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AFFIDAVIT

I, the undersigned, Akhil Antony Konkoth, hereby declare that the work presented in this thesis is my own work, carried out under the scientific supervision of Professors Romaric Lacroix and Rory Koenen, in accordance with the principles of honesty, integrity, and responsibility inherent in the research mission. The research work and the writing of this manuscript were carried out in compliance with both the national charter of ethics for research professions and the Aix-Marseille University charter on the fight against plagiarism.

This work has not previously been submitted in France or abroad in an identical or similar version to an examining body.

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LIST OF PUBLICATIONS AND PARTICIPATION IN CONFERENCES

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RÉSUMÉ

Les microvésicules (MVs) sont une population hétérogène de vésicules extracellulaires liées à une membrane phospholipidique et constitutivement sécrétées par tous les types de cellules en cas de stimulation ou d'apoptose. Les MVs transportent de nombreuses cargaisons fonctionnelles sous forme de protéines, d'acides nucléiques et de lipides, ce qui leur permet de délivrer ces cargaisons fonctionnelles aux cellules cibles réceptrices. En raison de leur rôle dans les communications inter- et intracellulaires, les MVs ont été impliquées dans plusieurs conditions physiologiques et pathologiques, y compris l'hémostase et la thrombose, et servent de biomarqueurs potentiels dans des conditions pathologiques. Le concept initial des MVs comme entités strictement procoagulantes a été modifié avec la découverte de la capacité fibrinolytique de certains sous-ensembles de MVs d'origine leucocytaire et endothéliale. En effet, la fonction hémostatique en termes de propriétés procoagulantes et profibrinolytiques des MVs dépend de leurs cellules parentales et de l'état d'activation des cellules. Des études antérieures ont seulement tenté de décrire le potentiel procoagulant ou profibrinolytique de toutes les sous-populations de MVs mais n'ont pas pris en considération la contribution qui peut provenir de chaque sous-population de MVs. Dans ma recherche, j'ai effectué une évaluation complète du potentiel procoagulant et/ou profibrinolytique de sous-ensembles individuels de MVs provenant de cellules sanguines et vasculaires humaines dans le contexte d'un milieu inflammatoire ou d'une maladie septique. À cette fin, j'ai purifié non seulement des MVs générées *in vitro* à partir de neutrophiles, de monocytes, de plaquettes et de cellules endothéliales humaines, mais aussi différents sous-ensembles de MVs provenant de la circulation sanguine humaine (*ex vivo*) par séparation immunomagnétique (IMS). L'analyse fonctionnelle des MVs a démontré qu'elles véhiculent un potentiel procoagulant et/ou profibrinolytique et qu'elles présentent donc un équilibre coagulolytique distinct en fonction de leurs cellules parentales et du milieu inflammatoire ou de la maladie septique. Ainsi, alors que les MVs de monocytes étaient la source prédominante d'activité procoagulante, les MVs de neutrophiles présentaient le potentiel fibrinolytique le plus important. L'équilibre coagulolytique des MV peut représenter un biomarqueur prometteur et supérieur dans les contextes cliniques, reflétant le déséquilibre hémostatique dans les conditions de maladies thrombo-inflammatoires comme le sepsis, plutôt que les activités procoagulantes et profibrinolytiques des MV prises indépendamment. Les MVs ayant une activité fibrinolytique élevée sont particulièrement intéressantes non seulement parce qu'elles contrebalancent l'état procoagulant, mais aussi en raison de leur potentiel inhérent à être développées en tant qu'agent

thrombolytique pouvant être adapté aux besoins des patients individuels présentant une coagulopathie de sepsis.

SUMMARY

Microvesicles (MVs) are a phospholipid membrane-bound heterogeneous population of extracellular vesicles constitutively secreted by all the cell types upon stimulation or apoptosis. MVs carry numerous functional cargo in the form of various proteins, nucleic acids, and lipids, allowing them to deliver these functional cargoes to recipient target cells. Due to their role in inter- and intra-cellular communications, MVs have been implicated in several physiological and pathological conditions including hemostasis and thrombosis, and serve as potential biomarkers in pathological conditions. The initial concept of MVs as strictly procoagulant entities has been changed with the discovery of fibrinolytic capacity on certain subsets of MVs of leukocyte and endothelial cell origin. Indeed, the hemostatic function in terms of procoagulant and profibrinolytic properties of MVs depends on their parental cells and the activation status of the cells. Previous studies have only attempted to describe either the procoagulant or profibrinolytic potential of all the subpopulations of MVs but have not taken the contribution that may arise from each subpopulation of MVs into consideration. In my research, I performed a comprehensive evaluation of the procoagulant and/or profibrinolytic potential of individual subsets of MVs from human blood and vascular cells in the context of an inflammatory milieu or sepsis disease. Towards this end, I purified not only *in vitro* generated MVs from human neutrophils, monocytes, platelets, and endothelial cells, but also different MV subsets from the human blood circulation (*ex vivo)* by immunomagnetic separation (IMS). Functional analysis of MVs demonstrated that they convey procoagulant and/or profibrinolytic potential and thus display distinct coagulolytic balance according to their parental cells and inflammatory milieu or sepsis disease. Accordingly, while monocyte MVs were the predominant source of procoagulant activity, neutrophil MVs displayed the most fibrinolytic potential. The MV coagulolytic balance may represent a promising biomarker in the clinical settings reflecting the hemostatic imbalance in thromboinflammatoy disease conditions like sepsis rather than procoagulant and profibrinolytic activities of MVs taken independently. MVs with high fibrinolytic activity are particularly compelling not only because they counterbalance the procoagulant state but also due to their inherent potential to be developed as a thrombolytic agent that can be tailored according to the needs of individual patients with sepsis coagulopathy.

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LIST OF ABBREVIATIONS

ABC: ATP binding cassette

Apo-A1: apolipoprotein A1

ApoBDs: Apoptotic bodies

ARF6: ADP ribosylation factor 6

ARRDC1: arrestin domain-containing protein 1

AT: antithrombin

ATP: adenosine triphosphate

CAD: coronary artery disease

DAMPs: damage-associated molecular patterns

DDi: D-dimers

EDMVs: Endothelial cell-derived microvesicles

EGFR: epithelial growth factor receptor

EpCAM: epithelial cell adhesion molecule

ERK: extracellular signal-regulated kinase

ERK1/2: extracellular signal-regulated kinase 1/2

ESCRT: endosomal sorting complex required for transport

EVs: Extracellular vesicles

FGF: fibroblast growth factor

GPI: glycosylphosphatidylinositol

HMEC: human microvascular endothelial cell line

Hsps: heat shock proteins

ILVs: intraluminal vesicles

IBD: inflammatory bowel disease

iNOS: inducible NO synthase

ISEV: International Society on Extracellular Vesicles

LBS: lysine binding sites

SNARE: soluble N ethylmaleimide-sensitive factor activating protein receptor

LDL-C: low-density lipoprotein cholesterol

MAPK: mitogen-activated protein kinase

MDMVs: Monocyte-derived microvesicles

miRNA: micro-RNA

MMPs: matrix metalloproteinases

MLC: myosin light chain kinase

MVBs: multivesicular bodies

NDMVs: Neutrophil-derived microvesicles

NEC:necrotizing enterocolitis

NET: neutrophil extracellular traps

NF-kB: nuclear factor-kappa B

NLRs: nod-like receptors

NO: nitric oxide

p38MAPK: p38 Mitogen-activated protein kinase

PA: plasminogen activators

PAMPs: pathogen-associated molecular patterns

PC: phosphatidylcholine

PC3: prostate cancer

PDGF:platelet-derived growth factor

PDMVs: Platelet-derived microvesicles

PE: phosphatidylethanolamine

PGE_2: prostaglandin E_2

PL: phospholipids

PS: phosphatidylserine

ROCK: Rho-associated protein kinases

SOFA: sepsis-related organ failure assessment score

SIRS: Systemic inflammatory response syndrome

SIRT1: sirtuin1

SSC: Scientific and Standardization Committee

TBI: traumatic brain injury

TF: tissue factor

TFPI: Tissue Factor Pathway Inhibitor

TLRs: Toll-like receptors

TM: Thrombomodulin

tPA: tissue-type plasminogen activators

TXA2: thromboxane A2

uPA: urokinase-type plasminogen activators

VAMP3: vesicle-associated membrane protein 3

VEGF: vascular endothelial growth factor

PART I: BIBLIOGRAPHIC REVIEW

CHAPTER 1: INTRODUCTION TO EXTRACELLULAR VESICLES

1.1. DEFINITIONS

Extracellular vesicles (EVs) comprise a heterogeneous population of tiny phospholipid membrane-encapsulated vesicles released by all types of cells. Depending on the size, biogenesis, and composition, these EVs are generally categorized into three types. (Figure 1 and Table 1)

1.1.1. EXOSOMES

Exosomes are small lipid bilayer vesicles of endosomal origin with a size ranging from 30-150nm. Exosomes are constitutively generated intracellularly within the lumen of endosomes[1]. Endosomes mature into multivesicular bodies (MVBs); during this process, the invagination of late endosomal membranes develops exosomes as intraluminal vesicles (ILV) involving particular sorting machinery. MVBs subsequently fuse to the cell membrane to release exosomes into extracellular space or are directed to lysosomes for degradation. Formation of MVBs and the exosomes within them requires complex sorting machinery called endosomal sorting complex required for transport (ESCRT). The discovery of ESCRT machinery involved in MVB formation made it possible to unravel the mechanism behind exosome biogenesis. This machinery consists of four distinct multiprotein complexes (ESCRT)- 0,-I,-II,-III, along with associated accessory proteins (e.g., Alix and VPS4) that take part in various processes involving MVB formation[2]. The ESCRT complex works sequentially as ubiquitinated membrane cargo is captured and sorted on microdomains of MVBs by ESCRT-0, I, and II while ESCRT-III performs budding and dissociation of ILVs. The formation of exosomes independent of ESCRT complex proteins is also a possible scenario and this is supported by the observation that MVBs that are CD63+ can still be generated even after the depletion of four complex proteins in the ESCRT system. Exosomes are generally enriched with tetraspanins such as CD63, CD81, and CD9 which were widely considered exosome-specific markers, however, other EVs including microvesicles (MVs) such as MVs of platelet origin have been found to carry these tetraspanins [3].

1.1.2. MICROVESICLES

MVs comprise another heterogenous class of tiny bilayer lipid particles resulting from the budding directly from the plasma membrane and having a size range of 100-1000nm. MV biogenesis relies on the rapid redistribution of several surface phospholipids (PL) and the well-organized contraction of actomyosin filaments. The functional role of MVs depends on the cellular origin and the activation status of the parental cells. Whilst the majority of the MVs in the peripheral blood are represented by the platelet origin, a small percentage of MVs represented by leukocytes, endothelial, and erythrocyte origin [4, 5, 6].

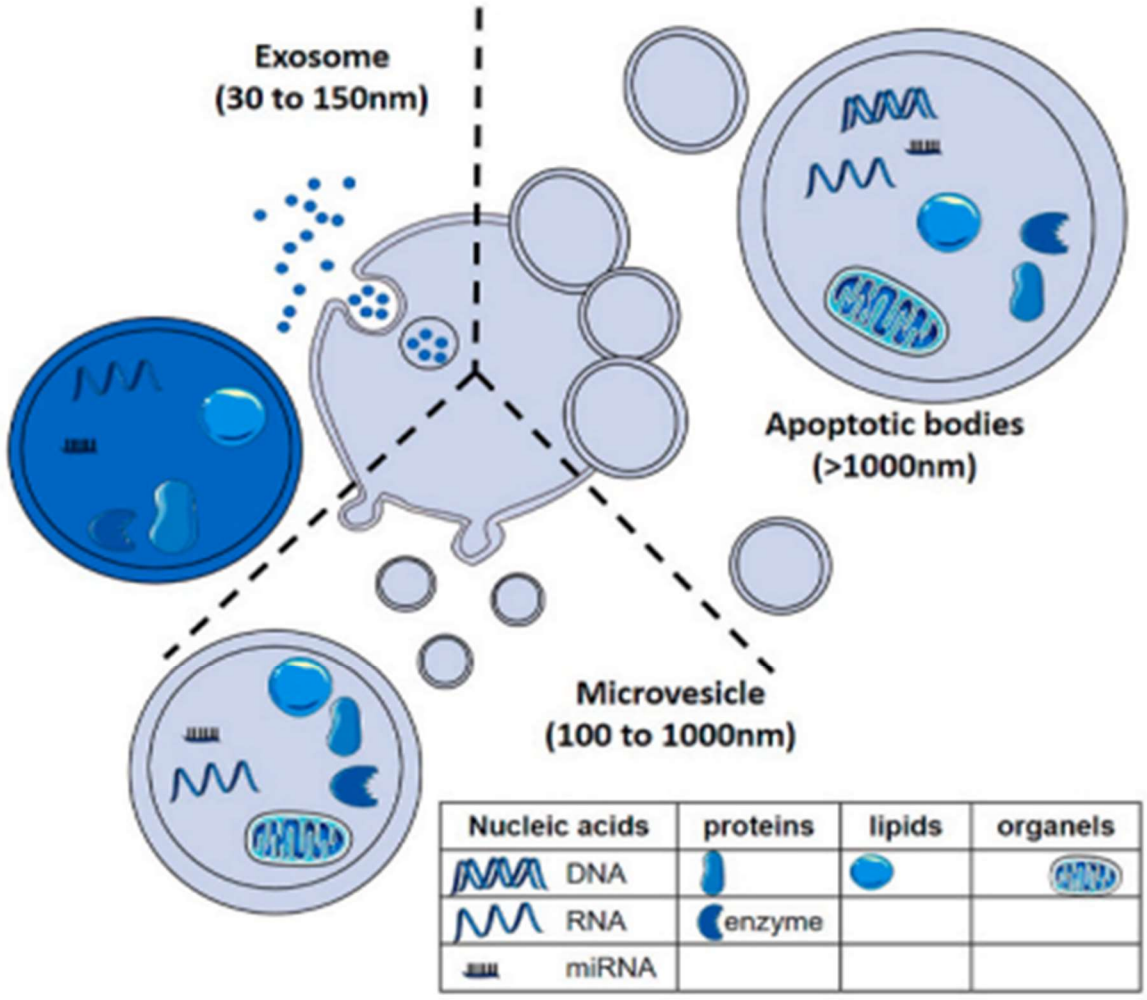

Figure 1: Heterogeneity of extracellular vesicles

(Excerpt from: Konkoth et al., Atherosclerosis, 2020)

1.1.3. APOPTOTIC BODIES

Apoptotic bodies (ApoBDs) are a type of extracellular vesicles secreted by the cells undergoing the process of apoptosis[7]. Unlike exosomes and MVs, apoptotic bodies are large vesicles and appear in significantly varying sizes (1000nm-2µm). Despite being formed when cells undergo apoptotic disintegration, ApoBDs play a crucial role in intercellular communication as they contain a variety of cargos such as intact organelles, degraded proteins, DNA fragments, and cytosolic components[8]. However, the impaired release of ApoBDs or inefficient clearance can lead to inflammation and autoimmunity[9].

Table 1: Characteristics of extracellular vesicles

	Exosomes	**Microvesicles**	**Apoptotic bodies**
Size	30-150nm	100-1000nm	1000nm-2µm
Origin	Exocytosis by fusion of multivesicular bodies	Budding of plasma membrane	Apoptosis
Composition	Nucleic acids, proteins, and lipids	Nucleic acids, proteins, and lipids	Nucleic acids, proteins, lipids, and cell organelles
Methods of detection	Electron microscopy	Flow cytometry Electron microscopy	Flow cytometry Electron microscopy
Preferred methods of quantification	Tunable resistive pulse sensing (TRPS) Nanoparticle tracking analysis (NTA)	Tunable resistive pulse sensing (TRPS) Nanoparticle tracking analysis (NTA)	Flow cytometry
Methods of purification	Ultracentrifugation Density gradient centrifugation using sucrose Polymer-based precipitation Immuno-magnetic separation	Ultracentrifugation Size exclusion chromatography (SEC) Immuno-magnetic separation	Centrifugation

1.2. NOMENCLATURE

Although the first use of the term ‘extracellular vesicles’ initially appeared in the literature in 1971, the standardization in the reporting of what generally constitutes EVs continued to be a greater challenge for several decades because of the inconsistent use of exosomes and microvesicles in the literature. The need for standardization or scientifically established

appropriate nomenclature to address what constitutes EVs was required as several published works in the literature failed to include proper characterization of EVs. Towards this end, in 2003, a Scientific and Standardization Committee (SSC) on vascular biology within the ISTH emphasized the need for standardization for the use of MVs in clinical settings[10]. International Society on Extracellular Vesicles (ISEV) then agreed upon, based on the scientific consensus recommendation, to adopt the term 'extracellular vesicles', as the generic term to indicate lipid-bound vesicles that are naturally secreted by the cells and cannot replicate.

Since clear differentiation of exosomes or MVs based on surface markers or biogenesis is difficult, based on the recommendation it is appropriate to use:

- Physical characteristics such as size ("small/large EV") with defined ranges, for example, sizes smaller than 100 or 200 nm corresponding to small vesicles, and greater than 200 nm would correspond to large vesicles or the density (by defining a low, medium or high density)

-The antigenic composition (EV containing CD63 or CD81 or even EVs labeled with Annexin V)

- Cellular origin or experimental vesiculation conditions (podocyte EVs, hypoxic EVs, large oncosomes, LO)

In this thesis, we chose to use the term 'MVs' to delineate large vesicles, that can be labeled with Annexin V or carry a specific marker that represents their cellular origin but for the sake of publication, I used the term 'EVs' in my research work.

1.3. BIOGENESIS AND RELEASE OF MICROVESICLES

MV biogenesis (Figure 2) involves outward blebbing of the plasma membrane surface that subsequently leads to the release of the formed vesicles into the extracellular space. This budding precedes a rapid reorganization of lipid rafts in the cell membrane and peripheral cytoskeleton resulting in different protein and lipid enrichment while modifying the membrane curvature. This unique enrichment process might explain the differences in characteristics between MVs and exosomes.

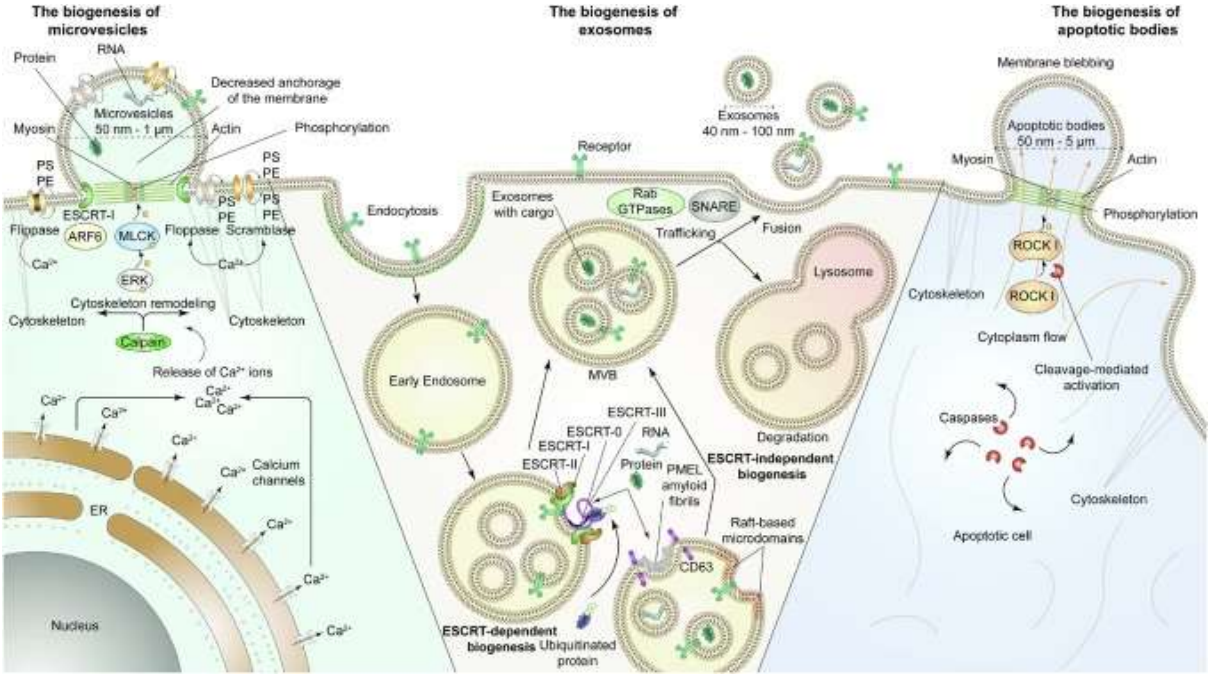

Figure 2: Biogenesis of extracellular vesicles

Legend: ARF6: ADP ribosylation factor 6; ERK: extracellular signal-regulated kinase; ESCRT: endosomal sorting complex required for transport; MLC: myosin light chain kinase; MVB: multivesicular body; PE: phosphatidylethanolamine; PMEL: premelanosome protein; PS: phosphatidylserine; ROCK: Rho-associated protein kinase; SNARE: soluble N ethylmaleimide-sensitive factor activating protein receptor.

(Excerpt from: Tarasov et al., Semin Cancer Biol, 2019)

1.3.1 MECHANISM OF BIOGENESIS

The process of MV generation entails two crucial mechanisms: dynamic shuffling of membrane lipids leading to membrane asymmetry and peripheral cytoskeleton reorganization leading to membrane budding.

1.3.1.1. REMODELLING OF MEMBRANE LIPIDS

The typical eukaryotic bilayer cell membrane has a thickness of 5nm and contains hundreds of different kinds of lipids that are distinctively distributed in the inner and outer leaf membranes. The cell membrane is primarily composed of glycerophospholipids, sphingolipids, and cholesterol but also contains proteins including transmembrane-proteins, caveolins, and glycosylphosphatidylinositol (GPI)-anchored proteins. Cholesterol and lipid molecules in the plasma membrane help to maintain the membrane fluidity and stability [11, 12].

The asymmetrical distribution of various lipids is an important characteristic of plasma membranes. This asymmetry is maintained by multiple factors and has important functional

consequences. Normally, cationic phospholipids such as phosphatidylethanolamine (PE), phosphatidylserine (PS), and phosphoinositides are located in the inner leaflet, whereas phosphatidylcholine (PC) and sphingomyelin are distributed in the outer leaflet of the plasma membrane. Conversely, cholesterol is distributed in both leaflets but more on the outer leaflets [11, 12]. This asymmetrical distribution of lipids within the plasma membrane is an energy-dependent process and therefore requires ATP hydrolysis. It includes 1) ATP binding cassette (ABC) proteins that actively translocate and shuttle phospholipids across both membrane leaflets and 2) ATP-dependent flippases proteins that specifically 'flips' certain phospholipids to the inner leaflet 3) floppases that are ATP-dependent regulate flip-flop movement of phospholipids across the membrane leaflets 4) scramblases disturb the normal distribution of phospholipids and allows randomization [11, 12, 13].

Perturbance in these proteins helps in dissipating the membrane asymmetry and has been implicated in several pathological processes including several hemostatic conditions. For example, defective scramblase activity in platelets accompanied by a functional failure to expose the PS to the outer plasma membrane is the central molecular characteristic of Scott's syndrome, a rare bleeding disorder associated with impaired procoagulant activity in certain types of blood cells[14, 15, 16]. This Ca^{2+}-dependent scramblase activity is essential for PS exposure to the external leaflet and is critical for the assembly of the tenase complex and prothrombinase complex that precedes the initiation of the coagulation cascade. Interestingly, this deficient scramblase activity has also been involved in the MV release. Further investigations led to the identification of the molecule responsible for the deficient scramblase activity. Recently, a transmembrane protein called TMEM16F was shown to be involved in the defective phospholipid scrambling activity in platelets in Scott's syndrome patients [17]. In these patients, the TMEM16F gene carried homozygous mutations (IVS12-1G→T and IVS6 + 1G→A), resulting in the absence of the exon 13 region from the TMEM16mRNA due to premature termination of gene translation[17, 18]. This phospholipid scrambling activity emphasizes the critical role of platelets in thrombotic conditions [19].

1.3.1.2. MEMBRANE PROTEIN ASSEMBLY

It has been demonstrated that MVs utilize endosomal machinery for the budding of plasma membranes. Like viral budding, the formation of MVs requires a special interaction of arrestin domain-containing protein 1 (ARRDC1) with TSG101 and some other protein components of

the ESCRT system at the cell membrane [20]. The translocation of TSG101 protein to the membrane mediated by ARRDC1 prompts the release of MVs that are catalyzed by VPS4 ATPase. This results in the presence of both ARRDC1 and TSG101 in released MVs but not exosomal markers[20].

1.3.1.3. MEMBRANE LIPID ASSEMBLY

Recruitment of various lipids to the membrane and unequal distribution of lipids initiates membrane curvature and release of MVs[21]. The unequal distribution of lipids at the surface is mediated by the recruitment of enzymes like lipases and flippases[22].

1.4. SELECTIVE ENRICHMENT OF CARGO TO MICROVESICLES

MVs contain myriad bioactive molecules such as cargo that reflect the type of cells and the activation status.

1.4.1 PROTEIN CARGO

In addition to the TSG101 and ARDDC1 regulatory proteins involved in the vesiculation process, selective enrichment of MVs with various cargos facilitated by small GTPases like ARF6 (ADP ribosylation factor 6) has been discovered. ARF6-dependent pathway promotes shedding of MVs enriched with MHC class I proteins, integrin β1, and membrane type-1 matrix metalloprotease (MT1-MMP), along with ARF6 [23]. The selective enrichment promoted by ARF6 also incorporates SNARE proteins like vesicle-associated membrane protein 3 (VAMP3) to the budding MVs[23]. Experiments conducted on tumor cells under hypoxic conditions demonstrated the involvement of hypoxia-inducible transcription factors in MV biogenesis. Elevated expression of RAB22A in hypoxia conditions induces increased MV shedding from tumor cells[24]. Moreover, cellular stress or injury has been shown to induce the release of endogenous danger signals such as ATP. Stimulation with endogenous ATP drives packaging TNF into budding vesicles in macrophages, a process that involves sphingomyelinase sphingomyelinase-dependent pathway[25].

1.4.2 NUCLEIC ACID CARGO

Besides protein cargos, significant work has demonstrated the presence of various forms of nucleic acids enriched in MVs. A recent study done by Vagner et al. demonstrated that MVs isolated from prostate cancer cell lines and patient plasma contained intact fragments of chromosomal DNA and the presence of DNA fragments reflected genetic aberration within the cells[26]. In cultured prostate cancer cell lines and experimental prostate cancer mice models, Reis-Sobreiro et al. demonstrated that disruption of inner nuclear membrane protein emerin resulted in the release of MVs that contain nuclear DNA[27].

Similar to the nuclear DNA cargo, recent studies have reported MVs with micro-RNA (miRNA) cargo. Clancy et al. described a molecular trafficking pathway that transports miRNAs to MVs mediated by activation of the ARF6 pathway[28]. The authors demonstrated that activation of ARF6 I tumor cell lines facilitates the active trafficking of miRNAs to the budding MVs by linking them with a nuclear exporter receptor called exportin-5. In another study, Lee et al. delineated a pathway linking caveolin-1 and the direct incorporation of miRNAs into MVs under oxidative stress conditions[29]. Lung epithelial cells under oxidative stress triggered interaction between phosphorylated caveolin-1 and an RNA-binding protein hnRNPA2B1 leading to shuttling of miRNAs to the MVs.

Taken together, these studies demonstrate that under normal and pathologic conditions formation of MVs is promoted, and depending on the cells and type of stimuli, a specific niche of cargo gets incorporated into the MVs providing them functional capacity during cell-cell communication.

1.5. FUNCTIONS OF MICROVESICLES

Although MVs were initially described as mere cellular waste, a mechanism to remove or recycle cellular products, now they are considered key players in several biological functions. MVs role in the regulation of vascular tone, inflammation, angiogenesis, cell survival, and immune cell modulation in the context of the cardiovascular system will be briefly discussed in this session and a comprehensive description of MVs role in hemostasis and sepsis pathophysiology will be detailed in chapter 2 and 3 (**MVs role in the context of atherosclerosis have been addressed in a review published during my thesis**).

1.5.1. ROLE IN VASCULAR TONE

MVs participate in the regulation of vascular tone by modulating the production of nitric oxide (NO) by endothelial cells[30]. It has been demonstrated that some MVs contain NO synthase or inducible NO synthase (iNOS) and they take part in vascular dysfunction by producing a large amount of NO [31]. Although NO is essential for maintaining vascular homeostasis[32], sustainable and excess production of NO by various cellular sources as a result of systemic inflammation leads to endothelial damage[32]. Conversely, mice mesenteric arteries exposed to MVs isolated from diabetic patients have been shown to impair vascular dilation[33], while endothelial MVs reduced acetylcholine-induced endothelium-dependent smooth muscle cell relaxation of the aortic ring as a consequence of impaired NO production and increased superoxide production[34]. Thromboxane A2 (TXA2) is another molecule that can modulate vascular tone. While NO is crucial for vascular relaxation, TXA2, a metabolite of arachidonic acid, contributes to vasoconstriction and hemostasis[35]. Endothelial transfer of arachidonic acid can induce production of TXA2 on platelet MVs and incubation of these MVs with aorta and pulmonary sections induced contraction.

1.5.2. ROLE IN INFLAMMATION

The potential role of MVs in immune modulation and vascular inflammation (Figure 3) has been reported in several studies. For example, neutrophil MVs released after an inflammatory stimulus have been shown to induce the secretion of proinflammatory cytokines like IL-6 and the expression of ICAM-1, VCAM-1, and E-selectin by the endothelial cells[36]. Treatment with neutrophil MVs can upregulate various proinflammatory genes in endothelial cells such as IL-1β, CCL3L3, and STAT3[37]. Endothelial cells displayed increased ICAM-1 expression that was correlated with the uptake of neutrophil-derived MVs and subsequent transfer of miR-155 to endothelial cells in a murine atherosclerosis model. This increase in endothelial ICAM-1 expression enhanced local inflammation in the vascular wall and supported monocyte transendothelial migration[38]. Similarly, platelet-derived MVs showed significant proinflammatory potential by delivering the chemokine RANTES/CCL5 directly to endothelial cells and enhanced monocyte accumulation to atherosclerotic endothelium in a mice carotid artery ex vivo perfusion model[39]. Smooth muscle cells acquired a proinflammatory phenotype with increased IL-6 secretion when incubated with platelet MVs and facilitated strong monocytic adhesion [40, 41]. Besides, increased adherence of neutrophils to endothelial cells that were pretreated with platelet MVs was observed in a flow model. In a recent study, MVs from

activated monocytes and platelets were shown to induce oxidative stress endothelial cells and upregulated proinflammatory markers such as TNF-α, IL-6, and NF-κB and also facilitated monocyte adhesion through ICAM-1 expression[42]. Finally, EVs derived from the endothelial cells following the treatment with ox-LDL were able to induce monocyte activation to a proinflammatory phenotype through miR-155 delivery to monocyte cells[43].

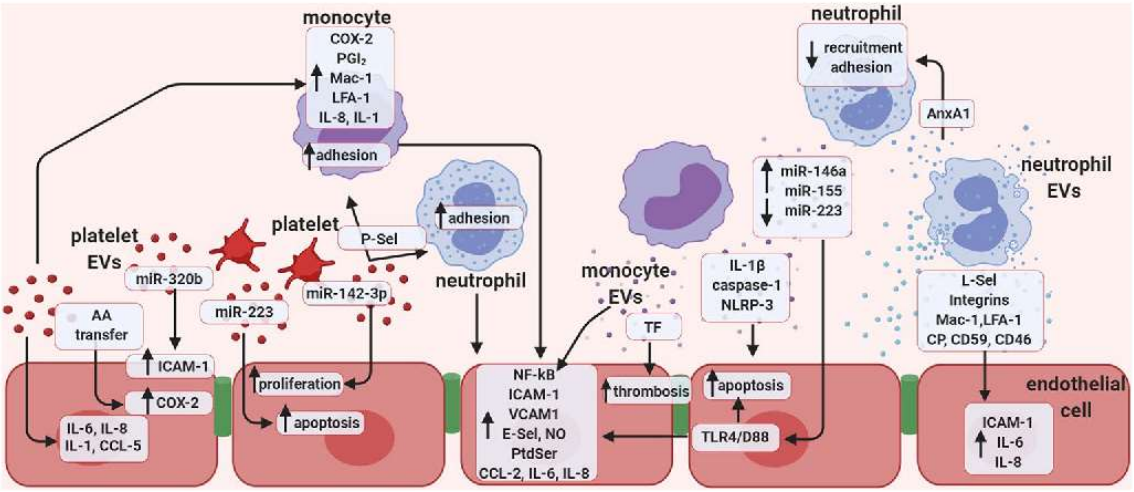

Figure 3: Functions of microvesicles in inflammation

(Excerpt from: Oggero et al, Front. Pharmacol., 2019)

1.5.3. ROLE IN ANGIOGENESIS

A recently published review from our team elucidated the complex role of MVs in the angiogenesis process[44]. Under normal physiological conditions, the process of angiogenesis involves several steps. However, as illustrated in Figure 4, the key process of angiogenesis modulated by MVs can be summarised in 3 steps.

1) Extracellular matrix degradation and remodeling – regulated by several proteases enriched by MVs [45].

2) Recruitment and Differentiation of angioblasts – as exemplified by Leroyer et al MVs induced differentiation of endothelial progenitor cells, conveyed by an oxidative stress-dependent mechanism [46].

3) The migration and proliferation of endothelial cells induced by MVs as demonstrated in using *in vitro* matrigel and *in vivo* mice models [47, 46].

In addition to this, various types of MVs are known to carry various bioactive proangiogenic molecules, ligands, and micro RNAs (miRNAs) such as vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), and tissue factor (TF) [48, 49, 50, 51, 44].

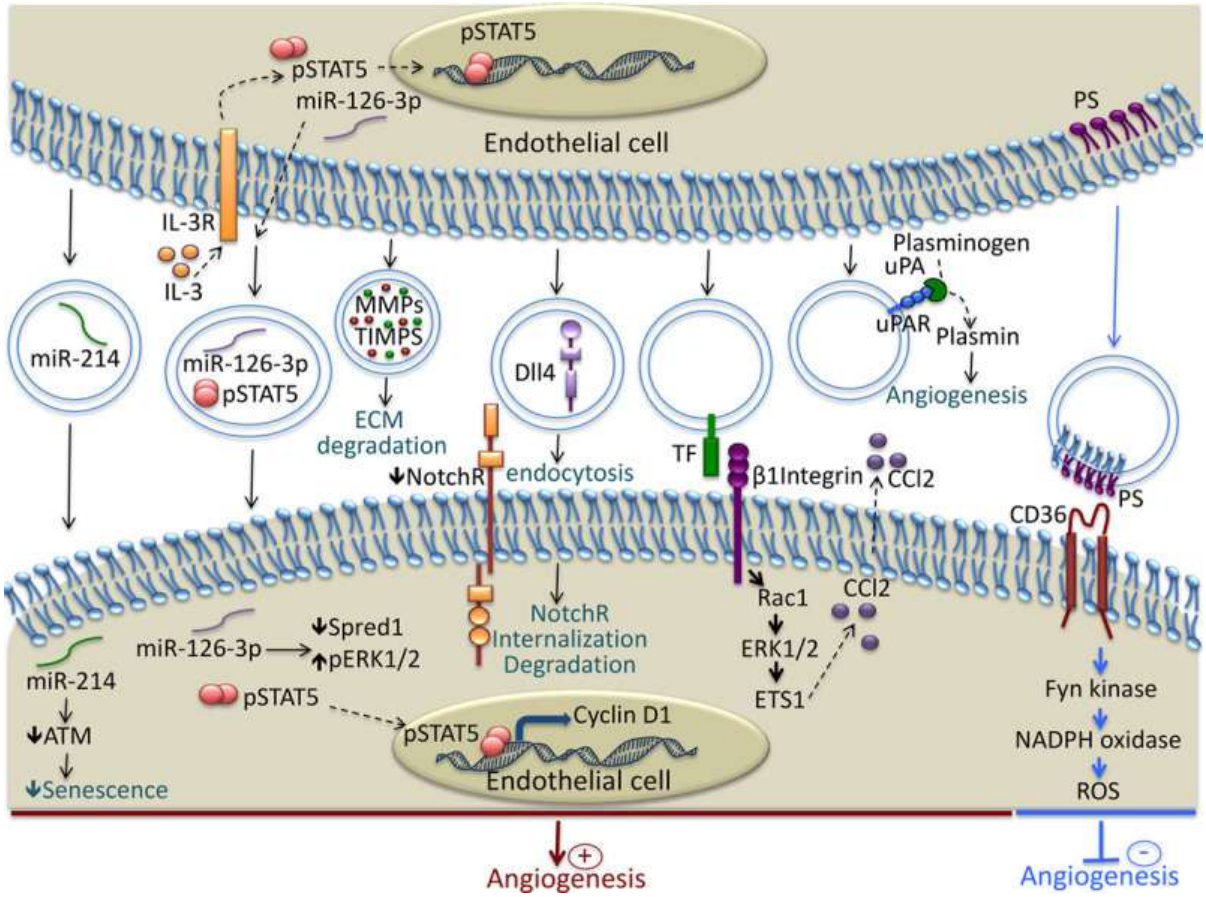

Figure 4: Mechanisms involved in the modulation of angiogenesis by MVs

ATM: ataxia telangiectasia mutated; CCl2: chemokine c-c motif ligand 2; Dll4: Delta-like 4; ECM: extracellular matrix; ERK1/2: extracellular signal-related kinase 1 and 2; ETS1: avian erythroblastosis virus E26 homolog-1; IL-3R: interleukin-3 receptor; MMPs: matrix metalloproteinases; Notch R: Notch receptor; PS: phosphatidylserine; Rac1: Ras-related C3 botulinum toxin substrate 1; ROS: reactive oxygen species; TF: tissue factor; TIMPS: tissue inhibitor of metalloproteinases; uPA: urokinase plasminogen activator; and uPAR: urokinase plasminogen activator receptor.

(Excerpt from: Todorova et al., Circ Res, 2017)

1.5.4. ROLE IN CELL SURVIVAL

Cells can employ MVs as tools to protect themselves from harmful elements to survive. Polymorphonuclear leukocytes and K562 cells are known to employ the vesiculation process as a mechanism for the removal of complement attack complex to rescue them from lysis[52, 53, 54]. This vesiculation process also involves the removal of FAS ligands through MVs by tumor cells as a way to evade activated T cell-induced apoptosis[55]. Similarly, endothelial cells were able to protect themselves from apoptosis through the secretion of caspase-3 MVs thus preventing Caspase-3 accumulation in the cells. Certain cancer cells use this vesiculation mechanism to achieve rapid removal of chemotherapeutic agents like doxorubicin thus contributing to treatment resistance[56]. Chemoresistance can also be achieved by intercellular

exchange and rapid distribution of ABCB1 protein released through MVs by cancer cells following chemotherapy[57]. Additionally, platelet-derived MVs have been shown to promote the survival and differentiation of neuronal stem cells thus driving neurogenesis and functional improvement in a rat brain ischemia model.

1.5.5. ROLE IN IMMUNE CELL MODULATION

The ability of MVs to modulate immune response to promote immune tolerance has been reported in several studies. During normal pregnancy, trophoblast evades the maternal immune system by constitutively secreting FasL through MVs[55]. Antigen-presenting cells like B lymphocytes and splenic antigen-presenting dendritic cells can secrete MVs containing MHC I and MHC II molecules, and these vesicles can induce an antigenic response in T cells [58, 59]. MVs transferred to resting dendritic cells from activated dendritic cells were capable of generating MVs and elicited T cell response through antigen presentation [60]. This antigen presentation can also be seen in platelet-derived MVs as they can participate in T cell immune modulation through molecules like CD154 [61]. Conversely, MVs generated from endothelial cells elicited T cell response indirectly by inducing plasmacoid dendritic cells[62].

1.6. THERAPEUTIC POTENTIAL OF MVS

MVs carry numerous bioactive molecules on their surface as well as within them. Generally, MVs carry surface molecules from their parental cells and their cytosolic compartment contains various bioactive molecules like proteins, nucleic acids, and lipids. By delivering these bioactive molecules to recipient cells (Figure 5), MVs can mediate either normal physiologic response or facilitate the pathophysiological progression of diseases including cardiovascular pathologies. Therefore, MVs appear to be a potential therapeutic tool to target various cell types to manipulate their functional activities to alter the course of pathological conditions.

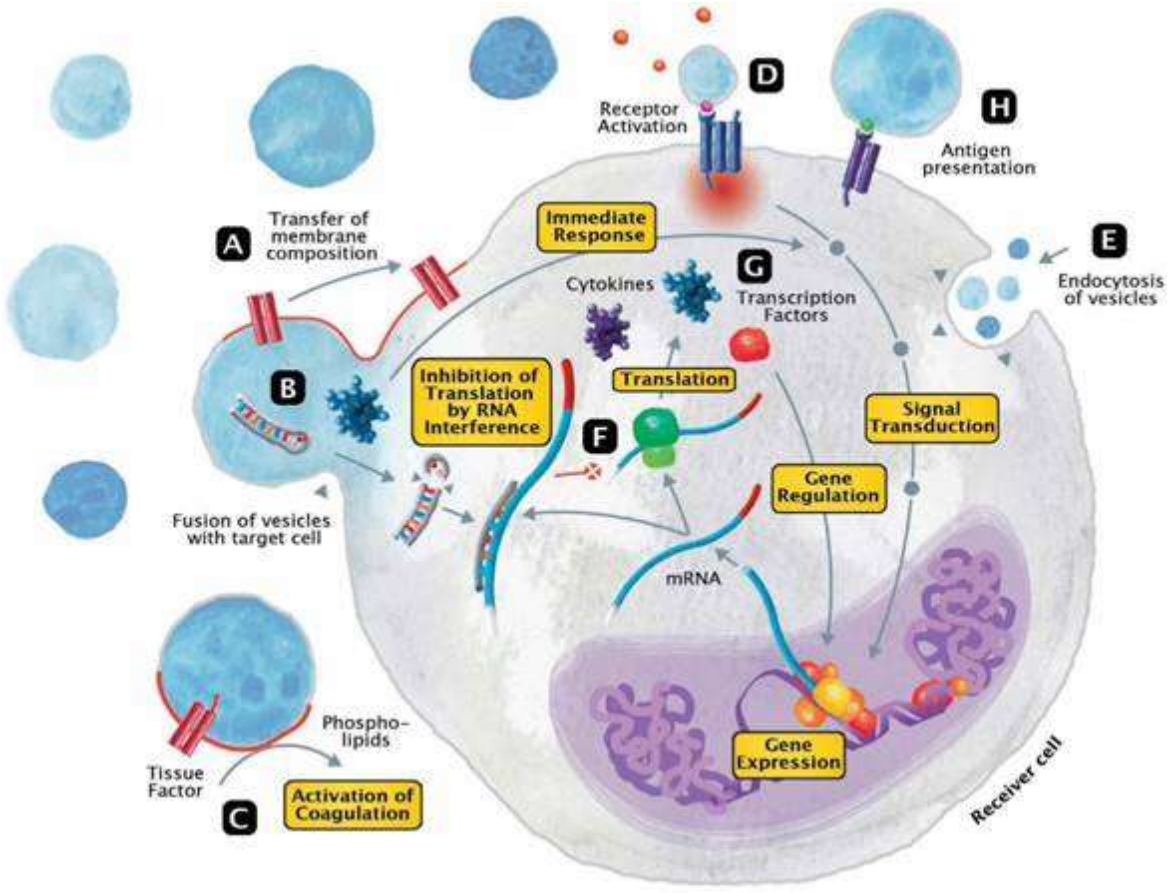

Figure 5: Modes of interaction of MVs with recipient cells

A) MVs modify the membrane composition of recipient cells upon fusion
B) Deposition of bioactive cargo molecules from MVs to the cytoplasm of recipient cells
C) MVs disseminate procoagulant molecules such as TF and PS from cells under systemic inflammation and cell activation
D) Components of MVs function as cellular ligands and modify cellular response
E) Internalization of MVs by the recipient cells to modulate cellular functions
F) Modification of cellular transduction machinery by delivering RNAs
G) Modification of posttranslational machinery by delivering miRNAs
H) Trigger cytokine release

(Excerpt from: Raeven et al., Theranostics, 2018)

The prospects of utilizing MVs as therapeutic agents in clinical settings have been explored using several in vitro and in vivo studies. MVs obtained from various biological fluids and in vitro conditions have shown to be promising therapeutic agents to treat several diseases[63]. MVs from plasma contain several bioactive molecules that have been demonstrated to exert anti-inflammatory, anti-oxidant, anti-apoptotic, and pro-angiogenic properties on several conditions such as cardiovascular, metabolic, and orthopedic problems[64]. Vicencio et al demonstrated that exosomes derived from the plasma modulate several protein kinase signaling pathways

including extracellular signal-regulated kinase 1/2 (ERK1/2), p38 mitogen-activated protein kinase (p38MAPK), and TLR-4 to exert cardioprotective function in an *in vivo* rat model of ischemia-reperfusion[65]. In a canine *ex vivo* model, plasma-derived engineered exosomes had a beneficial role in tendon repairing and healing by mitigating the inflammatory effect by reducing TGF-β and IL-6 expression and by increasing the expression of type III collagen from fibroblasts[66]. MVs from maternal breast milk contain several functional cargos that can modulate the functional aspects of recipient cells to improve infant health[67]. Exosomes containing miR-148a-3p from human breast milk demonstrated a mitigating effect on inflammation in a mice model of necrotizing enterocolitis (NEC) by significantly downregulating the expression of p53 and upregulating the expression of sirtuin 1 (SIRT1) in intestinal epithelial cells[68]. Similarly, miR-148a-3p contained in human milk exosomes mediated an anti-tumor effect by targeting PTEN and DNMT1 expression in an *in vitro* colon cancer cell model [69].

MVs obtained from culture conditions also have therapeutic potential. Most of the studies involved with in vitro obtained MVs come from mesenchymal stem cells (MSCs) in culture. Bone marrow MSC-derived MVs (BM-MSC-MVs) promote cartilage regeneration in osteoarthritis by inhibiting inflammatory mediators such as TNF-α, and pro-inflammatory interleukins and by upregulating the production of proteoglycans and type II collagen[70]. BM-MSC-MVs had a beneficial role in tendon healing and repair in rats by stimulating the growth of tendon stem cells and reducing the number of proinflammatory macrophages [71]. miR-146a-3p contained BM-MSC-MVs exerted an anti-inflammatory effect by significantly inhibiting the suppressing NF-kB activity in a rat model of inflammatory bowel disease (IBD)[72]. EVs from adipose tissue-derived MSCs (AD-MSC-MVs) mitigated the inflammatory effect on senescence by reducing the production of IL-6 and prostaglandin E_2 (PGE_2) in osteoarthritis[73]. In a mice model of allergic asthma, AD-MSC-MVs reduced pro-inflammatory mediators like IL-4 and IL-5 in lung tissue[74].

In a mice model of traumatic brain injury (TBI), exosomes enriched in miR-873a-5p significantly attenuated LPS-induced pro-inflammatory changes in microglia by reducing phosphorylation of ERK and NF-kB signaling[75]. Similarly, miR-124 enriched exosomes from microglia induced an M2 phenotype in microglia by inhibiting TLR-4 signaling and induced hippocampal neurogenesis in a rat TBI model[76].

1.7. PHARMACOLOGICAL INHIBITION OF MVs AS THERAPEUTIC TARGET

As MVs have been implicated in the development and pathophysiological progression of several diseases manipulating MV secretion utilizing pharmacological interventions appears to be an excellent strategy for therapeutic modulation of pathological conditions. So far, several pharmacological agents have been developed and tested as therapeutic tools to block the release of certain subpopulations of MVs. Recognizing and inhibiting specific subpopulation(s) of MVs that are involved in the pathological process but simultaneously avoiding adverse effects on normal physiological processes is important in clinical settings. The main pharmacological agents that have been tested for inhibiting MV secretion include Calpeptin, Y27632, Pantethine, Imipramine, and GW4869[77].

Calpeptin is a protease inhibitor that specifically blocks calpain, a calcium-dependent that is involved in cytoskeleton remodeling in cellular processes like apoptosis, cell proliferation, migration, and tumor metastasis. Due to calpain's role in cytoskeleton remodeling, they appear to promote MV release from cells. Fox et al demonstrated that intracellular activation of calpain upon platelet stimulation with collagen or thrombin increased procoagulant MV shedding, and this increased MV secretion was blocked by Calpeptin treatment[78]. More recently, MV shedding by activated platelets by various agonists like collagen, thrombin, calcium ionophore A23187, and prion proteins was inhibited by Calpeptin[79]. Inhibition of MV release by blocking calpain has been explored in other cell types including cancer cells.

Y27632 is a cell-permeable selective inhibitor of Rho-associated protein kinases (ROCK), a serine-threonine kinase involved in the regulation of cell movement and shape by modulating cytoskeletal remodeling. The potential role of Y27632 in the inhibition of MV shedding by blocking ROCK kinases has been evaluated using several cell models including cancer cells and endothelial cells. In a breast cancer and ovarian cancer cell model, increased MV secretion by constitutive activation of RhoA was significantly reduced by Y2763[80]. A similar inhibition of MVs was observed in the thrombin-stimulated human microvascular endothelial cell line (HMEC). ROCK-mediated substantial increase in MV shedding by thrombin-stimulated HMEC was prevented by pre-incubation with Y27632[81].

Pantethine is a natural derivative of pantothenic acid (Vitamin B5) that is synthesized in the body and has a tremendous effect on lipoprotein metabolism by lowering cholesterol and triglyceride synthesis. As reported by several studies, Pantethine has significant clinical

significance due to its effect on reducing serum cholesterol, triglycerides, low-density lipoprotein cholesterol (LDL-C), apolipoprotein A1 (Apo-A1), and Apo B in hyperlipidemic patients [82]. Since lipid metabolism is integral to maintaining membrane fluidity, lowering various lipids especially cholesterol may reduce MV shedding. Kavian et al demonstrated that pantethine treatment significantly reduced oxidative stress in endothelial cells and fibroblasts by inhibiting MV secretion in vitro and in an in vivo mice model of systemic sclerosis[83]. Using Pantethine, Penet et al investigated the effect of MVs in cerebral malaria. In TNF-α stimulated mouse brain endothelial cells (MBECs) and human umbilical vein endothelial cells HUVECs), Pantethine treatment reduced MV secretion[84]. Similarly, Pantethine infusion prevented cerebral malaria symptoms in a mice model with *Plasmodium berghei* ANKA infection by blocking MV secretion from endothelial cells and by maintaining the blood-brain barrier integrity [84].

Imipramine is a tricyclic anti-depressant medication used for treating depression. Imipramine can prevent hydrolysis of sphingomyelin to ceramide by blocking an enzyme called acid sphingomyelinase (aSMase) thus affecting the cell membrane fluidity and secretion of exosomes and MV biogenesis. A study that investigated the effect of Imipramine on MV secretion demonstrated that Imipramine treatment substantially blocked ATP-induced MV shedding from microglia and astrocytes by inhibiting aSMase[85]. In another study involving the human prostate cancer (PC3) cell lines, Kosgodage et al investigated the effect of Imipramine on MV secretion. PC3 cells when treated with Imipramine markedly reduced not only MV release but also exosomes[86].

GW4869 is a non-competitive inhibitor of neutral sphingomyelinase (nSMase). Like aSMase, nSMase is also involved in the synthesis of ceramide lipids thus influencing membrane lipid composition. Membrane fluidity rendered by these lipids is crucial for the formation of membrane curvature during the biogenesis of exosomes and MVs. In a study done by Lyu et al, the release of exosome enriched with pro-hypertrophic molecules from cardiac fibroblasts upon activation with angiotensin II (AngII) was markedly attenuated by GW4869 treatment[87]. The in vivo extension of this research using a mice model, GW4869 infusion profoundly reduced cardiac hypertrophy and fibrosis [87]. Similarly using GW4869, Charrier et al demonstrated that exosomes enriched with fibrotic mediator connective tissue growth factor (CCN2) released from human and mouse hepatic stellate cells (HSCs) transfected with CCN2 were significantly blocked following GW4869 treatment thus reducing the transfer of these exosomes to control HSCs[88].

These studies demonstrate that pharmacological inhibition of MVs represents therapeutic possibilities in the context of pathological conditions. However, further studies are warranted with *in vitro* and *in vivo* settings to rule out the safety of various pharmaceutical agents tested for inhibiting MV release and to prove their efficacy in blocking specific sets of subpopulations of MVs.

1.8. MVs AS BIOMARKERS

Since MVs play a crucial role in inter- and intra-cellular communications in normal physiology and pathophysiological conditions, the prospect of using MVs as reliable biomarkers has been expanded substantially. As MVs are widely detectable in several biological fluids, they have emerged as an excellent alternative source of biomarkers that reflect the underlying condition thus allowing us to stratify the patients according to the disease state and to predict the prognosis following therapeutic interventions. The goal here is to briefly demonstrate the clinical relevance of using MVs as biomarkers in human disease conditions.

The presence of several types of cargos such as nucleic acids, proteins, and lipids carried by MVs that are representative of the disease state and of specific cellular origin have been well established in several diseases and make them promising candidates for biomarkers in liquid biopsies. The minimally/non-invasive techniques allow us to gather a great deal of information on diseases by investigating MVs present in various biological fluids and circulation. The levels of certain subsets of MVs and MV-associated cargo changes in circulation and other body fluids due to underlying pathological conditions including diseases such as cardiovascular, metabolic, thrombo-inflammatory, and cancer in response to cell activation or injury[89, 90, 91, 92, 93]. In the context of these diseases, MVs with procoagulant, profibrinolytic, and /or proinflammatory activity have garnered considerable interest due to their involvement in coagulopathy and inflammatory response.

Levels of MVs from leukocyte, platelet, and endothelial origin are altered in the context of a variety of cardiovascular diseases like coronary artery disease (CAD), myocardial infarction, and atherosclerosis[94]. On the other hand, a more comprehensive listing regarding the association of MVs with sepsis is given in Chapter 3. Circulating levels of endothelial MVs demonstrate an important correlation between cardiovascular disease and endothelial dysfunction[95]. For example, CAD patients with diabetic mellitus (DM) had significantly elevated levels of CD144+ (VE-cadherin) endothelial MVs which was directly correlated with endothelial dysfunction[96]. Higher levels of endothelial MVs had a better predictive value of

future negative cardiovascular events in a CAD cohort of 378 patients [97]. Werner et al demonstrated that increased circulating levels of CD31+/Annexin V+ endothelial MVs have a direct association with endothelial apoptosis and CAD[98]. In another study, dialyzed chronic kidney patients with increased circulatory levels of CD31+/CD41- endothelial MVs had a higher probability of future cardiac events and worse prognosis[99]. Increased circulatory levels of leukocyte MVs also make an important association with cardiovascular events. Studies performed in atherosclerotic patients have demonstrated a positive correlation between elevated levels of leukocyte MVs in plasma with atherosclerotic plaque instability and reflected inflammatory microenvironment in the plaque area[100, 101]. In another study, a similar association was reported between elevated plasma levels of CD11b+/CD66+ leukocyte MVs and unstable plaque in patients with carotid stenosis[100].

Liquid biopsy, involving analysis of MVs in circulation and other body fluids has provided new prospects for investigating molecular signatures of cancer for better diagnosis and treatment strategies. MVs from cancer cells carry molecular signatures like epithelial growth factor receptor (EGFR), and epithelial cell adhesion molecule (EpCAM) [102, 103], making them superior candidates for early cancer detection than the circulating cancer cells. In glioma cancer patients, the detection of EGFR+ MVs in serum by flow cytometry allowed better distinction between low and high-grade patients and demonstrated potential effectiveness as a diagnostic and prognostic marker[104]. On the other hand, analysis of pleural fluids from patients with malignant pleural effusions provided EpCAM+ MVs as a promising biomarker.

The clinical relevance of using MVs as biomarkers in pathological conditions has gained tremendous attention. However, the detection, measurement, and characterization of MVs still pose a major challenge, and the lack of sensitivity and specificity of characterization methods even with great progress in the technical development and standardization of the methods continue to be a major limitation.

