesis, and coagulation cascades, and affect vascular function through interactions with endothelial cells[20]. Due to their retention of some membrane characteristics from platelets, P-EVs are considered to have potential targeting capabilities for damaged blood vessels[21], thus attracting increasing attention in the field of drug delivery in recent years.

Despite the growing interest in the potential applications of P-EVs in basic research and translational medicine, there is currently a lack of standardized methods for P-EV isolation, characterization, labeling, and functional assessment[22]. Additionally, most studies on P-EVs rely heavily on fresh platelet sources, which increases experimental variability and limits standardization, reproducibility, and large-scale application[23]. Therefore, developing stable, storable, and readily available alternative sources of platelets is crucial for advancing P-EV-related research and its potential medical applications.

EV heterogeneity and endothelial inflammation

EVs exhibit significant heterogeneity in their molecular composition and biological function[24]. This heterogeneity is influenced not only by the cellular origin of the EVs but also by the activation state of their parent cells, the surrounding microenvironment, and various pathological conditions. As a result, even EVs from the same cell type may carry different cargoes in response to different stimuli, leading to diverse biological effects[25]. Increasing evidence suggests that the role of EVs in endothelial activation is dependent on their cellular origin and the content they carry[26] (Figure 1). Studies have shown that EVs derived from inflammatory monocytes can enhance the expression of adhesion molecules and chemokine in endothelial cells, thereby contributing to endothelial inflammation[27,28]. Conversely, EVs generated under paeonol treatment may transfer anti-inflammatory microRNAs, which inhibit STAT3-mediated

inflammatory signaling and subsequent endothelial injury.[29]. Similarly, P-EVs have been reported to exert a bidirectional effect on endothelial cell function. While some studies indicate that P-EVs may alleviate endothelial inflammation by delivering regulatory microRNAs[30,31], other research has found that P-EVs can promote leukocyte recruitment and endothelial cell inflammation through transferring chemokines and adhesion-associated proteins[32,33].

This significant functional heterogeneity complicates the interpretation and comparison of various research findings. Most previous studies have focused on single-source EVs, and the experimental conditions have varied considerably[34]. As a result, the relative contributions of EVs from different cell sources to endothelial inflammation remain unclear. Therefore, a systematic comparison of the effects of EVs from various sources on endothelial function under standardized conditions is necessary to better understand their roles in vascular inflammation and endothelial dysfunction.

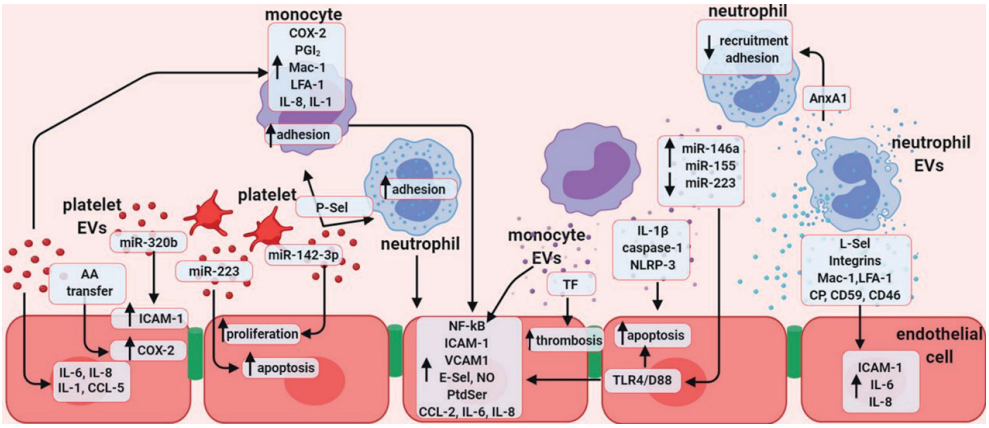


Figure 1. Extracellular vesicles promote endothelial cell activation.

Schematic overview of the potential effects of extracellular vesicles from different cellular origins on endothelial activation and vascular inflammation. EV cargo, including proteins, lipids, and microRNAs, may differentially regulate endothelial inflammatory signaling, leukocyte recruitment, thrombosis, and apoptosis. AA, Arachidonic acid; CCL5, C-C motif chemokine 5 (also known as RANTES); COX-2, cyclooxygenase type 2; ICAM-1, intercellular adhesion molecule 1; LFA-1, leukocyte function-associated antigen 1; NO, nitric oxide; PGI2; P-Sel, P-selectin; PtdSer, phosphatidylserine; TF, tissue factor; TLR-4/D88, toll like receptor 4; CP, ceruloplasmin. IL=interleukin.
(Excerpt from: Silvia Oggero., Front Pharmacol, 2019)[26]

EVs in chronic inflammatory cardiovascular diseases

Chronic kidney disease (CKD) is closely linked to an increased risk of cardiovascular disease, making cardiovascular complications one of the leading causes of death in patients undergoing peritoneal dialysis (PD)[35]. CKD is a systemic disease characterized by chronic low-grade inflammation[36]. While PD treatment helps improve uremia-related metabolic disorders, it may also maintain or worsen the inflammatory state due to factors such as long-term glucose exposure, bioincompatible stimuli, and local inflammation within the peritoneum[37]. Therefore, PD patients serve as an important clinical model for studying cardiovascular damage associated with chronic inflammation.

Heart failure with preserved ejection fraction (HFpEF) is a common cardiovascular complication in PD patients and is closely associated with endothelial dysfunction[38]. Furthermore, endothelial dysfunction and chronic inflammation are recognized as key pathological processes in both CKD and HFpEF[39-41].

In PD patients, the peritoneal microenvironment experiences low-grade chronic inflammation, characterized by the infiltration of inflammatory cells, cell activation, and the release of pro-inflammatory factors[37]. In this context, various peritoneal and circulating cells can release substantial amounts of EVs, resulting in peritoneal dialysis effluent-derived EVs (PDE-EVs) with specific inflammatory properties[42].

Given the crucial role of EVs in intercellular communication[11], PDE-EVs may play a part in transmitting inflammatory signals to the vascular system and regulating endothelial cell function. Therefore, PDE-EVs are potentially associated with endothelial dysfunction and cardiovascular complications in patients with PD. However, research on whether PDE-EVs directly contribute to PD-related endothelial injury and the development of HFpEF is still limited.

Further investigation into the effects of PDE-EVs on inflammatory responses and functional impairment in endothelial cells will enhance our understanding of the mechanisms underlying cardiovascular complications related to PD.

Aim and outline of this thesis

This thesis aims to investigate the functional heterogeneity of extracellular vesicles and their role in endothelial inflammation and activation of cardiovascular disease. In addition, this thesis explores methodological challenges associated with platelet-derived extracellular vesicle research in order to improve standardization and reproducibility.

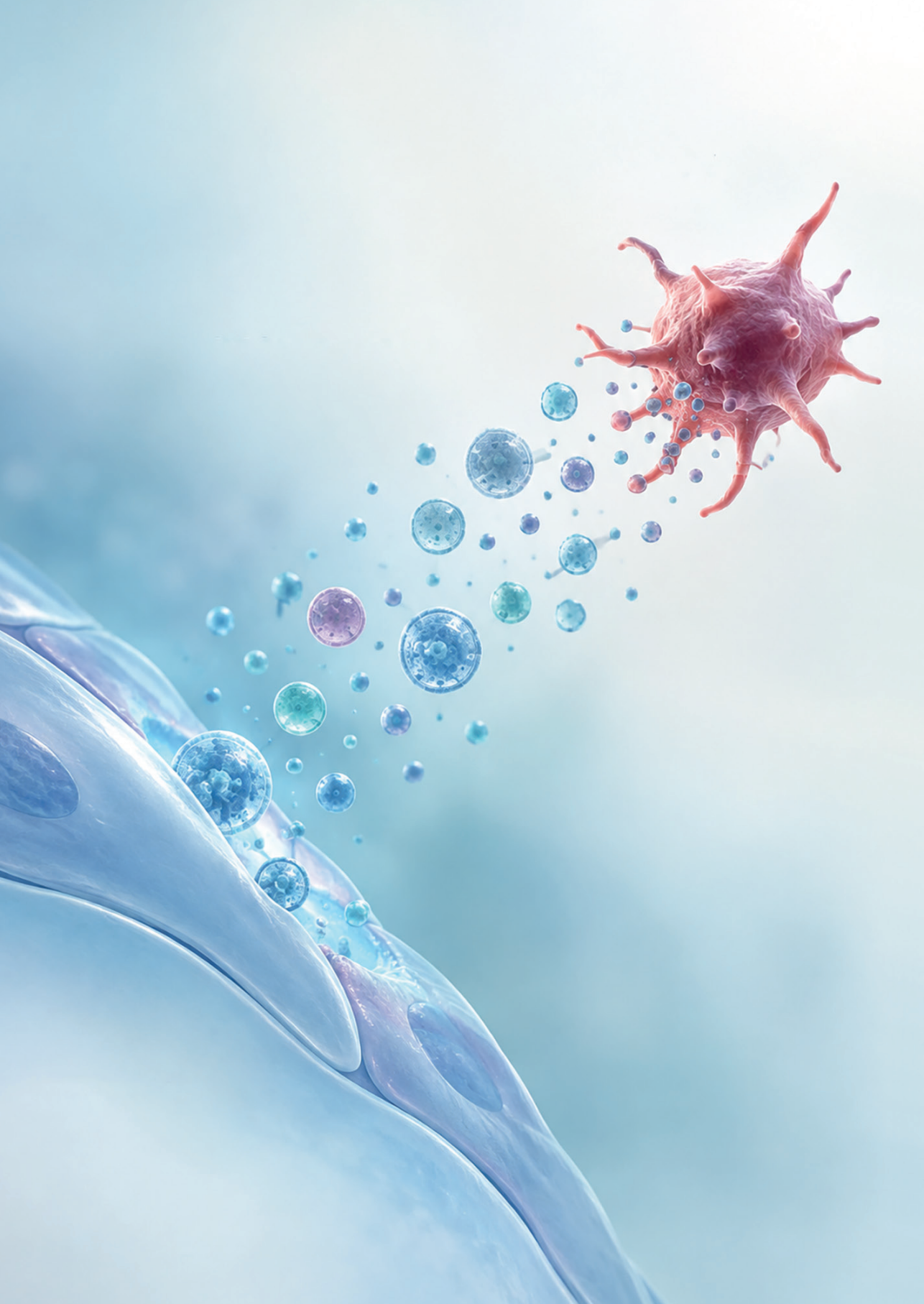
Chapter 1 offers a general introduction to the various topics and processes covered in this thesis. **Chapter 2** reviews the role of P-EVs in various cardiovascular diseases, including atherosclerosis, myocardial infarction, ischemia-reperfusion injury, and heart failure, and explores the potential of P-EVs as therapeutic targets and drug delivery vehicles. **Chapter 3** focuses on methodological challenges in P-EV research. It examines the generation and characterization of P-EV analogues derived from fresh and lyophilized platelets and compares different fluorescent labeling methods and their effects on EV properties and cellular uptake behavior. This chapter aims to establish a more stable and standardized platform for P-EV research. **Chapter 4** investigates the impact of EVs derived from various cell types on endothelial inflammation. It systematically compares the effects of EVs from different cell sources on endothelial cell function under both resting and inflammatory conditions, including endothelial activation, inflammatory factor release, monocyte adhesion, and apoptosis, to better understand the relative contributions of different EV subsets to vascular inflammation. **Chapter 5** expands EV-related research into clinical contexts. It examines the effects of peritoneal dialysis effluent derived EVs on endothelial cell inflammation and dysfunction. Additionally, it analyzes the relationship between these effects and the duration of peritoneal dialysis, as well as the presence of HFpEF, to explore the potential role of chronic inflammation-related EVs in cardiovascular complications among patients undergoing peritoneal dialysis. **Chapter 6** discusses the key results of this thesis in a broader perspective.

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Chapter 2

Assessing platelet-derived extracellular vesicles for potential as therapeutic targets in cardiovascular diseases

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Abstract

Introduction - Cardiovascular disease (CVD) is the leading cause of death worldwide. Platelet-derived extracellular vesicles (P-EV) have attracted extensive attention in cardiovascular disease research in recent years because their cargo is involved in a variety of pathophysiological processes, such as thrombosis, immune response, promotion or inhibition of inflammatory response, promotion of angiogenesis as well as cell proliferation and migration.

Areas covered - This review explores the role of P-EV in various cardiovascular diseases (such as atherosclerosis, myocardial infarction, ischemia-reperfusion injury, and heart failure), with relation to its molecular cargo (nucleic acids, bioactive lipids, proteins) and aims to provide new insights in the pathophysiologic role of P-EV, and methods for preventing and treating cardiovascular diseases based on P-EV.

Expert opinion - Studies have shown that the cargo of P-EV may be dysregulated during cardiovascular disease and delivery to tissues can result in detrimental pathophysiologic effects. Counteracting this process might have the potential to inhibit inflammation, promote angiogenesis, and inhibit cardiomyocyte death. In addition, P-EV have potential as biocompatible and autologous drug carriers. Therefore, better research on the mechanisms how P-EV act during cardiovascular disease and could be implemented as a therapeutic will provide new perspectives for the treatment of cardiovascular disease.

Keywords - Platelet-derived extracellular vesicles; cardiovascular disease; MicroRNA; Cell-cell communication; Therapeutics

1 Introduction

Cardiovascular disease (CVD) has been the leading cause of death worldwide for the past few decades, accounting for approximately 28.6% of all global deaths. As the mortality rate continues to rise each year, effective treatment options are urgently needed [43]. Platelets are known to play a significant role in cardiovascular diseases, including arterial thrombosis, vascular inflammation, and immune regulation [44,45]. Platelets secrete extracellular vesicles (P-EV) during aging and long-term storage [46,47] and after activation [48]. P-EV may act as a communication medium to mediate communication and interaction between platelets and other cells [17]. P-EV have specific surface markers (most notably CD41 and CD42b), and protein and genetic content similar to platelets [49], suggesting that they may also play a comparable role in the occurrence and development of CVD, which distinguishes them from extracellular vesicles (EV) derived from other cell types. At the same time, as a natural cellular biomaterial delivery carrier, P-EV can be implemented to selectively target sites of inflammation and vascular injury and have potential use as natural tools for diagnosis and treatment [50,51].

The above examples illustrate that P-EV may participate in intercellular communication and signal transduction through various mechanisms and may play a regulatory role in different physiological and pathological processes [52,53]. There is increasing evidence that P-EV is closely related to the occurrence and development, but also in the diagnosis and treatment of cardiovascular diseases [54]. P-EV contain many miRNAs and proteins that were found to play important roles in cardiovascular diseases [55,56]. The aim of this article is to review the research progress of the role of P-EV in the development and major manifestations of cardiovascular diseases that have high incidence and significant impact, focusing on the involvement of P-EV in the physiological and pathological processes of these diseases, as well as its potential use in treating cardiovascular diseases.

2 Definition, classification and biology of P-EV

As all extracellular vesicles, P-EV are enclosed by phospholipid bilayer and have a spherical structure with a diameter ranging from 40 nm to as large as 1 μm [57]. They are formed through platelet activation or spontaneously by aging and contain surface reservoirs similar, but not identical, to platelets [58]. Based on the "Minimal Information for Studies of EV" (MISEV) classification, P-EV are classified as exosomes (<0.1 μm) and platelet microparticles (PMP) (>0.1 μm). PMP are generated by shedding from the surface of platelets, while exosomes are derived from multivesicular endosomes (MVE) and α-granule exocytosis [52,59,60].

Among the various extracellular vesicles, P-EV account for over 70% of EV in blood [61] and are thus the most abundant type of circulating EV [48,58,62]. P-EV have been implicated in a variety

of key pathophysiological mechanisms [63]. Circulating P-EV are released by activated platelets or by megakaryocytes and carry platelet-specific proteins (such as CD41 and P-selectin) and genetic material (e.g. mRNAs and notably micro RNAs (miRNAs)) [64,65]. Due to these specific proteins and RNAs, P-EV can exert some functions of platelets, including hemostasis, immune regulation, and tumor progression [66-70]. Because of their large repertoire of adhesion receptors, P-EV may be unique compared to other EVs in their ability to mediate interactions with numerous different cell types, including immune cells and non-immune cells [71,72].

Given their abundance in circulation, it is not surprising that they are involved in various physiological and pathological processes [73]. P-EV can generally transport its contents (such as proteins, nucleic acids, and lipids) directly from the site of their formation to (distal) target cells, thereby regulating their biological functions [74]. They mainly interact with target cells through three mechanisms: (i) ligands on the P-EV membrane bind to receptors on the target cell membrane to activate signaling pathways, (ii) direct endocytosis, and (iii) membrane fusion [75]. Endocytosis allows exogenous P-EVs to enter the typical endosomal path and reach the MVE. Fusion of the MVE with the lysosome may lead to P-EV degradation, while P-EVs on the MVE plasma membrane or boundary membrane may release their contents into the recipient cell cytoplasm by fusion [76]. The biologic consequences of the cargo of P-EV are highlighted by a number of studies. For example, nucleic acids carried by the P-EV play a significant role in their functions. For example, miR-223 delivered by P-EV promotes lung cancer cell invasion by targeting tumor suppressor EPB41L3, an effect that could be counteracted by an antimir against miR-223[77]. Additionally, P-EV can also directly participate in intercellular communication by the action of their surface proteins. For instance, integrin β3 (ITGB3) expression is upregulated in the P-EV of patients suffering from nasopharyngeal carcinoma (NPC) and promotes the metastasis of NPC cells by inhibiting ferroptosis, a form of programmed cell death involving iron and peroxidated lipids [78]. But also lipid species in P-EV can influence the course of disease, for example lysophosphatidylcholine (LPC) in P-EV was shown to contribute to platelet activation, spreading, migration, aggregation, and might be a marker for plaque instability in cardiovascular disease [79]. The cellular effects of P-EV are summarized in Figure 1. The following section will provide detailed information on the specific biological functions of P-EV in manifestations of cardiovascular disease.

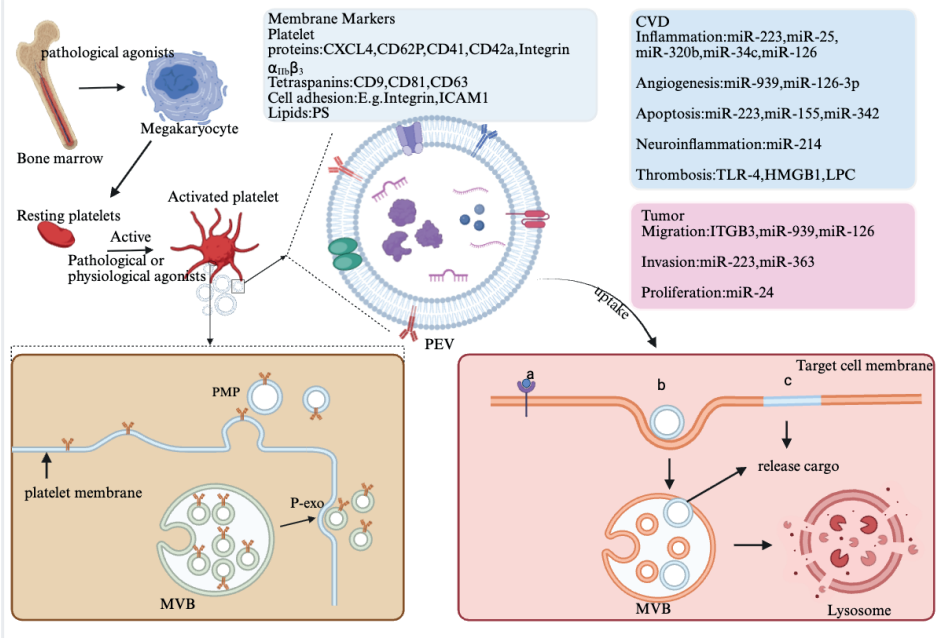


Figure 1 P-EV - biogenesis, fate, and cargo

P-EVs are released by activated platelets, mainly through platelet surface shedding and MVE exocytosis, and they interact with target cells mainly through three mechanisms: a. ligands on the P-EV membrane bind to receptors on the target cell membrane to activate signaling pathways, b. direct endocytosis, and c. membrane fusion. Endocytosis allows exogenous P-EVs to enter the typical endosomal pathway and reach MVEs. Fusion of MVEs with lysosomes may lead to P-EV degradation. At the same time, P-EVs on the MVE plasma membrane or boundary membrane may release their contents into the recipient cell cytoplasm by fusion. Various molecules exist on the surface or inside P-EVs, including nucleic acids, lipids, and proteins. Different platelet activation pathways affect P-EV cargo content. Created with BioRender.com.

3 P-EV in cardiovascular diseases

3.1 Atherosclerosis

Atherosclerosis is the leading cause of a variety of cardiovascular diseases, such as myocardial infarction, peripheral artery disease and ischemic stroke [80-82]. It is a chronic inflammatory disease characterized by the formation of atherosclerotic plaques, which are characterized by accumulation of fat, cholesterol, (micro)calcification, inflammatory cells, smooth muscle cells, fibroblasts, and fibrotic tissue in the walls of the larger arteries [83-85]. The formation mechanism of atherosclerosis involves endothelial cell dysfunction, abnormal proliferation and migration of vascular smooth muscle, and infiltration and activation of inflammatory cells [86-88]. Within the developing plaques, infiltrated monocyte matures into macrophages

that take up locally accumulated oxidatively modified low-density lipoproteins (ox-LDL) and ultimately transform into foam cells [86-90]. Although the role of platelets in the acute clinical manifestation of atherosclerosis has been well elucidated, the role of EVs released by platelets in the development and presentation of atherosclerosis remains to be further characterized.

Regarding the study of P-EV in atherosclerosis, researchers usually use P-EV derived from healthy donor platelets activated by thrombin as a model for EV release by platelets. P-EV from this source protects the vasculature in atherosclerosis mainly by blocking the classic inflammatory signaling pathways NF-κB and MAPK signaling pathways [91,92]. Thrombo-inflammation has emerged as an important pathophysiological mechanism for the development of atherosclerosis in the past two decades [93-95]. An important adhesion molecule in the development of atherosclerosis is Intercellular Adhesion Molecule 1 (ICAM-1, CD54), which can mediate inflammatory cell recruitment and infiltration, enhanced endothelial cell necrosis, and accelerated thrombotic reactions [96-99]. For example, one study has shown that EVs released from platelets from healthy volunteers activated by thrombin transfer miR-223 to endothelial cells, block NF-κB and MAPK signaling pathways by inhibiting the phosphorylation of p38, JNK, and ERK, and then negatively regulate the expression of ICAM-1 in endothelial cells, thereby attenuating the inflammatory response of endothelial cells [91]. The observation that P-EV downregulate ICAM-1 expression in endothelial cells was also made in a preceding study, but this effect was ascribed to the action of miR-320b [100]. On the other hand, a different study found that miR-223 transferring to endothelial cells by P-EV promoted HUVEC apoptosis induced by advanced glycation end products, by downregulating the expression of insulin-like growth factor 1 receptor [92]. Transfer of miR-320b to HUVEC led to enhanced HUVEC proliferation and migration, as well as to an inhibition of oxidative stress and inflammatory responses by targeting the electron transfer flavoprotein α subunit (ETFA), involved in flavoprotein electron transfer [101].

Further studies have found that EVs released from thrombin-activated mouse platelets down-regulate the expression of sialoglycoprotein podocalyxin (PODXL), a glycocalyx constituent, in human coronary artery endothelial cells (HCAECs) by transferring mir-34c-5p, inhibiting the inactivation of the p38 MAPK pathway and potentiating the inflammatory response of HCAECs [102]. The action of P-EV on endothelial cells might also be mediated by LPC, an abundant lipid component of P-EV, interacts with platelets, activates platelets, and promotes thrombosis [79].

Taken together, the action of P-EV on endothelial cells likely involves multiple miRNAs and might depend on the experimental context *in vitro*, and the pathophysiologic situation *in vivo*.

Next to endothelial cells, also vascular smooth muscle cells (VSMC) and immune cells can be influenced by P-EV. For example, P-EV that were harvested from stored platelets were able to induce a phenotypic shift in VSMC from a quiescent contractile to a synthetic and

proliferative phenotype [17], which is a process that occurs during the arterial remodeling and calcification [103]. In addition, like platelets, P-EV also direct the differentiation of immune cells [104,105], as is exemplified by the observation that they can polarize the differentiation of CD4+ T-cells towards regulatory T cells, thereby contributing to immune tolerance [106]. Monocytic cells can also be influenced by P-EV, as co-incubation of monocytes with P-EV led to a directed differentiation towards professional phagocytes [47], and the phagocytic potential of macrophages was increased by P-EV through the action of miR126-3p [107]. Of note, in the former study, the expression of the scavenger receptors CD68 and CD36 were increased after incubation with P-EV [47].

CD36 is a multi-ligand scavenger receptor on the surface of many cells, such as platelets, monocytes/macrophages, endothelial cells, and VSMC [108-110]. CD36 on the surface of monocytes/macrophages has been shown to play a vital role in developing atherosclerotic lesions by binding and endocytosing ox-LDL [111,112]. Thus, as a non-specific scavenger receptor for lipids, CD36 is associated with the formation of foam cells [113]. On megakaryocytes, the uptake of CD36 is important for their maturation and for the production of platelets [114]. On platelets, CD36 is also known as glycoprotein IV, and can mediate the adhesion of platelets to oxidized LDL [115]. Previous studies have shown that P-EV can inhibit platelet activation and thrombosis in a mouse carotid artery thrombosis model, possibly by promoting the ubiquitination and degradation of CD36 protein on the surface of platelets and macrophages, thereby inhibiting platelet activation, ox-LDL absorption, and foam cell formation [116]. Recent studies have found that patients with hypertension had high expression levels of miR-320b in plasma, which was ascribed to secretion into P-EV as a consequence of increased platelet activation. But P-EV can also be directly internalized by platelets through Toll-like receptor 4 (TLR-4), a process that may encourage thrombosis through an increase of platelet aggregation potential [117]. In addition, lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) exists on the surface of various vascular cells, mediating the uptake of ox-LDL, leading to forming foam cells, and promoting the occurrence and development of atherosclerosis [89,118,119]. Studies have found that the expression of miR-186-5p in circulating EVs of patients with acute myocardial infarction (AMI) is reduced, which in turn leads to a decrease in the inhibitory effect of miR-186-5p on LOX-1, promotes lipid accumulation, and further promotes the occurrence and development of atherosclerosis [120]. The above examples illustrate that P-EV can influence the development of atherosclerosis in multiple different ways (also summarized in figure 2). It would be interesting to unravel the exact contribution of the cargo and the binding and uptake mechanisms in order to design specific therapeutics.

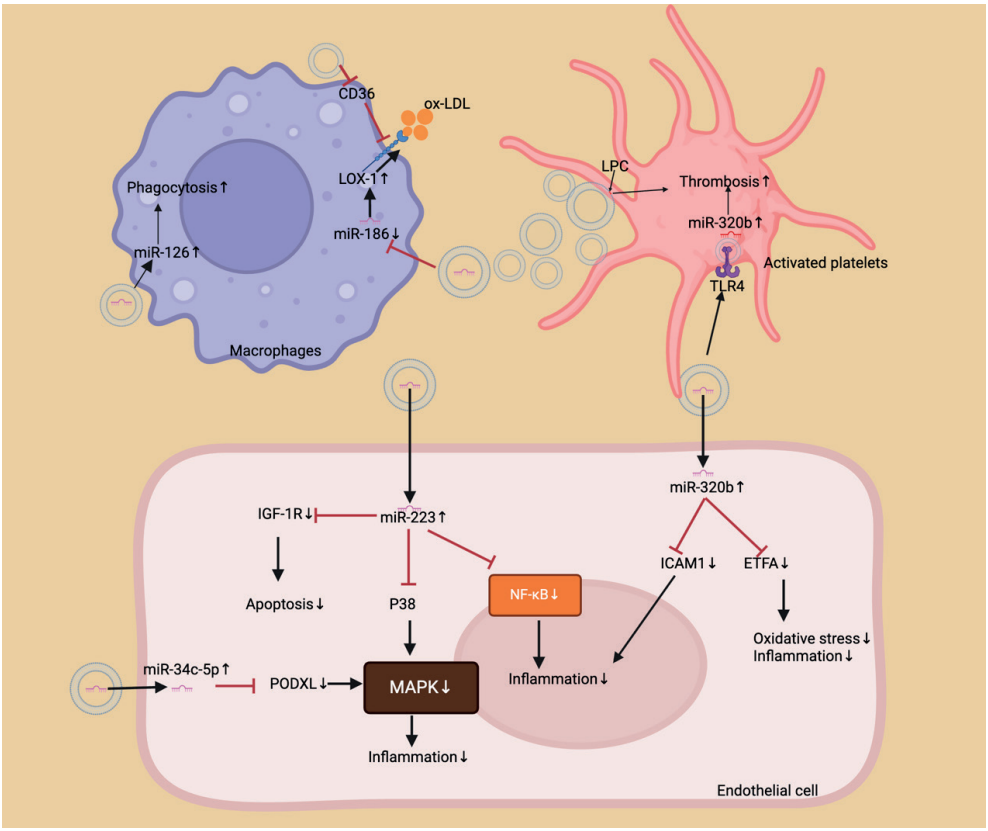


Figure 2 Effects of P-EV on the development of atherosclerosis.

Macrophages: Macrophages can ingest P-EVs containing miR-186 and miR-126. This process regulates the phagocytosis of ox-LDL by macrophages, which in turn affects the development of atherosclerosis. Platelets: P-EVs activate platelets and promote thrombosis through miR-320b and LPC. Endothelial cells: P-EVs transfer miR-223 to endothelial cells, inhibiting inflammation by blocking the MAPK and NF-κB signaling pathways and inhibiting endothelial cell apoptosis by targeting IGF-1R. Similarly, miR-34c-5p in P-EV modulates inflammatory responses by inhibiting the MAPK signaling pathway by targeting PODXL. Furthermore, miR-320b in P-EVs reduces endothelial inflammation by negative regulation of ICAM1 and inhibits oxidative stress and inflammation by targeting ETFA. Created with BioRender.com.

3.2 Myocardial infarction and cardiac ischemia-reperfusion injury

Cardiovascular diseases, including MI and ischemia-reperfusion injury, remain leading global causes of morbidity and mortality, imposing significant public health burdens [121-123]. The heart is a complex muscle pump that is responsible for maintaining normal blood pressure and providing nutrients and oxygen to the whole body through rhythmic contraction and relaxation [124], and dysfunction affects the entire organism. AMI leads to the loss of countless cardiomyocytes and triggers an extensive inflammatory response [125,126]. The cardiomyocytes

in adult mammals have very limited ability to regenerate [127] and lost heart muscle cells are replaced by non-contractile scar tissue [128]. Maintaining cardiac function after a heart attack involves reducing cell death, controlling inflammation, regulating scar tissue formation, and promoting angiogenesis [129-133]. Research has shown that exosomes in the blood of healthy individuals can protect the heart during reduced blood flow conditions [14]. This is mainly accomplished by delivery of specific molecular content.

For example, in a study by Ying Yang et al. it was found that P-EV in the blood of patients with AMI delivered miR-4306 to human monocyte-derived macrophages, inhibiting the migration of HMDMs by inhibiting the VEGFA/ERK1/2/NF-kB signaling pathway, thereby reducing the number of macrophages in the heart tissue of mice with MI [134]. But also proteins are transferred, high mobility group box 1 (HMGB1), a member of the damage-associated molecular patterns (DAMPs) family abundantly present in platelets [135], can activate immune cells, promote the production of proinflammatory factors, and promote blood clotting and thrombosis [136-139]. Recent research has considered MI as an abnormal sterile inflammatory process, and high levels of HMGB1 in human coronary artery blood clots are linked to poor clinical outcomes [140]. Studies have found increased HMGB1 expression in P-EV of patients with sepsis [141], in ST-elevation MI [142], and diabetes [143]. HMGB1 promotes blood clotting and inflammation and worsens blockages in the small blood vessels in the heart [142], and is also a driver of the formation of neutrophil extracellular traps, neutrophil-expelled chromatin that can serve as a scaffold for thrombotic reactions [144]. Solute carrier family 7 member 11 (SLC7A11) is a cystine-glutamate antiporter that mediates cystine transport to promote reduced glutathione and glutathione peroxidase 4 (GPX4) synthesis and plays a crucial role in inhibiting ferroptosis, a form of programmed cell death characterized by the iron-dependent accumulation of peroxidized lipids [145]. Zhao et al. discovered that EV derived from adipose tissue macrophages influence ferroptosis in cardiac injury caused by obesity through the transfer of mIR-140-5p, that targets and downregulates SLC7A11 [146]. In addition, a related study found that the expression of miR-26b-5p in plasma-EVs of patients with AMI was reduced, and the ability to target and inhibit the downstream ferroptosis marker SLC7A11 was weakened, thereby inhibiting ferroptosis after myocardial infarction, providing a promising target for the treatment of AMI [147].

