Microvesicles in vascular homeostasis and diseases

Position Paper of the European Society of Cardiology (ESC) Working Group on Atherosclerosis and Vascular Biology

Victoria C. Ridger[1*]; Chantal M. Boulanger[2,3*]; Anne Angelillo-Scherrer[4,5]; Lina Badimon[6,7]; Olivier Blanc-Brude[2,3]; Marie-Luce Bochaton-Piallat[8]; Eric Boilard[9]; Edit I. Buzas[10]; Andreas Caporali[11]; Francoise Dignat-George[12,13]; Paul C. Evans[1]; Romaric Lacroix[12,13]; Esther Lutgens[14,15]; Daniel F. J. Ketelhuth[16]; Rienk Nieuwland[17]; Florence Toti[18]; Jose Tuñon[19,20]; Christian Weber[15,21]; Imo E. Hoefer[22]

REVIEWERS: Gregory Y. H. Lip (review coordinator)[23]; Nikos Werner[24], Eduard Shantsila[23], Hugo ten Cate[25], Mark Thomas[23], Paul Harrison[26]

[1]Department of Infection, Immunity and Cardiovascular Disease, Faculty of Medicine, Dentistry and Health and the INSIGNEO Institute for In Silico Medicine, University of Sheffield, Sheffield, UK; [2]INSERM UMR-S 970, Paris Cardiovascular Research Center – PARCC, Paris, France; [3]Université Paris Descartes, Sorbonne Paris Cité, Paris, France; [4]Department of Hematology and Central Hematology Laboratory, Inselspital, Bern University Hospital, University of Bern, Bern, Switzerland; [5]Department of Clinical Research, University of Bern, Bern, Switzerland; [6]Cardiovascular Research Center (CSIC-ICCC), IIB-Sant Pau, Barcelona, Spain; [7]Cardiovascular Research Chair, UAB, Barcelona, Spain; [8]Department of Pathology and Immunology, Faculty of Medicine, University of Geneva, Geneva, Switzerland; [9]Centre de Recherche du CHU de Québec, Université Laval, Department of Infectious diseases and Immunity, Quebec City, Quebec, Canada; [10]Semmelweis University, Department of Genetics, Cell- and Immunobiology, Budapest, Hungary; [11]University/British Heart Foundation Centre for Cardiovascular Science, The Queen's Medical Research Institute, University of Edinburgh, Edinburgh, UK; [12]Aix-Marseille University, INSERM, VRCM, UMR-S1076, UFR de Pharmacie, Marseille, France; [13]Department of Hematology and Vascular Biology, CHU La Conception, APHM, Marseille, France; [14]Department of Medical Biochemistry, Academic Medical Center, University of Amsterdam, The Netherlands; [15]Institute for Cardiovascular Prevention, Ludwig-Maximilians-University, German Centre for Cardiovascular Research (DZHK), partner site Munich Heart Alliance, Munich, Germany; [16]Cardiovascular Medicine Unit, Center for Molecular Medicine, Department of Medicine, Karolinska Institute, Karolinska University Hospital, Stockholm, Sweden; [17]Laboratory of Experimental Clinical Chemistry, Vesicle Observation Center, Academic Medical Center, University of Amsterdam, Amsterdam, the Netherlands; [18]Faculty of Pharmacy, UMR CNRS 7213, University of Strasbourg, Strasbourg, France; [19]IIS-Fundación Jiménez Díaz, Madrid, Spain; [20]Autónoma University, Madrid, Spain; [21]Cardiovascular Research Institute Maastricht (CARIM), 6229 ER Maastricht, The Netherlands; [22]Laboratory of Clinical Chemistry and Hematology, UMC Utrecht, Netherlands; [23]Institute of Cardiovascular Sciences, University of Birmingham, Birmingham, UK; [24]Medizinische Klinik und Poliklinik II, Universitätsklinikum Bonn, Bonn, Germany; [25]UNS 50, University Medical Center, Maastricht, The Netherlands; [26]Institute of Inflammation and Ageing, College of Medical and Dental Sciences, University of Birmingham, Birmingham, UK

Summary

Microvesicles are members of the family of extracellular vesicles shed from the plasma membrane of activated or apoptotic cells. Microvesicles were initially characterised by their pro-coagulant activity and described as "microparticles". There is mounting evidence revealing a role for microvesicles in intercellular communication, with particular relevance to hemostasis and vascular biology. Coupled with this, the potential of microvesicles as meaningful biomarkers is under intense investigation. This Position Paper will summarise the current knowledge on the mechanisms of formation and composition of microvesicles of endothelial, platelet, red blood cell and leukocyte origin. This paper will also review and discuss the different methods used for their analysis and quantification, will underline the potential biological roles of these vesicles with respect to vascular homeostasis and thrombosis and define important themes for future research.

Keywords

Atherothrombosis, cell-cell interactions, inflammatory mediators, macrophage

Correspondence to:
Victoria Ridger, PhD
Department of Infection, Immunity and Cardiovascular Disease
Faculty of Medicine, Dentistry and Health
University of Sheffield, Sheffield, UK
E-mail: v.c.ridger@sheffield.ac.uk
or
Chantal M. Boulanger, PhD
INSERM UMR-S 970
Paris Cardiovascular Research Center – PARCC
56 rue Leblanc, 75015 Paris, France
E-mail: Chantal.boulanger@inserm.fr

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1. Introduction

Microvesicles (MVs) belong to the family of extracellular vesicles (EVs), including exosomes and apoptotic bodies, shed from activated or apoptotic cells. These extracellular vesicles are distinguished on the basis of their subcellular origin, their size, their content and the mechanism leading to their formation.

MVs were initially characterised by their pro-coagulant activity. Seminal work demonstrated that platelet-deprived plasma could support coagulation and that its clotting property was abrogated

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by high-speed centrifugation (1, 2). Wolf identified the phospholipid-containing material, derived from platelets and capable of supporting coagulation, as *platelet dust*, subsequently called microparticles (2, 3). For these historical reasons, the term *microparticle* is the most commonly employed to refer to extracellular vesicles generated from platelets in the fields of haemostasis and thrombosis. The term *microvesicle*, however, is utilised within this review for consistency, and refers to extracellular vesicles produced by cytoplasmic membrane blebbing and shedding (2, 4), and is distinguishable from the vesicles stored in multivesicular bodies or alpha-granules, called exosomes (5, 6).

Today, MVs are interesting biomarkers with potential prognosis value as their content (proteins, active lipids, miRNA) (7, 8) varies with the inducer that initiated their shedding and with the severity of the disease (9–14). In addition, MVs emerge as new regulators of cellular crosstalk, in particular in vascular biology.

The present Position Paper, rather than systematically reviewing the literature, will critically summarise the current knowledge on the mechanisms of formation of MVs, their cellular origin, their composition, and the methods used for their analysis and quantification; potential pitfalls in MV quantification and functional effects will be discussed. In addition, this review will enlighten the potential biological roles of these vesicles with respect to vascular homeostasis and thrombosis.

2. Microvesicle formation

2.1. Phospholipid transbilayer and phosphatidylserine (PS) exposure

The asymmetry of phospholipid distribution across the plasma membrane is a common feature of resting eukaryotic cells. Conversely, loss of asymmetry is a driving force for plasma membrane remodelling, i. e. lipid motion and membrane deformation. Modification of the monolayer content in diffusible molecules alters lipid packing and causes membrane instability, regulated by sphingomyelinases that produce diffusible ceramides and prompt PS and phosphatidylcholine translocation and membrane budding (15–17). Whether ceramides and inner-leaflet interacting annexins also prompt agonist-driven MV release is unknown (18, 19). Other membrane adaptors, like arrestin-domain-containing-protein 1, appear to mediate the release of small plasma membrane vesicles of undetermined composition (20). The recently identified crowding effect of integral asymmetric proteins with large ectodomains and smaller intracellular ones also favours membrane bending in liposomes (21). Whether integrins that are highly distributed in platelets, endothelial cells or leukocytes take part in crowding-driven budding effects remains unknown (22, 23).

In resting cells, spontaneous phospholipid transbilayer transport is very slow (1 lipid/24 hours) (15, 24). Asymmetry is the result of the opposing activities of ATP-dependent phospholipid transporters governing inward (flippases) or outward (floppases) translocation and of non-specific bi-directional ATP-independent lipid transporters (scramblases). During agonist-induced calcium cell stimulation, PS exposure results from i) calcium-dependent inhibition of flippase and ii) its rapid translocation exerted by floppase(s) and/or scramblase(s) (25–27). The calcium-dependent proteolysis of the cytoskeleton leads to an eventual transient imbalance in phospholipid density between the two leaflets driven by the swift PS egress and a lower phosphatidylcholine and sphingomyeline reverse transport (28, 29). This triggers local instability of the plasma membrane and shedding of MVs that are released upon raft clustering (30–32). The calcium-dependent channel TMEM16F (ANO6), an anoctamin, has recently been demonstrated to play a pivotal role in calcium-induced phospholipid scrambling in the release of MVs exposing PS (25, 33–36). Interestingly, TMEM16F is mutated in Scott Syndrome, a rare human inherited bleeding disorder caused by defective platelet PS membrane exposure and MV shedding also evidenced in red cells and leukocytes (34, 37–40).

The existing data in isolated cells support the concept of MVs exposing PS as a consequence of membrane randomisation. PS translocation and MV release are both defective in response to procoagulant agonists in Scott syndrome, suggesting that PS exposure is a prerequisite to MV shedding (41, 42). Moreover, annexin-5, a high affinity PS ligand, inhibits MV release from stimulated cells (43). Finally, PS exposure and PS-positive MV shedding show similar time and concentration dependence whilst agonists induce distinct MV protein signatures (8, 44–47).