In the forthcoming sessions, we will describe the multifaceted functional aspects of MVs in the context of hemostasis.

CHAPTER 2: ROLE OF MICROVESICLES IN HEMOSTASIS

Irrespective of their cellular origin, MVs play an integral role in hemostasis. In this session, after a brief introduction to the hemostasis, we will lay out different aspects of MVs' role in hemostasis with regards to thrombosis and fibrinolysis, we will introduce the concept of coagulolytic balance and the role of MVs in vascular physiology and pathology.

2.1. BACKGROUND TO HEMOSTASIS

Hemostasis comprises a coordinated set of physiobiochemical events that helps to restore the blood vessel integrity upon vessel damage while maintaining the blood in its fluid form. In the event of vessel wall damage, platelets get activated, adhere to the damaged vessel wall, and aggregate to seal off the injured site (primary hemostasis) by forming a primary hemostatic plug to prevent blood loss. Subsequently, secondary hemostasis begins with the production and activation of coagulation factors along with platelets reinforcing the earlier formed platelet hemostatic plug by forming a large amount of fibrin without disrupting the blood flow.

2.1.1. PRIMARY HEMOSTASIS

Intact endothelium actively secretes or expresses a myriad of molecules that constantly help to maintain the integrity of endothelium by providing necessary vasoprotection and anticoagulant effects. These molecules include prostacyclin (PGI2), nitric oxide (NO), and ecto-adenosine diphosphate (ADPase) that actively suppress the interaction of platelets with the vessel wall and anticoagulant molecules such as thrombomodulin, heparin-like molecules, von Willebrand factor, protein S, and tissue factor pathway inhibitor. Upon vessel wall damage or endothelial cell activation, the vessel protective or anticoagulant mechanism is compromised initiating a series of coagulation cascades through three distinct but overlapping processes.

2.1.1.1. ADHESION

Upon vascular damage, various components of the subendothelial matrix including von Willebrand factor (vWF) get exposed to the bloodstream which leads to the binding of platelets to the endothelium through the strong interaction of platelet GPIb-V-IX complex and vWF[105]. The initial tethering of platelets through this interaction facilitates much stronger engagement

of platelets with other subendothelial proteins like collagen via platelet GPVI and GPIa/IIa (integrin α2β1)[106, 107].

2.1.1.2. ACTIVATION

Platelet adhesion to the subendothelial proteins leads to the subsequent activation of platelets and degranulation through a series of biochemical pathways. Activated platelets release important soluble factors from their cytoplasmic granules such as thromboxane A2 (TXA2) dense-granule ADP, and ATP which binds to G-protein coupled thromboxane receptor and purinergic $P2Y_1$ and $P2Y_{12}$ receptors on platelet surface to further potentiate the platelet activation [108, 106, 109]. Activated platelets support thrombin generation and formation of fibrin by exposing negatively charged phospholipids on their surface and assembly of coagulation factors. Thrombin generated by the platelet surface then further accelerates the irreversible activation of platelets by proteolytic cleavage of PAR1 and PAR4 receptors on their surface[110]. By the mechanisms listed above, activated platelets cause reciprocal activation of several integrins on the surface of platelets especially GPIIaIIIb (or αIIbβ3) which enables bidirectional inside-out and outside-in signaling through these integrins for the continuous activation of the platelets[111].

2.1.1.3. AGGREGATION

Continuous activation of platelets at the site of vascular injury leads to aggregation of platelets via platelet-platelet interaction at the site of vascular injury and helps in thrombus growth. Platelet aggregation causes the formation of white thrombi that are rich in platelets due to the high shear blood flow over the injured vessel area but the continuous formation of fibrin at the low-shear areas gets filled with red thrombi.

2.1.2 COAGULATION

The coagulation cascade involves a series of proteolytic steps that advance through two distinct pathways called extrinsic and intrinsic pathways (Figure 6). These two proteolytic pathways converge to form a common pathway. This whole process requires a procoagulant surface provided mainly by the platelets and tissue factor (TF) that are expressed on the cell membrane. Based on the widely used cellular models, hemostasis occurs in three distinct steps: 1) the initiation phase 2) the amplification phase, and 3) the propagation phase (Figure 7).

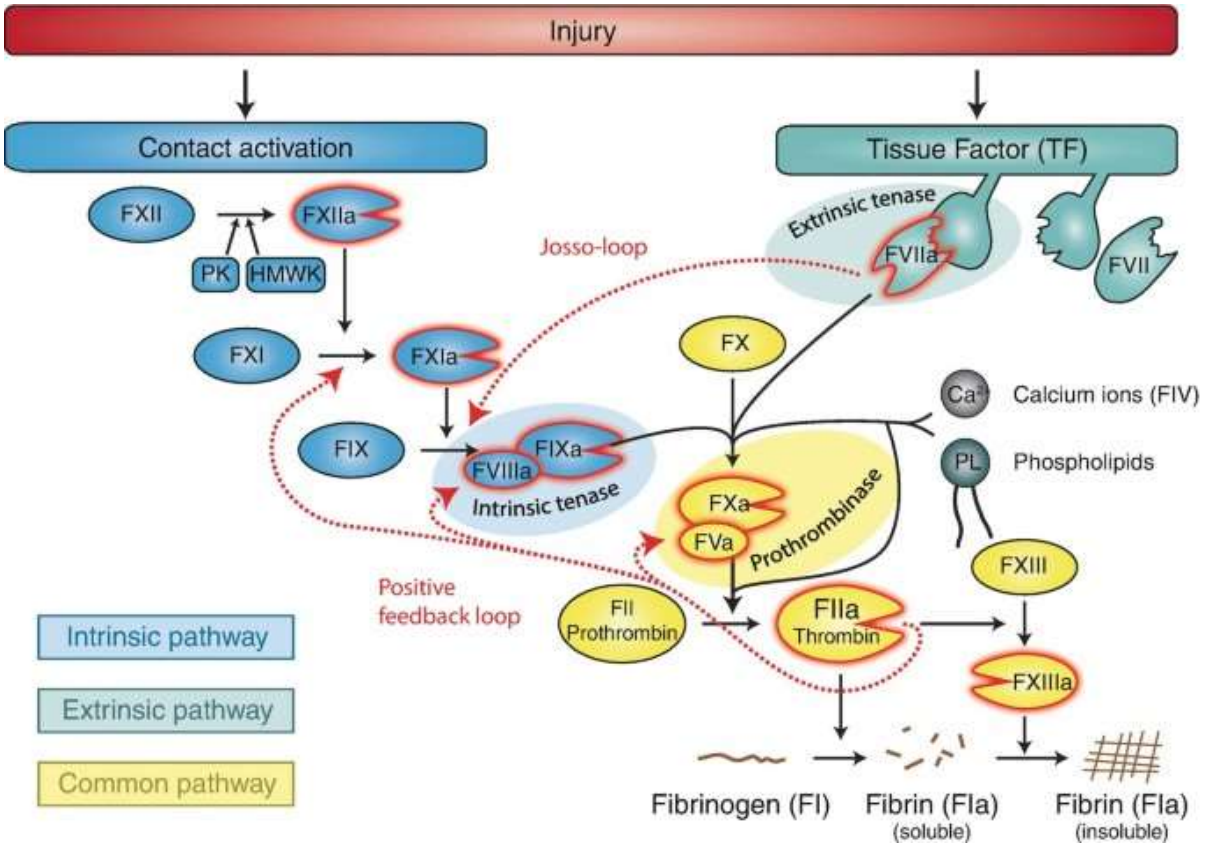

Figure 6: Coagulation cascade model

(Excerpt from: Mussbacher et al., Fundamentals of Vascular Biology, 2016)

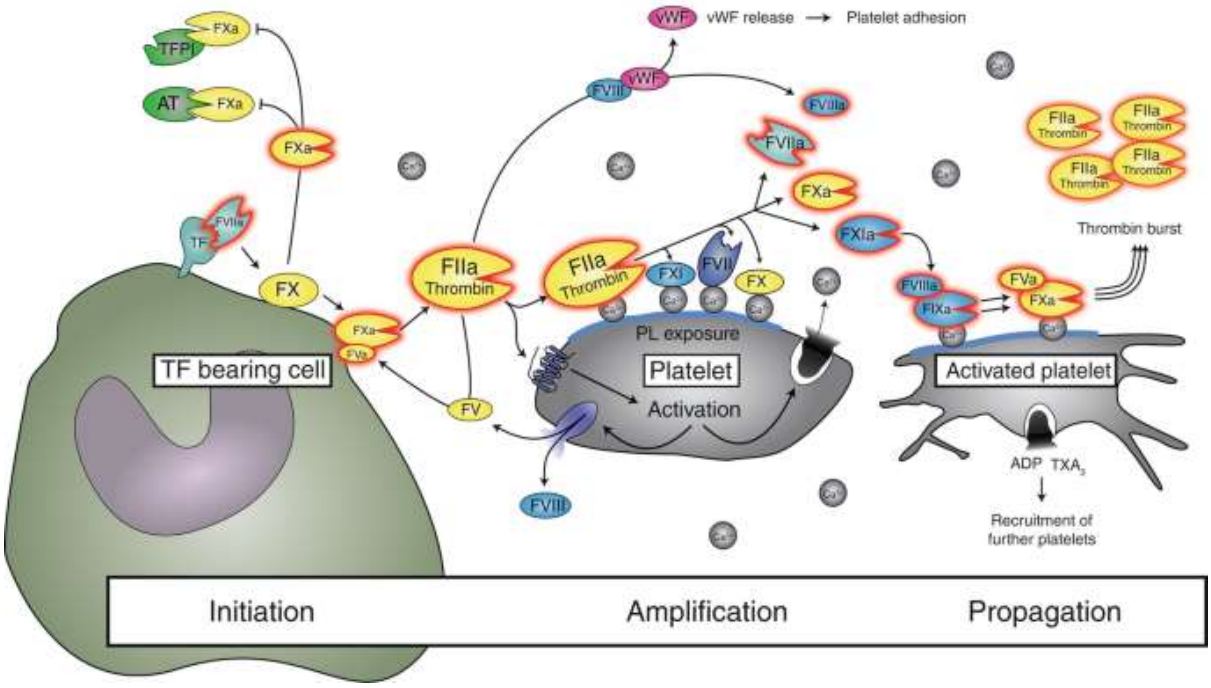

Figure 7: Cellular model of coagulation

(Excerpt from: Mussbacher et al., Fundamentals of Vascular Biology, 2016)

2.1.2.1. TISSUE FACTOR

Tissue factor is an integral membrane protein and the principal initiator coagulation cascade. TF is mainly carried in the blood by the cells and MVs and is also expressed in perivascular cells. I shall briefly describe this molecule.

TF belongs to the class two cytokine receptor superfamily and fibronectin type III family with mature protein consisting of 263 amino acids[112]. The mature TF contains an extracellular domain with two fibronectin type III domains at the NH-2 terminal part enabling binding of FVIIa, a transmembrane domain to anchor TF to the membrane, and a cytoplasmic COOH-terminal domain which is important for the signal transduction. The function of the extracellular domain of the TF is to facilitate the binding of FVII in a membrane surface-dependent manner and enhance the proteolytic cleavage of coagulation factors FVII, FX, and FIX [113].

TF is expressed in many organs including the brain, placenta, lungs, heart, kidney, and intestines[114]. Similarly, blood cells such as monocytes and macrophages express very low levels of TF[114], and rapidly increased expression is observed upon cell stimulation with bacterial endotoxins or inflammatory cytokines[113]. In contrast, quiescent endothelial cells express very little or no TF, but inflammatory conditions considerably elevate the expression of TF[115]. TF may have an inactivated 'encrypted' conformation in the normal blood that serves as a mechanism to limit unwanted activation of TF[116]. This encrypted conformation transforms to a 'decrypted' or activated form, facilitated by the exposure of PS during the event of significant cell stimulation, and further enhances the formation of the TF-FVIIa complex[117]. The decryption of TF may also be regulated by the action of protein disulfide isomerase (PDI) by introducing a double bond at Cys186-Cys209[118]. Several in vitro and in vivo studies have demonstrated that PDI significantly enhances TF-dependent procoagulant activity [119, 120, 121, 122]

2.1.2.2. INITIATION PHASE

The initiation step in the hemostasis begins with TF in the extrinsic pathway. TF is a key 'exogenous protein' that is mainly expressed in its inactive 'cryptic' form outside the blood vessel but also expressed by the vascular smooth muscles and fibroblasts. Cryptic TF, when exposed to the bloodstream during vascular injury, gets activated and forms a complex with FVIIa[123]. TF-FVIIa complex generates a small amount of activated FX (FXa) and FIX (FIXa) by proteolytic cleavage. Activated FX (FXa) then cleaves prothrombin to thrombin, which then converts inactive FV to active FV (FVa)[124, 125]. FXa associates with FVa to form a

prothrombinase complex on the TF expressing cells which act as an amplification loop for generating more thrombin. FXa activity is constrained locally on the cell surface mainly due to protease inhibitors like tissue factor pathway inhibitor (TFPI) and antithrombin (AT)[126]. TFPI and AT inhibit FXa that gets dissociated from the cell membrane thus preventing its diffusion to distant places. However, FXIa that gets dissociated from the TF-bearing cell membrane can diffuse to distant places as it cannot be targeted by TFPI.

2.1.2.3. AMPLIFICATION PHASE

The proteolytic activity of the TF-FVIIa complex and prothrombinase complex continuously generates a small amount of thrombin. This thrombin has several functions. One of the functions of thrombin includes activation of the platelets through PAR1 and PAR4 receptors. Platelet activation by the thrombin renders more procoagulant activity to platelets that are adhered at the site of vascular injury[127]. Thrombin also activates more FV and FVIII on the surface of platelets thus feeding into the generation of more activated coagulation factors. Similarly, thrombin also activates inactive FXI to FXIa[128].

2.1.2.4. PROPAGATION PHASE

In the propagation phase, procoagulant platelets play a central role rather than TF-expressing cells. FXIa generated during the initiation and amplification phase cleave FIX to FIXa. FIXa forms a complex with FVIIIa (tenase complex) on the platelet surface. Tenase complex on the platelet surface can rapidly generate FXa, which then binds to factor Va to create more prothrombinase complex and generate a minute amount of thrombin that then activates platelets. Tenase and prothrombinase complexes on the highly procoagulant surface of platelets feed into the generation of large amounts of thrombin, which is essential for fibrin formation and reinforcement of fibrin fibers in the clot by the FXIIIa [129].

2.1.3. FIBRINOLYSIS

2.1.3.1. GENERAL INFORMATION

The regulatory role fibrinolytic system is necessary to maintain the blood flow by removing the clot from the blood vessels which is governed by a series of proteases and protease inhibitors. The purpose of the fibrinolytic system is thrombolysis by the generation of a key proteolytic enzyme that is plasmin from the circulating plasminogen (Figure 8) by its primary plasminogen activators (PAs): urokinase-type PA (uPA) and tissue-type PA (tPA)[130].

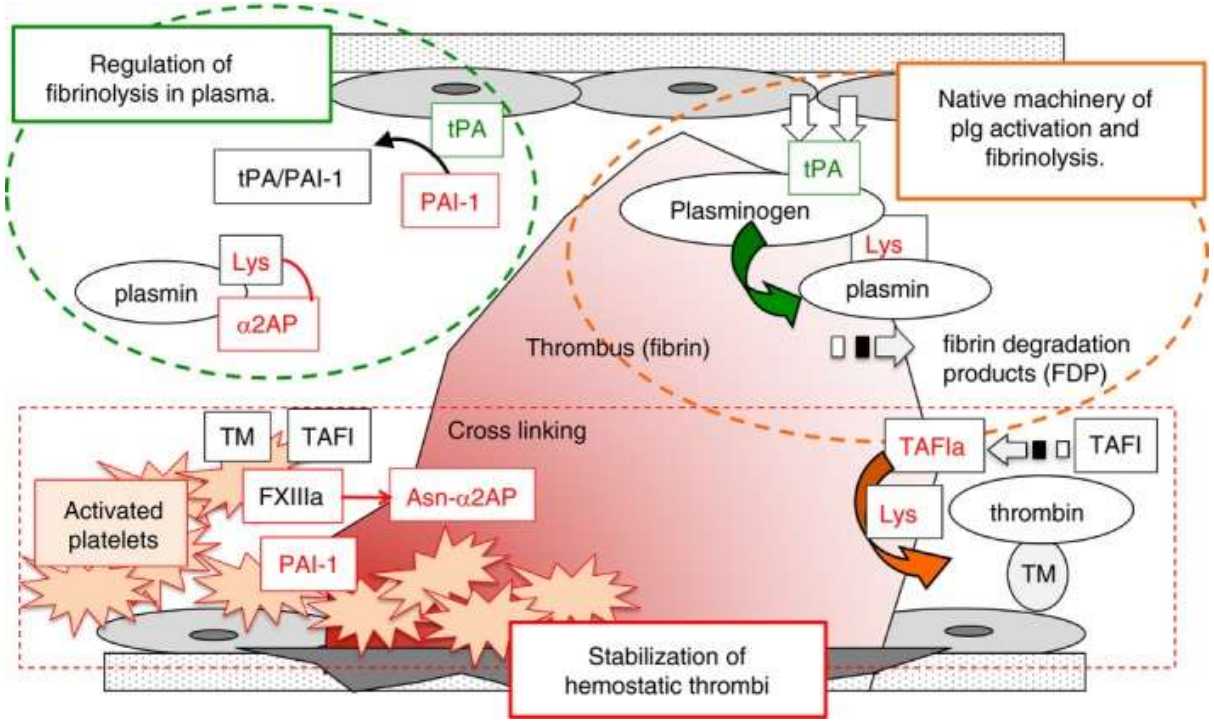

Figure 8: Activation of plasminogen and fibrinolysis and its modification by vascular endothelial cells and platelets

(Excerpt from: Urano et al., JTH, 2018)

2.1.3.2. THE PLASMINOGEN

Human plasminogen is a 90 kDa glycoprotein that is primarily synthesized by the liver in the form of zymogen[131, 132]. Plasminogen circulates in the plasma predominantly in the form of glu-plasminogen at a concentration of 2µM/mL. Plasminogen naturally exists in two isoforms: plasminogen type 1, which carries glycosylation at Asn289 and Thr346, and plasminogen type 2, which only carries glycosylation at Thr346[133]. These two isoforms have differences in their functional properties when it comes to activation status[133, 134], binding to certain monocytic type cells[134], and modulation of interaction kinetics with tPA[135]. Plasminogen is initially synthesized in the liver as 810-amino acid long polypeptide protein but later cleaved into a mature form that contains 791-amino acids. This mature form of glu-plasminogen comprises 7 domains from the N-terminus (heavy chain) to the carboxyl-terminus (light chain) with 561 and 260 amino acids, respectively, and is linked by two disulfide bonds. Between the N-terminus and carboxyl-terminus contains the five Kringle (K; k1-k5) domains with 80 amino acids in each domain which are stabilized by disulfide bonds by linking each domain together. Glu-plasminogen exists as a closed loop structure (tight or T-form) which shields the catalytic sequence Lys50, Arg68, and Arg70 of the Pan-apple domain in the N-terminus by the linker region between K3-K4 while Lys-plasminogen, a lesser version of the plasminogen that

displays more of a relaxed (R form) conformation[131, 130]. The T-form of the native Glu-plasminogen is attributed to the existence of Cl^- but can acquire the R-form when the lysine binding sites (LBS) interact with ω-amino acids (for example., ε-aminocaproic acids [EACA]) causing the catalytic sequence to be exposed[130].

Fibrinolysis is the process of proteolytic degradation of the fibrin networks which formed during the coagulation. The whole mechanism of fibrinolysis is precisely regulated by a series of serine proteases and the activators and inhibitors of these serine proteases. Fibrin is formed by the polymerization of the molecular fibrinogen. Molecular fibrinogen is a dimer that consists of three distinct polypeptide chains (Aα, Bβ, and ϒ) linked together by five disulfide bonds at the E domain of the N-terminal[136]. The lysine binding sites of the plasminogen mediate the binding of plasminogen to fibrin to initiate the degradation of fibrin [137, 138].

2.1.3.3. TISSUE PLASMINOGEN ACTIVATOR (tPA)

Tissue plasminogen activator (tPA) is a key serine protease that catalyzes the conversion of plasminogen to plasmin and, therefore, plays an important role in fibrinolysis. tPA in the circulation is mainly produced by endothelial cells, hepatocytes, monocytes, and lymphocytes[139]. Besides, tPA is also synthesized by brain cells, smooth muscle cells, and epithelial cells[139]. tPA is secreted as a single chain form and requires substrates like fibrin for the conversion of plasminogen to plasmin[130]. Under normal physiological conditions, tPA is inhibited by plasmin activator inhibitor type-1 (PAI-1), however, in the presence of either fibrin or plasminogen, tPA proteolytic function supersedes the inhibitory activity of PAI-1[130].

2.1.3.4. UROKINASE PLASMINOGEN ACTIVATOR (uPA) and RECEPTOR (uPAR)

Urokinase-type (Urokinase) plasminogen activator (uPA) is an important serine protease activator that cleaves plasminogen to plasmin. Though the fibrinolytic activity of uPA was initially discovered in the urine[140], it was later detected in many body fluids and extracellular spaces[141]. uPA is initially secreted as a low potent pro-uPA form consisting of a single polypeptide chain in a glycosylated zymogen structure but undergoes hydrolysis of Lys158-Ile159 to produce highly potent double chain form, albeit these two forms demonstrate no differences in the affinity towards uPA receptor (uPAR) binding[142]. The formation of a highly potent double-chain form of uPA is critical for the conversion of plasminogen to plasmin[143]. Moreover, the double-chain uPA exists in a high molecular weight (54 kDa) and low molecular weight (33 kDa) form depending on the presence or absence of a 135-amino acid fragment following the proteolytic cleavage between Lys135 and Lys136[144]. Only the high molecular

weight form of double-chain uPA binds to uPAR despite both forms being capable of plasminogen conversion to plasmin[144]. uPA exhibits more potent fibrinolytic activity than tPA even in the absence of uPAR making it highly efficient in fibrin clearance[145].

uPAR (CD87) is encoded by the PLAUR gene and is expressed by several cell types including monocytes, neutrophils, T cells, endothelial cells, epithelial cells, fibroblasts, and many cancer cell types. It's formed of a single polypeptide chain glycoprotein comprised of 313 amino acids with D1, D2, and D3 domains that are crossed by two flexible linker sequences[146]. uPAR can act as a receptor for both pro-uPA and active uPA[142]. However, uPAR is not just limited to the binding of uPA but also interacts with other structures including integrins, vitronectin, and various types of transmembrane receptors to initiate downstream signaling[147].

2.1.3.5. MATRIX METALLOPROTEINASES (MMPs)

Matrix metalloproteinases (MMPs) are proteases that belong to the family of zinc-containing endopeptidases responsible for ECM degradation[148]. It has been demonstrated that plasmin can modulate the activity of MMPs and fibrinolytic MVs of endothelial and leukocyte origin can enhance the functional activity of MMPs by catalyzing the conversion of plasminogen to plasmin[47]. Apart from its obvious role in the remodeling of ECM, certain MMPs like MMP-10 have been demonstrated to be directly involved in the fibrinolytic process by degrading fibrinogen or by inhibiting the activation of thrombin activable fibrinolysis inhibitor (TAFI) [149, 150]. Conversely, while it has been shown that increased expression of MMP-2 and MMP-9 significantly correlated with plaque instability in atherosclerosis, inhibition of MMP-13 increased plaque size by a mechanism involving increased collagen deposition[151, 152].

2.2. ROLE OF MICROVESICLES IN THE COAGULATION PROCESS

MVs have long been regarded as procoagulant entities largely because they express anionic phospholipids like phosphatidylserine (PS) and/or tissue factor (TF). However, this strictly procoagulant role of MVs has been challenged with the discovery of MVs with anticoagulant and fibrinolytic properties. Several animal and clinical models have demonstrated elevated levels of MVs with procoagulant and/or profibrinolytic properties in circulation in response to inflammatory conditions. Conversely, some recent studies have suggested that inflammatory conditions also increase MVs with profibrinolytic properties. These studies have demonstrated a direct correlation between an increase in MVs with the procoagulant and profibrinolytic properties and disease progression associated with thromboinflammatory conditions such as sepsis, atherosclerosis, and COVID-19 [153, 154]. In my research work, I investigated the

procoagulant and /or profibrinolytic properties of different subsets of MVs in the context of inflammatory stimuli/sepsis pathology. Similarly, how MVs with procoagulant and profibrinolytic properties affect disease progression in atherosclerosis has been addressed in the review written during my thesis work.

I have briefly addressed the functional determinants of procoagulant and profibrinolytic properties of MVs in the subsections below.

2.2.1. MOLECULAR DETERMINANTS OF PROCOAGULANT ACTIVITY

Procoagulant activity of mainly dependent on the expression of anionic phospholipids like phosphatidylserine (PS) and/or tissue factor (TF). TF is the principal initiator of coagulation and along with PS, TF can augment MV-dependent procoagulant activity significantly (Figure 9).

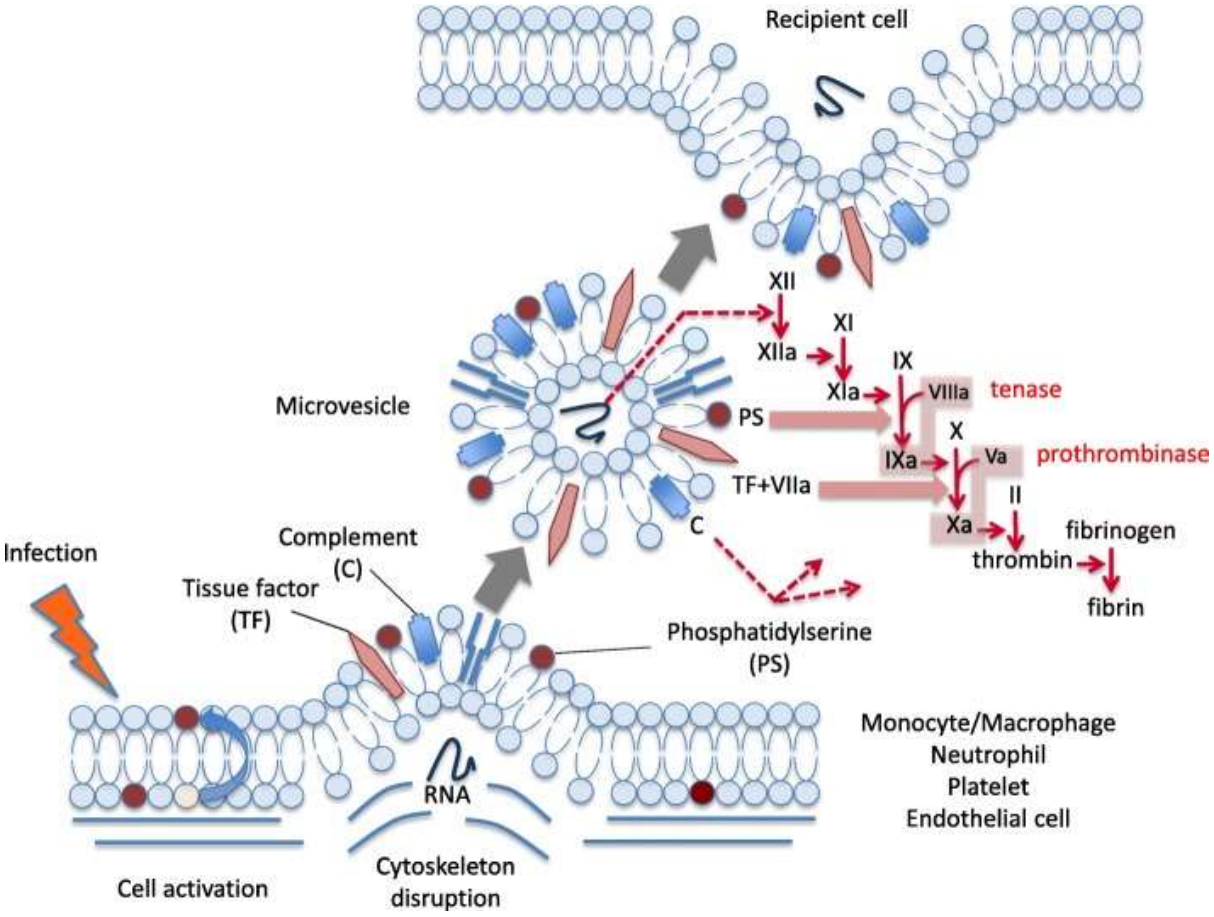

Figure 9: Molecular determinants of MV - dependent procoagulant activity

(Excerpt from: Iba et al, J Int care, 2018)

2.2.1.1. PHOSPHOLIPID-DEPENDENT PROCOAGULANT ACTIVITY

As explained earlier, phospholipids like phosphatidylserine have an asymmetrical distribution across the inner and outer leaflet of the plasma membrane, and when this asymmetry is lost, it leads to the externalization of phosphatidylserine and eventual shedding of MVs. Phosphatidylserine aids in the assembly of various coagulation factors on the membrane, eventually forming prothrombinase and/or tenase complex and generating thrombin during the event of thrombus formation[155]. It has been shown that the procoagulant activity of such MVs can be significantly enhanced by several methods including stimulation with endotoxins. The importance of PS in the procoagulant activity by MVs by enhancing the TF activity has been explored in several studies[156, 157]. Conversely, the existence of PS-negative MVs produced by platelets observed by electron microscopy indicates an alternative method of MV formation and MVs lacking procoagulant activity[158].

2.2.1.2. TF-DEPENDENT PROCOAGULANT ACTIVITY

MVs with the highest procoagulant activity express PS and TF. As discussed before, procoagulant cells and MVs can generate thrombin and fibrin by the assembly of prothrombinase complex on their surface. Participation of TF-bearing MVs in the thrombus formation has been investigated in numerous animal models of thrombosis including mice models[159, 160]. These animal models showed us the potential utilization of procoagulant MV measurement in clinical settings[161]. Several methodologies are currently being used to analyze and measure procoagulant MVs in clinical settings. For example, a flow cytometry instrument that employs immunologic methods can be used for antigenic characterization and enumeration of MVs that correspond to their cellular origin, whereas functional assays allow us to measure the procoagulant activity of these MVs[162]. Procoagulant activity of MVs varies depending on the cellular source, expression of PS and/or TF, and the agonists that trigger vesiculation[163, 112, 164]. Under physiological conditions, PS-positive MVs typically exhibit minimal to no tissue factor (TF) expression. However, under specific pathological conditions, such as cancer, cardiovascular disorders, sepsis, or COVID-19, a notable shift occurs in terms of their TF expression [165, 166, 154].

Blood cells in particular monocytes produce MVs with significant TF-dependent procoagulant activity upon activation with endotoxins such as LPS or proinflammatory cytokines[167, 168]. A study that directly compared the TF-dependent MV procoagulant activity demonstrated that MVs from LPS-stimulated monocytes had profound thrombin generation capacity and this

activity was significantly blocked using an anti-TF antibody but had no effect on the thrombin generation induced by the platelet MVs[169]. A similar study also compared the MV-TF-dependent procoagulant activity and found that only monocyte MVs but not platelet MVs supported thrombin generation and promoted fibrin formation [170].

A recent work that investigated the TF-dependent procoagulant effect of various subsets of MVs showed an elevated FXa generation and increased propagation of coagulation by MVs of monocyte, THP-1, and endothelial cell origin but not platelet and erythrocyte MVs[171, 172]. Previous studies have reported the release of TF-expressing MVs by endothelial cells upon stimulation[161]. A study that evaluated the procoagulant effect of endothelial-derived MVs analyzed by the rotational thromboelastometry (ROTEM) found that upon stimulation with TNF-α, endothelial cells released an increased quantity of TF+ MVs that significantly reduced clotting time (CT) and clot formation time (CFT), and moderately increased maximum clot firmness (MCF)[173].

In addition to the MVs originating from the blood cells and cells in the vascular compartments, MVs originating from certain cancer cells also demonstrate TF-dependent coagulation activity and have a great role in pathological thrombosis. It has been demonstrated that certain cancer cell lines such as pancreas adenocarcinoma cells (BXPC3) and human breast cancer cells (MCF7) produce MVs with profound TF activity as shown by the thrombin generation assay[174, 175].

Similar to *in vitro* studies, several *in vivo* studies have also explored the impact of TF-expressing MVs on thrombus formation using arterial and venous thrombosis models. The findings of these studies have been listed in a recent review[176]. Major findings demonstrate that TF-expressing MVs from various cellular sources profoundly support thrombus formation and some studies also emphasize the importance of membrane lipids such as PS in thrombus formation. The most noteworthy studies have been concluded in Table 2.

Table 2: Summary of in vivo findings on the role of procoagulant MVs on thrombus formation

Legend: UC: ultracentrifugation; IVC: inferior vena cava; WT: wild type; PDI: protein disulfide isomerase; TF: Tissue Factor; TFPI: Tissue Factor Pathway Inhibitor; PSGL-1: P-selectin glycoprotein ligand-1

Reference	Source of MVs	Purification method	Thrombosis model/Animal	Route of MV administration	Interaction of MVs with thrombus and outcome
Falati et al., 2003	Human and Mice Whole blood cells	ultracentrifugation	Mice/Laser-induced arterial thrombosis		Monocyte-derived MVs bound to P-selectin on platelet thrombi via PSGL-1. TF-bearing MPs derived from monocytes required p-selectin and PSGL-1 interaction for their role in thrombus development
Davila et al., 2006	MDA-MB-231, MCF-7, Or L3.6pl cancer cell lines	ultracentrifugation and ultrafiltration	Mice	Tail vein	Injected microparticles were profoundly procoagulant in a TF and thrombin-dependent manner.
Reinhardt et al., 2008	Human platelets and monocytes		Carotid artery ligation and wire-induced arterial thrombosis		MVs enhanced fibrin generation and coagulation in WT mice but not in mice pretreated with anti-PDI antibodies.
Ramacciotti et al., 2009	WT Mice whole blood (Leukocytes and platelets)		IVC ligation, Mice		MVs from platelets but not from leukocytes enhanced thrombus formation in a TF and p-selectin-dependent manner
Thomas et al., 2009	SOJ-6, Panc02 and LLC1 cell lines	ultracentrifugation	FeCl3 and Laser-induced arterial thrombosis	IV – Jugular vein, Mice	Injected MVs interacted with growing thrombus in a PSGL-1-dependent manner. MVs bearing PSGL-1 and TF were profoundly correlated with growing thrombus in vivo
Wang et al. Blood., 2012	Human pancreatic and colorectal cancer cell lines	ultracentrifugation	IVC ligation and $FeCl_3$-induced saphenous vein thrombosis	IV	TF+ MVs-derived cancer cell lines significantly increased thrombin-antithrombin complex and enhanced coagulation.

Mezouar et al., 2014	Panc02 cell line	ultracentrifugation	Laser-induced arterial Injury		Injected MPs bound to platelet fibrin by integrin receptor. High TF-bearing MVs enhanced thrombus formation in platelet dependent manner compared to Low TF bearing MVs with TFPI
Thomas et al., 2015	Panc02 cell line	ultracentrifugation	IVC ligation	IV – Jugular vein	Injected MPs did not require either p-selectin or GPIb for their interaction with thrombus. TF-bearing MPs adhered to neutrophil extracellular traps (NET) to enhance the thrombosis But did not require P-selectin or GPIb for their coagulation activity
Geddings et al., 2016	Human pancreatic adenocarcinoma Cell lines (BxPc-3 and L3.6pl)	ultracentrifugation	Femoral and IVC ligation	IV – Tail vain	BxPc-3 and L3.6pl derived TF+MVs enhanced thrombus formation in a TF-dependent manner by activating platelets
Hisada et al., 2016	BxPc-3 cell line	ultracentrifugation	IVC ligation		Injection of TF+ MVs activated platelets and promoted venous thrombosis.
Stark et al., 2018	Human whole blood, Human (FG, L3.6 pl and mice (KPC) pancreatic adenocarcinoma cell lines	ultracentrifugation	IVC ligation	IV – Tail vain	MVs from FG, L3.6 pl, and KPC cell lines significantly enhanced thrombus growth and also reduced clotting time compared to MVs obtained from the whole blood

2.2.1.3. ANTICOAGULANT FUNCTION OF MICROVESICLES

Recent evidence suggests that certain subsets of MVs contain anticoagulant functions that can effectively counterbalance the procoagulant activity of these vesicles. Under normal physiological conditions, cells in the blood and vasculature express various anticoagulant molecules, such as endothelial protein C receptor (EPCR)[177], thrombomodulin (TM)[178, 179] or tissue factor pathway inhibitor (TFPI)[180] are also expressed on the MVs[181, 182, 183]and has a regulatory and protective role in the coagulation process.

2.2.2. FIBRINOLYTIC ROLE OF MICROVESICLES

In addition to the expression of procoagulant and anticoagulant molecules, the expression of fibrinolytic molecules by some subsets of MVs makes their involvement in the coagulation process more complex. The functional ambivalence of MVs in coagulation that comes from the expression of these distinct molecules may have significance in the pathophysiology of cardiovascular and immunothrombosis disorders like sepsis and atherosclerosis.

2.2.2.1. MICROVESICLES WITH FIBRINOLYTIC ACTIVITY

The discovery of fibrinolytic properties in some subsets of MVs added more complexity to the process of hemostasis. In 1997, Ginestra et al demonstrated the presence of uPA/uPAR complex on the surface of MVs secreted by the human fibrosarcoma cell lines[184], and later, similar results were observed in other cancer cell lines[185, 186, 187]. Moreover, the exposure of the uPA/uPAR system has been well documented in endothelial cells. In 2002, Brodsky et al demonstrated the expression of uPA/uPAR complex on MVs from cultured HUVECs[188]. The plasmin generation capacity of the uPA/uPAR system expressed on endothelial MVs was first demonstrated in our lab[47]. It has been shown that uPA expressed by the monocyte and endothelial cells was able to effectively catalyze the conversion of plasminogen bound on the surface of platelets and fibrin. As MVs can bind fibrin, the uPA/uPAR system expressed on the MV membrane along with tPA could participate in the lysis of fibrin clots and plaque rupture in atherosclerosis (Figure 10). Finally, our team demonstrated that MVs of endothelial and leukocyte-origin plasma exhibited plasmin generation capacity[189]. Since some subsets of MVs demonstrate fibrinolytic capacity, they may participate in the pathophysiological process of diseases such as cardiovascular disorders, thrombo-inflammatory conditions, and cancers[190].

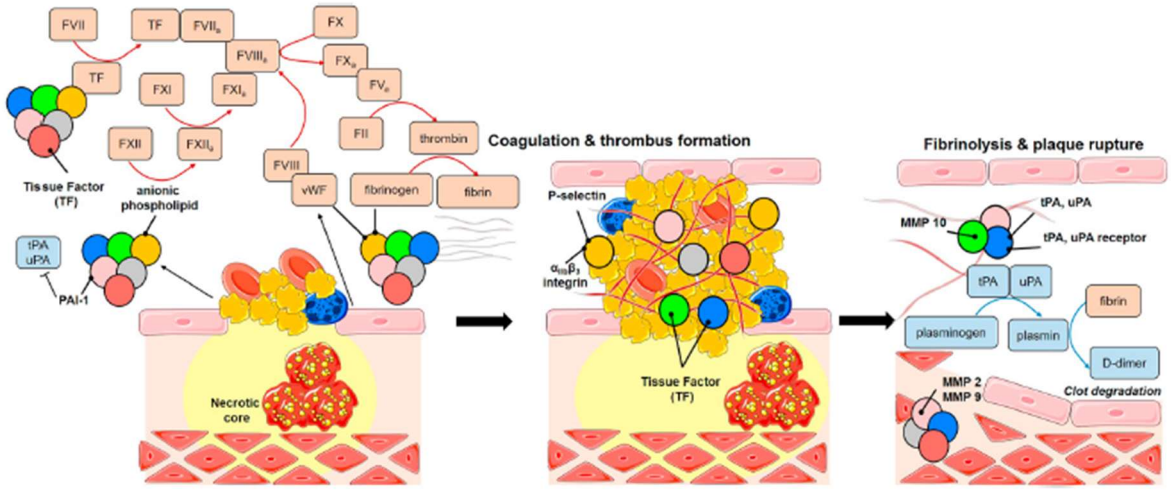

Figure 10: Involvement of MVs in hemostasis during atherosclerosis

(Excerpt from: Konkoth et al, Atherosclerosis, 2020)

2.3. METHODS TO PURIFY MICROVESICLES

Several methods can be employed to purify the MVs (Figure 11) from the cell culture supernatants or various biological fluids such as plasma. Although different methods have differential abilities to purify MVs to avoid contamination with various soluble molecules, it is imperative to use appropriate and efficient method(s) to ensure maximum purity of MVs in order to use them for biomarker studies or to investigate functional aspects. Many recently published reviews have elucidated various methods employed for MV purification[191, 192, 193, 194]. Ultracentrifugation, filtration, and affinity separation are the most commonly used methods for MV purification.

Ultracentrifugation is one of the widely used purification methods for MVs[191]. Differential centrifugation methods exploit the density, size, and shape to purify MVs (10, 000/20,000g for large EVs/MVs and ≥100,000g for small EVs) from the culture supernatants or biological fluids. However, purification of MVs using ultracentrifugation is not only time-consuming but also greatly diminishes the purity and yield of the MVs prepared because of the co-isolation of protein aggregates[195]. Additionally, cross-contamination from other types of MVs is also an issue when using only ultracentrifugation for the purification and viscosity of samples like plasma negatively affects the efficacy of this method[196]. Alternatively, the use of density gradient centrifugation (sucrose gradient or iodixanol gradient) allows the fraction of various types of EVs based on the density of vesicles[195].

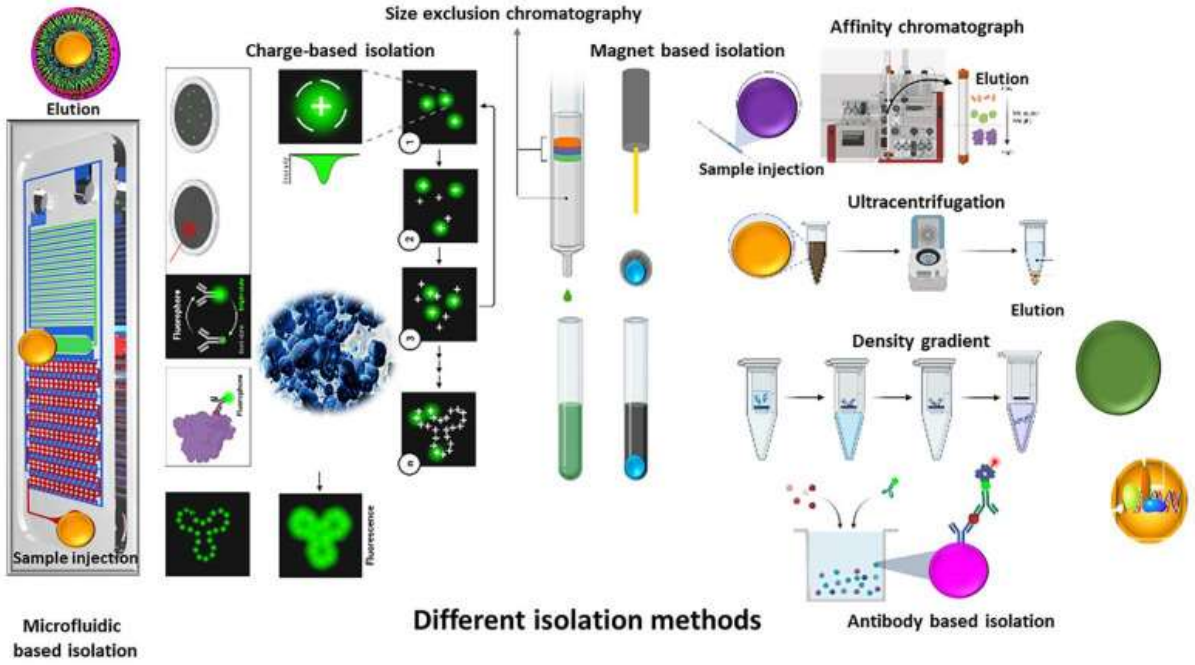

Figure 11. Methods to purify extracellular vesicles

(Excerpt from: Akbar et al, Cells, 2022)

A combination of methods can be used in conjunction with ultracentrifugation for the purification of MVs and to eliminate potential contaminants from the MV samples. Ultrafiltration and size exclusion chromatography (SEC) are commonly used size-based approaches along with ultracentrifugation to improve the yield and purity of MVs. Ultrafiltration columns use porous membranes with various molecular weight cut-offs (MWCO) (e.g., Vivaspin Turbo 15 PES, 100kDa MWCO) to concentrate large quantities of MVs while eliminating small contaminants like secreted molecules or proteins[197]. Using the SEC column following the ultrafiltration step allows the collection of high-quality MVs by separating them from the soluble contaminants while the sample is moving down through the porous compound packed inside columns [198, 199]. In addition to ultrafiltration and SEC methods, another size-based purification method that can be used is field-flow fractionation (FFF). This method is performed with a tiny separating chamber and upon sample injection, the parabolic laminar flow of the fluid within the chamber carries the sample forward while force by a crossflow separates different types of EVs based on the size[192].

Precipitation is another method employed in the purification of MVs by either introducing a water-excluding polymer or lectin into the sample. The commonly used water water-excluding polymer is polyethylene glycol (PEG) to precipitate MVs from the sample. Precipitated MVs can be pelleted by ultracentrifugation. Although the precipitation method is faster to perform

and does not require advanced skills, this method causes the aggregation of different types of vesicles and contaminant molecules[199]. Apart from PEG precipitation, lectin also can be used for the precipitation of MVs[199].

Immunomagnetic-based capture is another method to purify different types of EVs. The immunomagnetic method utilizes streptavidin-magnetic beads coated with biotinylated specific antibodies to capture specific subsets of MVs of interest from the sample[200]. Compared to the ultracentrifugation method, immunomagnetic capture of MVs is faster and can be performed with a small sample volume.

Finally, the microfluidics approach offers a great advantage to purify different types of EVs based on their physical and biochemical properties. The microfluidics devices can efficiently isolate MVs from low volumes of samples within a short time[201]. Microfluidics devices can be fabricated to incorporate various types of EV separation modalities either through passive or active processes[201]. For example, immunoaffinity-based microfluidics devices use microfluidics devices with inner channels modified with specific antibodies or decorated with magnetic beads coated with specific antibodies to capture specific subset(s) of MVs while the sample is flowing through the microchannels[202]. Although the development of specific antibodies for this purpose appears to be a limitation of this technique this limitation can be rectified with the development of microchips to isolate MVs based on size.

2.4. MEASUREMENT OF PROCOAGULANT AND PROFIBRINOLYTIC ACTIVITIES OF MICROVESICLES

2.4.1 MEASUREMENT OF PROCOAGULANT ACTIVITY

The various procoagulant MV tests in use have been included in Table 3. These assays include antigenic, functional, or a combination of both to detect TF and PS or procoagulant activity rendered by these two.

- **Antigenic methods**

In the antigenic assay, flow cytometry (FCM) is the primarily utilized method to analyze the procoagulant molecules such as PS and TF on the surface of MVs. The presence of PS on MVs can be detected with annexin V or lactadherin and TF by specific antibodies. Although FCM has been used routinely for the analysis of MVs <300nm, inadequacy of sensitivity and specificity remains a challenging issue. Moreover, the functional activity of PS, TF, or TFPI cannot be measured using FCM. On the other hand, detection of fibrin+ MVs may provide an

alternative source to measure thrombotic activity in conditions like cancer. Recently, a study by Sachetto et al. evaluated the potential of commercially available four ELISA kits for the detection of TF+ MVs isolated from the cell culture supernatant of pancreatic cancer cell line and MV-TF in the plasma from LPS-stimulated whole blood or acute leukemia patients. While Imubind and Quantikine were able to detect TF+ MVs isolated from the cell supernatant, they could not detect low levels of TF-expressing MVs in the plasma. Interestingly, Quantikine was able to detect high levels of MV-TF in the plasma of acute leukemia patients [203]. These data demonstrate that the major issue with antigenic-based detection of TF-expressing MVs is insufficient sensitivity to produce a signal in the samples that contain low levels of TF-expressing MVs, including the most recent study using ExoView chip-based system that failed to detect MV-TF in plasma from trauma and ovarian cancer patients due to limited sensitivity but detected high procoagulant TF+ MVs in culture supernatant of BxPC3 cancer cell lines[204].

Table 3: MV-dependent procoagulant assays

(Adapted from Lacroix et al, Semin Thromb Haemost, 2019)

	Test	Target	Method	Preanalytical	EV commercial kit	References
Antigenic	Flow cytometry	PS, TF, TFPI	Immunofluorescence	PFP	No	Poncelet et al, Trasci, 2015, Nolan et al, Platelets, 2017
	Flow cytometry	Fibrin	Immunofluorescence	PFP	No	Mege et al, Oncotarget, 2017
	Scanning confocal microscopy	TF	Immunofluorescence	PFP/Centrifugation	No	Hisada et al, Thromb Res, 2017
	ELISA	TF		PFP	Yes	Sachetto et al., 2023
Functional	Calibrated automated thrombogram	Functional PS or TF	Thrombin generation	PFP	Yes	Hemker et al, Pathophysiol. Haemost. Thromb., 2002
	STA-Procoag PPL	Functional PS	Coagulation time	PFP	Yes	Exner et al, Blood Coagul. Fibrinolysis, 2003
	FXa generation assays	Functional TF	FXa generation	Centrifugation	No	Khorana et al, JTH, 2008; Woei-A Jin, BJ Cancer, 2016; Vallier et al, Thromb Haemost, 209
	Fibrin generation assay	Functional PS and TF	Fibrin generation	Centrifugation	No	Berckmans et al, Blood, 2011
Combined	Zymuphen MP Elisa kit	PS	Thrombin generation	Immunocapture (PS) on PFP	Yes	Laroche et al, Platelets, 2017
	Zymuphen-MP TF	TF	FXa generation	Immunocapture (PS) on PFP	Yes	Laroche et al, Platelets, 2017

Legend: ELISA: Enzyme-linked Immunosorbent Assay; PFP: Platelet-Free Plasma; TF: Tissue Factor; TFPI: Tissue Factor Pathway Inhibitor.

- **Functional Assays**

MV-dependent procoagulant activity can be evaluated using functional assays that measure the contributions of PS and/or TF. STA-Procoag-PPL assay is a standardized automated test that analyses the procoagulant activity of PL-rich MVs in plasma by measuring FXa generation. On the other hand, a highly sensitive FXa generation assay[205, 200] developed in our lab in collaboration with BioCytex Biotech company can be performed on MVs purified from culture supernatants or biological fluids using either ultracentrifugation (Figure 12) or by immunomagnetic separation strategy (IMS) (Figure 13) to measure the PS and TF-dependent

generation FXa activity in a buffer system in the presence or absence of antibodies that block PS and/or TF.

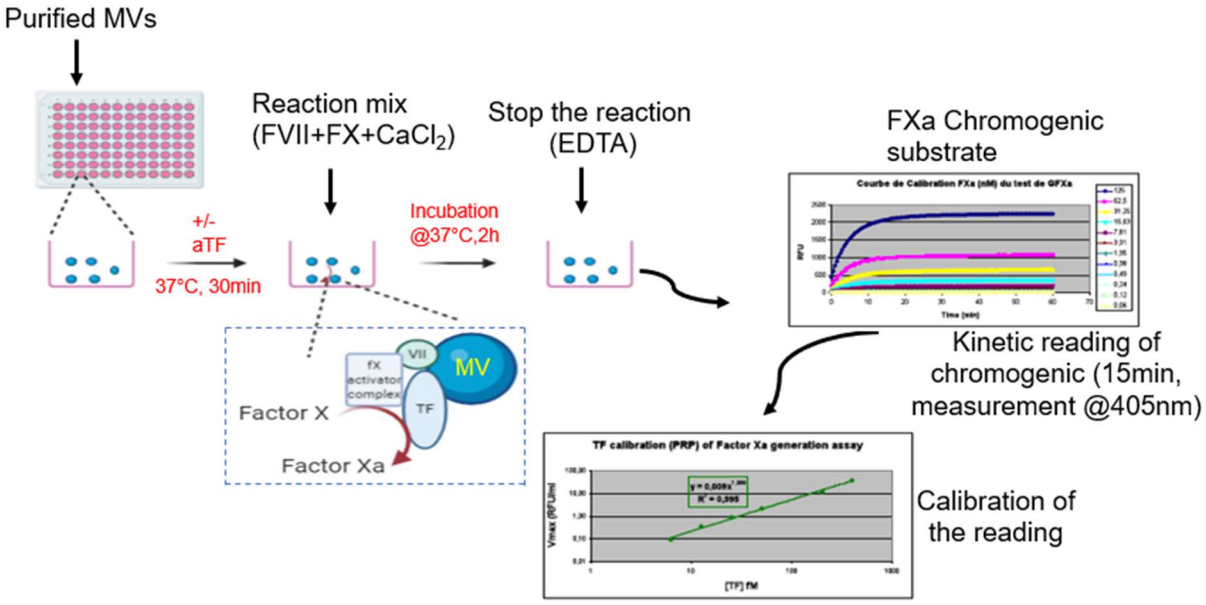

Figure 12. Method to measure MV-TF dependent FXa activity on purified MVs by ultracentrifugation

Legend: PFP: platelet-free plasma; IMS: immunomagnetic separation; TF: tissue factor

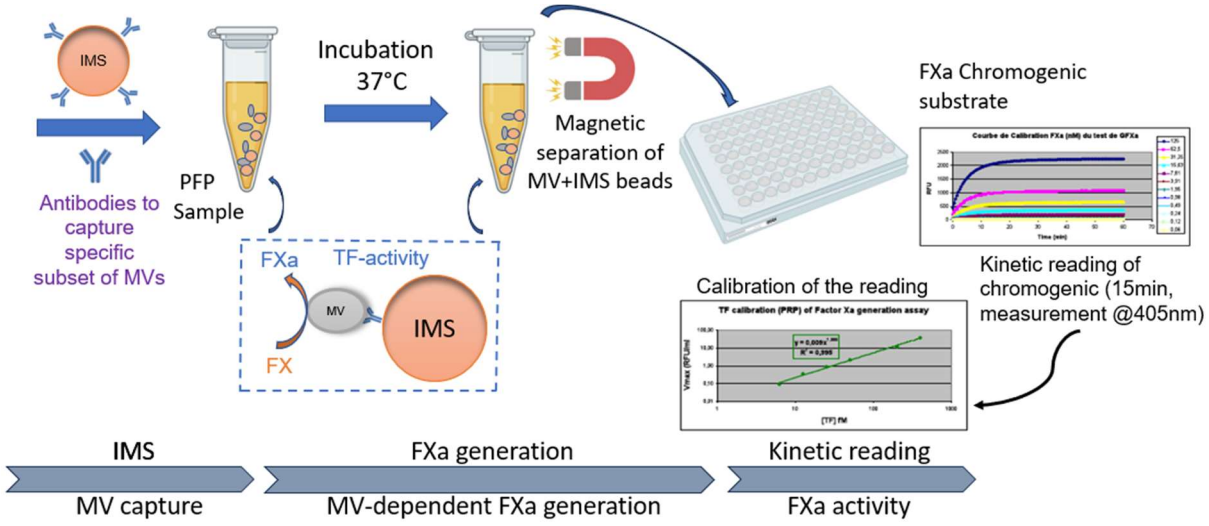

Figure 13. Method to measure MV-TF dependent FXa activity on purified MVs from PFP by IMS

Legend: PFP: platelet-free plasma; IMS: immunomagnetic separation; TF: tissue factor

Alternatively, a calibrated automated thrombogram (CAT), an assay that was originally developed by Hemker et al to measure thrombin generation in platelet-rich and platelet-poor plasma can be used to evaluate thrombin generation supported by PS+/TF+ MVs in PFP. CAT

is highly sensitive in that it can detect thrombin generation initiated by spiking purified procoagulant MVs in the PFP even without the addition of any external PL or TF.

- **Combined functional and antigenic assays**

A combination of antigenic and functional TF assay or CAT assay (Zymuphen MP-TF) was developed to measure MV-TF activity[206, 207]. However, lower sensitivity and specificity are an issue for this assay.