Numerous studies have confirmed that nitric oxide (NO) is a protective molecule for endothelial cells, promoting cell growth and migration while reducing cell death [148,149]. Expression of miR-939-5p in serum-EVs of patients with myocardial ischemia was found to be reduced, while the downregulation of miR-939-5p reversely upregulated the expression and activity of inducible nitric oxide synthases (iNOS), enhanced the production of endothelial NO, and thus promoted angiogenesis [150]. Furthermore, serum EVs in patients with AMI promoted angiogenesis by transferring miR-126-3p to endothelial cells, targeting and inhibiting tuberous sclerosis complex 1 (TSC1) and activating the mTORC1/HIF-1α signaling pathway, constituents of the hypoxia

response [151]. This effect might also be attributed to the release of angiogenic chemokines such as CXCL12 [152].

At present, the optimal treatment strategy for AMI is to restore cardiac blood supply as quickly as possible [153-156]. However, during reperfusion, irreversible myocardial damage caused by an abnormal inflammatory response, excessive oxidative stress, and cell death is called ischemia-reperfusion injury [157-159]. *In vitro* and *in vivo* studies indicate that circulating extracellular vesicles, primarily P-EVs, have a cardioprotective role during ischemia-reperfusion injury [160]. Additionally, related studies have highlighted the therapeutic potential of EVs in modulating inflammation in ischemia-reperfusion injury [161,162].

Livkisa and colleagues isolated EVs from clinical-grade human platelet concentrates to evaluate the protective effects and mechanisms of P-EV on myocardial ischemia-reperfusion. In a mouse ischemia-reperfusion model, P-EV could improve cardiac function, reduce scar formation, and promote angiogenesis after ischemia-reperfusion. In experiments *in vitro*, they have protective cardiomyocyte activity, maintain cell metabolic activity, reduce cell apoptosis, and stimulate human microvascular endothelial cell tube formation [163]. Similarly, studies have found that EVs from healthy male blood have a cardioprotective effect in ischemia-reperfusion injury, mediated by heat shock protein 70 (HSP70) on the surface of EVs, stimulating TLR4 signaling, leading to the activation of ERK1/2 and p38MAPK, and subsequent HSP27 phosphorylation in cardiomyocytes, playing a protective role by resisting oxidative stress and apoptosis, and maintaining sarcomere structure [164]. Studies have found that exercise-induced circulating EVs also play a cardioprotective role by activating ERK1/2 and HSP27 phosphorylation [165]. On the contrary, studies have found that EVs from the blood of a mouse ischemia-reperfusion model promote ischemia-reperfusion injury by transferring miR-155-5p to cardiomyocytes, inhibiting E3 ubiquitination ligase neural precursor cell expressed developmentally downregulated protein 4 (NEDD4) protein expression and the tagging of cyclophilin D (CypD), a peptidyl prolyl-cis-trans isomerase and a regulator of the mitochondrial permeability transition pore, for degradation by ubiquitination [166].

It is well known that remote ischemic preconditioning (IPC), e.g. by temporarily constricting blood flow in the limbs, can protect the myocardium from ischemia-reperfusion injury, and many studies have found that circulating EVs are involved in this process [167,168]. Studies have found that after remote IPC treatment, the number of circulating EVs in rats increased, and EV treatment can significantly reduce myocardial damage by inhibiting endoplasmic reticulum stress [169]. Wen Minghua and colleagues isolated EVs from rat blood after IPC in rats and found that miR-24 expression increased in IPC-EVs. This miRNA can negatively regulate the expression of Bim, a pro-apoptotic inducer of cell death, and reduce cardiomyocyte apoptosis, alleviating myocardial ischemia-reperfusion injury[170].

After establishing an ischemia-reperfusion model in rats, Ma Fang and colleagues extracted P-EV from collected blood [171]. Venous infusion of P-EV ischemia-reperfused rats led to an increase of the P-EV concentration in blood, and a decrease of the infarcted area. The protective effects of P-EV on rat myocardium sharply reduced after 6 hours. This phenomenon may be explained by a quick clearance of P-EV in the circulation, and the half-life of P-EV in the systemic circulation is less than 6 hours[171,172].

3.3 Heart failure and myocarditis

Chronic heart failure (CHF) is a leading cause of mortality and morbidity worldwide and has become a growing public health problem[173]. Heart failure refers to insufficient perfusion of organs throughout the body due to the heart's inability to pump blood effectively[174,175].

Previous studies have shown that circulating EVs during reperfusion are thought to play a therapeutic role in heart failure[176]. For example, Zhaofei Luo and colleagues found that EVs induced by ischemic preconditioning in normal rats retained protection against I/R injury in the failing heart after infarction by activating the pro-survival kinases ERK1/2 and Akt [177].

The number and activation state of peripheral blood mononuclear cells (PBMCs) in patients with heart failure increases [178], promoting the release of inflammatory factors (such as TNF-α, IL-10, CCL2, and ICAM1) and thereby promoting the progression of heart failure[179,180]. Studies have found that EVs from healthy donor plasma can inhibit the expression of miR-126 in PBMCs of patients with CHF, thereby reducing the release of inflammatory factors and exerting a protective effect on the heart [181].

Sympathetic nerve hyperexcitation plays an essential role in the progression of CHF [182,183]. It is reported that the rostral ventrolateral medulla (RVLM) is a crucial area for sympathetic nerve regulation, plays a vital role in regulating the cardiovascular system, and is closely related to the occurrence and development of heart failure [184-186]. Due to the existence of the blood-brain barrier (BBB), EVs play an essential role as a bridge for communication between the central and peripheral systems [187-189]. Studies have found that the expression of miR-214-3p in circulating EVs and RVLM of CHF rat models is upregulated, and miR-214-3p enhances the inflammatory response in the RVLM by inhibiting the expression of let-7g-5p and let-7i-5p, which may further promote sympathetic nerve hyperexcitation in CHF [190].

Viral infections are the leading cause of myocarditis worldwide, including severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), coxsackievirus B (CVB), influenza and parvovirus [191-194]. Patients with viral myocarditis are at risk for sudden death from acute heart failure, and susceptible individuals develop dilated cardiomyopathy (DCM) and chronic heart failure [195-197].

Recent studies have found that P-EV derived from healthy donors can significantly reduce myocarditis and improve cardiac function. P-EV contain miRNAs that reduce viral replication, TLR4 signaling, and T cell activation, leading to decreased cytokine production (interleukin 1β (IL-1β), transforming growth factor-β 1, matrix metalloproteinases) and mast cell degranulation [198]. Interestingly, administration of P-EV did not inhibit pericardial inflammation, primarily characterized by the presence of macrophages and mast cells [198].

4 P-EV as drug delivery vehicle

P-EV has many advantages as therapeutic biomaterials, such as their nanoscale structural properties that enable them to circulate in the human body for extended periods; therefore, P-EV used for drug loading can prolong drug release [21,199,200]. In addition, P-EV can penetrate tissue barriers such as the synovium, lymph, and bone marrow to achieve specific therapeutic effects [201]. Since P-EV are derived from platelets, they have significant biomarkers on the membrane surface, such as CD41 and CD62P, which mediate P-EV targeting local inflammatory and tumor sites [21,202-205]. In addition, sources of P-EV, such as PC, platelet lysate and platelet-rich plasma have been widely used in clinical practice [206]. Since platelets are anucleate, there is no risk of unnecessary proliferation or mutagenesis after injection; therefore, the overall safety risk of P-EV may be inherently low [207]. In recent years, there has been widespread attention on the use of P-EV as drug carriers or guiding agents of stem cells.

There are various strategies for obtaining P-EV for research and drug targeting purposes. Most commonly, platelets are activated by agonists and subsequently separated from the EV by differential centrifugation combined with (ultra-)filtration [208]. P-EV are also formed spontaneously during longer term storage, and can be likewise harvested by centrifugation and filtration. Care needs to be taken that preparations are not contaminated by lipoproteins, that may sediment as contaminants and might perturb subsequent biologic assays [209]. For drug delivery purposes, platelet membranes can be prepared by extrusion or sonication.

For example, platelet nanovesicles prepared by sonication were associated with cardiac stem cells and aided the guidance of these cells to sites of cardiac injury in a model of AMI, which led to a reduction of myocardial damage and improved cardiac function [205]. IL-1β plays a central role in the sterile inflammatory response caused by AMI. Therefore, IL-1β blockade is a promising strategy for the treatment of AMI [210,211]. However, traditional IL-1β blockers lack targeting specificity. This increases the risk of severe side effects. Ke Chengs's team manufactured P-EV with anti-IL-1β antibodies and injected them intravenously into a mouse model of myocardial infarction. The results confirmed the targeting ability of P-EV and inhibited inflammation and cardiomyocyte apoptosis to exert cardioprotective effects [212]. Likewise, Qingle Ma and colleagues loaded IL-1β inhibitors onto P-EV and infused them into mice with atherosclerosis.

The results showed that P-EV loaded with IL-1β inhibitors reduced local inflammation and plaque size in mice, exerting anti-atherosclerotic effects [213]. In addition, similar studies found that platelet membranes fused with EVs secreted by mesenchymal stem cells and extracellular vesicles derived from M2 macrophages, respectively, and both exerted cardioprotective effects in mice with atherosclerosis [214,215].

5 Expert opinion

Cardiovascular disease has always been a research hotspot due to its widespread prevalence and high mortality and hospitalization rates. Its related pathophysiological mechanisms include cell death, inflammation, angiogenesis, fibrosis, and lipid metabolism, which all are important to control or counteract. Many studies have confirmed the critical role of miRNA in various cardiovascular diseases and circulating miRNAs are mainly carried by EV. Since platelets are the primary source of circulating EV and contain miRNAs [216,217], it is conceivable that many circulating miRNAs are carried in platelet EVs. Platelets play a crucial role in atherosclerotic thrombosis and other inflammatory-related cardiovascular diseases, and P-EV may contain a part of the proteins and genetic information as their platelet parents. Therefore, P-EV may be involved in the occurrence and development of cardiovascular diseases. Platelets release a large amount of P-EV when subjected to physiological or pathological stimulation. Studies have confirmed that P-EV increases the blood circulation of patients with cardiovascular disease. The cargo in platelets, and likely also the P-EV, from patients and healthy individuals is different [74,218]. Some studies have confirmed that P-EV from healthy individuals has a more substantial protective effect on the heart than P-EV from patients. Thus, the cargo and the effects that platelets and P-EV might have on the target cells and organs might depend on the pathophysiological conditions. Indeed, studies have confirmed that P-EV play a role in atherosclerosis. Interestingly, P-EV surface proteins and lipids appear to promote atherosclerosis, while the contained miRNAs protect from it. Since the manner of activation of platelets appear to influence the cargo of the P-EV [74], it would be of key interest to distinguish “beneficial” from “detrimental” manners of platelet activation in future research in order to tilt P-EV actions towards healing. This might even be in reach in the near future, given the many clinically approved platelet antagonists on the market.

Platelets are equipped with many adhesion receptors to interact with their molecular and cellular environment, and P-EV can be implemented as a biocompatible drug delivery vehicle to target specific sites of inflammation and damage. When equipped with rationally chosen bioactive agents, inflammation can be curbed and tissue healing promoted. Platelets are readily accessible as sources of P-EV, in the form of platelet lysates, concentrate, and (autologous) platelet-rich plasma, all of which are established clinical products. Given the above considerations, it can be

concluded that P-EV are interesting regulators of human cardiovascular diseases and also have excellent potential for their understanding, diagnostics, and treatment.

6 Highlights:

a) Platelet-derived extracellular vesicles are increasingly established as vital communication mediators in the cardiovascular system.
b) The cargo of platelet-derived extracellular vesicles may play an important role in pathological processes such as thrombosis, inflammation, angiogenesis, and tissue healing.
c) Platelet-derived extracellular vesicles have potential both as drug delivery vehicles and therapeutic targets for cardiovascular diseases in clinical practice.

7 Author contributions

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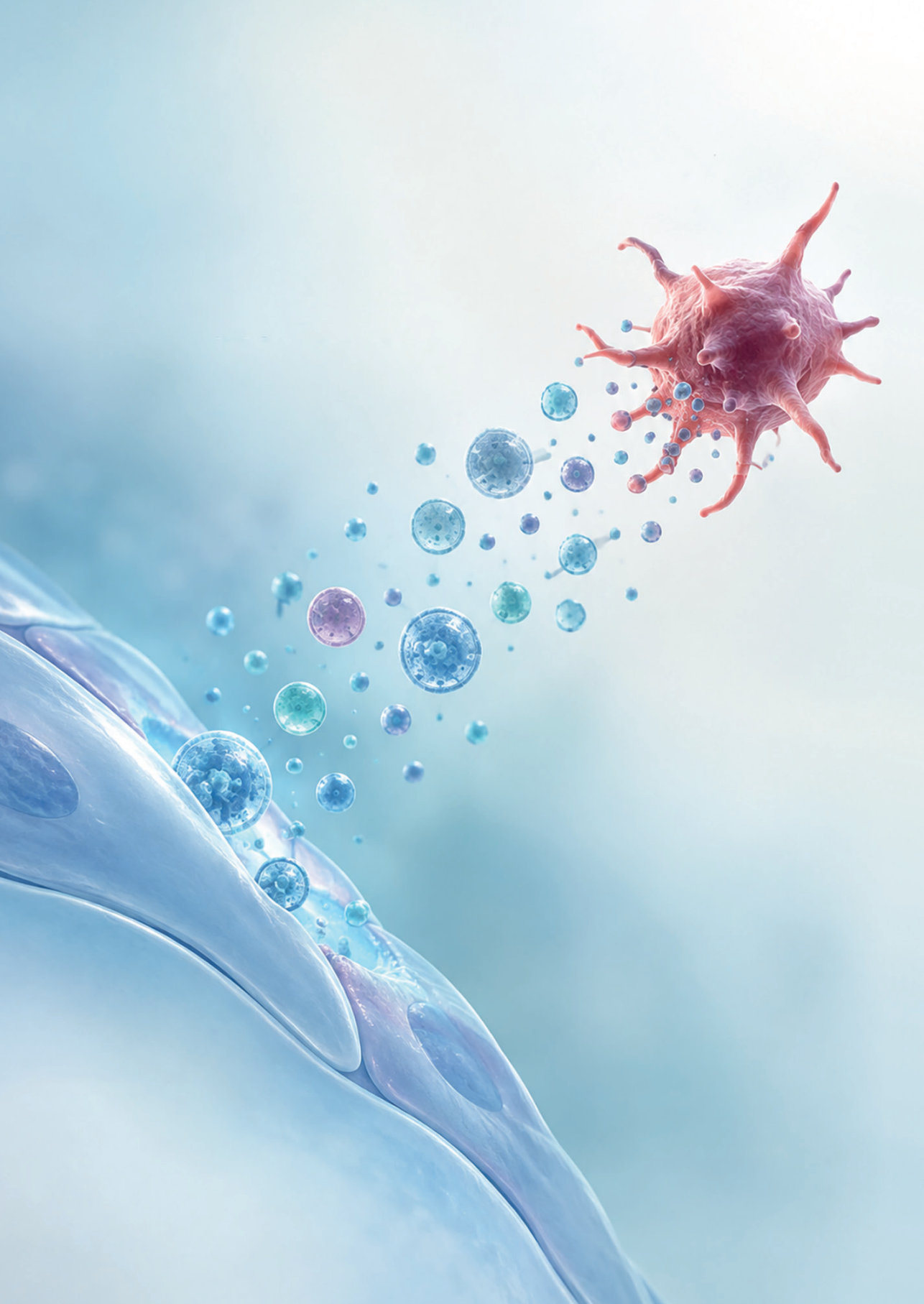
These interesting studies explored the content and dynamics of nucleic acids in platelets in health and disease.

Table 1. The role of P-EV cargo in cardiovascular disease

EV type	Cargo	Disease	Interaction	Target cell	Isolation	Characterization	Quantification method	Effect	Ref
P-EV	miR-223	AS	NF-kB/MAPK	HUVECs	UC	TEM, NTA, IF	qRT-PCR	Inflammation ¯	[91]
	miR-223	AS	IGF-1R	HUVECs	UC	–	qRT-PCR	Apoptosis	[92]
	LPC	AS	G2AR	DAMI	UC	FC	LMS	Thrombosis	[79]
	miR-34c-5p	AS	PODXL/MAPK	HCAECs	UC	FC, TEM, NTA, WB	qRT-PCR	Inflammation ¯	[102]
	poly-ubiquitin	AS	CD36	Platelet	UC	TEM	FC	Foam cell formation -	[116]
	miR-186	AS	LOX-1	RAW264.7 macrophage cells	UC	TEM, NTA , WB	qRT-PCR	Foam cell formation	[120]
	miR-4306	MI	VEGFA/NF-KB	HMDMS	UC	FC, WB	qRT-PCR	Migration ¯	[134]
	HMGB1	MI	TLR4	HCEMC	UC	FC	FC, WB,Elisa	Inflammation Coagulant	[142]
	miR-26b-5p	MI	SLC7A11	H9C2	EQ	WB, TEM, NTA	qRT-PCR	Ferroptosis ¯	[147]
Circulating EV	miR-939-5p	MI	iNOS	HUVEC	UC	WB, TEM, NTA	qRT-PCR	Angiogenesis	[150]
	miR-126-3p	MI	TSC1/mTORC1/ HIF-1α	HUVEC	UC	WB, TEM, NTA	qRT-PCR	Angiogenesis	[151]
	miR-155	MI-IR	NEDD4/CypD	primary cardiomyocytes	EQ	WB, TEM, NTA	qRT-PCR	Apoptosis	[166]
	HSP70	MI-IR	TLR4	primary cardiomyocytes	UC	WB, TEM, NTA,FC	WB	Apoptosis¯ Oxidative stress¯	[164]
	miR-24	MI-IR	Bim	H9C2	EQ	WB,TEM,NTA	qRT-PCR	Apoptosis¯	[170]
	miR-214-3p	CHF	let-7g-5p, let-7i-5p	PC12	TE	NTA,TEM	qRT-PCR	Neuroinflammation	[190]

AS: Atherosclerosis

MI: Myocardial infarction
IR: Ischemia-reperfusion
CHF: Chronic heart failure
UC: Ultracentrifugation
EQ: ExoQuick Kit
TE:TransExo® Exosome Isolation Kit
FC: Flow cytometry
WB:Western blot
LMS:Lipid mass spectrometry
TEM:transmission electron microscopy
NTA:NanoSight-tracking analysis
IF:Immunofluorescence staining
IGF-1R: Insulin-like growth factor 1 receptor
LPC: Lysophosphatidylcholine
PODXL: podocalyxin
LOX-1: lectin-like oxidized low-density lipoprotein receptor-1
HMGB1: High mobility group box 1
TLR4: Toll-like receptor 4
SLC7A11: solute carrier family 7 member 11
TSC1: tuberous sclerosis complex 1



Chapter 3

Generation and characterization of platelet-derived extracellular vesicle analogues from lyophilized platelets

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Abstract

Platelet-derived extracellular vesicles (P-EV) are thought to facilitate the transfer of information from platelets to target cells, playing a role in both physiologic and pathophysiologic processes, particularly in regulating immune responses and healing processes. In addition, P-EV show promise as drug carriers and biomarkers for disease. However, the procedures for isolation, purification and fluorescent labeling of P-EV remain unstandardized. Moreover, the requirement to use freshly obtained platelets for generating EV presents a logistical challenge for their study. In this study, we isolated, characterized, and compared P-EV analogues by sonication of freshly obtained and lyophilized platelets, investigated fluorescent labeling methods, and monitored cellular uptake. We found that P-EV analogues derived from fresh or lyophilized platelets showed similar characteristics regarding size, surface proteins and content. Among the fluorescent labeling methods, CFSE and DiO-C6 were most effective in labeling P-EV analogues from both fresh and lyophilized platelets. All labeling methods led to an increase in P-EV analogue's size, with CFSE and DiO-C6 resulting in the smallest increase. The addition of P-EV analogues to cultured immortal endothelial cells revealed that P-EV analogues were effectively internalized and directed to the lysosomal compartment. The results indicate that P-EV analogues from lyophilized platelets have similar functional properties as those from freshly isolated platelets and these are retained after labeling with CFSE. Thus, lyophilized platelets can serve as a source of P-EV analogues for functional studies.

Keywords: platelets, extracellular vesicle, isolation, labeling, endothelial cell, internalization

1 Introduction

Platelet-derived extracellular vesicles (P-EV) are phospholipid bilayer-encapsulated vesicles secreted by platelets upon activation or during prolonged storage [1-3]. Like their platelet origins, they contain a rich variety of molecular cargoes, including proteins, lipid mediators, and microRNAs, which can be transferred to target cells, thus playing an important role in intercellular communication [1,4,5]. Since P-EV have characteristics of their parental cells, including many platelet adhesion receptors on their surface, they have great potential as drug delivery vehicles that use these adhesion receptors to specifically target to sites of vascular and tissue injury [6-9].

Platelets have limited storage times, leading to a rapid decline in their quality, and the carefully controlled conditions required for platelet purification make it logistically challenging to perform functional studies on e.g. P-EV obtained from patients. Studies have shown that freeze-dried platelets could serve as a viable alternative to fresh platelets for obtaining functional P-EV [21]. However, whether extracellular vesicles from freeze-dried platelets (LP-EV) and extracellular vesicles from fresh platelets (FP-EV) have the same characteristics has not yet been conclusively investigated.

A common technique for studying EV biodistribution and target cell interaction involves labeling of EV with fluorescent dye molecules. There are various approaches for EV labeling. The most used method is the incubation of isolated EV with lipophilic fluorescent or lumen-binding dyes. However, the fluorescent labels typically have notable drawbacks such as the labeling efficiency of dyes. In addition, lipophilic dyes (e.g., PKH67) are prone to self-aggregation, forming micelles that closely resemble EV in size, structure, and fluorescence intensity. Moreover, the dyes may aggregate in EV, forming oligomers that increase the size of the EV [11-14]. Successful removal of unbound dyes from EV is a further critical step in most EV studies, as fluorescent dyes that are not associated with EV can compromise study results or even lead to false conclusions about EV function.

In this study, we compared P-EV analogues from two lyophilized and freshly stored platelets via sonication, compared the properties of P-EV analogues labeled with four common fluorescent dyes, and demonstrated the uptake of P-EV analogues and their fate in endothelial cells.

2 Material and method

2.1. Isolation of P-EV analogues

The isolation, processing and analysis of P-EV analogues was performed taking the recommendations of MISEV into account [15]. Fresh blood was collected from 8 healthy male

or female donors (aged 20–30 years), after obtaining informed consent, and was mixed with sodium citrate solution in a 9:1 volume ratio. The whole blood was centrifuged at 350 g for 15 min at room temperature (RT) to obtain platelet-rich plasma. The supernatant was collected and anticoagulant citrate buffer (at a volume ratio of 1:15) was added to maintain platelet quiescent. The platelets were subsequently precipitated by centrifugation at 1240 g for 15 min at RT, and two washing steps with Hepes buffer pH 6.6 (10 mM Hepes pH 6.6 ,136 mM NaCl, 2.7 mM KCl, 2 mM $MgCl_2$, 0.1% bovine serum albumin, 0.1% glucose, 200 U/ml apyrase and 2 µM prostaglandin E1). After pelleting, platelets were resuspended in Hepes buffer pH 7.45 (10 mM Hepes pH 7.45, 136 mM NaCl, 2.7 mM KCl, 2 mM $MgCl_2$, 0.1% bovine serum albumin and 0.1% glucose) at a concentration of 1×10^9 platelets/ml. Half of these platelets were lyophilized in the presence of 35 mM trehalose according to previous studies [16]. For the isolation of P-EV analogues, fresh and lyophilized platelets were sonicated for 5 min using a tip probe sonicator at a frequency of 20 kHz to disperse the platelets in Hepes buffer pH 7.45 at 1×10^8 platelets/ml. The sonicate was centrifuged at (1240 g) for 10 min, supernatants were transferred to a clean tube and filtered through a 0.8 µM filter. EV were pelleted by centrifugation at 20,000g for 1 hour at 4 ℃. Then P-EV analogues were purified by size exclusion chromatography (SEC) using Sepharose CL-2B (GE Healthcare, Cat. No.10276541), followed by elution in Hepes buffer pH 7.45. The eluate was collected in 20 sequential fractions of 0.5 ml. The fractions that eluted between 2 and 6 ml were pooled for experiments. Pooled P-EV analogues were measured by nanoparticle tracking analysis (NTA, ParticleMetrix, Inning am Ammersee, Germany), snap frozen in liquid nitrogen and stored at −80°C until use.

Table 1 - Buffer compositions

Buffer name	Composition and Concentration
Wash buffer	10 mM HEPES pH 6.6 ,136 mM NaCl, 2.7 mM KCl, 2 mM $MgCl_2$, 0.1% bovine serum albumin, 0.1% glucose, 200 U/ml apyrase and 2 µM prostaglandin E1, pH 6.6
Buffer A	100 mM NaCl, 10 mM KCl, 10 mM EGTA, 10 mM imidazole, 10 mM prostaglandin E1, 35 mM trehalose, pH 6.8
lyophilization buffer	9.5 mM HEPES, 142.5 mM NaCl, 4.8 mM KCl, 1 mM $MgCl_2$, 30 mM trehalose, 1% human serum albumin
Hepes buffer	10 mM HEPES buffer, 2 mM $CaCl_2$, 2.7 mM KCl, 2 mM $MgCl_2$ supplemented with 0.1% BSA and 0.2% glucose, pH 7.45

2.2. Size distribution analysis

The EV size distribution was measured by NTA. EV sample and labeled EV were diluted in filtered PBS to an acceptable concentration according to manufacturer's instructions. Samples were analyzed for size and concentration using NTA video capture for 0.5 s at 11 positions.

The acquisition parameters were set as follows: Sensitivity=80 nm/s, Shutter speed =100 milliseconds, Frame rate=30 frames per second, cycle number=2, Minimum brightness=30, Maximum size =1000 nm, Minimum size=10 nm, Tracelength=15. The videos were analyzed using ZetaView software (ParticleMetrix). Measurements were performed on at least three biological replicates, with two technical replicates each.

2.3. Cryo-transmission electron microscopy (EM)

A 2.5 µl sample containing 5 × 10^{10} EV /ml was applied to a glow discharged R2/2 200 µm grid (Quantifiol) to form a thin water film. These films were vitrified using a Vitrobot (FEI, Eindhoven, The Netherlands) by plunging the grid into ethane kept at the melting point of liquid nitrogen and at 100% humidity. Images were taken at −180 to −190 °C using a 4,096 × 4,096-pixel Arctica 200 kV (FEI Corporation) and Falcon III (FEI Corporation) camera as described previously [17].

2.4. Flow cytometry

P-EV analogues were characterized and quantified by flow cytometry (BD Accuri). P-EV analogues were stained with 1 µg/ml annexin A5-FITC (Annexin Pharmaceuticals), 1.25 µg/ml mouse monoclonal anti-human CD41a-PE (eBioscience) and 1.25 µg/ml mouse monoclonal anti-human CD62P-Ab-APC (eBioscience) in Hepes buffer containing 0.5% BSA and 5 mM $CaCl_2$ for 30 min protected from light. As a negative control, P-EV analogues were stained with 1 µg/ml annexin A5-FITC, 1.25 µg/ml mouse IgG-PE isotype control (eBioscience) and 1.25 µg/ml mouse IgG-APC isotype control (eBioscience) in Hepes buffer containing 0.5% BSA and 2 mM EDTA. Single staining controls were prepared accordingly for fluorescence compensation. The samples were measured by flow cytometry (BD Accuri). Data was analyzed using FlowJo software (Ashland, OR, USA) and the online Floreada.io tool (https://floreada.io).

2.5. Western blot

Total protein was extracted from P-EV analogues using RIPA lysis buffer (Thermo Fisher Scientific, Cat. No. 89900) containing 1% protease inhibitor (Thermo Fisher Scientific, Cat. No. 78430). Protein concentration was determined using a Micro BCA™ Protein Assay Kit (Thermo Fisher Scientific, Cat. No. 23235) according to manufacturer's protocol, and the protein concentration of the isolate was diluted to (2 µg/µl in 10 µl). The protein SDS-PAGE sample was prepared by adding SDS loading buffer prior to denaturation in a thermal block at 95 ℃ for 10 min. Samples were loaded on a 10% SDS-gel, resolved using SDS-PAGE under reducing conditions, and transferred to polyvinylidene fluoride (PVDF) membranes using 1×transfer buffer (Bio-RAD, Hercules, CA, Cat. No. 10026938) with 10% ethanol by Trans-Blot Turbo system (Bio-RAD). The membranes were blocked with 5% skimmed milk for 1h at RT. After blocking, PVDF membranes were incubated with the primary antibodies diluted in 5% BSA (Sigma, Cat. No. A9647) overnight on a rocking table at 4 ℃. The specific primary antibodies used were anti-integrin β3 (1:1000; Cell Signaling Technology, Danvers, MA, USA, Cat. No. 4702S), anti-TSG-101 (1:2000; Cell Signal, Cat. No. 72312), anti-CD63(1:1000; Cell Signal, Cat. No. 52090), anti-CXCL4 (1:1000;

Thermo Fisher Peprotech, Cat. No. P500-P05), anti-β-actin (1:1000; Cell Signal, Cat. No. 52090). Subsequently, the membranes were washed three times with 1×TBST and incubated with the HRP conjugated secondary antibody for 1 hour at RT. Pierce™ ECL Western Blotting Substrate (Thermo Fisher Scientific, Cat. No. 32106) was used to detect the protein signals. The relative density of protein bands was measured using ImageJ (https://imagej.net).

2.6. Culture of the EA.Hy926 endothelial cell line

EA.Hy926 cells (ATCC® CRL-292) were cultured in Dulbecco's Modified Eagle Medium (DMEM; Thermo Fisher Scientific, Cat. No. 31966021) supplemented with 10% fetal bovine serum, 1× HAT (5 mmol/L sodium hypoxanthine, 20 µM aminopterin, and 0.8 mmol/L thymidine) and 100 µg/ml penicillin/ streptomycin at 37 °C with 5% CO_2 as described in [18]. The culture medium was exchanged every 2 days. Cells were harvested with 0.05% trypsin-EDTA (Thermo Fisher Scientific, Cat. No. 25300) and sub-cultured upon reaching 70-85% confluency. Cells were used for experiments up to passage 5.

2.7. P-EV labeling

P-EV analogues in PBS were stained by incubation with freshly prepared dye solutions as follows: 5(6)-Carboxyfluorescein diacetate *N*-succinimidyl ester (CFSE, Sigma, Cat. No. 150347-59-4), 5-Chloromethylfluorescein diacetate (CMFDA, Celltracker™ green, Cayman Chem, Cat. No.136832-63-8), 2',7'-Bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, Acetoxymethyl ester (BCECF, AM, Thermo Fisher Scientific, Cat. No. B1170) and 3,3'-Dihexyloxacarbocyanine iodide (DiO-C6, Thermo Fisher Scientific, Cat. No. D273), or CellMask™ Deep Red (Thermo Fisher Scientific, Cat. No. C10046) staining stocks were made following the manufacturer's instructions resulting in 5 mM.

To stain EV with luminal dyes, three portions of 150 µl EV-containing PBS solution were added to 150 µl of 32 µM CFSE, 8 µM CMFDA and 32 µM BCECF solution, respectively, and incubated for 30 min, 1 h, or 2 h at 37 ℃. To stain EV with DiO-C6, 150 µl of EV-containing PBS solution was added to 150 µl of16 µM DiO-C6 in PBS and incubated for 30 min at 37 ℃ protected from light. To remove unbound dye molecules, the samples were centrifuged at 20000×g for 60 min at 4 ℃ and resuspended in equal volumes of PBS.

2.8. Cellular uptake of labeled P-EV analogues

To investigate P-EV analogues internalization, labeled P-EV analogues were incubated with EA.Hy926 cells. Cells were seeded in 6 well plates at densities of 5×10^5 cells/well. After 24 h of culture, EV were resuspended in fresh, fully supplemented media at a concentration of 5×10^8/ml, or an equal volume of PBS containing the dye (5×10^8/ml EV, 16 µM dye, negative control) and added to cells overnight. Nuclei were stained with Hoechst 33342 (Tocris, Cat. No. 875756). The samples were visualized using an EVOS M5000 Imaging system (Thermo Fisher Scientific) and in some experiments with a Cytation imager (BioTek, Agilent, Santa Clara, CA, USA) as described

[19]. For flow cytometry analysis, cells were rinsed and collected for uptake quantification using flow cytometry (BD Accuri). Data was analyzed using FlowJo software.

2.9. Lysosomal escape analysis

After incubation with P-EV analogues for up to 24 hours, EA.Hy 926 cells were treated with Cresyl violet at a concentration of 1 µM for 5 minutes according to Ostrowski et al. [20]. The samples were visualized using a Zeiss microscope. Colocalization analysis was performed by quantifying the number of overlapping green and red pixels within the cells in Image J Software. Pearson and Mander colocalization coefficients were calculated in Image J using the JACoP plugin [21].

2.10. Statistical Analysis

Values were shown as the mean ± standard deviation in GraphPad Prism 9.0 software. Statistical analyses were performed with Student's t-test or one-way analysis of variance (ANOVA). A P value below 0.05 was considered statistically significant.