Important proportions of MVs lacking externalised PS, but expressing cellular markers, have been reported in cell supernatants and in plasma (4, 46, 48–50). However little is known about the nature and mechanisms of these vesicles. These observations may result from the heterogeneous nature of MVs. They may also be the result of altered membrane fluidity by exogenous proteins in addition to differences in detection thresholds (15, 43, 51). However, one cannot exclude artefacts in MV assessment/labelling, including contamination with other extracellular vesicles or proteins, absence of calcium that dampen PS detection (annexin, lactadherin, Del-1) (43, 52, 53) or prompt fusion with other vesicles or proteins (54–57). This does not rule out the existence of these MVs derived from an, as yet, undetermined mechanism of membrane release.

The use of annexin V positivity to identify MV populations in flow cytometry analysis is widespread. However, annexin V labeling could be skewed due to the presence of phosphatidylserine on lipoproteins (58). Furthermore, the possibility of the existence of PS negative MVs may mean that a proportion of MVs are excluded from analysis should this criteria be applied. This population may indeed differ in proportion and function depending on the parent cell. The possibility remains that by excluding PS negative MVs (or those MVs that bind annexin V below the detection limit of flow cytometers) an important sub-population of MVs will remain ill-described and unstudied. In addition, it is well known that apoptotic bodies have exposed PS on their surface and caution must be taken when analysing PS positive MV populations to ensure there is no contamination with vesicles released via apoptosis.

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2.2 Cytoskeleton reorganisation and MV formation

The cytoskeleton and hydrostatic pressure equilibrate plasma membrane tension whereas curvature instability of the bilayer drives membrane shape fluctuations and budding. In resting platelets, αIIbβ3-mediated destabilisation of the actin cytoskeleton promotes the release of procoagulant MVs, confirming that cytoskeleton integrity is critical for membrane asymmetry (59). The role of cytoskeleton reorganisation in MV shedding was ascertained by inhibition of calcium-dependent proteases or actin depolymerisation that abolish MV release in stimulated platelets and megakaryocytes (60, 61) (►Figure 1). Depending on the cell lineage, agonist-induced Ca^{2+} influx prompts the protease activity of caspases and/or calpains that cleave typical cytoskeleton proteins like filamin, gelsolin, talin and myosin (60, 62). Calpains are critical for platelet or neutrophil shedding, caspases for MV generation in vascular cells under apoptotic and non-apoptotic conditions (30, 63). Caspases mainly act through Rho kinase-dependent phosphorylation of the myosin light chain kinases (MLCK) (64–67), although Rho-kinase dependent endothelial MV formation can be independent of caspase activation (68). The release of neutrophil MVs is caspase-8 dependent and requires phosphokinase A and MLCK (69). In tumour cells, the Rho–driven cytoskeleton reorganisation and its relevance to exaggerated MV shedding identifies the pivotal role of small GTP-binding proteins, such as Rab22A (70) or ARF6 (71). Finally, caspase-3 directly triggers Xkr8, a putative scramblase or caspase transducer that promotes PS exposure in the membrane of apoptotic cells (72).

3. Microvesicles methodology update

3.1 Detection of MV

Methodologies to study MVs can be classified based on detection of single or multiple MVs. The most common methods to study single MVs are flow cytometry, tunable resistive pulse sensing (TRPS), and nanoparticle tracking analysis (NTA). Methods to study multiple MVs include immunocapture-assays, functional assays, and hybrid assays that involve capture followed by function or phenotype testing. The latter, "bulk assays" offer potential for high-throughput processing of clinical samples, may be sensitive enough to take the functional contribution of small MVs into account, and can be more cost-effective and user-friendly than single vesicle detection-based methods. However, single MV detection methods offer information on size or cellular origin of single MV, and thus may be more suitable to determine the presence of rare subtypes of MVs in a mixed population.

More recently, surface plasmon resonance imaging combined with protein microarray technology has been applied to MVs (73). An array of antibodies are printed on a gold chip, and antibody captured MVs induce a change in refractive index. Potentially, surface plasmon resonance imaging may be useful for parallel and multiple analysis of MVs in clinical body fluids (74).

3.2 Pitfalls in MV measurements

Among the single particle-based method, flow cytometry remains the most commonly used technique with the highest potential to determine the cellular origin of single MV (75–77). Over the past few years, significant improvements have been made regarding the sensitivity of flow cytometry to detect single vesicles with a diameter of < 300 nm, which have further established this methodology as the most promising tool for routine enumeration of MV subsets (78–80). Because a method has been developed to derive information on absolute size (diameter) and refractive index of single MVs and similar-sized particles from flow cytometry light scatter signals, the comparison of absolute measurement results on MV between instruments and institutes may come within reach (81, 82). However, it is important to note that accurate detection of MVs by flow cytometry is still a challenge, in particular for circulating MV analysis (56, 83–85).

NTA measures the Brownian motion of single particles in a laser beam (86) and is a valuable tool for the measurement of size distribution of MVs. The light scattered from single particles is visualised by microscopy, and the movement of single particles is monitored and recorded in time. Although NTA detects particles with a diameter < 100 nm, the resolution is low (75). The main limitation of NTA, however, is the inability to distinguish MV from similar-sized particles in suspension (i.e. debris). The ability to use the fluorescent mode offers the opportunity to specifically identify MVs. An alternative method, TRPS, is based on impedance to monitor individual MVs with a diameter of 80–1,000 nm or more, as they move through tunable nanopores (87). Particles passing the pore generate a change in the electric resistance, thus providing information on diameter, surface charge, concentration, and zeta potential of single particles. The major disadvantage of TRPS is that it cannot distinguish between MVs and similar sized particles such as chylomicrons or protein aggregates.

Each of the above methods has advantages and disadvantages. Therefore, to confirm the presence of MVs, ISEV recommends the use of a second independent method (88) [also see *Section 9*]. As an independent method, often transmission electron microscopy is used, because this technique has a high resolution and can distinguish intact MVs from non-MVs at the level of a single particle.

3.3 Standardisation of MV measurements

Standardisation is mandatory to allow evaluation of the true clinical relevance of MV at a multicentre level, and should be accompanied by continuous improvement of methods. Flow cytometry is the first method to benefit from standardisation efforts coordinated by the International Society on Thrombosis and Haemostasis (ISTH) Standardisation Committee on Vascular Biology. Thus, several bead-based comparison studies have been performed to standardize light scatter gates for MV selection between instruments (76, 89). At present, additional standardisation strategies are being tested based on either absolute vesicle size approximation or fluorescence (90). In 2015, a collaborative workshop was initiated to standardise MV detection by flow cytometry

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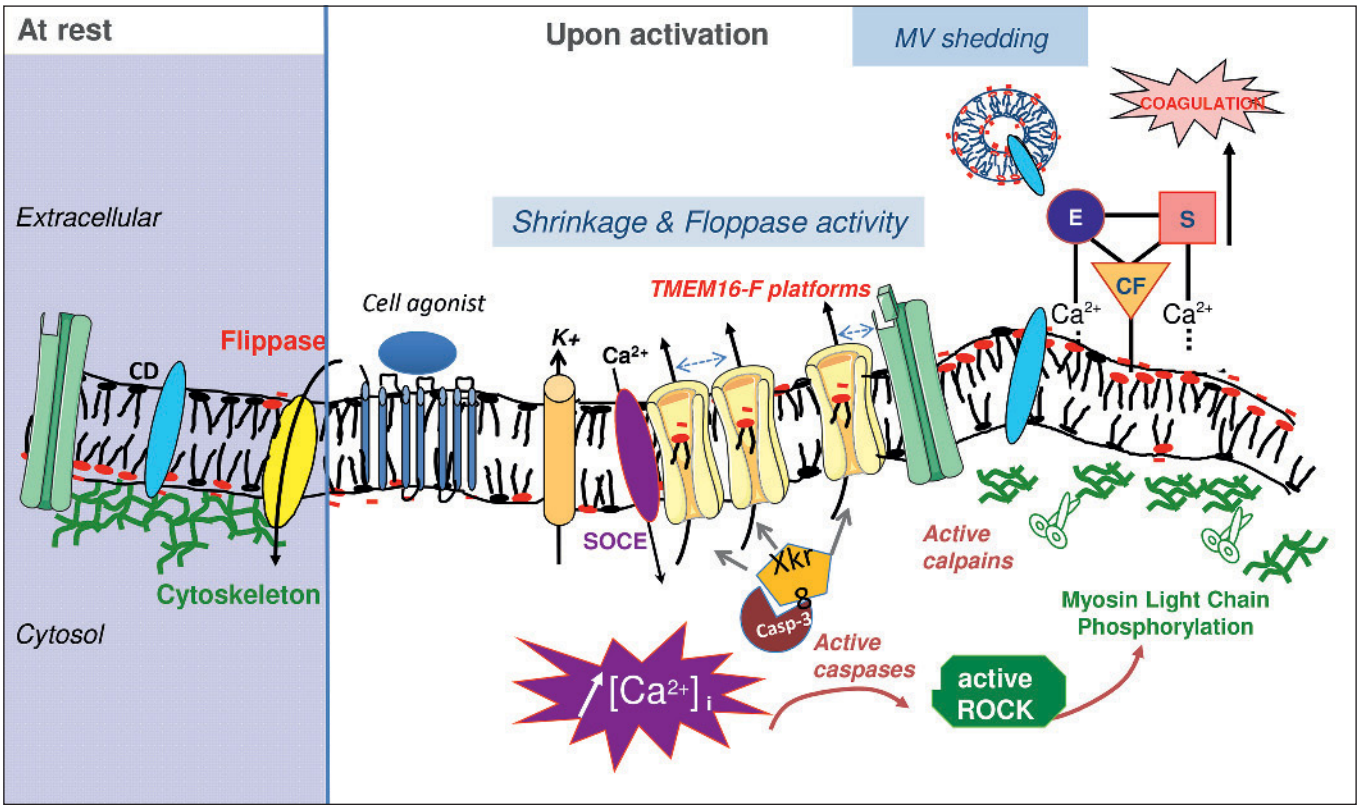

Figure 1: Floppase activity and facilitated transport of phosphatidylserine by TMEM-16 F (ANO-6) and procoagulant MV shedding. At rest, phosphatidylserine (PS) is translocated to the inner leaflet by flippase activity. Right panel: Upon cell activation and calcium-dependent flippase inhibition, PS translocation to the outer leaflet is driven by TMEM-16F (yellow shape) and local K^+ efflux prompts cell shrinkage and re-shaping. High calcium concentration promoted by Stored Operated Channels (SOCE) favours the constitution of TMEM16-F platforms by oligomerisation or interaction with other receptors like P2XR in the case of long term exposure to Ca^{2+} (green shape). Transient phospholipid imbalance between leaflets and the proteolysis of cytoskeleton by calpains and/or caspases lead to facilitated procoagulant MV shedding. Putative scramblase transducers as Xkr8 are activated by caspases and would trigger enhanced floppase activity as described in apoptotic cells (see text for details). Exposed PS catalyses the assembly of blood coagulation complexes at cell and MV surface (E: Enzyme, S: Substrate, CF cofactor).