2.4.2 MEASUREMENT OF PROFIBRINOLYTIC ACTIVITY

The fibrinolytic activity of MVs mainly measured either by evaluating MV-dependent plasmin generation capacity (MV-PGC) using a plasmin generation assay or by visualizing the lysis fibrin network. In the plasmin generation assay that was developed in our lab in collaboration with BioCytex Biotech company, MVs are incubated in a buffer containing plasminogen to generate plasmin[208]. Plasmin generated this way can be measured by adding a chromogenic substrate that is specific to plasmin[47]. This assay works with MVs isolated using ultracentrifuge or purified by immunomagnetic separation (IMS) (Figure 14). IMS is an excellent technique to purify a specific subset of MVs (for example, granulocyte MVs) from plasma that is otherwise impractical to do with ultracentrifugation[208].

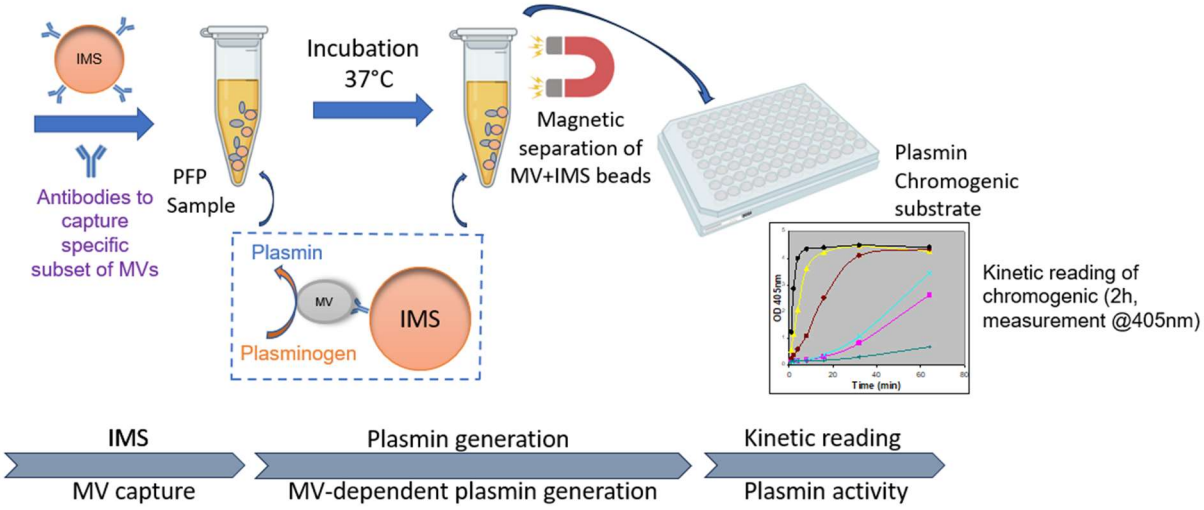

Figure 14. Method to measure MV-dependent plasmin generation on purified MVs from PFP by IMS

When it comes to the degradation of the fibrin fibers, the lysis of the fibrin network can be analyzed using fibrin zymography or the clot front lysis retraction test. Fibrin zymography is an excellent method to study the fibrinolytic activity of PAs[209, 210]. Fibrin zymography is an SDS-polyacrylamide gel-based technique where MV lysate is placed on the fibrin gel in the

presence of plasminogen. Fibrinolysis can be visualized as a clear band against a blue-colored Coomassie dye background which represents nondegraded fibrin[47].

Recently, a paper published in Blood by Cointe et al. from our group demonstrated how profibrinolytic MVs can lyse fibrin using a clot front lysis retraction test. When incubated with high fibrinolytic MVs from granulocytes, a fluorescent clot prepared using MV-depleted PFP was lysed in a uPA/uPAR-dependent manner[211].

2.5. CONCEPT OF COAGULOLYTIC BALANCE OF MICROVESICLES

The ambivalent nature of microvesicles with the expression of both procoagulant and profibrinolytic molecules affecting the coagulation process has been established. This functional ambivalence of MVs in the hemostatic process can be termed "MV coagulolytic balance" (MV-CLB) (Figure 15). As we described earlier, the coagulolytic balance of MVs dependent on the properties of parental cell it comes from the trigger that induce their release. Coagulolytic balance is a ratio between the procoagulant activity and the fibrinolytic activity which we measured using MV-TF dependent FXa generation and plasmin generation assays.

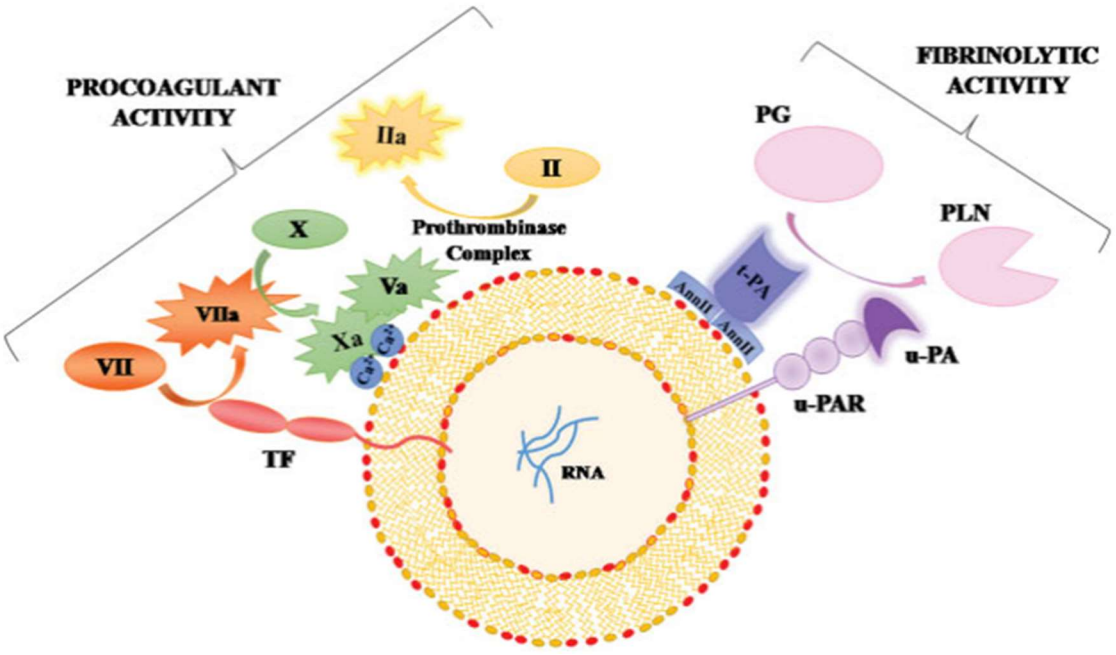

Figure 15: Concept of coagulolytic balance of microvesicles

Legend: Ann II: annexin A2; PG: plasminogen; PLN: plasmin; RNA: acid ribonucleic; TF: tissue factor; tPA: tissue plasminogen activator; u-PA: urokinase-type plasminogen activator; u-PAR: uPA receptor.

(Excerpt from: Vallier et al., Semin Thromb Hemost, 2016)

2.6. Conclusion

The initial concepts of MVs as mere cellular debris have been changed with significant research findings overwhelmingly demonstrating MVs' role in hemostasis and thrombosis. Under normal physiological conditions, the presence of various hemostatic molecules that govern procoagulant, anticoagulant, profibrinolytic, and antifibrinolytic properties of MVs (Figure 16) contributes to maintaining the balance between procoagulant and profibrinolytic function. This functional equilibrium in the context of hemostatic activity can be lost in immunothrombosis conditions with a prevailing procoagulant state. Previous studies from our lab have provided some insight into hemostatic functional changes associated with fibrinolytic activity of MVs in septic shock (SS) patients. In these preliminary studies, we demonstrated that as compared to non-survived SS patients, survived SS patients had significantly higher levels of MV-dependent fibrinolytic potential that counterbalanced the procoagulant state and may have contributed to the removal of microthrombi in patients. This observation was further consolidated with the findings that showed decreased thrombus formation in the kidneys and lungs of the SS mice model that led to increased survival of mice upon receiving infusion MVs (granulocyte MVs) with high fibrinolytic activity. Although these data demonstrate that increased fibrinolytic activity can counterbalance the procoagulant state, a comprehensive picture of MV hemostatic function with regards to procoagulant and/or profibrinolytic activity that may arise from individual subset of MVs in circulation has not been demonstrated. In my research study, I performed a comprehensive analysis of the procoagulant and/or profibrinolytic potential of MVs of neutrophil, monocyte, platelet, and endothelial cell origin in the context of inflammatory milieu/sepsis pathology.

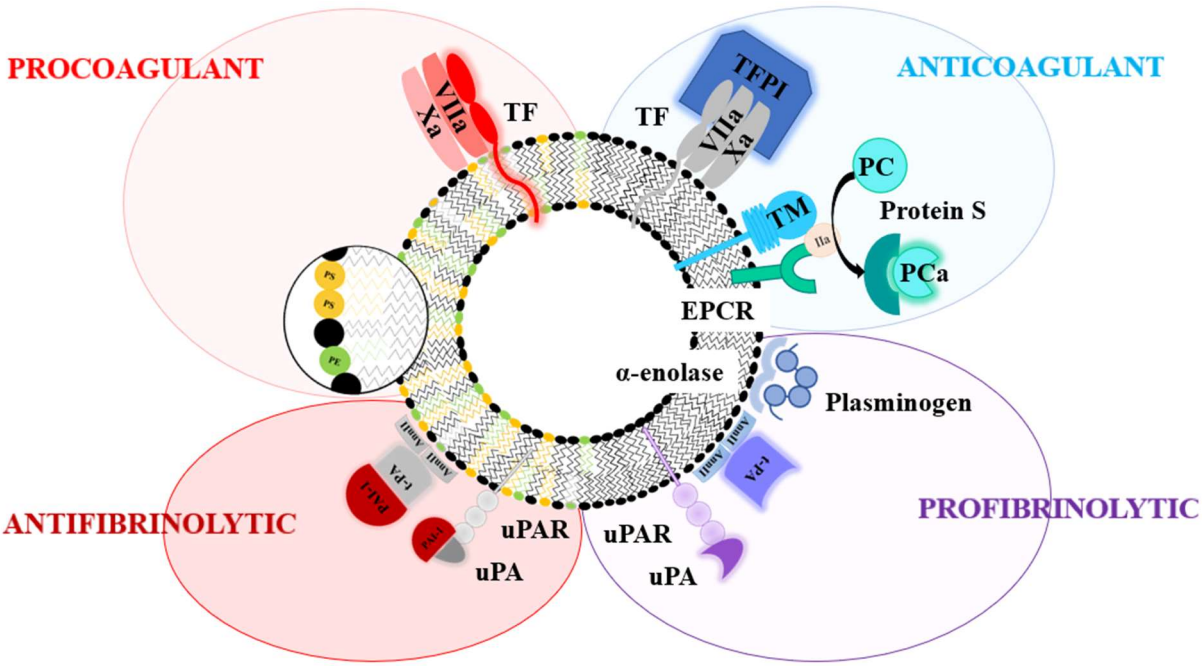

Figure 16: Prothrombotic and antithrombotic molecules of MVs

Legend: PS: phosphatidylserine; PE: phosphatidylethanolamine; TF: tissue factor; TFPI: tissue factor pathway inhibitor; TM: thrombomodulin; t-PA: tissue-type plasminogen activator; uPA: urokinase plasminogen activator; uPAR: urokinase-type plasminogen activator receptor; EPCR: endothelial protein C-receptor.

CHAPTER 3. ROLE OF MVs IN HEMOSTATIC IMBALANCE IN SEPSIS

Sepsis manifests as a result of exacerbated systemic immune response to certain types of infection, which then leads to acute organ dysfunction. The level of acute organ dysfunction varies from patient to patient and the type of pathogen causes it however, most often it is characterized by the collapse of respiratory and cardiac systems that may ensue death of the host. The exacerbated immune response triggers prolonged proinflammatory changes in the host, which leads to heightened coagulation with dysregulated fibrinolysis leading to excess fibrin deposition in the vasculature and disseminated intravascular coagulation (DIC). Although our current understanding of MVs' role in sepsis pathophysiology continues to evolve, we try to elucidate the link between these two entities and how MVs contribute to hemostatic imbalance in sepsis.

3.1. DEFINITION OF SEPSIS AND SEPTIC SHOCK

Multiple organ failure is the primary cause of death of the host in septic shock. A scientific consensus meeting was convened in Paris in 1994 by the European Society of Intensive Care Medicine and the Society of Critical Care Medicine (ESICM) to create a standardized scoring system for the assessment of clinal representation of sepsis-related organ failure to provide better clinical interventions to patients. This standardized score was called the sepsis-related organ failure assessment score (SOFA) and was introduced to clinical practice for improved prediction of sepsis-related patient outcomes[212]. Since its first introduction, several updates have been added to the criteria for the assessment of organ failure in patients (Table 4).

Although individual variability does exist in sepsis and septic shock conditions, according to the third international consensus[213] on sepsis and septic shock (SS), sepsis patients likely to have alterations in mental status with a systolic blood pressure of ≤ 100mm Hg and respiratory rate ≥22/min, while septic shock patients can be identified with persisting hypotension ≥65mm Hg along with serum lactate level of >2mmol/L (18mg/dL). Besides this, a SOFA score of ≥2 can be generally reflective of the deleterious effect of infection.

Table 4: Calculation of SOFA score

(Excerpt from: Singer et al, JAMA, 2016)

Table 1. Sequential [Sepsis-Related] Organ Failure Assessment Score[a]

	Score				
System	0	1	2	3	4
Respiration					
Pao_2/Fio_2, mm Hg (kPa)	≥400 (53.3)	<400 (53.3)	<300 (40)	<200 (26.7) with respiratory support	<100 (13.3) with respiratory support
Coagulation					
Platelets, $\times 10^3/\mu L$	≥150	<150	<100	<50	<20
Liver					
Bilirubin, mg/dL (μmol/L)	<1.2 (20)	1.2-1.9 (20-32)	2.0-5.9 (33-101)	6.0-11.9 (102-204)	>12.0 (204)
Cardiovascular	MAP ≥70 mm Hg	MAP <70 mm Hg	Dopamine <5 or dobutamine (any dose)[b]	Dopamine 5.1-15 or epinephrine ≤0.1 or norepinephrine ≤0.1[b]	Dopamine >15 or epinephrine >0.1 or norepinephrine >0.1[b]
Central nervous system					
Glasgow Coma Scale score[c]	15	13-14	10-12	6-9	<6
Renal					
Creatinine, mg/dL (μmol/L)	<1.2 (110)	1.2-1.9 (110-170)	2.0-3.4 (171-299)	3.5-4.9 (300-440)	>5.0 (440)
Urine output, mL/d				<500	<200

Abbreviations: Fio_2, fraction of inspired oxygen; MAP, mean arterial pressure; Pao_2, partial pressure of oxygen.

[a] Adapted from Vincent et al.[27]

[b] Catecholamine doses are given as μg/kg/min for at least 1 hour.

[c] Glasgow Coma Scale scores range from 3-15; higher score indicates better neurological function.

3.2. EPIDEMIOLOGICAL PREVALENCE OF SEPSIS

As far as the mortality rates are concerned, sepsis-related mortality accounted for 11 million out of reported 48.9 million sepsis cases and represented 19.8% of all deaths incidents in 2017 [214]. The number of sepsis incidents in 2017 showed a decline of 18.8% as compared to the total reported incidents of 60.2million in 1990. In a study published in 2017 [215], sepsis accounted for a third of all adult general ICU admissions between 2011 and 2015. Despite recent advances in medical sciences and access to better health care, sepsis remains one of the leading causes of mortality in developed countries[216]. The economic burden associated with sepsis treatment and rehabilitation is significantly high because of relatively high incident rates[217]. This warrants more investigation to better understand the disease pathophysiology and biomarker discovery which would enable us not only for early diagnosis but for improved treatment strategies.

3.3. ETIOLOGY OF SEPSIS

Sepsis is a condition with a dysfunctional immune system with exacerbated immune response to an underlying infection that can arise from various pathogens such as bacteria, viruses, or fungi. Bacteria is the most prevalent cause of sepsis and among all the bacteria. Although sepsis incidence prevalence is more associated with gram-negative bacteria (62.2%), recent evidence

shows that gram-positive bacteria (46.8%) have also turned into a significant source of infection[218]. While Escherichia coli, Pseudomonas, Enterobacteriaceae, and Acinetobacter species of gram-negative bacteria account for most of the gram-negative bacterial infections, Streptococcus aureus and streptococcus pneumonia gram-positive bacterial infection cases have also been reported in samples collected from patients [218, 219]. Irrespective of what bacteria, all bacterial infections share a common mechanism in the development of sepsis. Bacteria secrete endotoxins like LPS and other pathogen-associated molecular patterns (PAMPs) that cause Toll-like receptors (TLRs) to get activated and initiate complex intracellular signaling cascades that result in the secretion of pro-inflammatory mediators and infiltration of immune cells to the site of infection[220].

Several predisposing risk factors make some people more prone to sepsis infection. Age, gender, and race constitute general risk factors for sepsis with higher incidents in older individuals and males. More specific risk factors comprise chronic underlying health conditions (for example: cancer, AIDS, liver diseases, diabetes, cardiovascular complications, etc.), poor socioeconomic status, and use of immunosuppressive medications[221]. Out of all the risk factors, the immunocompromised stage is a common factor in people who are most susceptible to infection.

3.4. PATHOPHYSIOLOGY OF SEPSIS/SEPTIC SHOCK

Sepsis and septic shock are the result of overwhelming prolonged immune response to an underlying infection which leads to total shutdown of multiple organ systems in the host. Exaggerated immune response involves several cellular actors which constitute endothelial cells, monocytes or macrophages, neutrophils, and platelets, which activate numerous molecular factors and finally culminate in full-blown system damage even at microvascular levels (Figure 17). Sepsis pathophysiology involves a multifaceted response from the host that disrupts equilibrium between the systems of inflammation and anti-inflammation as well as coagulation and fibrinolysis leading to extensive activation of cross-talk between inflammation and coagulation pathways.

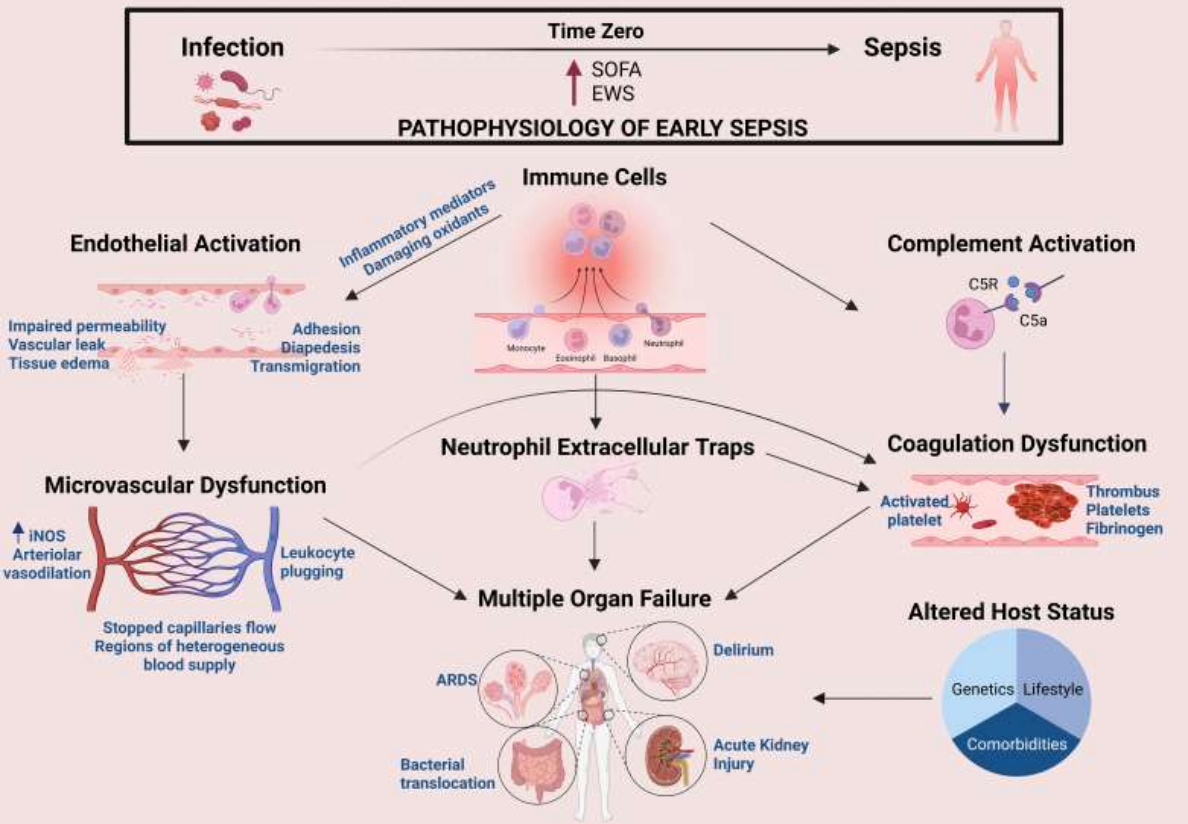

Figure 17: Pathophysiology of sepsis

(Excerpt from: Arora et al., Am J Physiol Regul comp Physiol., 2023)

3.4.1. IMMUNE RESPONSE AND ORGAN DYSFUNCTION IN SEPSIS

In the event of infection, innate immune cells such as macrophages, monocytes, neutrophils, and natural killer cells recognize the microbial products in the host through pattern recognition receptors (PRRs) expressed on these immune cells[222]. PRRs expressed on the cell surface primarily interact with pathogen-associated molecular patterns (PAMPs) on the microbial membrane or damage-associated molecular patterns (DAMPs) that arise from the damaged or dead host cells resulting from the infection. Activation of PRRs through this interaction is necessary for the proper response to infectious agents and the host's survival. While PAMPs include lipoproteins, peptidoglycan, lipopolysaccharide (LPS), flagellin, lipoteichoic acid, and nucleic acids from various pathogenic organisms, DAMPs comprise endogenous danger signals such as HMGB1 (high mobility group box 1), ATP (adenosine triphosphate), S100, heat shock proteins (Hsps), hyaluronic acid, uric acid, and DNA (deoxyribonucleic acid)[223]. Recognition of PAMPs and DAMPs by PRRs and subsequent activation of downstream signaling cascade plays a crucial role in the development of sepsis pathology.

Toll-like receptors (TLRs), nod-like receptors (NLRs), and RIG-I-like receptors (RLRs) are the main receptors that recognize PAMPs. In contrast, C-type lectin receptors (CLRs), and

RAGE proteins act as receptors for DAMPs. TLR signaling through the identification of molecular patterns primarily involves two adapter protein pathways, either MyD88 (Myeloid differentiation primary response 88) or TRIF (toll-interleukin 1 receptor domain-containing adaptor protein inducing interferon beta)[224]. TLR activation and adaptor protein engagement lead to the activation of mitogen-activated protein kinase (MAPK), nuclear factor-kappa B (NF-kB), interferon regulatory factor 3/7 (IFN3/7), resulting in the secretion of proinflammatory cytokines, type 1 IFN, chemokines, and antimicrobial peptides to neutralize pathogens.

Increased cell stress or cell damage during the event of an overwhelming inflammatory response to sepsis pathogens causes cells to continually release HMGB1, Hsps, S100, and DNA. High plasma concentration of HMGB1 is known to diminish neutrophil-associated bacterial killing and decrease NADPH oxidase thus potentiating organ damage associated with sepsis[225, 226]. In contrast, elevated levels of Hsps and S100 in the plasma further activate TLR signaling and potentiate inflammatory cytokine release which leads to further organ injury[227228]. However, in sepsis pathophysiology, there are two phases of inflammatory responses: an initial hyperinflammatory response or systemic inflammatory response syndrome (SIRS) with elevated levels of proinflammatory cytokine storm and a second phase of immunosuppressive response or compensatory anti-inflammatory response (CARS) that is associated with increased levels of anti-inflammatory cytokines such as IL-10, IL-1 receptor antagonist (IL-1ra) (Figure 18).

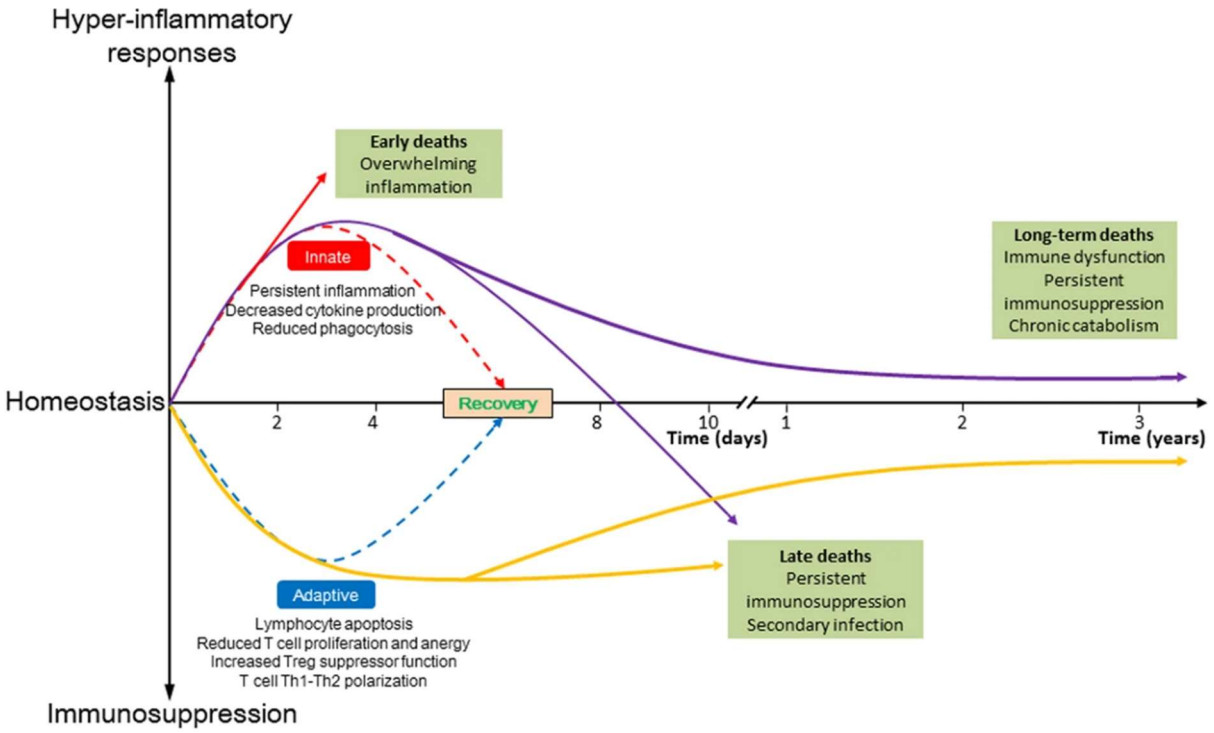

Figure 18: Immune response in sepsis

(Excerpt from Cao et al, Cell death & disease, 2019)

3.4.2. CARDIOVASCULAR DYSFUNCTION

The inflammatory milieu in SS induces the release of several proinflammatory cytokines and in particular, TNF-α and IL-1β in the blood are highly cardio-depressive and lead to cardiac dysfunction in SS patients[229]. Several converging studies with animal models [230, 231, 232, 233] and human cohorts have unraveled severe myocardial aberrations and cardiac output changes that correlated with high levels of TNF-α and IL-1β in SS. The combination of these two cytokines affects the release of other potent factors such as nitric oxide (NO) that further potentiate its effect on myocardial contractility[234235]. Administration of an anti-TNF-α antibody to SS patients saw significant improvement in cardiac function but did not improve mortality [236, 237].

The compounding effect of primary inflammatory mediators like TNF-α[238] and IL-1β[239] and secondary cytotoxic factors like NO [240], and oxygen-free radicals [241] contribute to accelerated cardio-depression in SS. High levels of proinflammatory mediators in SS lead to the expression of NO synthase (iNOS) in the myocardium [242, 243] which then augments the production of NO. Heightened NO production facilitates myocardial dysfunction by enhancing the myofilament sensitivity to $Ca2^{+}$ and the generation of free radicals[244].

Primary proinflammatory mediators along with NO and other cytotoxic mediators can exert a myocardial effect through their influence on the mitochondrial function by diminishing the activities of complex I and II of the mitochondrial respiratory system in SS [245, 246, 247]. Similarly, extracellular histones and HMGB1 are known to interact with TLR2 and TLR4 in the cardiomyocyte and cause mitochondrial dysfunction by depleting mitochondrial ATP levels, and membrane potential and by increased ROS production [248]. All these effects eventually cause severe impairment in the cardiac-excitation (EC) coupling, increased intravascular fluid leakage, and leukocyte transmigration due to increased vascular permeability (Figure 19) by disrupting endothelial integrity[248, 249]. In conclusion, various proinflammatory cytokines and cytotoxic mediators can exert myocardial damage which leads to cardiac depression in SS patients.

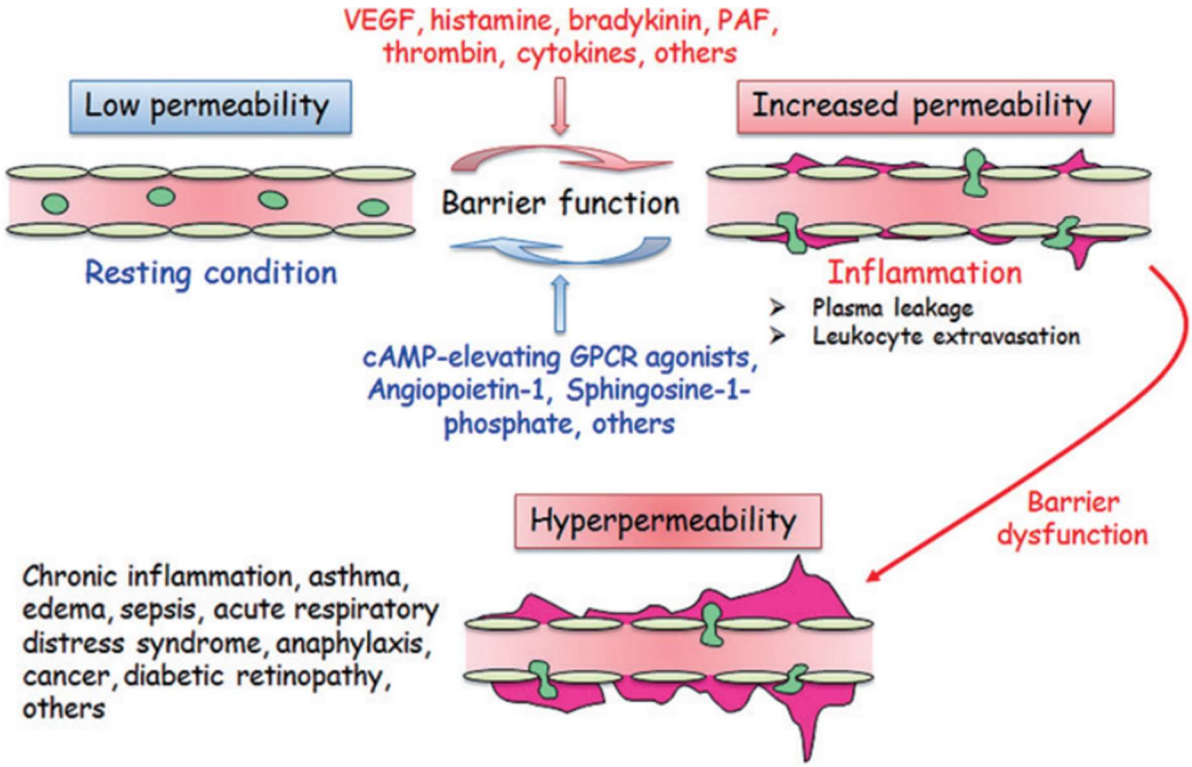

Figure 19: Endothelial barrier dysfunction and increased permeability in sepsis/septic shock

(Excerpt from: Rho et al,2017, J Nippon Med Sch. 2017)

3.4.3. HEMOSTATIC IMBALANCE DURING SEPTIC SHOCK

Hemostasis and inflammation are a well-regulated physiological process and are highly interconnected to each other. However, during uncontrolled infection, the crosstalk between hemostasis and inflammation may become highly dysregulated causing thrombo-inflammatory responses in the host. Dysregulated immune response flare-ups release several proinflammatory cytokines which can cause systemic activation of coagulation and

subsequently lead to disseminated intravascular coagulation (DIC) characterized by the development of microthrombi in the vasculature and bleeding at the systemic level [250]. The loss of hemostatic equilibrium between procoagulant and profibrinolytic components[251] plays a critical role in septic shock. In this session, we try to describe the impairment in hemostatic equilibrium in the development of SS.

3.4.3.1. ACTIVATION OF COAGULATION

Several cellular and molecular factors are involved in the activation of coagulation in sepsis. Although activation of immune cells is required for the proper host response in the event of infectious conditions, the immune cell detection of PAMPs and DAMPs through PRRs may result in the overwhelming activation of monocytes and neutrophils[252]. This activation increases thrombosis by triggering the expression of TF on monocytes[253, 254] and the release of NETs from neutrophils[255, 256]. Moreover, endothelial activation and subsequent activation of TF have been demonstrated in several *in vitro* models following stimulation with bacterial endotoxins or inflammatory cytokines such as TNF-α or IL-1β [257, 258, 259]. A similar TF expression by endothelial cells and monocytes has been observed *in vivo* in sepsis patients [260, 261]and healthy subjects after the administration of TNF-α [262, 263].

TF is the principal initiator of coagulation and plays a crucial role in coagulopathy associated with sepsis[264]. Formation of the TF-FVII complex is required for the activation of the coagulation system. This has been demonstrated in several animal models of sepsis and humans by administering agents that block the TF-FVIIa complex could significantly attenuate coagulation and mortality [265, 266, 267]. Similar observations were made in genetically modified mice models of bacterial endotoxemia with low levels of TF or FVII [254, 268]. Furthermore, endothelial dysfunction also has been implicated in sepsis-related organ dysfunction and mortality [269, 270, 271, 272]. Drake et al demonstrated significantly increased expression of TF in the lung and renal epithelium detected by immunofluorescence in a baboon model of sepsis[273]. In a similar model, up-regulation of TF, increased fibrin deposition and vascular permeability were observed in the arterial branched segments that were correlated with higher shear stress[274]. In contrast, the expression of TF in monocytes increases in response to elevated levels of C-reactive protein and acute-phase protein[275]. Alternatively, proinflammatory cytokines induce secretion of soluble TF isoform, an alternatively spliced tissue factor with truncated exon 5 [276] by endothelial cells, and in sepsis, the procoagulant activity of plasma increased by the release of soluble TF that is either associated with MVs or soluble TF isoform[277].

In conclusion, TF can be used as a valuable biomarker or prognostic tool to evaluate sepsis severity or mortality[278].

3.4.3.2. DYSFUNCTIONAL ANTICOAGULANT MECHANISM

Anticoagulant pathways play an important role in the regulation of coagulation and inflammation. The principal mediators that comprise the anticoagulant system are antithrombin (AT), Thrombomodulin (TM)/activated protein C (APC), and tissue factor pathway inhibitor (TFPI). Under normal physiology, they play a crucial role in controlling abnormal coagulation.

Antithrombin levels get significantly decreased in sepsis patients because of its rapid consumption for the thrombin formation in the event of DIC, reduced synthesis by the dysfunctional liver, or increased proteolysis by neutrophil elastase [279, 280, 281]. A deficiency of antithrombin levels accelerates the formation of fibrin due to inadequate inhibition of thrombin and is correlated with the severity of sepsis and mortality[282].

Like antithrombin, the TM/APC pathway plays an important role in regulating coagulation and inflammation. Functional deficiency of the TM/APC pathway has been implicated in DIC caused by sepsis. The various factors that contribute to the compromised TM/APC pathway in sepsis include decreased synthesis by endothelial cells and monocytes and reduced expression by these cells induced by TNF-α or increased cleavage by neutrophil enzymes[283, 284]. Thrombin/TM complex facilitates the conversion of protein-C to activated protein C. APC in the presence of protein S enzymatically degrades Va and VIIIa to prevent amplification of coagulation by inhibiting thrombin formation. Administration of recombinant human APC to sepsis patients with reduced levels of protein C[285, 286] may be a promising strategy to improve prognosis and reduce mortality[287].

Another important factor in the anticoagulation pathway is TFPI. TFPI is the primary inhibitor of the TF-FVIIa complex. Decreased levels of functional TFPI have been reported in sepsis patients with DIC[288], however, administration of recombinant TFPI to sepsis patients did not have any influence on mortality[289].

3.4.3.3. DEFICIENCY OF FIBRINOLYTIC SYSTEM

Inflammation in sepsis leads to coagulopathy which is manifested by hyperactivation of the coagulation system with dysfunctional anticoagulant and fibrinolytic system. In the initial stages of septic shock, in parallel with hyperactivation of coagulation, the fibrinolytic system is turned on with the acute release of plasminogen activators like t-PA and u-PA mainly by the

endothelium[251]. However, this initial surge in the fibrinolytic system with high plasmin generation is subsequently shut down by a sustained rise in plasma levels of plasminogen activator inhibitors such as PAI-1 and TAFI[290]. Bacterial endotoxin-induced sepsis animal models have demonstrated that TAFI is generated mainly in the presence of thrombomodulin[291]. However, decreased expression of thrombomodulin by endothelial cells in sepsis negatively impacts the generation of TAFI but not PAI-1[290]. This indicates a dominant role of PAI-1 in inhibiting fibrinolysis.

This shift towards a more procoagulant state in sepsis causes increased deposition of fibrin in microvasculature that eventually results in DIC and organ failure.

3.4.3.4. DISSEMINATED INTRAVASCULAR COAGULATION (DIC)

Disseminated intravascular coagulation (DIC) is an acquired syndrome characterized by excess intravascular activation of the coagulation system with a dysfunctional anticoagulant system at the systemic level leading to microvascular complications and organ dysfunction[292]. Acute systemic inflammatory response in sepsis leads to severe damage to the endothelium which initiates coagulopathy with amplified consumption of platelets and coagulation factors. These events are associated with increased thrombin generation and fibrin deposition in the microvasculature manifesting in multiple organ damage due to reduced organ perfusion (Figure 20). However, in some patients, continued consumption of platelets and coagulation factors caused by DIC may result in profuse bleeding[293].

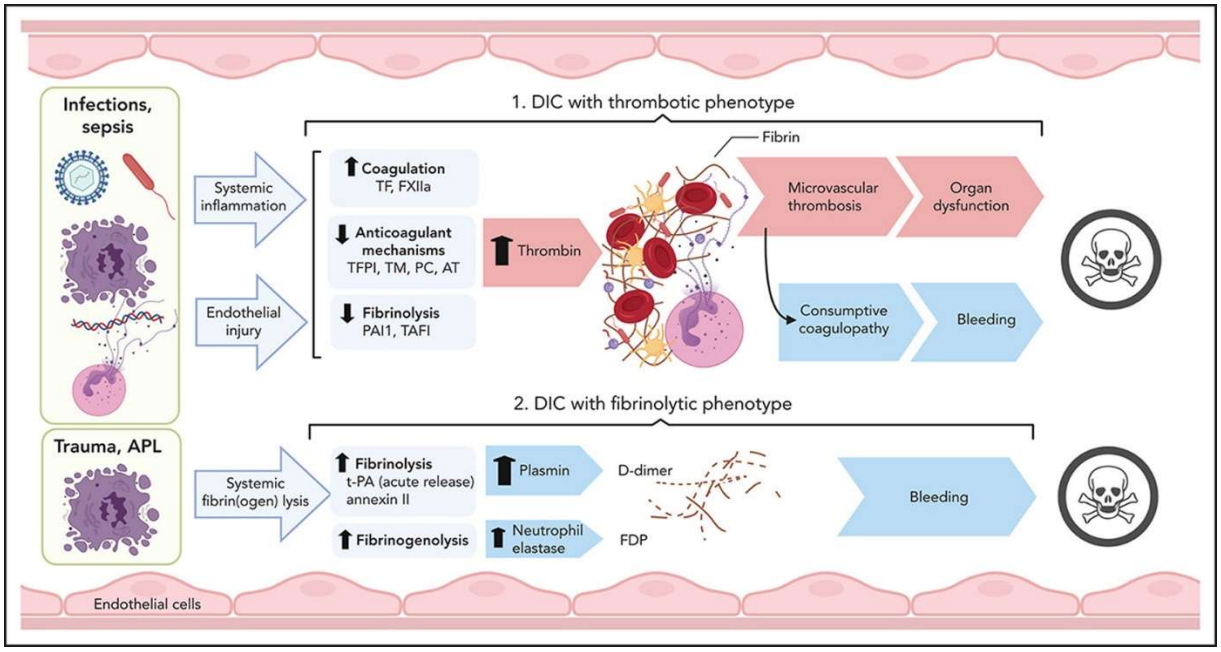

Figure 20: Pathogenesis of thrombotic and fibrinolytic phenotypes of DIC

(Excerpt from: Popescu et al, Blood, 2022)

3.4.5 ROLE OF MICROVESICLES IN SEPSIS

MVs in sepsis are represented by their multifaceted function (Figure 21). Overwhelming activation of coagulation/inflammation in sepsis is associated with increased release of MVs that may have either damaging or protective effects depending on their cellular source. Here, I would like to elucidate the potential role of MVs in the pathophysiology of sepsis.

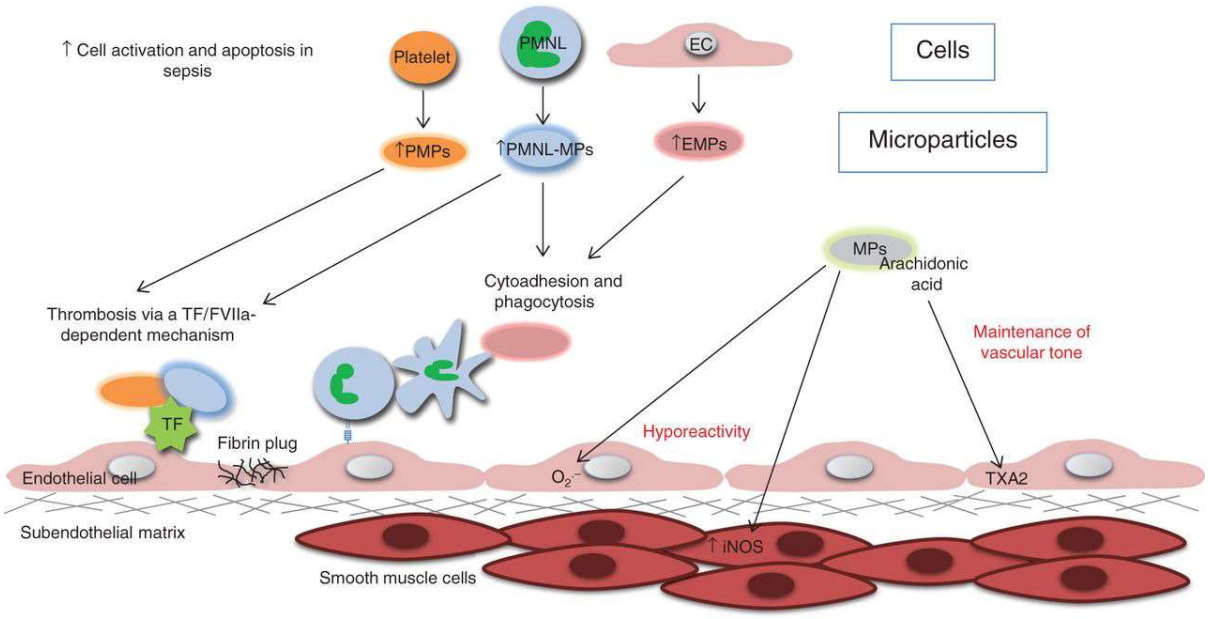

Figure 21: Role of microvesicles in sepsis

(Excerpt from: Reid et al. Br J of Anaesth, 2012)

3.4.5.1. EFFECT OF MICROVESICLES ON INFLAMMATION

MVs play an important role in the crosstalk between inflammation and coagulation. In sepsis, the level of MVs in the circulation increases, and depending on the cell type it originates from, they can have either proinflammatory (Figure 18) or anti-inflammatory effects by modulating the activity of target cells. Activated or damaged cells secrete DAMPs such as histones, HMGB1, nucleosomes, and NETs into circulation, and MVs are known to transfer them to target cells to modulate their function[294295]. On the other hand, MVs also exert immunomodulatory effects on target cells. Complementary factors carried by MVs modulate inflammation and cellular damage, thus they contribute to a balance between inflammatory and anti-inflammatory conditions[296]. MVs from an LPS-injected mouse enhanced the proliferation and migration of lymphocytes and induced differentiation of naïve T-cells to T-helper (TH)-1 and TH2 cells[297]. Following intraperitoneal injection of MVs from a CLP mouse caused elevated neutrophil migration to the peritoneal cavity[298]. Similarly, MVs from septic patients induced migration neutrophil MVs and they in turn upregulated IL-8 expression by monocytes[299].

MVs also mediate their immunomodulatory effect through miRNAs. MVs from both sepsis patients and animal models carry several miRNAs that are responsible for proinflammatory effects [298,300]. In contrast, neutrophil MVs released at the site inflammation carried some anti-inflammatory MiRNAs[300]. Additionally, platelet MVs enriched with MiR-223 had anti-

inflammatory effects by reducing the expression of ICAM-1 on endothelial cells [301, 302] and interaction between endothelial cells and leukocytes[302].

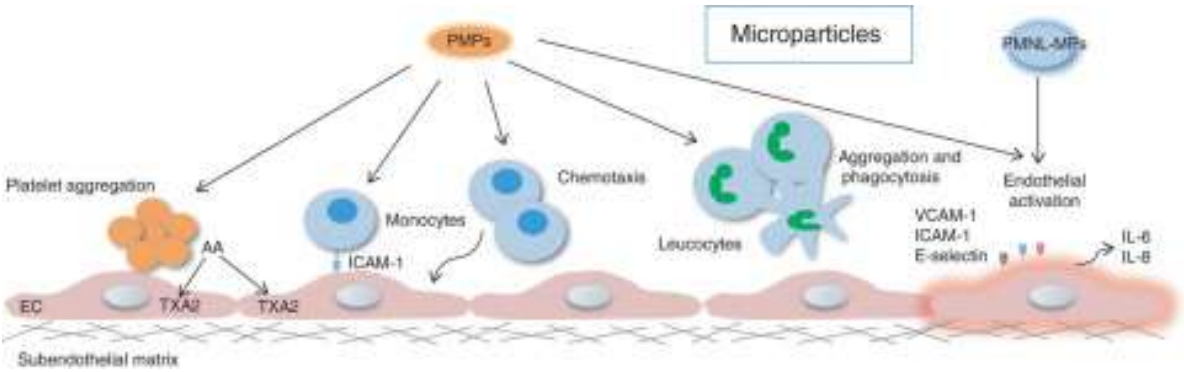

Figure 22: Proinflammatory effect of MVs

(Excerpt from: Reid et al. Br J of Anaesth, 2012)

3.4.5.2. EFFECT OF MICROVESICLES ON COAGULATION

Activation of coagulation plays an important role in infection to prevent bacterial spreading and thus fend off further damage that may be caused by the infectious agent. However, in response to infection during sepsis, the immune system becomes overwhelmed leading to the development of DIC due to heightened inflammatory reaction, systemic activation of coagulation, downregulation of anticoagulant system, and inhibition of fibrinolysis[303].

During sepsis, continued inflammatory response triggers the release of procoagulant MVs from various cellular sources. Converging documented evidence from animal studies and clinical cohorts demonstrates increased levels of PS+ procoagulant MVs as a result of sepsis [304, 305, 306, 307, 308, 309]. An increase in PS+ MVs was associated with increased thrombin generation through the activation of extrinsic and intrinsic coagulation pathways thus resulting in significantly enhanced coagulation[306, 308, 304, 307]. Since platelet MVs make up the predominant class of MVs in circulation, they display a prominent role in the hemostasis and coagulation process. Platelet MVs are mostly procoagulant which is mostly attributed to the exposure of PS on their surface. Several studies have demonstrated platelet MV's role in sepsis progression by promoting coagulation. For example, in a mice model of CLP, PS+ platelet MVs induce thrombin generation and activate extrinsic and intrinsic pathways of coagulation[310]. In a similar model, Zafrani et al,[307] the involvement of procoagulant platelet MVs in sepsis-associated coagulation and genetic overexpression of calpastatin, an endogenous calpain-specific inhibitor not only significantly reduced the release of procoagulant MVs but reversed systemic inflammation and intravascular coagulation. Besides, the reduction in procoagulant MVs in circulation significantly improved the survival of the animals by reducing organ dysfunction. On the other

hand, an increase in PS+ platelet MVs in patients with sepsis was significantly correlated with DIC and acute kidney injury (AKI)[311].

Expression of functional TF along with exposure to PS increases the MV-dependent procoagulant activity by several folds. Elevated levels of TF-bearing MVs have been reported in sepsis patients[312]. Since monocytes are the predominant source of TF activity in the circulation[313], MVs from monocytes appear to contribute to sepsis-associated coagulopathy. in the blood in a mice model of sepsis induced by the Methicillin-resistant *Staphylococcus* aureus (MRSA) showed an increase in monocyte MVs and was associated with TF-dependent robust thrombin generation[314]. In an endotoxemia mice model, LPS induced higher MV-TF-dependent procoagulant activity as compared to the control group, and the TF activity was significantly blocked by an anti-TF antibody[315]. Similarly, increased procoagulant activity by MVs from monocytes was dependent on TF in the human endotoxemia model[316]. In contrast, consistent with other endotoxemia models, LPS infusion augmented MV-TF activity but activation of intrinsic pathway also played a significant role as evidenced by accelerated thrombin generation that was independent of MV-TF activity[305]. Furthermore, DIC in sepsis patients with meningococcal infection had robust thrombin generation in plasma that was mediated by elevated levels of TF expressing MVs of monocytic origin[304].

Infection in sepsis dramatically increases neutrophil MVs and the presence of neutrophil MVs has been reported in the areas of inflammation that are affected in sepsis patients[317]. These MVs are carriers of cellular DNA, histones, and other procoagulant components of the parent cell that can potentially modulate procoagulant activity[318]. The tangled network of histones and antimicrobial peptides in the neutrophil extracellular traps (NET) is capable of preventing microbial dissemination by trapping them, however, they can massively influence coagulation pathways due to their procoagulant activity[319]. In a mice model of sepsis, Nair et demonstrated that LPS infusion can generate free histones and histones associated with MVs[320]. Histones associated with MVs have been demonstrated to be involved in inflammation and coagulation. Neutrophil MVs can also exert their impact on the endothelial cell layer inducing endothelial dysfunction. When stimulated with calcium ionophore, neutrophils generated MVs with high myeloperoxidase activity, and these MVs induced loss of endothelial cell integrity and morphological changes[321].

Additionally, some subsets of MVs are known to have anticoagulant and fibrinolysis properties[322, 47, 323]. Endothelial MVs express TM and endothelial protein C receptor (EPCR)

and the secretion of these MVs is upregulated along with procoagulant MVs in sepsis[322]. Besides their procoagulant function, erythrocyte MVs may provide a suitable surface for the anticoagulant reactions of protein C to balance the coagulation cascade[324].

In our lab, we demonstrated leukocyte and endothelial MVs have potential fibrinolytic and proteolytic capacity because they carry uPA/uPAR or tPA/uPAR system on their surface[47, 325, 189]. These MVs provide a catalytic surface for the conversion of plasminogen to plasmin, and this may help in the clot lysis[211]. This fibrinolytic mechanism of certain subsets of MVs can act as a counterbalance to procoagulant condition sepsis to protect from the deleterious effects of SS.

3.6. CLINICAL SCORES IN SEPSIS/SEPTIC SHOCK

Sepsis as a major public health crisis requires robust clinical criteria for the course and prognosis of the disease. Various clinical diagnosis and prognosis tools have been established for this purpose that can be used by clinicians and other health care professionals.

Earlier, based on the 1991 consensus meeting convened by the American College of Chest Physicians (ACCP) and the Society of Critical Care Medicine (SCCM), sepsis was termed a Systemic inflammatory response syndrome (SIRS), organ failure associated with sepsis was termed as 'severe sepsis' that would eventually progress towards 'septic shock' with arterial hypotension despite adequate resuscitation[326]. However, inadequacy related to these definitions led to revisions in the existing criteria.

A new Simplified Acute Physiology Score (SAPS II): This scoring system was developed from the previous Acute Physiology and Chronic Health Evaluation score used in the (APACHE II) by Gall et al[327]. SAPS II includes 17 criteria: Age, type of admission, heart rate, blood pressure, temperature, the ratio between partial pressure of oxygen (PaO_2), and the fraction of inspired oxygen (FiO_2), urine output, serum urea level, leukocyte count, serum potassium level, serum sodium level, serum bilirubin level, Glasgow coma score, and chronic health conditions.

Sequential (sepsis-related) organ failure assessment (SOFA) score is being widely used the assess the extent of organ failure to initiate clinical interventions[212]. The sequential increase in the SOFA score is associated with a high probability of mortality in patients[212]. The SOFA score generally accounts failure of 6 organ systems: Respiration (PaO_2/FiO_2), Coagulation abnormalities (thrombocytopenia), Cardiovascular dysfunction (hypotension), neurological

abnormalities (Glasgow coma score), renal function (increased serum creatine level or urine output), and liver function (increased bilirubin).

Besides these clinical scores, fibrin-related markers like D-dimers (DDi) and fibrin monomers (FM) may also be used in regular clinical practice as these hemostatic parameters directly reflect hemostatic imbalance with more intravascular fibrin formation and can be used for better evaluation of prognosis and for predicting mortality[328].

3.7. BIOMARKERS IN SEPSIS/SEPTIC SHOCK

Several biomarkers can be used to evaluate the patient's condition under sepsis disease. While the list of biomarkers continues to evolve, there are no ideal biomarkers that cannot be used to determine the diagnosis or prognosis of patient status. However, there are a few reliable biomarkers that are routinely used for patient diagnosis. As sepsis causes a significant imbalance in immune response and coagulation process that eventually leads to organ damage, we need to identify robust biomarkers that would reflect these underlying problems and plan various treatment options. Therefore, many currently used biomarkers in clinical settings for the management of sepsis or septic shock are discussed in Table 5:

Table 5: Biomarkers used for diagnosis of sepsis

(Adapted from Raveendran, J R Coll Physicians Edinb, 2019)

Biomarkers	Pathophysiology	Clinical relevance	References
CRP	Acute-phase reactant produced by the liver in response to inflammation or tissue damage	Commonly used, low specificity	Coelho et al, 2007
PCT	Calcitonin prohormone. Produced in response to infection	Commonly used and correlated with severity, helps to discriminate between infectious and non-infectious systemic inflammation. Useful as a test diagnostic and as a prognostic marker, as well as a marker of sepsis severity and associated mortality. Helps assess response to treatment.	Hur et al, 2014

ChT activity	Secreted by activated macrophages	Needs assessment	Raveendran et al, 2019
Presepsin	Produced in response to bacterial infection	High specificity and positive predictive value of bacterial infection in critically ill pediatric patients	Limongi et al, 2018
IL-27	Produced by antigen-presenting cells in response to microbial products and inflammatory stimulus	High specificity and positive predictive value of bacterial infection in critically ill pediatric patients	Hanna et al, 2015
Hepcidine	Produced from the liver. Synthesis is induced by IL-6, produced in response to inflammation	Reliable marker of early and late neonatal sepsis	Wu et al, 2012
SAA	an apolipoprotein. The major protein in the acute phase	Equivalent to or more sensitive than the CRP	Raveendran et al, 2019
MIF	Produced in response to microbial products, hypoxia, and proliferative signals	Increase in septic shock. Helps differentiate survivors from nonsurvivors.	Bozza et al, 2004
LBP	Reactive acute phase	Not an impressive marker due to highly variable levels in the patients	Opal et al, 1999
Pentraxine 3	Binds to a specific pattern of fungi, bacteria, and viruses inducing phagocytosis. Secreted by various cells, such as leukocytes and endothelial cells	Correlation with sepsis severity and coagulation/fibrinolytic dysfunction associated with sepsis. In patients with febrile neutropenia after chemotherapy, high pentraxin 3 values predict the development of septic shock and bacteremia	Mauri et al, 2010, Huttunen et al, 2011
Cytokines IL-6 receptor antagonist, IL-8, TNF-α and IL 1	Immunomodulating agent produced from nucleated cells	Correlates with severity and outcome of sepsis	Raveendran et al, 2019
HMGB1	Released by an activated macrophage	Not a predictor of mortality in patients with sepsis	Karlson et al, 2008

Protein-C	Coagulation biomarker	Low levels in neutropenic patients predict severe sepsis and septic shock	Raveendran et al, 2019
BPW analysis	Based on activated partial thromboplastin time	For the diagnosis of sepsis	Delannoy et al, 2009
sTREM-1	Member of the super immunoglobulin family. Increased expression in the presence of bacteria or fungi	Superior to CRP and PCT as an indicator of sepsis. Indicates the severity of sepsis	Li et al, 2013
suPAR	Expressed in different cells. Participates in various immunological functions	Less useful in sepsis	Eugen-Olsen et al, 2011
Midregional proadrenomedullin	Potent vasodilating and bactericidal agent	A good predictor of severity and outcome of CAP	Christ-Crin et al,2006
Polymorphonuclear CD64 index	Upregulation of CD64 expression is an early immune response to bacterial infection	Early detection of sepsis in neonates	Icardi et al, 2009
Mean neutrophil volume	Volume, conductivity, and scatter parameters of neutrophils are a useful tool to identify bacterial infection	Very accurate and sensitive method compared to the manual method of identifying bacterial infection	Raveendran et al, 2019
Mannan and antimannan antibodies	Mannan is present in the cell walls of invasive fungal organisms	Diagnostic marker of sepsis that is due to fungal infection6	Arendrup et al, 2009
IP-10/CXCL10	Proinflammatory chemokine	Useful as a biomarker for diagnosing viral infections	Ng et al, 2007

3.7.1. MICROVESICLES AS BIOMARKERS IN SEPSIS

The prospect of using MVs as biomarkers has been explored in several studies. These existing studies strongly suggest that MVs can be used as reliable biomarkers in the diagnosis/prognosis of sepsis. These clinical studies are included in Table 6.