3 Results

3.1 Characterization of P-EV analogues from fresh and lyophilized platelets.

Platelets were isolated from fresh anticoagulated venous blood, washed, and split into an immediately processed "fresh" (FP) and a "lyophilized" (LP) part, as outlined in the methods section. After sonication, coarse platelet debris was removed by centrifugation and filtration, as shown in Figure 1. All associated buffers are listed in table 1. The resulting EV were separated from proteinaceous content using gel filtration as described [22,23], resulting in two respective peaks containing EV and proteins as main contents (Figure 2A). Whereas the "fresh" FP-EV and the "lyophilized" LP-EV somewhat differed in concentration and protein contents (Figure 2B, C), the mean diameter of the FP-EV and the LP-EV was comparable, both in NTA analysis and electron microscopy (EM, Figure 1D,E). In addition, EM revealed a phospholipid bilayer structure typical of microvesicles, with mean diameters of 115.1 ± 45.6 and 109.3 ± 31.5 nm for FP-EV and LP-EV, respectively. To further study whether lyophilized platelets could still be induced to vesiculate through platelet activation, we compared P-EV derived from fresh and lyophilized platelets via sonication or by Ca^{2+}-ionophore stimulation and found that both types exhibited comparable size distribution (Supplementary Figure S1A-D). EV produced by sonication contained slightly more protein than those generated through Ca^{2+}ionophore stimulation (Supplementary Figure S1F). Sonication induced a markedly higher yield of EV compared to stimulation with calcium ionophore (Supplementary Figure S1E). Notably, the amount of EV released from lyophilized platelets upon Ca^{2+} ionophore stimulation was comparable to that from fresh platelets, indicating preserved responsiveness post-lyophilization.

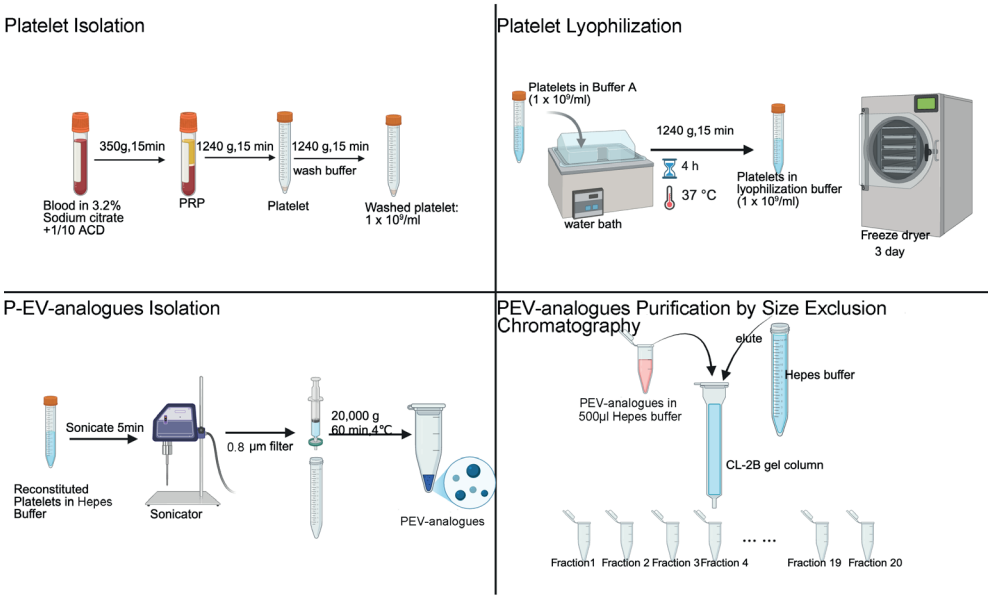


Figure 1 - Workflow for the Generation and Purification of P-EV analogues

Platelets were isolated from human peripheral blood and either used directly or subjected to lyophilization. P-EV analogues were subsequently generated from both platelet types via sonication stimulation and purified by SEC. PRP: Platelet rich plasma. Created in BioRender.

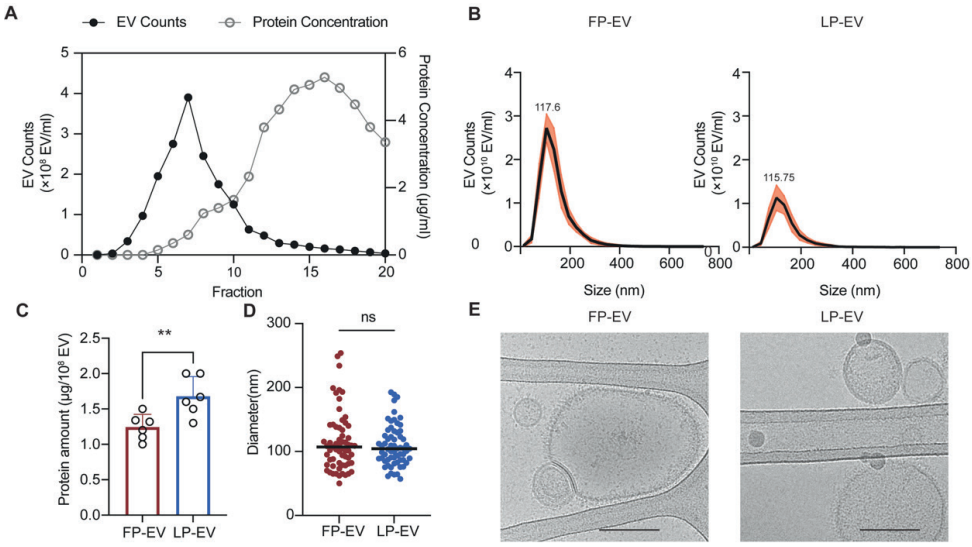


Figure 2 - Isolation of P-EV analogues from freshly obtained (FP) and lyophilized (LP) platelets.

(A) Dual-axis graph showing LP-EV (P-EV analogues derived from lyophilized platelets) counts (left Y axis) and protein concentrations (right Y axis) for each eluted fraction (0.5 ml) after size exclusion chromatography. The average of two batches is shown. (B) NTA plots of eight batches of FP-EV (P-EV analogues derived from fresh platelets) and LP-EV. Black line: mean, red area: SD. (C) Protein amount in FP-EV and LP-EV preparations (Mean ± SD, n = 6) (D) P-EV diameter based on EM images. (E) Representative EM images of FP-EV and LP-EV. N = 60 EV were measured from two batches.

3.2 Composition of P-EV analogues from fresh and lyophilized platelets.

Using flow cytometry and western blotting, platelet surface markers, as well as cytoplasmic and EV-specific proteins were determined. The expression of the well-established platelet receptors CD41a (integrin a_{IIb}) and CD62P (P-selectin) was similar on the surface of FP-EV and LP-EV (Figure 3A, B), this was paralleled by a similar expression of integrin b_3 and CXCL4 (platelet factor 4) by western blotting (Figure 3C). However, EV induced by Ca^{2+} ionophore exhibited slightly reduced CD41a and CD62P expression relative to those generated by sonication (Supplementary Figure S2A, B). In addition, the contents of CD63 and TSG-101 (PAN-EV markers) were comparable as well (Figure 3D), also supporting the EV-features of the generated vesicles. Furthermore, Ca^{2+} ionophore-induced P-EV showed comparable expression of integrin α_{IIb}, CXCL4 and CD63 to those generated by sonication, while TSG101 levels were marginally elevated (Supplementary Figure S2C-D).

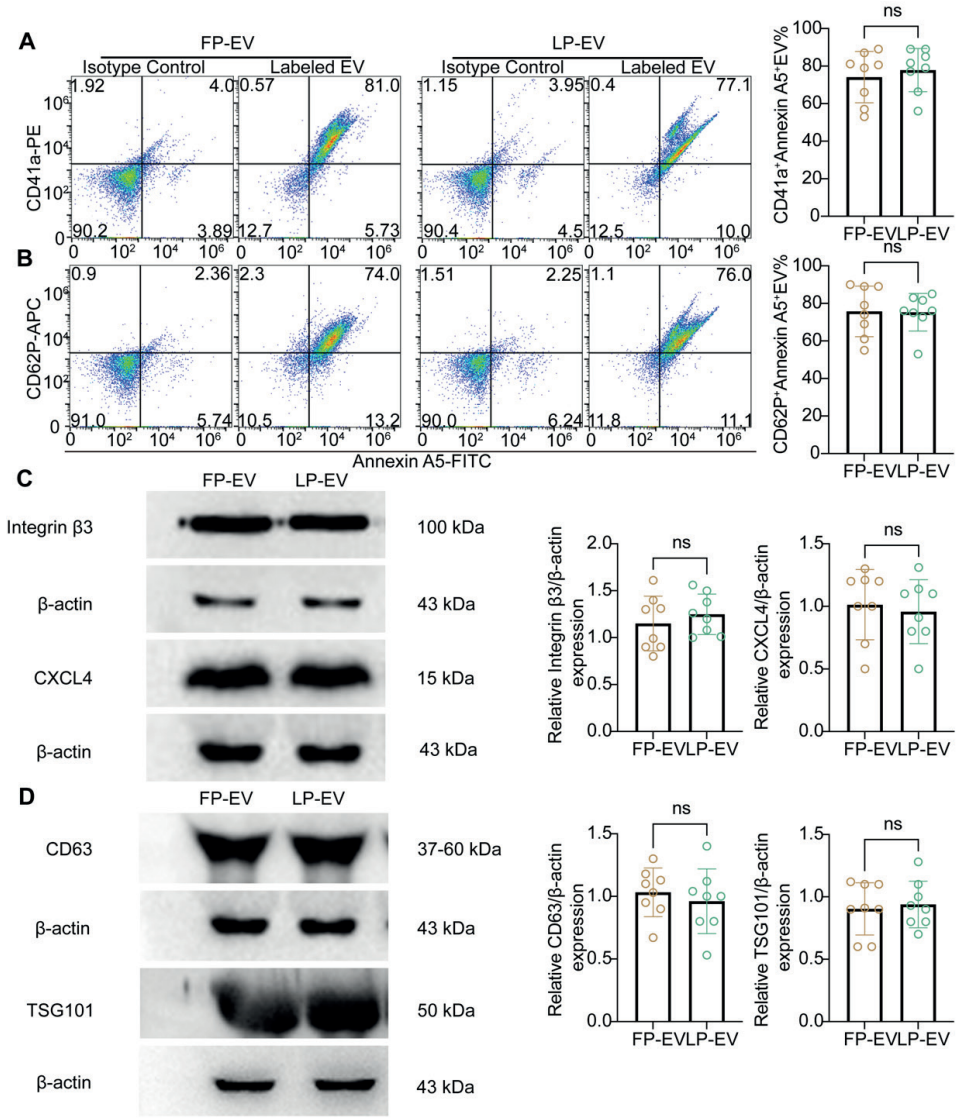


Figure 3 - Characterization of P-EV analogues from freshly obtained and lyophilized platelets.

Flow cytometric analysis of the expression of CD41a (A) and CD62P (B) on the surface of P-EV analogues. Western blot analysis of the expression platelet-associated proteins (C), and of PAN-EV-markers (D) in P-EV analogues, along with b-actin as loading control (30 µg total protein per lane). ns: not significant, (Mean±SD, n=8).

3.3 Fluorescent labeling of P-EV analogues from fresh and lyophilized platelets.

The investigation of effects of EV on target cells, such as uptake and subcellular trafficking, is facilitated by fluorescent labeling. However, this inevitably affects the properties of the EV, depending on the (chemical) nature of the dye. In addition, different coupling chemistries or fluorescent moieties also determine the fluorescent brightness of the labeled EV. In this study, the labels chosen were DiO-C6 (lipophilic membrane dye), CFSE (amine-reactive surface and intracellular dye), BCECF (intracellular, pH-dependent dye), CMFDA (intracellular dye) and all emit green fluorescence after excitation, amenable to flow cytometry and microscopy applications. Flow cytometry analysis showed that labeling with DiO-C6 resulted in high intensity labeling of both FP-EV and LP-EV, followed by CFSE (Figure 4A-C). Analysis using NTA revealed that labeling efficiency was moderate, with approximately 20% of all EV showing green fluorescence when labeled with DiO-C6 or CFSE, and less than 10% when labeled with BCECF and CMFDA (Figure 4D). Moreover, all fluorescent labeling methods led to a significant increase in mean vesicle size, with BCECF and CMFDA treatment almost tripling mean EV size (Figure 4E). The proportion of fluorescently labeled EV relative to the total EV counted before washing appeared low measured by NTA, potentially due to particle loss during the washing steps or degradation during the 37°C incubation for dye labeling. As the NTA results showed relatively low labeling efficiency of CFSE (~20%), we compared it with CellMask™ Deep Red, a widely recognized and efficient membrane dye. In addition, to rule out potential effects caused by sonication, we compared sonication-induced FP-EV with Ca^{2+} ionophore induced P-EV. Flow cytometry analysis showed that the fluorescence positivity was comparable between CFSE and CellMask™ Deep Red labeling, regardless of whether the particles were generated by sonication or Ca^{2+} ionophore stimulation. Moreover, no significant difference in labeling efficiency was observed between the two EV types (Supplementary Figure S3A-C). Furthermore, we labeled both types of EV with CD41 Alexa Fluor™ 488 (CD41-AF488) and analyzed them by NTA. Compared to CD41-AF488, CFSE demonstrated higher labeling efficiency, while no significant differences were observed between the two EV types (Supplementary Figure S3D).

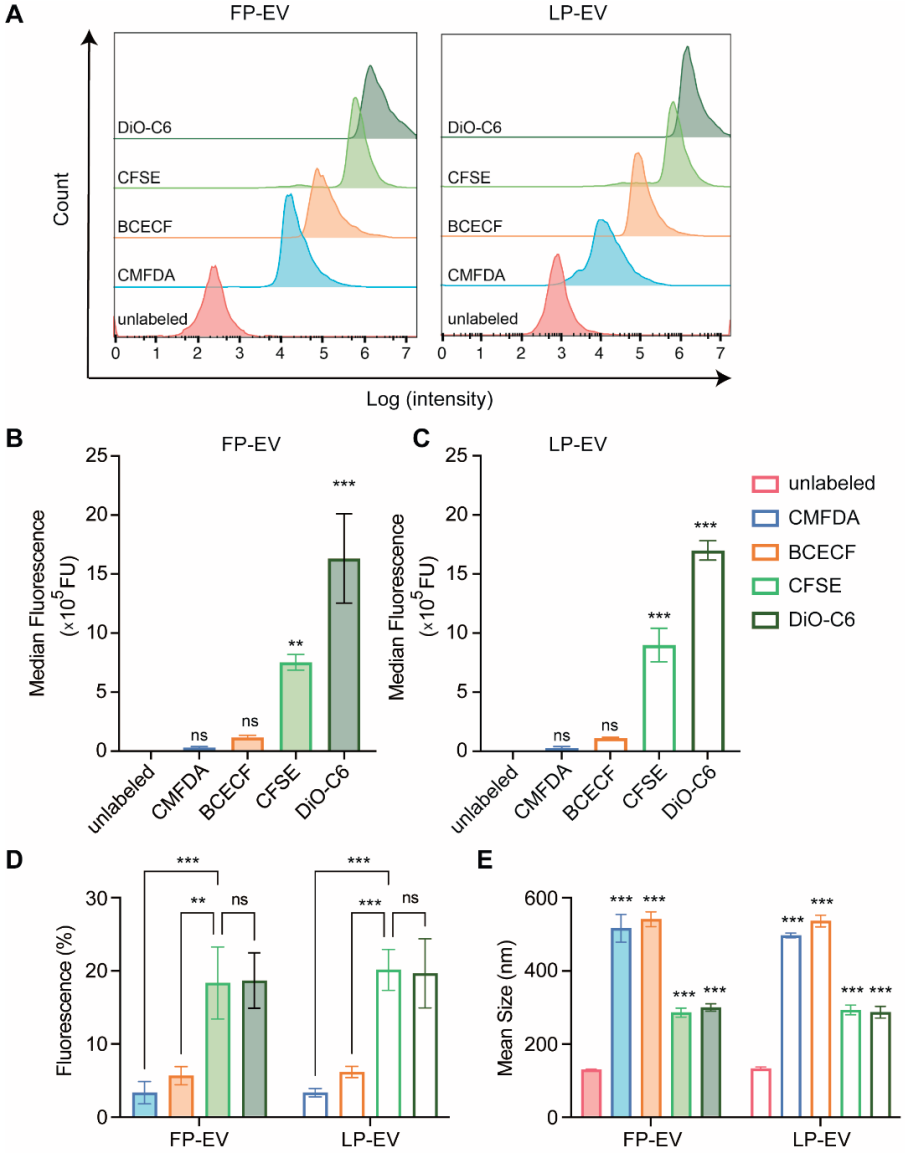


Figure 4 - Labeling of P-EV analogues with various fluorophores.

The fluorescence intensity of the labeled P-EV analogues was measured by flow cytometry (A) and expressed as median fluorescence (B-C). Comparisons vs. unlabeled EV. The fluorescence efficiency (D), expressed as the number of fluorescence P-EV analogues divided by the number of total P-EV analogues, and size (E) of labeled P-EV analogues was measured using NTA. Comparisons vs. PBS, *P<0.05; **P<0.01; ***P<0.001, ns: not significant (Mean ± SD, n = 3).

3.4 Cellular uptake of fluorescent P-EV analogues.

In order to investigate the cellular uptake of fluorescently labeled “fresh” and “lyophilized” P-EV analogues, and to assess the performance of the dye-labeling in flow cytometry and microscopy applications, the P-EV analogues were incubated with the endothelial cell line EA.Hy926 for up to 24 hours. Uptake was confirmed to be time-dependent (Supplementary Figure S4) and P-EV analogues were shown to be intracellular (Supplementary Movie 1). Flow cytometry revealed that FP-EV and LP-EV were taken up by the EA.Hy926 cells to a similar extent, and independent of the label implemented (Figure 4 and Supplementary Figure S5). Cells incubated with CFSE-labeled P-EV analogues showed a high uptake of the vesicles with a low mean background fluorescence (uptake of free dye) and clearly measurable fluorescent intensity both in flow cytometry and by microscopy (Figure 5A-C). While P-EV analogues labeled with DiO-C6 resulted in the highest mean fluorescence of the recipient cells, not all cells were positive for EV uptake in the flow cytometry experiment. Additionally, the background fluorescence was higher for DiO-C6 than for all other labels used (Figure 5D-F). P-EV analogues that were labeled with BCECF and CMFDA were also effectively taken up by the EA.Hy926 cells, but overall fluorescence intensity was lower (Supplementary Figure S5) than for DiO-C6. The absence of P-EV analogues by EA.Hy926 cells incubated at 4°C suggests that cellular uptake is an active process (Supplementary Figure S6). Interestingly, inhibition of integrin $a_{IIb}b_3$, a platelet adhesion receptor to endothelial cells, using the clinically used antagonist eptifibatide only slightly lowered uptake of P-EV (Supplementary Figure S6).

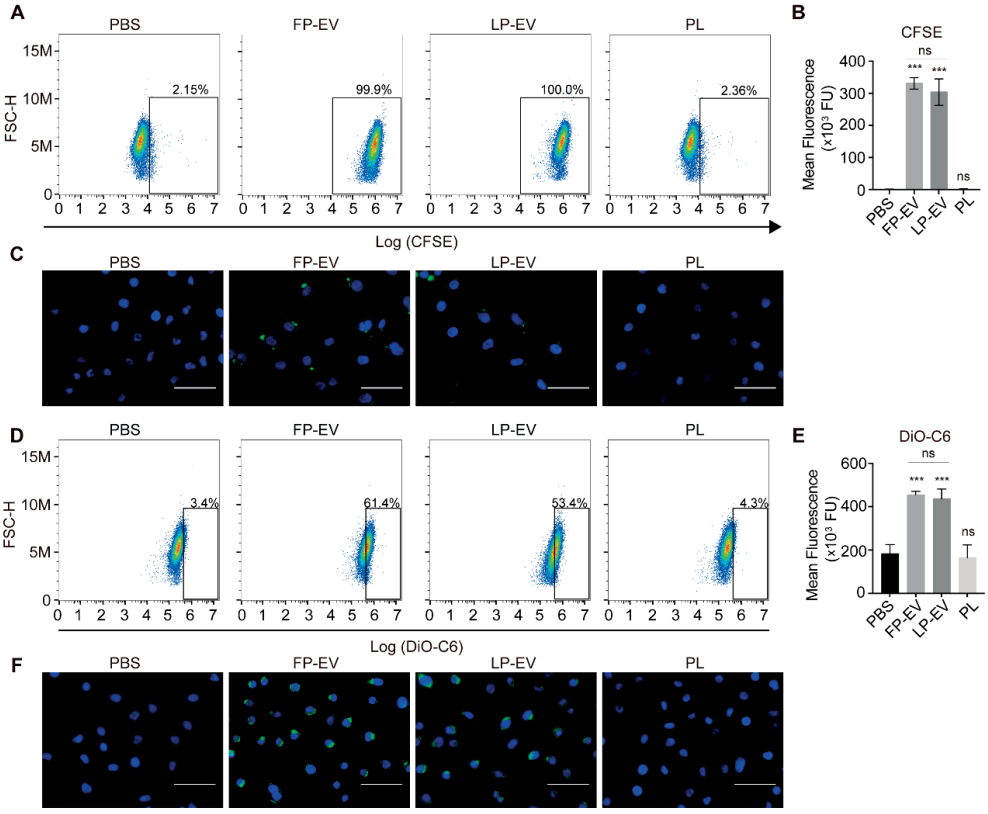


Figure 5 – Cellular uptake of P-EV analogues labeled with CFSE or Dio-C6.

Flow cytometric analysis (A,B,D,E) and representative microscopy images (C,F) of the uptake of FP-EV, LP-EV, and negatively charged synthetic phospholipid vesicles (PL), labeled with CFSE (A-C) and DiO-C6 (D-F) by cultured EA.Hy926 cells after overnight incubation. The dye dissolved in PBS was used as a control**.** Quantification of flow cytometry mean fluorescent intensity in units (FU). Comparisons vs. PBS. Scale bar=75 µm *P<0.05; **P<0.01; ***P<0.001, ns: not significant (Mean ± SD, n = 3).

3.5 Intracellular fate of P-EV analogues internalized by endothelial cells.

To investigate the intracellular fate of the P-EV analogues after uptake, the lysosomes were visualized using cresyl violet [231]. Colocalization of green P-EV analogues' fluorescence with red lysosomal fluorescence was not evident 1 and 4 hours after addition to EA.Hy926 cells (Figure 6A, B, unmerged images in Supplementary Figure S7). However, after 24 hours, the colocalization of P-EV analogues with lysosomes increased, indicating that the P-EV analogues target to the lysosomal compartment over time (Figure 6A, B). Further, to assess dose-dependent uptake, cells were incubated with increasing concentrations of P-EV analogues (0, 10, 100, and 1000 EV/cell). No detectable uptake or lysosomal colocalization was observed at 0 or 10 EV/cell. At 100 EV/cell, approximately 24% of internalized EV were colocalized with lysosomes, which increased to ~70% at 1000 EV/cell. These findings indicate a dose-dependent EV uptake and lysosomal localization (Figure 6E-G). In addition when associated with the lysosomes, the fluorescence of the P-EV analogues appeared to localize around the nucleus (Figure 6C). The green and red signals showed a high spatial correlation after 24 hours at 1000 EV/cell, further supporting colocalization of intracellular P-EV analogues with lysosomes (Figure 6D).

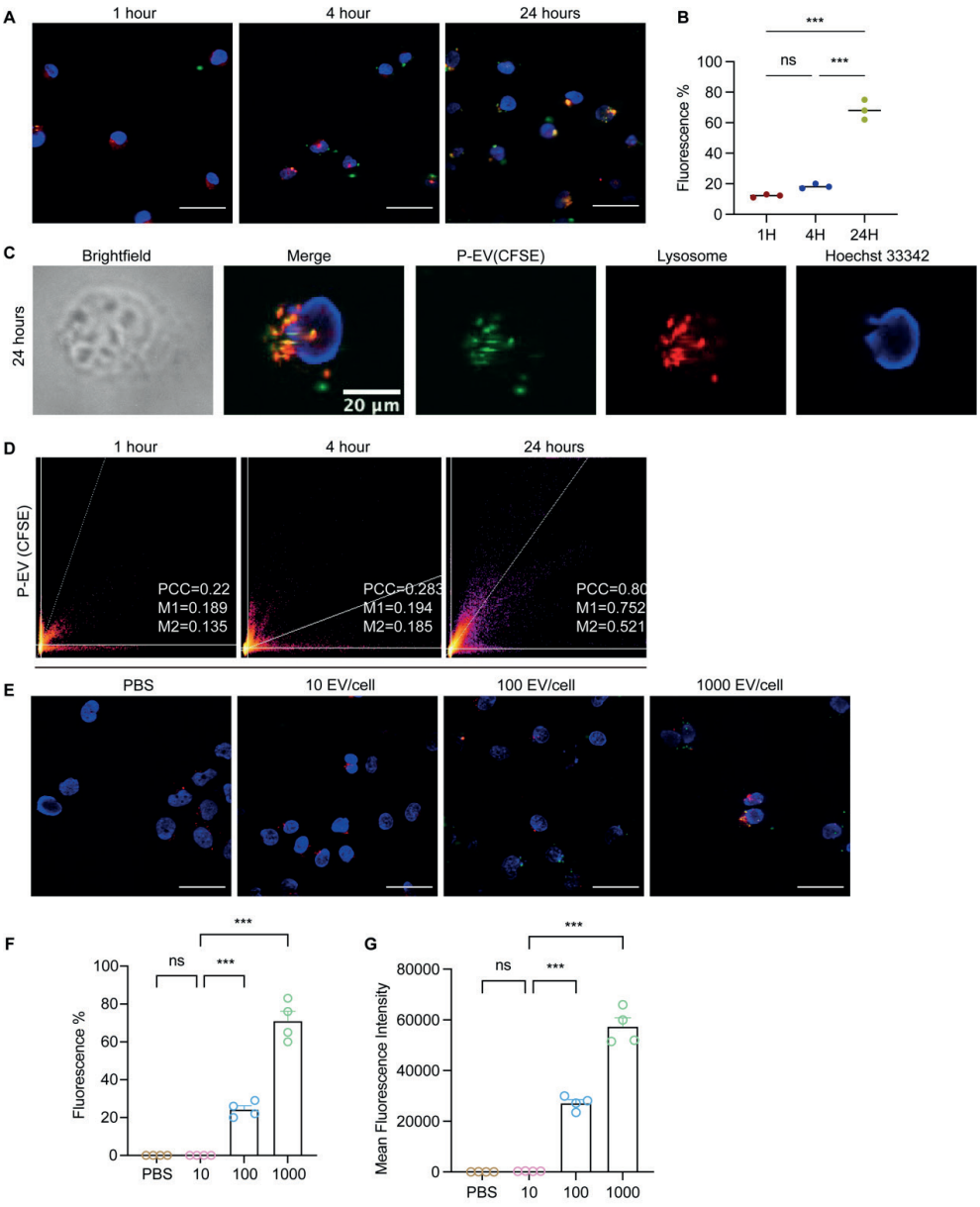


Figure 6 – Colocalization of LP-EV with lysosomes.

Lysosomes were stained with cresyl violet (red fluorescence) and EA. hy926 cells were subsequently incubated with CFSE-LP-EV (green fluorescence) for up to 24 hours. Efficiency of lysosomal escape was measured as the percentage of the green CFSE-LP-EV signal that colocalized with the red lysosome signal. **(A)** Representative fluorescence images of EA.Hy926 cells after treatment with P-EV analogues at different timepoints. Scale bar: 50µm. **(B)** Percentage of CFSE-LP-EV colocalized with the lysosomal compartment at indicated times after addition of CFSE-LP-EV(n=3). **(C)** Representative fluorescence images of EA.Hy 926

cells after treatment. Scale bar: 20μm. **(D)** Colocalization analysis of lysosomes with CFSE-LP-EV by Image J. Pearson's correlation coefficient (PCC) of CFSE-LP-EV colocalized with the lysosomal compartment after 1, 4, and 24 hours. The PCC quantifies colocalization, with values ranging from 1 to –1. A value of 1 indicates perfect correlation, -1 indicates complete exclusion, and 0 indicates a random relationship. M1: fraction of green fluorescence overlapping red fluorescence. M2: fraction of red fluorescence overlapping green fluorescence. **(E)** Representative fluorescence images of EA.Hy926 cells after treatment with P-EV analogues at different dose. Scale bar: 50μm. **(F)** Percentage of CFSE-LP-EV colocalized with the lysosomal compartment at indicated dose after addition of CFSE-LP-EV overnight(n=4). **(G)** Green fluorescence intensity (EV) after treatment with different doses of CFSE-LP-EV. *** P<0.001, ns: not significant, Mean ± SD.

4 Discussion

There is growing evidence that P-EV play an important role in various pathophysiological processes, including regulating inflammation, hemostasis, tumor progression, and immune regulation [24-26]. Furthermore, when prepared as nanovesicles with parental properties, P-EV can target sites of inflammation, cardiac ischemia and vascular injury [6,7,27]. In addition, since P-EV can be produced from autologous blood posing low safety risks, they have garnered significant attention as potential drug delivery carriers. However, the study of P-EV faces several challenges. First, due to their limited storage time and strict storage conditions, the quality of platelets degrades rapidly [28], and fresh high-quality human platelet products are in short supply in clinical practice. This makes freeze-dried platelets a promising source for studying P-EV and obtaining functional P-EV, as platelets can be stored for indefinite times at -80°C [16]. This is particularly useful for studying P-EV from patient samples, as it facilitates experimental logistics and portability of samples. Subsequent sonication of platelets warrants a condition- and stimulus-independent generation of uniform P-EV for experimentation, lowering individual variations of activation potential and response to stimuli, whilst preserving receptor presentation and cell binding properties [7,10,29]. Given that this method bypasses natural vesiculation processes, the resulting vesicles are more accurately referred to as P-EV analogues.

Beyond P-EV generation, the isolation method can significantly affect their cargo contents [30]. Therefore, establishing a standardized procedure for P-EV isolation is essential for the reproducibility of EV research and its future clinical applications. In this study, SEC, filtration, and centrifugation were employed to isolate P-EV analogues. Subsequently, characterization was conducted using NTA, cryo-transmission EM, flow cytometry, and western blotting to identify the isolated P-EV analogues and for comparing P-EV analogues obtained from fresh or lyophilized platelets. According to NTA and EM measurements, the average sizes of the two types of P-EV analogues were comparable. Analysis of the molecular markers confirmed that both types of P-EV analogues expressed (activated) platelet markers such as CD41a, phosphatidylserine, CD62P, integrin β3, and CXCL4, as well as exosome markers CD63 and TSG-101 [31]. Interestingly, the expression levels of those markers in LP-EV and FP-EV from the same individual were similar. Although CD63 is well-established as a marker for exosomes, it is also a platelet activation

marker localized within the α-granules of platelets and associates with integrin β3 and the tetraspanin CD9 on the platelet surface [32-34]. Furthermore, to evaluate whether lyophilized platelets are capable of releasing EV upon activation, we compared their response to Ca^{2+} ionophore stimulation with that of fresh platelets. Ca^{2+} ionophore is a well-established stimulus that triggers EV release from platelets by triggering an intracellular calcium flux. Following stimulation, the number of EV released per platelet from lyophilized platelets, as well as the size and surface marker expression of EV, were comparable to those of fresh platelets, suggesting that the lyophilized platelets retain capacity for stimulus-induced vesiculation. Our previous studies revealed that integrin β3 is expressed at higher levels and in an active conformation on the surface of P-EV analogues after sonication, compared to P-EV isolated from platelets activated with physiologic triggers [29]. This indicates that P-EV analogues from sonicated platelets have characteristics of activated platelets (active integrin β3, CD62P, PS exposure), yet without the closure mechanism of integrin β3 (leading to a reduction of surface expression) that is required for the EV release from platelets stimulated by physiologic triggers such as thrombin or collagen [35].