(www.evflowcytometry.org). In this workshop the knowledge from three international societies is combined: International Society on Extracellular Vesicles provides knowledge on *vesicles*, International Society on Advancement of Cytometry provides knowledge on *detection of MV by flow cytometry*, and the International Society on Thrombosis and Haemostasis contributes with expertise on *blood collection, handling and storage*. Similarly, attempts are on-going to standardize TRPS measurements and coagulation.

For a long time measurement of (single) vesicles was considered as measuring "the tip of the iceberg". Because the sensitivity of vesicle detection is improving rapidly, there is now an urgent need for developing novel guidelines regarding collection, handling and storage, as required for reliable biorepositories. Recently, a start has been made in developing such guidelines for human blood, urine and saliva using sensitive MV detection methods ((91); www.metves.eu), but more research is clearly required. Furthermore, labelling protocols for flow cytometry require updates and improvements, and standards and validation protocols need to be developed. Finally, training and education are now being developed, such as the online course developed by ISEV on the "Basics of Extracellular Vesicles", which is open access (https://www.courserа.org/learn/extracellular-vesicles). In addition, the ISTH Academy has recorded two webinars on MVs. Both courses include up-to-date information on isolation and detection of MVs.

3.4 MV isolation and pitfalls in testing their functional effects

One of the most important considerations when isolating MVs from blood is the presence of contaminating platelets. When comparative studies are performed, sample preparation must be identical in order that any artefact is present in all samples (91). In most studies, MVs are isolated and/or concentrated using protocols involving either initial low or intermediate multiple centrifugations, combined with ultracentrifugation, density gradient centrifugation, or combinations thereof. These MV populations are impure and contaminated by plasma or serum proteins hence affecting the outcome of functional or –omics measurements (▶Table 1). To overcome these limitations, size exclusion chromatography has been adopted recently by many investigators to separate MVs

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Table 1: Measuring the efficiency of MV isolation procedures.

		Starting material	Isolated MV
Recovery MV	Measure MV	+	+
Confirm MV identity	Electron Microscopy	N/A*	+
Contamination	Measure protein Measure lipoprotein	+ +	+ +
Enrichment of MV	EV/protein ratio EV/lipoprotein ratio	+ +	+ +

Starting material, for example plasma or conditioned cell culture medium, will contain MVs, proteins and lipoproteins. To determine the recovery of MVs, measure MVs before (starting material) and after isolation, and express the recovery as [concentration isolated MV]/[concentration MVs in starting material] × 100 %. Confirm the identity of MVs in obtained fraction(s) by electron microscopy. Finally, to determine the (relative) enrichment of isolated MVs compared to the starting material, measure the concentration of protein and lipoprotein before and after isolation, and calculate the ratio of [MV concentration]/[protein concentration] and [MV concentration]/[lipoprotein concentration]. *MVs cannot be visualised directly in most fluids due to the presence of proteins and other contaminants.

from soluble proteins in cell preparation ((92); www.metves.eu), thereby facilitating for example detection of MVs by electron microscopy as well as –omics studies of EVs. More quantitative information is clearly needed about the extent of MV recovery for each isolation method (93).

Several questions may arise when testing MV functional effects either *in vitro* or *in vivo*. First, the choice of MV concentrations should be of physiological or pathological relevance. Surprisingly, few studies evaluate the effects of more than one concentration of MVs, although previous reports point out the paradoxical effects of different MV concentrations (94). Second, the experimental design should include robust controls, including the supernatant above the isolated MV pellet. Control experiments using saline-buffered solutions may be also an additional control. When cytokines or inhibitors are used to trigger MV release *in vitro*, caution should be taken to remove these compounds from the enriched vesicle preparation, for example by dialysis or size exclusion chromatograph, to avoid experimental bias. The presence of large numbers of MVs and exosomes in serum used for culture experiments should also be taken into account. Similarly, presence of lipoproteins, hormones and other mediators in plasma should also be taken into account when designing adequate control experiments for testing functional effects of circulating MVs. Finally, *in vivo* effects of MV of human origin need to be tested in suitable animal models in order to avoid immune reaction to human biological material that would mask MV functional effect.

4. Microvesicles as regulators of cell communication

In 1996, Raposo et al. demonstrated that EVs could be transferred between cells (95). This concept is based on the observation that EVs released from a given cell type interact through specific receptor ligand with other cells, leading to trigger cell stimulation directly or by transferring the surface receptors (96). Since then, numerous studies confirmed that EVs are an important mode of intracellular communication and cargo delivery between cells, including platelets, endothelial cells, and monocytes. Hence, it is now recognised that EVs are an integral part of the intercellular microenvironment and act as regulators of cell-to-cell communication (►Figure 2).

Ratajczak et al. proposed that MV-mediated cell-to-cell communication emerged very early during the evolution as one of the first communication mechanisms (97). The first evidence for MV-mediated protein transfer was observed by Barry et al. where bioactive lipids were functionally transferred via platelet MVs to endothelial cells leading to specific biological effects (98). Another example is the transfer of arachidonic acid between activated and resting platelets that results in the modulation of their procoagulant responses (99).

Since then, significant progress has been made in the field of MV transfer. However, the study of MV release and uptake within *in vitro* and *in vivo* settings remains challenging, as there are no reliable detection methods to discriminate cells that uptake EVs from cells that do not. Recently, a promising novel tool using Cre-loxP system has been developed to directly identify the fluorescently marked Cre-reporter cells that take up EVs released from Cre recombinase–expressing cells (100).

4.1 MV uptake and clearance

The mechanisms controlling vesicle uptake and internalisation are still a matter of debate. Various mechanisms have been proposed, including endocytosis (101), phagocytosis (102) and plasma or endosomal membrane fusion (103). Their molecular mechanisms have been validated using antibodies to test the role of specific ligands or receptors, and chemical inhibitors to block specific uptake pathways. A recent study has shown specific differences between exosomes and MVs for transferring genetic information (104). In particular, MVs, but not exosomes, can functionally transfer loaded reporter molecules to recipient cells. These results have significant implications for the understanding of EVs role in cellular communication and further development of EVs as vehicles for macromolecule delivery.

Several studies have also demonstrated rapid clearance of MVs of different cellular origin from the circulation, mainly by liver and spleen phagocytes (55, 105–107). However, there is no definitive mechanism regarding the cell types involved in uptake and the potential consequences of such uptake. The presence of externalised PS is of vital importance for effective uptake through glycoproteins Del-1 or lactadherin bridging MV's PS and cellular integrins on endothelial cells and macrophages, respectively (106, 108, 109).

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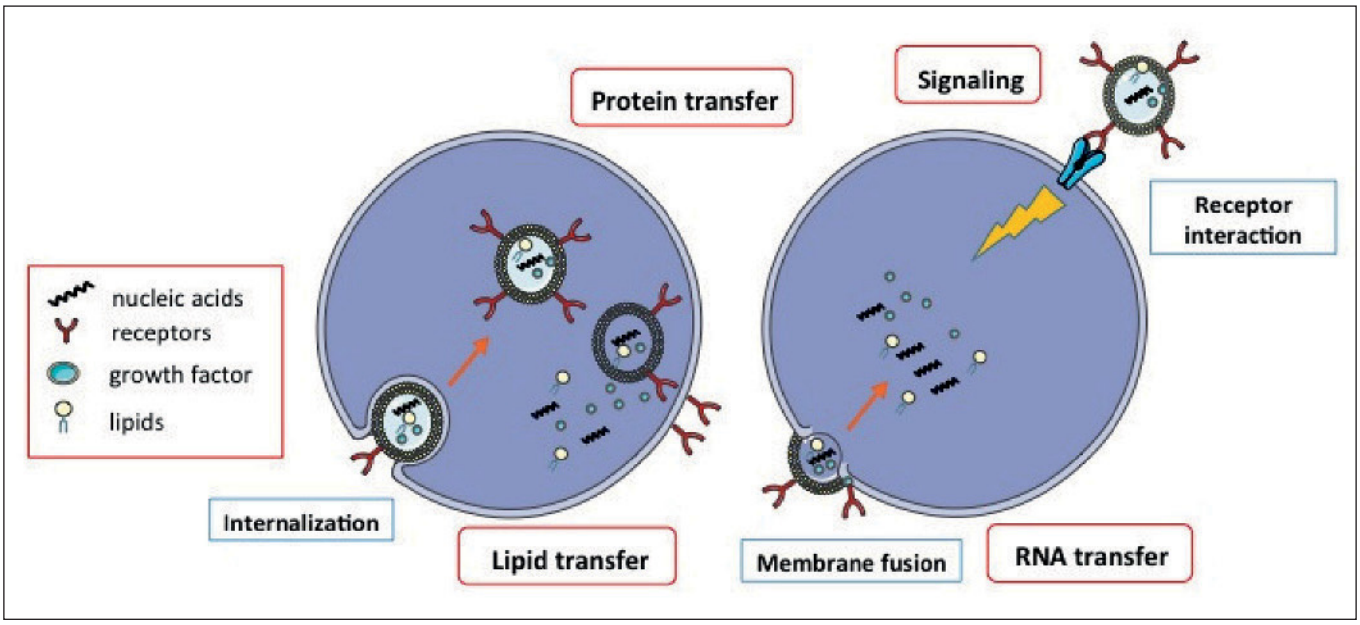

Figure 2: Molecular component and pathways used by MVs to regulate cell communication. MVs may transfer membrane components and cytosolic and nucleic acids to the target cell by internalisation or following membrane fusion. MVs interactions with membrane-associated receptors may induce specific responses in target cells. Uptake of some mediators, such as miRNA, induces reprogramming of target cells. Receptors present on MV surface could be recycled and presented on the surface of the target cell.