Table 6: Clinical studies evaluating the role of extracellular vesicles in sepsis

(Completed from Raeven, Theranostics, 2018)

Legend: AKI: acute kidney injury; AnnV +/-: Annexin V positive/negative; APC: activated protein C; BAL: broncho-alveolar lavage; CAP: community-acquired pneumonia; CD: cluster of differentiation; DIC: disseminated intravascular coagulation; EC: endothelial cell; EEVs: endothelial cell-derived EVs; EPCR: endothelial protein C receptor; EryEVs: erythrocyte-derived EVs; EVs: extracellular vesicles; GEVs: granulocyte-derived EVs; ICU: intensive care unit; IL: interleukin; LEVs: leukocyte-derived EVs; LPS: lipopolysaccharide; MEVs: monocyte-derived EVs; PEVs: platelet-derived EVs; PhtdSer: phosphatidylserine; rhAPC: recombinant human APC; ROS: reactive oxygen species; SIRS: non-infectious systemic inflammatory response syndrome; TF: tissue factor; TNF: tumor necrosis factor.

Study	Diagnosis	Cohort size	EV sources	Summary of findings
Larsson (1996)	Gram-negative sepsis in ICU	Sepsis (n=4)	Platelets	3 out of 4 septic patients showed high PEV count (no statistics).
Lundahl 1996	Mixed ICU population	ICU patients (n=19), healthy (n=20)	Platelets	Increased PEV (no statistics) in 2 out of 8 patients who died shortly after sampling.
Newland 2000	Meningococcal septic shock	Septic shock (n=7), healthy (n=5)	Platelets, ECs, granulocytes, monocytes, erythrocytes	Only PEV and GEV increased in septic vs. healthy on admission. Only GEV decreased within 10 hours of admission.
Joe 2001	Mixed severe sepsis	Severe sepsis (n=9), healthy (n=14)	Platelets, erythrocytes, ECs, granulocytes, TF+ Cells	AnnV+/CD61+ higher, AnnV-/CD61+ lower, AnnV- / glycophorinA+ higher, AnnV-/ CD62E+ lower, AnnV+/ CD66b+ and AnnV-/ CD66b+ higher, AnnV+/CD142+ lower in sepsis vs. healthy.
Ogura 2001	Mixed sepsis, trauma + SIRS, all with C protein >10mg/dL	Sepsis (n=14) SIRS (n=12) healthy (n=12)	Platelets	PEV/platelet count higher in sepsis vs. healthy, no difference in trauma vs. sepsis.
Fujimi 2002	Mixed sepsis with CRP>10 mg/dL	Sepsis (n=21), healthy (n=21)	Granulocytes	GEV counts are higher in septic vs. healthy. Enhanced expression of CD11b in sepsis vs. healthy on GEV < 1 μm but not GEV > 1 μm
Janiszewski	Septic shock	Septic shock (n=16), healthy (n=6)	Platelets, leukocytes, monocytes, granulocytes, ECs	PEVs exposed p22phox and gp91phox, exhibited intrinsic ROS production and enhanced apoptosis (vs. healthy volunteers) after incubation with ECs or vascular smooth muscle cells. Effects reversible by the addition of ROS antagonists.
Soriano 2005	Mixed severe sepsis	Severe sepsis (n=35), healthy	ECs, platelets	EEV, not PEV, and EEV-monocyte conjugate count was higher in septic

		(n=45)		patients on day 1 vs. healthy controls. EEV monocyte conjugates at all time points were higher in non-survivors vs. survivors
Gambim Diagnostic 2007	Mixed septic shock	Septic shock (n=12), healthy (n=10))	Platelets	EV from human platelets were similar to septic patients derived PEV after *in vitro* exposure to NONOate and LPS, but not to TNF-alpha or thrombin, generated superoxide and nitric oxide. ECs incubated with EVs underwent caspase-3 activation/apoptosis, inhibited by ROS antagonists.
Azevedo 2007	Septic shock	Septic shock (n=55), healthy (n=12)	Platelets	Exosomes from septic patients decreased maximal DT and positive dT/dt in rat papillary muscle preparations and positive dT/dtmax in isolated rabbit hearts pre-exposed to LPS. Exosomes from septic patients showed intrinsic NO production and induced myocardial NO content. No effects of exosomes from septic patients on isolated rabbit heart not pre-exposed to LPS and rat aortic ring contractility.
Huisse 2008	Severe sepsis	Severe sepsis (n=14), heat stroke (n=18), healthy (n=18)	Total EVs, platelets, monocytes, **granulocytes,** ECs	Severely septic patients showed lower PEVs, increased GEVs and MEVs and similar EEVs vs. healthy volunteers
Mostefai 2008	Mixed septic shock	Septic shock (n=36), ICU non-septic (n=18)	Total EVs, platelets, ECs, leukocytes, **granulocytes,** monocytes, erythrocytes	Total EVs, PEVs, EEVs, L-Selectin+EVs and P-Selectin+EVs were increased, LEVs decreased, and GEVs, MEVs, EryEVs and AnnV+ EVs were not different in septic vs. non-septic ICU patients.
Perez-Casal 2009	Severe sepsis, rhAPC treatment	Severe sepsis with rhAPC (n=4), severe sepsis without rhAPC (n=4)	EPCR+ EVs	Co-localization of EPCR and APC on EVs from septic patients during, but not before, rhAPC infusion. APC on EPCR+ EVs was higher during vs. before (individual controls) rhAPC treatment and vs. non-rhAPC-treated controls.
Forest 2010	SIRS, mixed sepsis or septic shock	<50y SIRS (n=26), <50y sepsis (n=27), ≥75y SIRS (n=31), ≥75y sepsis (n=27)	ECs, erythrocytes, Platelets	Lower EEVs, but the same EV procoagulant activity in older vs. younger patients. Lower EEVs in sepsis vs. SIRS in young patients. Lower EV procoagulant activity in sepsis vs. SIRS in both old and young patients. Elderly sepsis non-survivors had higher EEVs vs. elderly survivors.
Rank 2011	Sepsis	Hematopoietic stem-cell transplantation (n=19), incl. infection/sepsis (n=15)	Erythrocytes	EryEVs level was affected only by the development of graft versus host--disease but not conditioning therapy, total body irradiation, high-dose chemotherapy, *in vivo* T cell depletion, aplasia, or engraftment in uncomplicated patients, infectious complications, and/or sepsis.
Perez-Casal 2011	Severe sepsis (Pneumonia or Intraabdomina l infection), rhAPC indication	Severe sepsis with rhAPC (n=25) or without rhAPC (n=25), healthy	EPCR+EV, APC+EV, myeloid cell line	Increased circulating EV carrying EPCR and APC expression after rhAPC. Decrease of endothelial permeability by APC+EVs via PAR-1.

		(n=6)		
Prakash 2012	Septic peritonitis, mixed ICU with suspected ventilator associated pneumonia or lung donors	Septic peritonitis (n=3), control abdominal lavage fluid from non septic laparotomy (n=4), ICU suspected pneumonia BAL (n=33), control BAL from lung donor (n=2)	Granulocytes, platelets, ECs, erythrocytes	GEV was only present in inflamed foci. THP-1 cells were activated by phagocytosis of GEV.
Woth 2012	Mixed severe sepsis	Severe sepsis (n=33), healthy (n=20)	Platelets	Elevated AnnV+ EV and PEV in septic vs. healthy on admission. Higher AnnV+ EV and PEV in fungal vs. non-fungal septic patients on day 1. CD42+ EV increased in fungal vs. non-fungal sepsis at all times and PAC1+ EVs at day 1 and day 5.
Timár 2013	S aureus bacteremia and fever	Bacteremia (n=12), healthy (n=6)	Granulocytes	GEV 5-6-fold higher in serum and bacterial-EV aggregates larger with serum of bacteremic patients vs. healthy.
Tőkés-Füzes 2013	Severe sepsis	Severe sepsis (n=37) or non septic ophthalmic patients (n=20)	Platelets, monocytes, myeloid cell line	Increased total, CD41+, CD42a+, and PAC1+ EVs in septic vs. healthy. Increased total, CD41+, and CD13+ EVs on admission in septic patients with renal dysfunction. A negative correlation of CD42a+ PEVs with blood urea nitrogen and creatinine concentrations in sepsis.
Mostefai 2013	Septic shock	Septic shock (n=16)		Septic EVs augmented histamine-induced contraction in human tissue-engineered vascular media. Septic EV treatment increased cyclo-oxygenase-1 and IL-10 expression of IL-10 (but not IL-1α, IL-1β, IL-6).
Vanlerssel 2013	Mixed severe sepsis or septic shock	Severe Sepsis (n=30), healthy (n=15)	ECs	EEVs were not different between septic and healthy.
Delabranche 2013	Mixed septic shock with DIC	Septic shock (n=92) incl. (n=40) with DIC	Procoagulant EVs (prothrombinase assay), ECs, leukocytes	CD11a+ and CD105+ EVs were increased in DIC at admission. Increased CD105+ EVs had higher and CD31+ EVs lower odds ratios for DIC.
Dalli 2014	Sepsis caused by CAP	Sepsis survivor (n=25), sepsis non-survivor (n=25), healthy (n=15)	Granulocytes	Alpha-2-macroglobulin+ EVs were higher in sepsis survivors vs. nonsurvivors and healthy volunteers. High alpha-2- macroglobulin+ EVs correlated with better outcome
Hellum 2014	Meningitis with or without shock	Meningitis and septic shock (n=13) and meningitis (n=10), healthy (n=6)	PhtdSer+ EVs	Faster and more efficient thrombin generation by EVs in shock vs. no shock and strong correlation with LPS level in shock.
Exline 2014	Sepsis	Sepsis (n=34), non-infected critically ill controls (n=16)	Total EVs	Higher EV-derived caspase-1 activity on day 1 and day 3 in septic vs. non-septic patients. EVs from septic patients on day 1 induced lymphocyte apoptosis.
Woei-A-Jin 2014	Community-acquired febrile Ecoli urinary tract infection	Febrile urinary tract infection (n=215), healthy volunteers (n=19)	Monocytes	Higher TF+ EV activity on admission in patients with higher APACHE II score categories, but weak correlation with soluble E-selectin, soluble vascular cell adhesion molecule, thrombin-

				antithrombin complexes, and procalcitonin. TF+ EV activity was higher in patients vs. healthy.
Matsumoto 2015	Mixed severe Sepsis + DIC	Severe sepsis (n=24), trauma SIRS (n=12), cerebral haemorrhage SIRS (n=6), healthy (n=23)	ECs	TF+ EEV, TM+ EEV, and EPCR+ EEV were increased in both septic shock and trauma vs. healthy; lack of correlation with APACHE II and sequential organ function score; moderate-to-strong correlation with the International Society of Thrombosis and Haemostasis DIC score
Herrmann 2015	Sepsis (Gramand Gram+, possibly polymicrobial infections, 62.5% pulmonary, 37.5% abdominal)	Sepsis (n=14), SIRS (n=8)	Granulocytes	CD11β+/CD18+ and CD11β+/CD177+ EVs were higher in sepsis vs. control. Similar IL-6 plasma release by septic vs. control. Procoagulant activity increased, clotting time decreased, and bacteria aggregation activity was higher in sepsis vs. control.
Zhang 2016	Mixed sepsis	Septic (n=15), healthy (n=10)	Platelets, ECs, erythrocytes, leukocytes, granulocytes, T lymphocytes, monocytes	All PhtdSer+ EVs were increased in sepsis vs. healthy. EVs induced coagulation at PhtdSer-exposing sites in vitro; partly reversed by PhtdSer antagonism.

Trepesch 2016	Mixed sepsis, severe sepsis, septic shock, survivor vs. non-survivors	Sepsis (n=6), severe sepsis (n=4), septic shock (n=20)	PhtdSer+ EVs	The amount of PhtdSer+ EVs was not associated with mortality and organ dysfunction
Delabranche 2016	Mixed septic shock	Septic shock (n=259) incl. with DIC at admission (n=61), with DIC within first 24h (n=32)	Procoagulant (PhtdSer+) EV, leukocytes, apoptotic ECs, platelets monocytes/macrophages	Increased CD105+ EVs and decreased CD31+ EVs were associated with DIC. Increased CD11a+ EVs/leukocytes supported leukocyte activation.
Lehner 2016	Septic shock	Septic shock (n=30), healthy (n=18)	ECs, leukocytes, platelets	Low but increased counts of EEVs (CD144+, CD62E+, CD106+) and increased CD31+/CD41- EVs vs. healthy. Correlation between CD31+/CD41- EVs and leukocytes. Increased levels of CD41+ EVs and CD31+/CD41-/AnnV+ EVs in 48h non-survivors. CD144+, CD62E+, and CD106+ EVs are not different between DIC and non-DIC.
Stiel 2016	Septic shock with and without DIC	Septic shock: with DIC (n=35), without DIC (n=65)	Granulocytes	CD66+ GEV/neutrophilic granulocyte count is higher in septic shock with DIC vs. without DIC.
O'Dea 2016	Major burn injury vs. severe sepsis	Major burn injury (n=15), severe sepsis (n=15), healthy (n=12)	Leukocytes, monocytes, granulocytes, ECs	EVs increase on admission in burns vs. healthy. Only CD45+/ Cd14+ MEVs and CD66b+/CD11b+ GEVs increased in sepsis vs. healthy. CD45+/Cd14+ MEVs and CD105+ EEVs are lower in sepsis vs. burns. CD45+ LEVs and CD66b+/CD11b+ GEVs increased in dying vs. surviving burn patients. No difference between dying and surviving sepsis patients.

Matsumoto 2017	Mixed SIRS vs severe sepsis	Trauma patients (24) with severe sepsis (n=25), healthy (n=23)	Monocytes	Increased AnnV+/CD13+/CD142+ EVs in both trauma and severe sepsis vs. healthy. Moderate association between AnnV+/CD13+/ CD142+ EVs and APACHE II score, IL-6, Injury Severity Score (in trauma), and International Society of Thrombosis and Haemostasis DIC score (in sepsis)
Panich 2017	Sepsis, septic shock	Sepsis with AKI (n=79), sepsis without AKI (n=60), healthy (n=8)	Exosomes	Activating transcriptional factor-3+ exosomes increased on day 1 of admission in sepsis with AKI vs. sepsis without AKI Area under receiver operating curve for AKI 0.84.
Reithmair 2017	Sepsis, septic shock	Sepsis (n=22), healthy (n=23)	miRNA in serum, blood cells, and EVs (exosomes, Tumor susceptibility gene 101+) by next-generation sequencing	Compartment-specific (serum, cellular, extracellular) differences over time.
Lehner 2017	Severe sepsis or sepsis shock, medical ICU	Severe sepsis or septic shock (n=12)	Microparticles expressing platelet endothelial cell adhesion molecules (from platelets, leukocytes, and/or ECs) and platelets.	Increase of CD31+/CD41- EVs post-filter vs. pre-filter. Decreased total PhtdSer-exposing EVs and decreased TF+ EVs postfilter vs. pre-filter. No PhtdSer-exposing EVs and no TF activity measurable in the ultrafiltrate.
Lashin 2017	Sepsis due to CAP or fecal peritonitis	CAP (n=60), fecal peritonitis (n=40), healthy (n=10)	Granulocytes, monocytes, T lymphocytes, platelets, erythrocytes, and ECs	Circulating GEVs, MEVs, T-lymphocyte-derived EVs, and alpha-2-macroglobulin+ EVs (from all studied cell types) at day 1 were higher in CAP vs. fecal peritonitis and healthy volunteers, EEVs only vs. healthy, PEVs and EryEVs were not different. Alpha-2-macroglobulin+ EVs were higher in survivors of CAP, but not in fecal peritonitis.
Puskarich 2018	Adults presenting to the emergency department (ED) with suspected infection, ≥2 SIRS criteria, and either systolic blood pressure 4ÿmmol/L were eligible	184 samples	Platelets (PS+, CD41+ and CD61+)	Similar to patients enrolled later in the intensive care unit, PS expressing PMPs are lower in emergency department sepsis non-survivors. These changes primarily reflect the degree of thrombocytopenia, and an independent prognostic role was not observed. Future studies should control for platelet count in the assessment of PMP prognosis in sepsis.
Cointe 2018	Septic shock patients	34 samples	CD15-MV associated MV PGC	Plasmin generation values were significantly lower for non-survivors compared to survivors both at inclusion and 24h later. combining both MV-PGC and PAI-1 in a ratio significantly improved the predictive value of PAI-1.
Kawamoto 2019	SIRS, sepsis	27 SIRS without sepsis, 27 sepsis, and 18 healthy	Expression of 3 major integrins β1, β2, β3 PD-L1 and PD-L2	The EV expression of β2 integrin and PD-L2 was significantly increased in sepsis patients compared with healthy controls. EV expression of PD-L1 was not elevated in sepsis and SIRS; however, circulating soluble PD-L1 levels were significantly higher in sepsis. Furthermore, EV expression of β2 integrin in sepsis patients correlated

				with hypotension and reduced kidney function.
Boscolo 2019	Sepsis shock	Septic shock (n=40), healthy (n=40)	AnnV+, E-selectin+, thrombomodulin (TM+), leukocytes (CD45+, CD36+) Platelets	Septic patients showed higher levels of all MP considered compared to controls. TM+MP was significantly lower in more severe sepsis, while CD36+MP was significantly increased in patients requiring longer vasoactive support and mechanical ventilation. increased E selectin+ MP in subjects who developed AKI were observed. PMP/ platelets ratio at baseline was significantly associated with longer vasoactive support longer mechanical ventilation and DIC occurrence.
Chen 2020	Sepsis, septic shock	Sepsis (n=40) septic shock (n=40)	Neutrophils	Significantly increased levels of MPs from neutrophils in sepsis and septic shock patients as compared to healthy controls.
Morris 2020	Sepsis patients admitted to the emergency department with either suspected infection, SIRS criteria, organ dysfunction such as lactate >2 mg/dL or hypotension with < 90 mmHg of Systolic blood pressure	Sepsis or septic shock (n=7) and healthy (n=5)	Exosomes from plasma	Significantly elevated expression levels of Serum Amyloid A-1 (SAA-1), C-reactive protein (CRP), and Serum Amyloid A-2 (SAA-2) in sepsis patients.
Im 2020	Critically ill sepsis or septic shock patients admitted to intensive care unit (ICU)	sepsis patients (n=145), septic shock (n=75) and healthy (n=20)	Plasma exosomes	Significant increase in exosomes in septic shock patients as compared to sepsis patients and healthy controls. Exosome levels in plasma were highly correlated with sepsis symptom severity and mortality of patients.
Fendl 2021	Sepsis patients	Sepsis patients (n=30), and healthy (n=5)	PS+/CRP+ EVs, or EVs with erythrocyte, platelet, monocyte, or leukocyte markers	Septic patients demonstrated significantly higher levels of PS+/CRP+ EVs compared to healthy controls. The majority of EVs were platelet origin (CD41+).
Qiu 2022	Sepsis patients admitted to intensive care unit (ICU), Sepsis-3 criteria (Infection and an acute increase ≥2 in SOFA score)	Sepsis (n=37), and healthy (n=25)	Plasma EVs	Plasma from sepsis patients presented a higher number of EVs and total EV proteins as compared to the healthy controls. EVs from sepsis patients had significantly elevated expression of three miRNAs, miR-483-3p, let-7d-3p, and miR-92b-5p, involved in injury and inflammation.
Puga 2022	Sepsis/septic shock patients admitted to ICU	Sepsis/septic shock (n=10), and healthy (n=10)	Total MVs (Annexin V+), leukocyte (CD45+), neutrophils (CD66b+ and CD16+), monocytes (CD14+), platelets (CD41+), and	Elevated levels of total MVs (Annexin V+) in sepsis/septic shock patients as compared to healthy controls. Levels of neutrophil MVs (CD66b+ and CD16+) were higher in sepsis/septic shock patients who were discharged from the hospital as compared to dead patients.

			erythrocytes (CD235+)	
Cointe 2022	Septic shock patients	Septic shock (n=34), and healthy (n=18)	CD15-MV associated MV PGC	Septic shock patients demonstrated heterogeneity in terms of CD15-MV derived plasmin generation allowing stratification of patients based on PGC
Lacroix et al., 2024	Septic shock patients	Septic shock (n=225)	MV-CLB was measured as a ratio between procoagulant and profibrinolytic activity	Patients who died showed a significantly higher MV-CLB compared to patients who survived. MV-CLB was a better predictor of patient mortality than the procoagulant and profibrinolytic activities taken separately.

3.8. CONCLUSION

Sepsis is a life-threatening situation with an exacerbated immune response that leads to systemic inflammation with DIC and organ damage. Early diagnosis and therapeutic intervention are critical for the appropriate management of septic patients to prevent severe morbidity and mortality. Several biomarkers are being used in clinical settings for early diagnosis and prognosis purposes. While most used CRP and PCT biomarkers in conjunction with neutrophil count and sTREM-1 can be helpful, diagnosis and prognosis of SS seem to be still challenging. Although existing biomarkers have been beneficial in clinical settings, there is no single ideal biomarker that can potentially be used to stratify patients according to severity or treatment response. During sepsis, the procoagulant and profibrinolytic balance that maintains the hemostatic equilibrium becomes dysfunctional. As MVs have been demonstrated to be an important player in the hemostasis process, assessing MV procoagulant and/or profibrinolytic potential could provide a more comprehensive picture of patient status. Therefore, MVs could be a useful potential biomarker that could be used in conjunction with clinical characteristics of patients for better diagnosis and treatment assessment.

PART II: PERSONAL WORK

The initial concepts of MVs as mere cellular debris have been changed with significant research findings overwhelmingly demonstrating that MVs mediate intra- or inter-cellular communications and are implicated in several cellular/molecular functions including hemostasis. MV's undisputable role in hemostasis was first discovered with the blood clotting experiments performed by Chargaff and West in the 1940s. A series of observations demonstrated the existence of sedimentable structures with high clotting capacity in plasma that significantly shortened clotting time and was later described as 'platelet dust' by Peter Wolf[329]. The biological significance of these observations later laid the foundations for research in the field of MVs or EVs in general.

MVs have long been regarded as procoagulant entities largely because they express anionic phospholipids like phosphatidylserine (PS) and/or tissue factor (TF). MVs that express both PS and TF have profound procoagulant potential. However, this strictly procoagulant role of MVs has been challenged with the discovery of MVs with anticoagulant and fibrinolytic properties. Previous reports from our lab have demonstrated that leukocytes and endothelial MVs have a fibrinolytic capacity and they can cleave plasminogen to plasmin through the uPA/uPAR system or tPA. Under normal physiological conditions, the presence of various hemostatic molecules that modulate procoagulant and profibrinolytic properties of MVs contribute to maintaining the balance between procoagulant and profibrinolytic function. This normal functional ambivalence of MVs in the hemostatic process can be termed "coagulolytic balance of MVs" (MV-CLB). This functional equilibrium in the context of hemostatic activity can be lost in thrombo-inflammatory conditions with a prevailing procoagulant state.

MVs generally contain a complex mixture of bioactive molecules which reflects the molecular signatures of their parental cell and activation status. Under an inflammatory milieu, cells in the blood and vascular compartment accelerate the secretion of MVs with procoagulant molecules that may potentiate the crosstalk between inflammation and coagulation process leading to thrombo-inflammatory complications in cardiovascular disorders, sepsis, and COVID-19.

Sepsis is a thrombo-inflammatory life-threatening condition that arises from an exacerbated immune response to underlying infection leading to systemic inflammation, coagulation, and organ dysfunction. Excess activation of coagulation pathways along with dysfunctional

anticoagulant and fibrinolytic system leads to increased fibrin deposition in microvasculature that is characterized by disseminated intravascular coagulation (DIC) and multi-organ damage. Generally, sepsis or septic shock (SS) is associated with profound procoagulant activity. However, previous work from our lab has reported increased fibrinolytic activity in some subsets of SS patients, providing a mechanism that counterbalances the procoagulant state. we observed that SS patients who survived had elevated levels of MV-dependent fibrinolytic activity that is specific to granulocyte MVs. This observation further consolidated the findings that demonstrated increased clot lysis mediated by uPA/uPAR of granulocyte MVs in vitro and decreased thrombus formation in kidneys and lungs of the SS mice model that led to increased survival of mice upon receiving infusion granulocyte MVs.

Although these data demonstrate that increased fibrinolytic activity can counterbalance the procoagulant state, a comprehensive picture of MV hemostatic function with regards to procoagulant and/or profibrinolytic activity that may arise from individual subsets of MVs in circulation in the context of their cellular source and inflammatory stimuli/sepsis pathology has not been demonstrated. This would not only allow us to develop MV-based biomarkers and therapeutic vehicles based on their hemostatic function but also to manipulate the secretion of specific subsets of MVs to modify disease conditions.

In addition to the evaluation of the MV-CLB in the context of sepsis pathology, modulation of the MV-CLB in a different pathophysiological context also was evaluated. Activation of different blood cells during the extracorporeal treatment procedures has been observed. We investigated the ability of biopolymer filter membranes used in the rheopheresis session to induce neutrophil activation and generation of MVs with fibrinolytic capacity.

This thesis has two parts:

1) **Analysis of procoagulant and/or profibrinolytic potential of different subsets of MVs to evaluate MV-CLB as a mediator in sepsis pathophysiology and a potential biomarker (Article 1; this is the major part of my thesis work).**

2) **Generation of MVs with high fibrinolytic potential by neutrophils induced by biopolymer membrane filters (Article 2).**

The goal was to purify in vitro generated human neutrophil, monocyte, platelet, and endothelial cell-derived (NDMVs, MDMVs, PDMVs, and EDMVs, respectively) to evaluate their coagulolytic balance and compare this to major MV subsets of MVs isolated from the human plasma using immunomagnetic separation (IMS). Purified MVs were analyzed with a panel of functional assays to test the hypothesis that different subsets of MVs have distinct procoagulant and/or profibrinolytic properties and thus display distinct coagulolytic balance according to their cellular source and inflammatory stimuli/pathological conditions. A comprehensive analysis of MVs demonstrated that they display distinct coagulolytic balance according to their parental cells and inflammatory milieu or sepsis disease. coagulolytic balance can be used in clinical settings as a promising biomarker that reflects the hemostatic imbalance in coagulopathy associated with diseases like sepsis.

In addition, we evaluated the levels of different subsets of MVs in the plasma of patients who received rheopheresis treatment for microvascular thrombosis complications. There was a significant increase in NDMVs in these patients that was associated with high plasmin generation activity. Moreover, *in vitro* treatment with plasmapheresis membranes induced the activation of neutrophils and generated a significant quantity of NDMVs with fibrinolytic potential. This result indicates that depending on the stimuli, cells can generate MVs with significant procoagulant or profibrinolytic potential that may modulate MV-CLB.

Article 1

Extracellular vesicles display a distinct coagulolytic balance according to their cellular origin and activation status: Application in sepsis coagulopathy

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Abstract

Cellular activation triggers the release of extracellular vesicles (EVs) that convey not only procoagulant but also fibrinolytic properties. Therefore, their effect on hemostasis may result from an equilibrium between these two activities, which we termed the "EV-coagulolytic balance" (EV-CLB). However, the variations in the EV-CLB according to cellular origin and the activation status of parental cells have never been investigated. The aim of the present study was to investigate the EV-CLB of the main circulating subsets of EVs using EVs derived from human monocytes (MDEVs), endothelial cells (EDEVs), neutrophils (NDEVs), and platelets (PDEVs) generated either *in vitro* from stimulated *versus* unstimulated cells or directly isolated by immunomagnetic separation (IMS) from the plasma of septic patients. EV-dependent procoagulant and profibrinolytic activities were assessed by a panel of functional assays, including factor Xa, thrombin, and plasmin generation assays. EV-induced changes in clot structure were investigated by scanning electron microscopy and fibrinography. MDEVs and EDEVs from stimulated cells showed the highest levels of procoagulant EV-CLB. This procoagulant profile was more pronounced under inflammatory conditions. Clots induced by these EV subpopulations were denser and displayed greater resistance to fibrinolysis. MDEVs and EDEVs from sepsis patients display an EV-CLB procoagulant profile. In contrast, NDEVs

were the only subpopulation displaying a profibrinolytic profile characterized by significantly high plasmin generation capacity, both *in vitro* and in sepsis patients, with a correlation with markers of coagulopathy. Finally, PDEVs showed low procoagulant activity and were devoid of profibrinolytic activity compared to other EV subsets at the same concentration. This work provides the first demonstration that the cellular origin and activation status of parental cells drive the EV-CLB profile. This profile defines a specific signature of a single EV subpopulation that can be used as a new biomarker reflecting the hemostatic imbalance associated with sepsis or other infectious coagulopathies.

Keywords:

Coagulation, coagulopathy, extracellular vesicles, fibrinolysis, sepsis.

Introduction

Extracellular vesicles (EVs) are a heterogeneous population of small and large nanovesicles enclosed in a phospholipid bilayer that are released by all types of cells. EVs carry diverse cargo, including proteins, lipids, and nucleic acids, and play a crucial role in several physiological functions, including the regulation of hemostasis (Ridger et al., 2017; Yáñez-Mó et al., 2015). Although the major source of circulating EVs in the peripheral blood of healthy humans predominantly originates from platelets (70-90%), other less represented EVs released by leukocytes and endothelial cells have gained considerable attention due to their involvement in regulating vascular hemostasis (Das et al., 2021; Holstein et al., 2020; Suades et al., 2022; Tripisciano et al., 2017).

EVs have long been regarded as procoagulant entities largely because they expose membrane anionic phospholipids such as phosphatidylserine (PS) and may express functional tissue factor (TF) according to the cell driving their biogenesis (Zifkos et al., 2021). When externalized, PS on the EV surface substantially enhances the procoagulant activity of EVs in the presence of TF by facilitating the assembly of coagulation factors, leading to the initiation of the coagulation cascade *via* tenase and/or prothrombinase complexes (Tripisciano et al., 2017; Zifkos et al., 2021). However, EVs with anticoagulant and fibrinolytic activities leading to the dissolution of clots have also been described (Lacroix et al., 2013). The major anti-coagulant proteins present on the EV surface include TF pathway inhibitor (TFPI) and the endothelial cell protein C receptor (EPCR). Moreover, some subsets of EVs harbor urokinase plasminogen activator receptor (uPAR, CD87) and can effectively generate plasmin via a process mediated

by urokinase plasminogen activator (uPA) (Cointe et al., 2022; Lacroix et al., 2012; Vallier et al., 2016). The opposing distinct functional effect of EVs on hemostasis depends on their cellular origin and may be viewed as a balance between the procoagulant and profibrinolytic properties of EVs, which we refer to as the "EV-coagulolytic balance" (EV-CLB) in the present study.

The well-regulated hemostatic equilibrium can be lost under thrombo-inflammatory conditions such as sepsis (Levi & Van Der Poll, 2017). Several studies have demonstrated that significantly elevated levels of EVs in sepsis correspond with the activation of cells in an inflammatory environment (Ogura et al., 2004; Piccin et al., 2007; Reid & Webster, 2012). In sepsis, accumulated evidence in the literature has shown that these EVs can exert either a deleterious effect through the expression of procoagulant or proinflammatory properties (Hellum et al., 2014; Zhang et al., 2016) or a protective effect mediated by anti-inflammatory properties or fibrinolytic properties (Bao et al., 2022; Cointe et al., 2018, 2022; Guervilly et al., 2011; Mostefai et al., 2008; Youn et al., 2021). This suggests that distinct determinants could therefore tune the EV-dependent coagulolytic balance and influence coagulopathy associated with sepsis and other diseases associated with thrombo-inflammation where the well-regulated procoagulant and profibrinolytic EV balance is lost.

Therefore, we hypothesized that the cellular origin and pathological context driving the biogenesis of EVs could determine the distinct functional profiles of EV-CLB. We performed a comprehensive evaluation of the procoagulant and/or profibrinolytic properties of EVs generated from human monocytes, endothelial cells, neutrophils, and platelets, under inflammatory conditions. We provided a proof of concept that these cell-specific coagulolytic signatures can be detected *ex vivo* using subpopulations of EVs isolated from patients with sepsis.

Methods

Sepsis patients: blood collection and plasma preparation

Blood samples from septic shock (SS) patients were collected in accordance with the guidelines provided by the International Sepsis Definition Conference (Levy et al., 2003). In the present study, a total of 10 patients were included from a large cohort of patients. Blood samples were collected within the first 24 h of admission to the intensive care unit. Age-matched (49 ± 8 years) and sex-matched healthy controls (n = 8) were also included in the comparative analysis.

Ethical approval was granted by a local medical ethics committee (CPP Sud Méditerranée N° 13-67) in accordance with the Declaration of Helsinki, and written informed consent was obtained from either patients or close relatives. Blood samples were collected and processed according to the guidelines of the International Society on Thrombosis and Hemostasis (Coumans et al., 2017). Briefly, a light tourniquet was applied, and samples were drawn from the antecubital vein using a butterfly device with a 21-gauge needle. Blood was collected into 5-ml vacutainer tubes containing 0.129 mol/L sodium citrate (BD Diagnostics, Franklin Lakes, NJ, US), and the first few milliliters were discarded. The samples were subjected to two successive centrifugations (2,500 × g for 15 min at room temperature with no brake), and platelet-free plasma (PFP) was prepared. The PFP was homogenized, aliquoted, and stored for a maximum period of one year at -80°C until use.

Human coronary artery microvascular endothelial cells (HCMECs)

Primary HCMECs were purchased from PromoCell (C-12285, PromoCell GmbH, Heidelberg, Germany) and cultured in gelatin-coated flasks containing endothelial growth medium (Endothelial Cell Basal Medium MV2, C-22221, PromoCell GmbH, Heidelberg, Germany) supplemented with C-39221 (PromoCell GmbH, Heidelberg, Germany) and 5% EV-depleted fetal calf serum (FCS). EV-depleted FCS was prepared by centrifugation twice at 100,000 × *g* for 90 min. Cells in culture were expanded to 70-80% confluency at passage 9. The cells were washed with PBS twice and treated with TNF-α (10 ng/ml) (Miltenyi Biotec, Bergisch Gladbach, Germany) for 24 h in fresh medium, after which the conditioned media was collected for EV isolation. Conditioned media from untreated passages 7 to 9 (before TNF-α stimulation) were collected to isolate EVs, which were used as controls.

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.

Isolation of platelets and platelet-derived EVs (PDEVs)

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).

Isolation of EVs from neutrophils, monocytes, and endothelial cells (NDEVs, MDEVs, and EDEVs)

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 (Théry et al., 2018). 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 ($0.5x10^5$ EVs/µl). We have submitted all relevant data from our experiments to the EV-TRACK knowledgebase (EV-TRACK ID: EV240012) (EV-METRIC: 50-56%) (Van Deun et al., 2017).

Immunomagnetic separation of EVs from plasma

Immunomagnetic separation (IMS) was performed to capture different subsets of EVs directly from platelet-free plasma (PFP) as previously described by Cointe *et al.* and Franco *et al.* (Cointe et al., 2018; Franco et al., 2020) with some modifications. To perform the immunomagnetic separation of EVs directly from the PFP, all the tested IgG-mAbs were biotinylated and coated on streptavidin beads (kindly provided by BioCytex, Marseille). These IgG antibodies included anti-CD15 (clone: FR4A5), anti-HLA-DR (clone: BRA-30), anti-CD41 (clone: PL2-49), and anti-CD146 (Ref: S-Endo 1), and a nonspecific irrelevant anti-DNP IgG1 was used as a negative control; all antibodies were provided by Biocytex (Marseille, France). Quality control of the beads was performed by flow cytometry to confirm the coating of IgG antibodies on the beads with an anti-mouse IgG fluorescein (FITC) conjugate. The beads were separated from the PFP supernatants using a magnet (DynaMag, Invitrogen/Dynal), washed once with PBS-BSA buffer, and resuspended in either HEPES-Ca^2 + buffer (150 mM

NaCl, 20 mM HEPES, and 0.1% NaN_3, 50 mM $CaCl_2$, pH 7.4; 0.22 µm filtered) or PBS/BSA–azide buffer (PBS, 0.1% BSA, and 0.1% NaN_3 buffer; filter, 0.22 µm).

Characterization and quantification of EVs by flow cytometry

A detailed description of EV staining is provided in the supplemental file and our previous work (Bonifay et al., 2022). For the quantification and purity assessment of EVs after isolation, 30 µl of EV suspension was incubated with 2.5 µl of the specified antibody(ies) and 10 µl of annexin V-FITC (AnnV, Beckman Coulter) in the dark at RT for 30 min. After incubation, the samples were diluted with 250 µl of AnnV binding buffer. EV enumeration was performed using highly sensitive FCM (Cytoflex LX, Beckman Coulter). Violet SSC (405 nm) was used to maximize the collection of size-related resolution of EVs.

For EVs derived from human PFP, 10 µl of pre/post-IMS plasma was incubated with a saturating amount of the specific antibody(ies) and 4 µl of AnnV in the dark at RT for 30 min. After incubation, the samples were diluted with 250 µl of AnnV binding buffer and 10 µl of MP Count Beads (Biocytex, Marseille, France) for counting in absolute values.

All FCM data were analyzed either with Kaluza Analysis 2.1 software or CytExpert 2.3 software.

Characterization of EVs by transmission electron microscopy

Transmission electron microscopy (TEM) was performed on pellets of EVs following the final purification step with ultracentrifugation. Briefly, EV pellets were fixed in 2.5% glutaraldehyde and 2% PFA in PBS for 30 min at RT and stored in Hanks' Balanced Salt Solution at 4°C. Then, they were postfixed with 2% osmium tetroxide (OsO_4) in PBS for 1 h on ice, dehydrated in a gradient series of acetone baths, and embedded in epoxy resin. Ultrathin sections of EV pellets were then prepared with a UC7 ultramicrotome (Leica, Wetzlar, Germany), and the sections were stained with 1% aqueous uranyl acetate (10 min) and lead citrate (4 min). The grids were observed at 80 kV on an FEI Morgagni transmission electron microscope (Thermo Fisher, Waltham, USA), and images were acquired using a MegaView3 camera (Emsis, Muenster, Germany).

Characterization of EVs by tunable resistive pulse sensing

The EV size distribution was analyzed using tunable resistive pulse sensing (TRPS). Measurements were taken using an IZON qNano (IZON, Oxford, England) with NP150 Nanopore membranes and CPC200 beads as a calibrant. Samples and calibrant beads were

diluted in filtered (0.22 µm) PBS. The NP150 nanopore was stretched between 44 and 48 mm, the pressure was set at 12 mbars, and the voltage was set at 0.78 V. The samples were measured, and the data were analyzed with the IZON control suite V3.3.3.2001 software in classic capture mode.

Characterization of EVs by western blot

Western blot characterization of EVs was performed to determine the expression of integrin α5, and serum albumin contamination of EV samples was assessed. In brief, EV pellets were lysed in RIPA buffer and centrifuged at 10,000 × g for 10 min. Protein lysate was collected, mixed with 4X loading buffer (NuPAGE LDS Sample Buffer, Pierce, Thermo Fisher and 10X denaturing agent (NuPAGE Reducing Agent, Thermo Fisher) and preheated at 70°C for 10 min. Protein lysate was loaded into each well of a 4-12% nitrocellulose (NUPAGE) gel under SDS. Migration was performed at 180 V for 60 min, and transfer to a nitrocellulose membrane (Amersham Protran, Merck Sigma–Aldrich, St Quentin Fallavier, France) was performed for 2 h at 60 V. The membranes were briefly washed with 1× Tris-buffered saline (TBS)-Tween solution (TBS, ET220B, Euromedex, Souffelweyersheim, France) and blocked with 3% BSA/TBS for 1 h at RT. The membranes were then incubated with the primary antibodies anti-integrin α5 (1:1000; Cell Signaling Technology) and anti-albumin (1:1000; MA532531; Thermo Fisher) overnight at 4°C on a rocker. Next, the membranes were washed and treated with HRP-conjugated polyclonal rabbit anti-mouse or goat anti-rabbit secondary antibodies (1:3000; Thermo Fisher) for 1 h at room temperature. The membranes were visualized by chemiluminescence (ECL or ECL Femto, Pierce, Rockford, IL, USA) and imaged using a G-BOX imaging system (GeneSys, Cambridge, UK).

EV-TF-dependent factor Xa generation assay

EV-TF-dependent procoagulant activity was measured using a factor Xa (FXa) generation assay based on a previously published method (Vallier et al., 2019). Briefly, EVs were diluted in 70 µl of HEPES buffer and incubated with either an inhibitory anti-TF monoclonal antibody (aTF: clone SBTF-1, 10 µg/mL final, Biocytex, Marseille, France), an anti-TFPI antibody, recombinant annexin V (10 µg/mL, 556416, BD Biosciences, France) to block phosphatidylserine (PS), or an IgG DNP control antibody (10 µg/mL final, clone aDNP 2H11-2H12, Biocytex, Marseille, France) for 30 min in a 96-well flat bottom plate. Next, 8 µl of a reaction mixture containing purified FVIIa and FX (FVIIa, 10 nM final; FX, 190 nM final; Stago BNL, JV Leiden, Netherlands) in HEPES-Ca^{2+} buffer (150 mM NaCl, 20 mM HEPES

and 0.1% NaN_3, 50 mM $CaCl_2$, pH 7.4; 0.22 µm filtered) was added to the EVs to initiate FXa generation by incubating for another 2 h at 37°C. For some sets of experiments, FVIIa was not added to the EVs. The reaction was stopped by adding 8 µl of HEPES-EDTA buffer (150 mM NaCl, 20 mM HEPES, 0.1% NaN_3, and 200 mM EDTA, pH 7.4). Finally, FXa was measured by adding a chromogenic substrate. The chromogenic reading was performed for 15 min at an absorption wavelength of 405 nm, and activity was expressed as Vmax-mOD/min.

Thrombin generation assay

Thrombin generation (TG) by EVs was measured using a calibrated automated thrombogram (CAT) in EV-depleted plasma (EVs were removed by centrifugation at 3 × 100,000 × g for 90 min) as described by Hemker *et al*. (Hemker et al., 2003) with some modifications. To account for the known variability of the CAT assay, tests were performed in triplicate. In brief, EVs (at a final concentration of $16x10^3$ EVs/µl per well) were preincubated in 25 µl of HNBSA buffer (50 mM HEPES, 175 mM NaCl and 5 mg/ml BSA) supplemented with either an inhibitory anti-TF monoclonal antibody (aTF: clone SBTF-1, 10 µg/mL, Biocytex, Marseille, France) or an isotype control antibody (10 µg/mL) in a microplate for 30 min at 37 °C. Next, 80 µl of EV-depleted plasma was added to each well containing EV samples ($16x10^3$ EVs/µl final concentration). For the positive control, 25 µl of HNBSA containing 10 pM TF (Innovin, Dade Behring, Marburg, Germany) and 4 µM phospholipid (PL) (PS/PE/PC 20/20/60% Avanti Polar Lipids, Inc., Alabaster, USA) were added to 80 µl of EV-depleted plasma. The calibrator well contained 80 µl of EV-depleted plasma, 15 µl of calibrator containing the α2-macroglobulin/thrombin complex, and 10 µl of HNBSA buffer. Then, the reaction was initiated by adding 20 µl of FluCa fluorogenic substrate (Z-Gly-Gly-Arg-AMC; Fluobuffer, Stago) $CaCl_2$ to each well. TG was measured over 80 min, and the data were analyzed using Thrombinoscope software version 5.0 (Maastricht, Netherlands). The software analyzed different TG parameters, including lag time, endogenous thrombin potential (ETP), the velocity index, the peak level of thrombin formation, and the time to peak (ttP).

EV-plasmin generation capacity (EV-PGC)

EV-dependent PGC was detected using a plasmin generation assay to measure the fibrinolytic potential of EVs. In brief, 50 µl of PBS/BSA–azide buffer (0.1% BSA, and 0.1% NaN_3 buffer) containing 50 µl of a mixture of 1 µM plasminogen and ε-aminocaproic acid (ε-ACA, 5 mmol/L, Sigma–Aldrich, St. Louis, MO, US) was added to various concentrations of EVs (0.5; 1 and 2 x 10^6 EVs) for 24 h in a 96-well flat bottom plate. Following the incubation, 80 µl of the selective chromogenic substrate of plasmin (methyl-malonyl)-hydroxypropylarginine-p-

nitroaniline (CBS0065: Stago, Asnière, France) at 0.75 mM was added to the EV mixture. The buffer and EV supernatant from the last EV wash were used as negative controls. Plasmin generation activity was recorded by taking the chromogenic reading for 120 min at an absorption wavelength of 405 nm at 37°C, and the activity was expressed as mOD/min.

Fibrin clot formation and visualization by scanning electron microscopy (SEM)

Fibrin clots were formed by spiking EVs ($6.6x10^4$ EVs/µl) into EV-depleted plasma in the presence of $CaCl_2$ (2.4 mmol/L) at 37°C for 2 h. Clots were washed in PBS (3 x15 min) with rotation, fixed in 2.5% glutaraldehyde and 2% PFA in PBS, postfixed with 2% OsO_4 in PBS, dehydrated with graded ethanol, and finally sputter-coated with gold. Fibrin clot structures were then observed with an FEI Teneo SEM instrument (Thermo Fisher). Morphometric analysis of clots via SEM images was performed by measuring the fibrin fiber diameter and porosity corresponding to the space between the fibrin fibers using DiameterJ, a well-defined open-access plugin tool that can be incorporated into ImageJ/FIJI software (Hotaling et al., 2015). Five random areas of SEM images of each clot were segmented using the DiameterJ segmentation algorithm to produce binary (black and white) images of the fibrin network. Fiber thickness and porosity were measured in 5 randomly selected areas.

Fibrinography of EV-induced clot

Fibrinography is derived from the turbidimetric clotting assay of the EuroCLOT study (Carter et al., 2007). Fibrin formation and lysis induced by EVs in plasma were analyzed using Fibrinography. Briefly, EV-depleted plasma (400µl) was spiked with EVs (25x103 EVs/µl) and clotting was initiated with the addition of TF, PL, and CaCl2 at a final concentration of 2 pM, 4µM, and 16.7mM respectively. Fibrinolysis was initiated in the presence of tissue plasminogen activator (tPA, 0.132 µg/mL). The protofibril numbers were recorded during fibrin formation and lysis and calculated from the optical density (OD) at 540nm and 780nm (C. Dassi et al., 2019) every 2 seconds for 30 min at 37°C on a STA®-R prototype instrument (D. Stago). In kinetic mode, the following parameters of the curve were quantified: Clotting Time (CT), Time to fibrin polymerization Plateau (TP), Optical Density variation (DOD) at TP and Lysis Time (LT) corresponding to 50% maximal DOD during lysis.

Statistical analysis

All the data were analyzed using GraphPad Prism 8.04 software (GraphPad Software, La Jolla, CA, USA). All quantitative experimental data are presented as the mean±SEM of three (n = 3) independent biological replicates, except for IMS experiments, in which more than three (n =

8, healthy individuals and n = 11, sepsis patients) independent biological replicates were used. The statistical analyses were performed using either analysis of variance (ANOVA) with Tukey's test for comparisons between multiple groups or the Mann–Whitney test for comparisons between two groups. Spearman's nonparametric test was performed for correlation analysis. A p value less than 0.05 was considered to indicate statistical significance.

Results

Characterization of EV subsets isolated from the different cell types

EVs were first purified from the conditioned media of activated platelets, nonactivated or activated neutrophils, monocytes, and endothelial cells and activated platelets using a combination of ultrafiltration, SEC, and ultracentrifugation (**Figure 1A**). EV subsets were further characterized in accordance with the MISEV guidelines (Théry et al., 2018). First, TEM images demonstrated the presence of lipid bilayer-enclosed for all the EV subset (**Figure 1B**). Second, flow cytometry analysis revealed small events in which both anionic phospholipids (Ann V+) and lineage-specific antigens were expressed under nonactivated and activated conditions: AnnV+/CD15+ for NDEVs, AnnV+/CD18+ for MDEVs, AnnV+/CD146+ for EDEVs and AnnV+/CD41 for PDEVs (**Figure 1C**). The cross-contamination level from other types of EVs in each purified EV batch was less than 5% **(Supplemental Figure 1**). Third, western blot analysis revealed that all the EV subsets expressed the integrin α5 subunit, confirming the origin of the EVs from the cell membrane. Western blot analysis further confirmed the purity of the EV preparations in the absence of soluble proteins such as albumin in the NDEVs, MDEVs, and EDEVs, while the PDEVs had negligible amounts of albumin due to the absence of SEC purification for this subset of EVs (**Figure 1D**). Fourth, analysis of the size of EVs by TRPS (qNano) showed that the EV populations displayed a median size between 150-177 nm (**Figure 1E**) and a homogenous distribution (**Supplemental Figure 2**) across all EV subsets irrespective of the stimulation status of the cells.

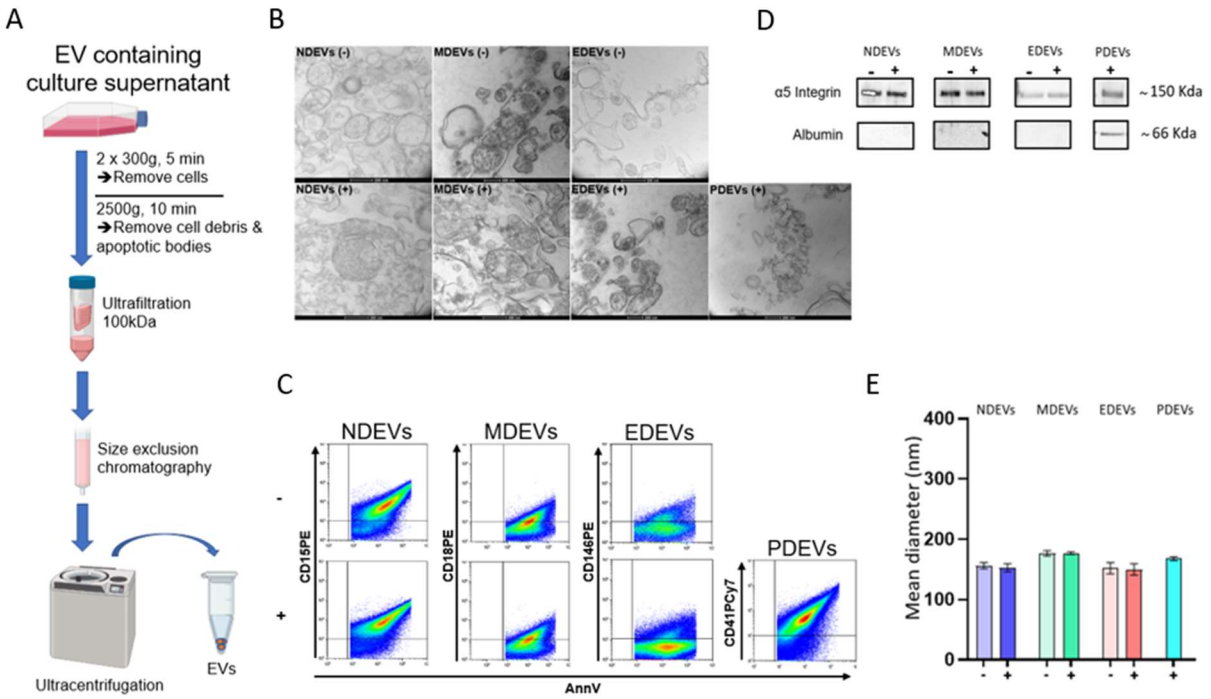

Figure 1. Purification and characterization of subsets of EVs from cells. A) Schematic workflow of EV production and purification from cells: EV-rich supernatants were collected from their respective cell culture (both unstimulated (-) and stimulated (+) cells). Then, a series of centrifugations were performed (to remove cells, cell debris, and apoptotic bodies), subjected to ultrafiltration, and size exclusion chromatography, and finally EVs were pelleted by ultracentrifugation at 100,000g. B) Representative TEM images of lipid bilayer encapsulated EVs. C) Representative western blots for integrin α5 subunit of integrin and albumin in the different EVs subsets in unstimulated (-) and stimulated (+) conditions. D) Histograms representing the relative size of EVs analyzed by TRPS (qNano) from three independent experiments. PDEVs were isolated and directly pelleted by ultracentrifugation from the platelet supernatant following stimulation with thrombin. Bars represent standard deviation.

Monocyte and endothelial EVs display a procoagulant phenotype that is more pronounced under inflammatory stimulation

To evaluate the procoagulant activity of the different subsets of EVs, we used an FXa generation assay and compared the procoagulant activity of EVs from nonstimulated and stimulated cells. As illustrated in **Figure 2 (A&B)**, non-stimulated MDEVs and EDEVs demonstrated low but detectable FXa generation activity that significantly increased under inflammatory conditions (MDEVs: 5 ± 0.57 mOD/min *vs.* 22 ± 2.64 mOD/min, unstimulated and stimulated, respectively; p = 0.0018; and EDEVs: 6.33 ± 2.84 mOD/min *vs.* 17.33 ± 1.33 mOD/min, unstimulated and stimulated, respectively; p = 0.02). Interestingly, FXa generation by MDEVs and EDEVs was inhibited using an anti-TF antibody (SBTF-1) or in the absence of FVIIa, indicating a dependence on the TF pathway. Furthermore, FXa generation by EVs was also inhibited in the presence of Ann V, indicating that PS also contributes to FXa generation. The inhibitory effect of Ann V on MDEVs was more pronounced than that on EDEVs. However, the addition of an anti-TFPI antibody (aTFPI) did not modify EV-TF-

dependent FXa generation, suggesting that purified EVs do not carry active TFPI. Taken together, these data indicate that MDEVs and EDEVs support TF- and PS-dependent procoagulant activity. In contrast, no FXa generation was detectable neither in NDEVs in both unstimulated and stimulated conditions nor in PDEVs in stimulated conditions (**Figure 2 C&D)**. Interestingly, these specific activities were consistent with those measured in the cells of origin (**Supplemental Figure 3**) confirming that the functions and contents of EV are greatly influenced by their parental cells.