Another significant challenge in P-EV research lies in effectively labeling these vesicles to study their biodistribution and interactions with target cells. Currently, fluorescent dyes are the most commonly used method for molecular labeling. However, these dyes present notable limitations. They are prone to aggregation, which can lead to the formation of EV-like nanoparticles that may be absorbed by cells. Furthermore, the dyes can cause an increase in EV size due to membrane fusion or insertion [14]. In our study, we tested several dyes for their suitability to support downstream applications, including the lipophilic carbocyanine dye DiO-C6, amine-reactive dye CFSE and cell-permeable CMFDA, and the pH-dependent dye BCECF. DiO-C6 is a synthetic compound that features two C-6 hexyl chains, allowing it to be effectively incorporated into lipid membrane layers. This dye emits weak fluorescence in aqueous solutions, and its emission becomes bright green only when the alkyl chains are incorporated into a membrane [36]. While P-EV analogues labeling with DiO-C6 resulted in robust green fluorescence, the size of the P-EV analogues was almost tripled. The intracellular dye BCECF showed a similar increase in vesicle size, but fluorescence labeling efficiency was considerably lower. P-EV analogues labeling with CFSE and CMFDA resulted in a (twofold) increase in vesicle size, and only CFSE labeling resulted in a bright fluorescence of the P-EV analogues. The observed size increase might be explained by the small initial size of the P-EV analogues combined with the sheer number of relatively large fluorescent molecules that are coupled to it. Thus, there is an obvious tradeoff between labeling efficiency and the influence of the label on the physical properties of the EV that requires specific optimization for each application, as was also shown previously for other dyes [12,13]. To further evaluate the staining characteristics of CFSE, we compared it with CellMask™ Deep Red and CD41-AF488. Also in comparison with these labels, CFSE consistently demonstrated favorable labeling performance. Additionally, we compared EV generated by Ca^{2+} ionophore stimulation with those produced by sonication-induced P-EV analogues. The two populations

showed comparable fluorescence intensities, suggesting that the process of sonication induction did not affect the fluorescent labeling of the vesicles. These findings support the use of CFSE as a reliable marker for EV visualization.

The CFSE dye was chosen to investigate the uptake of P-EV analogues into the immortal endothelial cell EA.Hy926, because of its minimal impact on P-EV analogues size and its strong, stable fluorescence. A time-dependent uptake of CFSE-labeled P-EV analogues by EA.Hy926 cells was observed, indicating an active, energy-dependent process that only took place at 37°C. Confocal microscopy revealed that P-EV analogues were internalized by these cells. Interestingly, blocking the integrin b3 receptor did not reduce the uptake of P-EV analogues, suggesting that endothelial receptors such as ICAM-1 are unlikely to play a role in this process. While the uptake of platelets and P-EV analogues by endothelial cells, along with the transfer of their contents, has been observed by others [37-39], the molecular mechanism of this cellular uptake remains poorly characterized. The fluorescent labeling of P-EV analogues represents a valuable tool to advance future investigations into the uptake mechanism. Twenty-four hours after internalization, the P-EV analogues were, at least in part, directed to the lysosomal compartment, indicating that they are eventually marked for destruction [40]. Nevertheless, the intracellular presence of the P-EV analogues for the initially observed 4-hour timespan might harbor sufficient possibilities to release the biomolecular cargo into the intracellular space of the recipient cells. The perinuclear localization of the P-EV analogues observed in this study may be indicative of such transfer, but this needs to be confirmed in future studies. Importantly, our dose–dependent experiments showed that even at relatively low concentrations (100 EV/cell), a measurable degree of lysosomal colocalization (~24%) was observed. This proportion increased with higher EV doses, reaching approximately 70% at 1000 EV/cell. These findings indicate a dose-dependent uptake behavior, consistent with previous reports [41], but also suggest that biologically relevant responses may still occur at lower, more physiologically appropriate EV concentrations. Thus, careful consideration of dosing is essential in evaluating EV function.

To further evaluate the biological activity of the P-EV analogues, we examined their effect on endothelial cell activation. As shown in Supplementary Figure S8, neither type of P-EV analogues induced upregulation of ICAM-1 expression, suggesting that lyophilization does not confer pro-inflammatory properties to P-EV analogues.Taken together, this work demonstrates that lyophilization is a feasible method to facilitate the processing of platelets for downstream processing, that functional P-EV analogues can be effectively generated from these lyophilized platelets, and that CFSE labeling is a feasible approach to enable investigation of uptake and intracellular fate of P-EV analogues. This work provides a proof-of-concept for such studies.

Author contributions

X.X. Performed experiments, analyzed data, created figures, drafted the manuscript, obtained funding; I.D. Provided intellectual input, supervised project; T.M.H. Provided intellectual input, supervised project; R.R.K. Obtained funding, finalized manuscript, provided intellectual input, supervised project.

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Conflict of Interest Declaration

The authors declare no conflict of interest.

AI usage statement

No AI technology was used in creating the content of this article.

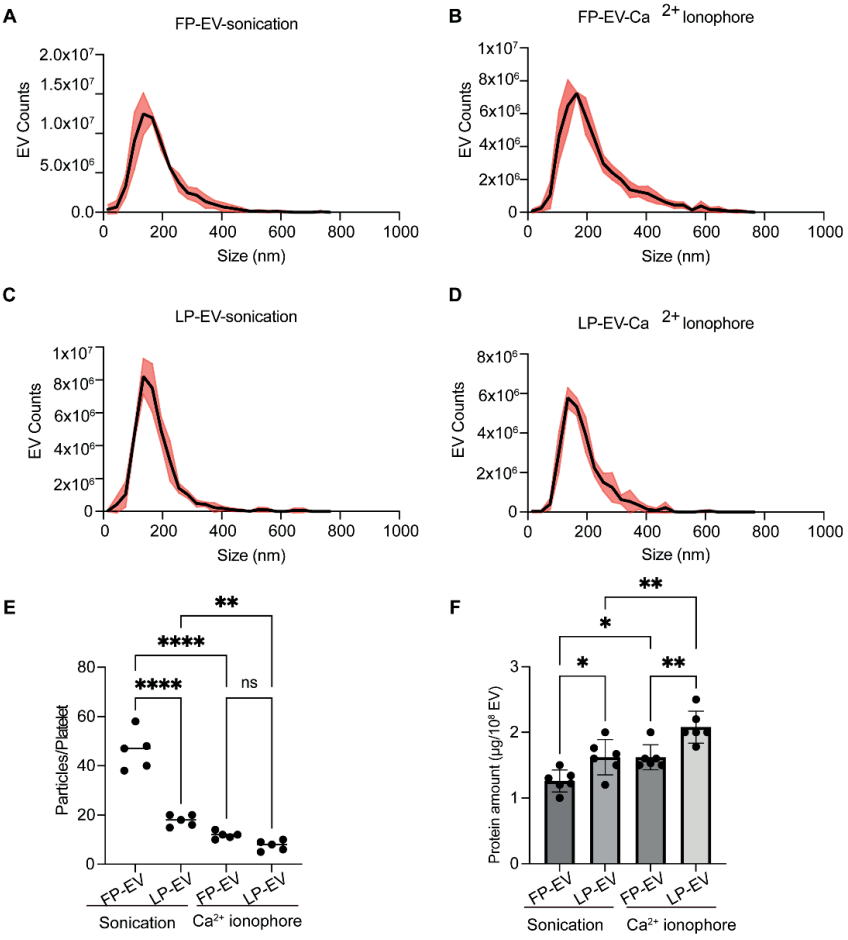
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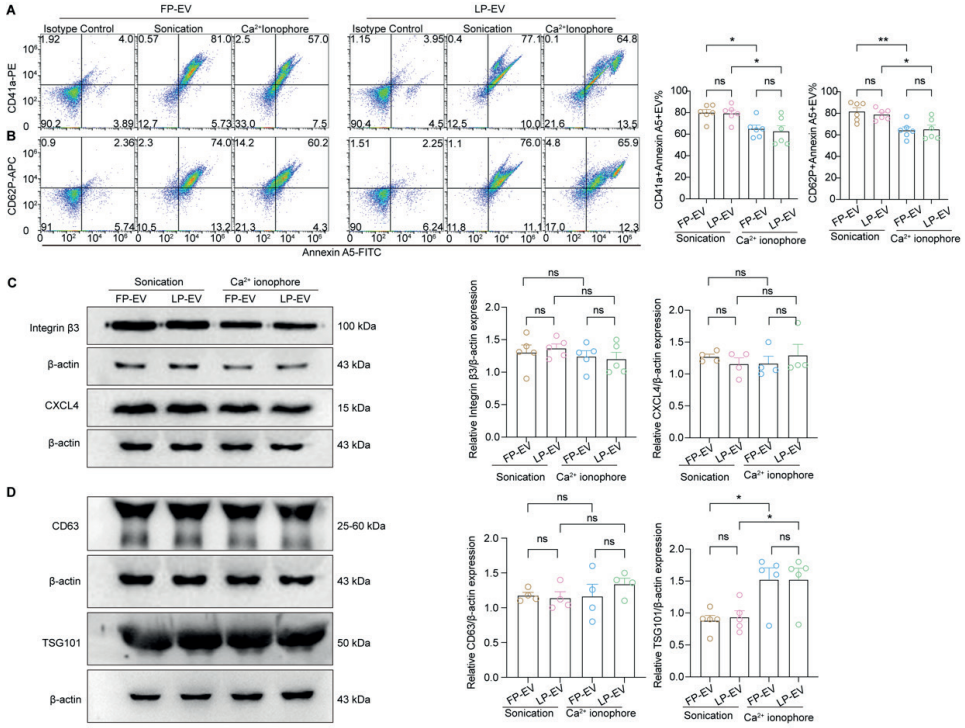
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Supplementary data



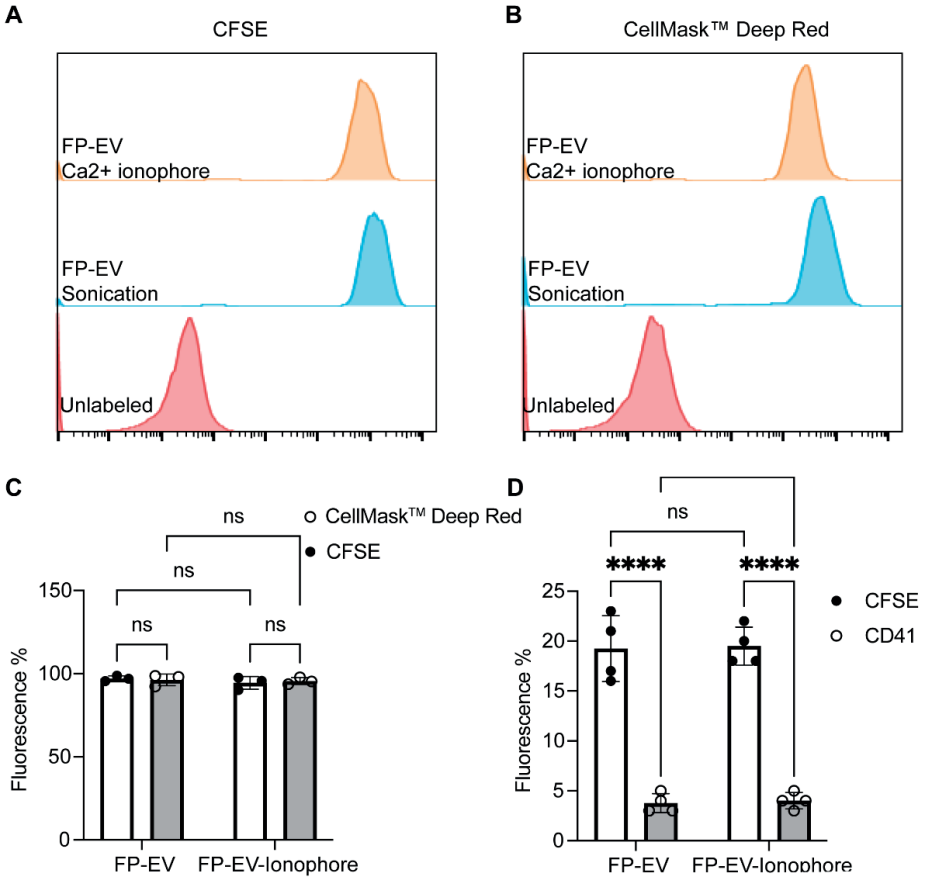
Supplementary Figure S1 - Isolation of P-EV/ P-EV analogues from freshly obtained (FP) and lyophilized (LP) platelets following stimulation by either sonication or Ca^{2+} ionophore.

(A-D) NTA plots of five batches of four EV types. Black line: mean, red area: SD. (E) Quantification of EV generated per single platelet(n=5). (F) Protein concentrations in four EV types of preparations (n=6). *P<0.05; **P<0.01; ****P<0.0001; ns: not significant, Mean ± SD.



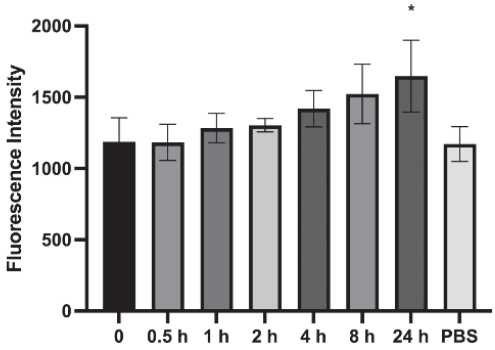
Supplementary Figure S2 - Characterization of P-EV/ P-EV analogues from freshly obtained and lyophilized platelets following stimulation by either sonication or Ca^{2+} ionophore.

Flow cytometric analysis of the expression of CD41a (A) and CD62P (B) on the surface of P-EV/ P-EV analogues(n=6). Western blot analysis of the expression platelet-associated proteins (C), and of PAN-EV-markers (D) in P-EV/ P-EV analogues, along with *β*-actin as loading control (30 µg total protein per lane) (n=4-5). *P<0.05; **P<0.01;ns: not significant, Mean±SD.

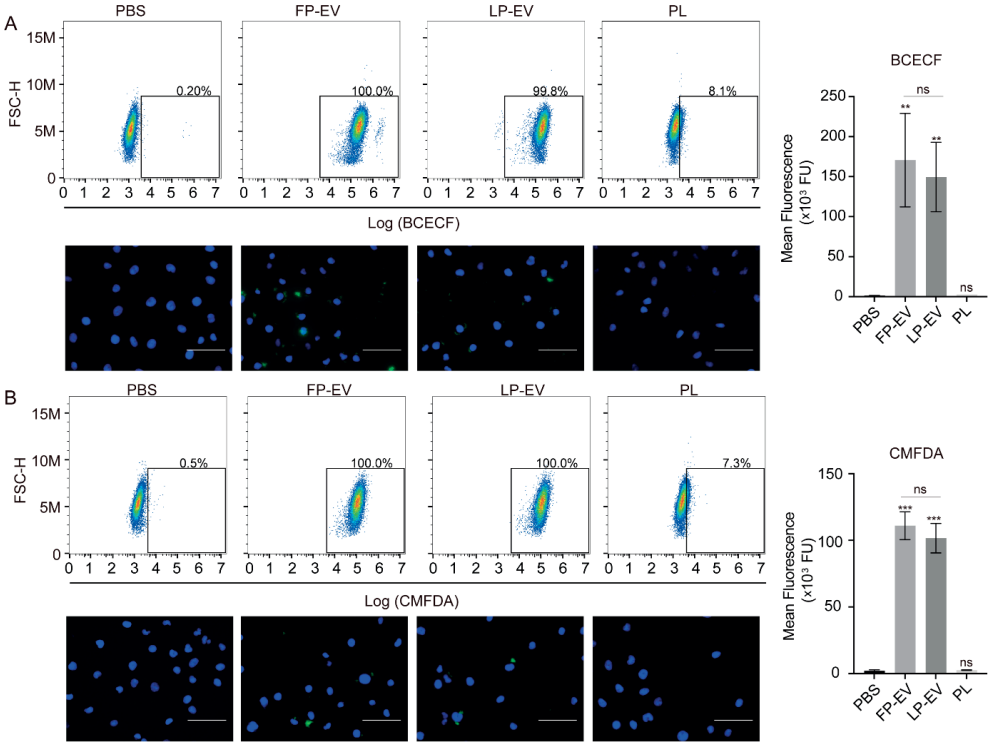


Supplementary Figure S3 - Labeling of P-EV/P-EV analogues with various fluorophores.

The fluorescence intensity of the labeled P-EV/P-EV analogues was measured by flow cytometry (A-B) and expressed as fluorescence %(C)(n=3). The fluorescence efficiency (D), expressed as the number of fluorescence EV divided by the number of total EV, and size (E) of labeled P-EV/P-EV analogues was measured using NTA(n=4). ****P<0.0001; ns: not significant, Mean ± SD.

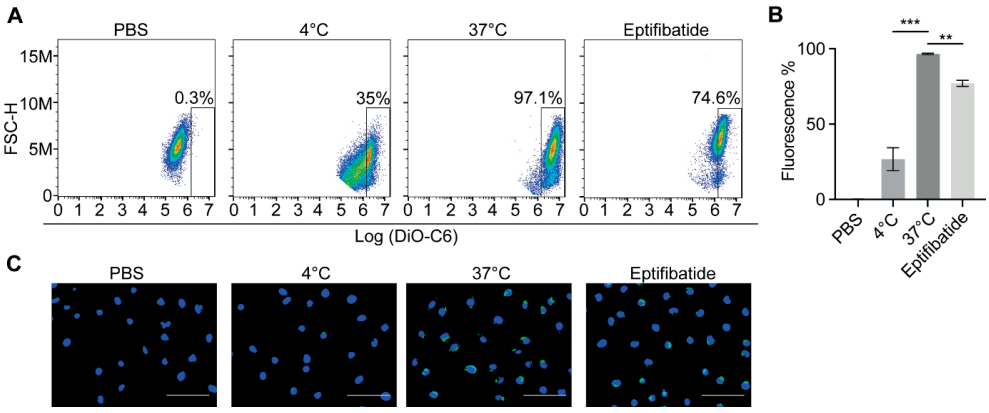


Supplementary Figure S4 - Endothelial cell line EA.Hy926 was incubated with LP-EV that labeled with CFSE for different time periods and fluorescence was measured using a Cytation imager (Mean ± SD, n = 3), Comparisons vs. PBS. *P<0.05.



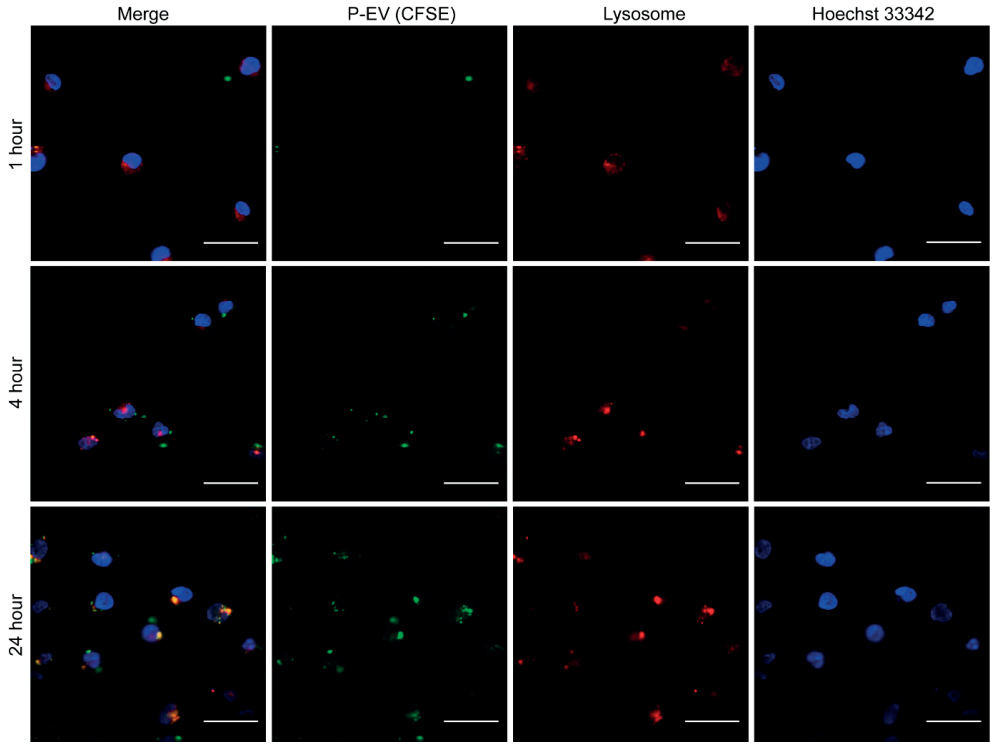
Supplementary Figure S5 - Cellular uptake of P-EV analogues labeled with different fluorescent dyes by EA. Hy926 cells.

Flow cytometric analysis and representative microscopy images of the uptake of FP-EV, LP-EV, and PL, labeled with BCECF (A) and CMFDA (B) by cultured EA.Hy926 cells. The dye dissolved in PBS was used as a control. Right panels: Quantification of flow cytometry mean fluorescent intensity in units (FU). Comparisons vs. PBS. *P<0.05; **P<0.01; ***P<0.001, ns: not significant (Mean ± SD, n = 3).

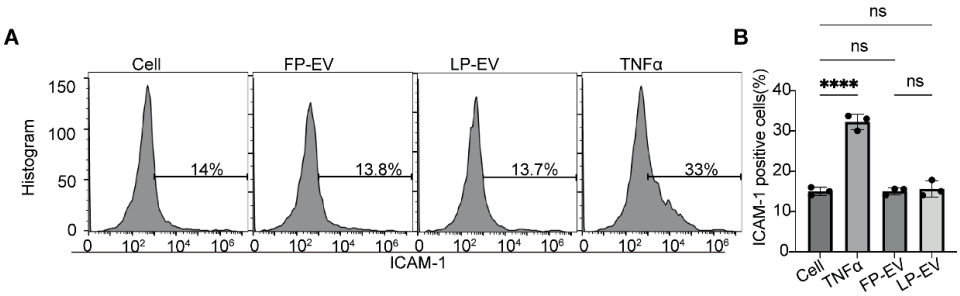


Supplementary Figure S6 - EA. Hy926 cells were incubated with P-EV analogues at low temperature or after treatment with integrin inhibitors.

(A) Flow cytometric analysis and (B) quantification of flow cytometry mean fluorescent intensity in units (FU). (C) Representative microscopy images of the uptake of FP-EV, LP-EV, and PL, labeled with DiO-C6 by cultured EA. Hy926 cells, incubated at 4°C or at 37°C, in the absence or presence of 50 µg/ml integrin $\alpha_{IIb}\beta_3$ inhibitor eptifibatide. Comparisons vs. PBS. Mean ± SD, **P<0.01; ***P<0.001 n=3.

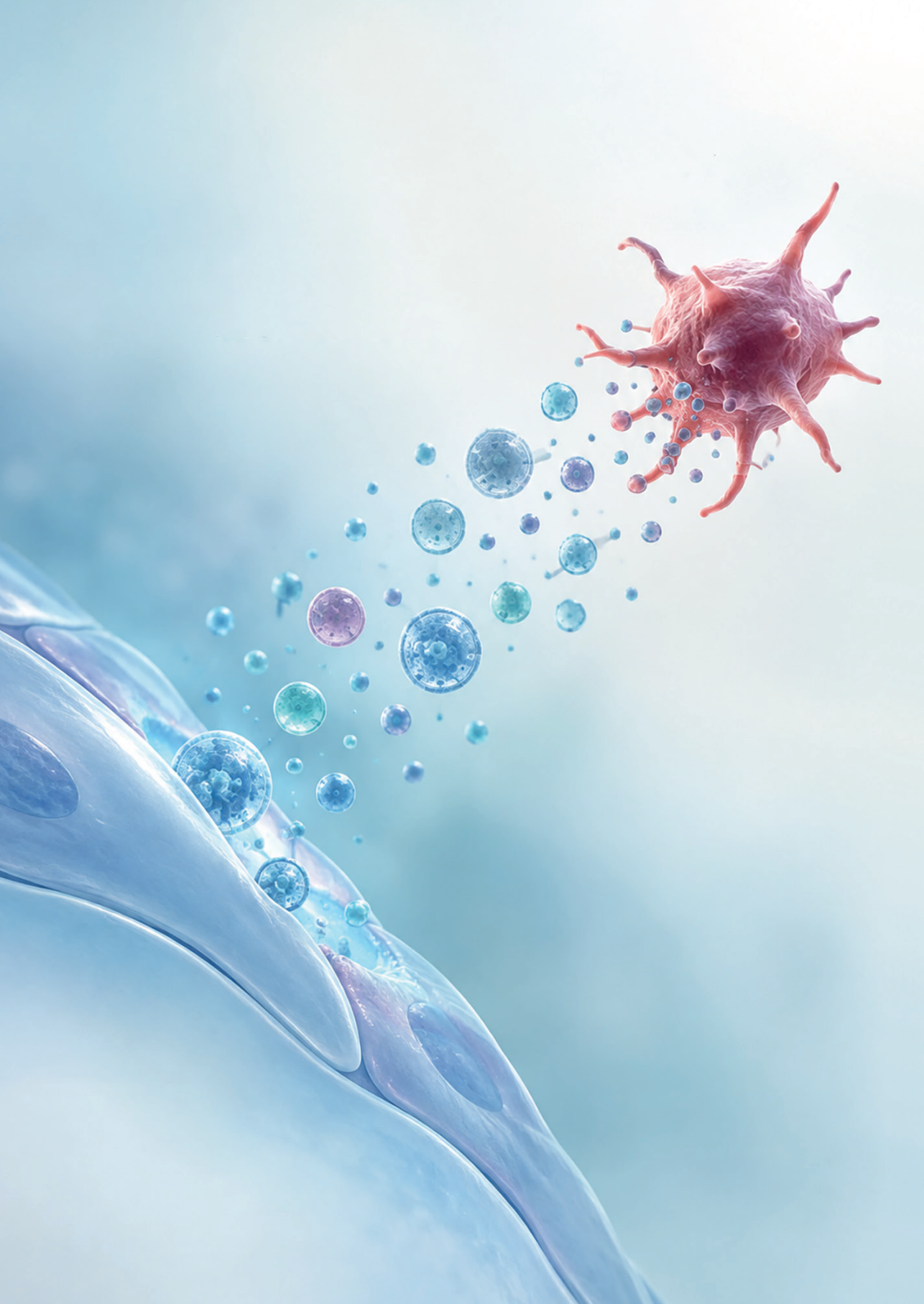


Supplementary Figure S7 - Lysosomes were stained with cresyl violet (red fluorescence) and EA.Hy926 cells were subsequently cultured with CFSE-LP-EV (green fluorescence) for 1, 4, and 24 hours. Representative fluorescence images of EA.Hy926 cells after treatment with P-EV analogues at different timepoints. Scale bar: 50µm.



Supplementary Figure S8 - ICAM-1 expression in EA.hy926 cells after overnight treatment with two types of EV (500 EV/cell).

(A) Representative flow cytometry plots. (B) Percentage of ICAM-1–positive cells. ****P<0.0001; ns: not significant, mean ± SD, n=3.



Chapter 4

Differential effects of extracellular vesicles from distinct cellular origins on endothelial inflammation and dysfunction

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Manuscript in preparation.

Abstract

Various vascular and immune-related cells contribute to the initiation and progression of atherosclerosis by involving endothelial inflammation. Extracellular vehicles (EVs) have emerged as key mediators of intercellular communication in vascular inflammation. Although previous studies have reported that EVs from specific cell types can regulate endothelial inflammation, a systematic comparison of the relative contributions and functional heterogeneity of EVs from different vascular and immune-related cell sources to endothelial inflammation remains lacking. This study systematically compared the effects of EVs derived from different cell sources under resting and inflammatory conditions on endothelial cell activation. The inflammatory cytokine profiling revealed that EVs exhibited cell type-dependent inflammatory cargo characteristics. Functionally, EVs induced rapid transient Ca^{2+} responses in endothelial cells in a source-dependent manner. Consistently, inflammatory EVs induced endothelial activation to varying extents, as evidenced by increased Intercellular adhesion molecule-1 (ICAM-1) expression and chemokine (C-C motif) ligand 2 (CCL2) secretion. These changes resulted in enhanced monocyte adhesion and migration. Furthermore, prolonged exposure to EVs promoted endothelial cell apoptosis, which is associated with mitochondrial dysfunction. Our findings demonstrate that EVs derived from different cellular sources differentially regulate endothelial cell function, accompanied by distinct inflammatory cargo profiles and calcium signaling responses.

Key words: Extracellular Vesicles, Endothelial Inflammation, Atherosclerosis, Monocyte, Neutrophil, Platelet.

1 Introduction

Atherosclerosis (AS) is a chronic inflammatory vascular disease closely linked to endothelial dysfunction [1,2]. As a key early event in AS, endothelial inflammation can lead to the increased expression of adhesion molecules and chemokines, promoting the recruitment of inflammatory cells and triggers local inflammatory responses, thereby contributing to the formation and progression of atherosclerotic plaques [3,4]. Increasing research indicates that various vascular and immune-related cells, including platelets, neutrophils, monocytes, and endothelial cells, play a role in this complex inflammatory microenvironment [5-7]. These cells work together to regulate vascular inflammatory responses through intercellular communication[8].

Extracellular vesicles (EVs) are membrane-bound nanoparticles released by almost all cell types that mediate intercellular communication according to their cargo (e.g. lipid, protein, and nucleic acids) [9]. EVs have been implicated in vascular inflammation, AS, and thrombosis [10,11]. Emerging evidence indicates that both the cellular origin and activation state of parent cells influence EV cargo and thereby modulate their effects on recipient endothelial cells [12,13].

Previous studies have reported the roles of EVs derived from various cell types in vascular inflammation[14]. For example, monocyte-derived EVs (M-EVs) can induce the expression of inflammatory molecules in endothelial cells, such as Intercellular Adhesion Molecule-1 (ICAM-1), Vascular Cell Adhesion Molecule-1 (VCAM-1), and Interleukin 6 (IL-6), which in turn promotes monocyte adhesion and exacerbates endothelial inflammation[15]. Similarly, neutrophil-derived EVs (N-EVs) are linked to endothelial activation, monocyte recruitment, and increased vascular inflammation [16,17]. On the other hand, endothelial cell-derived EVs (E-EVs) can amplify local inflammatory signals by transporting pro-inflammatory microRNAs (miRNAs) [17,18]. In contrast, platelet-derived EVs (P-EVs) exhibit a complex regulatory effect; some studies suggest they can reduce endothelial inflammation by transferring functional miRNA [19,20], while others indicate they may enhance leukocyte recruitment and endothelial activation by delivering chemokines and adhesion factors to endothelial cells [21,22].

Current research has mainly focused on EVs derived from single-cell sources, while systematic comparisons of EVs derived from different vascular and immune cells remain limited, particularly regarding their roles in endothelial inflammation. In this study, we compared the effects of EVs from platelet, monocyte, neutrophil and endothelial cell on endothelial inflammatory activation under both resting and inflammatory conditions. In addition, we analyze their inflammatory factor profiles and calcium signaling responses.

2 Methods and materials

2.1. Cell culture

Human umbilical vein endothelial cells (HUVECs; PromoCell, Cat. No. C-12203) and the human monocytic leukemia cell line THP-1 (DSMZ, Cat. No. ACC-16) were cultured in Endothelial Cell Growth Medium 2 (PromoCell, Cat. No. C-22011) and RPMI 1640 medium (Gibco, Cat. No. 11875-093), respectively. RPMI 1640 medium was supplemented with 10% fetal bovine serum (FBS; Gibco) and 1% penicillin-streptomycin (Gibco). Cells were maintained at 37°C in a humidified incubator containing 5% CO_2. For HUVEC culture, plates were precoated with rat tail collagen type I (Sigma-Aldrich, Cat. No. 08-115) diluted in phosphate-buffered saline (PBS) for 30 min at 37°C. HUVECs up to passage 5 were used for all experiments.

2.2. Isolation of neutrophils and monocytes

Human neutrophils and monocytes were isolated from whole blood collected from healthy donors (French Blood Bank, Marseille) using density gradient separation and positive selection by immunomagnetic separation. All steps were performed under sterile conditions at 4°C to prevent contamination and premature cell activation. Briefly, neutrophils and monocytes were isolated from whole blood using density gradient centrifugation (1.077 g/ml Lymphocyte separation medium, Eurobio, France) at 400 × *g* for 10 min. Following centrifugation, the upper layer of plasma was discarded, and the peripheral blood mononuclear cell (PBMC) layer was collected. The remaining neutrophil–red blood cell fraction was treated with 50 mM ammonium chloride buffer for 40 min to lyse the red blood cells. Neutrophils and monocyte suspensions were processed separately, washed twice by centrifugation at 300 × *g* for 10 min in PBS-BSA buffer (PBS without Ca^{2+} and Mg^{2+}; 0.5% bovine serum albumin (BSA) and 100 µM EDTA), and resuspended in the same buffer. Neutrophils and monocyte cell suspensions were incubated with anti-CD66b-APC (B15091, Beckman Coulter, France) and anti-CD14-APC (HCD14, BioLegend, Amsterdam 1105 BM, Netherlands) primary antibodies, respectively, in the dark at 4°C for 20 min. The cells were subsequently washed in PBS-BSA buffer and incubated with anti-APC microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) in the dark at 4°C for 15 min. Following the incubation, the cells were washed and passed through a magnetic column (MACS, Miltenyi Biotec, Bergisch Gladbach, Germany) to collect the neutrophil and monocyte cell fractions. The cell purity was greater than 95%, as determined by flow cytometry. Neutrophils and monocytes were cultured in RPMI medium supplemented with 10% EV-depleted FCS with or without stimulation with TNF-α (10 ng/ml) (Miltenyi Biotec) and LPS (10 µg/ml) (Merck Sigma–Aldrich, St Quentin Fallavier), respectively, for 18 h.