The receptor tyrosine kinases Tyro3, Axl and Mer and their ligands protein S and Gas6 are also involved in the PS-dependent uptake of MV by macrophages and endothelial cells (110). In addition, lipid raft–mediated endocytosis contributes to MV internalisation in human brain endothelial cells, but not in those originating from human umbilical vein (109, 111). MV uptake also depends upon how they are produced. For instance, stimulation of human aortic endothelial cells with tumour necrosis factor α (TNF-α) leads to the formation of two populations of MVs, with distinct miRNA content (112). MVs rich in miRNAs are taken up much faster than the other MV population, suggesting that endothelial cells could differentially recognize MVs generated upon different molecular cues.

4.2 Cargo transfer

MVs influence behaviour of target cells in multiple ways: by directly activating cell surface receptors via protein and bioactive ligands, by transfer of cell surface receptors or delivery, including transcription factors, mRNA and non-coding RNA. The bioactive ligands exposed on the MV surface are responsible for several important regulatory processes; for instance, direct stimulation of endothelial cells with MV- associated CD40 ligand (CD40L) stimulates angiogenic responses *in vivo* (113). Moreover, MVs can transfer the adhesion molecule CD41 (Integrin alpha-IIb) from platelets to endothelial cells, conferring the latter pro-adhesive properties (114). Transfer of the chaemokine CCL5 (RANTES) exposed on platelet MVs to target endothelial cells by GPIIb/IIIa and JAM-A dependent mechanisms contributes to monocyte recruitment (115). Furthermore, intercellular adhesion molecule-1 (ICAM-1) is transferred, by a PS-dependent mechanism, from MVs isolated from atherosclerotic human plaques and functionally integrated into endothelial cells following membrane fusion, resulting in increased monocyte adhesion and transmigration (116).

The presence of functional mRNA in EVs was first described in 2006 for murine stem cell-derived vesicles (117). EVs, however, could also transport mRNA fragments (118), long non-coding RNA (119), miRNA (120, 121), ribosomal RNA (rRNA) (119) and fragments of tRNA-, vault- and Y-RNA (122). This concept that non-coding RNA, and specifically miRNA, are transported into extracellular spaces, together with the evidence that exchange of miRNAs between cells can be accomplished through EVs, led to a revolutionary hypothesis of the existence of a miRNA vesicle-mediated communication system. Embedding of miRNAs in EVs could explain their resistance to nucleases when released outside the cell (123, 124). A large fraction of miRNAs exported by cells also associates with the Argonaute (Ago) protein family (125). Some studies report absence of RISC complex proteins (including Ago2) in the exosomes sub-group of EVs (126), whereas others report presence of Ago2 protein (127). In this regard, RISC proteins in EVs could process precursor microRNAs (pre-miRNAs) into mature miRNAs, inducing the cell-independent microRNA biogenesis (128). This is an exiting novel area of research that requires caution due to numerous potential pitfalls in the interpretation of the data as changes in miRNA content may not result in functional changes in the target cell.

Endothelial EVs can stimulate repair by functionally influencing endothelial target cells. For instance, endothelial MVs and apoptotic bodies can transfer functional miR-126 to target en-

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Table 2 : Plasma MV changes in subjects with cardiovascular risk factors and in patients with atherosclerotic vascular diseases.

	MV subpopulation	Changes	References
Cardiovascular risk factors			
Age	Endothelial, TF+MV, PS+MV	↑, ↓	(294, 295)
Female gender	Endothelial, Platelet, PS+MV	↑	(296)
Hypertension	Endothelial, platelet	↑	(297, 298)
Hypercholesterol-aemia	Endothelial, lymphocyte, leucocyte, platelet	↑	(249, 299)
Hypertriglyceridaemia	Endothelial	↑	(298, 300)
Smoking, Pollution	Endothelial, platelet, leucocyte	↑	(148, 301–303)
Obesity	Endothelial, platelet	↑	(304–307)
Diabetes	Endothelial, platelet, leucocyte, TF+MV	↑	(308–310)
Metabolic syndrome	Endothelial	↑	(298, 311)
Family history CVD	ND	ND	ND
Physical inactivity	Endothelial	↑	(312)
Atherosclerotic vascular diseases			
Subclinical athero-sclerosis	Leucocyte, platelet, lymphocyte, endothelial	↑	(249, 253, 299, 313)
Coronary calcification	Endothelial, Platelet	↑	(314)
Acute coronary syndrome	Endothelial, Platelet, monocyte	↑	(13, 310, 315–319)
Stable coronary disease	Endothelial, Platelet	↑	(316, 320)
Cardiac sudden death	Endothelial	↑	(321)
Acute stroke	Endothelial, platelet, leukocyte	↑ or no change	(189, 322–324)
Cerebrovascular atherosclerosis	Endothelial	↑	(325)
Peripheral artery disease	Platelet	↑	(326–328)
End-stage renal disease	Endothelial, platelet, erythrocyte	↑	(329, 330)

CVD: cardiovascular diseases; TF: tissue factor; PS^+ MVs: MVs expressing phosphatidylserine; ND : Not determined.

dothelium to promote CXCL12-mediated angiogenic cell recruitment for atheroprotection or re-endothelialisation after endothelial injury in mice by stimulating endothelial migration and proliferation, due to downregulation of RGS16 and SPRED1, respectively (121, 129). Endothelial p75 neurotrophin receptor activates the NF-κB signalling, inducing miR-503 transcription and the shedding of endothelial MVs by triggering the expression of Rho kinase (130). Intriguingly, miR-503-containing endothelial MVs are taken up by pericytes *in vivo* leading to, downregulation of miR-503 target genes, EFNB2 and vascular endothelial growth factor A (VEGFA), and increased vessel permeability (130). Furthermore, miR-143 and miR-145 packaged in endothelial EVs released under shear stress are taken up by smooth muscle cells, where they downregulate target genes, inducing atheroprotective effects (131). Finally, circulating MVs from patients with coronary artery disease are deficient in Del-1, a glycoprotein mediating their endothelial uptake. *Ex vivo*, this led to a reduced uptake of MV-associated miRNAs (miR-17, miR-19a, miR-21, miR-92a, miR-146a, miR-222, and miR-223) in recipient cultured endothelial cells (132).

5. Endothelial microvesicles

The suggestion that endothelial MVs are causative agents in vascular pathology has arisen from their numerical increase in a range of diseases that have been extensively reviewed (133–135). Endothelial derived MVs carry endothelial proteins such as adhesion molecules (VE-cadherin, platelet endothelial cell adhesion molecule 1, intercellular adhesion molecule 1 (ICAM-1), E-selectin, αv integrin), growth factors (Endoglin, S-Endo 1 (CD146), VEGF receptor 2 (VEGF-R2, haemostatic molecules (von Willebrand factor, TF, TF pathway inhibitor (TFPI), tissue plasminogen activator, plasminogen activator inhibitor 1, endothelial protein C receptor (EPCR)) or active components (Endothelial NO synthase, urokinase type plasminogen activator) (136). They have also been reported in human and murine plasma (137, 138), vitreous fluid (139) and in inflammatory lesions such as the atherosclerotic plaque or ischaemic tissues (140) (►Table 2). The composition of endothelial MVs depends upon the stimulus triggering their biogenesis, and their components originate from the plasma membrane, the cytosolic fraction, the cytoskeleton or from mitochondria (8). Elevated numbers of endothelial MVs were first reported in 1999 in disease populations, and were thus considered as potential diagnostic and prognostic biomarkers (137) (►Table 2).