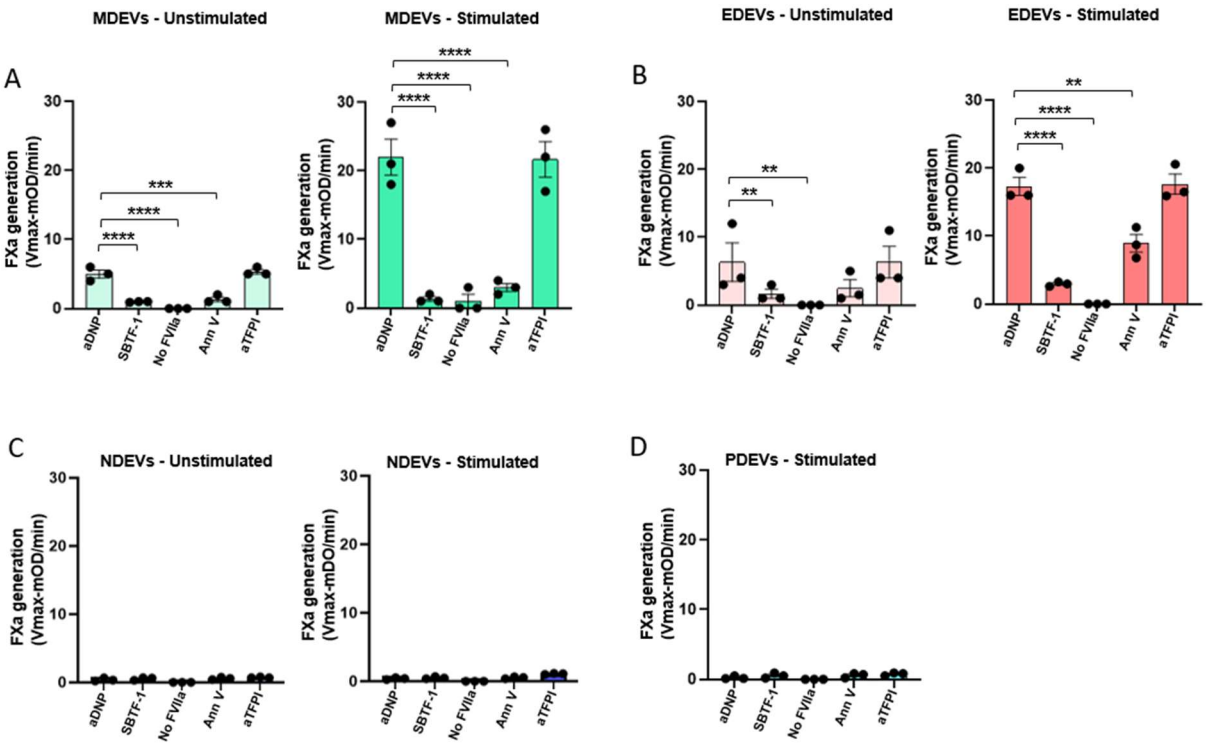

Figure 2. MDEVs and EDEVs support TF-dependent FXa generation. FXa generation by (A) MDEVs, (B) EDEVs, (C) NDEVs, and (D) PDEVs was measured using a TF-dependent chromogenic assay. FXa generation was assessed in the presence of either a control DNP antibody (aDNP), anti-human tissue factor antibody (SBTF-1 Ab, aTF), anti-tissue factor pathway inhibitor (aTFPI) antibody, annexin V, or in the absence of FVIIa (No FVII). Buffer was used as blank and the data shown is subtracted from the buffer value. Data show mean ± SEM of 3 independent EV samples. Asterisks represent significant *p ≤ 0.05, **p ≤ 0.01, and ***p ≤ 0.001 compared to aDNP condition.

The procoagulant activity of different subsets of EVs was further evaluated with a thrombin generation assay by spiking purified EVs into EV-depleted plasma in the absence of any external triggers, such as PL or TF. As shown in Figure 3A, the levels of thrombin generated by MDEVs and EDEVs from unstimulated cells increased under stimulated conditions. MDEVs from stimulated cells induced a significant increase in the thrombin peak (9.33 ± 2.4 nM *vs.* 106.7 ± 13.5 nM, unstimulated and stimulated, respectively, p = 0.0007) and a

significant reduction in the lag time (15 ± 3.2 nM *vs.* 1.7 ± 0.33 nM, unstimulated and stimulated, respectively, p = 0.01), which were abolished in the presence of the anti-TF antibody (SBTF-1) (**Figure 3B**). EDEVs from stimulated cells induced a nonsignificant increase in the thrombin peak (30 ± 6.11 nM *vs.* 55 ± 13.79 nM, unstimulated and stimulated, respectively, p = 0.17) and a significant reduction in the lag time (14 ± 3 nM *vs.* 5.33 ± 0.33 nM, unstimulated and stimulated, respectively, p = 0.04), which was also abolished in the presence of an anti-TF antibody (SBTF-1) (**Figure 3C**). In contrast, in the absence of any external trigger, neither NDEVs nor PDEVs were able to produce detectable thrombin (**Supplemental Figure 4 A&B)**.

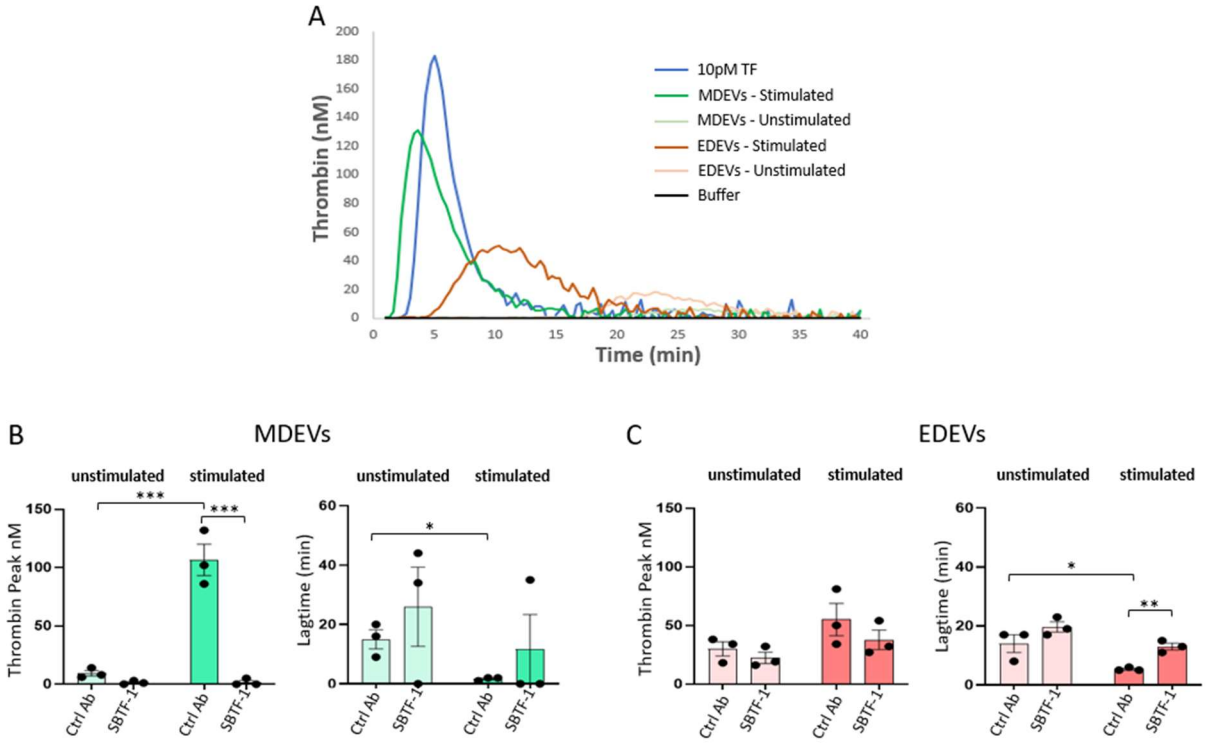

Figure 3. MDEV and EDEV-dependent thrombin generation were determined using CAT. A) TG experiments were performed by spiking $16x10^3$EVs/µl in re-calcified EV-depleted plasma in the presence of a control antibody (Ctrl Ab) or anti-human tissue factor (SBTF1 Ab, aTF). Innovin (10 pM) was used as the positive control and EV-depleted plasma mixed with buffer was used as the negative control. (B and C) Histograms representing thrombin peak nM concentration and Lagtime of B) MDEVs and C) EDEVs. Data show mean ± SEM of 3 independent EV samples. Asterisks represent significant *p ≤ 0.05, **p ≤ 0.01, and ***p ≤ 0.001 compared to the control condition.

We also examined the ability of the different subsets of EVs to influence fibrin clot structure after spiking EVs into recalcified EV-depleted plasma without any other external triggers (PL or TF). As illustrated in Figure 4A, SEM images revealed ultrastructural differences between the clots formed by each subset of EVs. Compared with EVs from unstimulated cells, MDEVs

and EDEVs from stimulated cells induced the formation of denser and more compact clots with more branched fibers (**Figure 4A**). This observation is supported by a significant decrease in the fibrin fiber diameter associated with MDEVs (0.12 ± 0.003 µm *vs.* 0.18 ± 0.005 µm, stimulated and unstimulated, respectively, p = 0.008) (**Figure 4B**) and a significant reduction in the clot porosity for both MDEVs and EDEVs (MDEVs: 0.18 ± 0.018 μm^2 *vs.* 0.08 ± 0.005 μm^2, p = 0.006 and EDEVs: 0.15 ± 0.002 μm^2 *vs.* 0.10 ± 0.009 μm^2, unstimulated and stimulated, respectively, p = 0.01) (**Figure 4C**). In contrast, clots formed by NDEVs and PDEVs had thicker fibrin fibers and larger pores than those formed by MDEVs and EDEVs from stimulated cells, indicating reduced procoagulant activity.

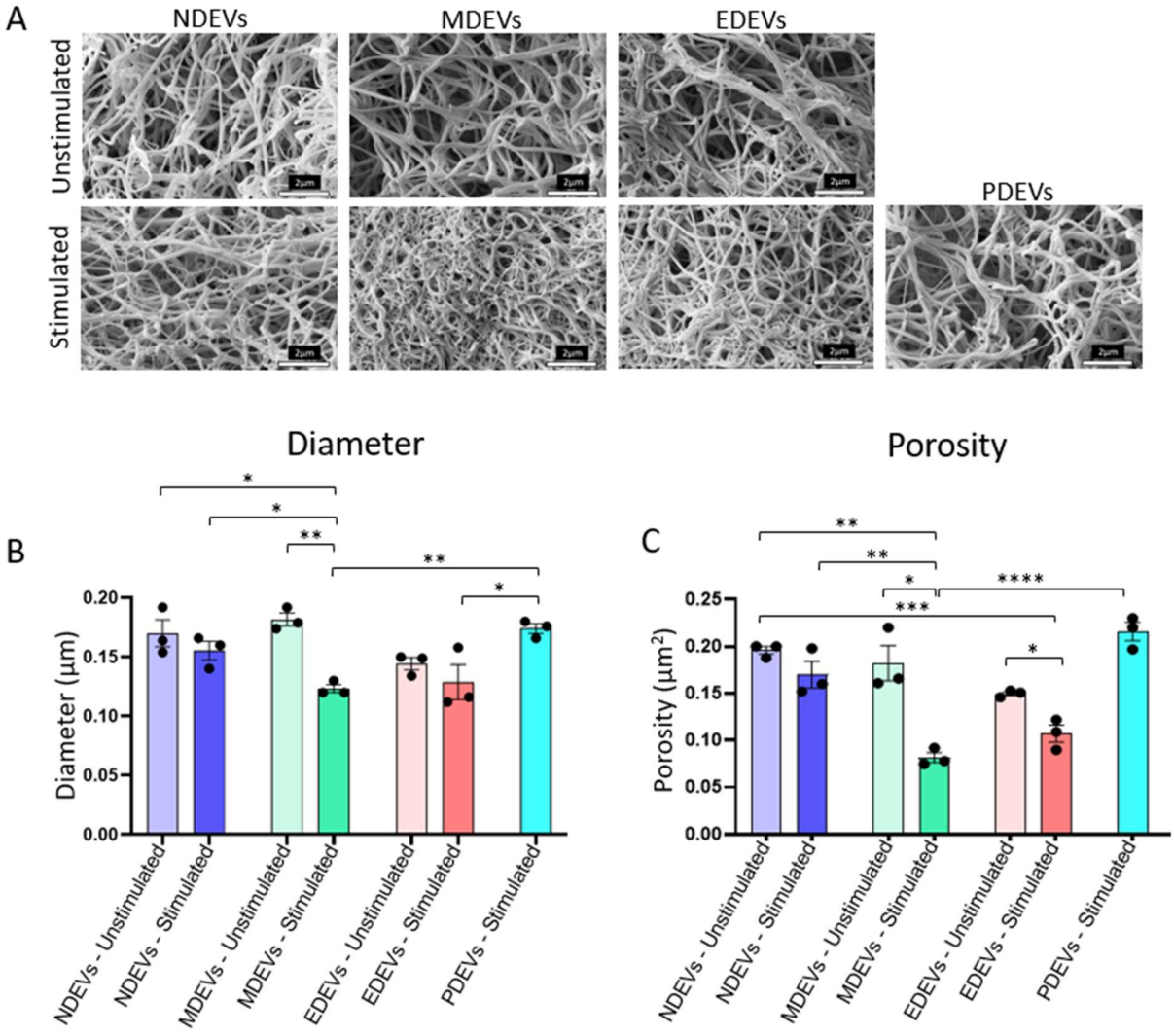

Figure 4. EV-induced clot structure analyzed by scanning electron microscopy. A) Representative SEM images of clots formed by spiking different subsets of EVs into EV-depleted plasma. Histograms representing B) the diameter of fibrin fiber and C) the porosity of the clot. Data show mean ± SEM of 3 independent EV samples. Asterisks represent significant *p ≤ 0.05, **p ≤ 0.01, and ***p ≤ 0.001 compared to the control condition.

These data were confirmed using fibrinography after spiking the different subsets of EVs into EV-depleted plasma in the presence of TF and tPA. As shown in Figure 5A, no significant difference was observed between different subsets of EVs from unstimulated cells. In contrast, compared with the plasma control, MDEVs and EDEVs from stimulated cells induced a significant decrease in coagulation time (80.7 ± 0.3 s (plasma control) *vs.* 35.3 ± 5.8 s (MDEVs) or 65 ± 1.15 s (EDEVs), p = 0.002 and p = 0.04, respectively) (**Figure 5A&C)** and a significant increase in resistance to clot lysis (1130 ± 17 s (plasma control) *vs.* 1449 ± 71 s (MDEVs) or 1291 ± 13 l s (EDEVs), p = 0.004 and p = 0.04, respectively) (**Figure 5B&D**). Moreover, we observed fewer protofibrils on MDEVs from the stimulated cells, suggesting an increase in procoagulant activity. These results demonstrate that MDEVs and EDEVs derived from the stimulated cells and exhibiting high procoagulant activity and originate from stimulated cells, modulate fibrin polymerization and contribute to the formation of clots that were more resistant to lysis.

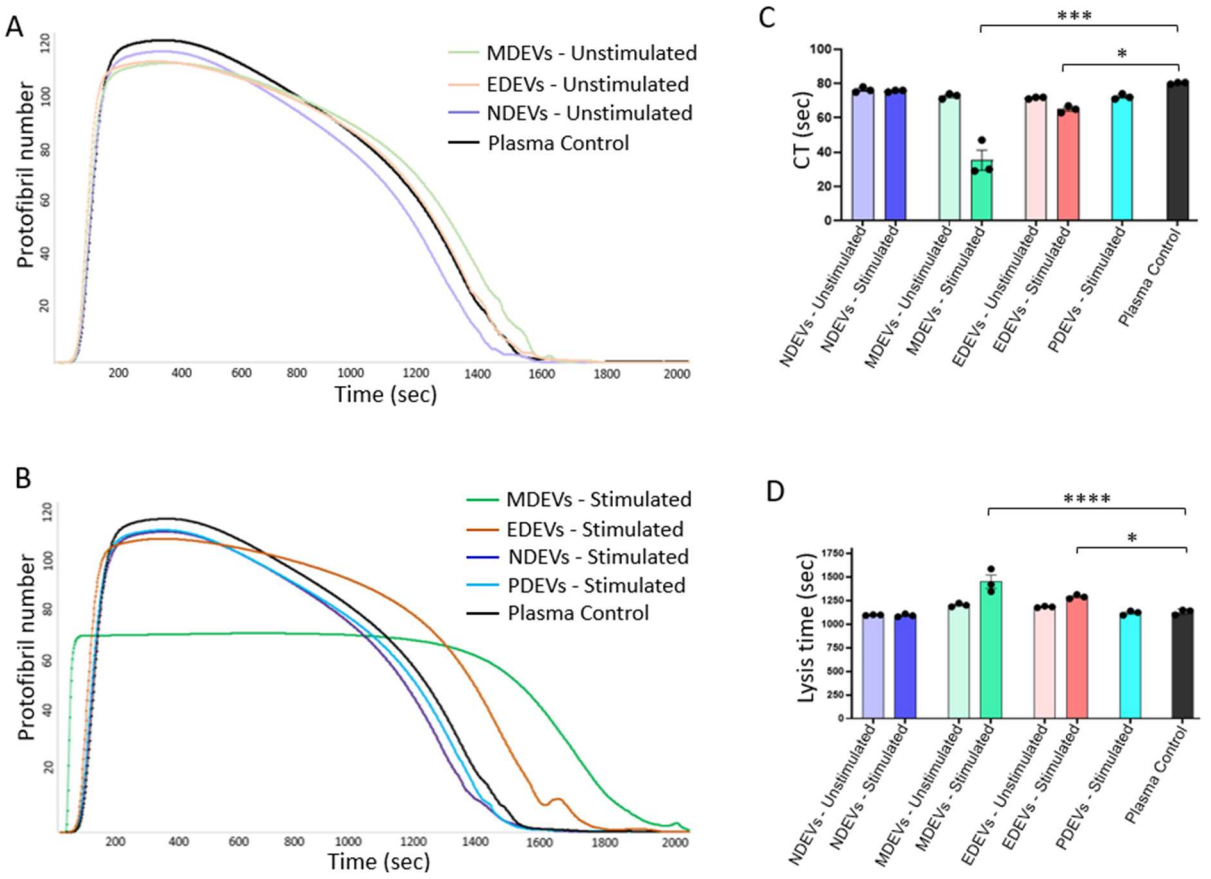

Figure 5. MDEV and EDEV influenced clot formation and lysis profiles analyzed by fibrinography. Fibrinography was performed by spiking EVs into EV-depleted plasma in the presence of 2 pM TF, 4µM PL, 16.7mM $CaCl_2$, and tPA (0.32µg/mL) Protofibril packing and temporal evolution of a clot induced by the EVs from (A) stimulated and (B) unstimulated cells. Histograms representing (C) Lysis time and (D) coagulation time (TC) of EV-induced

clot. Data show mean ± SEM of 3 independent EV samples. Asterisks represent significant *p ≤ 0.05, **p ≤ 0.01, and ***p ≤ 0.001 compared to the control condition.

Profibrinolytic potential of different subsets of EVs purified from cells

To evaluate the profibrinolytic activity of the different subsets of EVs, we used a plasmin generation assay. NDEVs, MDEVs, and EDEVs from both unstimulated and stimulated cells effectively cleaved plasminogen to plasmin in a concentration-dependent manner (**Figure 6A-C**). Interestingly, the amount of plasmin generated by NDEVs under unstimulated conditions (12.6 ± 2.08 mOD/min, Figure 6A) was greater than that generated by MDEVs (6.6 ± 0.39 mOD/min, Figure 6B) and EDEVs (8.6 ± 3.4 mOD/min, Figure 6C). In contrast, PDEVs did not have any significant plasmin generation activity for all the considered EV quantities (Figure 6D). Interestingly, unlike procoagulant activity, stimulation of the cells did not have any significant impact on the fibrinolytic activity induced by the EVs except for a trend to decrease with EDEVs.

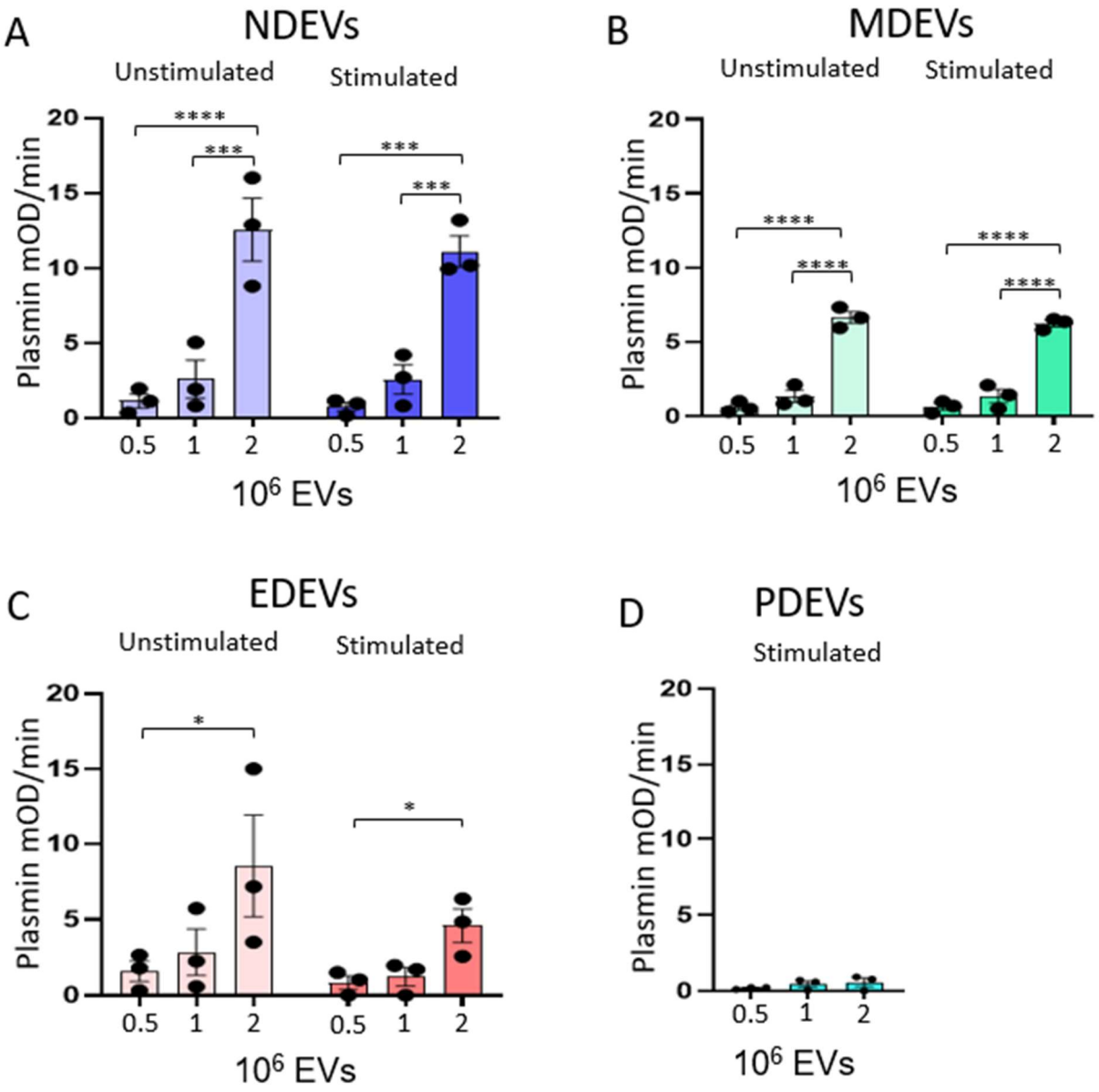

Figure 6. EV-PGC profile varies with subsets of EVs. Histograms representing (A) NDEVs, (B) MDEVs, (C) EDEVs, and (D) PDEVs dependent plasmin generation. Buffer was used as the negative control and the data shown is subtracted from the buffer value. Data show mean ± SEM of 3 independent EV samples. Asterisks represent significant *$p \leq 0.05$, ***$p \leq 0.001$, and ****$p \leq 0.0001$ compared to the control condition.

Altogether, the results showed that the different subsets of purified EVs display distinct CLB profiles according to the cellular origin and activation status of their parental cells. MDEVs and EDEVs were more profibrinolytic than procoagulant under unstimulated conditions, but their CLBs switched toward a more procoagulant profile under inflammatory conditions. In contrast, the CLB profile of NDEVs was more profibrinolytic under both unstimulated and stimulated conditions. On the other hand, purified PDEVs were devoid of any procoagulant or profibrinolytic activity.

Procoagulant and profibrinolytic profile of EVs derived from the plasma of sepsis patients

To check the EV-CLB profiles *in vivo,* we immuno-captured the different subsets of circulating EVs from the plasma of healthy donors (n = 8) and sepsis patients (n = 10) as models of unstimulated and inflammatory conditions. The sepsis patient's characteristics are available in **supplemental Table 1**. Flow cytometry analysis revealed a different pattern of EV distribution between healthy donors and sepsis patients, with a greater number of NDEVs and EDEVs in sepsis patients (**Supplemental Figure 5**). NDEVs, MDEVs, EDEVs, and PDEVs were isolated using IMS beads coated with anti-CD15, anti-HLA-DR, anti-CD146, and anti-CD41 antibodies, respectively. As illustrated in **Supplemental Figure 6**, HLA-DR-IMS, CD41-IMS, and CD146-IMS-specific IMS achieved more than 85% depletion of the respective EV populations, while CD15-IMS achieved approximately 60% depletion; the nonspecific depletion of EVs by the control DNP-IMS remained very low (<5%).

We then evaluated the procoagulant activity of the immunocaptured EVs. In healthy donors, a low but significant level of TF activity was detected only with MDEVs in comparison to the control DNP (MDEVs, 15 [6.25-38.75] fM *vs.* control, 5 [5-6.75] fM, p = 0.01) (**Figure 7A**). Compared with that in healthy donors, MDEV-dependent TF activity was increased in sepsis patients (MDEVs, 40.5 [23.25-76.25] fM *vs.* MDEVs, 15 [6.25-38.75] fM, p = 0.05). Interestingly, TF activity on EDEVs, NDEVs, and PDEVs was also detectable in some patients. The TF-independent procoagulant activity was also calculated from the remaining FXa generation after TF inhibition and removal of the FXa background noise (FXa, DNP control beads). As shown in **Supplemental Figure 7A,** this activity was significantly increased in sepsis patients and was mainly supported by MDEVs and PDEVs. When each subset of EVs was normalized according to the concentration measured by flow cytometry, MDEVs showed the highest TF-independent procoagulant activity among the subsets of EVs in sepsis patients **(Supplemental Figure 7B)**.

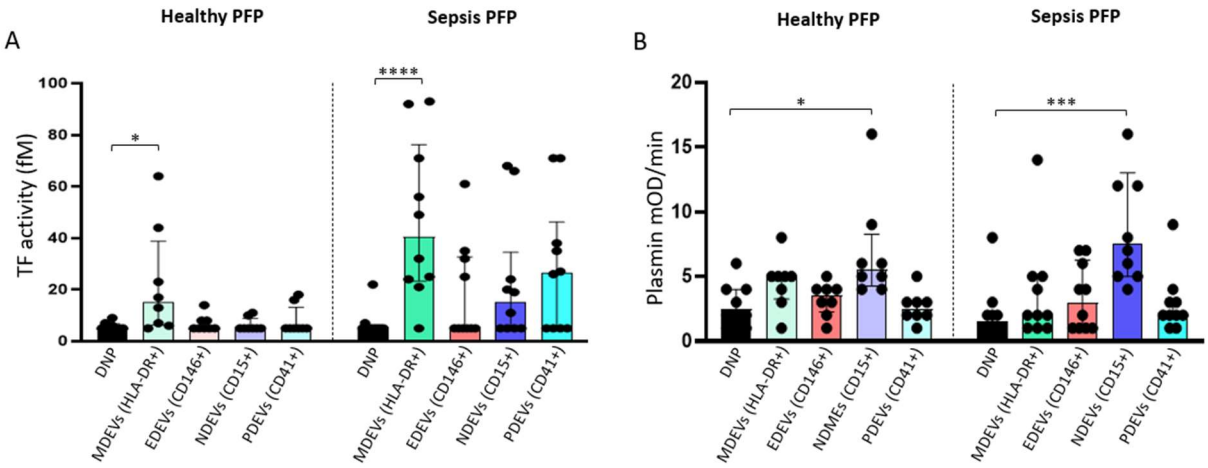

Figure 7. EVs captured from sepsis PFP display a heterogeneous profile of EV-TF and EV-PGC. Histograms representing (A) EV-TF and (B) EV-PGC activity of circulating MDEVs, EDEVs, NDEVs, and PDEVs were measured after IMS from plasma of healthy subjects (n = 8) and Sepsis patients (n = 10). DNP-IMS was used as a negative control. Asterisks represent significant *p-value <0.05, **p-value <0.01, and ***p-value <0.001 compared to the control condition.

Furthermore, we determined the fibrinolytic activity of different subsets of immunocaptured EVs. A significant increase in fibrinolytic activity was detected only with NDEVs in comparison to the control DNP (CD15-IMS, 5.5 [4.25-8.25] fM *vs.* DNP-IMS, 2.5 [1.25-4] plasmin mOD/min, p = 0.01) **(Figure 7B).** In sepsis patients, NDEV-dependent plasmin generation was still detectable but more heterogeneous than that in healthy donors. In most patients, no fibrinolytic activity was detectable for other EV subsets.

Given the different proportions of the different subsets of EVs in the circulation of healthy donors and sepsis patients, we normalized the TF-dependent procoagulant and profibrinolytic activity according to the concentration of each subset. The results are presented for the same concentration of the EVs used for *in vitro* FXa and plasmin generation ($0.5x10^6$ EVs) (**Supplemental Figure 8**). The results obtained with the EDEVs are not interpretable due to their very low concentration. Similar to the inflammatory conditions *in vitro* data, the TF-dependent procoagulant activity of MDEVs significantly increased in sepsis patients. Surprisingly, NDEVs also displayed TF-dependent procoagulant activity both in healthy donors and in sepsis patients, but this subset of EVs had the highest plasmin generation capacity. Compared to other subsets of EVs, PDEVs displayed very low TF-dependent activity

at the same concentration and were devoid of fibrinolytic activity in both healthy donors and sepsis patients.

Finally, we calculated the EV-CLB for each subset as the ratio between TF and plasmin generation activity measured in the same volume of plasma (100 µL). MDEVs showed the most procoagulant CLB profile which was significantly increased under inflammatory conditions in (sepsis) patients. EDEVs showed a balanced CLB profile in healthy donors but shifted toward a more procoagulant profile under inflammatory conditions. Similarly, PDEVs also acquired a procoagulant CLB in sepsis patients. In contrast, NDEVs exhibited the lowest CLB among the other subsets of EVs, indicating superior fibrinolytic activity (**Table 1)**. Interestingly, the NDEV-CLB was heterogeneous among sepsis patients, was positively correlated with fibrin monomers (r^2 = 0.77, p = 0.014) and was negatively correlated with fibrinogen and platelets (r^2 = -0.72, p = 0.02 and r^2 = -0.61, p = 0.06, respectively). The CLB profile of NDEVs was the only one that correlated with sepsis coagulopathy.

CLB (arbitrary unit)	Healthy (n = 8)	Sepsis (n = 10)	p-value
MDEVs	4 [2.56-5.37]	13.35 [6.05-25.38]	0.01
EDEVs	1.83 [1.61-3.25]	5 [1.3-19.38]	0.22
NDEVs	1 [0.68-1.25]	1 [0.65-5.5]	0.80
PDEVs	2.5 [1.67-4.55]	5 [3.25-19.63]	0.13

Table 1: CLB profile of different subsets of EVs in healthy donors and sepsis patients.

MDEVs: monocyte-derived extracellular vesicles; EDEVs: endothelial cell-derived extracellular vesicles; NDEVs: neutrophil-derived extracellular vesicles; PDEVs: platelet-derived extracellular vesicles.

Data are median [25th to 75th percentile]. p-value ≤0.05 was considered significant.

Discussion

Previous studies have demonstrated that cellular activation triggers the release of EVs that convey procoagulant and/or profibrinolytic properties. However, neither the simultaneous procoagulant and profibrinolytic activities of a single EV subset nor the impact of its cellular

origin and pathological environment on the EV-CLB have been considered. This work provides the first demonstration that the cellular origin and activation status of parental cells are able to drive the CLB profile of each EV subpopulation. In this study, we showed that MDEVs display a procoagulant CLB profile that significantly increases under inflammatory conditions both *in vitro* and in sepsis patients. EDEVs display a neutral CLB profile at the basal level that switches toward a more procoagulant CLB profile under inflammatory conditions. In contrast, NDEVs were the only subpopulation displaying a profibrinolytic CLB profile characterized by significantly high plasmin generation capacity, which was strongly correlated with markers of coagulopathy in sepsis patients. Finally, PDEVs showed low procoagulant activity and were devoid of profibrinolytic activity compared to other EV subsets at the same concentration. The selective feature of these EV subpopulation CLB profiles was demonstrated both *in vitro* after inflammatory stimulation and *ex vivo* in sepsis patients.

Resting monocytes from healthy donors have been reported to express low levels of TF (Egorina et al., 2005), which was significantly upregulated upon stimulation with LPS (Butenas et al., 2005) and increases the TF-dependent procoagulant activity of MDEVs *in vitro* (Aleman et al., 2011; Basavaraj et al., 2012; Khaspekova et al., 2016; Lipets et al., 2020; Shustova et al., 2017). Moreover, MDEVs demonstrated a greater ability to induce coagulation and fibrin network formation with enhanced clot stability mediated through TF (Aleman et al., 2011; Lipets et al., 2020). Additionally, TF+ MDEVs accumulated into a growing thrombus in a mouse model of laser-induced arterial thrombosis (Falati et al., 2003). Furthermore, TF+ MDEVs measured by flow cytometry were correlated with procoagulant activity in sepsis patients (Hellum et al., 2014; Nieuwland et al., 2000; Zhang et al., 2016). In agreement with these results, we showed that well-purified MDEVs obtained via SEC display both PS- and TF-dependent procoagulant activity. Interestingly, using an immunomagnetic strategy, we confirmed that compared with those from healthy donors, MDEVs isolated from sepsis patients display increased PS- and TF-dependent procoagulant activity. On the other hand, our group previously showed that the fibrinolytic activity of MDEVs generated from human monocytes after LPS stimulation is dependent on the activation of the uPA/uPAR system (Lacroix et al., 2012). This work showed that this activity is detectable in circulating MDEVs. This fibrinolytic activity is not affected by LPS stimulation but does not seem to compensate for the high procoagulant activity conveyed by MDEVs. As a result, the CLB profile of MDEVs remains procoagulant, with a higher CLB ratio than that of all other circulating subsets of EVs in both healthy donors and sepsis patients.

Previous studies have demonstrated that TNF-α stimulation significantly increases the procoagulant activity of EDEVs *in vitro* (Combes et al., 1999; Holnthoner et al., 2017; Szotowski et al., 2005). In addition, an increase in circulating levels of TF+ EDEVs has already been reported in sepsis patients (Matsumoto et al., 2015). Here, we confirmed that purified EDEVs display procoagulant activity that is mainly detectable after inflammatory stimulation. In addition, despite the low proportion of EDEVs among total circulating EVs, we demonstrated that EDEVs specifically isolated from the blood of sepsis patients display TF-dependent procoagulant activity. The possibility that TF activity originates from another source of EVs bound to EDEVs cannot be excluded but is unlikely because of the low level of the nonspecific depletion of CD146 beads (< 5%). Regarding the fibrinolytic CLB profile, our group previously demonstrated using endothelial cell lines or human endothelial primary cells that EDEVs can effectively support plasmin generation via either the uPA/uPAR system or tPA (Lacroix et al., 2007, 2012). Interestingly, the present work revealed a reduction in EDEV fibrinolytic activity under inflammatory conditions, which may contribute to the switch toward a procoagulant CLB profile.

Accumulating data in the literature suggest that neutrophils support procoagulant activity not only through NETosis but also through the vectorization of TF (Darbousset et al., 2012; Ritis et al., 2006; Savchenko et al., 2014; Von Brühl et al., 2012). However, the expression and activity of TF in human neutrophils remain controversial (Noubouossie et al., 2019). Little data are available regarding the potential procoagulant activity of NDEVs. One *in vitro* and *in vivo* study reported the detection of TF+ NDEVs by flow cytometry (Kambas et al., 2014), but the sensitivity and specificity of this method are controversial (Mackman et al., 2022). Here, we were unable to detect any TF-dependent activity on purified NDEVs from human neutrophils *in vitro* even after inflammatory stimulation. This procoagulant activity was also undetectable in healthy donors. Nevertheless, we detected significant TF-dependent procoagulant activity in half of the sepsis patients. Therefore, in the complex setting of inflammatory disorders, we cannot exclude the possibility that NDEVs can acquire TF-dependent activity after stimulation with agonists other than TNF-α. It is also possible that NDEVs acquire TF after transfer from other cellular sources. On the other hand, consistent fibrinolytic activity dependent on the uPA/uPAR system has already been described in NDEVs from sepsis patients (Cointe et al.,

2018, 2022). We demonstrated here that among the other EV subsets, NDEVs displayed the most profibrinolytic CLB profile.

Previous studies have demonstrated that EVs from platelets support thrombin generation (Ferreira et al., 2020; Guerreiro et al., 2024; Tripisciano et al., 2017, 2020). This procoagulant activity is highly dependent on PS exposure or the contact pathway (Ferreira et al., 2020; Guerreiro et al., 2024; Tripisciano et al., 2017, 2020; Van Der Meijden et al., 2012). In contrast, unlike other subsets of EVs, we were not able to detect any significant procoagulant activity on purified PDEVs using either FXa and thrombin generation or fibrin structures. The difference in the literature may be explained by the lower concentration of PDEVs used in this study. Moreover, the type of agonist used for platelet stimulation (thrombin) may have influenced the results. Indeed, while not demonstrated for EVs, it has been shown that platelet activation upon treatment with a combination of collagen and thrombin or collagen alone produced more procoagulant platelets than stimulation with thrombin alone (Hua et al., 2015; Pasalic et al., 2018). When directly captured from the plasma of healthy donors and sepsis patients, we showed that significant TF-dependent TF activity was observed in some sepsis patients. The level of this activity remains very low considering the high concentration of circulating PDEVs compared to the other subsets of EVs. One hypothesis is that the low TF activity of PDEVs in sepsis patients may result from the transfer of TF from other cellular (Eichhorn et al., 2023) sources, as shown by De Conde *et al*. (Del Conde et al., 2005), or from the formation of EV aggregates between platelets and leukocyte EVs, as recently shown in COVID patients (Eichhorn et al., 2023). Consistent with the literature, PDEVs were devoid of fibrinolytic activity (Lacroix et al., 2012). Therefore, the resulting PDEVs-CLB remained mainly neutral, except for those in some sepsis patients in whom we observed a shift toward a more procoagulant CLB.

In this work, we proposed EV-CLB as a means to integrate both the procoagulant and fibrinolytic activity of each subset of EVs rather than only looking at these activities independently. This strategy may have interesting applications regarding the potential of EVs as biomarkers in thrombo-inflammatory diseases. Given the heterogeneous profile of the EV-CLB according to EV subsets, it may be advantageous in some clinical settings to focus on the resulting CLB profile of one EV subset rather than global activity. In line with this hypothesis, we found that only the NDEV-CLB was significantly correlated with coagulopathy markers in sepsis patients. Although we successfully demonstrated that the CLB profile varies according to different subsets of EVs both *in vitro* and *ex vivo*, our study has several limitations. First, we

calculated the EV-CLB by the ratio between the TF-dependent procoagulant and the fibrinolytic activity (uPA/uPAR). Although there is cumulative evidence that these two pathways play a critical role in hemostasis (Aleman et al., 2011; Cointe et al., 2022), they do not recapitulate all possible mechanisms involved, such as the contact pathway. Second, our study did not include erythrocyte-derived EVs, a major subset of circulating EVs that have been reported to display procoagulant activity (Avenick et al., 2021; Lacroix et al., 2012; Noubouossie et al., 2020).

This work provides the first demonstration that the cellular origin and activation status of parental cells drive the EV-CLB and defines a selective signature at the single level of EV subsets. EV-CLB represents a potential new biomarker reflecting the hemostatic imbalance associated with sepsis coagulopathy and other coagulopathies.

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Author contributions

Akhil Konkoth: conceptualization; methodology; investigation; data curation; formal analysis; writing-original draft; and review & editing; **Amandine Bonifay:** methodology; and review & editing; **Corinne Chareyre:** methodology; visualization; and review & editing; **Evelyne Abdili:** methodology; visualization; and review & editing; **Stella Thomassen:** methodology; and review & editing; **Sandra Ghayad:** reviewing and editing the manuscript; **Lea Plantureux:** methodology; and review & editing; **Stéphane Robert:** methodology; visualization; and review & editing; **Corentin Franco:** methodology; and visualization; **Tarik Bouriche:** methodology; visualization; and resources; **Sylvie Cointe:** methodology; and review & editing; **Philippe Poncelet:** conceptualization; funding acquisition; project administration; resources; and formal supervision; **Françoise Dignat-George:** conceptualization; funding acquisition; resources; and review & editing. **Rory R. Koenen:** funding acquisition; project administration; resources; formal supervision; and review & editing. **Romaric Lacroix:** conceptualization; funding acquisition; project administration; resources; formal supervision; and review & editing.

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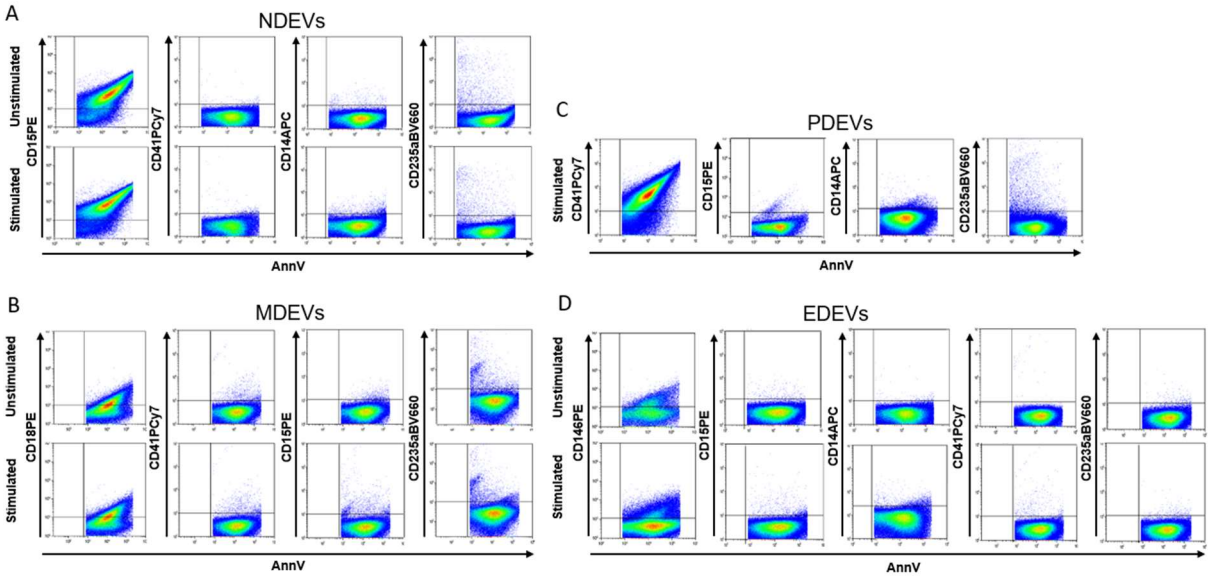
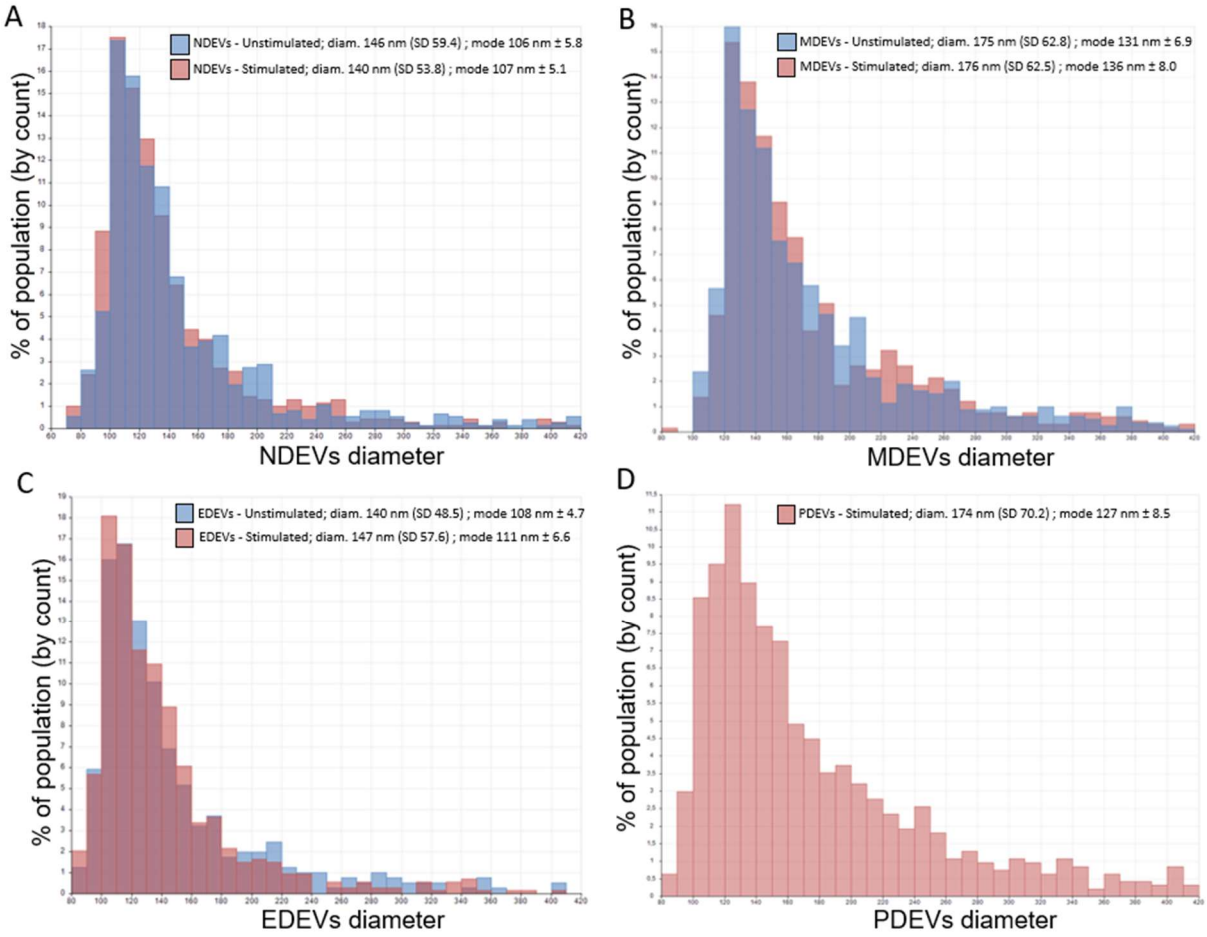
Supplementary data

Purity Analysis of EV subsets by flow cytometry (Supplemental Figure 1)



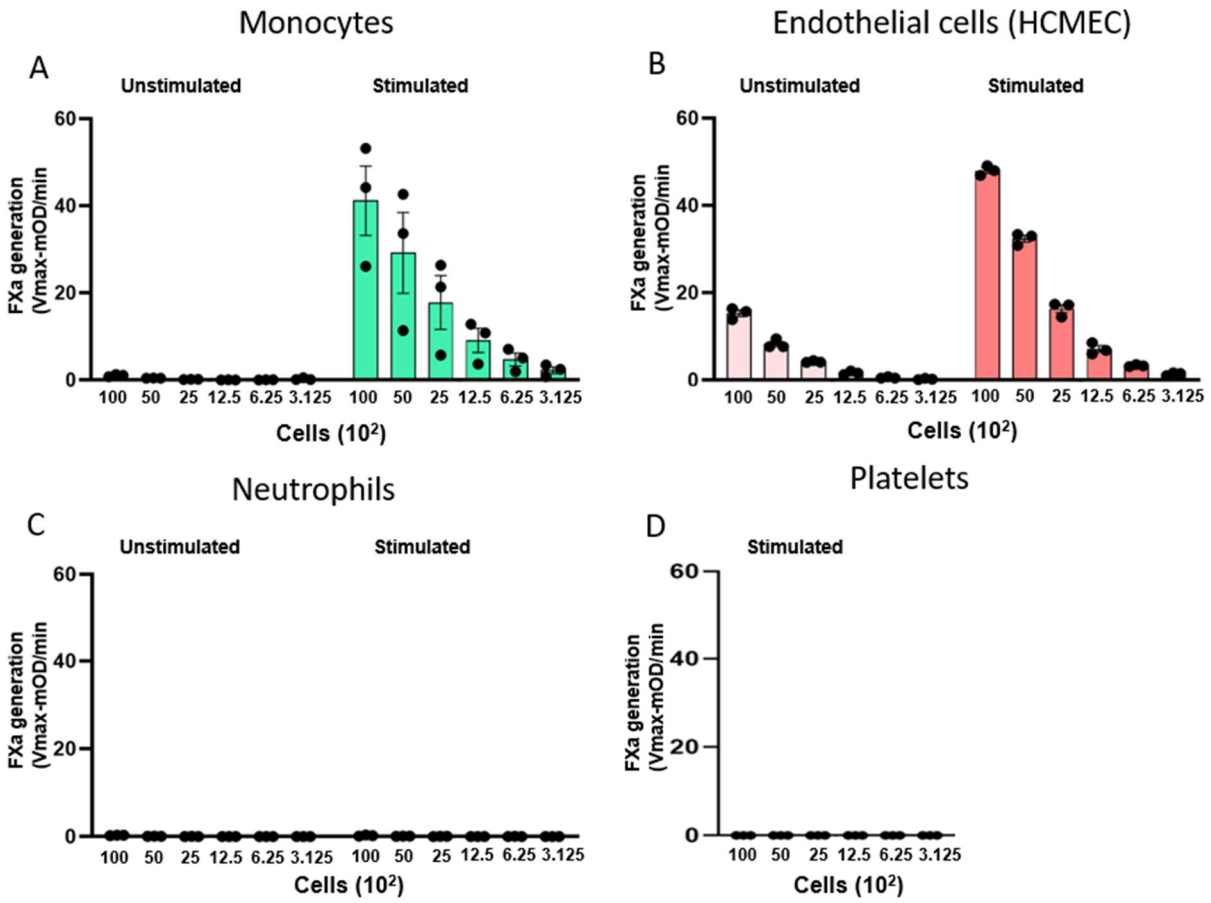
Supplemental Figure 1. Purity analysis of EV subsets performed by flow cytometry. Representative FCM graphs for (A) CD15+/AnnV+ NDEVs, (B) CD18+ and/or AnnV+ MDEVs, (C) CD41+/AnnV+ PDEVs and (D) CD146+ and/or AnnV+ EDEVs. The cross-contamination level from other types of EVs in each purified EV batch was less than 5%.

Size distribution of EVs analyzed by qNano (Supplemental Figure 2)



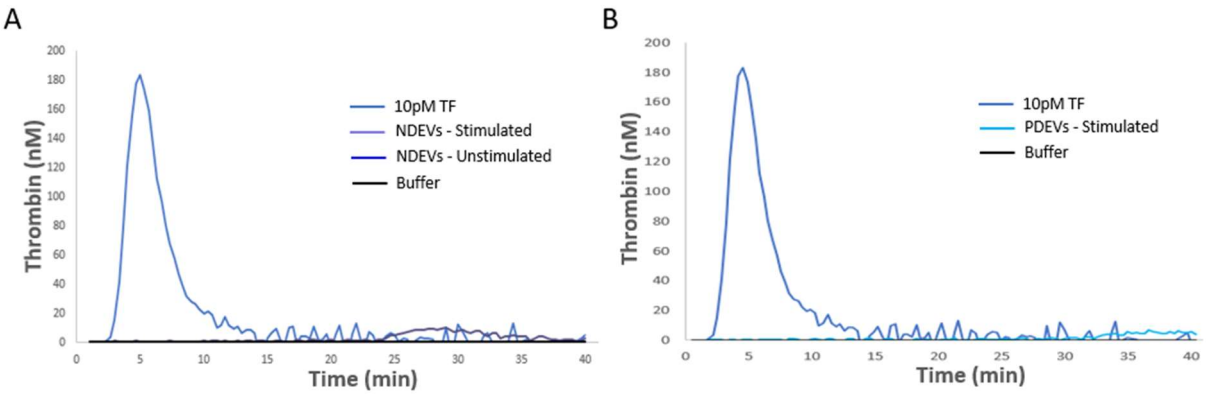
Supplemental Figure 2. Size distribution of EV subsets analyzed by tunable resistive pulse sensing (TRPS). A) NDEVs, (B) MDEVs, (C) EDEVs, and (D) PDEVs.

FXa generation by cells (Supplemental Figure 3)



Supplemental Figure 3. Monocytes and endothelial cells support FXa generation. Histograms representing FXa generation by (A) monocytes, (B) endothelial cells (HCMECs), (C) neutrophils, and (D) platelets were measured using a chromogenic assay. Buffer was used as the negative control and the data shown is subtracted from the buffer value. Data show mean ± SEM of 3 independent experiments.