2.3. Isolation of platelets and platelet-derived EVs (P-EVs)

Platelets were isolated from peripheral venous blood collected from healthy volunteers. Briefly, venous blood was drawn into 5-ml sodium citrate vacutainer tubes (BD Diagnostics) from the antecubital vein using a 21-gauge needle. Blood was centrifuged for 13 min at 200 × *g* to collect

the PRP. PRP was mixed at a 1:1 ratio with Tyrode-BSA 0.2% buffer (137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 0.2 mM Na_2HPO_4, 12 mM $NaHCO_3$, 5.5 mM D-glucose; filter, 0.22 µm) containing 20 U/ml apyrase (Merck Sigma–Aldrich, St Quentin Fallavier, France) and 1 mM prostaglandin I2 (PGI2) (Merck Sigma–Aldrich). The tubes were centrifuged at 900 × *g* for 13 min at 28°C; the platelet-poor plasma (PPP) was discarded, and the platelets were washed twice and resuspended in 0.2% Tyrode–BSA containing apyrase/PGI2 solution. Washed platelets were labeled with an anti-CD41-PCy7 antibody and quantified by flow cytometry. The cell suspension was then incubated with $CaCl_2$ and $MgCl_2$ for 30 min at 37°C. The platelet suspension was then treated with 1 U/ml thrombin (Merck Sigma–Aldrich, St. Quentin Fallavier, France) for 1 h with slow rotation at RT. Following thrombin treatment, the cell suspension was centrifuged at 4500 × *g* for 20 min to remove the platelets and cell debris. The EV-rich supernatant was centrifuged at 100,000 × *g* for 90 min at 4°C (JA-30.50 Ti fixed-angle rotor, k-factor: 280, Beckman Coulter) to pellet the EVs, which were then resuspended in HEPES buffer (150 mM NaCl, 20 mM HEPES, and 0.1% NaN_3, pH 7.4; filter, 0.22 µm) and stored at -80°C in small aliquots ($0.5x10^5$ EVs/µl as determined by flow cytometry).

2.4. Isolation of EVs from neutrophils, monocytes, and endothelial cells (N-EVs, M-EVs, and E-EVs)

We isolated and purified EVs from the EV-rich culture supernatant of HCMECs, neutrophils, and monocytes. Samples were collected and processed for EV purification and analysis according to international guidelines [23]. In brief, the collected conditioned media was centrifuged twice at 300 × g for 5 min to remove the cells, once at 2500 × g to remove cell debris and apoptotic bodies, and then once by ultracentrifugation at 100,000 × g for 90 min at 4°C. The resuspended EV pellet was passed through a 100-kDa ultrafiltration tube (VS15T41 Vivaspin Turbo 15 PES, Sartorius, Goettingen, Germany) by centrifugation at 1300 × g to concentrate the EVs. The EV-rich retentate was then subjected to size-exclusion chromatography (SEC) using IZON qEVoriginal columns (IZON Science, Oxford, England) with 70-nm pore sizes by eluting the EV sample with PBS buffer. Purified EVs were pelleted by ultracentrifugation at 100,000 × g for 90 min (JA-30.50 Ti fixed-angle rotor, k-factor: 280, Beckman Coulter), resuspended in HEPES buffer, and stored at -80°C in small aliquots (0.5x105 EVs/µl). We have submitted all relevant data from our experiments to the EV-TRACK knowledgebase (EV-TRACK ID: EV240012) (EV-METRIC: 50-56%) [24].

2.5. Luminex assay

The cytokine content of EV preparations was analyzed using a multiplex bead-based immunoassay (Luminex platform) according to the manufacturer's instructions. Briefly, EV samples were incubated with antibody-conjugated beads specific for a panel of inflammatory cytokines. After washing, detection antibodies and streptavidin-PE were added, and fluorescence intensity was measured using a Luminex analyzer. Data acquisition and analysis were performed using xPONENT software.

2.6. Intracellular Ca^{2+} measurement

Intracellular Ca^{2+} signaling in HUVECs was measured using 5 µM calcium-sensitive fluorescent dye Fluo-4 AM (Invitrogen, Cat No. F14217). Fluorescence measurements were performed using a FlexStation microplate reader. Baseline fluorescence was recorded prior to EV stimulation, and intracellular Ca^{2+} responses were continuously monitored for 5 min with a reading interval of 0.18 s. Thrombin (10 nM) was used as a positive control. Changes in intracellular Ca^{2+} levels were quantified based on peak fluorescence intensity.

2.7. Flow cytometric analysis of ICAM-1 expression

HUVECs were seeded into 12-well plates at a density of 5×10^4 cells/well and cultured for 24h, then treated with EVs (200 EV/cell) or vehicle control for 15 h. Following treatment, cells were subsequently resuspended in FACS buffer (PBS supplemented with 1% FBS and 1 mM EDTA) and incubated with 2.5 µg/ml anti-human ICAM-1 monoclonal antibody (eBioscience, Cat. No. BMS108) for 30 min at 4°C. After washing, cells were incubated with Alexa Fluor™ 647-conjugated goat anti-mouse IgG secondary antibody (Thermo Fisher Scientific, Cat. No. A-21235) for 15 min at 4°C in the dark. Cells were analysed using BD AccuriTM C6 Plus flow cytometer by collecting a total of 10000 events.

2.8. Enzyme linked-immunosorbent-assay (ELISA)

CCL2 levels in HUVEC culture supernatants were quantified using a sandwich enzyme-linked immunosorbent assay (ELISA). HUVECs were treated with EVs or vehicle control for 15 h, and cell culture supernatants were collected and centrifuged to remove cell debris.

Ninety-six-well ELISA plates were coated overnight at 4°C with anti-human CCL2 capture antibody (clone E10051, Thermo Fisher Scientific). After blocking, samples and recombinant human CCL2 standards (PeproTech®, Cat. No. 300-04) were added and incubated for 2 h. Bound CCL2 was detected using biotinylated anti-human CCL2 detection antibody (PeproTech®, Cat. No. 500-P34BT). Signal development was performed using HRP-conjugated streptavidin and TMB substrate solution. Absorbance was measured at 450 nm using a microplate reader, and CCL2 concentrations were calculated based on the standard curve.

2.9. Flow chamber adhesion assay

For flow chamber adhesion, HUVECs were seeded in 35-mm dishes coated with Collagen Type I, Rat Tail (30 µg/mL, Sigma-Aldrich, Cat. No. 08-115) and cultured to confluence. Cells were then incubated with PBS, IL-1β (10 ng/mL, Thermo Fisher Scientific, Cat. No. 200-01B), or PDE-derived EVs (100 EVs per cell) for 15 h. THP-1 cells were labeled with 1 µM SYTO™ Green Fluorescent Nucleic Acid Stains (Thermo Fisher Scientific, Cat. No. S7575) for 30 min at 37°C, washed, and perfused over the endothelial monolayer in Hanks' balanced salt solution (HBSS, pH 7.45) containing 10 mM HEPES, 3 mM $CaCl_2$, 2 mM $MgCl_2$, and 0.2% BSA for 2–6 min at a shear stress of 3 dyn/cm². Adherent fluorescent cells were manually counted in at least six random fields and expressed as cells/mm².

2.10. Cell migration assay

Migration of THP-1 cells towards the MVs were assessed by using a 12-well chamber with track-etched polycarbonate membranes (5 µm pore size, Neuroprobe, Gaithersburg, MD). EVs or CCL2 (10ng/ml final concentration, positive control) were added to the lower wells and THP-1 cells ($5x10^5$ cells) in the upper chamber. Cells were allowed to migrate for 90min at 37°C. Following the migration, non-migrated cells were carefully removed by pipetting, and a wetted Q-tip, and the membrane was fixed in methanol for 10 seconds, stained with Eosin and Hematoxylin, respectively for 20 seconds. Stained cells were manually counted using a light microscope.

2.11. Apoptosis assay

For apoptosis assays, HUVECs were seeded in 12-well collagen-coated plates (30 µg/mL) and incubated with PBS, EVs (100 EVs per cell) for 15 h, or with 1 µM raptinal for 30 min as a positive control. Apoptosis was assessed by Annexin A5/propidium iodide (PI) staining. Cells were harvested, washed with PBS, and resuspended in Annexin A5 binding buffer (10 mM HEPES, pH 7.4, 2.5 mM $CaCl_2$, 140 mM NaCl). Annexin A5 (1 µM, in-house prepared) and PI (1 µM, Thermo Fisher Scientific) were added, and samples were incubated for 15 min at room temperature protected from light. Flow cytometric analysis was performed using a BD Accuri cytometer, and data were analyzed with FlowJo software. A minimum of 10,000 events were acquired per sample. Cells negative for both Annexin A5 and PI were considered viable. Early apoptotic cells were defined as Annexin A5-positive and PI-negative, whereas late apoptotic cells were Annexin A5- and PI-positive. Necrotic cells were defined as PI-positive and Annexin A5-negative. The percentage of total apoptotic cells was calculated as the sum of early and late apoptotic populations.

2.12. Measurement of mitochondrial membrane potential (ΔΨm)

Mitochondrial membrane potential (ΔΨm) was assessed using the JC-1 (Cell Signaling Technology, Cat. No. 92891) according to the manufacturer's instructions. HUVECs were seeded in collagen-coated 6-well plates (30 µg/mL collagen) and incubated with vehicle control or EVs (200 EVs per cell) for 24 h. Following treatment, cells were washed once with PBS and incubated with 1 mL JC-1 working solution for 30 min at 37°C in the dark. After incubation, cells were washed twice with JC-1 assay buffer and immediately imaged using a fluorescence microscope (EVOS, Thermo Fisher Scientific) at ×200 magnification. JC-1 aggregates (red fluorescence) and JC-1 monomers (green fluorescence) were analyzed, and mitochondrial membrane potential was quantified as the ratio of red to green fluorescence intensity.

2.13. Statistics

Data were analyzed using GraphPad Prism software. Statistical significance was determined using one-way analysis of variance (ANOVA), followed by Tukey's multiple comparisons post hoc test. Data were considered statistically significant when $p < 0,05$. All data are presented as mean ± SD.

3 Result

3.1. EVs exhibit cell type–dependent inflammatory cargo profiles

Our team successfully isolated and identified EVs from monocytes, neutrophils, platelets, and endothelial cells under both resting and inflammatory conditions. To characterize the inflammatory cargo of EVs, we performed multiplex cytokine profiling using the Luminex platform. This analysis revealed distinct inflammatory profiles among EVs from different cell types. Under inflammatory conditions, the levels of pro-inflammatory cytokines in EVs derived from endothelial cells and monocytes were significantly higher than in the resting state, with the most pronounced changes observed in monocyte. In contrast, neutrophil-EVs showed minimal differences between resting and inflammatory states (Figure-1). Interestingly, the expression patterns of certain cytokines differed. For example, the anti-inflammatory cytokine IL-10 was found at higher levels in resting EVs of endothelial cells compared to those under inflammatory conditions. Additionally, CCL5 was present at lower levels in monocyte-EVs under inflammatory conditions than in resting EVs (Figure-1). Corresponding quantitative data for individual cytokines are shown in Supplementary Figure S3. These results suggest that the regulation of inflammatory cargo in EVs is cell type-specific and depends on activation state.

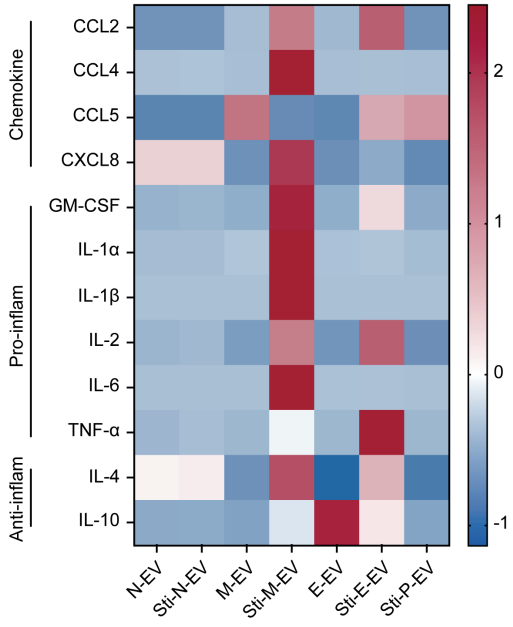


Figure 1. Inflammatory cargo profiling of EVs derived from different cellular origins.

Heatmap showing the relative expression levels of inflammation-related factors in EVs derived from different cell types under resting and inflammatory conditions. Inflammatory mediators were grouped into chemokines, pro-inflammatory cytokines, and anti-inflammatory cytokines. Color scale from blue to red indicates low to high expression levels.

3.2. EV-Triggered Rapid Ca^{2+} Signaling in Endothelial Cells

To evaluate the immediate effects of EVs on endothelial cells, we monitored intracellular calcium dynamics using Fluo-4 AM. Stimulation with EVs caused a rapid, transient increase in intracellular Ca^{2+} levels, peaking within 60 seconds post-exposure. This response resembled that induced by thrombin (positive control) but exhibited a lower amplitude (Figure 2A). Quantitative analysis of the Ca^{2+} peak response revealed that under inflammatory conditions, EVs derived from neutrophils, endothelial cells, and monocytes induced significantly higher calcium responses compared to the vehicle group. In contrast, EVs obtained from platelets did not show a significant difference from the vehicle group. Among the EVs tested, inflammatory EVs derived from monocytes triggered the strongest calcium response, which was significantly higher than their resting counterparts and P-EVs (Figure 2B). These findings suggest that the calcium signaling in endothelial cells induced by EVs is dependent on the cellular origin of the EVs and is notably enhanced in a cell-type-specific manner under inflammatory conditions.

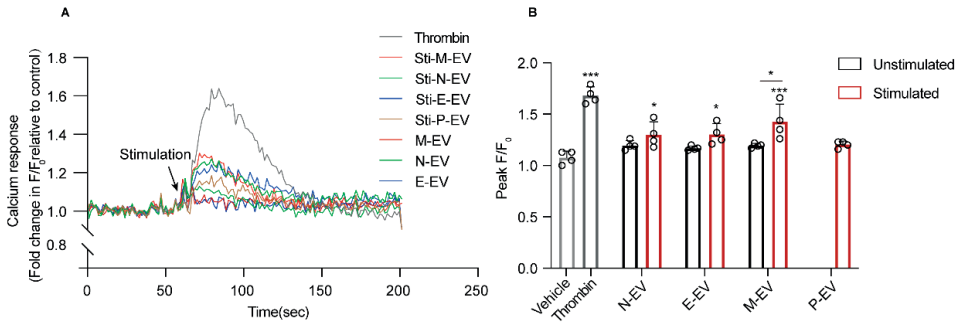


Figure 2. EVs induce rapid intracellular Ca^{2+} signaling in HUVECs.

(A) Representative intracellular Ca^{2+} traces in HUVECs following stimulation with EVs derived from different cell types. Thrombin was used as a positive control.

(B) Quantification of peak fluorescence intensity showing rapid cytosolic Ca^{2+} elevation upon EV stimulation. Data are presented as mean ± SD (n = 4). *P < 0.05, ***P < 0.001 versus vehicle group. Horizontal bars indicate comparisons between the indicated groups. ns, not significant.

3.3. EVs differentially regulate inflammation in HUVECs

Based on the observed differences in Ca^{2+} signaling induced by EVs, we further investigated whether these differences were associated with endothelial inflammatory activation. EVs derived from different cellular origins differentially induced ICAM-1 expression in HUVECs. Compared with the vehicle group, EVs derived from monocytes, endothelial cells, and neutrophils under inflammatory conditions significantly increased ICAM-1 expression. Compared with their corresponding resting EVs, inflammatory EVs generally showed a higher tendency to induce ICAM-1 expression, although statistical significance was reached only in M-EVs (Figure 3A).

Notably, the degree of ICAM-1 induction was consistent with the magnitude of the calcium response observed across different EV groups (Supplementary Figure S1).

Further analysis of endothelial CCL2 secretion showed that inflammatory EVs exhibited a higher tendency to induce MCP-1 production. Compared with the vehicle group and their corresponding resting EV groups, inflammatory M-EVs significantly increased MCP-1 secretion levels, and also induced significantly higher MCP-1 levels than other inflammatory EV groups (Figure 3B). The effect of EV treatment on leukocyte recruitment was further evaluated under flow conditions. Monocyte adhesion assays demonstrated that, compared with the vehicle group and the corresponding resting EV groups, all inflammatory EVs significantly promoted monocyte adhesion. Among these groups, inflammatory M-EVs induced the strongest adhesion response, which was significantly greater than that induced by other inflammatory EV groups (Figure 3C-D).

Monocyte migration assays further indicated that inflammatory EVs generally promoted monocyte migration across the endothelium in a dose-dependent manner (Supplementary Figure S2). Compared with the vehicle group and the corresponding resting EV group, inflammatory EVs derived from monocytes and platelets significantly enhanced monocyte transendothelial monocyte migration (Figure 3E). Overall, these findings suggest that inflammatory EVs differentially promote endothelial inflammatory activation and leukocyte recruitment, with inflammatory M-EVs consistently exhibiting the strongest pro-inflammatory profile across multiple functional assays.

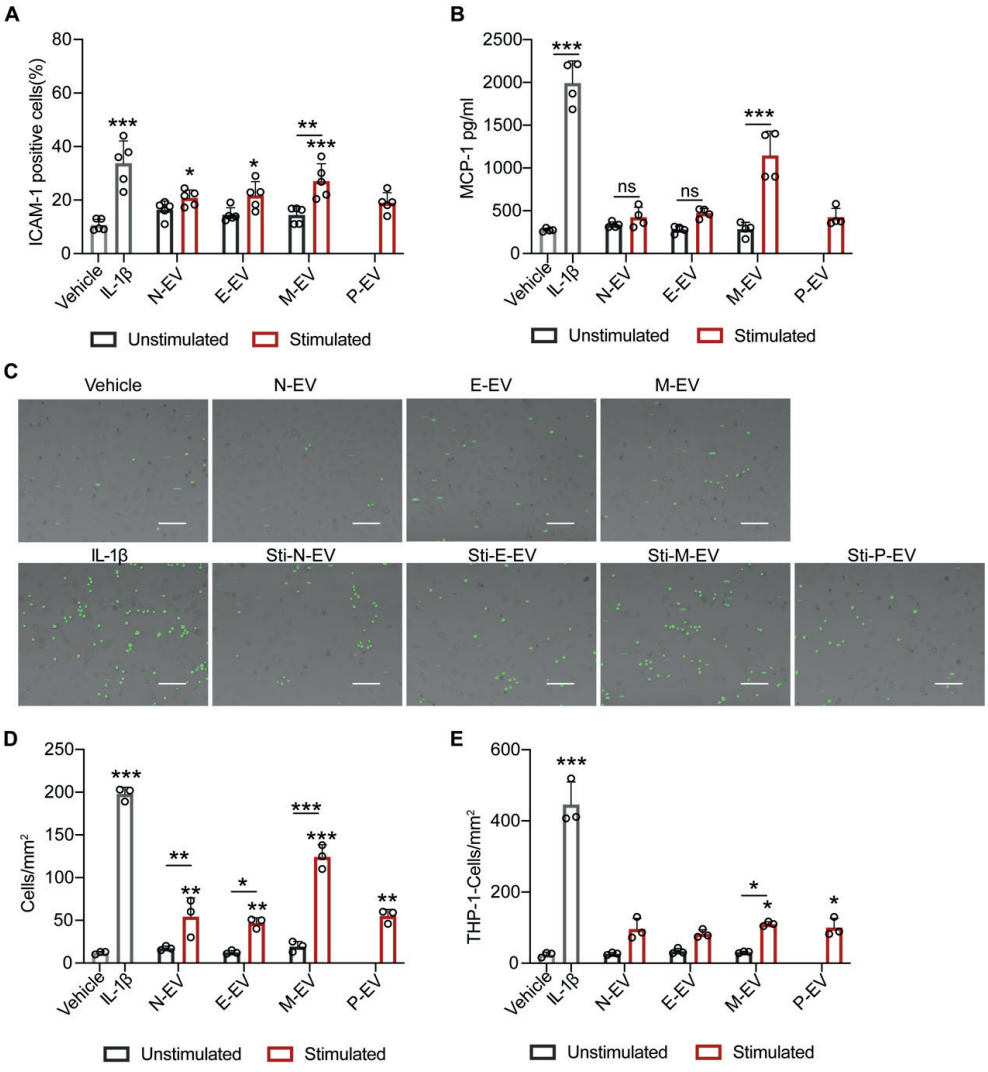


Figure 3. Inflammatory EVs promote endothelial activation and monocyte recruitment.

(A) ICAM-1 surface expression in HUVECs determined by flow cytometry. (B) MCP-1 levels in HUVEC culture supernatants measured by ELISA. (C) Representative images of THP-1 cells adherent to EV-treated HUVECs under flow conditions. (D) Quantification of THP-1 cell adhesion to HUVECs. (E) Quantification of THP-1 transendothelial migration. Data are presented as mean ± SD (n = 3-5). *P < 0.05; **P < 0.01; ***P < 0.001versus vehicle group. Horizontal bars indicate comparisons between the indicated groups. ns, not significant. Scale bar = 100 μm.

3.4. EVs differentially induce apoptosis and mitochondrial dysfunction in HUVECs.

To evaluate the effects of prolonged EV exposure on endothelial cell viability, apoptosis was assessed after 24 h of incubation. Flow cytometry analysis showed that EV treatment significantly increased endothelial apoptosis compared with the vehicle group. Among the different EV groups, inflammatory M-EVs induced significantly higher levels of apoptosis than their corresponding resting EVs and P-EVs (Figure 4A-B).

To further explore the potential mechanisms underlying EV-induced apoptosis, mitochondrial membrane potential (ΔΨm) was evaluated. Compared with the vehicle group, inflammatory EVs derived from monocytes, endothelial cells, and neutrophils significantly reduced mitochondrial membrane potential, indicating mitochondrial dysfunction. Consistent with the apoptosis data, inflammatory M-EVs induced a greater reduction in ΔΨm than resting M-EVs and P-EVs (Figure 4C-D). These findings suggest that EV-induced endothelial apoptosis may be mediated, at least in part, through mitochondrial depolarization.

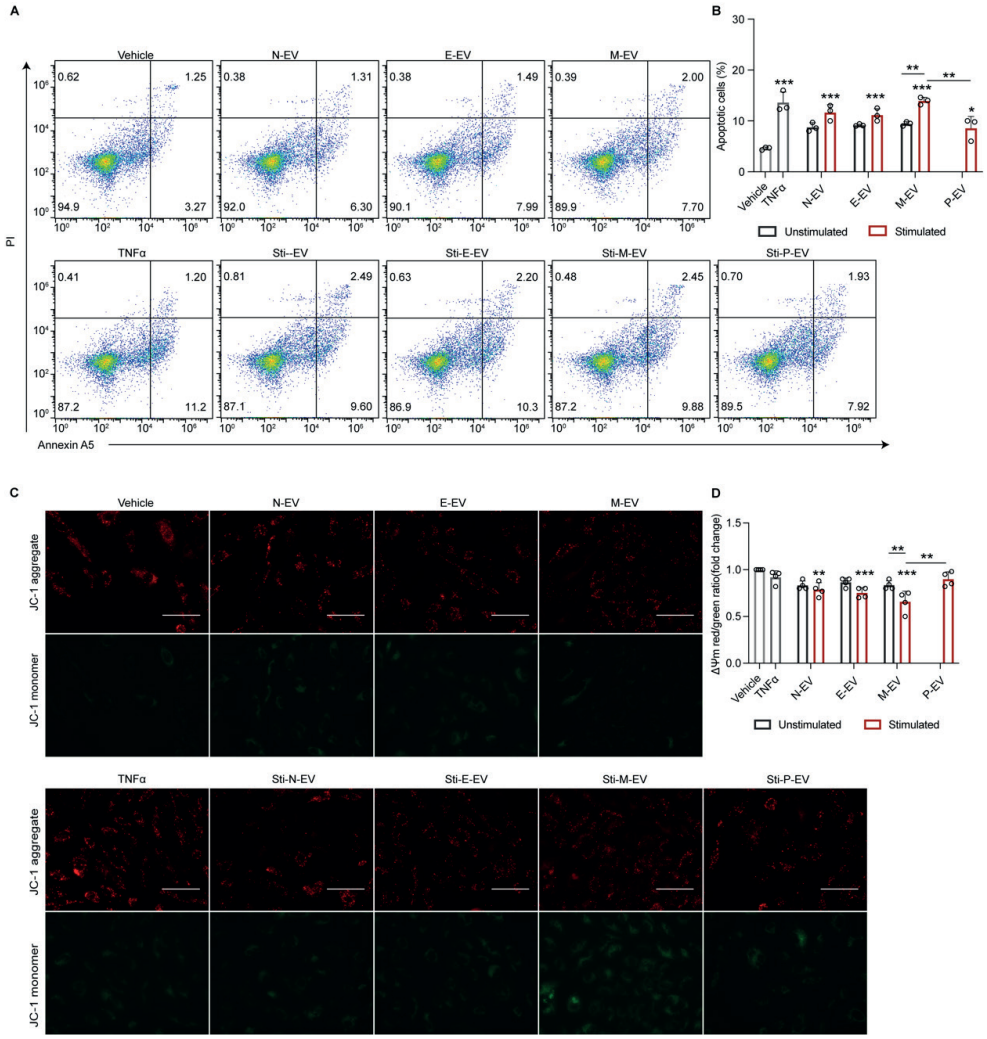


Figure 4. EVs induce endothelial apoptosis and mitochondrial dysfunction.

(A) Representative flow cytometry plots of Annexin A5/PI staining in HUVECs following EV treatment. (B) Quantification of total apoptotic cells, including early apoptotic (Annexin $A5^+/PI^-$) and late apoptotic (Annexin $A5^+/PI^+$) populations. (C) Representative JC-1 fluorescence images showing mitochondrial membrane potential (ΔΨm) in HUVECs after EV treatment. Red fluorescence indicates JC-1 aggregates, whereas green fluorescence indicates JC-1 monomers. (D) Quantification of the JC-1 red/green fluorescence intensity ratio. Data are presented as mean ± SD (n = 3–4). *P < 0.05, **P < 0.01, ***P < 0.001 versus vehicle group. Horizontal bars indicate comparisons between the indicated groups. ns, not significant. Scale bar = 75 μm.

4 Discussion

Accumulating evidence suggests that EVs play a crucial role in the development and progression of vascular inflammation and atherosclerosis by mediating intercellular communication [25-27]. This study systematically compared the effects of various EVs released by vascular and immune cells under both resting and inflammatory conditions on endothelial cell function. Our results indicate that the ability of EVs to promote endothelial cell activation, leukocyte recruitment, and endothelial dysfunction is significantly dependent on their cell of origin and inflammatory status. Notably, EVs derived from monocytes that were stimulated with LPS exhibited the most pronounced pro-inflammatory effects across multiple experiments. Additionally, the inflammatory effects induced by different EV populations were generally consistent with the intensity of the rapid Ca^{2+} signaling response they elicited in endothelial cells.

Endothelial inflammation is a crucial feature of early atherosclerotic lesions, characterized by increased expression of adhesion molecules and chemokines, thereby promoting inflammatory cell recruitment and vascular inflammation [1,28]. In this study, inflammatory M-EVs significantly enhanced the expression of ICAM-1 and CCL2, which in turn promoted monocyte adhesion and transendothelial migration. Research has demonstrated that inflammatory M-EVs can induce endothelial cells to express inflammatory molecules such as ICAM-1, VCAM-1, and IL-6 through NF-κB-related pathways and the mechanism may involve NLRP3 activation and the transport of functional microRNAs [14,29,30]. In contrast, while TNFα-treated neutrophil and endothelial cell-derived EVs significantly increased ICAM-1 expression and monocyte adhesion, their effects on CCL2 secretion and monocyte transendothelial migration were relatively limited. It has been suggested that ICAM-1 is primarily involved in the firm adhesion of leukocytes to the endothelial surface [31,32], whereas chemokines, like CCL2, are more involved in subsequent transendothelial migration [33,34]. Thus, these findings imply that inflammatory N-EVs and E-EVs are more likely to enhance the expression of endothelial adhesion molecules and promote leukocyte adhesion, but they may not fully activate the signaling pathways related to chemotactic migration. Furthermore, previous studies have shown that neutrophil- and endothelial cell-derived EVs can promote the expression of endothelial adhesion molecules and leukocyte adhesion through miRNA and NF-κB-related signaling [15,18,35,36]. Under specific inflammatory conditions, such as N-formylmethionyl-leucyl-phenylalanine (fMLP) stimulation, N-EVs have been reported to further promote monocyte migration [37]. These differences indicate that the inflammatory cargo carried by EVs may vary depending on different stimuli, leading to inconsistent regulatory effects on various stages of endothelial activation and leukocyte recruitment. Interestingly, while inflammatory P-EVs have limited effects on ICAM-1 and CCL2 expression, they still promoted monocyte adhesion and migration. Previous studies have shown that P-EVs can enhance interactions between leukocyte and endothelial cells through P-selectin, CCL5, and GPIbα-related mechanisms [21,22,38]. Therefore, the leukocyte recruitment effect observed in this study may not be entirely dependent on the upregulation

of ICAM-1 or CCL2. These findings further suggest that EV-mediated endothelial-leukocyte interactions are highly complex and influenced by multiple regulatory factors [39].

The functional differences observed among various populations of EVs may be linked to the different inflammatory components they carry. Our cytokine profiling analysis revealed significantly different inflammatory molecular characteristics in EVs from different sources. Inflammatory M-EVs were rich in various chemokines and pro-inflammatory factors, consistent with their stronger ICAM-1 induction and leukocyte recruitment effects. This aligns with previous reports that EVs under inflammatory conditions can accumulate pro-inflammatory cytokines, lipid molecules, and RNAs regulating endothelial function [40,41]. In contrast, EVs derived from neutrophils and platelets showed relatively fewer detected inflammatory factors. Furthermore, we observed that M-EVs retained certain levels of anti-inflammatory factors (IL-4, IL-10), suggesting that EV-mediated inflammatory signaling is not simply pro-inflammatory but may also contain certain negative regulatory components.

We found that EV stimulation rapidly induced a transient increase in intracellular calcium ions (Ca^{2+}) in endothelial cells. Previous research has shown that Ca^{2+} signaling is involved in the regulation of endothelial cell inflammation activation and inflammation-related pathways such as NF-κB [42-44]. The intensity of the Ca^{2+} response induced by different EV populations was generally consistent with the degree of subsequent inflammatory activation, suggesting that Ca^{2+}-dependent signaling may be involved in EV-mediated endothelial inflammation regulation. Although the study did not explore the specific molecular mechanisms involved, the findings indicate that Ca^{2+} signaling may represent an early functional response following EV stimulation, contributing to subsequent inflammatory responses [45].

Additionally, we discovered that prolonged exposure to EVs can induce endothelial cel apoptosis and a decrease in mitochondrial membrane potential, suggesting mitochondrial dysfunction as a downstream consequence of EV mediated inflammatory activation. Notably, EVs derived from inflammatory monocytes had the strongest effects, indicating that sustained Ca^{2+}signaling and endothelial inflammation may contribute to progressive endothelial injury.

However, the study has some limitations. First, it primarily utilized an in vitro model of endothelial cells, which may not fully capture the complexity of the in vivo vascular microenvironment. Second, while Ca^{2+} signaling is linked to endothelial cell activation, the causal relationships and downstream signaling pathways involved require further investigation. In summary, our study demonstrates that EVs from different vascular and immune cell sources exert varying effects on endothelial inflammation and dysfunction. EVs derived from inflammatory monocytes displayed the strongest pro-inflammatory characteristics and were associated with enhanced Ca^{2+} signaling, leukocyte recruitment, and endothelial injury. These findings offer new insights into EV-mediated intercellular communication during vascular inflammation and atherosclerosis.