5.1 Regulation of endothelial MV formation

Circulating levels of endothelial MVs are thought to reflect a balance between cell stimulation, proliferation, apoptosis and other forms of cell death (136). Biological factors that are pertinent to vascular health and haemostasis are involved in the generation of endothelial MVs (►Figure 3). Among them, inflammatory and coagulation factors (such as TNF-α and other inflammatory cytokines, bacterial lipopolysaccharides (LPS), reactive oxygen species (ROS), plasminogen activator inhibitor, thrombin, camptothecin, C-reactive protein and uraemic toxins, oestrogens) are able to induce *in vitro* endothelial MV generation (66, 136, 141–145). Interestingly, endogenous nitric oxide (NO) and oxidised lipids (146) also impact on MV generation by cultured cells. Although little is known on the precise mechanisms involved in endothelial MVs release *in vivo*, the role of arterial shear stress has recently been demonstrated (68, 147). Generation of endothelial MVs from the

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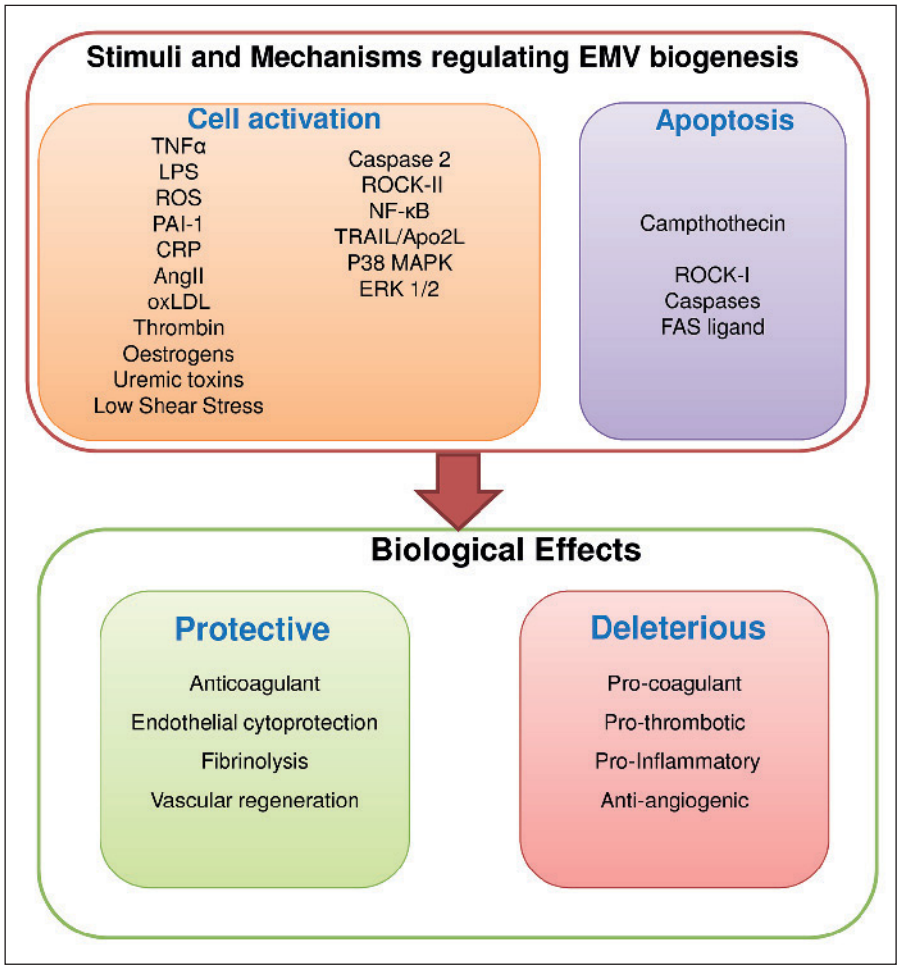

Figure 3: Biogenesis and biological effects of endothelial MVs. Endothelial MV release can be triggered following cell activation or apoptosis. The resulting remote biological effects of endothelial MVs can be either protective or deleterious.

endothelium may be considered a hallmark event reflecting the beginning of endothelial dysfunction. For instance, passive exposure to cigarette smoke rapidly (within 30 minutes) increases circulating EMVs in healthy subjects concomitantly with impaired endothelial function (148).

Evaluating the intracellular transcriptional events leading to endothelial MV formation in response to thrombin evidenced an early step involving genes linked to cytoskeleton re-organisation, such as Rho-kinase ROCK-II, followed by a second step mediated by TNF related apoptosis-inducing ligand (TRAIL)/Apo2L, a cytokine belonging to the TNF-α super-family (149). The transcription factor NF-κB was required both for the early and late production of endothelial MVs. The activation of the p38 mitogen-activated protein kinase, as well as the activation of Rho kinase and extracellular signal-regulated protein kinases 1 and 2 by low shear stress were also identified as critical pathway in the production of endothelial MV (68, 136), thus providing a paracrine loop enhancing the endothelial response to inflammation (▶Figure 3).

5.2 Multifaceted roles of endothelial MVs in vessel wall homeostasis

The expression of anionic phospholipids, especially PS, able to bind and activate coagulation factors contributes to the procoagulant potential of endothelial MVs (137, 150). Moreover, different agonists induce the generation of endothelial MVs expressing tissue factor (137, 146, 150, 151). Interestingly, endothelial MVs bind to monocytes and induce tissue factor expression and activity (152). However, the limited evidence of endothelial MV contribution *in vivo* frustrates our knowledge of their role in coagulation and thrombosis. The thrombogenic activity of endothelial MVs has been demonstrated in a mouse model after exogenous injection of TF positive endothelial MV (153). However, selective deletion of tissue factor in endothelial cells has no effect on coagulation activation in a murine model of endotoxaemia (154). Therefore, the increased levels of tissue factor positive endothelial MV detected in diseases such as sickle cell or sepsis (155, 156), suggests that TF positive endothelial MV may be selectively associated to certain disease states, and possibly to subsets of endothelial MVs that remain to be characterised. Interestingly, other studies have provided evidence that endothelial MVs can also exhibit antico-

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agulant and vasculo-protective activity: they can deliver miR-126 or thromboxane A2 to the vessel wall (157), and can expose EPCR and activated protein C (APC). APC positive endothelial MV display anticoagulant and cytoprotective properties effects on endothelial cells through the reduction of apoptosis (158). Moreover, endothelial MV also behave as a catalyst, supporting plasmin generation by plasminogen (94), which confer them fibrinolytic properties with a pivotal role for clot dissolution. These findings illustrate the broad scope of mechanisms involving endothelial MVs in haemostasis and thrombosis, and their potential beneficial capacity to influence the evolution of the disease (►Figure 3).

5.3 Role of endothelial MVs in inflammation

Inflammatory mediators enhance endothelial MV biogenesis *in vitro*. An increase in endothelial MVs is evident in many inflammatory–type diseases such as atherosclerosis, diabetes or autoimmune conditions (►Table 2). There is a direct correlation between endothelial MV number and IL-6, both *in vivo* and *in vitro*, implying a close relationship between endothelial vesiculation and classic inflammatory pathways of cytokine production (159, 160). Endothelial MVs may not only reflect the activation status of the cells but also confer further systemic activity, indicating that they are not only the consequence, but could also be involved in regulating inflammation. In addition, interaction between endothelial MV and naïve endothelial cells triggers pro-inflammatory responses assessed by up-regulation of ICAM-1 mRNA expression and soluble ICAM-1 shedding from targeted cells, an effect that was no longer observed using endothelial MV from un-stimulated endothelial cells (159). Endothelial MVs injected intravenously in mice lead to increased systemic and pulmonary levels of IL-1β and TNFα, correlating with an increase in neutrophils in the lung (161).

5.4 Endothelial MVs, angiogenesis and vessel remodelling

The angiogenic effect of endothelial MVs is highly dependent on their composition. Some studies reported that endothelial MVs impair angiogenesis, but the underlying mechanism remains unclear. The role of ROS may be important as *in vitro*, angiogenesis impairment can be rescued with a cell permeable superoxide dismutase mimetic (162). Physiological and pathological concentration of endothelial MVs injected into $LDLR^{-/-}$ mice on a high fat diet inhibited angiogenesis in the heart with effects on endothelial nitric oxide synthase and NO generation (163). This inhibitory effect on tube formation was also reported by MVs from diabetic patients with coronary artery disease (164). Overall it would appear that endothelial MVs inhibit angiogenesis. However, endothelial MVs from human microvascular endothelial cells were shown to induce angiogenesis at low concentration, through plasmin generation, whereas higher concentrations have opposite effect (94). Within the atherosclerotic plaque, CD40L positive MV enhance endothelial proliferation, promoting *in vivo* neovessel formation and thus favouring intra-plaque haemorrhage (113). Ligation of endothelial CD40 with CD40L positive endothelial MVs modulate VEGF and PI3K: AKT activation and cell proliferation (113). Importantly, since some endothelial MVs can both promote or inhibit angiogenesis, appropriate animal models are required in order that the effects of endogenous release can be assessed.

5.5 Conclusion

Endothelial MVs are multifaceted biological vectors playing a role in both physiological and pathological conditions. Their pleiotropic roles identify them as active intercellular communicators potentially contributing to the regulation of vascular homeostasis. Dysregulation of endothelial MV biogenesis and its biological activities may be an surrogate marker of vascular dysfunction and as such provide potential biomarkers of endothelial dysfunction (►Table 2). Although this prospect is challenging, a fully understanding of endothelial MV biogenesis and *in vivo* demonstration of their role in pathophysiology is required and will undoubtedly uncover new areas of vascular biology.

6. Platelet microvesicles

Platelets are anucleated fragments released in the bloodstream from their cellular precursor, the megakaryocyte (165). Outnumbered in blood by red blood cells (RBCs) only, platelets are recognised for their role in haemostasis and thrombosis. With their broad content in mediators and expression of receptors for immune regulatory functions, evidence also supports their contribution to immunity, inflammation and tissue repair (166, 167). Hence, studies reveal that vesicles released by platelets also convey an elaborate set of cargo and might play roles other than the support of coagulation and thrombosis, such as angiogenesis, cancer, cardiovascular diseases and inflammation (4, 134, 168, 169) (►Table 2).