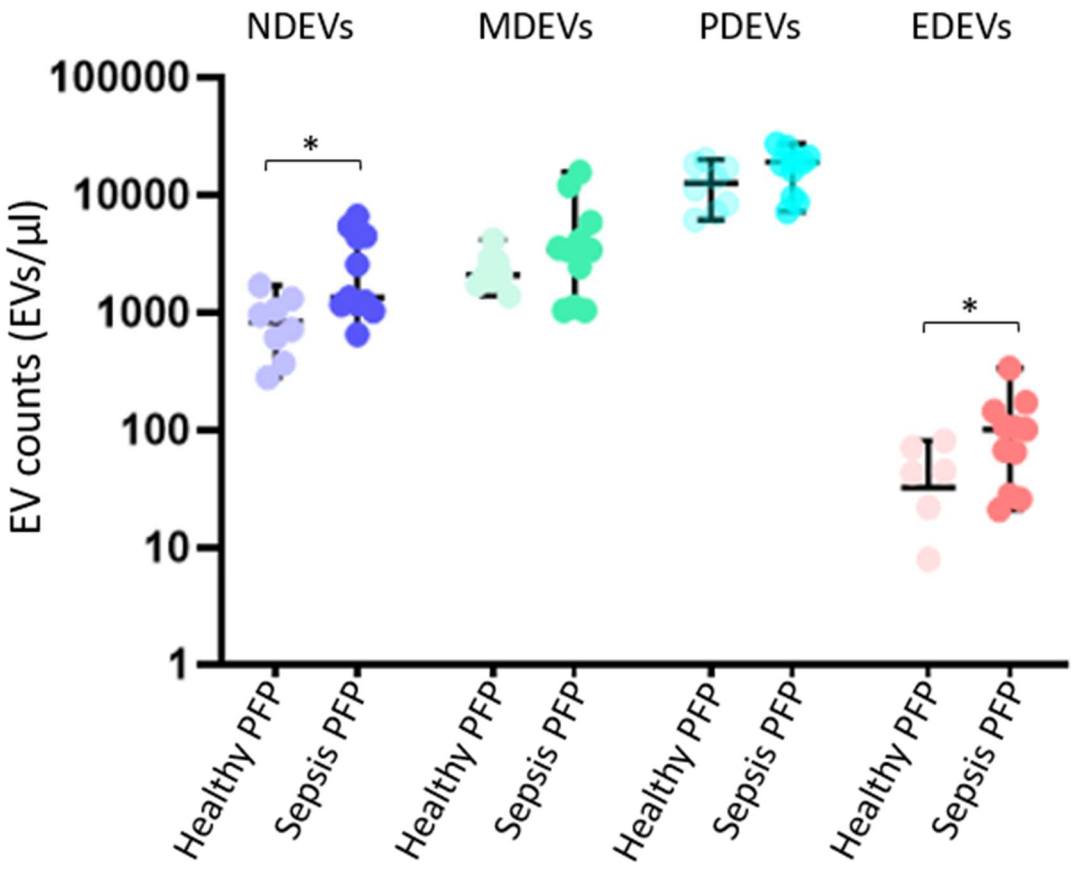
Thrombin generation by NDEVs, and PDEVs (Supplemental Figure 4)



Supplemental Figure 4. NDEV and PDEV-dependent thrombin generation was determined using CAT. TG experiments were performed by spiking $16x10^3$EVs/µl of (A) NDEVs and (B) PDEVs in re-calcified EV-depleted plasma in the presence of a control antibody or anti-human tissue factor (SBTF-1 Ab). Innovin (10 pM) and PL

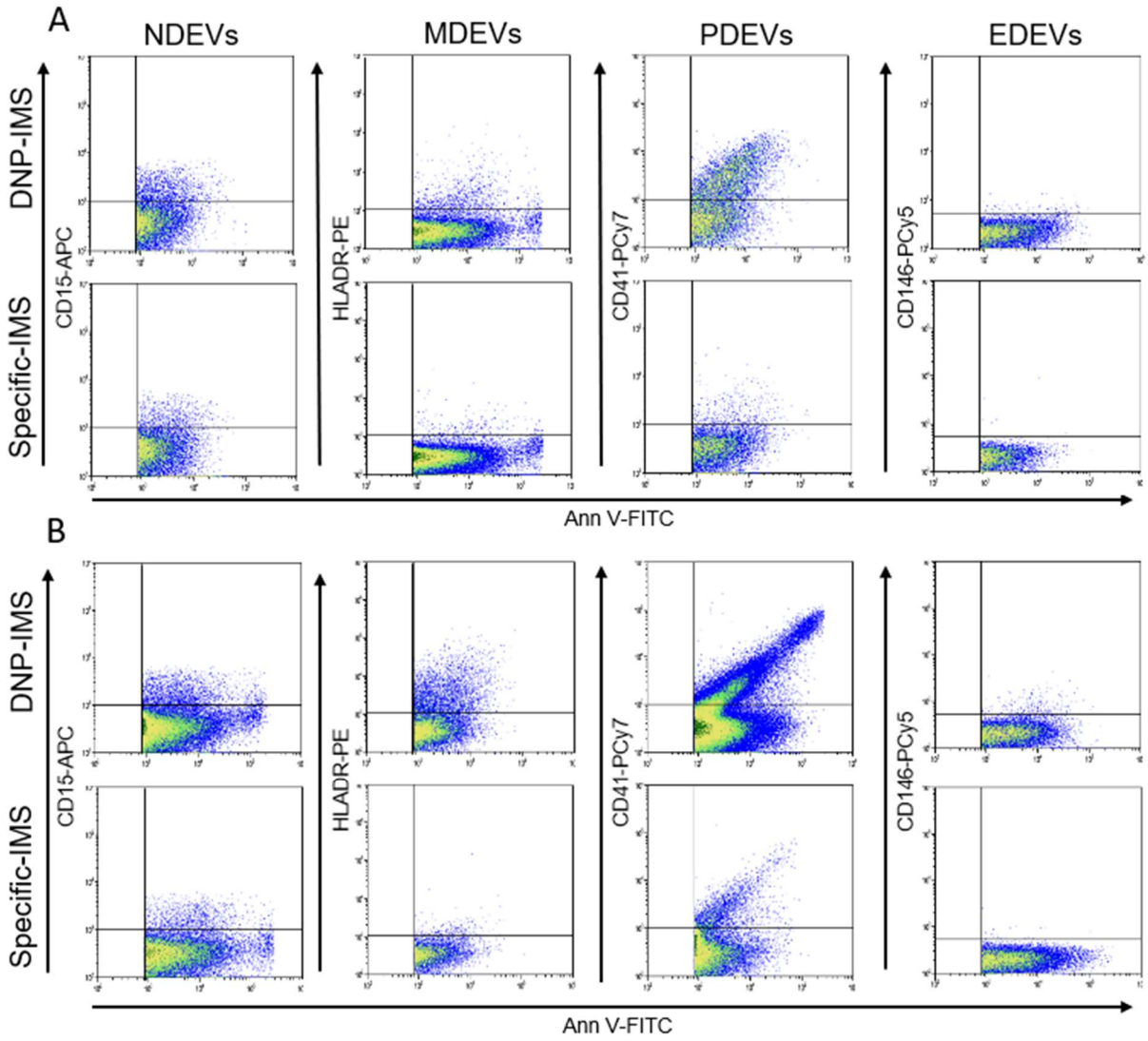
(μM) were used as the positive control and EV-depleted plasma mixed with buffer was used as the negative control.

Circulating levels of EVs in sepsis patients and healthy controls (Supplemental Figure 5)



Supplemental Figure 5. Levels of EVs in sepsis patients and healthy controls. Counts of NDEVs, MDEVs, PDEVs, and EDEVs in healthy control PFP (n = 8) and sepsis PFP (n = 11) were analyzed by flow cytometry. Data show mean ± SEM.

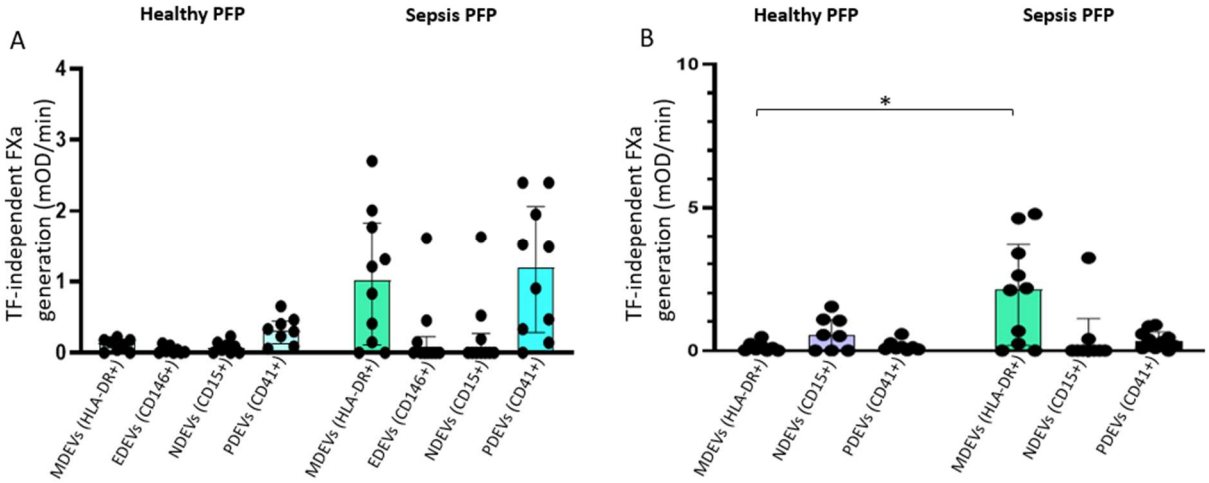
IMS depletion of EVs by DNP-IMS and CD29-59 IMS (Supplemental Figure 6)



Supplemental Figure 6. IMS efficiently captures specific circulating EVs from plasma. Representative graphs of flow cytometric detection of residual NDEVs (CD15+/AnnV+), MDEVs (HLA-DR+/AnnV+), PDEVs (CD41+/ AnnV+), and EDEVs (CD146+/ AnnV+) post-IMS depletion in plasma of (A) healthy individuals and (B)

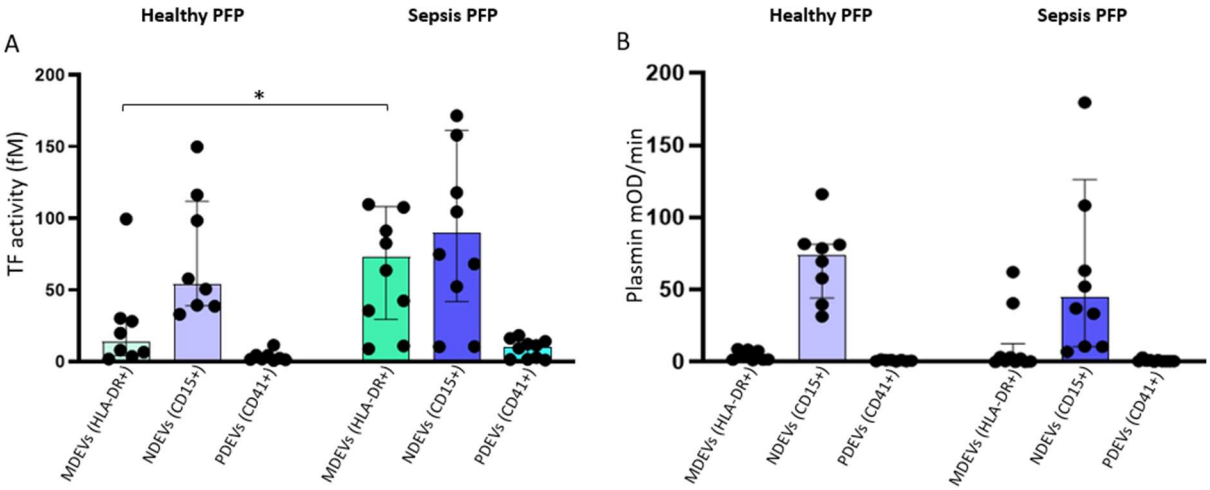
sepsis patients. 1. DNP-IMS; and 2. Specific-IMS (CD15-IMS, HLA-DR IMS, CD41-IMS, and CD146-IMS). All analyses were performed on CytoFlex LX.

TF-independent FXa generation of EVs (Supplemental Figure 7)



Supplemental Figure 7: (A) The TF-independent procoagulant activity was calculated from the remaining FXa generation after TF inhibition and removal of the FXa background noise (FXa, DNP control beads). (B) TF-independent procoagulant activity was normalized in each subset of EVs according to the concentration measured by the flow cytometry.

TF-dependent FXa and plasmin generation of EVs (Supplemental Figure 8)



Supplemental Figure 8: TF-dependent procoagulant and profibrinolytic activity was normalized according to the concentration of each subset. The results were expressed for the same concentration of the EVs *in vitro* (A) FXa and (B) plasmin generation ($0.5x10^6$ EVs).

Supplemental Table 1:

	Healthy Donors (n = 8)	Sepsis Patients (n =10)	p-value
Sex (male): n, (%)	5 (62.5)	7 (70)	NS
Age (years, SD)	49 ± 8	59 ± 15	NS
Fibrinogen (g/L)	-	7.1 [4.4-9.2]	-
DDI (µg/mL)	-	3 [2.25-7.03]	-
Fibrin monomer(mg/L)	-	5 [5-37.85]	-
Lactate (mmol/L)	-	1.9 [1.55-2.37]	-
Troponin (ng/mL)	-	0.01 [1.55-2.37]	-
Germs: n, (%) **Gram-negative bacilli** **Gram-positive cocci** **Other**	-	 3 (30) 4 (40) 3 (30)	

Sepsis patient characteristics: Clinical and biological parameters of septic shock patients upon patient admission (Day 1): total population (n=10). Data are n (%), mean SD, or median [25th to 75th percentile]. P value ≤0.05 was considered significant.

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Article 2

Rheopheresis induces activation of neutrophils and generation of extracellular vesicles with fibrinolytic potential

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Abstract

Background: Extracellular vesicles (EVs) are lipid bilayer-enclosed nano-particles generated by cells in association with various stimuli or pathophysiological conditions. Rheopheresis is a double-filtration plasmapheresis technique administered to eliminate high-molecular-weight proteins or inflammatory mediators to improve hemorheology in patients with severe microvascular ischemic complications. Biocompatibility issues associated with the biopolymer filters used in rheopheresis may induce the activation of blood cells and generate EVs. Aim: Investigate whether biopolymer filters used in the rheopheresis trigger cell activation and generation of EVs with fibrinolytic potential.

Methods: Plasma levels of different subsets of EVs in patients pre- and post-dialysis and rheopheresis sessions were analyzed using highly sensitive flow cytometry. The procoagulant and/or profibrinolytic potential of EVs in plasma was evaluated using EV-dependent TF assay and plasmin generation assay. Additionally, the biopolymer filter membrane-induced neutrophil activation and generation of neutrophil-derived EVs (NDEVs) with plasmin generation capacity were analyzed.

Results: A total of thirteen patients hemodialysis patients who received rheopheresis treatment and a control group of ten hemodialysis patients who did not receive rheopheresis sessions were analyzed. A significantly elevated level of NDEVs that was associated with high plasmin generation was observed in patients who received rheopheresis. Plasmapheresis biopolymer filter membranes induced neutrophil activation with significant CD11b expression ($p = 0.05$) and increased release of NDEVs ($p = 0.03$). Conclusion: This result

indicates that biopolymer filter membranes induce neutrophil activation and generate EVs with high fibrinolytic potential.

Introduction

Apheresis (Heamapheresis) is an extracorporeal medical intervention commonly employed in clinical practice that allows the separation of plasma to remove/collect certain components from the plasma to treat critically ill patients. Activation of blood cells has been observed in patients who undergo various apheresis procedures including rheopheresis. Rheopheresis is an extracorporeal apheresis procedure used for therapeutic purposes in clinical settings to eliminate a specific spectrum of high molecular weight proteins from the blood such as fibrinogen, α-2-macroglobulins, LDL cholesterol, lipoproteins, immunoglobulins, CRP, von Willebrand factor, and proinflammatory cytokines thus significantly improves hemorheology, tissue microcirculation, and mitigate inflammatory complications in several chronic thromboinflammatory conditions. Rheopheresis is a recommended treatment choice for diseases that affect microcirculation therefore, exceptionally effective in age-related macular degeneration, peripheral arterial disease, calciphylaxis, hearing loss, and critical limb ischemia (Klingel et al., 2000; Padmanabhan et al., 2019; Robert et al., 2020; Solignac et al., 2022).

While apheresis techniques including rheopheresis may provide critical treatment benefits to patients, the biocompatibility of the filter membranes used during the procedure seems to be an important issue that may cause blood cell activation. The most biological responses elicited during blood-membrane interactions are the adsorption of plasma proteins to the membrane and the activation of platelets and leukocytes. Although these biological responses with regard to hemodialysis (HD) membranes have been reported extensively (Bonomini et al., 2022; Bowry et al., 2021; Kohlová et al., 2019), biocompatibility studies on plasmapheresis membranes are inadequate (Bouhler et al., 1991; Krieter et al., 2021). Platelets and immune cells respond to different types of stimuli and direct contact with biomaterials may cause the activation and adhesion of these cells, triggering increased coagulation-inflammation cross-talk and release of extracellular vesicles (EVs). We have demonstrated that under an inflammatory milieu and in sepsis patients, blood cells and endothelial cells release EVs with distinct procoagulant and/or profibrinolytic potential. Interestingly, the high profibrinolytic potential of neutrophil EVs may help to counteract the deleterious effect of major procoagulant activity of monocyte EVs in sepsis patients. In the present study, we wanted to examine the

impact of biopolymer filter membranes used in patients who underwent rheopheresis sessions on the release of EVs with procoagulant and/or profibrinolytic potential.

The purpose of this study was to investigate the effect of biopolymer filter membranes on the activation of neutrophils and the release of EVs with fibrinolytic potential.

Material and Methods

Patients

Hemodialysis patients (n = 13) who underwent rheopheresis sessions for treating the peripheral arterial disease-induced chronic limb-threatening ischemia (PAD-CLTI) and calciphylaxis at the dialysis center of the Hospital of La Conception (Marseille, France) were included in the study. Additionally, hemodialysis patients not treated with the rheopheresis session (n = 10) for peripheral artery disease also were included in the study.

Rheopheresis Treatment

Rheopheresis sessions were performed on patients using an automated machine (Plasauto Σ marketed by Hema T Medical, Ramonville-Saint-Agne, France) manufactured by Asahi Medical Co, Ltd. (Tokyo, Japan) in the presence of anticoagulation with either citrate or heparin (depending on the prescription). Rheopheresis consists of two tandem-placed hollow fiber filters, the first one for plasma separation (plasmapheresis; Plasmaflo OP-05W[L]) from blood cells, and the separated plasma is sent to the second filter (Rheofilter ER-4000) to remove the specific spectrum of high molecular weight proteins from the plasma (Figure 1). Patients received rheopheresis sessions in parallel with the dialysis (the duration of each session lasted approximately 1-2h depending on the plasma volume target of between 40-60 mL/kg) with anticoagulated blood pumped into tandem placed dialysis and rheopheresis membranes.

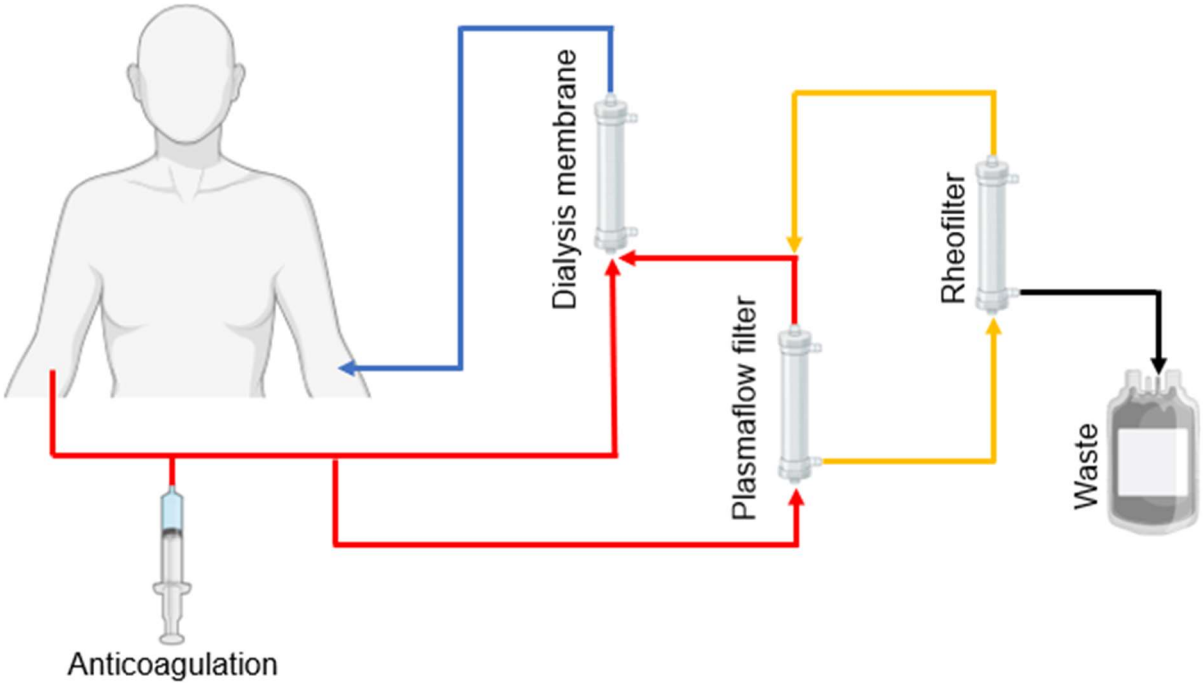

Figure 2. Schematic representation of rheopheresis session in tandem with dialysis. Blood from the vascular access is pumped to the dialysis membrane and the plasmapheresis filter following the anticoagulation. Plasma is then pumped to the rheofilter to remove specific molecular cut-off proteins.

Treatment purified neutrophils with small pieces of plasmapheresis membranes

Since blood cells come in contact only with the Plasmaflo filter membrane, we used only plasmapheresis membranes to investigate the contact activation of neutrophils. Intact plasmapheresis membranes (stored in saline water at 4°C; approximately 23 cm) were split open lengthwise to expose the lumen and cut into small pieces (approximately 1-2mm in length) (Koga et al., 2018). Cut pieces of Plasmapheresis membranes (equivalent to two full-length membranes) were washed twice with PBS and added to 4x10^6 neutrophils suspended in 400µl of EV-depleted plasma. Incubated neutrophils (4x10^6 cells) in EV-depleted plasma with or without plasmapheresis membrane for 1hr at 37°C. Mixed neutrophils gently by pipetting (including the tubes without membranes) every 10 min to ensure proper mixing with the membranes.

Flow cytometry analysis of EVs and neutrophil activation

For the quantification of EVs in patient PFP following dialysis and rheopheresis sessions, we used the following primary antibodies: anti-CD15-PE (IM1954U, Beckman Coulter, France) for neutrophils-derived EVs (NDEVs), anti-HLA-DR-PE for monocytes-derived EVs

(MDEVs), anti-CD41-PCy7 (6607115, Beckman Coulter) for platelet-derived EVs (PDEVs), anti-235a-BV660 for erythrocyte derived EVs (EryEVs), and anti-CD146-PE (A07483, Beckman Coulter) for endothelial cells-derived EVs (EDEVs). Briefly, 10µl of PFP was incubated with 0.7µl of specified antibody and 4µl of AnnV in the dark at RT for 30 minutes. After the incubation, samples were diluted with 250µl of AnnV binding buffer and 10µl of MP Count Beads (Biocytex, Marseille, France) for counting in absolute values. NDEVs, MDEVs, PDEVs, EryEVs, and EDEVs were defined as CD15+/AnnV+, HLA-DR+/AnnV+, CD41+/AnnV+, CD235a+/AnnV+, and CD146+/AnnV+ events, respectively.

All FCM data were analyzed either with Kaluza Analysis 2.1 software or CytExpert 2.3 software.

To check the activation status of the neutrophils, purified neutrophils treated with/without plasmapheresis membranes were labeled with 8µl of CD11b-PE or isotype-matched control antibodies by incubating at RT for 20min. Following the incubation, neutrophils were analyzed with Cytoflex flow cytometer (Beckman Coulter) by gating in forward scatter vs side scatter (FSC/SSC) density plot. The mean fluorescent intensity (MFI) of labeled neutrophils (5000 cells in total events) was analyzed.

NDEVs were pelleted by ultracentrifugation at 100,000g after removing cell debris and apoptotic bodies by centrifugation once at 2500g. Pelleted NDEVs were stained with 2.5 µl of CD15-PE, isotype-matched control antibodies, and 4µl of Annexin V (Ann V) by incubating at RT for 30min. After the incubation, samples were diluted with 250µl of AnnV binding buffer. Following the incubation, NDEVs were analyzed with Cytoflex flow cytometer, and NDEVs were defined as CD15+/AnnV+ events.

All FCM data were analyzed either with Kaluza Analysis 2.1 software.

Evaluation of plasmin generation capacity of NDEVs

NDEVs were pelleted by centrifuging the plasma at 100,000g for 90min and resuspended EVs in 30µl HEPES buffer. 100µl plasminogen mix (50% of 2µM Plasminogen with PBS-BSA-Azide buffer and 5mM final concentration of EACA) containing $3x10^6$ NDEVs (in duplicate) incubated for 24hrs in the 96-well flat bottom plate. Plasminogen mix without EVs was used as control. Following the incubation, 80µl of the selective chromogenic substrate of plasmin (methyl-malonyl)-hydroxypropylarginine-p-nitroaniline (CBS0065: Stago, Asnière, France) at 0.75 mM was added to the EV mix. Buffer and EV supernatant from the last washing of EVs

were used as a negative control. Plasmin generation activity was recorded by taking the chromogenic reading for 120 minutes at 405nm wavelength of absorption at 37°C and the activity was expressed in mOD/min.

Results

Study population

The main clinical and biological parameters of the patients who underwent hemodialysis with/without rheopheresis sessions have been summarized in Table 1.

Table 1: Main clinical and biological characteristics of patients

	HD Group with Rheopheresis (N = 13)	HD Group without Rheopheresis (N = 10)
Age (years old)	68 (59–71)	78.5 (73.5–80.5)
Male gender	8 (62%)	8 (80%)
HD Vintage (Months)	33 (18–39)	20 (16–35)
BMI (kg/m^2)	26.0 (23.2–29.5)	30.1 (27-71)
Diabetes	8 (62%)	8 (80%)
Hypertension	13 (100 %)	8 (80%)
Dyslipidemia	11 (85%)	6 (60%)
Smoking history	7 (54%)	7 (60%)
Coronary disease	7 (54%)	6 (60%)
Ejection fraction (%)	65 (53.7–65)	50 (50-62.5)
Peripheral occlusive arterial disease	9 (69%)	9 (90%)
Stroke history	0	1 (10%)
Arteriovenous fistula	9 (69%)	6 (60%)
Indication of rheopheresis: PAD-CLTI Calciphylaxis	 8 5	

Number of sessions	18 (12–30)	
Albumin (g/L)	31.9 (28.4–35.2)	35.8 (33.2-39.5)

Extracellular vesicle-dependent tissue factor and plasmin generation activity in patients pre-and post rheopheresis and dialysis sessions

To examine the effect of hemofiltration membranes on the different subsets of EV quantity, we quantified the different subsets of EVs in the plasma of patients following dialysis sessions and rheopheresis sessions using flow cytometry. We observed a significant increase in the levels of NDEVs and MDEVs in the plasma following rheopheresis sessions (Figure 2A&B). However, there was no significant difference in counts of PDEVs, EryEVs, and EDEVs (Figure 2C, D,&E). In contrast to the rheopheresis, there was no significant difference in the quantity of all the subsets of EVs analyzed.

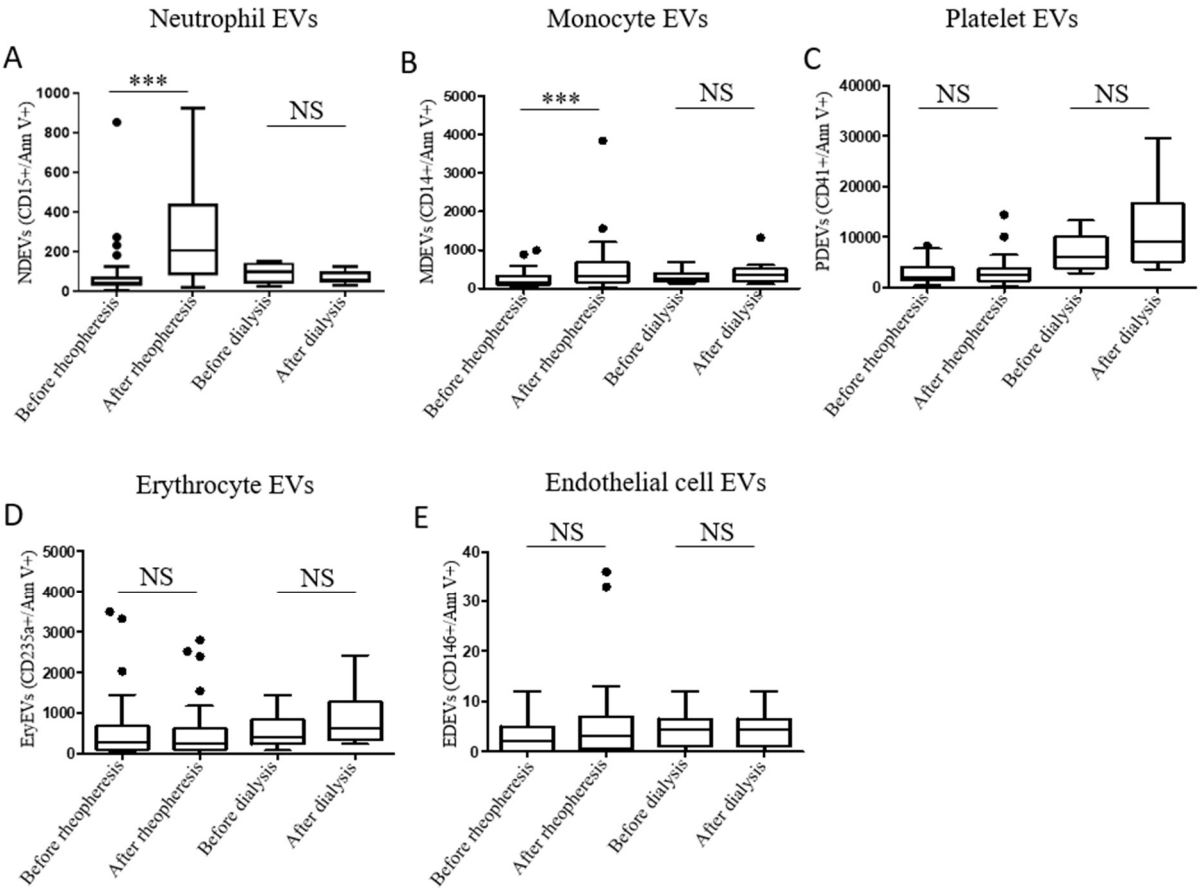

Figure 2. Plasma levels of NDEVs (CD15+/AnnV+), MDEVs (HLA-DR+/AnnV+), PDEVs (CD41+/ AnnV+), EryEVs (CD235a+/Ann V+), and EDEVs (CD146+/ AnnV+) in patients pre-and post rheopheresis and dialysis sessions.

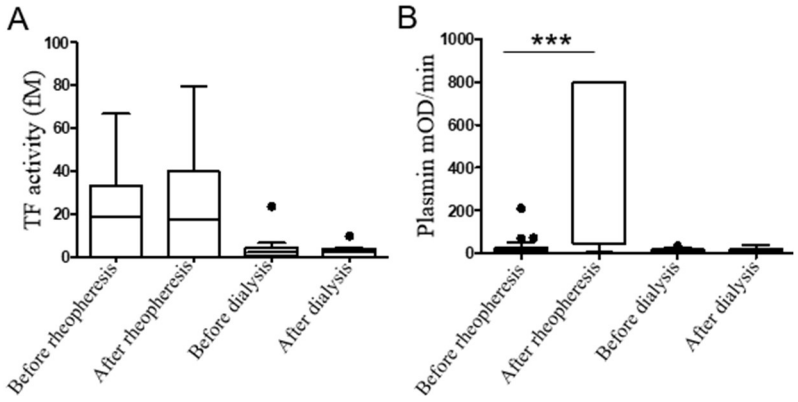

Figure 3. TF and plasmin generation activity of EVs of neutrophil origin following rheopheresis and dialysis sessions in chronic kidney patients. (A) TF activity of NDEVs in the plasma (B) Plasmin generation potential of NDEVs in plasma. NDEV-dependent plasmin generation significantly increased following the rheopheresis session but not after dialysis.

Next, we measured the procoagulant and profibrinolytic potential of different subsets of EVs. As illustrated in Figure 3, EV-dependent TF and plasmin generation activity was observed in the patient plasma. Although there was no major difference in the TF activity pre- and post-rheopheresis sessions, a significant increase in the EV-dependent plasmin generation activity was observed. Moreover, plasmin generation activity was exclusively associated with NDEVs, not other EV subsets. In contrast, there was no TF and plasmin generation activity observed in patient pre- and post-dialysis sessions.

Activated neutrophils express CD11b and release EVs

Next, we investigated the impact of plasmapheresis membranes on neutrophil activation and microvesicle release using purified neutrophils. Neutrophils isolated from the whole blood were incubated in EV-free plasma for 1hr in the presence or absence of small cut pieces of plasmapheresis membranes. Surface expression of CD11b on neutrophils was measured as an indicator of the activation of cells. As illustrated in Figure 4, incubation of neutrophils with the plasmapheresis membranes significantly increased the cell surface expression of CD11b (Fig. 2B,C), an indication of cell activation. Next, we quantified the NDEVs present in the EV-free plasma after removing the plasmapheresis membranes, neutrophils, and apoptotic bodies. Flow cytometry analysis detected the presence of CD15+/Ann V+ NDEVs with significantly increased quantity (Fig. 2D,E) following the treatment of neutrophils with membranes.

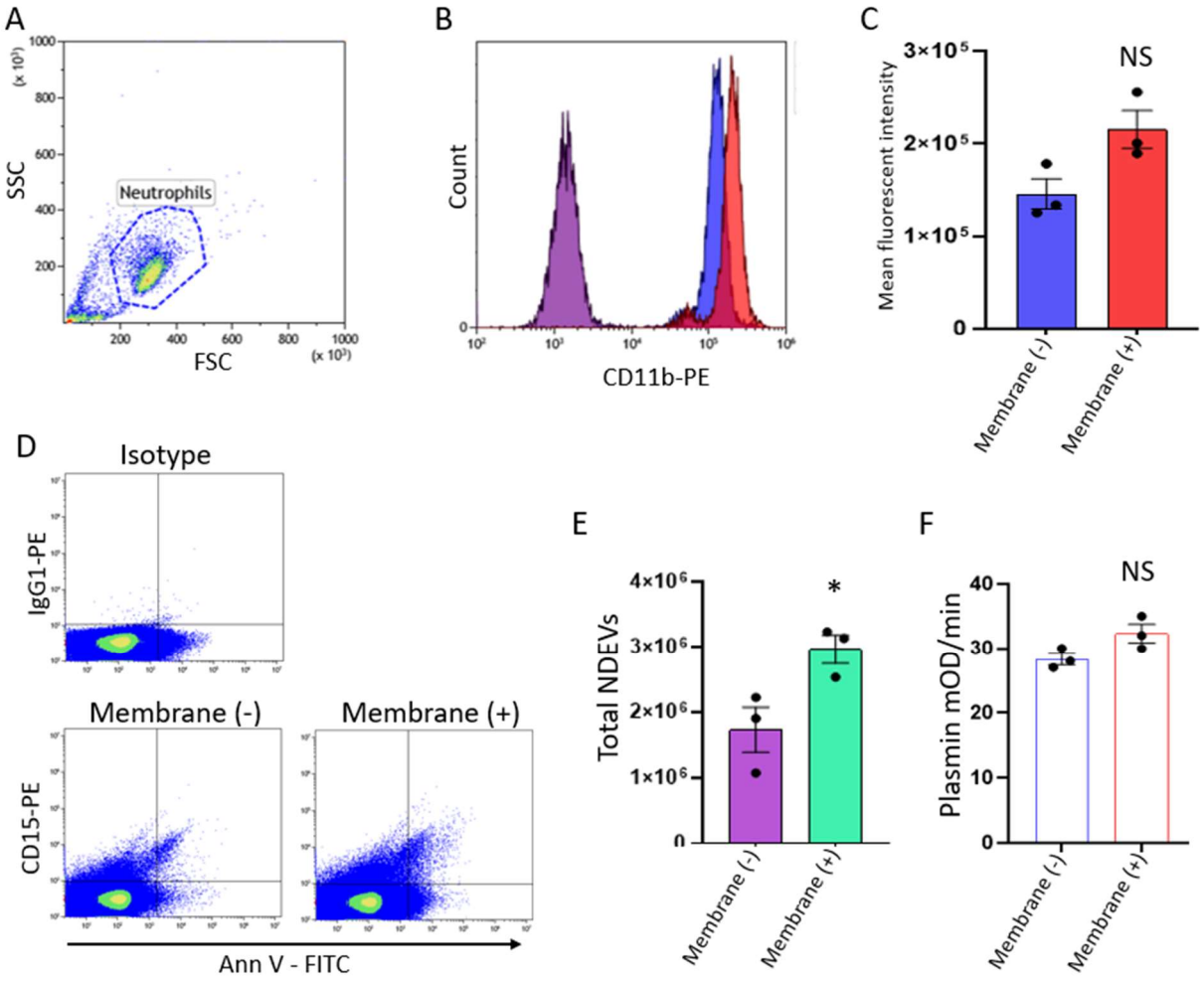

*Figure 4. Activation of neutrophils and release of NDEVs induced by plasmapheresis membrane (polyethylene hollow filter membrane coated with ethylene vinyl alcohol copolymer) in normal pooled EV-free plasma. Neutrophils isolated from whole blood incubated for 1hr in EV-depleted plasma with or without small cut pieces of plasmapheresis membrane. Neutrophil activation and EV quantification were performed with flow cytometry (CytoFlex LX). (A) Neutrophils were gated in FSC/SSC density plot. (B) A representative flow cytometry histogram (n=3) showing neutrophil activation with CD11b expression. Purple peak, neutrophils with isotype control antibody; Blue peak, neutrophils without plasmapheresis membrane; Red peak, neutrophils with plasmapheresis membrane. (C) CD11b expression on neutrophils (p = 0.05). (D) Representative flow cytometry density plot of neutrophil-derived EVs (NDEVs). NDEVs with isotype control antibody; CD15+ NDEVs from neutrophils incubated in EV-free plasma with and without plasmapheresis membrane. (E) Total NDEVs quantified by flow cytometry. (F) Plasmin generation capacity (PGC) of NDEVs (0.5.10^6 EVs) from neutrophils incubated in EV-depleted plasma with and without plasmapheresis membrane. Data show mean (±SEM, n=3) of 3 independent biological replicates. Flow cytometry data was analyzed with Kaluza 2.1 software. *NS, not significant.*

Finally, we evaluated the plasmin generation activity of NDEVs. NDEVs from plasmapheresis membrane-treated neutrophils had slightly increased plasmin generation activity (Figure 4F), however, no significant difference was observed as compared to EVs from untreated cells. Taken together, these data suggest that the plasmapheresis membrane activates neutrophils and releases a significant quantity of EVs with high fibrinolytic potential. Altogether, these data

indicate that biopolymer filter membranes used in the rheopheresis treatment can induce neutrophil activation and generate EVs with high fibrinolytic activity.

Discussion

The therapeutic efficacy of biopolymers in clinical practice for extracorporeal treatment is determined by biocompatibility, where filter membranes may trigger biochemical reactions upon contact with blood components. To our knowledge, this is the first study to look into the impact of biomaterial filter membranes used in extracorporeal therapies on neutrophil activation and the release of NDEVs with fibrinolytic potential. Here, we demonstrated a significant increase in the NDEVs and markedly increased plasmin generation that is dependent on NDEVs in patients following rheopheresis treatment. We also demonstrated *in vitro* activation of neutrophils upon contact with plasmapheresis biopolymer filter membranes and the release of NDEVs with plasmin generation capacity.

The properties of the synthetic polymers and their surface modifications used in the fabrication of membrane filters may elicit some adverse biochemical reactions upon contact with blood components during the apheresis procedure thus affecting the biocompatibility of the membranes (Bacal et al., 2021). Over the past few decades, several synthetic polymers with or without surface modifications have been developed with the objective to significantly reduce adverse biochemical reactions. Although modern synthetic polymers and surface modifications have considerably improved biocompatibility, complete prevention of undesirable biological responses to membranes is still elusive. The most used materials in membrane-based apheresis include cellulose diacetate, polysulfone, polypropylene, polyvinylalcohol, ethylene-vinyl alcohol, and polymethyl methacrylate (PMMA) (Bacal et al., 2021). The most biological responses elicited during blood-membrane interactions are the adsorption of plasma proteins to the membrane and the activation of platelets and leukocytes.

While our preliminary data do indicate that plasmapheresis hollow membranes fabricated of polyethylene (and the inner wall of the membrane tube coated with ethylene vinyl alcohol)) induce activation of neutrophils and EV secretion, this research needs to be extended further with mechanistic studies to investigate the possible mechanism behind neutrophil activation and increased EV secretion in the context of apheresis procedures. Bio(haemo)compatibility issues and surface characteristics of the polymers utilized in the fabrication of filter membranes have been known to elicit the activation of blood cells at varying degrees during extracorporeal

treatment procedures (Belloni et al., 1993; Jørstad, 1987). Direct interaction of blood components to the biopolymer membranes leads to the activation of platelets and leukocytes thus subsequently leading to thromboinflammatory response (Bonomini et al., 2022).

The principal trigger for the activation of platelets and leukocytes is the biofouling effect caused by the adsorbed fibrinogen, fibronectin, and complement components with altered structural properties (Gorbet et al., 2019; Kizhakkedathu & Conway, 2022). A few studies have demonstrated the activation of blood cells including neutrophils and generation of EVs upon interaction with biopolymer filter membranes used for dialysis as well as apheresis procedures (Daniel et al., 2006; Itoh et al., 2006; Weiss et al., 2017). The activation of leukocytes may trigger coagulation and the release of EVs. We found significantly elevated levels of NDEVs and MDEVs in the plasma of patients following the rheopheresis session, suggesting the activation of their parent cells and EV generation. A profound change in the plasmin generation activity following the rheopheresis session may have been caused by the neutrophil activation and EVs generated during the rheopheresis procedure in response to the activation of other leukocytes and coagulation pathways. As to the plasmin generation capacity, there were no major differences in the plasmin generation capacity of NDEVs from neutrophils treated or untreated with plasmapheresis membranes. This suggests changes in the plasmin generation observed following the rheopheresis procedure are largely influenced by the significant increase in the NDEVs as demonstrated *in vitro* results and may not be necessarily associated with an increase in NDEV-dependent fibrinolytic potential. However, it is important to demonstrate the activation of neutrophils in patients following the rheopheresis session to further validate the in vitro results obtained. These results are in line with our previous research findings that according to the stimuli or pathophysiological context, different cells generate EVs with distinct procoagulant and/or profibrinolytic potential thus influencing the EV-coagulolytic balance (Cointe et al., 2022; Lacroix et al., 2007, 2012).

In conclusion, we demonstrated for the first time that biopolymer membrane filters used in the rheopheresis procedure induce neutrophil activation and generate a significant quantity of EVs with fibrinolytic potential. However, further studies are warranted to understand the cellular/molecular mechanism behind the neutrophil activation and generation of EVs induced by these membrane filters.

Author contributions

Akhil Konkoth: Conceptualization; methodology; investigation; data curation; formal analysis; writing-original draft; review & editing, **Evelyne Abdili:** methodology; investigation, **Françoise Dignat-George:** Funding acquisition; resources; review & editing. **Romaric Lacroix:** Funding acquisition; project administration; resources; formal supervision; review & editing.

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PART III: DISCUSSION AND PERSPECTIVES

Although previous studies have reported procoagulant or profibrinolytic potential of all the subpopulations of MVs in the context of thrombo-inflammatory conditions like sepsis, none of these studies have attempted to investigate hemostatic functional heterogeneity that may be contributed by the individual subpopulation of MVs. The general objectives of this study were 1) to delineate the impact of cellular sources and stimuli on the hemostatic functional properties of different subsets of MVs, 2) to understand the impact of different subsets of MVs on hemostatic imbalance associated with sepsis to develop MVs as potential biomarkers 3) to identify the potential targets of MVs as therapeutic agents in thrombotic conditions.

We isolated *in vitro* generated MVs from human neutrophils, monocytes, platelets, and endothelial cells and used a novel immuno-magnetic separation strategy to capture circulating MV subsets from the plasma of healthy individuals and sepsis patients. A comprehensive analysis of these MVs demonstrated distinct hemostatic functional signatures of procoagulant and/or profibrinolytic potential i.e., coagulolytic balance according to the cellular origin and pathological thromboinflammatory context.

The initial concept of MVs' role in hemostasis as procoagulant entities has evolved with the discovery of MVs with potential fibrinolytic properties. The opposing distinct functional effect of MVs on hemostasis depends on a balance between the procoagulant and profibrinolytic properties of the MVs which we can refer to “coagulolytic balance”. The procoagulant and profibrinolytic properties that maintain the hemostatic equilibrium can be lost in thrombo-inflammatory conditions such as sepsis[330]. Several documented pieces of evidence show significantly elevated levels of MVs in sepsis that correspond with the activation of cells in an inflammatory environment[331, 332, 333, 334]. Depending on the functional activity, these MVs can exert either a deleterious[335, 336] or a protective effect in sepsis[337, 338, 339, 340]. This suggests that distinct hemostatic determinants could therefore tune MV-dependent coagulolytic balance and therefore influence coagulopathy associated with sepsis and with other diseases associated with thrombo-inflammation where the well-regulated procoagulant and profibrinolytic EV balance is lost.

In this regard, the major work of my thesis was to perform a comprehensive analysis of the procoagulant and/or profibrinolytic potential of different subsets of MVs generated in vitro and purified directly from the plasma of healthy individuals and sepsis patients. A second and

smaller part of this thesis was dedicated to investigating the generation of NDMVs with fibrinolytic activity in patients who underwent therapeutic rheopheresis session for peripheral arterial disease and calciphylaxis.

1) Analysis of procoagulant and/or profibrinolytic potential of different subsets of MVs to evaluate MV-CLB as a mediator in sepsis pathophysiology and a potential biomarker.

This work focused on evaluating the procoagulant or profibrinolytic potential of major circulating subpopulations of MVs in the blood in the context of inflammatory milieu/pathology thus defining the MV-coagulolytic balance (MV-CLB). These opposing procoagulant and profibrinolytic functional properties of MVs in the hemostatic context are principally modulated by the TF and uPA/uPAR systems respectively. The TF expressed on the cell surface or MVs forms a complex with the activated FVII (FVIIa) catalyzing the generation of FXa and the subsequent thrombin generation is critical for the thrombus formation. On the other hand, the generation of plasmin from the plasminogen catalyzed by the uPA/uPAR complex is essential for the lysis of the thrombus.

In this study, we demonstrated that while MDMVs and EDMVs from stimulated cells displayed significant procoagulant activity, irrespective of the activation status of the cells, NDMVs, MDMVs, and EDMVs had high fibrinolytic capacity. In parallel, with the help of a novel IMS strategy, we could efficiently purify major circulating subsets of MVs in the plasma and identified MDMVs as the major source of procoagulant activity and NDMVs as the principal source of fibrinolytic activity in the sepsis plasma. Accordingly, different subsets of MVs convey distinct procoagulant and/or profibrinolytic potential, thus demonstrating distinct CLB according to their parental cell and the stimuli/pathology. MV-CLB may represent a potential biomarker reflecting coagulopathy in sepsis pathology.

Since pathological activation of procoagulant pathways and dysfunctional fibrinolytic pathways play an important role in the development of sepsis coagulopathy, it is important to understand the cellular source of the procoagulant and profibrinolytic activity. In our work, the use of a novel immunomagnetic separation strategy not only allowed us to isolate specific subsets of MVs but also made it possible to determine their procoagulant and/or profibrinolytic potential in sepsis patients. While the combination of SEC and ultrafiltration can be used efficiently to purify MVs from biological fluids with minimal contamination as compared to ultracentrifugation and precipitation methods, IMS seems to offer a great advantage over these

methods to purify specific subsets of MVs from a small volume of sample in a short time[341, 342, 208, 200]. Our study demonstrated that IMS in combination with highly sensitive TF and PGC assay could effectively purify cell-specific subsets of MVs to determine their procoagulant and/or profibrinolytic potential in sepsis patients.

In the context of sepsis, bacterial endotoxins and inflammatory cytokine response from immune cells induce an exacerbated inflammatory response thus establishing an extensive cross-talk between inflammation and coagulation pathways. Exacerbated innate immune response in sepsis significantly enhances the release of MVs with procoagulant properties into circulation which can potentiate coagulopathy associated with the disease[343, 344, 345, 331]. A similar hemostatic imbalance with elevated levels of procoagulant MVs has been observed in other thromboinflammatory diseases such as COVID-19[346, 347]. However, subsets of MVs with potential anti-inflammatory or fibrinolytic capacity may provide a beneficial effect on patients with thromboinflammatory diseases[337, 348].

As demonstrated in our results, different cell types under the inflammatory stimuli produce a heterogeneous population of MVs that convey distinct procoagulant and profibrinolytic properties. Host immune response during bacterial infection is critical for the containment of infectious pathogens to prevent their dissemination. In addition to the obvious role of monocytes/macrophages in warding off infectious agents, inflammatory response by the monocyte/macrophages through the release of proinflammatory mediators can trigger activation of the TF-dependent extrinsic pathway of coagulation. Activation of monocytes during the sepsis infection is highly relevant as the presence of LPS can upregulate the TF expression and enhance the secretion of TF-expressing MVs into the circulation thus constituting the predominant source of MV-TF activity[349, 350, 312]. Therefore, increased expression of TF on monocytes and elevated levels of TF-expressing MVs contribute to advancing systemic coagulopathy.

The use of highly sensitive TF that was developed in our lab and the thrombin generation assay was critical for the measurement of the procoagulant potential of MVs[351, 352]. We found that under inflammatory conditions, monocytes and endothelial cells and their MVs had profound FXa generation activity as compared to the unstimulated condition. MVs that express both TF and PS have significantly high procoagulant activity because TF is the principal initiator of the coagulation cascade and PS can further augment TF activity[353]. In the MV-TF experimental setting, pre-incubation of MVs with SBTF-1 anti-TF antibody and annexin V independently

and strongly blocked FXa generation thus confirming the functional significance of TF and phosphatidylserine in the procoagulant activity of MVs.

Similar to the monocyte activation and enhanced TF activity, endothelial dysfunction significantly contributes to the pathophysiology of sepsis coagulopathy. Secretion of proinflammatory cytokines such as TNF-α and IL-6 are known to induce endothelial dysfunction and induce a proinflammatory phenotype. Continuous activation of endothelial cells through proinflammatory mediators enhances procoagulant pathways, suppresses the anticoagulant and fibrinolytic pathways, and the release of procoagulant MVs[354]. Our results demonstrated that the stimulation with TNF-α not only increased FXa generation by the endothelial cells but also by the EDMVs.

Comparable to the in vitro results, ex vivo MV-TF activity indicates that MDMVs constitute the major source of procoagulant activity in the sepsis plasma[349, 312]. Moreover, although less represented compared to other major MV subsets in the circulation, we observed markedly elevated levels of EDMVs in sepsis plasma and some patients had increased MV-TF activity that is contributed by the EDMV[355, 306]. The increased TF expression and PS exposure by the monocytes, endothelial cells, and their MVs enhance their procoagulant properties and lead to increased thrombin generation. Our data demonstrated that both MDMVs and EDMVs from the stimulated cells had profound thrombin generation capacity. Elevated TF expression and thrombin generation are required for fibrin formation and increased clot stability. As indicated in our results, the clots produced highly procoagulant MVs from the stimulated monocytes and endothelial cells were highly resistant to clot lysis. Although we did not assess the clot formation and clot lysis characteristics directly in the sepsis plasma, some recent studies have demonstrated enhanced clot stability due to increased fibrin formation and prolonged clot lysis time in sepsis patients[356, 357, 358, 359, 360].

Increased expression of TF and thrombin generation are critical components of coagulopathy induced by the sepsis infection. Regardless of their important function in eliminating infectious pathogens, aberrant activation of coagulation factors like TF and thrombin during sepsis can trigger the activation of immune cells through their interaction with PAR receptors thus further amplifying procoagulant and proinflammatory pathways[361, 362, 363]. In addition to monocytes and endothelial cells, neutrophils and platelets also have been implicated in the aberrant inflammation-coagulation cross-talk despite it remains debatable as to whether TF is expressed or acquired by the neutrophils and platelets. The release of NETs from neutrophils is highly

procoagulant and further activates the coagulation cascade. Moreover, systemic activation of coagulation and inflammation triggers platelet activation and promotes platelet-mediated coagulation and inflammatory response.

Besides the obvious role of procoagulant components, dysfunctional fibrinolytic components have a pivotal role in sepsis coagulopathy [251]. The fibrinolytic system mediated by the tPA or uPA/uPAR complex is crucial for the plasmin generation and subsequent lysis and removal of clots. However, hyperactivation of procoagulant and proinflammatory pathways in sepsis DIC promotes impairment in the fibrinolytic system mediated by the upregulated expression of PAI-1, α-2 antiplasmin, and activated thrombin-activable fibrinolysis inhibitor (TAFIa)[364]. Moreover, the plasma levels of plasminogen and PAI-1 are heterogeneous in some sepsis patients[208, 356, 357, 359]. In contrast, a similar impairment in the fibrinolytic system has not been observed in other coagulopathies such as COVID-19[365, 360].

In this study, we demonstrated that while in vitro-generated NDMVs, MDMVs, and EDMVs were capable of generating plasmin, only NDMVs constituted the major source of plasmin generation in the plasma of sepsis patients. It has been demonstrated plasmin generation by leukocyte and endothelial MVs depends on the expression of the uPA/uPAR system or tPA[47, 189, 211]. Importantly, as indicated in our results, the profibrinolytic potential of NDMVs may help to counterbalance the deleterious procoagulant state in sepsis to render a protective response in patients[208, 211]. Therefore, plasmin generation supported by the neutrophil and NDMVs demonstrated how cellular origin represents a strong determinant factor in modulating the MV-CLB. Most importantly, only NDMV-CLB had a lower balance value (lower the value better the fibrinolytic activity) and demonstrated a significant correlation with coagulopathy markers in sepsis.

However, in contrast to the in vitro results, NDEVs captured from the septic patient plasma had some TF activity. Although functional TF expression by neutrophils is still debatable, some studies have demonstrated low levels of TF expression by neutrophils in vitro [366, 367]. Moreover, Darbousset et al demonstrated that neutrophils expressed TF on their surface and actively contributed to thrombus formation in a mice arterial thrombosis model by their interaction with injured endothelial cells [368]. When the interaction of TF-expressing neutrophils with endothelial cells was blocked with antibodies directed against the ICAM-1, neutrophil-associated thrombus formation was significantly reduced. Therefore, activated neutrophils in vivo could be a potential source of TF.

Similar to our study, some previous studies have explored the plasma levels of MVs and their potential utility as biomarkers in sepsis patients. However, unlike our study, these studies have only compared the levels of procoagulant MVs and MV-dependent procoagulant activity but have not explored the profibrinolytic aspects of these MVs [304, 369, 370, 306, 371]. Moreover, only a few of these studies performed correlation analyses between procoagulant MVs and biological or clinical parameters associated with sepsis [369, 370, 371]. Sepsis pathophysiology has a strong association with coagulation and fibrinolytic components that reflect the heterogeneous nature of patients. Although assessing the levels of procoagulant MVs and their procoagulant activity have important predictive value when it comes to excessive coagulation activation and mortality in sepsis DIC, measuring the profibrinolytic activity of MVs along with the procoagulant activity will further improve the diagnostic, prognostic, and biomarker potential of MVs. This is especially important because previous work from our team has demonstrated that although septic shock is generally associated with fibrinolytic dysfunction, some subsets of septic shock patients have MVs with fibrinolytic potential mediated by high plasmin generation capacity [348, 211]. Therefore, combining the procoagulant and profibrinolytic potential while evaluating the hemostatic activity of MVs may represent a promising tool that may reflect the actual depiction of patient status in sepsis conditions.

Our study revealed the complex interplay between MV-dependent procoagulant and profibrinolytic activity and their role in the development of sepsis. Importantly, our work demonstrated the significance of evaluating the MV-CLB in predicting coagulopathy in the context of sepsis pathology regardless of the small cohort we had. Moreover, this novel work will allow us to develop potential biomarkers based on MV-CLB in the context of coagulopathies associated with other diseases. This is especially important considering only NDMV-CLB had a significant correlation with the markers in sepsis coagulopathy. An extension of our work involving a multicenter prospective study on a cohort of 225 septic shock patients (PHRC MISS) showed survivors had NDMV-CLB with lower balance as compared to non-surviving patients. Moreover, NDMV-CLB demonstrated a superior ability to predict patient survival compared to the procoagulant and profibrinolytic activity taken independently. Additionally, MV-CLB allowed the stratification of sepsis patients based on the severity of the disease (correlation with SAPS-II, and hypoxia markers). Therefore, the implementation of MV-CLB as a biomarker in clinical settings would allow us to move to a tailored treatment strategy to improve patient outcomes in septic shock.

Another important aspect of our research was that we demonstrated the cellular source of procoagulant and profibrinolytic activity in vitro and ex vivo. This work would allow us to explore the hemostatic role of MVs from blood cells and cells in the vascular compartment in vivo. We intend to develop mice thrombosis models with conditional knockout of TMEM16F scramblase protein to manipulate the secretion of the specific subset(s) of MVs. This will enable us to evaluate the impact of infused endogenous MVs on hemostatic properties by compensating for the deficiency of a specific subset of the MV population.

In conclusion, this novel work demonstrated that depending on the cell type and stimuli/pathological conditions MVs display distinct procoagulant and/or profibrinolytic potential thus demonstrating distinct MV-CLB that has functional significance in the pathophysiology of thrombo-inflammatory conditions. As a translational extension, MV-CLB can serve as a potential biomarker reflecting the hemostatic imbalance in coagulopathies such as sepsis to develop novel treatment strategies.

2) Generation of MVs with high fibrinolytic potential by neutrophils induced by biopolymer membrane filters.