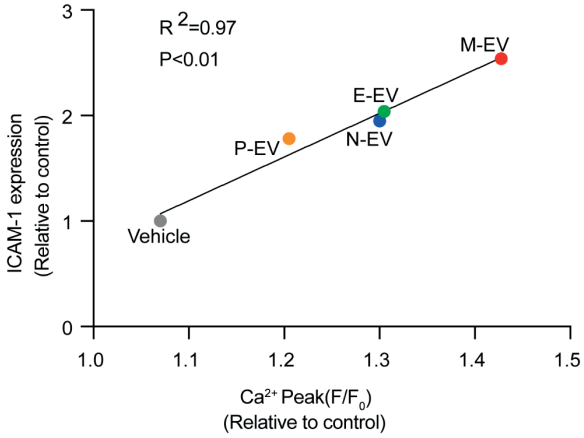
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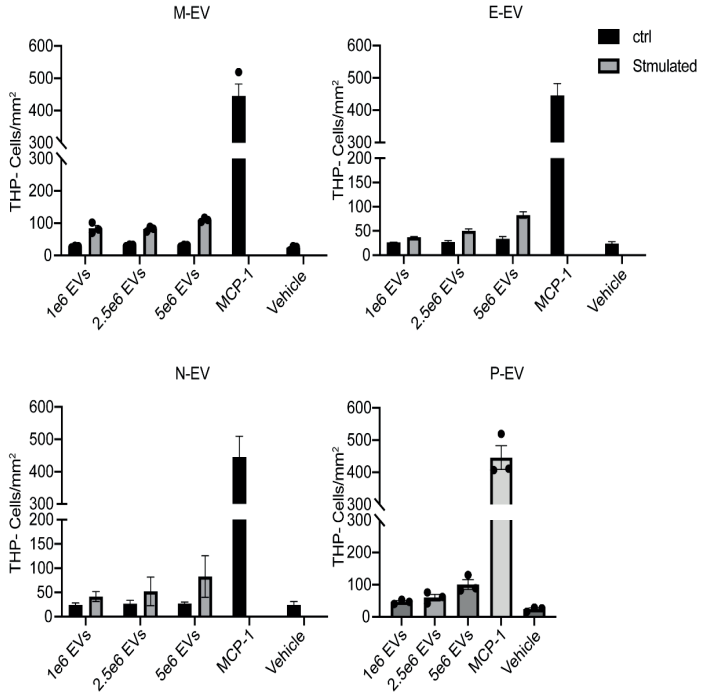
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Supplementary Data



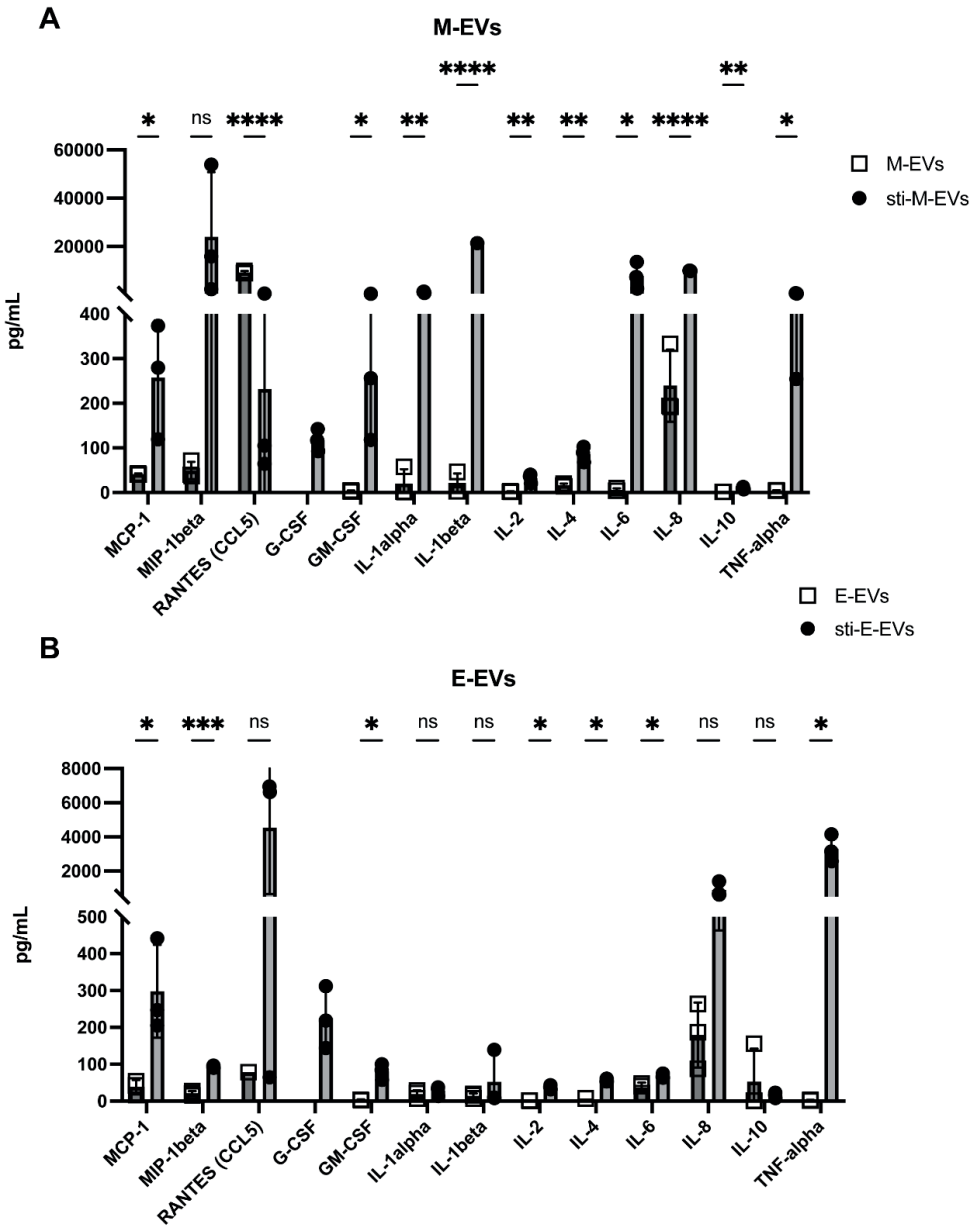
Supplementary Figure S1 Correlation analysis between EV-induced intracellular Ca^{2+} responses and endothelial ICAM-1 expression.

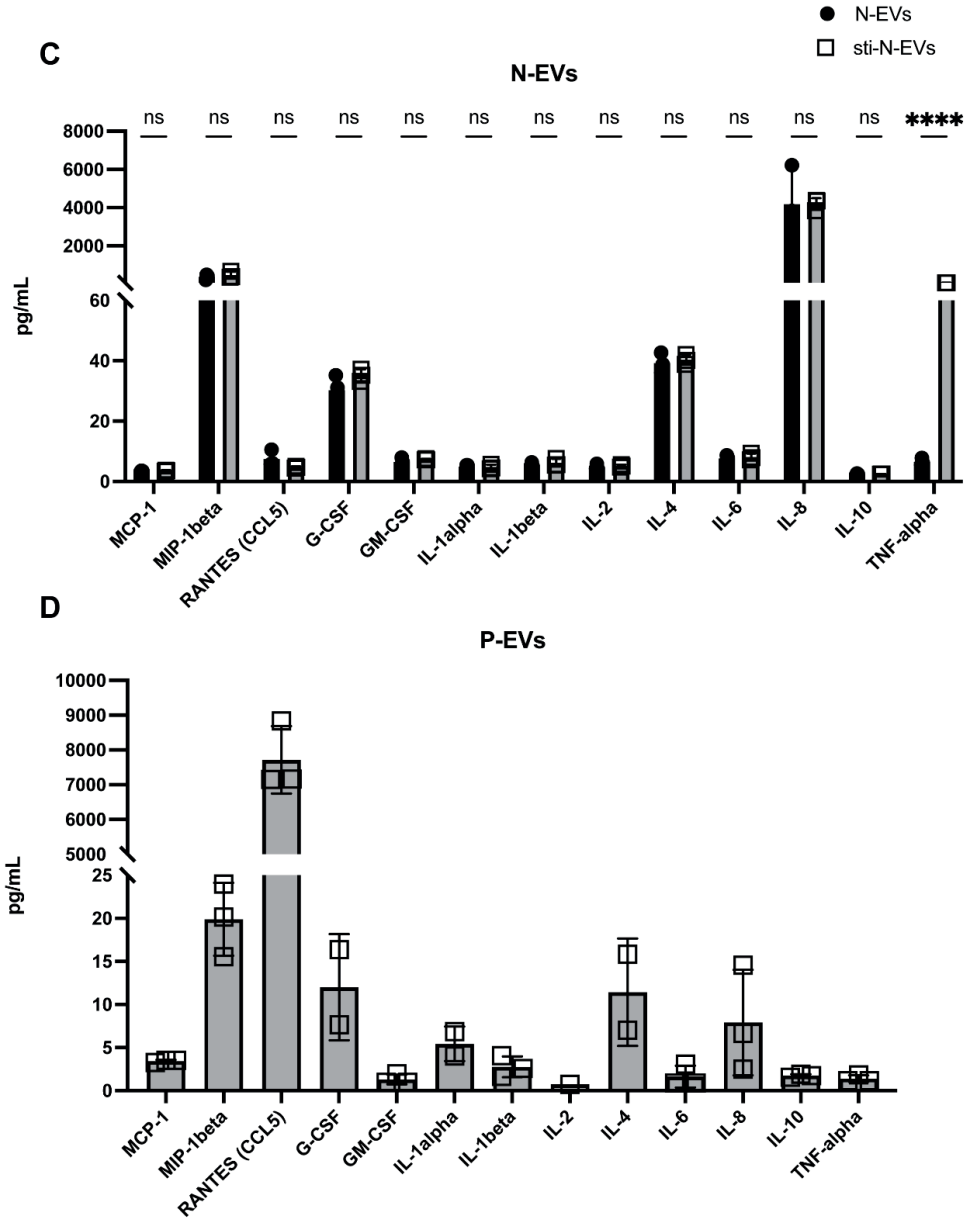
Peak Ca^{2+} fluorescence intensity and ICAM-1 expression levels were normalized to the vehicle control group. Linear regression analysis demonstrates a positive association between EV-induced Ca^{2+} signaling and endothelial inflammatory activation.



Supplementary Figure S2 Dose-dependent effects of EVs on monocyte transendothelial migration.

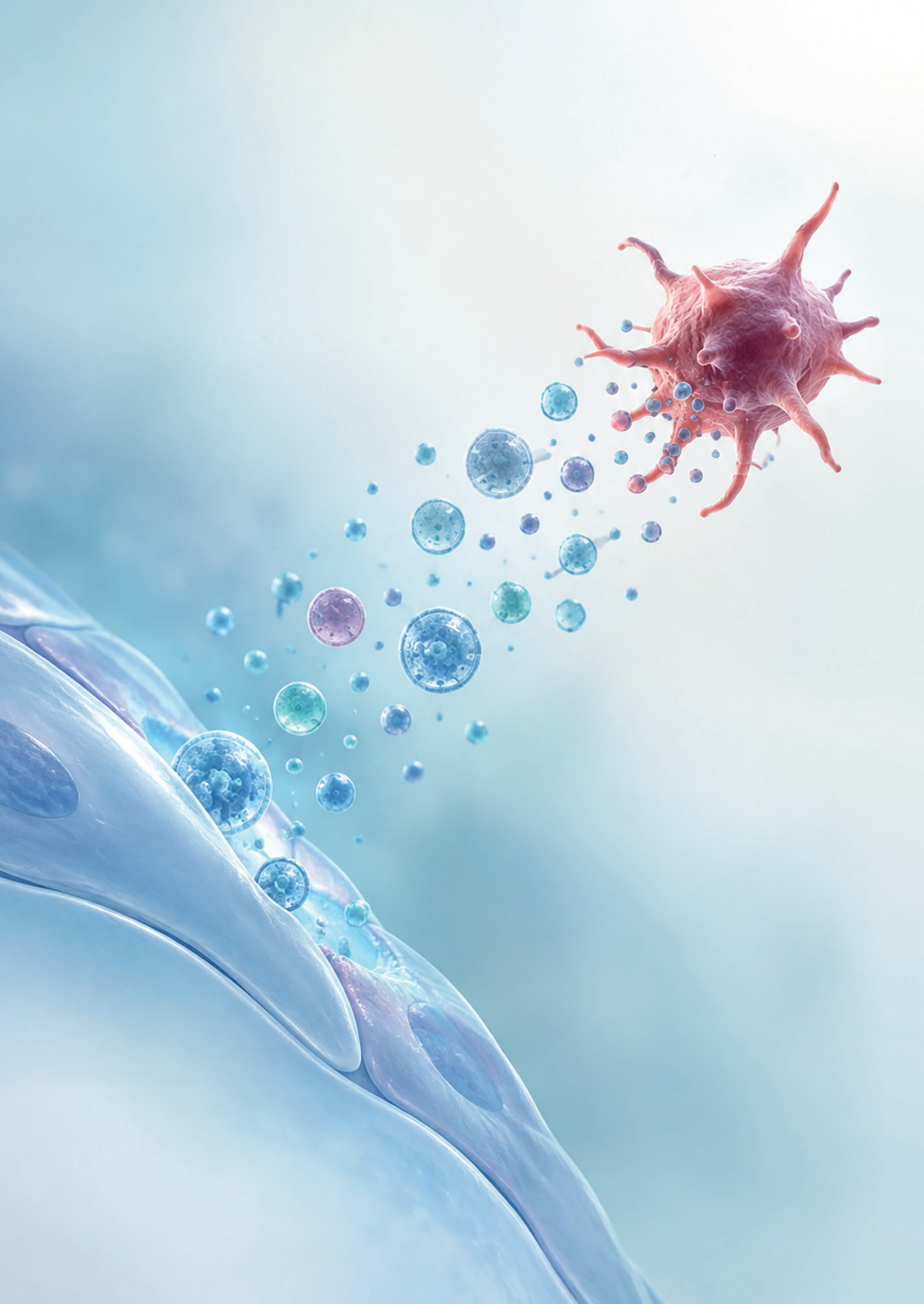
HUVECs were treated with increasing concentrations of EVs, followed by assessment of THP-1 cell migration across the endothelial monolayer. Data are presented as mean ± SD, n=3.


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MIP-1beta
RANTES (CCL5)
G-CSF
GM-CSF
IL-1alpha
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IL-2
IL-4
IL-6
IL-8
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RANTES (CCL5)
G-CSF
GM-CSF
IL-1alpha
IL-1beta
IL-2
IL-4
IL-6
IL-8
IL-10
TNF-alpha



Supplementary Figure S3 Quantitative analysis of individual inflammatory mediators corresponding to the cytokine heatmap shown in Figure 1.

Bar graphs represent relative expression levels of selected cytokines and chemokines in EV preparations derived from different cell types and stimulation conditions. Data are presented as mean ± SD, n=3, *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.



Chapter 5

Extracellular vesicles derived from peritoneal dialysis effluent exert differential effects on endothelial cells depending on dialysis duration and HFpEF status

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Manuscript in preparation.

Abstract

Peritoneal dialysis (PD) is linked to chronic inflammation and a significant risk of cardiovascular complications, particularly heart failure with preserved ejection fraction (HFpEF). Extracellular vesicles (EVs) have emerged as important mediators of intercellular communication and may contribute to vascular dysfunction. This study aimed to examine the effects of peritoneal dialysis effluent-derived extracellular vesicles (PDE-EVs) on endothelial cell function, considering different durations of dialysis and the presence of HFpEF. PDE-EVs were isolated and incubated with human umbilical vein endothelial cells (HUVECs). Various techniques, including flow cytometry, ELISA, and microscopy, were used to evaluate EV uptake, endothelial inflammation, and apoptosis. The findings revealed that PDE-EVs were effectively internalized by endothelial cells, leading to a pro-inflammatory phenotype. This was demonstrated by increased expression of intercellular adhesion molecule-1 (ICAM-1) and heightened secretion of interleukin-6 (IL-6), along with increased monocyte adhesion to the endothelium. Significant differences in the functional effects were noted among different patient groups. EVs from patients undergoing long-term PD complicated by HFpEF elicited the strongest inflammatory response. Notably, there were no significant differences in EV uptake among the groups, indicating that functional variability may arise from differences in the constituents carried by the EVs. In summary, these results suggest that PDE-EVs exert an inflammatory regulatory effect on endothelial cells.

Keywords: peritoneal dialysis effluent, extracellular vesicle, endothelial cell, inflammation, chronic kidney disease.

1 Introduction

Chronic kidney disease (CKD) represents a major global health burden and is frequently complicated by cardiovascular disease (CVD), which remains the leading cause of mortality in this population[1-4].

Patients with end-stage kidney disease (ESKD) require renal replacement therapies, including peritoneal dialysis, hemodialysis, or kidney transplantation[5]. PD is widely adopted as a home-based modality with favorable hemodynamic stability, however cardiovascular complications remain highly prevalent in this population. [6-8]. Notably, heart failure (HF) is one of the most common complications and leading causes of death in this population[8,9], with HFpEF accounting for more than 50% of HF cases[10,11]. The mechanisms underlying HFpEF development in PD patients remain poorly understood. Endothelial dysfunction and chronic inflammation are recognized as key pathological processes in both CKD and HFpEF[12-16]. In patients undergoing PD, there may be a persistent low-grade inflammatory environment in the peritoneum[17]. However, it remains unclear whether this inflammation is linked to systemic vascular endothelial damage and the development of cardiovascular complications, such as HFpEF.

Extracellular vesicles (EVs) are lipid bilayer-enclosed particles that mediate intercellular communication by transferring bioactive molecules[18,19]. Emerging evidence suggests that EVs play an important role in regulating endothelial inflammation and vascular function[20-22]. EVs present in peritoneal dialysis effluent (PDE) are mainly derived from mesothelial cells, endothelial cells, immune cells platelets, and may also contain components transported across the membrane from the blood, thus reflecting the dynamic state of the local peritoneal microenvironment.[23,24]. We hypothesize that PDE-derived EVs are involved in regulating vascular endothelial function and may play a role in cardiovascular complications related to PD. Therefore, this study aimed to investigate the effects of EVs derived from PD patients with different dialysis durations and HFpEF status on endothelial inflammation and function.

2 Methods and materials

2.1. Cell culture

Human Umbilical Vein Endothelial Cells (HUVECs, PromoCell, Cat. No. C12203) and the human monocyte leukemia cell line (THP-1, DSMZ, ACC16) were cultured in Endothelial Cell Growth Medium 2 (PromoCell, Cat. No. C-22011), or RPMI 1640 Medium (Gibco, Cat. No. 11875093), supplemented with 10% fetal bovine serum (FBS, Gibco) and 1% penicillin and streptomycin (Gibco), respectively. The cultures were maintained at 37°C in a humidified environment with 5% CO_2. For HUVECs, culture plates were precoated with rat tail collagen type I (Sigma-Aldrich,

Cat. No. 08-115) in phosphate-buffered saline (PBS) for 30 minutes. HUVECs were used for experiments up to passage 5.

2.2. EV isolation

Patients with chronic kidney disease (CKD) undergoing peritoneal dialysis (PD) were classified based on dialysis duration and cardiac function, as described in the supplementary data. Then patients were divided into four groups: short-term PD with normal cardiac function (short-normal), short-term PD with HFpEF (short-HFpEF), long-term PD with normal cardiac function (long-normal), and long-term PD with HFpEF (long-HFpEF), with six patients in each group.

Peritoneal dialysis effluent (PDE) was subsequently collected from each patient group and centrifuged for 10 min at 3,500 x g to remove debris and contaminants. PDE was concentrated using centrifugal ultrafiltration devices (100 kDa MWCO, Pall Corporation, Cat. No. MAP100C37) at 3,500 × g, 4℃ to final volume of 1 ml. EVs were purified using size exclusion chromatography (SEC). Briefly, concentrated PDE was loaded onto a 13 mL Sepharose CL-2B column (GE Healthcare, Cat. No. 17-0140-01) and eluted with PBS. Fractions of 0.5 mL were collected and the EV-containing fractions 5-9 were pooled, aliquoted and stored at −80℃. A schematic overview of the EV isolation workflow is shown in Figure 1A.

2.3. Protein quantification

PDE-derived EVs were lysed in RIPA buffer (Thermo Fisher Scientific, Cat. No. 89900) containing protease inhibitors (Roche Diagnostics, Cat. No. 11873580001) for 2h on ice. The lysates were then centrifuged at 12,000 x g for 15 min at 4 ℃ to remove insoluble debris. Protein concentrations were determined using the Micro BCA™ Protein Assay Kit (Thermo Fisher Scientific, Cat. No. 23235) according to manufacturer's protocol.

2.4. Nanoparticle tracking analysis

Nanoparticle tracking analysis (NTA) was conducted to assess the size distribution and concentration of the EV isolates. The protocol followed was identical to that used in our previous study[25]

2.5. Western Blot

For western blot analysis, 30 µg of protein lysates from either cells or EVs were mixed with Laemmli sample buffer (5× stock; 0.5 M Tris-HCl, pH 6.8, 10% SDS, 30% glycerol, 200 µM bromophenol blue, 5% β-mercaptoethanol) and denatured for 10 min at 96°C. The samples were then separated using SDS-PAGE under reducing conditions and transferred to a polyvinylidene fluoride (PVDF) membrane. The membranes were blocked overnight at 4℃ in a solution of 5% skim milk in TBS-T (0,1% Tween-20 in TBS). Next, the transferred proteins were probed with the following antibodies overnight at 4°C: anti-TSG-101 (1:2000; Cell Signal, Cat. No. 72312), anti-CD63 (1:1000; Cell Signal, Cat. No. 52090), anti-*β*-actin (1:3000; Santa Cruz Biotechnology,

Cat. No. SC-47778) and anti-ICAM-1(1:500, Santa Cruz Biotechnology, Cat. No. SC-7964). After incubation, the membranes were treated with their respective HRP-conjugated secondary antibodies—anti-mouse (1:3000; Agilent, Cat. No. P0447) and anti-rabbit (1:10,000; Abcam, Cat. No. ab97051)—for 60 minutes. Bands were visualized using the Western Blotting Detection kit (Advansta, Cat. No. K12045), and band intensity was quantified using ImageJ software.

2.6. Surface marker detection

PDE-derived EVs were characterized by flow cytometry using a BD Accuri cytometer, and data were analyzed with FlowJo software. For staining, PDE- derived EVs were incubated with Annexin A5–APC (1 µg/mL, in-house prepared), anti-human CD31–FITC (1.25 µg/mL; Beckman Coulter, Cat. No.IM1431U), and anti-human CD41a–PE (1.25 µg/mL; BD Pharmingen, Cat. No.560979) in HEPES buffer containing 0.5% BSA and 5 mM $CaCl_2$ for 30 min on ice protected from light. Negative controls included staining with corresponding isotype antibodies (IgG–APC, IgG–FITC, IgG–PE; eBioscience) in HEPES buffer containing 0.5% BSA and 2 mM EDTA. Single-staining controls were prepared for fluorescence compensation. The percentage of antibody-positive EVs was determined by subtracting the corresponding isotype control signal from the antibody-stained signal. Endothelial cells were stained with anti-human ICAM-1 (2.5 µg/mL, ThermoFisher, Cat. No. BMS108) and corresponding isotype control (eBioscience) for 30 min at 4°C in the dark. Following washing with PBS containing 0.5% BSA, cells were incubated with Alexa Fluor™ 647-conjugated goat anti-mouse IgG secondary antibody (1:1000, Thermo Fisher Scientific, Cat. No. A-21235) for 15 min at 4°C in the dark. After a final washing step, cells were analyzed by flow cytometry using a BD Biosciences BD Accuri flow cytometer.

2.7. Endotoxin test

The endotoxin content of EV preparations was determined using the ToxinSensor™ Chromogenic LAL Endotoxin Assay Kit (GenScript, Cat. No. L00350C) following the manufacturer's protocol. $5x10^7$ of EV samples were mixed with the LAL reagent in endotoxin-free tubes. After incubation at 37 °C, the chromogenic substrate was added, and the reaction was stopped with stop solution. Absorbance was recorded at 545 nm, and endotoxin levels were quantified using a standard curve.

2.8. EV uptake

Endothelial cells were seeded on glass coverslips for confocal microscopy analysis or in a 12-well culture plate for flow cytometric analysis and allowed to attach overnight. EV (and control PBS) were labelled using 3,3'-Dihexyloxacarbocyanine iodide (DiO-C6, Thermo Fisher Scientific, Cat. No. D273) 8 µM for 1 h at 37◦C.Next, cells were incubated with 200 EVs/cell labelled EV (or equivalent volume of control PBS) for 20 h. For confocal imaging, cells were fixed using 4% paraformaldehyde for 20 min and nuclei were stained with Hoechst. Images were taken using a Zeiss confocal microscope. Punctae per cell and the number of DiO-C6-positive cells were analyzed using ImageJ software. For flow cytometry, cells were trypsinized, washed with PBS

containing 1% BSA and fluorescence intensity was measured using the BD Accuri cytometer. Data were analyzed using FlowJo software.

2.9. Enzyme linked-immunosorbent-assay (ELISA)

Human ICAM-1/CD54 levels were measured using a commercially available ELISA kit (R&D Systems, Cat. No. DY720). Human IL-6 concentrations were determined using an ELISA Ready-SET-Go kit (eBioscience, Cat. No. 88-7066-22). Cell culture supernatants and plasma samples were analyzed according to the manufacturers' instructions.

2.10. Flow chamber adhesion assay

For flow chamber adhesion assays, HUVECs were seeded in 35-mm collagen-coated dishes (30 µg/mL) as previously described[26] and incubated with PBS, IL-1β (10 ng/mL), or PDE-derived EVs (100 EVs per cell) for 15 h. THP-1 cells were labeled with 1 µM SYTO 13 fluorescent nucleic acid stain for 30 min at 37°C, washed, and perfused over the endothelial monolayer in Hanks' balanced salt solution (HBSS, pH 7.45) containing 10 mM HEPES, 3 mM $CaCl_2$, 2 mM $MgCl_2$, and 0.2% BSA for 2–6 min at a shear stress of 3 dyn/cm². Adherent fluorescent cells were manually counted in at least six random fields and expressed as cells/mm².

2.11. Apoptosis assay

For apoptosis assays, HUVECs were seeded in 12-well collagen-coated plates (30 µg/mL) and incubated with PBS, PDE-derived EVs (100 EVs per cell) for 15 h, or with 1 µM raptinal for 30 min as a positive control. Apoptosis was assessed by Annexin A5/propidium iodide (PI) staining. Cells were harvested, washed with PBS, and resuspended in Annexin A5 binding buffer (10 mM HEPES, pH 7.4, 2.5 mM $CaCl_2$, 140 mM NaCl). Annexin A5 (1 µM, in-house prepared) and PI (1 µM, Thermo Fisher Scientific) were added, and samples were incubated for 15 min at room temperature protected from light. Flow cytometric analysis was performed using a BD Accuri cytometer, and data were analyzed with FlowJo software. A minimum of 10,000 events were acquired per sample. Cells negative for both Annexin A5 and PI were considered viable. Early apoptotic cells were defined as Annexin A5-positive and PI-negative, whereas late apoptotic cells were Annexin A5- and PI-positive. Necrotic cells were defined as PI-positive and Annexin A5-negative. The percentage of total apoptotic cells was calculated as the sum of early and late apoptotic populations.

2.12. Statistics

Data were analyzed using GraphPad Prism software. Statistical significance was determined using one-way analysis of variance (ANOVA), followed by Tukey's multiple comparisons post hoc test. Data were considered statistically significant when $p < 0,05$. All data are presented as mean ± SD.

3 Results

3.1. Characterization of peritoneal dialysis effluent derived extracellular vesicles

PDE-derived EVs were isolated and characterized consistently with the MISEV2023 recommendations[27]. NTA revealed a typical particle size distribution (Figure 1B-E), and the mean diameter of EVs from the four patients was comparable (Figure 1F). Western blot analysis demonstrates the presence of EV markers CD63 and TSG101 in EVs derived from peritoneal dialysis across all four patient groups and they were comparable (Figure 1G-I). Notably, the particle concentration in PDE (particles/mL) and the protein content per particle (fg/particle) varied among the four patient groups (Figure 2A-B). EVs from patients undergoing long-term PD exhibited significantly higher concentrations than those from short-term PD patients; however, heart function did not affect the EV yield. In contrast, heart failure did influence the protein content of EVs, within short-term group showing significantly higher protein content in HFpEF-EVs compared to the normal heart function group, while within long-term group showing significantly lower protein content in HFpEF group. Furthermore, the HFpEF group showed significantly lower protein content in long-term EVs compared to the short-term group. This indicates that the features of the EVs may be affected by both the duration of PD and by the occurrence of heart failure. To further characterize the EVs, we measured several associated cell-specific markers: platelet-specific marker CD41a, platelet- and endothelial cell-specific marker CD31, and phosphatidylserine as indicated by Annexin A5 (Figure 3A-C). For CD31 and CD41a, the long-term PD group with normal heart function exhibited the highest signals among all groups. For phosphatidylserine, the long-term group showed higher levels than the short-term group. In summary, these results confirm the successful isolation of EVs from patients undergoing peritoneal dialysis, demonstrating a corresponding size distribution and expression of relevant markers.

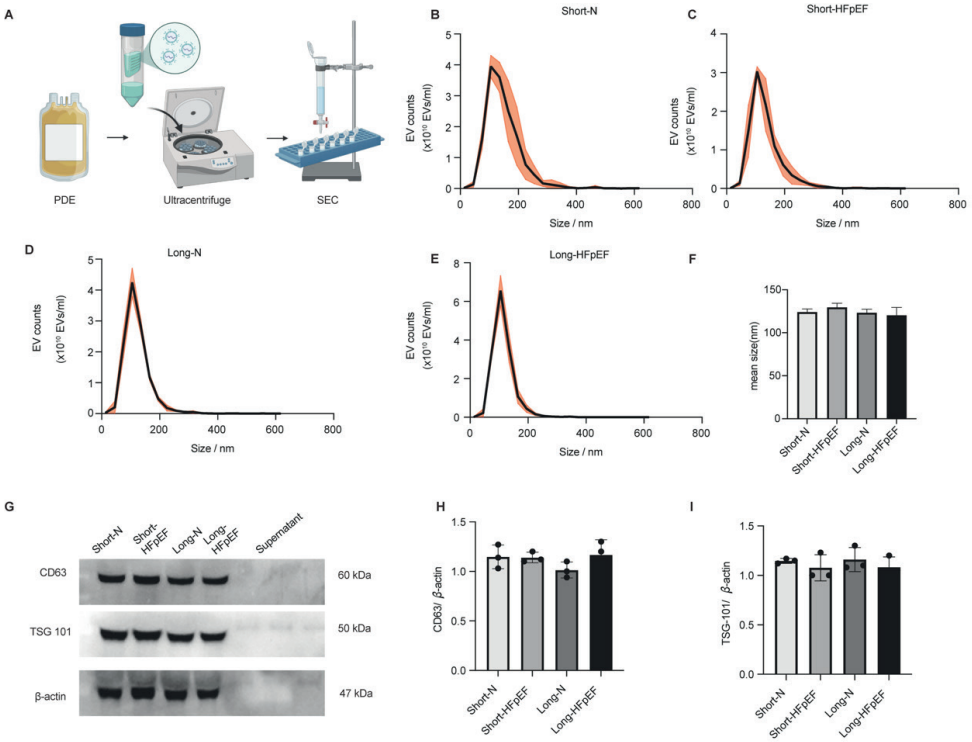


Figure 1. Isolation and characterization of PDE-derived EVs.

EVs were isolated from PDE of CKD patients classified by dialysis duration (short-term vs. long-term) and cardiac function (with or without HFpEF), using ultrafiltration followed by size-exclusion chromatography, in accordance with the MISEV2018 guidelines.

(A) Schematic representation of EV isolation and purification from PDE. Created in BioRender.

(B-E) Nanoparticle tracking analysis (NTA) demonstrates a typical EV size distribution.

(F) Quantification of average particle size. (G-I) Western blot analysis of CD63 and TSG101 expression in PDE-derived EVs from all four groups and corresponding supernatant fractions, along with β-actin as loading control (30 µg total protein per lane). Supernatant fractions showed minimal signal. Data are presented as mean ± SD (n = 3).

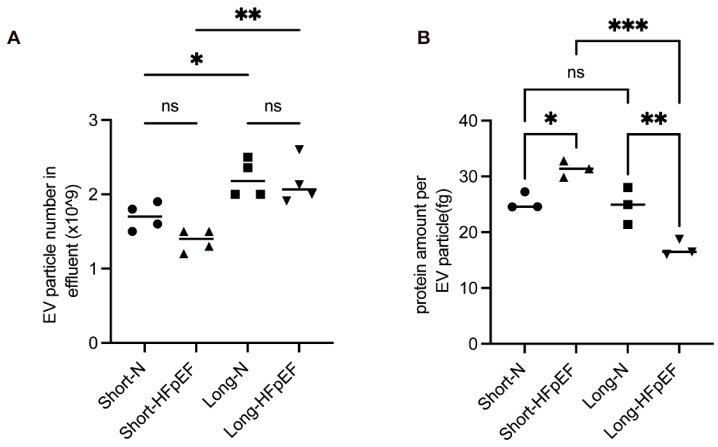


Figure 2. Quantification of PDE-derived EVs yield and protein content.

(A) Particle concentration in PDE (particles/mL) measured by NTA. (B) Protein content per particle (fg/particle), calculated by dividing total protein amount by particle number (10^9 particles per sample). Data are presented as mean ± SD (n = 3-4). *P < 0.05; **P < 0.01; ***P < 0.001; ns: not significant.