As the megakaryocyte is a large cell that can undergo several rounds of DNA replication without cellular division, its cytoplasm is particularly rich in miRNA and other factors, which are transferred to platelets during proplatelet formation. Thus, albeit anucleated, platelet cytoplasm contains miRNA and the miRNA machinery such as Ago2 (170), which have been reported to be encapsulated within MVs (171). Growth factors (e.g. platelet-derived growth factor, transforming growth factor β) (172), enzymes (e.g. 12-lipoxygenase, thromboxane synthase) (98, 173), cytokines (e.g. IL-1) (174, 175), transcription factors (173) and even functional mitochondria (173, 176) (►Figure 4) are found in platelet MVs, and can be efficiently internalised by other cells such as endothelial cells (106, 171), macrophages (55, 177), and neutrophils (173). Whereas the internalisation process in endothelial cells implicates MV's PS recognition by receptors such as developmental endothelial locus-1 (Del-1) and the interaction of the receptor tyrosine kinase Axl with its ligand Gas6 found at the surface of MVs (55, 110), the internalisation by neutrophils is also tightly regulated and requires 12-lipoxygenase activity present within MVs (173). Hence, platelet MVs are retrieved inside neutrophils in the joints

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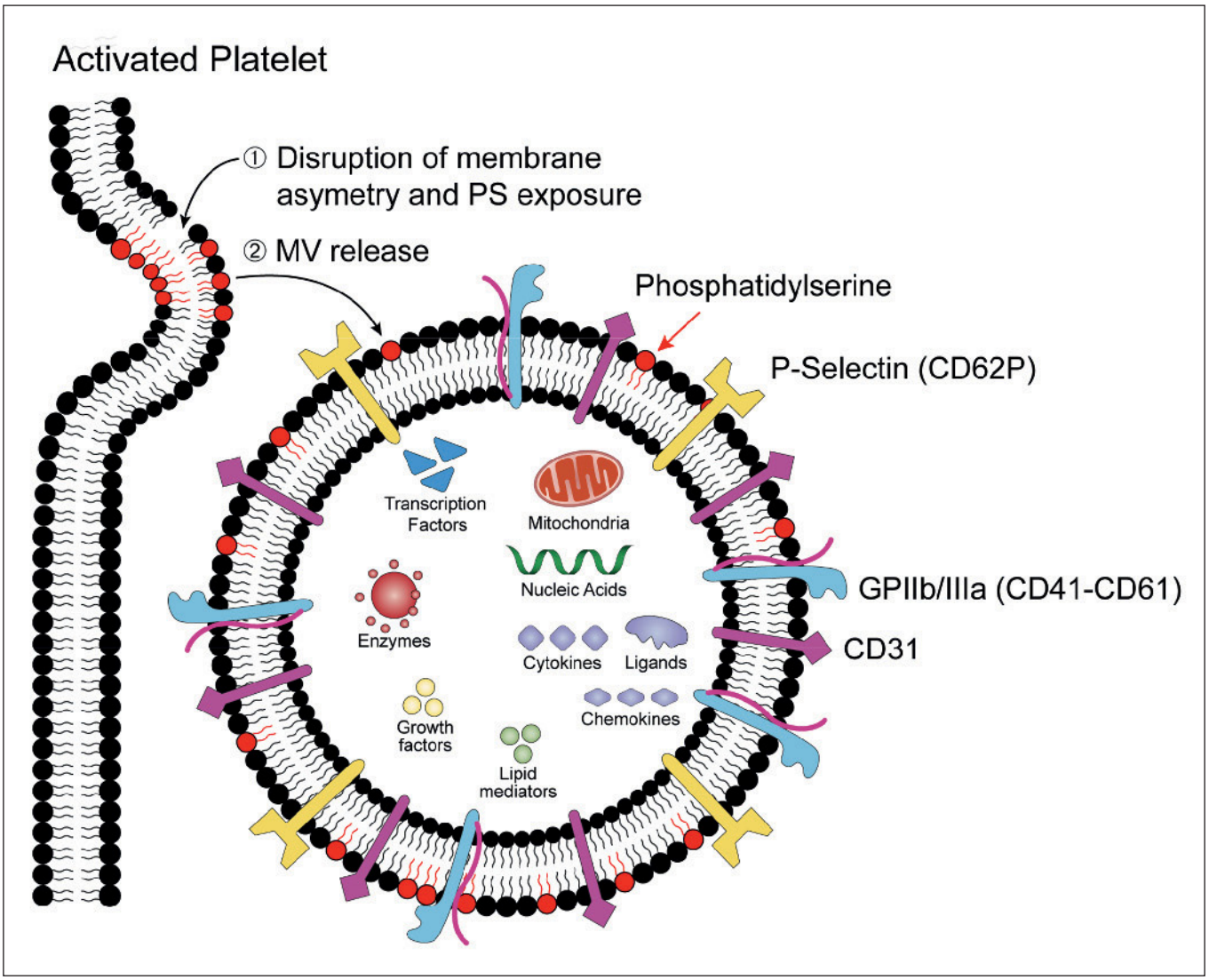

Figure 4: Composition of platelet-derived MVs. Platelet MVs are produced by activated platelets on disruption of membrane asymmetry and plasma membrane budding (steps 1 and 2). Although platelets are anucleated, they do contain a broad arsenal of molecules, which can be transferred to platelet MVs.

of arthritic mice, and the ablation of the gene coding for 12-lipoxygenase (ALOX12) abrogates the internalisation of MVs and consistently reduces inflammation.

6.1 Regulation of platelet MV production

In spite of their small size, approximately 3 µm in diameter, platelets contain an impressive membrane reservoir. The platelet plasma membrane comprises sinuous invaginations called the open canalicular system, which channels provide an important source of membrane permitting the formation of filopodia and spreading (up to 420%) on activation (178). Furthermore, impressively long (250 µm) membrane tendrils, called flow-induced protrusions, trail into the blood vessel from adherent activated platelets under flow conditions (179). Thus, although platelets are small, their abundance in blood and their important membrane content might explain how platelets represent a dominant source of MVs in blood in physiological conditions (49).

Different platelet stimuli can trigger platelet activation via different signalling pathways leading to an increase in intracellular calcium. Studies have shown distinct potencies at inducing MV release, e.g. Ca^{2+} ionophore > thrombin > the glycoprotein VI (GPVI) agonist cross-linked collagen related peptide (CRP-XL) > co-stimulation with thrombin and CRP-XL > collagen > LPS > thrombin receptor activating peptide > adenosine di-phosphate (6). Mechanistically, platelet agonists induce the rise in intracellular calcium concentration, which in turn triggers cytoskeleton cleavage through calpain activation and cellular contraction and blebbing (30, 180). In addition to platelet agonists, platelet MV formation can also be triggered by physical stimuli (shear stress, hypoxia) or prolonged storage.

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6.2 Platelet MVs in physiological and pathological conditions

A combination of cryo-electronic microscopy and flow cytometry determined that the blood at steady state contains approximately 10^7 platelet MVs exposing PS per ml, pointing to a constitutive production of MVs by platelets, potentially due to shear stress and platelet aging. However, studies suggest that instead, most of the MVs in blood in fact originate from megakaryocytes (4, 60, 181). Circulating MVs originating from megakaryocytes are distinguished from those derived from activated platelets by the presence of P-selectin, lysosomal associated membrane protein 1 and immunoreceptor-based activation motif (ITAM) receptors (4, 60, 181). However, in pathological context during which platelets are activated, such as in rheumatic diseases (182), at least part of MVs in blood circulation were proven to originate from platelets (181). Whether platelet MVs contribute to the increased risks of cardiovascular diseases and thrombosis in patients with rheumatic disorders (183), however, remains to be established.

Platelet activation also occurs in the presence of complement components (184) providing a possible explanation for the high concentrations of platelet MVs in diseases where complement-mediated platelet activation occurs, such as paroxysmal nocturnal haemoglobinuria and aplastic anaemia (185). This may indeed explain why therapy with eculizumab, a humanised monoclonal antibody acting as a terminal complement inhibitor, prevents thrombosis in patients with paroxysmal nocturnal haemoglobinuria (186). Hence, with their procoagulant potential, platelet MVs may play their part in venous and arterial thrombosis. Indeed, platelet MVs were demonstrated to play an important role in atherothrombotic process. Beyond being markers of platelet activation, platelet MVs in blood showed functional effects on atherothrombotic disease because they enhanced platelet and fibrin deposition on atherosclerotic arterial wall, promoting platelet adhesion, further recruitment of platelets and thrombus formation (187). Accordingly, elevated concentrations of platelet MVs are found in patients with acute coronary syndrome (188), transient ischaemic attacks and strokes (189, 190), diabetes with atherothrombotic disease and peripheral arterial disease (191), heparin-induced thrombocytopenia with thrombosis (192), as well as myeloproliferative neoplasms (193) (►Table 2). Platelet MVs also contribute to thrombosis in patients with haemoglobinopathies such as sickle cell disease and thalassaemia (194, 195). Interestingly, in polytransfused patients with thalassemia major, the number of platelet MVs augments following splenectomy, and consistently these patients have a higher tendency to develop thrombosis (196). Thrombosis is also prevalent in cancer pathophysiology, and studies suggest that it might be due, at least in part, to platelet MVs (197), although the cancer cells themselves represent an important source of MVs (150). Hence, platelet MV levels correlate with the aggressiveness of certain neoplasias, a high level being predictive of a poor clinical outcome (193, 197, 198).

Other molecules exposed in platelet MVs, in addition to phosphatidylserine, impact haemostasis and thrombosis. For instance, platelet MVs may bear activated protein C, a recognised anticoagulant protein, and thereby have a protective effect in early sepsis (199). While platelets were reported to express TF, studies by different investigators failed to detect TF in both resting and activated platelets (150, 200–202). Monocytes, however, are a major source of TF, which is stored in an encrypted form. It is suggested that monocyte-derived MVs harbor functional decrypted TF, and that the formation of highly thrombogenic platelet MVs and monocyte MVs hybrids might explain why TF has been reported on platelet MVs (150, 200–203). Hence, P-selectin harbored by platelet MVs targets MVs to the thrombi at site of injury, and injection of MVs containing P-selectin improved the kinetics of fibrin formation and could normalise the bleeding time in a haemophilia mouse model (204, 205). Importantly, P-selectin, through binding with P-selectin glycoprotein ligand 1 (PSGL-1), recruited monocyte-derived MVs expressing TF to the thrombus, and further amplified thrombosis (204, 205).