Imbalance in MV-CLB to the procoagulant state is common in immunothrombosis conditions. In the second part of this thesis, we evaluated the generation of MVs with fibrinolytic potential from neutrophils that are triggered by the biopolymer membrane filters used during the extracorporeal procedures. We observed an imbalance in the MV-CLB towards the fibrinolytic activity that is prompted by a different kind of stimuli in patients who underwent rheopheresis sessions.

The rationale behind the investigation on the modulation of the hemostatic function of MVs comes from a recent study that was published by our research group demonstrated that rheopheresis session markedly reduced proinflammatory markers while significantly increasing anti-inflammatory IL-10 in circulation in patients with microcirculation issues [372]. Moreover, the shift in inflammatory balance towards the anti-inflammatory side induced by the rheopheresis concomitantly improved endothelial markers and microcirculation by reducing the plasma viscosity in these patients (Figure 23). While we have no clear picture of what could be the possible mechanism behind this change in the inflammatory balance, it is plausible that rheopheresis establishes these effects through immune modulation by removing the

inflammatory markers from the circulation. Our preliminary results suggest that rheopheresis could also modulate the hemostatic functions of cells in circulation as indicated by the profoundly elevated levels of fibrinolytic MVs of neutrophil origin in patients. As shown by our in vitro results, the increase in plasmin generation in the patient's plasma may not be the result of the upregulation of the uPA/uPAR system at the membrane level but rather a sheer increase in NDEVs with plasmin generation capacity. As such, because of their natural fibrinolytic effect, increased circulatory levels of NDEVs may have a potential role in improving microcirculation in patients suffering from microischemia by eliminating thrombi from the vasculature.

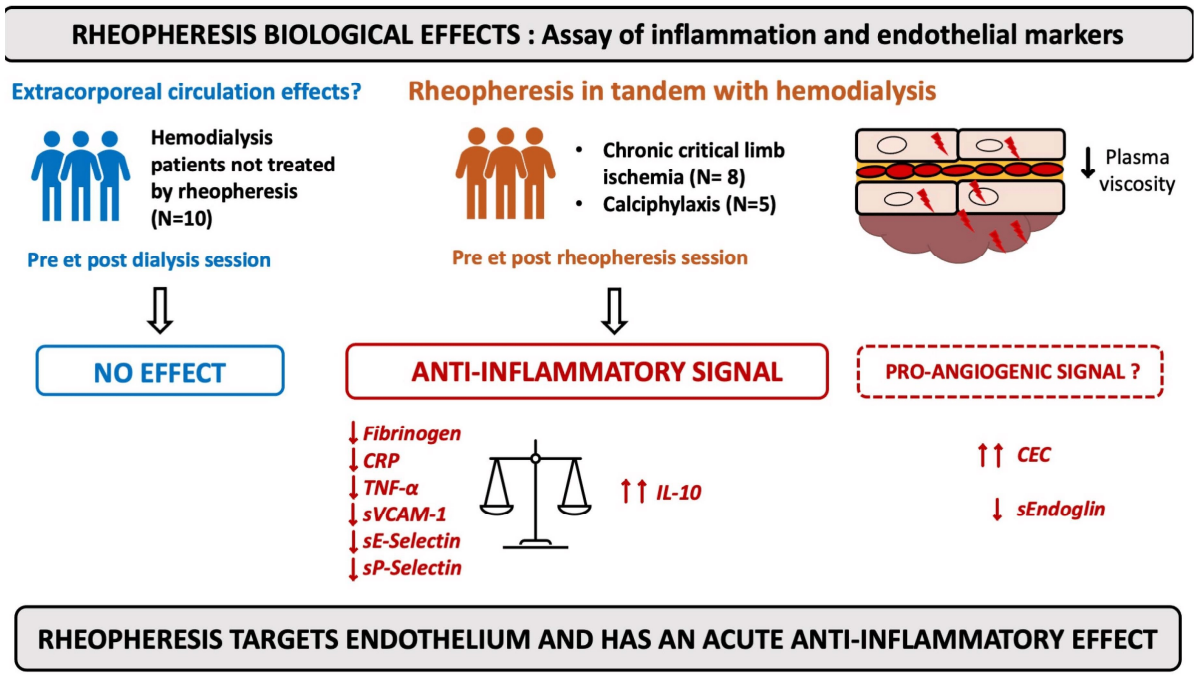

Figure 23. Biological effects of rheopheresis.

(Excerpt from: Solignac et al, J. Clin. Med, 2023)

Our preliminary data indicates that the biopolymer plasmapheresis membrane activates neutrophils and generates a significant quantity of MVs with high fibrinolytic potential that may modulate MV-CLB. While the cell activation driven by the biofouling effect with competitive adsorption of various proteins (Vroman effect) such as fibrinogen and fibronectin to the biomaterials may partially explain the cause behind cell activation[373], a clear understanding of cellular and molecular mechanisms behind cell activation is still lacking. A recent study by Koga et al demonstrated CD11b (αM subunit of integrin mac-1) mediated interaction of neutrophils with adsorbed fibrinogen to polysulfone hemodialysis membrane and

neutrophil activation[374]. Moreover, significant increases in the MVs of neutrophil and platelet origin have been reported in patients who underwent dialysis or lipid apheresis (a procedure to eliminate various LDL particles)[375, 376, 377]. However, to the best of our knowledge, no other study has investigated neutrophil activation and generation of NDMVs with fibrinolytic activity during extracorporeal treatment procedures. Since both uPAR and integrin αMβ2 can modulate neutrophil migration, adherence, and fibrinolytic activity[378], an increase in the CD11b (αM) demonstrated in our result implies that neutrophil activation may generate MVs that carry αMβ2 which may modulate MVs plasmin generation capacity. However, this needs to be investigated.

The preliminary results obtained would allow us to extend this work further to explore the following objectives: 1) Investigate the relationship between CD11b expression on neutrophils and fibrinogen adsorption to the plasmapheresis membrane by perfusing neutrophils over the membranes fixed in a flow chamber 2) Evaluate the expression of uPAR/uPAR on neutrophils and NDMVs by flow cytometry and plasmin generation assays following neutrophil treatment with plasmapheresis membrane 3) Evaluate the PLAUR (plasminogen activator, urokinase receptor) gene expression by activated neutrophils induced by the plasmapheresis membrane.

Plasmin generation mediated by the uPA/uPAR system is a critical player in the fibrinolytic system. Previous work from our team and the current work in this thesis have demonstrated that the cellular source and the stimulants that trigger MV generation are strong determinant factors in the plasmin generation and the MV-CLB. While our data indicate that MVs of neutrophil, monocyte, and endothelial origin support plasmin generation, unlike MVs of monocyte and endothelial origin, the high fibrinolytic potential of neutrophil MVs is not counteracted by the procoagulant activity. Moreover, the plasmin generation mediated by the neutrophil MVs can be significantly amplified by saturating the uPAR expressed on the surface of these MVs with exogenous uPA and infusion of these MVs into sepsis mice model reduced microthrombi in vital organs and enhanced the survival of the mice [211].

The remarkable aspect of these results is identifying neutrophils as a critical source of fibrinolytic MVs with the potential utilization as thrombolytic agents in thrombotic conditions. Importantly, as a part of the FIGRAVIS project funded by the national research agency, our lab seeks to produce large quantities of neutrophil MVs as potential thrombolytic agents to lyse the clot in the brain that is associated with sepsis. We currently investigating the possibility of mechanical extrusion in combination with various cell stimulation strategies to significantly

amplify the quantity of the MVs produced from neutrophils and to upregulate the uPA/uPAR system in these MVs. In addition, our team would evaluate the use of bioreactors with neutrophils differentiated from induced pluripotent stem cells (iPSCs) [379]. This would allow us to scale up the MV production and address the compatibility issues associated with the infusion of MVs.

In conclusion, this novel work has successfully demonstrated a previously unrecognized new mechanism or stimulus that has the potential to shift the hemostatic balance towards the fibrinolytic side. By identifying this new mechanism or stimulus, this research opens avenues for targeted interventions and therapeutic strategies focusing on generating a significant quantity of MVs with fibrinolytic capacity aimed at providing better thrombolysis treatment to patients.

In conclusion, MV-CLB opens up new avenues to understand the impact of MVs in the development of the pathophysiology of coagulopathies. Additionally, MV-CLB demonstrates substantial clinical utility as a potential biomarker reflecting hemostatic imbalance in immunothrombosis rather than the procoagulant or profibrinolytic activity taken into consideration individually. This work not only enhances our diagnostic capabilities but also stage for a paradigm shift in parsonalized thrombolytic therapy based on the MV profiles.

Annexes

Appendix 1

Multifaceted role of extracellular vesicles in atherosclerosis.

Konkoth A, Saraswat R, Dubrou, C, Sebatier F, Leroyer A, Lacroix R, Duchez A, Dignat-George F. Atherosclerosis. 2020 Feb; 319:121-131

Appendix 2

Blood Coagulation and Beyond: Position Paper from the Fourth Maastricht Consensus Conference on Thrombosis.

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Appendix 1

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Multifaceted role of extracellular vesicles in atherosclerosis

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ABSTRACT

Extracellular vesicles (EVs) are small vesicles released by the majority of cells in response to cell activation or death stimuli. They are grouped as small EVs or exosomes, large EVs such as microvesicles (MVs) and apoptotic bodies, resulting from distinct mechanisms of generation. EVs are released into the extracellular space, in most human biological fluids and tissues, including atherosclerotic plaques. They transport complex cargo of bioactive molecules, including proteins, lipids and genetic material and are therefore involved in pathophysiological pathways of cell-cell communication. Indeed, EVs are involved in several processes such as inflammation, coagulation, vascular dysfunction, angiogenesis and senescence, contributing to the initiation and progression of atherothrombotic diseases. Consequently, they behave as a determinant of atherosclerotic plaque vulnerability leading to major cardiovascular disorders. Over the last decade, the field of EVs research has grown, highlighting their involvement in atherosclerosis. However, limitations in both detection methodologies and standardisation have hindered implementation of EVs in the clinical settings. This review summarizes the effect of EVs in atherosclerosis development, progression and severity, with specific attention devoted to their ambivalent roles in senescence and hemostasis. This review will also highlight the role of MVs as multifaceted messengers, able to promote or to attenuate atherosclerosis progression. Finally, we will discuss the main technical challenges and prerequisites of standardization for driving EVs to the clinics and delineate their relevance as emergent biomarkers and innovative therapeutic approaches in atherosclerosis.

1. Introduction

Cardiovascular diseases (CVD) are the leading cause of death and disability worldwide. Among them, atherosclerosis is the underlying cause of myocardial infarction, stroke, unstable angina, and sudden cardiac death. Atherosclerosis is a progressive, chronic inflammatory disease affecting large blood vessels by triggering the build-up of plaque within the vessel wall. Plaque formation is characterized by an accumulation of lipids and fibrous elements. In the circulation, cholesterol is transported by lipoproteins, primarily as low-density lipoprotein (LDL). An abnormal excess of LDL-cholesterol is retained in the subendothelial space of the artery wall, leading to activation of endothelial cells (EC), and is the initiating event in atherosclerosis. Increased expression of adhesion molecules ICAM-1 on ECs promotes recruitment of inflammatory cells, such as monocytes [1] and neutrophils [2], to the intima. T lymphocytes, although being less abundant than monocytes, also penetrate the intima, and regulate the function of the innate immune cells, as well as the endothelial and smooth muscle cells (SMCs) [3]. Human atherosclerotic lesions contain T lymphocytes and display markers of adaptive immune activation [4]. Macrophages and SMCs engulf lipids and become foam cells. They release cytokines and reactive oxygen species (ROS) promoting inflammation, senescence, cell death such as apoptosis and necroptosis. The enlargement of the necrotic core and plaque instability lead to plaque rupture and promote thrombosis.

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

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2. Extracellular vesicles

EVs is a general term of bilipid layer membrane vesicles released from different eukaryotic and prokaryotic cells. Characterization and classification of EVs is a challenge and still debated [9,10]. Until now, EVs have been classified into three distinct populations: small (<100 nm), medium (100–1000 nm) and large EVs (>1000 nm) also called exosomes, microvesicles (MV) and apoptotic bodies (AB), respectively [9]. Small EVs (sEV) originate from intracellular multivesicular bodies while MVs are released by budding of plasma membranes. ABs are generated during vesiculation of apoptotic cells (Fig. 1A). The release of EVs and MVs is an active process involving the endosomal sorting complex required for the transport (ESCRT) system. Additionally, scramblase and flippase activities and the cytoskeleton participate in MV release [11,12]. EVs are detected in biological fluids in healthy and pathological conditions. In CVD such as atherosclerosis, circulating EV level is increased. They contribute to atherosclerosis development by promoting calcification, plaque progression and thrombus formation after rupture of the plaque [6,13,14]. EVs are well known for their involvement in intercellular communication [12] by establishing an exchange of bioactive molecules. Indeed, their cargo nucleic acids, proteins, lipids and organelles [15,16] can be transferred to target cells. Adhesion, internalization of EVs and transfer of their content induce different cellular processes such as inflammation [15,16], angiogenesis [17], coagulation [18] and senescence [19]. However, the protective role of EVs in atherosclerosis has been reported. EVs released from endothelial cells mediate shuttling of atheroprotective molecules like miRNAs to smooth muscle cells to restore communication with the endothelial cells [20]. Atherosclerotic plasma is rich in EVs and lipoproteins such as high density lipoprotein (HDL) and low density lipoprotein (LDL) (Fig. 1B). They share the same range of density and size. The isolation of exosome and MVs from plasma could be contaminated by these lipoproteins [21]. Therefore, it is difficult to separate the effect of lipoproteins and EVs isolated from plasma.

3. Source and role of extracellular vesicles in atherosclerosis

3.1. Involvement of extracellular vesicles in the initiation of atherosclerosis

The notion that EVs are key players in the initiation of atherosclerosis comes from the observation that increased levels of leukocyte and endothelial-derived MVs are associated with carotid remodelling in individuals even before atherosclerosis is detectable [22]. Indeed, there is substantial evidence that MVs are effectors of vessel wall inflammation, notably by regulating leukocytes adhesion to the endothelium and macrophage polarization to a pro-inflammatory phenotype. Moreover, platelet-derived EVs support monocyte recruitment in systemic inflammation, through the transfer of platelet GP1bα, a platelet adhesion receptor to monocytes, allowing monocytes recruitment in large and small blood vessels [23]. Futhermore, circulating platelet MVs may serve as a transcellular delivery system for RANTES, triggering monocyte arrest to the inflamed and atherosclerotic endothelium [24]. Thus, MVs can act as paracrine messengers that intensify inflammation during atherogenesis by stimulating vascular and inflammatory cells. Moreover, many types of MVs, in particular from leukocytes, endothelial cells and platelets, can induce expression of selectins and cell adhesion molecules involved in leukocyte diapedesis [25]. Neutrophil MVs, by delivering miR-155 [26], inflammatory adipocyte-derived EVs [27] or MVs from HFD-fed rats [28], can induce VCAM-1. Also, ICAM-1 can be induced by oxLDL-derived endothelial MVs, in a model of obesity [1,5]. These findings confirm that diet can influence MV release and subsequent effects on cardio-metabolic health [29]. Indeed, obesity is causally associated with atherosclerosis, and EVs derived from adipocytes might be involved in mediating CVD, specifically by promoting leukocyte attachment to vascular endothelial cells [27]. It has also been described that in a high glucose condition, increased NADPH oxidase activity in endothelial MVs promotes activation of the endothelium and development of diabetes-associated atherosclerosis [30]. Monocyte exosomes also induce adhesion molecules and cytokines via activation of NF-κB in endothelial cells [31]. In addition, mature DC derived-exosomes increase endothelial inflammation and atherosclerosis via TNF-α mediated NF-κB pathway [32] (Fig. 2).

3.2. Involvement of extracellular vesicles in lesion progression

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

For instance, human plaque MVs carry catalytically active TACE/ADAM17 and significantly enhance the cell surface processing of TACE/

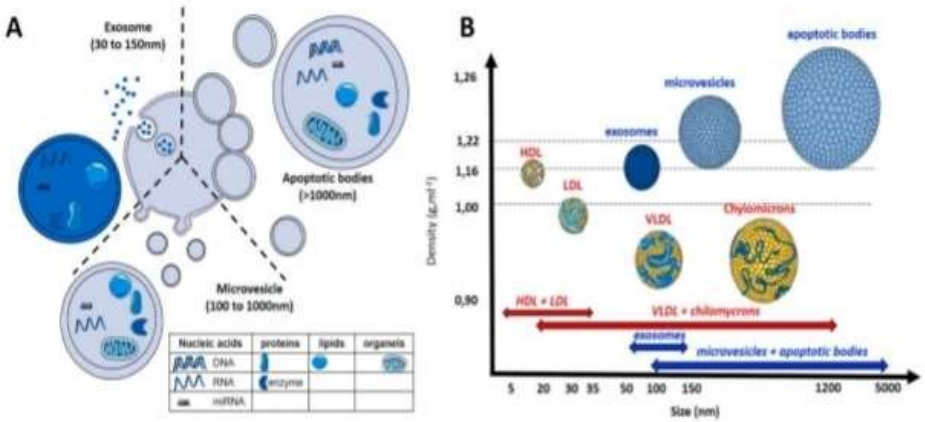

Fig. 1. Heterogeneity of extracellular vesicles.
(A) Extracellular vesicles are heterogeneous in size and content. The cargo of EVs is dependent on cell origin and stimuli (pathologic or physiologic). (B) EVs get along with lipoproteins in blood. High density lipoprotein (HDL); low density lipoprotein (LDL); very low density lipoprotein (VLDL).

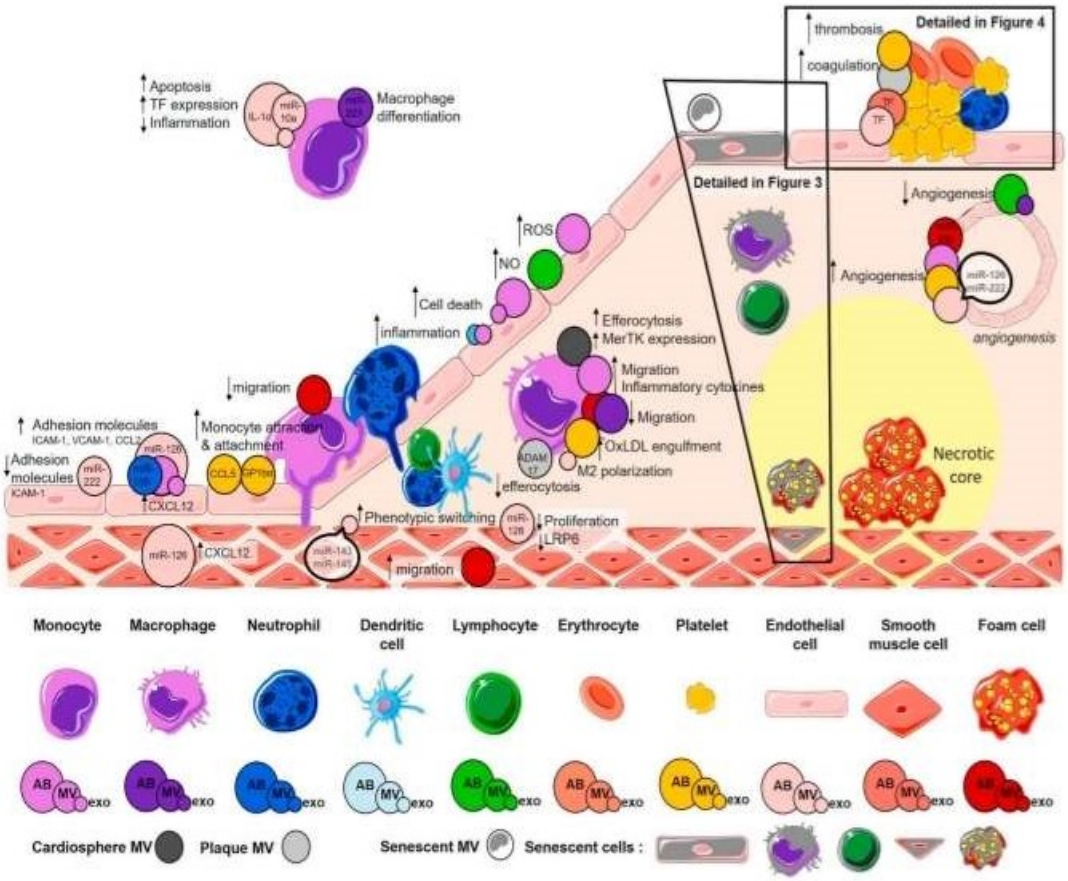

Fig. 2. Impact of extracellular vesicles during atherosclerosis.

ADAM17 substrates TNF, TNFR-1, and endothelial protein C receptor [37]. On the other hand, exosomal MALAT1 derived from oxLDL-treated endothelial cells [38] promotes M2 macrophage polarization, which can reduce plaque inflammation. Macrophage MVs also induce macrophage differentiation through delivery of miR-223 [39] and inhibit cell migration [40]. Similarly, mesenchymal stem cell exosomes polarize macrophage toward an anti-inflammatory phenotype through the miR-let7/HMGA2/NF-κB pathways [41]. Moreover, adipose tissue-derived exosomes may be implicated in the metabolic complications of obesity as they may have a proatherogenic effect by regulating macrophage foam cell formation and polarization [42] (Fig. 2). To summarize, circulating EVs are involved in endothelium activation leading to leukocyte infiltration and inflammation, which are the main initiative steps in atherosclerosis.

3.3. Involvement of extracellular vesicles in the advanced lesion

3.3.1. Relation between extracellular vesicles and the calcification process

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

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

3.3.2. Extracellular vesicles in angiogenesis during atherosclerosis

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

3.3.3. Connection between extracellular vesicles and cell death

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

years, necroptosis, a regulated form of cell death was described in advanced plaque [54]. Necroptosis is a caspase-independent death program regulated by phosphorylation of Receptor Interacting Protein kinase 1 and 3 that activate the phosphorylation of Mixed Lineage Kinase domain-like (MLKL) involved in the rupture of the plasma membrane. Several stimuli in the plaque, including high levels of oxidized LDL (oxLDL), oxidative stress, tumor necrosis factor-alpha (TNFα), induce necroptosis. Interestingly, the generation of EVs could also be mediated by MLKL and the ESCRT system [56]. Additionally, the recognition of dead bodies is mediated by different receptors such as MerTK and CD47 in atherosclerosis. MerTK can be cleaved by the metalloproteinase ADAM-17 from plaque EVs [37] that could lead to an efferocytosis defect. Moreover, MV from cardiosphere derived-cells induce MerTK expression on macrophage by transferring miR-26a, which inhibits ADAM-17 expression [57]. This process could enhance efferocytosis (Fig. 2). During apoptosis, cells release apoptotic bodies and MVs. These MVs are highly pro-coagulant and participate in atherogenicity [58,58,59]. However, EVs from apoptotic cells could contribute to survival mechanisms. Indeed, endothelial EV carrying EPCR and activated protein C (APC) could also promote cell survival by induction of cytoprotective and anti-inflammatory effects [60]. Furthermore, endothelial cells in culture could release EV containing caspase-3 and 8 [61]. Statins, a treatment to reduce CVD risks, increase the detachment of apoptotic cells and release of endothelial MVs. Thus, endothelial-derived MVs contribute to the sorting of several pro-apoptotic factors preventing cell detachment and apoptosis. This mechanism could explain the statins protective effect on the endothelium [61]. EVs from apoptotic platelets induce monocyte adhesion, and polarization into resident phagocyte macrophage [62]. This mechanism could be considered as beneficial. Taken together, EVs from cell in apoptosis or necrosis could have deleterious and protective effects on atherosclerosis progression.

4. The interplay between senescence, extracellular vesicles and atherosclerosis

Senescence affects several cells involved in the progression of atherosclerosis such as endothelial cells, SMCs, macrophages, foam cells and T cells [63,64]. Senescence is defined by an arrest of the replicative potential of cells, with telomere shortening and upregulation of cyclin-dependent kinase inhibitor [64–66] (bullet box 1). However, these cells are still metabolically active, they secrete a panel of pro-inflammatory cytokines and extracellular vesicles as part of the senescence-associated secretory phenotype (SASP) [64–66] (Key-point box 1). Since the early stage of atherosclerosis, endothelial cells, SMCs and macrophages become senescent [63,66] and senescent foam cells (SMCs and macrophages) accumulate in the necrotic core [63,64] due to defective clearance of senescent cells. Indeed, clearance of senescent cells (p16Ink4a positive cells) delays ageing disorders [67]. The vascular endothelium becomes senescent during atherosclerosis [68]. Growing evidence suggests the potential of senescent cells to transfer senescence to other cells through EVs [69–72]. One recent publication elegantly shows the involvement of senescent EVs in atherosclerosis [68]. MVs from the plasma of elderly patients with acute coronary syndrome induce senescence in endothelial cells *in vitro*, but also under low shear stress conditions. Eendothelial cell MVs from elderly plasma induce senescence of the endothelium and thrombogenicity through Ang II/AT1R/NADPH and the PI3-kinase pathways [68]. Many speculations of the involvement of senescent EVs in atherosclerosis could be done. For example, ADAM-17 expressed on MVs from plaque [37] is associated with senescence [73] and this enzyme cleaves also MerTK [57], a receptor involved in efferocytosis. This could explain the accumulation of senescent cells during atherosclerosis. Moreover, several miRNAs carried by MVs *in vivo* and *in vitro* target the senescence process and could be involved in atherosclerosis [19]. Indeed, miR-21 and miR-217 carried by plasma small EVs of elderly volunteers, induces endothelial senescence and could be applied to atherosclerosis [19]. Finally, EVs from elderly plasma induce vascular calcification, and this notion may exist in atherosclerosis [47]. Inter-regulatory loops that could interface endothelial senescence and thrombosis have also been described [74]. Interestingly, local production of activated factor X in atherosclerotic plaque was found to induce vascular smooth cell senescence [75] (Fig. 3).

5. Involvement of extracellular vesicles in hemostasis during atherosclerosis

Hemostasis involves molecular factors belonging to the coagulation and fibrinolysis systems, which are key actors not only in atherothrombosis but also in the earlier stage of atherosclerosis initiation and progression, in close interaction with the inflammation and angiogenesis processes [76]. EVs represent a biological surface promoting efficiently both procoagulant and fibrinolytic cascades [18,77] (Fig. 4). They are now identified as a key partner contributing to the different steps of atherosclerosis. Most studies have been focused on the procoagulant property of MVs. This explains the considerable number of studies that link the role of EVs to thrombosis formation and plaque rupture in atherosclerosis in the last two decades [59,78]. Briefly, the exposure of active Tissue Factor (TF) at the EV surface initiates the coagulation process in the presence of high concentrations of anionic

Key-point box 1
Senescence

- Senescence is a cellular program involved in different processes such as embryonic development, cancer-protection, ageing and CVD [64–66].
- Senescence is beneficial when it is transient (acute senescence) whereas aberrant accumulation of senescent cells (chronic senescence) is detrimental and leads to inflammation and decline of tissue functions.
- Therapies that target senescent cells (senotherapies) enhance CVD.
- Triggers of cellular senescence are diverse such as telomere shortening, DNA damage, proteotoxic stress, ROS, RAAS, hypertension, hyperglycaemia, secreted molecules, cytotoxic compounds, radiation, oncogene activation, mitotic stress, tumour-suppressor inhibition, secreted molecules [65].
- Cellular senescence is characterized by a permanent cell-cycle arrest and by a specific secretome named senescence-associated secretory phenotype (SASP).
- Intracellular markers of senescence used are senescence-associated β-galactosidase (SA-β-gal) activity at pH 6.0, proliferative marker Ki67, cyclin-dependent kinase inhibitors such as p21CIP1, p16INK4a and p19ARF, p14ARF, p27KIP1 or p15Ink4b; DNA damage response is detected with γ-histone H2AX or tumour suppressor p53-binding protein 1; senescence-associated heterochromatic foci is evaluated with chromobox protein homologue 3 (HP1-γ), histone H3K9me3 or core histone macro-H2A.
- SASP is mainly composed of cytokines ((IL)-1α, IL- 1β, IL-6, IL-8, IL-18, TNF-α), chemokines (CCL2), proteins (iNOS, PAI-1, MMP-1, -2, -3, -7, -8, -9, -10, −12, −13, and −14) and EVs (exosomes and microvesicles) [70,72].

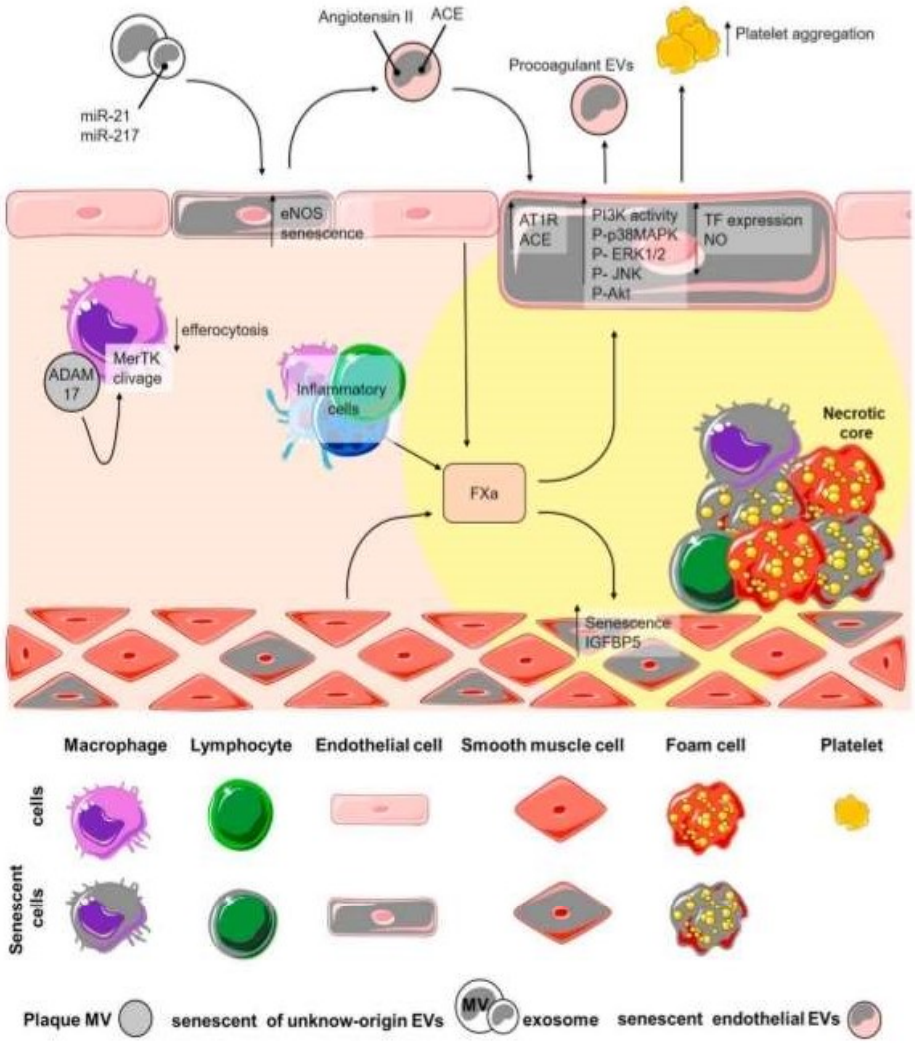

Fig. 3. Interplay between senescence, extracellular vesicles and atherosclerosis.

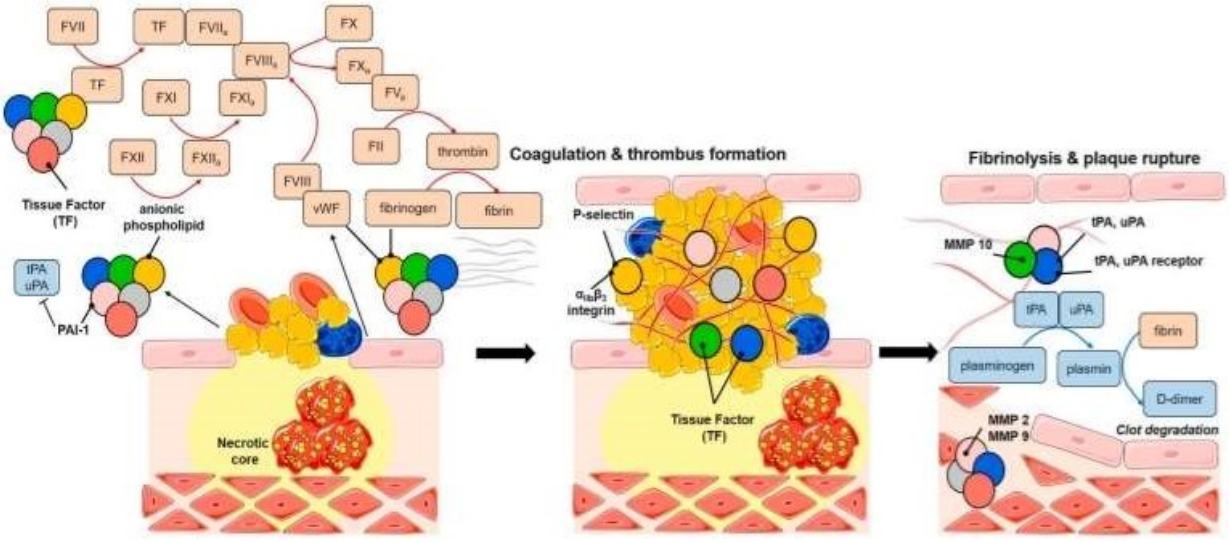

Fig. 4. Involvement of extracellular vesicles in hemostasis during atherosclerosis.

phospholipids such as phosphatidylserine (PS) (Key-point box 2 and Fig. 3). MVs derived from atherosclerotic plaque display a much higher thrombogenic potential compared to circulating MVs [59]. High expression of TF correlates with high concentration of EVs from leukocytes, SMCs, and erythrocytes within the plaque [59]. In addition to TF bearing MVs that originated from living cells, plaque also contains significant levels of PS and TF positive apoptotic MVs [78]. Tobacco smoke increases the athero-thrombotic effect of epithelial EVs with their pro-coagulant properties [79] and TF-positive MVs from macrophages [80].

Key-point box 2
Coagulation and fibrinolysis

- Blood clot is the result of coagulation.
- Coagulation cascade involves a series of proteolytic reactions. The activation of the coagulation process begins with the tissue factor VII pathway and results in thrombin generation. Thrombin converts fibrinogen to fibrin. Crosslinking of fibrin is mediated by Factor XIIIa (Fig. 4). Many actors play a key role in coagulation such as platelet, neutrophils with their neutrophil extracellular traps and endothelium [2].
- EVs are involved in coagulation thanks to the exposure of anionic phospholipids and tissue factor that mediate the assembly of coagulation factors and the formation of fibrin [77,87].
- Fibrinolysis is the result of clot lysis by degradation of fibrin [77,88].
- Tissue plasminogen activator (tPA) from endothelial cell and urokinase plasminogen activator (uPA) convert plasminogen to plasmin that degrades fibrin in thrombus.
- A subset of EVs carry fibrinolytic molecules like uPA and tPA, which convert plasminogen to plasmin and promote clot lysis by degrading fibrin in the thrombus [77,88].
- Proteolysis occurs to clot lysis through the action of matrix metalloproteinase (MMP-1,9,10).

As plaque MVs display greater thrombogenicity than plasma EVs, they confer a higher risk for thrombus formation. Indeed, when advanced plaques become unstable and break, they serve as a source of continuous procoagulant EVs released in the bloodstream. This might exacerbate coagulation and lead to ischemic stroke or myocardial infarction. Besides the data regarding MVs in plaque, there are well-documented data providing information on the strong interplay between circulating procoagulant MVs released from different sources and atherosclerosis progression. Activated platelets produce MVs with high-level expression of molecules such as PS, TF, αIIbβ3-integrin (CD41/CD61), glycoprotein IV (CD36), thrombospondin (TSP1), P-selectin (CD62P), P-selectin glycoprotein ligand 1 (PSGL-1), which promote thrombosis. These platelet-derived MVs have been positively correlated with the intima-media thickness of the carotid artery of obese patients and menopausal women [81,82]. This is also supported by the prognostic value of activated platelets, as it generates TF and thrombospondin-1 positive MVs along with a high number of TF positive monocyte MVs in young patients with high cardiovascular risk [83]. Further studies have demonstrated the critical role of platelet procoagulant MVs in promoting platelet and fibrin deposition to the damaged arterial wall and surfaces with exposed collagen under shear stress, as well as normal flow conditions [84]. Moreover, vessel wall injury can lead to enhanced formation of thrombogenic MVs from endothelial cells, which then amplify the coagulation cascade even further [78]. Several molecules have been shown to modulate the procoagulant potential of MVs in atherosclerosis. For example, atorvastatin treatment in patients with atherosclerosis reduces thrombin generation and expression of tissue factor, P-selectin and GPIIIa on platelet-derived MVs [85]. Conversely, interleukin-33 treatment results in increased production of TF-positive MVs and activity in monocyte subsets, with potential implications for cardiac events during atherosclerosis [86]. As all these studies provide an understanding of how MVs are linked to the coagulation process in atherosclerosis, they might guide the choice of drug target to modulate coagulation and atherosclerotic progress.

In addition to their thrombogenic activity, some EVs have fibrinolytic and proteolytic potential. Endothelial and leukocyte MVs carry tPA and uPA respectively, on their surface and can promote plasmin generation *in vitro* [87,88]. By conveying plasmin, endothelial MVs activate matrix metalloproteases (MMP), which are involved in extracellular matrix degradation and release of growth factors that play a crucial role in tissue remodelling and angiogenesis [77]. Among them, MMP10 is particularly crucial in fibrinolysis as it has been shown to prevent formation of fibrin by proteolytically degrading fibrinogen and may thus prevent thrombus formation [89]. On the other hand, MMP2 and MMP9 accelerate vascular remodelling by degrading matrix components, and high levels of these MMPs have shown to significantly correlate with plaque instability and rupture, resulting in the progression of atherosclerosis [89,90]. Similar to MMP2 and MMP9, smoke-induced EVs carrying MMP14 may contribute to plaque rupture as it has been shown to exhibit significant gelatinolytic and collagenolytic activity *in vitro* [91]. Interestingly, EVs not only activate pro-MMPs to active MMPs through plasmin generation, but can also directly vectorize MMP2, MMP9 and MMP10 [77] (Fig. 4). Whether fibrinolytic properties carried by EVs can play a role in atherosclerosis remains to be answered [77,87]. The fibrinolytic potential of EVs can be modulated by pharmacologic agents, such as rosuvastatin, found to increase the plasminogen level in EVs from the LDL plasma fraction [92]. However, the role of the fibrinolytic potential carried by EVs has not been directly investigated in atherosclerosis and future studies are needed to determine in which extent this may contribute to homeostatic imbalance and provide a potential target for therapeutic manipulation.

6. Translation of extracellular vesicle analysis to the clinic

6.1. Extracellular vesicles as biomarkers of atherosclerosis

Recognition of EVs as potential biomarkers of disease state is illustrated by the growing number of reviews on the topic [93–96]. However, the measurement of EV remains challenging. Preanalytical variables and technical limitations impact EV determination, therefore impeding sensitivity and repeatability of MV measurements and limiting comparisons between studies. An important step has been the publication of guidelines for sample preparation and analysis [9,10], resulting from the joint efforts of international scientific societies (The International Society of Extracellular Vesicles (ISEV) and the International Society on Thrombosis and Haemostasis (ISTH)). In practice, standardization of the pre-analytics is crucial since handling, delay and storage of plasma samples are the major sources of artefactual generation or loss of EVs [97]. Regarding the analytical methods available for EVs determination, some methods like electronic microscopy (EM), nanoparticle tracking analysis (NTA) and tunable resistive pulse sensing (TRPS) allow the measurement of particles of small size but they are not adapted to the clinic because they are time-consuming and not available in hospital laboratories. Flow cytometry is mostly used to enumerate and determine the cellular origin of EVs in clinical samples. However, detection and characterization of exosomes can be challenging, considering their small size, but possible with advanced flow cytometry techniques. Indeed, exosome can be detected after latex bead capture before analysis on flow cytometer [98]. The main disadvantage of these analysis comprises the EV separation with their heterogeneity in size and origin that contrasts with the isolation of EV based on the size. Since the last years, this method has benefited from considerable improvement, with the development of instruments with increase sensitivity and resolution [10]. Several standardized protocols and calibration tools are now available [9,10] and pave the way for multicentric studies allowing to delineate the clinical relevance of MVs. Besides flow cytometry, functional assays

are available to measure MV dependent procoagulant activity [10].

Several reviews summarized MVs as potential biomarkers for the early identification of subclinical manifestations of atherosclerosis [96,97,99,100]. MVs are clinically relevant biomarkers because they provide some useful and predictive information to stratify patients according to their cardiovascular risks, and to monitor the response to treatment [96,97,99,100]. MVs are detectable in several biological fluids and provide an instant picture of inaccessible tissues, such as the endothelium or atherosclerotic plaque. Moreover, these liquid biopsies may be a signature of the individual underlying atherosclerotic disease. They have the advantage to protect their content from degradation due to their bilayer lipid membrane and allow to perform longitudinal measurements. Levels of EVs in blood are increased in cardiovascular and cardiometabolic diseases, including diabetes mellitus, obesity, subclinical atherosclerosis, coronary artery diseases and myocardial infarction [93,97,101]. The concentration of endothelial and erythrocyte EVs is indeed related to Framingham risk scores for coronary artery disease in healthy patients [97]. Endothelial derived EVs level correlates with the severity of coronary artery disease in patients with diabetes [97]. Previous studies have nicely shown that atherosclerotic plaques contain large amounts of EVs, which could either contribute to plaque progression and be released into the circulation. These EVs, mainly derived from leucocytes, reflecting the local inflammatory environment, could serve as diagnostic and prognostic biomarkers in CVD [93,94]. Indeed, increased circulating leukocyte MVs positively correlate with high plaque burden in subclinical atherosclerosis [93]. Annexin V positive MVs, released into the circulation by the endothelial cells following vessel wall injury, could be used as a potential biomarker of endothelial damage during atherosclerotic complications [100]. Similarly, elevated endothelial MVs in the circulation of carotid patients may be used to predict plaque instability [102]. Annexin V positive MV originated from apoptotic endothelial cells have been demonstrated to increase significantly in the circulation of type 2 diabetes mellitus patients with asymptomatic atherosclerosis [103]. In HIV infected patients, elevated MVs associated TF activity remarkably correlates with the presence of plaque in the coronary artery [99]. The high circulatory levels of procoagulant MVs from platelet and endothelial cells are indicative of the severity of coronary calcification [95]. On the other hand, lymphocyte CD45 and CD3 derived MVs could be a biomarker of lipid-rich atherosclerotic plaques in familial hypercholesterolemia [96]. MVs are both actors of plaque development and potential promising biomarkers to monitor plaque progression. Altogether, these studies are consistent with the fact that EV detection could predict plaque instability and guide therapeutic options. Progression of the technical development for measuring MVs, standardization of methods in combination with efforts to define the most interesting subsets of MVs in different clinical contexts are issues that should be addressed in the future. Taken together, the recent development of sensitive and standardized methods will facilitate the introduction of circulating MVs as biomarkers in the clinic.

6.2. *Extracellular vesicles as therapeutics in atherosclerosis*

EVs might be used as therapeutics [20,55,104–107]. In the last 10 years, several clinical studies have suggested the potential use of EVs as therapeutic vectors by either modifying their cytosolic content or loading them with molecules that could be transferred to target cells through specific interactions. Several reviews related the clinical trials using EV to therapies in different diseases, especially CVD [6,108]. Two preclinical studies performed on murine models of atherosclerosis have demonstrated a decrease of atherosclerotic plaque size thanks to the injection of vesicles derived from human endothelial cells [20,55]. Apoptotic bodies from endothelial cells from atherosclerotic patients or *ApoE*$^{-/-}$ mice contain miR-126, that mediate the atheroprotective action through increased CXCL12 expression, decrease of V-CAM1, and mobilization and incorporation of Sca-1 positive progenitor cells to the plaque [55]. Endothelial cells could also release exosomes rich in miR-143/146 but not in miR-126. The uptake of these exosomes by SMC in the plaque reduces atherosclerotic lesions in the *ApoE*$^{-/-}$ model [20]. Other studies related to the treatment of myocardial infarction used EVs. Indeed, injection of EVs from iPSC-derived cardiomyocyte could exert a protective effect by regulating inflammation and angiogenesis [107]. On the same line, exosomes from stem cells could be used to promote cardiac function in the pathological heart [109].

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

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

7. Conclusions

EVs are potent vehicles of bioactive molecules, which influence various processes in atherosclerosis. Their involvement in vascular pathophysiological responses is more complex than initially thought and it demonstrates not only EVs diversity but also the complexity of the mechanisms they are involved in. Indeed, they play detrimental roles by promoting inflammation, thrombosis, vascular dysfunction and senescence but can also be protective, through their involvement in fibrinolysis and cell survival.

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

- to develop animal models defective for MV production, to better understand the role of EVs, the cellular and molecular determinant triggering their ambivalent involvement in the pathophysiological process. Since they can either promote or inhibit coagulation, fibrinolysis, angiogenesis and survival, they may consequently guide the evolution of the atherosclerotic diseases
- to identify the cargo components based on the heterogeneity of EVs and their subpopulation, thanks to the -omic technologies. This cargo of bioactive molecules, such as proteins, lipids, nucleic acid, could contribute to increase or resolve disease severity. Defining the molecular profiling of EVs will help identify atherosclerotic related specific signatures and exploit the full biological potential of MVs as emergent biomarkers
- to better understand mechanisms controlling the spatiotemporal release of EVs and their clearance. Indeed EVs, as professional in cell-cell communication, could be associated with, internalized or cleared by cells. In cardiovascular diseases such as atherosclerosis, the generation of EVs and their origin in circulation or tissues should be established, since they could originate from circulating cells or the vessel structure with endothelial cells, fibroblasts and smooth muscle cells. Thus, the EV clearance process or association with circulating

cells should be investigated since EVs in the circulation could be attributed to the increase or defect of EV generation and clearance
- to overcome the remaining challenges to drive MV in clinical practice, in particular (1) resolve technological limitations and develop standardization, two prerequisites to validate their clinical interest at a multicenter level and (2) validate the benefit of therapeutic modulation of MV in the prevention of future cardiovascular complications
- to establish whether EVs could be used as therapeutic agents or therapeutic vectors. Using the property of EVs (small vesicle, biomolecular cargo, great internalization) holds a promising avenue of therapeutics in cardiovascular disease with engineered vesicles. Moreover, EVs isolated from patients could also be transferred to another patient and resolve diseases. The bio-engineering vesicle field will expand in the future, employing new material, thus a new way of delivery, and different ways to produce them.

Declaration of competing interest

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

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Appendix 2

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Blood Coagulation and Beyond: Position Paper from the Fourth Maastricht Consensus Conference on Thrombosis

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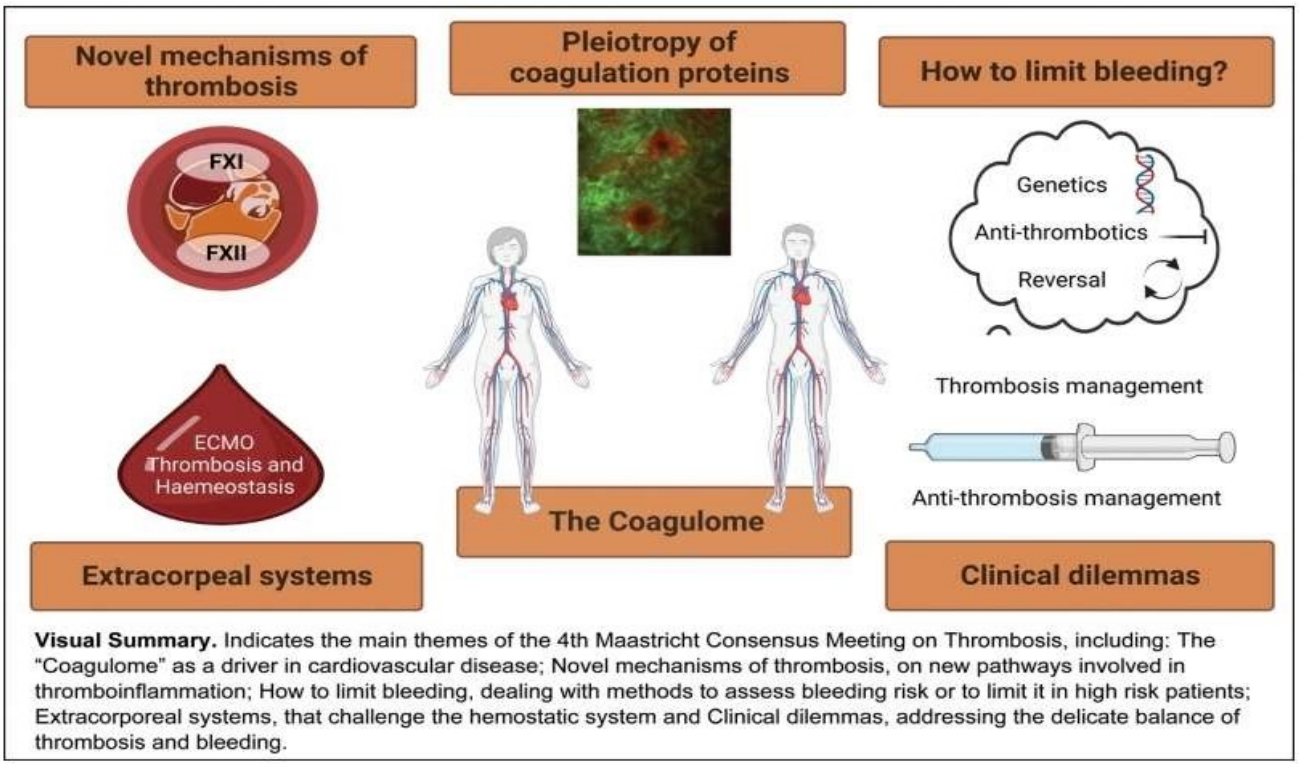
Abstract

The Fourth Maastricht Consensus Conference on Thrombosis included the following themes. Theme 1: The "coagulome" as a critical driver of cardiovascular disease. Blood coagulation proteins also play divergent roles in biology and pathophysiology, related to specific organs, including brain, heart, bone marrow, and kidney. Four investigators shared their views on these organ-specific topics. Theme 2: Novel mechanisms of thrombosis. Mechanisms linking factor XII to fibrin, including their structural and physical properties, contribute to thrombosis, which is also affected by variation in microbiome status. Virus infection-associated coagulopathies perturb the hemostatic balance resulting in thrombosis and/or bleeding. Theme 3: How to limit bleeding risks: insights from translational studies. This theme included state-of-the-art methodology for exploring the contribution of genetic determinants of a bleeding diathesis; determination of polymorphisms in genes that control the rate of metabolism by the liver of P2Y12 inhibitors, to improve safety of antithrombotic therapy. Novel reversal agents for direct oral anticoagulants are discussed. Theme 4: Hemostasis in extracorporeal systems: the value and limitations of ex vivo models. Perfusion flow chamber and nanotechnology developments are developed for studying bleeding and thrombosis tendencies. Vascularized organoids are utilized for disease modeling and drug development studies. Strategies for tackling extracorporeal membrane oxygenation-associated coagulopathy are discussed. Theme 5: Clinical dilemmas in thrombosis and antithrombotic management. Plenary presentations addressed controversial areas, i.e., thrombophilia testing, thrombosis risk assessment in hemophilia, novel antiplatelet strategies, and clinically tested factor XI(a) inhibitors, both possibly with reduced bleeding risk. Finally, COVID-19-associated coagulopathy is revisited.

Keywords

- antiplatelet agent
- artificial surfaces
- atherosclerosis
- oral anticoagulants
- thrombosis



Visual Summary. Indicates the main themes of the 4th Maastricht Consensus Meeting on Thrombosis, including: The "Coagulome" as a driver in cardiovascular disease; Novel mechanisms of thrombosis, on new pathways involved in thromboinflammation; How to limit bleeding, dealing with methods to assess bleeding risk or to limit it in high risk patients; Extracorporeal systems, that challenge the hemostatic system and Clinical dilemmas, addressing the delicate balance of thrombosis and bleeding.

Introduction

During the Fourth Maastricht Consensus Conference on Thrombosis (MCCT), held in April 2022, the main theme of the conference was "Blood coagulation and beyond" expressing the desire of the organizers to look beyond boundaries. A characteristic of this conference is the strong interaction among presenters and audience encouraged by the breakout sessions following presentations creating room for in-depth discussions among basic, translational, and clinical scientists from different backgrounds. The MCCT meeting focused on five different topics, to be addressed below. The authors comprise not only faculty but also PhD students that were actively involved in discussions as well as note taking of the discussion sessions; these notes and the summary of the presentations provided the basis for this article in which all actively involved act as contributory authors. This meeting was co-organized with the EU-Marie Curie International Training Network TICARDIO and all PhD students from this network were contributing to this article.

Theme 1: The "Coagulome" as a Critical Driver of Cardiovascular Disease

The Brain Coagulome

To briefly introduce the term coagulome, which we use in analogy to the previously used term "endotheliome" to describe a multifactorial approach to the endothelium,[1] assessing its multifunctional properties in health and disease by combining different methods, to obtain an integrated image of this pivotal cellular compartment, is essential.

Primary and secondary prevention of ischemic stroke (IS) benefits from antiplatelet and anticoagulant therapies.[2] However, compared with coronary heart diseases (CHDs), P2Y12 inhibitors other than clopidogrel have no clinical use in primary stroke prevention and can be contraindicated (prasugrel) in patients with a previous stroke for increased risk of intracranial bleeding.[3] Similarly, dual antiplatelet therapy (DAPT) is effective just in the early phases of the IS (21–30 days) before becoming useless or detrimental. In the case of vorapaxar, which is the only approved drug of a novel class antithrombotic agent acting on the protease-activated receptor-1 (PAR-1), trials directly assessing stroke management are lacking.[4] PAR-1 is fundamental for pleiotropy of coagulation factors in the central nervous system (CNS).[5] The main proteases that can activate PAR-1 are matrix metalloproteinase 9 (MMP-9)[5] and thrombin, whose activation state, concentration, and association with activated protein C (aPC) lead to differential pathway activation in physiology[6] as well as CNS pathologies.

Factor (F) XI has been shown to be involved in thrombus stabilization during stroke.[7] In a large population, elevated FXI was associated with the risk of IS and a FXI:C level <15 U/dL incurred protection against stroke.[8,9] The FXIa level was higher in subjects with previous stroke compared with those with a history of transient ischemic attack (TIA) (34 vs. 11.4%, $p < 0.0001$), suggesting that FXIa is associated with worse functional outcomes of cerebrovascular disease.[10] The related mechanism could be that inhibition of FXI(a) reduces

thrombin generation, activation of TAFI (thrombin activatable fibrinolysis inhibitor), and ultimately may enhance the lysis of clots that form or embolize into cerebral arteries.[11]

Consistent with those findings in human studies, in the mouse model of acute IS (temporal occlusion of the middle cerebral artery), administration of antibody 14E11 that blocks the activated FXII (FXIIa)-dependent activation of FXI resulted in a significant reduction in infarct size and a significant improvement in neurological function compared with the control group.[12] Clinical and experimental evidence demonstrated that coagulation proteins have pleiotropic effects on the CNS not limited to physiological repair of vascular damage and pathological ischemic/hemorrhagic stroke.

The different effects of antiplatelet and anticoagulant agents on the CNS can be in part due to the existence of a unique and complex interface represented by the neurovascular unit (NVU). Indeed, other organs can promptly differentiate their own blood vessels when repairing a lesion, or for metabolic reasons even without perturbing the tissue integrity, the same cannot be said for the CNS. The NVU is a unique integrated frontier in which the mesenchymal cells (endothelial cells [ECs], pericytes, smooth muscle cells, fibroblasts) do not originate from within the CNS tissue (purely ectodermal formed by neurons and macroglia) but penetrate without violating its integrity during embryogenesis. During CNS development through a clear contribution of coagulation factors such as tissue factor pathway inhibitor (TFPI), FV, FVII, and FX, the mesenchyma enters the nervous parenchyma.[13] The same happens for the resident immune cells, the microglia, which is a distinct population of myeloid cells, not differentiated from the bone marrow (BM), but originating from the yolk sac.[14] Hence the coagulation factors, as mentioned, do not limit their intervention to vascular repair and exert their function also on the nervous tissue, justifying their emerging role in neurological diseases other than stroke.