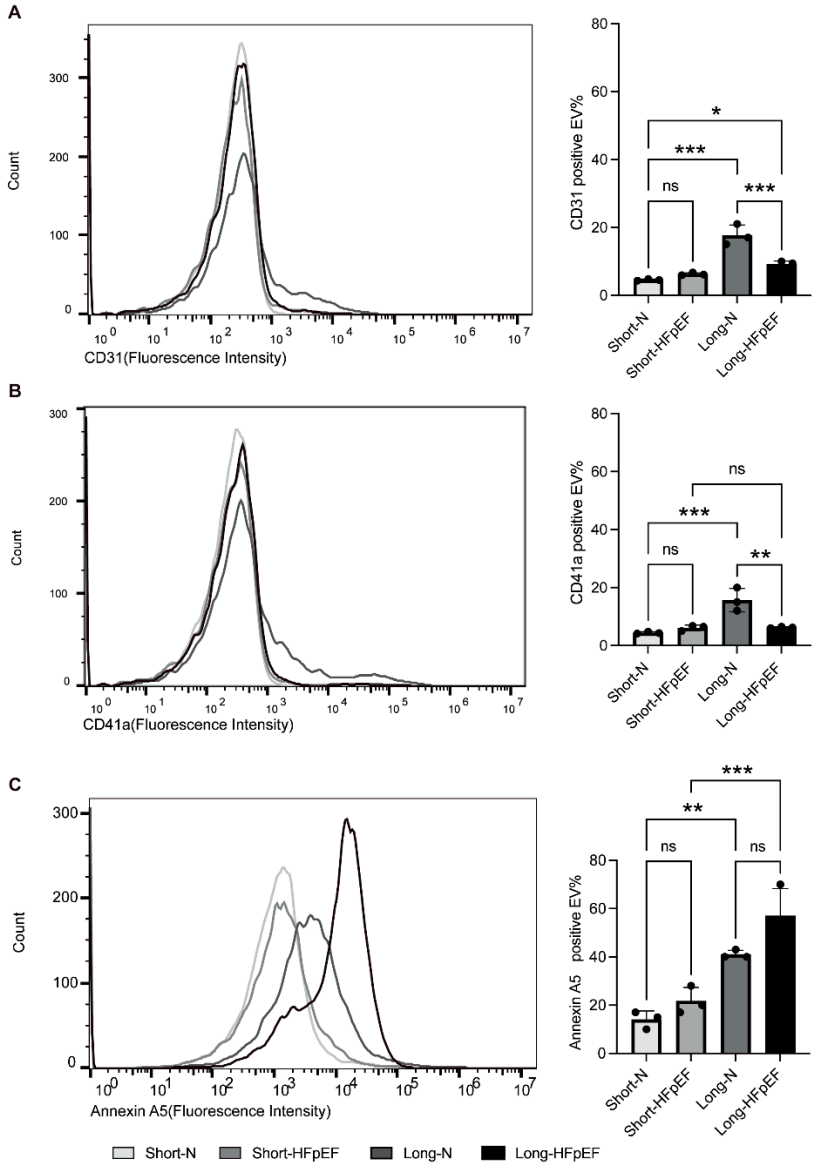


Figure 3. Surface marker expression of PDE-derived EVs analyzed by flow cytometry.

Representative overlaid histograms of CD31 (A), CD41a (B), and Annexin A5 (C) expression on EVs from different groups. Quantification of the percentage of marker positive EVs. Positive events were defined based on isotype controls. Data are presented as mean ± SD (n = 3). *P < 0.05; **P < 0.01; ***P < 0.001; ns: not significant.

3.2. Cellular uptake of fluorescent PDE-derived EVs

To determine whether PDE-derived EVs are internalized by HUVECs, flow cytometry and confocal microscopy were performed. EVs from all four patients' groups were efficiently internalized and exhibited a punctate intracellular distribution, predominantly localized in the perinuclear region (Fig. 4C). Quantitative analysis revealed no significant differences between the four groups in the percentage of EV-positive cells or puncta number per cell assessed by microscopy (Figure 4D-E), nor in mean fluorescence intensity measured by flow cytometry (Figure 4A-B). Z-stack confocal imaging further confirmed intracellular localization of EVs (Supplementary Figure S1A–D). To explore the mechanism of EV internalization, EV uptake was assessed under low-temperature conditions (4 °C) and in the presence of the dynamin inhibitor Dynasore (80 µM). EV uptake was markedly reduced at 4 °C, indicating that internalization is an energy-dependent process. In contrast, treatment with the dynamin inhibitor Dynasore did not significantly reduce EV uptake, suggesting that internalization may not depend on dynamin-mediated endocytosis (Figure 4F).

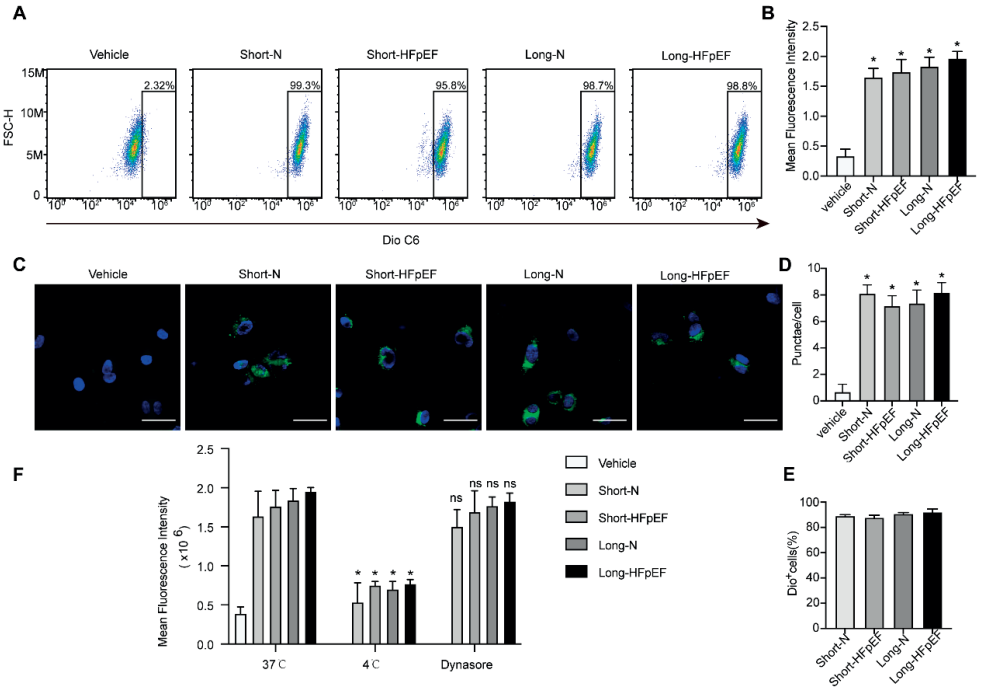


Figure 4. Cellular uptake of PDE-derived EVs labeled with DiO-C6.

PDE-derived EVs from four patient groups were labeled with DiO-C6 and incubated with HUVECs. (A) Flow cytometric analysis of DiO-C6–labeled EV uptake after overnight incubation. (B) Quantification of flow cytometry mean fluorescence intensity (FU). (C) Representative confocal images of punctate intracellular staining. (D) Punctae quantified per cell using ImageJ; ~60 cells analyzed per condition. (E) Percentage of DiO-C6–positive cells quantified; ~60 cells analyzed per condition. (F) EV uptake quantified by flow cytometry at different temperatures or in the presence of Dynasore. Data are presented as mean ± SD (n = 3). Data are presented as mean ± SD (n = 3). *P < 0.05; ns: not significant. Scale bar = 75 μm.

3.3. PDE-derived EVs promote inflammation in HUVECs

To investigate the effects of PDE-derived EVs on endothelial cell inflammation, we assessed the expression of ICAM-1 at three levels: membrane level by flow cytometry, total cellular level by Western blotting, and in culture supernatants by ELISA. We also measured the secretion levels of IL-6 using ELISA. In all experiments, the positive control group (IL-1β) consistently showed the highest expression of ICAM-1. Flow cytometric analysis demonstrated that all EV groups exhibited significantly increased ICAM-1 expression compared with vehicle group. Notably, EVs derived from long-term PD patients with HFpEF displayed significantly higher expression than the other EV groups, indicating that EVs from this patient had the strongest effect (Figure 5A). Consistent with these findings, the total cellular expression of ICAM-1 assessed by Western blotting was also highest in the long-HFpEF group (Supplementary Figure S1A).

Additionally, the analysis of secreted ICAM-1 levels showed significant increases in both the short-normal and long-HFpEF groups compared to vehicle controls. Importantly, secretion levels in the short-normal group were significantly higher than those in the short-HFpEF group (Figure 5B). Soluble IL-6 levels analysis also confirmed a significant increase in all EV groups compared to the vehicle group, with the long-HFpEF group showing significantly higher levels than the long-normal group (Figure 5C). Furthermore, measurement of plasma ICAM-1 and IL-6 levels indicated a trend consistent with the secretion pattern observed in HUVECs, showing elevated levels in both the short-normal and long-HFpEF patient groups (Figure 6A,B).

Moreover, we test the effects of PDE-derived EVs on the adhesion of monocytic cells to HUVEC under flow conditions. Firm adhesion of monocytic cells to HUVECs was significantly increased with all types of EV treatment when compared to the vehicle group. Remarkably, the Long-HFpEF group exhibited the highest adhesion effect among all EV groups (Figure 5D). To confirm that the observed effects were attributed to EVs, we performed an endotoxin test. The result shown the endotoxin levels of EVs from all four patients less than 0.02 EU/5x10^7 particles (Supplementary Figure S1B).

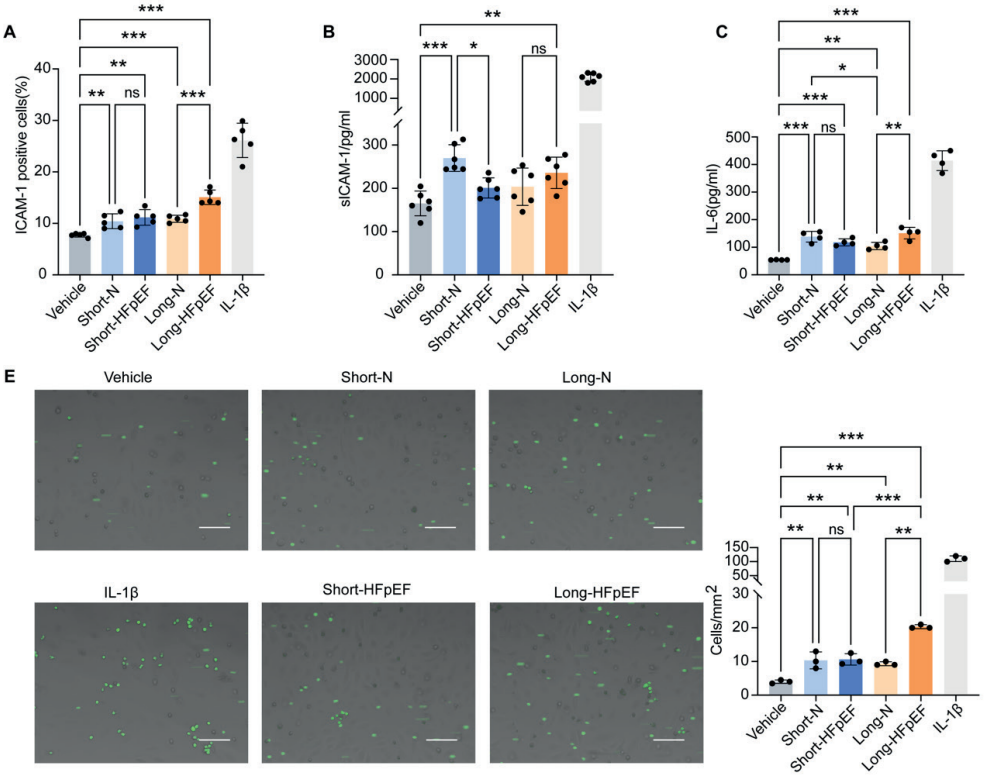


Figure 5. PDE-derived EVs promote inflammatory activation in HUVECs.

(A) Surface ICAM-1 expression on HUVECs measured by flow cytometry. (B, C) Levels of soluble ICAM-1 and IL-6 in HUVEC culture supernatants after EV treatment, determined by ELISA. (D) Representative images of THP-1 cells adherent to EV-stimulated HUVECs. (E) Quantification of THP-1 cell adhesion to HUVECs. Data are presented as mean ± SD (n = 3-6). *P < 0.05; **P < 0.01; ***P < 0.001; ns, not significant. Scale bar = 100 μm.

3.4. PDE-derived EVs promote HUVEC apoptosis

Flow cytometric analysis indicated that all EV groups significantly increased HUVEC apoptosis. Notably, the long-normal group showed a significantly higher rate of apoptosis compared to the long-HFpEF group. Additionally, raptinal-stimulated HUVEC demonstrated greatest pro-apoptosis effect among all EV groups (Figure 7A-B).

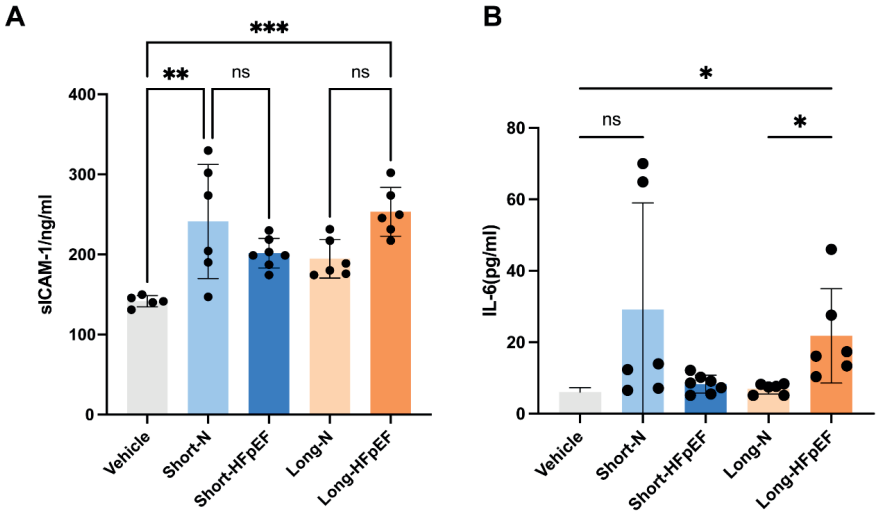


Figure 6. Circulating inflammatory marker levels in plasma of PD patients.

(A) Plasma levels of sICAM-1 measured by ELISA. (B) Plasma levels of IL-6 measured by ELISA. Data are presented as mean ± SD (n = 6). *P < 0.05; **P < 0.01; ***P < 0.001; ns, not significant.

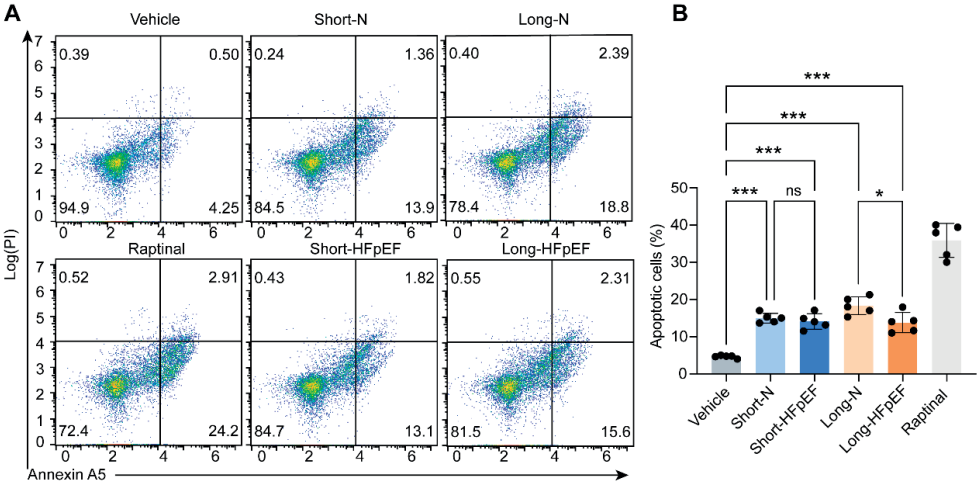


Figure 7. PDE-derived EVs promote apoptosis in HUVECs.

(A) Flow cytometric analysis of Annexin A5 and PI staining in HUVEC after EV treatment. (B) Quantification of total apoptotic cells, calculated as the sum of early (Annexin $A5^+/PI^-$) and late (Annexin $A5^+/PI^+$) apoptotic populations. Data are presented as mean ± SD (n = 5). *P < 0.05; ***P < 0.001; ns, not significant.

4 Discussion

Peritoneal dialysis is associated with inflammation and a high incidence of cardiovascular diseases, particularly heart failure, which remains a leading cause of mortality in this population[6,7,28]. In addition to classical modes of intercellular communication, EVs have emerged as important mediators of inflammation and vascular dysfunction[29-31].

In the present study, we demonstrate that PDE-EVs are internalized by endothelial cells and promote a pro-inflammatory phenotype, as evidenced by increased ICAM-1 expression and IL-6 secretion. Importantly, the effects of PDE-EVs differ according to dialysis duration and cardiac function of the donor patients. EVs from patients with long-term PD and HFpEF induced the strongest inflammatory responses, whereas EVs from long-term PD patients without HFpEF exhibited the most pronounced pro-apoptotic effects. These findings suggest that the functional properties of PDE-EVs are shaped by the clinical status of the donor and highlight their biological heterogeneity.

The diverse roles of PDE-EVs may be related to the differences in their molecular composition. Increasing evidence suggests that EVs isolated from peritoneal dialysis fluid contain various bioactive molecules associated with inflammation, fibrosis, and vascular stress, reflecting the biological state of the peritoneal environment. For example, EV-associated proteins like Glycoprotein 96 have been shown to activate the Toll-like receptor signaling pathway and its downstream NF-κB pathway, promoting inflammatory responses[32,33]. Additionally, EVs enriched with integrin-linked kinases (ILK) can activate the p38 MAPK signaling pathway, linking the composition of EVs to stress-related and fibrotic pathways[23]. Beyond protein-mediated signaling, EVs can also transfer regulatory RNAs that modulate key pathways such as Smad, ERK, and Wnt/β-catenin[34,35], which in turn can affect the phenotype and function of endothelial cells. These mechanisms may collectively contribute to the increased inflammatory and apoptotic responses observed in our study.

Our results further show that PDE-EVs can trigger inflammatory activation in endothelial cells. This is evidenced by increased release of ICAM-1 and elevated levels of IL-6, a well-known inflammatory marker. IL-6 is commonly used to gauge the inflammatory burden in peritoneal dialysis patients and is associated with negative clinical outcomes[36,37]. Similarly, high levels of ICAM-1 are associated with peritoneal injury and peritonitis[38]. Previous studies have shown that EVs derived from platelets and monocytes can modulate endothelial behavior and promote endothelial inflammatory responses through their bioactive cargo[39]. In addition, EV characteristics appear to differ depending on the underlying cardiovascular disease state, suggesting that EV composition may reflect disease-associated inflammatory processes[39,40]. Together, these findings support the idea that PDE-EVs can play a role in mediating endothelial inflammatory responses.

From a clinical standpoint, PDE-EVs may not only have the potential to serve as biomarkers that reflect the condition of the peritoneum and blood vessels but may also play an active role in regulating endothelial function. Unlike previous studies that primarily focused on circulating or urinary EVs[31,41,42], EVs derived from peritoneal dialysis fluid provide a more localized research perspective and readily available source. This study, which incorporates clinical stratification, has revealed differences in EV function among various patient groups, offering new insights into the mechanisms underlying cardiovascular complications related to peritoneal dialysis. However, this study has some limitations. First, the relatively small sample size may limit the generalizability of the results, necessitating validation in larger cohorts. Second, while we have confirmed functional differences, the specific molecular mechanisms underlying these differences have yet to be fully understood. Future research should combine proteomics and transcriptomics analyses with in vivo validation to further clarify the role of PDE-EVs in cardiovascular disease related to PD.

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Supplementary data / methods

Study design

To investigate the functional endothelial impact of peritoneal dialysis effluent (PDE)-derived EVs in the context of peritoneal dialysis and cardiac function, we stratified our cohort into four patient groups based on two clinical parameters: cardiac function (normal versus impaired, as assessed by left ventricular global longitudinal strain (LV-GLS) measured with echocardiography) and PD vintage (long-term vs. short-term). This yields the following patient groups: (1) normal cardiac function and short dialysis vintage (normal/short); (2) normal cardiac function and long dialysis vintage (normal/long); (3) impaired cardiac function and short dialysis vintage (impaired/short); and (4) impaired cardiac function and short dialysis vintage (impaired/short). Per patient group, PDE-samples were pooled to ensure sufficient EV yield for functional assays. In parallel, systemic inflammatory markers were measured in individual patient plasma samples, to evaluate whether systemic inflammation correlates with cardiac function and functional endothelial impact of PDE-EVs.

Study population

We used baseline clinical data, echocardiography images, plasma samples and PDE-samples of the echocardiography sub-study of the Renal Lifecycle Trial (acronym: STOP-HF-in-PD). The Renal Lifecycle Trial (NCT05374291; approved by the Medical Ethics Committee of the University Medical Center of Groningen on 3-4-2025, approval number METc2021/232), is a placebo-controlled randomized trial comparing impact of dapagliflozin versus placebo on cardiorenal outcomes in three patient-groups: 1) patients with kidney failure patients, including PD-treated patients; 2) patients with advanced CKD; and 3) kidney transplant patients.[43] Patient samples for the current study were included at six Dutch participating dialysis centers of STOPHFinPD between July 2023 and December 2024. In- and exclusion criteria of this trial are listed in Table 1. Specifically for this study, we additionally excluded patients with a recent peritonitis in the past six weeks. All baseline data were collected before prescription of the trial drug.

Table 1. STOP-HF-in-PD (echocardiography sub-study Renal Lifecycle Trial)[a] (cohort 1 and 2)

Inclusion criteria
- On peritoneal dialysis for kidney failure for ≥3 months
- ≥18 years
Exclusion criteria
• Mentally incapacitated and not able to give informed consent
• Diabetes mellitus type 1
• Currently using a sodium-glucose co-transporter 2 inhibitor
• ≥2 urinary or genital infections in the past 6 months
• Life expectancy of <6 months
• Scheduled kidney transplantation within 6 months
• Received systemic immunosuppression or intensification of a systemic immunosuppression treatment for a renal indication in the past 6 months
• Active malignancy (treated squamous cell or basal cell carcinoma of the skin excluded)
• Severe hypersensitivity or hepatic impairment Child-Pugh class C
• Known with severe non-adherence to medical regimens or unwillingness to comply with the trial protocol
• Pregnancy or breastfeeding
• An organ transplant besides a kidney transplant
• Severe lactose intolerance
• PD-related peritonitis in the past 6 weeks[b]

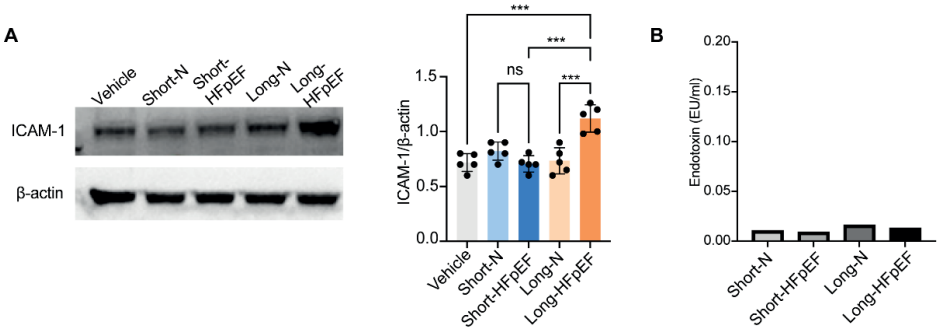
[a]Adapted from Bakker *et al.[43]* [b]Exclusion criterium specific for this study.

Data collection

During the baseline study visit we collected demographic data, biometrics, and clinical information regarding co-morbidities, as well as PD-related data on residual diuresis, PD vintage, modality and schedule and available recent (<6 months) peritoneal equilibrium tests and Kt/V measurements.

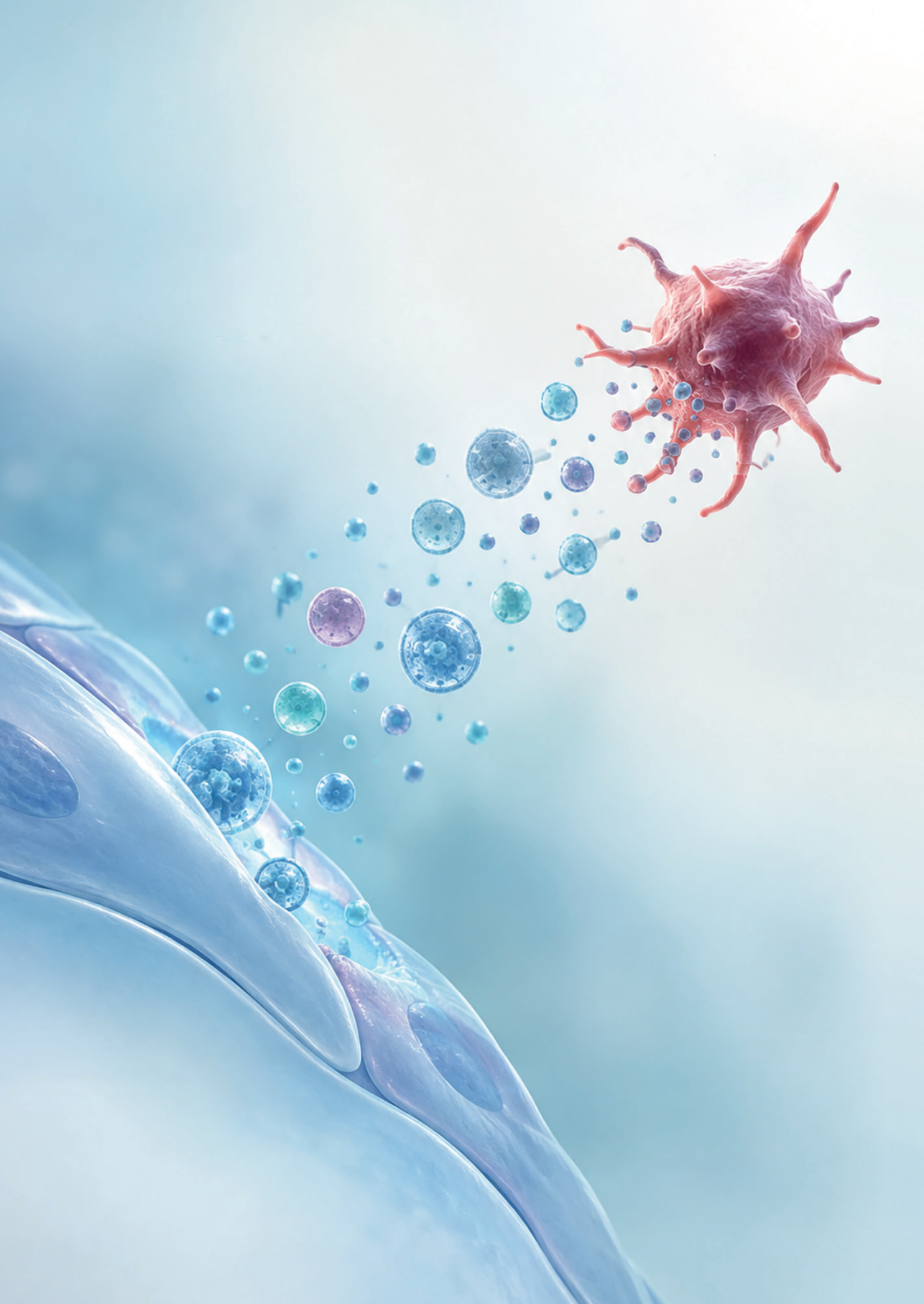
Transthoracic echocardiography

We performed a transthoracic echocardiography using site-specific equipment in accordance with a standardized study protocol, as described elsewhere (ref). We measured LV-GLS with speckle-tracking echocardiography. Analysis of the images was performed centrally at the Amsterdam UMC by a single operator.



Supplementary Figure S1 Additional characterization of endothelial ICAM-1 expression after EVs treatment and endotoxin levels in PDE-EV preparations.

(A) Representative Western blot and quantification of total ICAM-1 protein expression in endothelial cells following treatment with PDE-EVs. (B) Endotoxin assessment of PDE-EV preparations. Data are presented as mean ± SD (n = 3,5). *P < 0.05; ***P < 0.001; ns, not significant.



Chapter 6

General discussion

Platelet-derived extracellular vesicles

As described in Chapter 2, platelet-derived extracellular vesicles (P-EVs) play important roles in hemostasis, inflammation regulation, vascular repair, and immune modulation[1-3]. Furthermore, due to their ability to target damaged blood vessels[4,5] and the favorable safety profile of autologous blood preparation, P-EVs have attracted widespread interest as potential drug carriers. Consequently, P-EVs have become an important focus of research in the field of extracellular vesicles (EV). However, P-EV research faces numerous methodological challenges. Platelets are highly sensitive to external stimuli, which means that their isolation, storage, and processing can greatly influence the formation and properties of EVs[6,7]. In addition, the current EV research field lacks unified standards in vesicle isolation, characterization, fluorescent labeling, and functional experiments[8,9], which can affect the reproducibility and comparability of different studies.

In **Chapter 3**, we systematically evaluated several key methodological issues in P-EV research. First, we found that lyophilized platelets can serve as a stable and reproducible source of P-EV analogues. Compared to fresh platelets, P-EV analogues derived from lyophilized platelets showed high consistency in particle size, morphology, expression of platelet and EV markers, and cellular uptake behavior. Furthermore, lyophilized platelets still released EVs with similar characteristics after stimulation with calcium (Ca^{2+}) ionophores, suggesting that they retained their ability to induce vesicle formation.

In addition, we systematically compared various fluorescent labeling strategies. Fluorescent labeling of EVs has long presented a technical challenge in EV research, as fluorescent dyes can cause dye aggregation, non-specific signals, and changes in EV particle size[10,11]. Our findings indicate that different fluorescent dyes have different effect on the physical properties of P-EV analogues. Notably, 5(6)-Carboxyfluorescein diacetate N-succinimidyl ester (CFSE) stands out for maintaining a strong fluorescence signal while having a relatively minor impact on EV particle size, making it more suitable for subsequent studies on EV uptake.

Notably, we further analyzed its uptake and intracellular transport behavior in endothelial cells. The results showed that P-EV analogues are actively taken up by endothelial cells, and the internalization process was time-dependent, with the internalization rate at 37 °C being significantly higher than at 4 °C, indicating that this process is active, energy-dependent. Additionally, blocking integrin β3 did not significantly inhibit P-EV analogue uptake, suggesting that endothelial cell receptors are likely not involved in this process[12,13].

Our further investigation revealed that internalized P-EV analogues could partially enter the lysosomal pathway, exhibiting a significant time- and dose-dependent effect. As the exposure time and dose of EVs increased, the co-localization ratio of P-EV analogues with lysosomes also

gradually increased, consistent with previous reports[14]. This suggests that the intracellular fate of EVs is dynamically regulated after cellular uptake. Notably, even at relatively low concentrations of EVs that are closer to physiological conditions, some degree of lysosomal co-localization was still observed, suggesting that low-dose EVs may also have biological significance.

Additionally, P-EV analogues derived from healthy donors in this study did not induce significant endothelial inflammatory activation. This indicates that neither lyophilization nor sonication impart notable pro-inflammatory properties to P-EV analogues. However, P-EV cargo can change significantly under pathological conditions, therefore further research is needed to determine whether P-EVs derived from cardiovascular disease patients have different biological effects.

Functional heterogeneity of extracellular vesicles

Numerous research indicates that EVs exhibit significant functional heterogeneity[15,16]. In Chapter 4, we systematically compared EVs derived from various vascular and immune-related cells, including endothelial cells, monocytes, neutrophils, and platelets, under both resting and inflammatory conditions, and analyzed their effects on endothelial cells. The results showed significant differences among different EV populations in terms of their inflammatory cargo composition and functional effects. Inflammatory monocyte-derived EVs exhibited the highest expression levels of most detected inflammatory factors, which aligned with their stronger ability to induce inflammation in endothelial cells. Conversely, neutrophil- and platelet-derived EVs showed relatively lower expression levels of these inflammatory factors. Notably, neutrophil-derived EVs exhibited similar inflammatory factor profiles before and after inflammatory stimulation, however, the ability of inflammatory neutrophil-derived EVs to induce endothelial inflammation was significantly higher than that of the resting EV group. This suggests that the functional effects of EVs may not be entirely determined by the detected inflammatory factors alone but may involve other cargo components or membrane-related signaling mechanisms. Furthermore, we observed that inflammatory monocyte-derived EVs and endothelial cell-derived EV populations retained certain levels of anti-inflammatory factors, such as interleukin 4 (IL-4) and interleukin 10 (IL-10), suggesting that EV-mediated inflammatory signaling may simultaneously include pro-inflammatory and negative regulatory components.

This study further demonstrates that EVs from different cell types do not exert entirely the same regulatory effects on different stages of endothelial inflammation. EVs derived from inflammatory monocytes enhance intercellular adhesion molecule 1 (ICAM-1) expression, monocyte chemoattractant protein-1 (CCL2 / MCP-1) release, and monocyte adhesion and migration, while EVs from neutrophils and endothelial cells primarily promote adhesion between leukocytes and endothelial cells, with relatively limited impact on transendothelial migration.