Whereas most studies on platelet MVs implicate the study of blood, platelet MVs are found in lymph and in the synovial fluid of patients with rheumatoid arthritis (175, 206, 207), suggesting that they can reach locations outside blood vessels. While platelet MVs might display a local pro-inflammatory activity through their cytokine content, exposure of autoantigens and interactions with neutrophils (173, 175, 208), platelet MVs also deliver anti-inflammatory signals by the inhibition of IL-17 and IFN-gamma production by a particular set of regulatory T cells (209), reportedly present in rheumatoid arthritis (210), thereby enhancing the stability of the regulatory T cells in an inflammatory environment (209). It is not completely understood how platelet MVs egress the vasculature, but it might implicate transportation by leukocytes and vascular permeability (211). Hence, *in vivo* studies revealed gaps in the arthritic joint vasculature that permitted the accumulation of synthetic submicron microspheres outside blood vessels, pointing to a role of permeability in the process (211).

Constitutive platelet activation, such as seen in Stormorken's syndrome, also called "inverse Scott's syndrome", leads to thrombocytopenia and bleeding tendencies (212). Platelets from Stormorken's syndrome patients are in an activated state and therefore display PS on the outer surface due to a gain-of-function mutation in the sensing protein stromal interaction molecule 1 gene or a loss-of-function mutation of the gene coding for the calcium channel pore-forming protein ORAI1 (213, 214). As circulating platelet MVs are elevated in this pathology, it suggests, however, that platelet MVs might not suffice to prevent bleedings in this condition (212).

6.3 Conclusion

Platelet MVs contain and elaborate cargo, which can be transferred to recipient cells, thus suggesting that platelet MVs might be implicated in development, angiogenesis, wound healing, tissue regeneration and repair and remodelling, as well as cancer (169, 215–217). Continuous research on the topic and improvement of the detection methods will reveal the different roles that platelet MVs might play in health and diseases.

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7. Leukocyte microvesicles

Leukocyte MVs have been shown to express adhesion molecules PSGL-1, CD11b, ICAM-1) (63, 116, 218), IL-1β (219), tissue factor (220) and complement receptor 3 (221). In addition to containing IL-1β, MVs also carry active caspase 1 (222), an enzyme member of the inflammasome machinery needed to cleave proIL-1β and proIL-18 into their bioactive secreted forms. These inflammasome containing vesicles were shown to also mediate apoptotic cell death. EVs released by membrane budding of leukocytes are present in high amounts locally in inflamed tissues, and detected in the circulation. Immune cell activation, massive leukocyte death or lympho-proliferative processes have direct effects on the abundance of leukocyte-derived MVs. One general feature of circulating leukocyte-derived MVs is that their numbers in the circulation vary in broad ranges. This can reflect the ability of the triggered immune system to alter the number of its immune cells very dynamically depending on the innate and adaptive activating or suppressing signals. In line with this, the number of leukocyte-derived MVs is substantially elevated in haematological malignancies (223–225), after severe trauma (226) and in sepsis (227, 228). In addition, levels of circulating leukocytes-derived MVs increase with the presence of cardiovascular risk factors and in patients with atherosclerotic vascular diseases (►Table 2).

As for platelets (4), accumulating evidence supports molecular and functional heterogeneity of leukocyte-derived MVs (221, 229, 230). However, the scale and significance of leukocyte-derived MV diversity is probably underestimated, warranting further investigation. While cluster of differentiation antigens provide crucial information about the cellular origin of the MVs, currently no tools to distinguish between activation- and apoptosis-induced leukocyte MVs are available. Whether externalised PS on the surface of MVs is a specific indicator of apoptotic cell origin has not been definitively demonstrated.

7.1 Regulation of leukocyte MV production

Leukocyte activation is usually associated with increased cytoplasmic calcium levels. For example, the engagement of antigen-specific receptors (T cell receptor and B cell receptor) on lymphocytes, Fc gamma receptors on natural killer cells and mast cells, and cytokine and co-stimulatory receptors results in increased cytosolic calcium ion concentrations (231) (►Figure 5). *In vitro* leukocyte-derived MV release can be triggered by the calcium ionophore A23187 (232). Calcium signaling in immune cells is crucial for controlling a wide array of adaptive cell responses including proliferation, differentiation and various effector functions (e.g. cytokine production); this list can be extended to ”vesiculation” (i.e. MV release) as a process in the case of which elevated cytosolic calcium level is a major trigger (233–235)(also discussed in *Section 2* of this article). Under pro-inflammatory conditions (e.g. during infection or autoimmune diseases), immune cell activation is associated with a leukocyte-derived MV release. Importantly, in the extracellular space, the released MVs are present in concomitance with soluble pro-inflammatory mediators (e.g. cytokines), and thus, they can have combined effects on cells (►Figure 5). Indeed, T-cell derived MV-s were shown to synergise with TNF in inducing IL-8 expression by monocytes (236). The combined effects of different MVs with i) other EV subpopulations and/or ii) with soluble mediators are currently largely unexplored.

7.2 Role of leukocyte MV in inflammation

Inflammatory disease lesions are often hypoxic (232). Given that hypoxia is a known inducer of MV release (237), it may also contribute to the overall, pro-inflammatory condition-related induction of leukocyte-derived MV release. The released MVs in turn may amplify the inflammatory processes affecting different tissues (238). This is clearly exemplified during sepsis (a systemic inflammation) that leads to increased levels of circulating MVs released by granulocytes (227, 228). Due to their inherent heterogeneity overviewed recently (239), besides exerting pro-inflammatory effects on cells such as inducing cyclooxygenase 2, NF-κB or inducible NO synthase (240–243), MVs can also play regulatory/anti-inflammatory roles as well (244–248).

7.3 Evidence for leukocyte MVs in disease

Important evidence linking presence of leukocyte-derived MVs and disease *in vivo* comes from a study in which MVs were studied in plaque and plasma of 26 patients undergoing carotid endarterectomy. Atherosclerotic plaques contained MVs predominantly released by leukocytes (macrophages, lymphocytes and granulocytes) while in contrast platelet-derived MVs were found in plasma of the same patients (140). Further evidence connecting leukocyte-derived MVs with disease is that stable statin-treated heterozygous familial hypercholesterolaemia patients were found to have elevated numbers of lymphocyte- and monocyte-derived MVs. In addition, circulating MVs positive for T lymphocyte antigen determinants have been identified as markers of lipid-rich atherosclerotic plaques in familial hypercholesterolemia (249) (►Table 2). Accordingly, patients with high-grade carotid stenosis presented with high levels of leukocyte MVs (250) and a characteristic circulating leukocyte MV-signature containing lymphocytes, monocytes and activation markers (CD66b) in the systemic circulation reflected the formation of coronary thrombotic occlusions in patients with acute myocardial infarction (13), to whom monocyte MVs related to the long-term prognosis of cardiovascular death (251). Moreover, in a recent prospective five-year follow-up randomised, controlled, multicentre study, T lymphocyte-derived circulating MVs were found to be elevated in high cardiovascular risk subjects without clinical atherosclerosis who had a major cardiovascular event during the five-year follow-up period (252) (►Table 2). Finally, the role of leukocyte-derived MVs is further supported by the observation that circulating leukocyte-derived MVs are predictors of subclinical atherosclerosis burden in asymptomatic individuals (253) (►Table 2). Altogether, these findings suggest that leukocyte MV shedding relates to atherosclerotic CVD progression, providing a link between inflammation and thrombosis.

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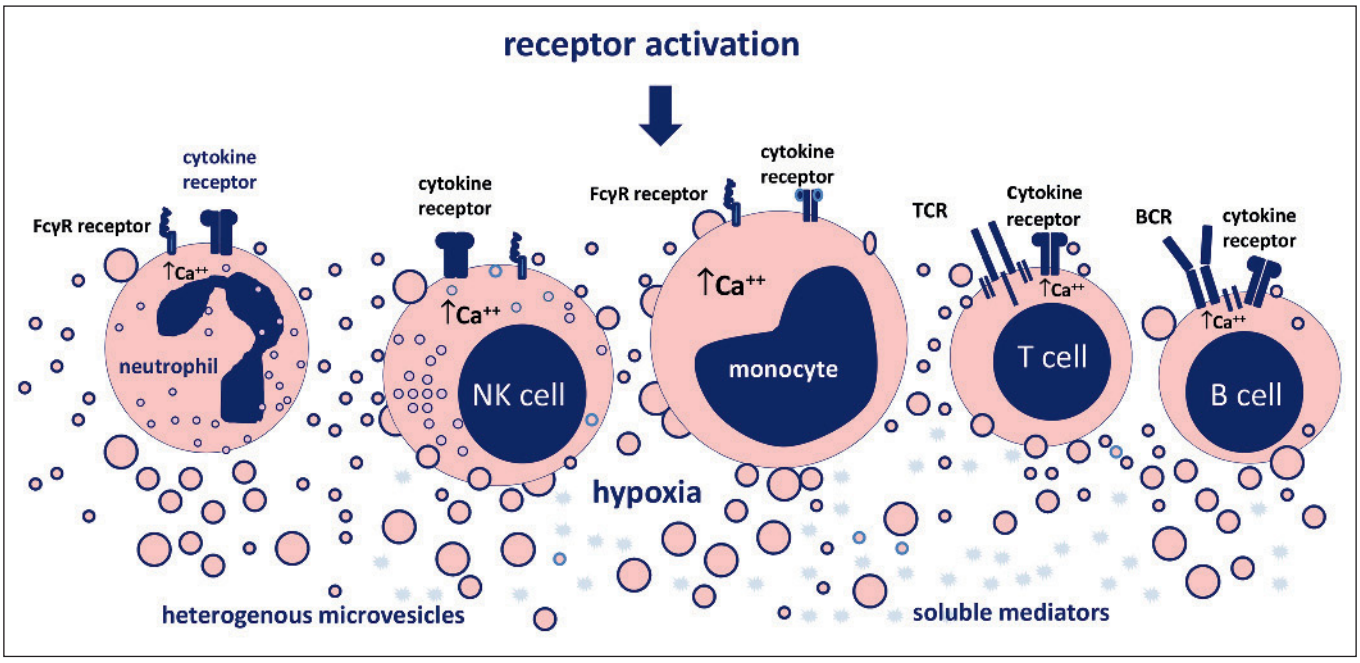

Figure 5: Leukocyte MV formation. MV release by leukocytes is triggered by i) engagement of various cell surface receptors by activating ligands that lead to increased cytosolic calcium ion concentration as well as ii) by hypoxic environment. MVs and conventional mediators (cytokines) are present simultaneously in the extracellular space and may exert combinatorial effects on cells. A few pathological conditions with confirmed role of leukocyte-derived MVs are listed.