This suggests that different EV populations may participate in different stages of leukocyte recruitment, rather than acting through identical mechanisms. Furthermore, Vasina found that the interaction between P-EV and monocytes triggers a significant response in calcium signaling, which is clearly dependent on the state of the vesicles. EVs from different sources or in various activation states demonstrate differing abilities to induce Ca^{2+} changes[26]. These fluctuations in Ca^{2+} not only reflect the early interactions between the EVs and recipient cells but may also influence subsequent inflammatory responses by modulating Ca^{2+}-dependent signaling pathways, such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB)[17], these findings align with our observations and further support the idea that Ca^{2+} signaling could act as an early event in the alterations of endothelial cell function induced by EVs. However, how EVs trigger Ca^{2+} signaling and its downstream regulatory mechanisms remain not fully understood and require further investigation. In addition to acute inflammatory activation, we found long-term exposure to inflammatory EVs can lead to increased endothelial cell apoptosis and decreased mitochondrial membrane potential, suggesting that EVs may further participate in the progression of chronic vascular injury and endothelial dysfunction.

The findings of this study reinforce the idea that EVs display considerable functional diversity and indicate that EV-mediated endothelial responses are highly dependent on their cellular origin and inflammatory state. In the next section, we will explore the potential impact of disease-related EV heterogeneity on chronic inflammatory cardiovascular complications.

Disease-associated heterogeneity of EVs in chronic inflammatory conditions

In Chapter 5, we further explored the potential role of disease-associated EV heterogeneity in chronic inflammatory cardiovascular complications. Patients undergoing peritoneal dialysis are in a state of chronic inflammation and are at a high risk of developing cardiovascular diseases, particularly heart failure with preserved ejection fraction (HFpEF)[18-20]. We found that peritoneal dialysis effluent-derived EVs (PDE-EVs) can be taken up by endothelial cells and induce pro-inflammatory phenotypes, including increased ICAM-1 expression and elevated IL-6 secretion. Additionally, EVs from different patient sources exhibited significantly different functional effects. EVs from long-term peritoneal dialysis patients with HFpEF induced the most significant endothelial inflammatory response, while EVs from long-term peritoneal dialysis patients without HFpEF showed a more pronounced pro-apoptotic effect.

Notably, there was no significant difference in EV uptake levels in endothelial cells among the different groups, suggesting that the functional differences observed among various EV populations are more likely related to variations in their cargo composition than differences in uptake efficiency. Previous studies have shown that the possible origins of PDE-EV include

peritoneal mesothelial cells, infiltrating immune cells, endothelial cells, platelets, and circulating EVs entering through vascular permeability or the systemic circulation[27,28], and these EVs can carry various inflammation-related proteins[21], fibrosis-related factors[22], and participate in the regulation of signaling pathways such as NF-κB, extracellular regulated protein kinases (ERK), and sma and mad-related proteins (Smad)[23-25]. The results of this study further support the idea that the disease microenvironment can shape the functional properties of EVs. Long-term inflammation, metabolic abnormalities, and cardiovascular complications may collectively alter the biological composition of EVs, thereby enhancing their inflammatory regulatory effects on endothelial cells. Compared to Chapter 4, this chapter further illustrates that EV heterogeneity not only originates from different cell types but is also dynamically influenced by disease progression and the pathological environment. From a clinical perspective, these results suggest that EVs may not only serve as biomarkers reflecting disease states but may also directly participate in the process of chronic inflammation-related vascular injury.

Conclusion and future perspectives

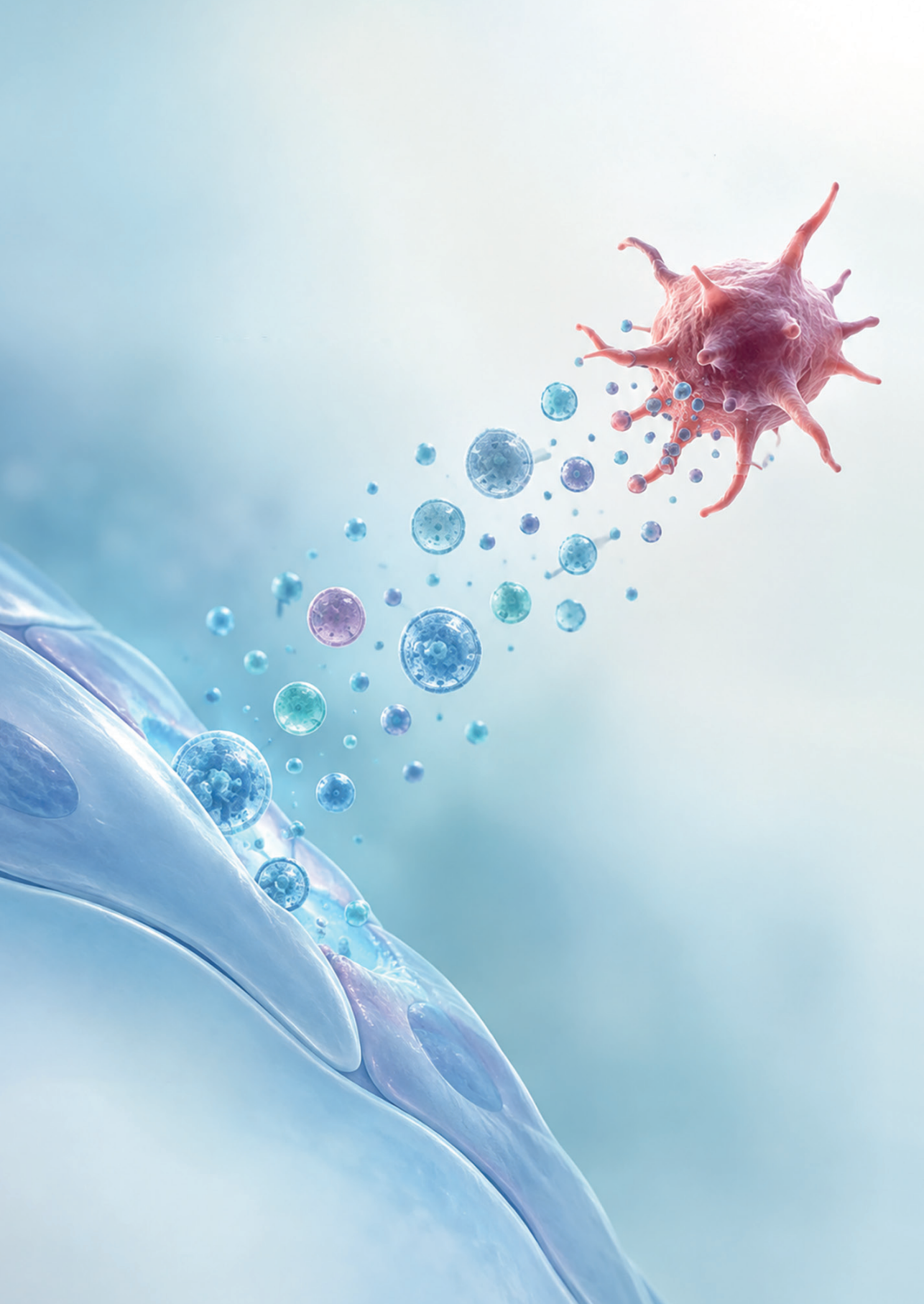
This thesis systematically investigates the role of EVs in endothelial inflammation, examining EV functional heterogeneity, EV-mediated endothelial inflammatory responses, and methodological aspects of P-EV research. Results show that different EV populations can induce varying degrees of endothelial inflammation, leukocyte recruitment, and endothelial damage. Specifically, it was found that EVs derived from inflammatory monocytes and PDE-EVs from patients undergoing long-term peritoneal dialysis with HFpEF exhibited more pronounced pro-inflammatory effects, suggesting that pathological states may influence EV cargo composition and biological function. Furthermore, this thesis systematically evaluates key methodological issues in P-EV research, including P-EV analogue generation, fluorescent labeling, and intracellular transport analysis, providing experimental evidence for improving the standardization and reproducibility of P-EV research.

Future research requires further analysis of the composition and function of different EV subsets, combining proteomics, transcriptomics, single-particle analysis, and in vivo disease models, to clarify their roles in chronic inflammation-related cardiovascular diseases. With the continuous improvement of EV research methods and standardization systems, EVs are expected to become new biomarkers and potential therapeutic targets for cardiovascular diseases. In particular, P-EVs as a naturally biocompatible EV population, may show potential application value in drug delivery and precision medicine.

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Chapter 7

Summary | Samenvatting | 总结

Summary

In this thesis, I focus on the role of extracellular vesicles (EVs) in endothelial and vascular inflammation and explores the functional heterogeneity of EVs and the methodological challenges associated with research on platelet-derived EV. Chapter 1 outlines the important roles of cardiovascular disease and endothelial inflammation in vascular lesions and reviews the research progress on EVs as mediators of intercellular communication in the context of vascular inflammation. Furthermore, this chapter summarizes EV heterogeneity, the status of platelet derived extracellular vesicles (P-EV) research, and the methodological challenges faced in EV research, and proposes the research objectives and overall framework of this thesis.

Chapter 2 reviews the role of P-EV in various cardiovascular diseases (such as atherosclerosis, myocardial infarction, ischemia-reperfusion injury, and heart failure), with relation to its molecular cargo (nucleic acids, bioactive lipids, proteins) and aims to provide new insights in the pathophysiologic role of P-EV, and methods for preventing and treating cardiovascular diseases based on P-EV.

To establish a stable and reproducible P-EV research system, **Chapter 3** systematically evaluated the feasibility of using lyophilized platelets as a source of P-EVs and compared different EV fluorescent labeling methods. Additionally, this chapter analyzed the uptake and intracellular transport behavior of P-EV analogues in endothelial cells. The results showed that lyophilized platelets can generate P-EV analogues with similar characteristics and cellular uptake behavior to those of EVs derived from fresh platelets, and CFSE was found to be a reliable labeling method suitable for studies involving P-EV uptake.

In **Chapter 4**, we systematically compared the effects of EVs secreted by different vascular and immune-related cells under resting and inflammatory conditions on endothelial cell activation. Our results showed that EVs derived from different cell types and under different inflammatory states exhibit distinct inflammatory cytokine profiles and functional characteristics. Among them, EVs derived from inflammatory monocytes showed the strongest pro-inflammatory effects, leading to increased Ca^{2+} signaling, elevated levels of ICAM-1 expression, and enhanced leukocyte recruitment. These results further support the concept of EV functional heterogeneity.

In **Chapter 5**, we examined the effects of EV derived from peritoneal dialysis effluent (PDE-EVs) on endothelial inflammatory, considering different dialysis durations and the presence of heart failure with preserved ejection fraction (HFpEF). Our results showed that PDE-EVs were effectively internalized by endothelial cells, which resulted in a pro-inflammatory response. This was evidenced by an increase in ICAM-1 expression, elevated secretion of IL-6, and enhanced adhesion between monocytes and endothelial cells. We observed significant differences in these functional effects across different patient groups. Specifically, EVs from patients undergoing long-

term peritoneal dialysis and experiencing HFpEF triggered the strongest inflammatory response. Importantly, there were no significant differences in the uptake of EVs among the groups, suggesting that the functional disparities may arise from variations in the specific components carried by the EVs. In conclusion, these findings imply that PDE-EVs play a regulatory role in the inflammatory response of endothelial cells.

Samenvatting

Dit proefschrift onderzoekt primair de rol van extracellulaire vesikels (EVs) afkomstig uit bloedplaatjes bij endotheel- en vasculaire ontsteking, en verkent hun functionele heterogeniteit en de methodologische uitdagingen in het onderzoek naar EVs afkomstig uit bloedplaatjes en van andere cellen. **Hoofdstuk 1** schetst de belangrijke rol van hart- en vaatziekten en endotheelontsteking bij vasculaire beschadigingen, en bespreekt de stand van het wetenschappelijke onderzoek over EVs als intercellulaire communicatiemediatoren bij vasculaire ontsteking. Verder vat dit hoofdstuk de heterogeniteit van EVs samen, de huidige stand van het onderzoek naar van bloedplaatjes afgeleide extracellulaire vesikels (P-EVs), en de methodologische uitdagingen in het EV-onderzoek, en formuleert het de onderzoeksdoelstellingen en het algemene kader van dit proefschrift.

Hoofdstuk 2 bespreekt de rol van P-EVs bij verschillende hart- en vaatziekten (zoals atherosclerose, myocardinfarct, ischemie-reperfusieschade en hartfalen), en de relatie met hun moleculaire lading (nucleïnezuren, bioactieve lipiden en eiwitten). Het doel is om nieuwe inzichten te verschaffen in de pathofysiologische effecten van P-EVs en op P-EVs gebaseerde preventie- en behandelmethoden voor hart- en vaatziekten.

Om een stabiel en reproduceerbaar P-EV-onderzoekssysteem op te zetten, evalueert **hoofdstuk 3** systematisch de haalbaarheid van gevriesdroogde bloedplaatjes als bron van P-EV en vergelijkt verschillende methoden voor het fluorescent markeren van EVs. Verder analyseert dit hoofdstuk de opname en het intracellulaire transport van deze zogenaamde “P-EV-analogen” in endotheelcellen. De resultaten tonen aan dat men uit gelyofiliseerde bloedplaatjes P-EV-analogen kan genereren die vergelijkbare eigenschappen en cellulair opnamegedrag vertonen als direct van verse bloedplaatjes afgeleide EVs, en dat de veelgebruikte fluorescente kleurstof CFSE een betrouwbaar label is voor het markeren van P-EV voor opnamestudies in levende cellen.

In **hoofdstuk 4** hebben we systematisch de effecten vergeleken van EVs die worden afgescheiden door verschillende vasculaire en immuuncellen onder normale en ontstekingsomstandigheden op de activering van endotheelcellen. De resultaten tonen aan dat EVs afkomstig van verschillende bronnen en onder verschillende ontstekingscondities verschillende profielen van inflammatoire cytokinen en functionele kenmerken vertonen. EVs afkomstig van inflammatoire (met lipopolysaccharide-geactiveerde) monocyten vertoonden het meest significante pro-inflammatoire effect en induceerden een sterkere intracellulaire calcium-signalering, hogere ICAM-1-expressieniveaus en meer leukocytenrekrutering. Deze resultaten ondersteunen verder het standpunt van functionele heterogeniteit van EVs.

In **hoofdstuk 5** hebben we het effect onderzocht van EVs afkomstig uit peritoneale dialysevloeistof op de inflammatoire respons van endotheelcellen, rekening houdend met verschillende dialysetijden en de aanwezigheid van hartfalen met behouden ejectiefractie (HFpEF). De resultaten toonden aan dat PDE-EV's effectief kunnen worden opgenomen door endotheelcellen, wat leidt tot een pro-inflammatoir fenotype in endotheelcellen. Dit werd ook ondersteund door verhoogde ICAM-1-expressie, verhoogde IL-6-secretie, en verbeterde adhesie tussen monocyten en endotheelcellen. Er werden significante verschillen in functionele effecten waargenomen tussen de EVs afkomstig uit de dialysevloeistof van de verschillende patiëntengroepen. EVs afkomstig van patiënten die langdurig peritoneale dialyse ondergingen en tegelijkertijd HFpEF hadden, veroorzaakten de sterkste ontstekingsreactie. Opvallend was dat er geen significant verschil was in de opname van EVs tussen de groepen, wat suggereert dat de functionele verschillen mogelijk voortkomen uit variaties in de componenten die door de EVs worden vervoerd. Kortom, deze resultaten suggereren dat PDE-EVs een regulerende rol spelen bij ontstekingen in endotheelcellen en dat deze mogelijk eigenschappen hebben die met de duur en ernst van de ziekte zijn gerelateerd.

总结

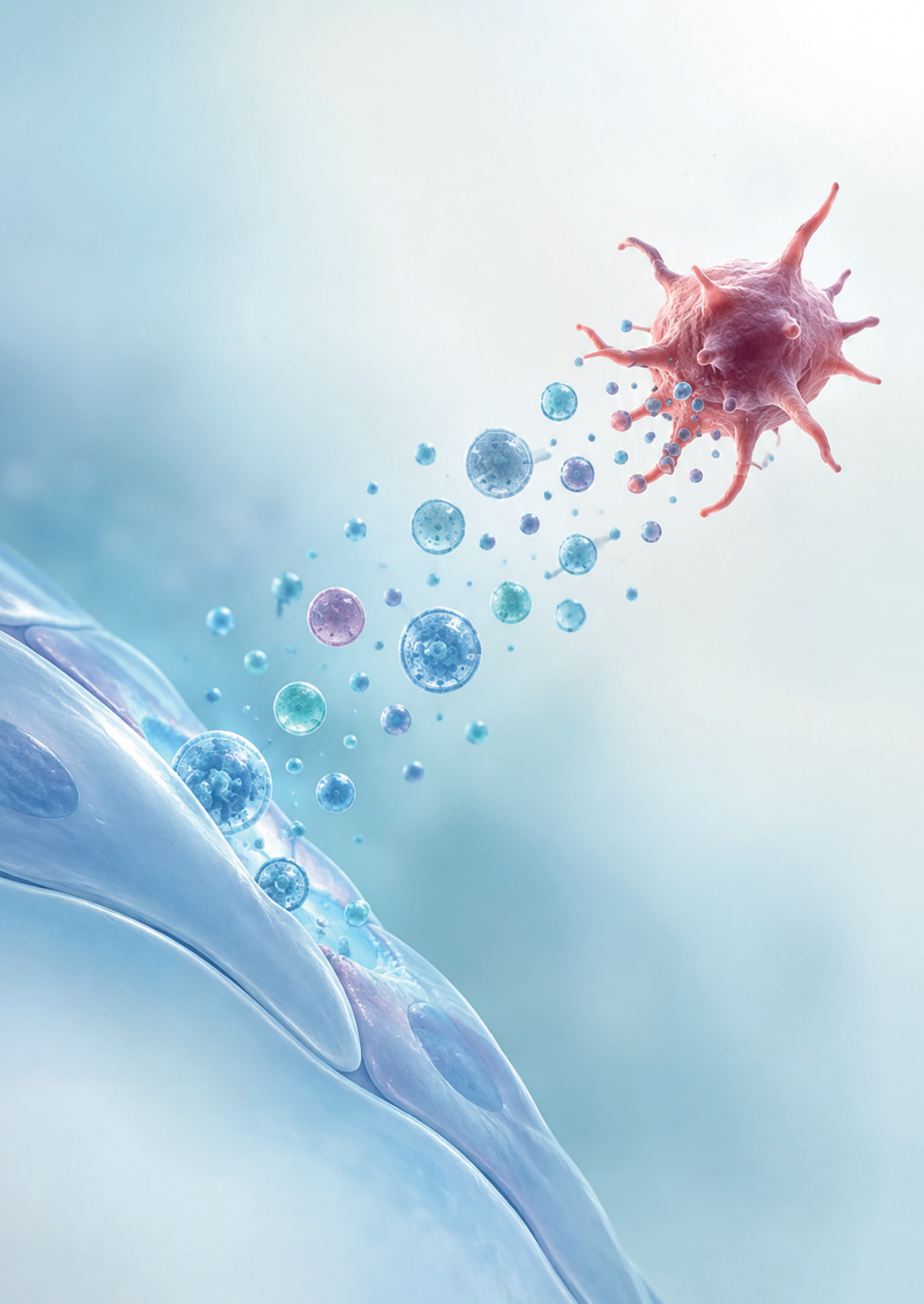
本论文主要研究EV在内皮炎症与血管炎症中的作用，并探讨EV功能异质性及血小板来源EV研究中的方法学问题。第一章概述了心血管疾病及内皮炎症在血管病变中的重要作用，并介绍了EV作为细胞间通讯介质在血管炎症中的研究进展。此外，本章还总结了EV异质性、P-EV研究现状以及EV研究中面临的方法学挑战，并提出了本论文的研究目的和总体框架。

第二章综述了血小板来源EV在各种心血管疾病（如动脉粥样硬化、心肌梗死、缺血再灌注损伤和心力衰竭）中的作用，以及其分子载荷（核酸、生物活性脂质、蛋白质）的关系，旨在为P-EV的病理生理作用以及基于P-EV的心血管疾病的预防和治疗方法提供新的见解。

为了建立稳定且可重复的P-EV研究体系，在第三章中，我们系统评估了冻干血小板作为P-EV来源的可行性，并比较了不同EV荧光标记方法。此外，本章进一步分析了P-EV类似物在内皮细胞中的摄取及胞内转运行为。研究结果表明，冻干血小板可生成与新鲜血小板来源EV具有相似特性和细胞摄取行为的P-EV类似物，而CFSE是一种适用于P-EV摄取研究的可靠标记方法。

在第四章中，我们系统比较了不同血管和免疫相关细胞在静息和炎症条件下分泌的EV对内皮细胞活化的影响。研究结果表明，不同来源及不同炎症状态下产生的EV具有不同的炎症因子谱和不同的功能特征。其中，炎症性单核细胞来源EV表现出最显著的促炎作用，并诱导更强的Ca^{2+}信号、ICAM-1表达水平和白细胞募集。这些结果进一步支持了EV功能异质性的观点。

在第五章中，我们研究了腹膜透析液来源EV对内皮细胞炎症反应的影响，并考虑了不同透析时间和HFpEF的存在。结果发现，PDE-EV能被内皮细胞有效内化，导致内皮细胞呈现促炎表型。ICAM-1表达增加、IL-6分泌增多以及单核细胞与内皮细胞黏附增强均证实了这一点。不同患者组的功能效应存在显著差异。长期接受腹膜透析治疗且合并HFpEF的患者来源的EV引发了最强的炎症反应。值得注意的是，各组间EV摄取量无显著差异，表明功能差异可能源于EV所携带成分的不同。总之，这些结果提示PDE-EV对内皮细胞具有炎症调节作用。



Chapter 8

Impact

Scientific impact

Extracellular vesicles (EVs) have recently been recognized as important carriers of intercellular communication, playing a significant role in inflammation, atherosclerosis, and cardiovascular diseases. However, the field of EV research faces two main challenges. First, the functional heterogeneity among different EV populations is not yet fully understood. Second, there is a lack of standardized methods for isolating, characterizing, and functionally analyzing EVs.

This thesis focuses on the role of EVs in vascular inflammation, demonstrating that EVs are not functionally homogeneous and that their biological effects are closely related to their cell origin, inflammatory state, and disease background. The study found that EVs from different cell types can induce varying degrees of endothelial inflammation, leukocyte recruitment, and cell damage. Notably, EVs derived from inflammatory monocytes and those from patients undergoing long-term peritoneal dialysis with concurrent heart failure with preserved ejection fraction (HFpEF) exhibited significantly stronger pro-inflammatory effects. These findings enhance our understanding of the functional heterogeneity of EVs and provide new experimental evidence regarding the mechanisms of EV-mediated vascular inflammation.

Methodological impact

In addition to functional studies, this thesis systematically evaluated several key methodological challenges in P-EV research, including P-EV analogue generation, EV isolation and purification, fluorescent labeling, and the analysis of cellular uptake and intracellular transport.

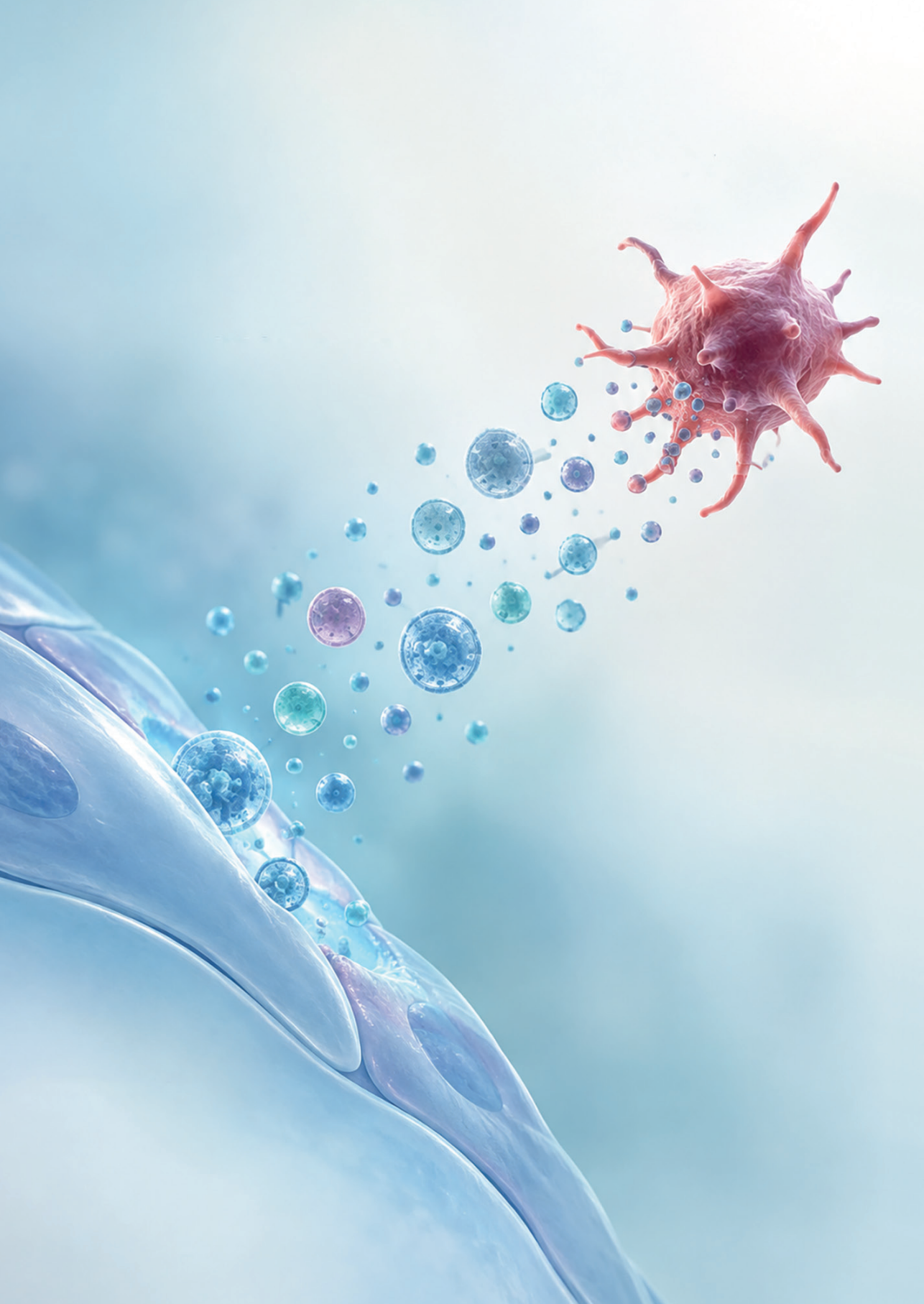
This study demonstrates that lyophilized platelets can serve as a stable and reproducible P-EV source, allowing for the generation of P-EV analogues that possess similar characteristics and cellular uptake behavior to fresh platelet-derived EVs. Additionally, this research compared various EV fluorescent labeling strategies and established a CFSE labeling method suitable for P-EV uptake studies. Furthermore, this study analyzed the time- and dose-dependent intracellular transport and lysosomal localization behavior of P-EV analogues in endothelial cells. These findings provide valuable experimental references for foundational P-EV-related research and contribute to the standardization, reproducibility, and comparability of different EV studies.

Translational and societal relevance

Cardiovascular disease is a leading cause of death worldwide, and chronic inflammation, particularly endothelial inflammation, plays a crucial role in its development and progression. EVs, which are commonly found in body fluids, can reflect the pathological conditions of cells

and tissues. As a result, they are gaining attention as potential biomarkers for diseases and therapeutic agents.

This study suggests that disease-state-related EVs may play a direct role in regulating vascular inflammation and can serve as indicators of various disease states. Notably, the relationship between peritoneal dialysis effluent-derived EV (PDE-EVs) and inflammatory responses related to HFpEF offers new insights into chronic inflammation and its cardiovascular complications. Additionally, due to their natural biocompatibility and ability to target damaged blood vessels, P-EVs may also be useful in drug delivery and precision medicine in the future.



Chapter 9

Curriculum vitae | Publications | Acknowledgements

Curriculum vitae

Xin Xin was born on August 1, 1992, in Liaoning Province, China. She obtained her bachelor's degree in clinical medicine from Shenyang Medical College in 2016. Following that, she pursued a master's degree in internal medicine at the Department of Cardiology at The First Hospital of China Medical University, where she studied under Prof. Dr. Dalin Jia, graduating in 2021. During her master's research, she focused on the molecular mechanisms underlying cardiovascular dysfunction associated with metabolic disorders, specifically exploring microRNA-mediated regulation of cardiomyocyte apoptosis and myocardial injury.

In January 2023, she started her Ph.D. research at the Department of Biochemistry, Cardiovascular Research Institute Maastricht (CARIM) at Maastricht University in the Netherlands. She is supervised by Prof. Dr. Rory Koenen, Prof. Dr. Ingrid Dijkgraaf, and Prof. Dr. Tilman M. Hackeng. Her doctoral research focuses on extracellular vesicles (EVs) and their potential roles in cardiovascular diseases. She investigates the biological functions of EVs derived from various cellular sources, particularly platelet-derived EVs, and their effects on endothelial inflammation and dysfunction. Additionally, she explores strategies for characterizing these vesicles and evaluating their potential applications in cardiovascular research.

Her research contributed to a better understanding of extracellular vesicle biology, particularly their involvement in endothelial inflammation and dysfunction in the context of cardiovascular diseases.

Conference

Netherlands Society for Extracellular Vesicles (NLSEV) 2023 **Rotterdam, the Netherlands.** **Oral presentation**	Optimization of platelet derived extracellular vesicles purification and labeling for therapeutic drug delivery
European Platelet Network (EUPLAN), 2025, **Würzburg, Germany.** **Poster presentation**	Differential Effects of Extracellular Vesicles from Distinct Cellular Origins on Endothelial Inflammation and Dysfunction.

Publication

1. **microRNA Expression Profiles in Myocardium of High-Fat Diet-Induced Obesity Rat**.
   Huimin Yang, **Xin Xin**, Hang Yu, Yandong Bao, Pengyu Jia, Nan Wu, Dalin Jia.
   Diabetes Metab Syndr Obes. 2020 Apr 15:13:1147-1159.

2. **miR-141-3p regulates saturated fatty acid-induced cardiomyocyte apoptosis through Notch1/PTEN/AKT pathway via targeting PSEN1.**
   Xin Xin, Lian Duan, Huimin Yang, Hang Yu, Yandong Bao, Dalin Jia, Nan Wu, Ying Qiao., Environ Toxicol. 2022 Apr;37(4):741-753

3. **Assessing platelet-derived extracellular vesicles for potential as therapeutic targets in cardiovascular diseases.**
   Xin Xin, Rory R Koenen.
   Expert Opin Ther Targets. 2025 Jan-Feb;29(1-2):17-28.

4. **Generation and characterization of platelet-derived extracellular vesicle analogues from lyophilized platelets.**
   Xin Xin, Ingrid Dijkgraaf, Tilman M. Hackeng, Rory R. Koenen.
   Extracellular Vesicle. Volume 6, December 2025, 100095

5. **Differential Effects of Extracellular Vesicles from Distinct Cellular Origins on Endothelial Inflammation and Dysfunction.**
   Xin Xin, Akhil A Konkoth, Amandine Bonifay, Corinne Chareyre, Evelyne Abdili, Sandra E. Ghayad, Lea Plantureux, Stéphane Robert, Corentin Franco, Tarik Bouriche, Sylvie Cointe, Philippe Poncelet, Françoise Dignat-George, Romaric Lacroix, Rory R. Koenen. Manuscript in preparation

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At that time, I was not confident about my English at all and felt extremely nervous during the interview, as I had very little experience speaking English before coming to the Netherlands. I still clearly remember you telling me that English is just a tool, and that communication and scientific thinking matter far more. Your kindness and encouragement gave me the courage to leave China and begin this journey in the Netherlands. Throughout these years, you have always been exactly the kind of supervisor you promised to be patient, supportive, approachable, and genuinely willing to help. Whenever I encountered difficulties, whether in experiments, writing, or daily life, you were always there with guidance and encouragement. Beyond your scientific knowledge and creativity, I deeply admire the way you treat people. You create an environment where everyone feels supported, respected, and motivated. Your enthusiasm for science, your ability to connect ideas and remember literature so effortlessly, and your rigorous scientific thinking have inspired me tremendously. I have learned so much from you, both scientifically and personally. I sincerely hope that in the future I can become someone as warm, reliable, and inspiring as you are. Thank you also for all the practical guidance in the laboratory, from extracellular vesicle isolation to NTA measurements and monocyte adhesion assays. I am also especially grateful for your tremendous dedication and guidance in academic writing. From published papers to manuscripts in preparation, to my graduation thesis and every presentation slide, you have provided meticulous revisions, suggestions, and feedback. Each revision process has not only improved my writing skills but also gradually taught me how to express scientific questions more clearly and rigorously. Your attention to detail, strict requirements for logic, and efficient work style have all benefited me immensely. I am deeply grateful for all the time, patience, and effort you invested in helping me grow over these years.

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