7.3 Lymphocyte MVs

Apoptosis is associated with the release of MVs (254), and atherosclerotic plaques have been reported to be enriched in apoptotic lymphocytes (255). Hence, lymphocyte MVs have been detected in atherosclerotic plaques (256). Furthermore, circulating T lymphocyte-derived MVs are elevated in familial hypercholesterolemia patients (249). Thus, the link between lymphocyte derived-MVs and atherosclerosis is supported by numerous reports. T cell-derived MVs were shown to impair endothelial function (257). On the other hand, vascular hyperreactivity was also documented and lymphocyte-derived MVs were shown to inhibit angiogenesis (258). The LDL receptor has been proposed to mediate the uptake of T cell-derived MVs and influence the VEGF pathway (259).

Also, data suggest a link between lymphocyte-derived MVs and preeclampsia. In preeclamptic women T cell- and granulocyte-derived MVs were increased compared with normal pregnancy (235). Furthermore, synovial fluid $CD8^+$ T cell-derived MV profiles were found to be characteristic for rheumatoid arthritis (206) suggesting that leukocyte MVs may potentially hint at locally activated immune cell populations.

Surprisingly, there are very few reports published on circulating B cell-derived MVs, mainly related to B cell malignancies (224, 260–262).

7.5 Conclusion

MVs derived from leukocytes can play effector roles in the pathophysiological mechanisms of different diseases. Recent advances in immunology have identified high number of immune cell subsets. From the MV research perspective, MVs secreted by these immune cell subsets, represent a highly promising, however, yet minimally explored area of research that deserves further attention.

8. Red blood cell microvesicles

RBCs are 700,000 to 5.2 million to a microlitre of blood. Any stimulus of RBC vesiculation, such as calcium influx and spicule extension (263–270), may thus trigger a significant storm of RBC-derived MVs (271). Indeed, RBC MVs are amongst the most common circulating vesicles, and they are particularly elevated during intravascular haemolysis, in ST-segment elevation myocardial infarction (272, 273) related to myocardial damage (273), in sickle cell disease (50, 274–277), thalassaemia (277–279) and during blood pocket storage and aging (280–283), among others. RBCs are also thought to release MVs spontaneously during reticulocyte maturation (284) and upon *Plasmodium falciparum* replication (285).

RBC-derived MVs display many characteristics and activities common to other MVs, including externalisation of PS, with subsequent pro-coagulant effects (50, 279, 286–288), complement pathway activation (289) and a pro-inflammatory impact on blood vessels (290). RBC-derived MVs, like their mother cells, are also unique due to the quantity of iron that they carry. Each RBC contains about 250 million molecules of haemoglobin, each with four prosthetic haeme groups and four iron ions. Leakage of haemoglobin out of only 0.1 % RBC results in an increase up to 2 µM in plas-

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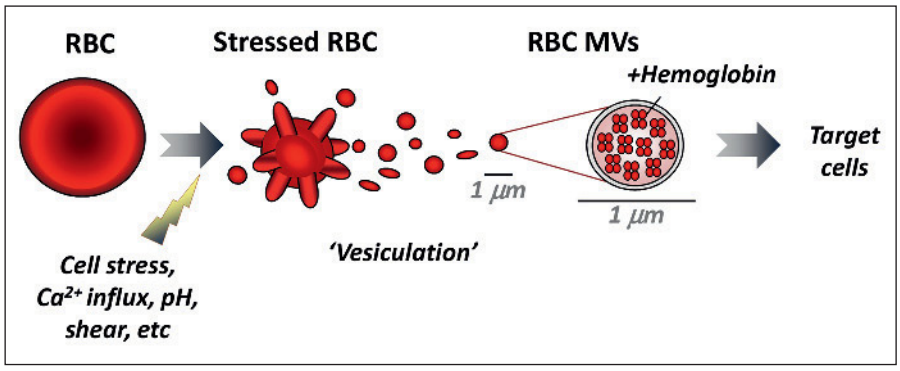

Figure 6: Red blood cell (RBC) MVs are unique transporters of haeme. RBCs release MVs under stress, probably from membrane buds called spicules. RBC MVs contain high amounts of haemoglobin originating from their parent cell cytoplasm. RBC MVs may thus transport unusually high amounts of haeme and iron, bringing these highly pro-oxidant molecules in close proximity of their target cell membranes, with a vast array of possible pathophysiological consequences, which remain to be explored.

ma haemoglobin. These significant concentrations of haemoglobin attracted attention with respect to vesiculation during intravascular haemolysis.

One relevant question relates to the retention of haemoglobin, haeme and iron by MVs during RBC vesiculation and haemolysis. MV depletion experiments using differential centrifugation or size fractionation of plasma suggest that 5% to 40% of extracellular haemoglobin associates with RBC membrane fragments during sickle cell disease (291), thalassaemia (279) and blood aging (281). Haemolysis, as defined today, is thus likely to comprise vesiculation as a mode of haemoglobin exit from RBC and a mode of presentation in plasma, beyond haeme association with classical partners such as haemopexin, haptoglobin, albumin and LDL.

A second question pertains to the redox state of MV-contained haemoglobin, haeme and iron. The pseudoperoxidase activity of these molecules is linked to the level of enzymatic control applied by porphyrins and globins onto the iron atoms. This may prove critical to the pathophysiological impact of RBC-derived MV. Healthy MVs may contain mostly intact haemoglobin, like inert RBCs. Pathological RBC MVs may contain excess haeme and trigger radical oxygen species production by vascular endothelial cells through TLR-4 signaling, like in sickle cell disease (291, 292).

Early biochemical analyses suggested that haemoglobin remains functional and able to exchange gases, judging by the NO-depleting and vaso-constrictive effects of RBC MV (281, 283, 291, 293). RBC MVs may contain a significant proportion of meth-haemoglobin (291), an oxidised metabolite prone to releasing free haeme. When RBC vesiculation is coupled to haemoglobin injury, MV may transport lipophilic, protein-free haeme (►Figure 6). This association would prove deleterious, as haeme embedded into membrane phospholipids is known to release iron and catalyze extensive oxidative degradation of nearby lipids and proteins. RBC MVs may thus sequester haeme away from classical recycling pathways, and mediate unique effects through a mixed load of haeme and lipid metabolites.

9. Limitations and future directions

During their formation process, MVs retain functional receptors, proteins, bioactive lipids, organelles and genetic material from the parental cells, and behave as active sensors, communicators, and effectors on their surrounding environment. There are a number of important points that need to be considered when designed and analysing experiments to investigate the presence and function of MVs:

- Numerous pitfalls could occur during their isolation procedure and their characterisation, such as the presence of contaminating protein. Therefore, one should rely on at least two different technical approaches to identify them, with consideration given to the limitations of each approach. We should also bear in mind that pharmacological interventions in patients can modulate MV circulating levels, either directly or indirectly.
- As highlighted in this review, there is an emerging role for MVs in vascular homeostasis and disease. However, further research is needed to pin down the molecular mechanisms involved to further elucidate MVs potential as therapeutic targets or vectors.
- To accelerate the progress with biomarker discovery, we need to enhance our understanding of the biological role played by specific cell derived MVs in haemostasis. For example, understanding the role of EMVs in vascular inflammation will enable

Abbreviations

APC: Activated protein C, CD40L: CD40 ligand, CRP-XL: cross-linked collagen related peptide, Del-1: developmental endothelial locus 1, EPCR: endothelial protein C receptor, EVs: extracellular vesicles, ICAM: intercellular cell adhesion molecule, IL: interleukin, LDLR: low-density lipoprotein receptor, LPS: lipopolysaccharide, miRNA: micro RNA, MVs: microvesicles, NF-kB: nuclear factor kappa-b, NTA: nanoparticle tracking analysis, PECAM: platelet endothelial cell adhesion molecule, pre-miRNAs: precursor microRNAs, PS: phosphatidylserine, RBC: red blood cell, ROCK: Rho-associated protein kinase, SOCE: Stored Operated Channels, SPRi: surface plasmon resonance imaging, TF: tissue factor, TNF-α: tumour necrosis factor α, TRAIL: TNF related apoptosis-inducing ligand, TRPS: tunable resistive pulse sensing, VEGF: vascular endothelial growth factor, VEGF-R2: VEGF receptor 2.

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routine quantification of EMVs in patients' samples to be interpreted in a more meaningful manner.
- MVs, and more generally extracellular vesicles, are becoming an intense area of research as therapeutic targets in heart diseases. In addition, improved characterisation of their content and surface molecule expression will aid in integrating information regarding their function in normal physiology with their pathological role.

Central to the above is the optimisation and standardisation of isolation and analysis of MVs and the use of robust experimental controls.

Disclosures

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