This pleiotropy has been demonstrated in various pathologies that have no strict vascular etiology, such as multiple sclerosis (MS), Parkinson's disease (PD), and Alzheimer's disease (AD).[5] TFPI was shown to be increased in the frontal cortex of AD brains compared with healthy controls.[15] In MS patients, TFPI levels were higher in the group of progressive MS compared with relapsing–remitting MS and healthy controls. Same results were obtained for plasminogen activator inhibitor-1 (PAI-1) expression in these groups.[16] In a randomized controlled trial (RCT) of recovering MS patients, it was shown that plasma levels of TFPI[17] and other coagulation inhibitors (e.g., protein S) increased with increasing recovery rate and patients with a generally low level of TFPI in earlier disease states showed better rehabilitation afterwards.[17]

As a neurodegenerative disease, AD is characterized by abnormal loss of cholinergic neurons in areas of the brain that are primarily responsible for cognition and memory. The key pathological elements in AD have been proven to be amyloid-β (Aβ) peptides and neurogenic fiber tangles. In animal studies, human amyloid precursor protein (hAPP) transgenic mice from line J20 (hAPP-J20 mice) are used to establish the AD model, and the results have shown that coagulation factors are involved in the metabolism of Aβ,[18,19] which can lead to the activation of FXII, resulting in FXI activation and thrombin generation, ultimately leading to a prothrombotic environment that contributes to the development of AD. These data are supported by decreased levels of plasmatic FXI in AD patients, with depletion of its inhibitor, suggesting a chronic activation with subsequent inactivation and clearance of FXI during the disease.[20] Moreover, in the same patients, activation of the intrinsic coagulation pathway is supported by elevated plasmatic fibrin levels.[20] Compared with cognitively healthy people or patients diagnosed with mild cognitive impairment, patients who are diagnosed with AD have significantly increased plasmatic levels of FXI.[21] FXI may therefore be a predictor of AD-type diagnosis, as an increase in FXI has been associated with a reduction in cognitive function.[21,22] Proteomic analyses of plasma and postmortem brain tissues (the inferior frontal cortex, superior frontal cortex, and cerebellum) from AD patients demonstrated a clear activation of complement coagulation cascade, in particular of FXII and FXIII, further corroborating this hypothesis.[23]

The pathogenesis of AD could be particularly sensitive to NVU disruption; fibrin deposition, possibly an end stage product resulting from the long-term dysfunction of the NVU, has been demonstrated in both large vessels and capillaries of AD patients and can have a great impact on metabolic coupling, particularly in the hippocampal region.[24] Parenchymal deposition of fibrin, as the last step of the coagulation cascade, could enhance the inflammatory state and contribute to the loss of integrity of the blood–brain barrier (BBB). In the dysfunctional NVU, astrocytic apolipoprotein E4 (APOE4), interacting with pericytic low-density lipoprotein receptor-related protein 1, through cyclophilin A (CypA) signaling, increases MMP-9 transduction and thrombin/PAR-1 signaling.[25] The BBB breakdown was more severe in carriers of APOE4, an identified genetic risk factor for AD with cognitive impairment, independently of AD biomarkers, both Aβ and tau. The BBB damage, measured in vivo by magnetic resonance imaging (MRI) as well as pericyte- and platelet-derived biomarkers such as soluble platelet-derived growth factor receptor β (sPDGFRβ) predicted the future cognitive status in carriers even after controlling the analysis for Aβ and tau levels.[25] These predictive biomarkers correlated with increased CypA - MMP9 activity in the cerebrospinal fluid (CSF) and are very promising for early diagnosis of AD. Fibrin–Aβ fibrils are not accessible to breakdown by plasmin, activate FXII, and inhibit microglia/macrophages scavenging through CD11b silencing.[26] Blockage of fibrin–Aβ interaction (as demonstrated through RU-505) could pave the way to overcome the failures in disease-modifying therapies for neurodegeneration.[27] Finally, FXIIa, high molecular-weight kininogen, and kallikrein activities, all thrombo-inflammatory mediators, are detected in AD and their effects can be experimentally attenuated by FXII depletion.[28]

The aforementioned data reinforce the idea of the vicious circle starting with the regional failure of the NVU and leading to protein deposition and neuroinflammation.

Potential areas for future investigation:

- Investigate the emerging pleiotropic role of coagulation cascade in the CNS with the central role of PAR1 interference.
- Explore the role of pericytes for NVU stability, for vascular tone, permeability, and metabolic regulation and as early CSF biomarkers of AD.
- Search for brain-specific biomarkers of the patient's thrombo-inflammatory state to develop noninvasive, easy-to-access diagnostic/prognostic tools.
- Diffuse homogeneous protocols for the evaluation of BBB integrity using standard MRI or PET-CT (positron emission tomography-computed tomography) scans, to be correlated with novel biomarkers (e.g., sPDGFRβ) and ATN (Amyloid, Tau, Neurodegeneration) classification in clinical settings.
- Targeting the fibrin/CD11b complex and inhibiting FXIa and FXII with novel or existing drugs in future clinical trials for neurodegeneration, especially AD.

The Cardiovascular Coagulome: Focus on Thrombin and Inhibition of Its Amplification

While the role of thrombin generation in CHD, including the process of atherogenesis and atherothrombosis, has been demonstrated in experimental and clinical studies, current research focuses on specific coagulation proteases, including FXI and the tissue factor (TF)/TFPI axis.

FXI, as a component of the intrinsic pathway of coagulation, is activated by FXIIa and then proceeds to the downstream coagulation cascade that eventually triggers thrombin generation. In addition, FXI can also be feedback-activated by thrombin, further accelerating fibrin formation. Over the past decades, many studies have attempted to investigate the role of FXI in thrombin generation and its relationship with thrombus formation.

In the animal model of atherosclerosis (ApoE knockout mice, ApoEko), knockout of FXI reduced peripheral atherosclerosis by up to 33%.[29,30] In another animal study, low-density lipoprotein receptor knockout ($Ldlr^{-/-}$) mice combined with high-fat diet were treated with anti-FXI antibody (14E11) or FXI ASO. Compared with controls, 14E11 and FXI-ASO both reduced the area of atherosclerotic lesions in the proximal aorta, and 14E11 also reduced aortic sinus lesions.[31] These data suggest that FXI plays a role in atherogenesis, and that depletion of FXI may reduce development of atherosclerosis. Another indicative factor for thrombogenicity in coronary lesions might be the co-localization of TFPI with TF. Tissue studies of coronary atherosclerotic plaques revealed expression of TFPI in ECs, macrophages, foam cells, and smooth muscle cells. Co-localization with TF only occurred in ECs and macrophages in the groups of highest severity and was also found in the necrotic lipid core.[32]

CHD is the result of partial or complete occlusion of the coronary arteries due to thrombosis, which impairs the blood supply to the heart muscle. Outcomes of the PRIME study including nearly 10,000 men showed that patients with a general low free TFPI plasma concentration had a more than twofold increased risk of developing CHD. This effect was increased to sevenfold, when von Willebrand factor (vWF) levels were increased.[33] Additionally, TFPI levels were generally higher in non-ST segment elevation myocardial infarction (NSTEMI) compared with ST-segment elevation myocardial infarction (STEMI) patients.[34] In another study, TFPI levels in hospitalized patients with acute coronary syndrome (ACS) were indicative for the severity of myocardial infarction (MI) but were not associated with mortality.[35]

Numerous polymorphisms of TFPI have been studied over the last decades that in part correlate with increased risk of cardiovascular disease,[36,37] but sometimes only shown to be related to altered TFPI plasma levels, but not to an increased risk for CHD.[38–40] However, studies reporting blood concentrations of coagulation should be carefully interpreted, since lower circulating levels could reflect both reduced production or increased consumption (or vice versa). This requires more research to understand the pathophysiology in the respective disease setting to improve applicability of a given coagulation factor as a putative biomarker.

Lorentz et al found that mice treated with an anti-FXI antibody, 14E11, had decreased myocardial infarct size in a model of ischemia/reperfusion (I/R) injury, indicating that FXI activation or activity might contribute to cardiac I/R injury.[41] Kossmann et al revealed that depletion of FXI could not only decrease a vascular coagulation–inflammatory circuit in angiotensin II-induced arterial hypertension, but also prevent arterial hypertension-induced end-organ damage.[42]

The role of FXI in acute MI (AMI) is less clear than in stroke. Patients with lower levels of FXI are at less risk of venous thromboembolism (VTE) and MI[43] and FXI level is correlated with MI risk among men in the study of Myocardial Infarction Leiden.[44] Butenas et al reported that plasma FXIa level could be quantified in most patients with ACSs, whereas it was undetectable in age-matched healthy controls.[45] However, conflicting data exist. Salomon et al reported similar incidences of AMI in patients with severe FXI deficiency and the general population and inherited FXI deficiency seems to be not protective against AMI.[46] Results from the Risk of Arterial Thrombosis in Relation to Oral Contraceptives (RATIO) case-control study showed that high levels of FXI are associated with IS, but are not or to a lesser extent associated with MI, in young women.[47] These data suggest that the contribution of FXI in thrombosis varies between vascular beds and sex. The question why the deficiency of FXI has disparate effects on acute IS and MI, and what the exact role of FXI on MI is, still requires further exploration.

Atrial fibrillation (AF) is the most common sustained cardiac rhythm disorder and is associated with a prothrombotic state. It was shown in a cohort study that in long-term follow-up, the FXIa level in circulating blood has been associated with poor prognosis such as IS and cardiovascular death in AF patients on anticoagulants.[48] Recently, the FXIa inhibitor asundexian at two doses (20 and 50 mg daily) showed lower bleeding rates than the active comparator, the FXa inhibitor apixaban 5 mg, in a phase II trial in AF at risk

for stroke. However, it still remains to be further investigated to what extent inhibition of FXI(a) is equally or more effective than established direct oral anticoagulants (DOACs) to prevent thrombotic events and if they could improve long-term prognosis of AF.[49] Current clinical studies testing the efficacy and safety of different types of FXI inhibitors, or FXI-lowering agents, is discussed further on in this article.

Potential areas for future investigation:

- A possible therapeutic target to prevent thrombo-inflammation occurring in the heart is the direct targeting of FXI or FXII that both can bind to platelets that concentrate both factors through their GPIbα and PAI-1 surface proteins and thereby increasing thrombin generation. A potential drawback of targeting in particular FXII is the increased risk for infection. Patients who are receiving FXI/FXII inhibitor treatment should therefore be monitored on a regular basis for markers of infection or inflammatory disease, such as concentration of complement fragment C1q in soluble plasma.
- It remains to be investigated in clinical trials what exactly the differences are between inhibition of FXI and FXII and whether there is any redundancy to targeting prekallikrein. Also, potential mechanisms of bypassing FIX activation should be elucidated beforehand.
- It is still unclear whether possible therapeutic options against cardiovascular thrombosis would also be suitable for treating or preventing thrombotic events in the management of aortic valve stenosis. One major risk factor for aortic valve stenosis patients is acquired vWF syndrome, which is directly related to disease severity. In this condition, vWF becomes proteolytically cleaved by high shear forces as it passes the stenotic valve. This results in a higher bleeding risk for patients of aortic valve stenosis that is not easy to measure.
- Another possible treatment strategy for preventing hypercoagulation in the heart might be drugs targeting TF or FVII, but to avoid bleeding, a safer approach is the targeting of TF signaling pathways. Also, inhibitors of TF/FVII, such as NAPc2,[50] could be repurposed as anti-inflammatory or antifibrotic drugs.

Bone Marrow: Role of Coagulation in Cell Trafficking

Following hematopoietic stem cell transplantation (HSCT), the blood and immune system take a long time to regenerate. This period is dangerous since patients have a low ability to mount an immune response and are at a high risk for life-threatening infections and internal bleeding. Therefore, finding novel ways to shorten the recovery time will reduce morbidity and mortality rates post HSCT. Previously the role of coagulation-associated pathways in the regulation of murine hematopoietic stem and progenitor cell (HSPC) maintenance within the BM has been described.[51–55] Importantly, these pathways also regulate the mobilization of human HSPC in healthy stem cell donors, and moreover, impact the neutrophil and platelet engraftment rates of patients post HSCT.[56] In particular, the involvement of PAR1, the major thrombin receptor in human HSPC regulation, was shown through analysis of peripheral blood samples obtained from 20 healthy HSPC donors before and after treatment with G-CSF. Overall, the baseline levels of PAR1 expression on circulating mononuclear cells (MNCs) before G-CSF treatment positively correlated with higher yields of total G-CSF-mobilized leukocytes and CD34+ HSPC. To further assess the requirement for functional PAR1 signaling in human HSPC mobilization, chimeric immune-deficient mice were utilized, pre-engrafted with human cord blood HSPC. Importantly, blocking PAR1 signaling by in vivo administration of a specific PAR1 antagonist inhibited G-CSF-induced mobilization of human white blood cells and CD34+ HSPC to the circulation of chimeric mice. Migration, homing, engraftment, and mobilization of human HSPC are dependent on the chemokine CXCL12, which is highly expressed in the BM, and its major receptor CXCR4, which is expressed by human HSPC. Importantly, in vitro migration of human HSPC toward a gradient of the chemokine CXCL12 was inhibited by blocking PAR1, suggesting a role in human HSPC migration and engraftment. Indeed, by following recovery parameters of patients transplanted with G-CSF-mobilized cells, accelerated neutrophil and platelet engraftment in patients transplanted with mobilized cells expressing higher PAR1 levels on MNC at baseline was demonstrated. Utilizing functional preclinical murine models, the importance of the thrombin/PAR1/nitric oxide (NO) axis as a crucial regulatory pathway mediating G-CSF-induced mobilization was demonstrated.[57] The most primitive, BM retained, long-term repopulating hematopoietic stem cells (HSCs) express endothelial protein C receptor (EPCR). Its major ligand, aPC, is also produced in the BM. Signaling via the APC/EPCR/PAR1 axis controls BM HSC adhesion and retention via NO inhibition and activation of adhesion interactions. In contrast, G-CSF activates NO generation in HSPC, EPCR shedding from their surface, which leads to their mobilization. Importantly, EPCR expression is essential for chemotherapy resistance of normal mouse[53] and human HSC[58] via adhesion interactions suggesting that, unfortunately, EPCR also protects human acute myeloid leukemia stem cells from radio- and chemotherapy treatments. To conclude, Nevo and colleagues identified a new player participating in the regulation of human HSPC, with potential to predict efficiency as well as clinical outcome of G-CSF-induced mobilization, homing, and engraftment kinetics as well as efficiency.

Potential areas for future investigation:

- Assess the clinical importance of PAR1 by validating its role in autologous HSPC transplantation setting, where the main difficulty is harvesting mobilized HSPC from heavily chemotherapy-treated patients.
- Manipulating PAR1 expression in human HSPC to improve the efficiency of mobilization and prognosis of HSPC-transplanted patients.
- Analyze the role of coagulation proteases in G-CSF-induced mobilization.

Kidney: The Coagulome in Kidney Disease

The loss of the microvasculature, also referred to as microvascular rarefaction, is a critical determinant in kidney disease states such as acute kidney failure, diabetic

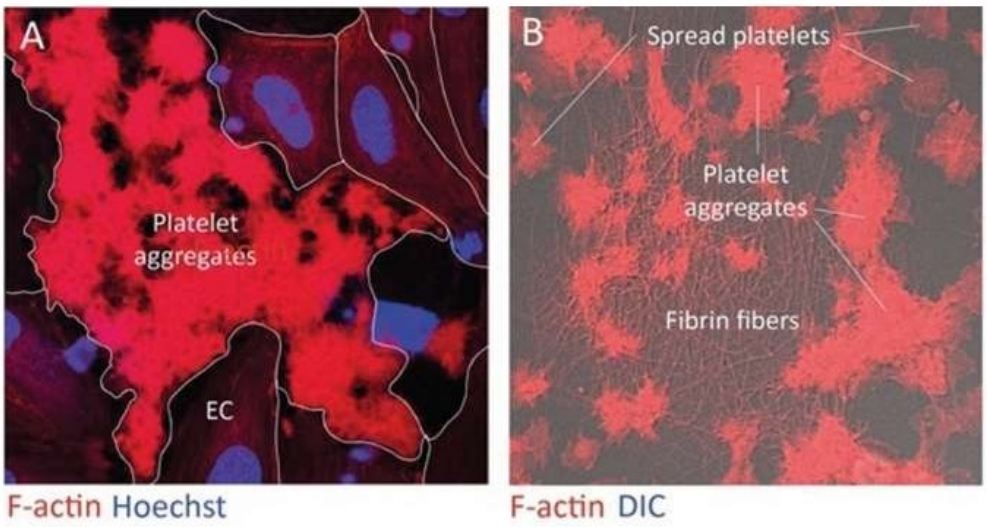


Fig. 1 Platelet-rich plasma rotation perfusions on TNFα-treated monolayers of human umbilical vein endothelial cells. After 15 minutes the cultures were fixed and stained for platelets (F-actin), nuclei (Hoechst), and (right panel) fibrin (antifibrinogen antibody). (**A**) Platelets selectively adhere to the extracellular matrix exposed in gaps that appeared between the endothelial cells upon overnight exposure to TNFα. Subsequent analyses demonstrated the platelets display all characteristics of "coated platelets." (**B**) Fibrin fibers confirm the activation of the coagulation system at the site of platelet adhesion. TNFα, tumor necrosis factor α.

nephropathy, or kidney transplant rejection.[59] The resulting ischemia is a driver for an inflammatory response that is associated with increased expression of profibrotic mediators such as TGFβ or CTGF (connective tissue growth factor; CNN2) that ultimately contribute to chronic kidney failure. Pericytes are essential functional components of the microvasculature stabilizing the capillaries through multiple reciprocal interactions. A key mechanism in microvascular rarefaction is the dissociation of pericytes from the capillary ECs[60] subsequent to inflammatory or pro-angiogenic stimuli[61] such as tumor necrosis factor-α, vascular endothelial growth factor, or a disbalance in the circulating levels of angiopoietin(ang)-2 over ang-1.[62] Conditions associated with ischemia can rapidly upregulate TF expression by vascular EC and elicit a pro-coagulant response through activation of the endothelial PARs. As a consequence, activated ECs lose their cell–cell contacts, dissociate from the pericytes, and engage in an angiogenic response, all processes that can promote microvascular rarefaction. For instance, in AF, the disbalance between supply and the excessive need for oxygen by the fibrillating myocytes leads to a state of hypoxia[5] that promotes subendothelial TF expression. Therefore, a role for the coagulome in the microvascular rarefaction that drives the pro-fibrotic substrate for AF is under active investigation. For instance, a recent paper by Dólleman et al explored the impact of DOACs on vascular integrity in vitro using platelet-free plasma in thrombin generation and endothelial barrier assays.[63] Interestingly, they demonstrated that while the anti-FXa DOAC rivaroxaban and the anti-thrombin DOAC dabigatran are both efficient in blocking their target proteases, rivaroxaban could preserve endothelial barrier function while dabigatran failed to protect endothelial integrity. The barrier disrupting effect of dabigatran could be prevented in the presence of a custom-made peptide that blocks thrombin's exosite-I. The take-home message of this study is that selective use of DOACs could well have a favorable impact on long-term (micro-)vascular health.

Many studies have shown that activation of the coagulation system and platelets go hand in hand. In mouse models of kidney I/R injury, platelets rapidly adhere to the ischemic (micro-)vasculature. Using an in vitro model, it was demonstrated that platelets predominantly adhere to the (TF-rich) EC matrix where gaps were formed resulting from the loss of EC–EC contacts in cultured monolayers (►**Fig. 1**). Using this model of perfusion of platelet-rich plasma, it was demonstrated that the adhered platelets markedly stimulated the generation of FXa depending on the presence of phospholipids, TF, and TFPI (Dolleman et al, manuscript in preparation). Subsequent studies revealed that the adhered platelets resemble the so-called coated platelets[64] that, due to dual activation, highly express P-selectin, TF, TFPI, and heparinase. These data strongly support a potential role for platelets in ischemia-driven microvascular rarefaction. This could be particularly relevant for patients with diabetic nephropathy. In fact, recent data show that platelets can be detected in the glomeruli of patients with diabetic kidney disease. Moreover, a direct relation was observed between platelet-derived extracellular vesicles and the degree of albuminuria in these patients.[65] Subsequent mechanistic studies in a mouse model for diabetic nephropathy demonstrated that the platelet P2Y12 inhibitor ticagrelor could counteract disease progression by lowering albuminuria, mesangial matrix expansion, macrophage infiltration, and fibrosis.[66] Future studies with selective platelet inhibitors such as GLP-1 analogues[67] could well augment our

therapeutic options in progressive ischemia-associated diseases of the kidney.

Potential areas for future investigation:

- While equally effective in anticoagulant activity, selective use of DOACs could have long-term beneficial effects for microvascular complications in chronic kidney disease patients. These in vitro findings should be validated by in vivo animal and clinical studies.
- The long-term benefit of the use of selective platelet inhibitors by patients with diabetic nephropathy warrants clinical investigation.

Coagulation in Endothelial Cell Barrier Function

Hyperlipidemia results in LDL/APOB-containing lipoprotein accumulation in the artery walls, promoting vascular inflammation, EC dysfunction, and localized loss of endothelial barrier function. Recent works have highlighted the extensive crosstalk between coagulation and inflammation in such diseases in which EC dysfunction serves as a hallmark.[68–72] Yet, the inciting factors for inflammation in hyperlipidemia remain unclear. Studies have shown that inhibiting FXI reduced inflammatory markers in mouse and nonhuman primate models of either acute and chronic inflammation.[31,73–75] Translating this to patients, it has recently been shown that pharmacological inhibition of FXI reduces inflammatory markers, including the hallmark biomarker C-reactive protein (CRP), in a clinical trial in end-stage renal disease patients on hemodialysis.[76] Follow-on studies are underway to evaluate whether use of FXI inhibition for the prevention of catheter-associated thrombosis similarly blunts the rise in CRP levels following placement of an indwelling catheter, which would provide further evidence of a link between the FXI activation and inflammation (ClinicalTrials.gov #NCT04465760). Continuing this theme, preliminary studies in a primate model of diet-induced hyperlipidemia show that the elevated CRP levels in an obese cohort were reduced by approximately 25% following 4 weeks of anti-FXI therapy. Defining the mechanisms by which FXI plays a role in propagating inflammation will provide insight into whether FXI inhibition has potential therapeutic anti-inflammatory benefits in cardiovascular disease and, in particular, hyperlipidemia.

Vascular endothelium serves as a site of catalysis for enzymatic reactions, while also facilitating multiple pathways that maintain blood cells in a quiescent state. As such, EC dysfunction is common in inflammatory diseases, such as atherosclerosis, and often appears early on in the course of the disease.[77,78] Recent observations have shown that FXI inhibition preserves endothelial barrier function in mice and primates in vivo,[31,75] suggesting that the EC surface may serve as a source or a sink for FXIa activity (►**Fig. 2**). Mechanistic studies discovered that the anticoagulant role of the endothelium includes sequestration of FXIa activity.[79] Next, it was determined that FXIa is inactivated by complex formation with vascular EC-derived PAI-1. It was found that FXIa–PAI-1 complexes were either released into the media or trafficked to EC endosomes and lysosomes in vitro (►**Fig. 2**). In a nonhuman primate model of lethal systemic inflammatory response syndrome (SIRS) associated with sepsis, the authors were able to detect FXIa–PAI-1 complexes in the circulation after a bacterial challenge.[79] In preliminary studies, it was found that inactivation of FXIa by PAI-1 on the EC surface may invoke a signaling pathway to increase vascular permeability by way of cleavage of EC VE-cadherin. Taken together, these data suggest that the kallikrein–kinin system, and, in particular, FXI, act as a nexus between the coagulation cascade, inflammation, and EC barrier function. This work holds promise to provide rationale for FXI inhibition as a useful approach for protecting barrier function in settings characterized by inflammation such as hyperlipidemia.

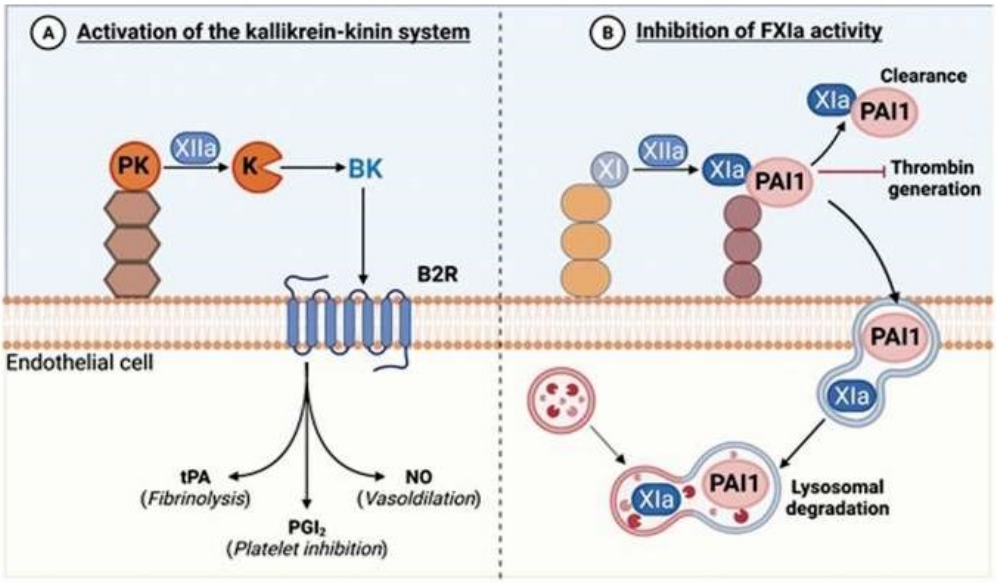


Fig. 2 Endothelial cells promote (A) the activation of the kallikrein–kinin system while (B) inhibiting FXIa activity.

Potential areas for future investigation:

- To determine whether the ability of FXI to act "upstream" and activate FXII contributes to activation of the kallikrein–kinin system to promote inflammation.
- To explore if FXI activation or activity directly regulates EC barrier (dys)function.

Theme 2: Novel Mechanisms of Thrombosis

The Relevance of Factor XII?

FXIIa is a serine protease consisting of a heavy and a light chain held together by a disulfide bond. It auto-activates upon contact with negatively charged compounds (e.g., glass, kaolin, and diatomaceous earth), as well as biological negatively charged molecules (e.g., DNA, RNA, misfolded proteins, polyphosphates). Substrates of FXIIa include proteins involved in coagulation, inflammation, fibrinolysis, and angiogenesis. Surprisingly, however, its deficiency in humans has not been associated with an overt pathological phenotype. Nevertheless, a cohort study found FXII levels to be inversely associated with overall mortality, although not for those at the lowest levels.[80] These apparently contradictory findings have stirred the debate on the physiologic functions of FXII (►**Fig. 3**).

Involvement of FXII in human hemostasis is based on its essential role in contact-activated in vitro coagulation assays. Moreover, its concentration in blood is higher than any other coagulation factor from the contact activation system (e.g., ~10-fold higher than FXI). This stands in stark contrast to the lack of a bleeding phenotype in FXII-deficient humans and knockout mouse models. Thus, the question arises: is FXII really a coagulation factor? To explain this discrepancy, it has been hypothesized that FXII might only be involved in hemostasis of soil-contaminated wounds, where it is activated by negatively charged silicates. This so-called "dirty wound theory" is based on the observation that marine animals lack FXII, in contrast to land-based animals.[81] From an evolutionary perspective, particularly the absence of FXII in sea mammals suggests its redundancy in wounds which are continually cleaned by surrounding water. This theory is supported by experiments in FXII-knockout mice, where hemostatic differences between clean and soil-

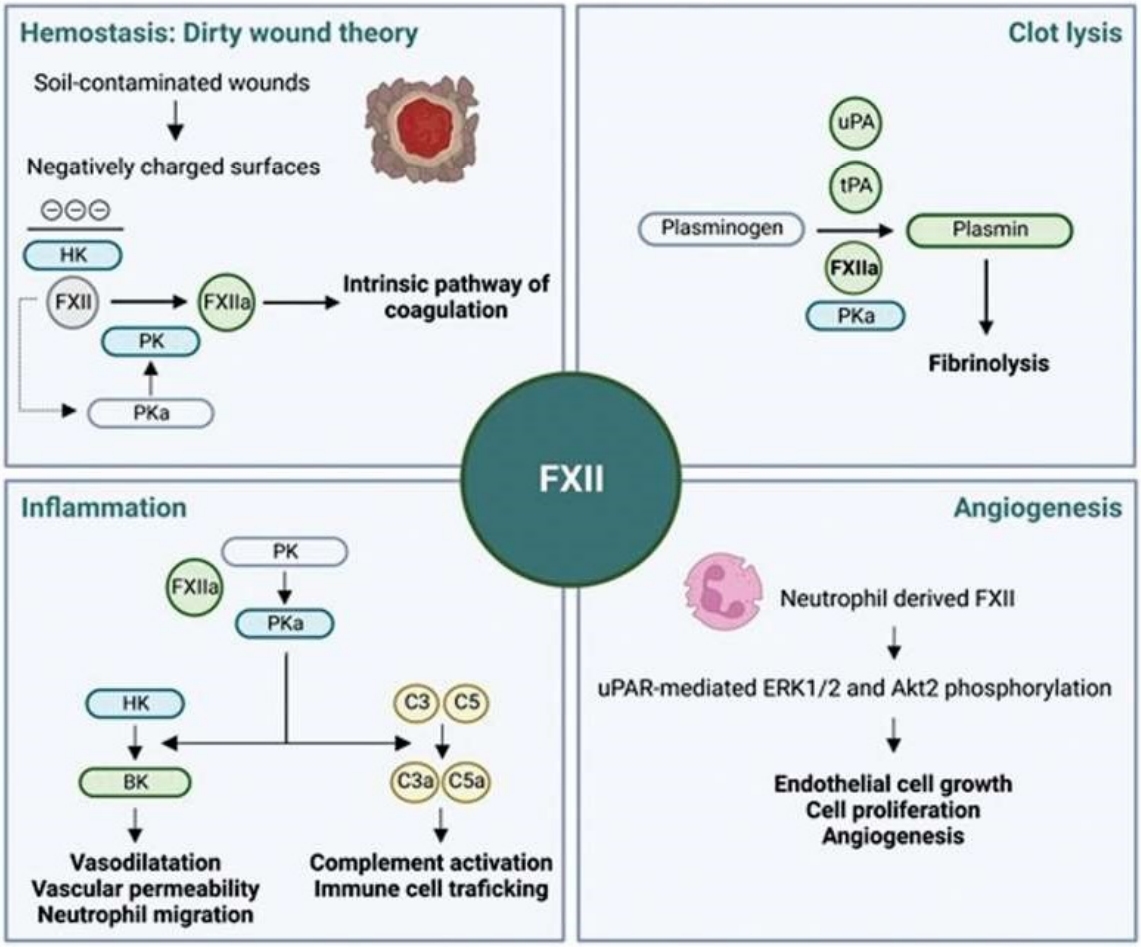


Fig. 3 Potential physiological role of factor XII. BK, bradykinin; C3(a), (activated) complement factor 3; C5(a), (activated) complement factor 5; FXII(a), (activated) coagulation factor XII; HK, high-molecular-weight kininogen; PK, plasma prekallikrein; PKa, plasma kallikrein; tPA, tissue plasminogen activator; uPA, urokinase plasminogen activator.

contaminated wounds were observed.[82] Future studies will have to establish if these differences also have physiological relevance in humans.

While its hemostatic role in wound healing remains uncertain, activated FXII is known to trigger the formation of kallikrein and bradykinin release, which stimulates vasodilation, vascular permeability, neutrophil migration, and complement activation contributing to the immune defense in the wound site. Interestingly, excess FXIIa levels are observed in a genetic disease called hereditary angioedema (HAE), characterized by recurrent episodes of severe edema due to extreme bradykinin release.[83] It is caused either by a FXII mutation causing increased autoactivation or a deficiency of its main inhibitor, C1 esterase inhibitor.[84] Another mutation of FXII resulting in spontaneous auto-activation has been identified as the cause of a rare disorder termed FXII-associated cold autoinflammatory syndrome (FACAS), which is characterized by cold-induced urticaria, arthralgia, chills, headaches, and malaise.[85] These phenotypes of HAE and FACAS both support the notion that FXII is mainly involved in regulating inflammation and vascular permeability.

Notably, patients with HAE or FACAS are not reported to have increased thrombosis risk, despite the underlying uncontrolled FXII activation. This begs the question: can FXIIa "choose" to have enzymatic activity for a certain substrate? Unraveling of this question will require further molecular insight into FXII. Currently, this protein is thought about as a "string of pearls" with five domains linked to the protease domain by a proline-rich region. However, the natural confirmation of FXII is most likely very different and our understanding of individual domains is limited. Molecular research will have to establish in what ways this protein can be activated and interact with its substrates, which might explain distinct enzymatic activity in different conditions.

Furthermore, FXII has been implicated in the fibrinolytic system based on its high degree of homology with tissue plasminogen activator (tPA). Indeed, in vitro experiments have shown that FXIIa can convert plasminogen to plasmin and enhance fibrinolysis, but its rate is much lower than that of tPA or urokinase plasminogen activator (uPA).[86] Therefore, the relevance of this enzymatic activity in vivo remains to be established. Conversely, however, plasminogen was found to influence pathways of FXII presenting as HAE in the setting of a rare plasminogen mutation (HAE-PLG).[87]

Finally, although FXII is mainly secreted by the liver, there is growing evidence for a separate pool of leukocyte-expressed FXII that contributes to wound healing and angiogenesis.[88] This was found to be mediated by unactivated FXII signaling through the uPA receptor, stimulating processes such as EC growth and proliferation. This more recent finding highlights the variety of roles FXII has in human physiology, some of which might still need to be uncovered.

In conclusion, although clinical data on FXII do not support a pivotal role in hemostasis or thrombosis, new perspectives regarding the role of FXII have been discovered in the last two decades. These include a role in inflammation, fibrinolysis, and angiogenesis, with novel pathways downstream of FXII still pending to be elucidated.

Potential areas for future investigation:

- To establish why gain-of-function mutations in FXII lead to an inflammatory, but not a thrombotic state.
- To further delineate the relationship between structure and function of FXII.

Biomechanics of Fibrin and Fibrin Clot Lysis

In both physiological and pathological conditions, thrombi are subjected to extreme mechanical forces such as wound stretch, clot contraction, or shear stress. Yet, thrombi manage to retain their structural integrity through a remarkable combination of compliance and resilience. These characteristics are understood to be provided by the fibrin network, which forms the primary scaffold of clots. Fibrin networks can reversibly stretch up to approximately 150%, resist elongation of several hundred percent, and stiffen by at least two orders of magnitude before rupture.[89,90] Biophysical studies over the past decade have shown that these unique mechanical features stem from the complex structure of fibrin fibers, which are bundles of protofibrils that are themselves double-stranded filaments of fibrin molecules. Consequently, fibrin networks undergo several phases of stretch at different structural levels (►**Fig. 4**).[91]

At first, stretch causes the natively disordered and hence flexible αC-domains of fibrin molecules to straighten, which allows elongation (i.e., strain) with almost no increase in resistance to deformation (i.e., elastic modulus). Next, the fibers gradually align in the direction of strain, which is accompanied by a strong increase of the elastic modulus. Finally, the strain is transferred to the folded domains of the fibrin monomers, which results in a further linear increase of the modulus. If the strain continues to be increased, however,

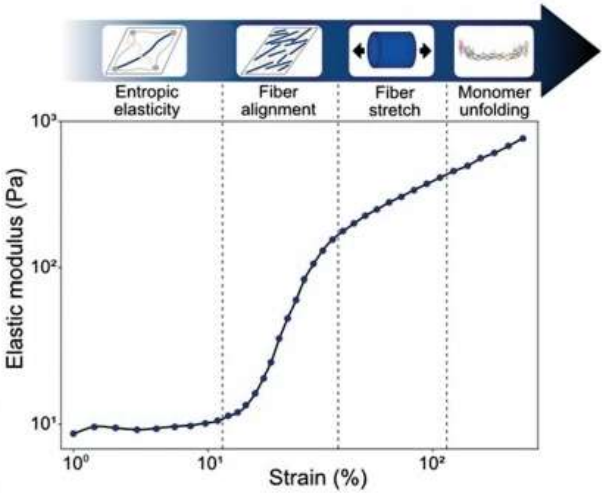


Fig. 4 Elastic modulus of a fibrin network as a function of strain, measured by shear rheology. Fibrin forms a soft elastic network at low strain, and stiffens 100-fold in various stages marked by the vertical dashed lines when the strain is increased. The stages correspond to entropic elasticity (low strain), strain-induced fiber alignment, fiber backbone stretching, and finally fibrin monomer unfolding.

the folded monomer domains start to unfold, which provides irreversible elongation, and eventually leads to rupture.

These insights into fibrin mechanics have only recently been acquired by applying novel methods based on rheology combined with in situ X-ray scattering or vibrational spectroscopy complemented with single-fiber and single-molecule stretching assays.[90–92] This mechanistic understanding of fibrin mechanics is ready to be used now to assess the role of mechanical forces in thrombotic and bleeding disorders. Thus far, clot characteristics have mainly been studied using microscopy. These studies found that patients with MI, IS, VTE, and recurrent episodes are characterized by in vitro fibrin clots with a dense network and thinner fibers.[93] Such clots are known to be less permeable, making them less susceptible to fibrinolysis, which could explain associations to adverse outcomes. However, dense clots are also known to be stiffer, which potentially increases thrombus obstructiveness or embologenicity, giving an alternative explanation for differences in outcomes. In contrast, patients with hemophilia A and B were found to have clots with loose networks and thick fibers, which might make them more prone to bleeding events due to the fragility of such clots.[94,95]

These findings suggest that mechanical phenotyping of clots is a promising avenue for future research. It might provide parameters that can contribute to more accurate diagnosis and risk stratification, mirroring the use of mechanical phenotyping for connective tissue disorders and cancer.[96] Also, it could give rise to novel therapies using pharmacological or mechanical interventions that influence thrombus mechanics to, for example, improve outcomes of endogenous or therapeutic thrombolysis. However, much still needs to be elucidated about clot mechanics and the role of the fibrin network in physiology and pathology. Experiments on fibrin have mostly been performed in purified systems. This means it is largely unknown how fibrin interacts with other clot components such as platelets and red blood cells, and hence how thrombus composition and spatially heterogeneous structure affect thrombus pathologies (e.g., platelet- vs. fibrin-rich thrombi).[97,98] In summary, the integration of biophysical research into the field of thrombosis and hemostasis is bound to bring fascinating fundamental insights and clinically relevant advances in the near future.

Potential areas for future investigation:

- The interplay of macromolecules including fibrin and different cell types (platelets, red blood cells) in clot mechanics and sensitivity to lysis.
- The relation between different mechanical properties of thrombi (stiffness, viscoelasticity, plasticity, rupture strength) and the risk of embolization and sensitivity of clots to lysis.
- Evaluate the potential of mechanical phenotyping of thrombi, either collected by thrombectomy or reconstituted from patient plasma, for diagnosis and risk stratification.
- Evaluate the potential of mechanical phenotyping of thrombi to assess the efficacy of novel therapies using pharmacological or mechanical interventions that influence thrombus mechanics to improve outcomes of endogenous or therapeutic thrombolysis.

The Microbiome and Thrombosis

In contrast to acute inflammatory conditions in case of viral infections, the gut microbiota is a driver of low-grade inflammation, chronically impacting vascular inflammation.[99] Dependent on host nutrition, microbiota-derived products constantly leak into the portal circulation, with signaling-active molecules and metabolites reaching the hepatic microcirculation.[100] Studies on germ-free mouse models clearly demonstrate that the transcriptome of the liver sinusoidal endothelium is broadly influenced by gut microbial colonization, with the sphingolipid synthesis pathway recently identified as one of the primarily affected microbiota-modulated pathways.[101,102] Furthermore, vWF expression in the hepatic endothelium is augmented by the presence of gut commensals.[103] Another example is the sensitivity of neutrophils toward lipopolysaccharide (LPS)-induced neutrophil extracellular traps (NET)osis, which was attenuated by the presence of gut commensals.[104] Importantly, several experimental and clinical studies unveiled the gut microbiota as a novel risk factor for cardiovascular disease and arterial thrombosis.[103,105–107] Interestingly, under low-cholesterol diet conditions, germ-free *Apoe*-deficient and germ-free *Ldlr*-deficient mice had elevated plasma cholesterol levels and *Apoe*-deficient mice presented increased atherosclerotic lesion size, an effect that was abolished at high-cholesterol diet feeding.[108–110] Interestingly, in the germ-free *Apoe*-deficient mouse atherosclerosis model, *Roseburia intestinalis*, due to its production of the short-chain fatty acid butyrate, has a protective role in atherogenesis.[111] Another microbiota-derived metabolite related to cardiovascular risk and arterial thrombosis is trimethylamine (TMA), a choline metabolite produced by TMA-lyase enzymes and converted to trimethylamine-N-oxide (TMAO) by flavin-dependent monooxygenase-3 in the liver.[112–114] The metaorganismal TMAO-pathway was demonstrated to promote arterial thrombus growth via multiple pathways, including the induction of platelet hyperreactivity and vascular endothelial TF expression.[106,115] Of note, in a translational pig model it was recently demonstrated that the reduction of dietary fat for a time period of 30 days, resulting in reduced plasma cholesterol levels, was able to revert dysbiosis of the fecal microbiome and to reduce plasma TMAO levels,[116] a predictive functional marker for adverse cardiac events.[117] Vascular innate immune signaling, triggered by microbial-associated molecular patterns derived from the intestinal microbiota, for instance by the activation of endothelial Toll-like receptor-2 signaling in the liver resulting in enhanced vWF synthesis, is an additional mechanism linking the gut microbiota with enhanced arterial thrombus growth.[5] In contrast to germ-free mice, colonized mice showed increased ADP-induced GPIIb/IIIa activation and elevated adhesion-dependent phosphatidylserine exposure, promoting arterial thrombus growth.[102,118] Intriguingly, gut microbial diversity might even affect

the disease, as has been reported in postmortem studies of patients with acute respiratory distress syndrome due to pathogens invading the respiratory tract and provoking an inflammatory response associated with acute lung injury.[147]

Immunothrombosis, if balanced, is a physiological role in host defense. The term describes the microvascular thrombotic response that facilitates microbe containment and elimination, a critical component of innate immunity.[148,149] The pathological entity from immunothrombosis is in situ pulmonary thrombosis which is a different entity from the embolic events from deep vein thrombi which are a net result of thromboinflammation.[150] As part of any inflammatory response to attenuate microbial invasion, microcirculatory thrombosis also produces multiorgan injury.[151,152] These important host defense mechanisms have been described, but with the ongoing pandemic and massive numbers of COVID-19 patients who manifested lung or multiorgan dysfunction, the concept of immunothrombosis was increasingly reported.[148] In summary, although thromboinflammation and immunothrombosis have many similarities, they should not be used as interchangeable counterparts, even if they have been used synonymously in the past.

Long COVID defined as long-lasting multiorgan symptoms that last for weeks to months after SARS-CoV-2 infection is associated with cardiovascular manifestations including peri-myocarditis. If and how in situ thrombosis does play a role in long COVID is still unanswered, and studies are ongoing. Currently there is no guided therapy for long COVID other than anecdotal reports and further studies are needed to unravel the underlying mechanisms.[153]

Potential areas for future investigation:

- Determine the viral or inflammatory triggers for either thrombosis and/or bleeding.
- The role of vascular bed-specific hemostasis in viral infections.
- Study the role of inflammatory components, i.e., virus-specific T-cells in the initiation and regulation of the hemostatic balance.
- Determine better ways of translating results from the homogeneous [experimental] models into clinical practice, or heterogeneous reality to improve the timing and type of therapeutic interventions.

Theme 3: How to Limit Bleeding Risks: Insights from Translational Studies

Genetics and Bleeding Disorders

Hemostasis is controlled by interplays between platelets, coagulation, and fibrinolysis; their normal function is to prevent bleeding. Genetic variants in genes that encode for regulators of these three processes are known to cause inherited forms of bleeding. The summary deals with the use of next-generation sequencing (NGS) approaches for diagnostic and gene discovery. To date, almost 100 curated disease-causing genes have been identified to cause inherited bleeding, platelet, or thrombotic disorders (www.isth.org/page/GinTh_GeneLists).[154] This is a dynamic list that is yearly updated as since 2011; 25 novel genes have been discovered using NGS approaches.[155] This gene list is useful for clinical laboratories that have implemented multigene panel tests to diagnose inherited bleeding disorders. This is a cost-effective approach to rapidly screen patients. The international study ThromboGenomics has shown that the diagnostic rates obtained for thrombocytopenia, platelet function, and coagulation disorders are 47.8, 26.1, and 63.6%, respectively, while this rate drops to 3.1% for patients with unexplained bleeding disorders (having normal laboratory test parameters) using a multigene panel test.[156] These differences can be explained by the inclusion criteria and the quality of the laboratory test that detects the abnormality. Patients with abnormal test data for (anti-)coagulation parameters or with low platelet counts are easy to identify, and genetic variants are often associated with such defects. Still, genetic variants were also detected in some patients with normal laboratory parameters where these assays were unable to detect the defect. In contrast, the genes for the platelet function disorder "storage pool disease" or having unexplained bleeding disorder are still unknown and therefore, screening with a multigene panel test is not useful as exemplified by causing a very low diagnostic rate in the ThromboGenomics study. Of interest is the unexpected finding of oligogenic inheritance where patients have more variants in more than one gene. Today, this field still struggles with the detection of numerous variants of unknown significance (VUSs) that cannot be used in clinical practice.[157] These VUSs require further functional and genetic studies to prove pathogenicity. Rapid screening models and data exchange with the community could improve the variant classification.

International studies BRIDGE-BPD and NIHR BioResource have used whole-exome sequencing (WES) and whole-genome sequencing (WGS) for the discovery of novel genes for bleeding disorders.[158,159] Success rates are typically high if screening consanguineous or very large pedigrees, or if more families have been recruited with similar gene phenotypes. Even if the genetic defect is discovered, it can take several years to understand the disease as illustrated for SRC-related thrombocytopenia.[154] Five years after the discovery of the SRC gain-of-function variant E527K, the same variant was detected in other pedigrees that helped to delineate the syndromic phenotype associated with thrombocytopenia and RNAseq provided evidence for defective interferon regulation as underlying cause.[154] Still many patients do not receive a diagnosis even though their complete genome has been analyzed. This can be explained by the fact that each genome contains numerous unique coding variants and the noncoding regions are very difficult to analyze due to the lack of information about regions of interest (promoter or regulatory regions) versus junk DNA. An additional layer of information will be critical to understand noncoding variation. Therefore, blood cell RNAseq will be performed for patients who do not have a diagnosis but from whom WGS data are available. Gene expression and splicing analysis will assist in the understanding of variants that influence these processes as the cause of a bleeding disorder.

Potential areas for future investigation:

- Oligogenic inheritance is unexplored in our field. It is currently not understood what the clinical relevance is of combining common and rare variants in different known genes that modify bleeding and thrombosis risks. This might be relevant for molecular diagnostics as it is known that single variants can result in a different clinical severity of a certain disorder.
- Some patients present with obvious clinical bleeding phenotypes but have normal laboratory test data. Genetic causes still remain unknown for such patients as it is very difficult to find causative genes if no idea of the underlying defective pathway is known. It might be necessary to develop better laboratory assays to study such patients and these should include ECs that are currently not studied.
- In addition to the currently used WES and WGS, other OMICS methods will be required to explore disease mechanisms and enhance gene discoveries. Novel statistical methods that can combine OMICS results will be required to address these needs.

Genetics and Antithrombotics: Toward Individual Drug Tailoring

Personalized Antithrombotic Therapy Based on Genetic Testing

Besides several other factors (i.e., body weight, diabetes, etc.) genetic polymorphisms play a role in the variable response of drugs in patients.[160] Therefore, genetic testing may influence the efficacy and/or safety of antithrombotic treatment, and thus optimize patients' outcomes.

Genetic Testing

When a nucleotide change in a gene is present in more than 1% of a population, it is called a genetic polymorphism. These polymorphisms often affect the drug-metabolizing cytochrome P450 (CYP450) enzymes, which play a role in activation or deactivation of a drug.[161]

In patients with ACSs or undergoing percutaneous coronary intervention (PCI), DAPT with aspirin (acetylsalicylic acid) and a P2Y12 inhibitor (ticagrelor, prasugrel, and clopidogrel) is the cornerstone of medical therapy to prevent the recurrence of thrombotic events including stent thrombosis.[162] Ticagrelor and prasugrel are much stronger than clopidogrel and have shown reduced thrombotic events in large outcome trials.[163,164] However, the reduced thrombotic risk is counterbalanced by an increased bleeding risk.[163,164] In addition, it is well known that major bleeding has a similar impact on patient outcome as a recurrent thrombotic event, e.g., MI.[165,166] Aspirin is metabolized by different enzymes, but up to now none of the genetic polymorphisms has impacted clinical outcome.[167] Ticagrelor is a direct-acting drug, while both clopidogrel and prasugrel need activation by cytochrome CYPP450 genes.[168] The active compound of ticagrelor is metabolized by CYP3A4, which can also directly bind to the P2Y12 receptor.[169] Prasugrel is metabolized mainly by CYP3A4 and CYP2B6, and to some extent by CYP2C9 and CYP2C19; however, polymorphisms in these genes are not related with a heightened thrombotic risk.[170] However, clopidogrel is very much affected by polymorphisms which lead to less response in 30% of patients.[171]

In the two-step activation of clopidogrel process, multiple CYP enzymes play a role (CYP2C19, CYP3A4/5, CYP1A2, CYP2B6, CYP2C9) (**► Fig. 5**).[172]

CYP2C19 plays a role in both steps and is the greatest contributor in this metabolic process. The prevalence of the CYP2C19 polymorphisms (*2 and *3) is approximately 25% of the Caucasian population.[168] There are much data demonstrating that carriers of CYP2C19 LoF-alleles have a diminished antiplatelet response and therefore higher platelet reactivity (HPR).[173–176] This HPR translates to higher risk for thrombotic events, including stent thrombosis.[173,177]

Clinical Evidence for a Genotype-Guided Antithrombotic Therapy

Many studies have assessed a CYP2C19 genotype-guided strategy (escalating or de-escalating) in patients with coronary artery disease (CAD).[178,179] De-escalation means switching from a more potent drug (ticagrelor or prasugrel) to the less potent clopidogrel in extensive metabolizers, while escalation means switching from clopidogrel to ticagrelor or prasugrel in intermediate or poor metabolizers. De-escalation can be used in ACS, where standard treatment is ticagrelor. Escalation can be done in chronic coronary syndrome patients undergoing PCI, stroke or peripheral artery disease, where clopidogrel is standard treatment.

In the RCT the Popular Genetics, a genotype-guided de-escalation strategy was tested versus usual care in 2,488 patients undergoing primary PCI for STEMI. All patients were treated with aspirin, but in the genotype-guided group, intermediate and poor metabolizers were treated with ticagrelor or prasugrel (39%), and extensive metabolizers with clopidogrel (61%). Patients in the control group were all treated with ticagrelor or prasugrel. Genotype-guided P2Y12-inhibitor treatment reduced the bleeding risk (9.8 vs. 12.5%, hazard ratio [HR]: 0.78, 95% confidence interval [CI]: 0.61–0.98, $p = 0.04$) and there was no increase in thrombotic events.

In the RCT TAILOR-PCI, 5,302 patients undergoing PCI for ACS or stable CAD were randomized to genotype-guided escalation or conventional therapy (clopidogrel).[180] In the genotype-guided group, intermediate or poor metabolizers were treated with ticagrelor (31%), and the other patients were treated with clopidogrel (68%). The primary analysis was only in patients who were intermediate or poor metabolizers and did not show a statistical difference in cardiovascular death, MI, stroke, stent thrombosis, and severe recurrent ischemia at 12 months (HR: 0.66, 95% CI: 0.43–1.02; $p = 0.06$), but the reduced event rate suggests a clinical benefit with the genotype-guided group. There was also no significant difference in bleeding between groups. Despite the fact that the trial was underpowered to detect an effect size less than the prespecified expected 50% relative risk reduction, it showed a promising reduction in thrombotic risk of genotype-guided therapy. A meta-analysis including

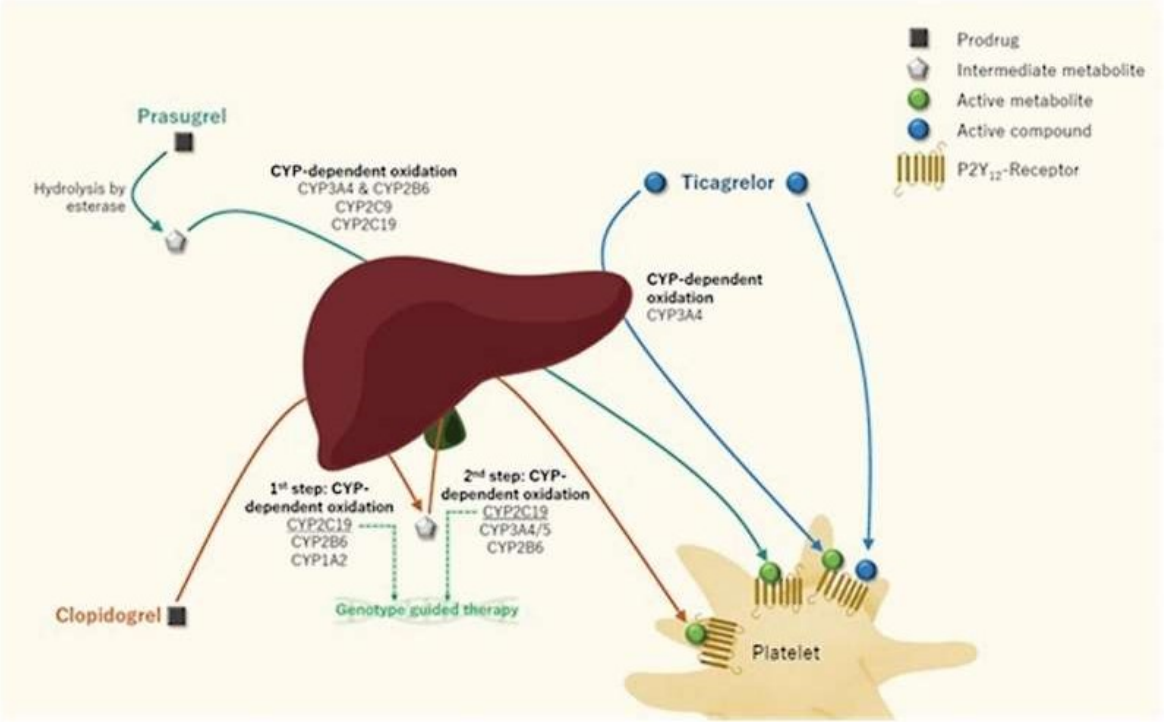


Fig. 5 Biotransformation and metabolization of the oral P2Y12-inhibitors. Antithrombotic therapy can be personalized by (1) using CYP2C19 genotype-guided therapy, which is the only genetic polymorphism for which a genotype-guided therapy is assessed in randomized clinical trials or (2) assessing the actual responsiveness to antiplatelet therapy by measuring on-treatment platelet reactivity, which is influenced by different modifiable and nonmodifiable factors.

15,949 patients with CAD showed that in intermediate or poor metabolizers, ticagrelor/prasugrel reduced thrombotic risk as compared with clopidogrel, but in extensive metabolizers there was no difference in thrombotic risk whether patients were treated with ticagrelor/prasugrel or clopidogrel.[178,179] Therefore, the Clinical Pharmacogenetics Implementation Consortium recommends to avoid clopidogrel in intermediate and poor metabolizers and use prasugrel or ticagrelor[178] (►**Table 1**). Nevertheless, genotype-guided antiplatelet therapy is not yet standard care in patients with CAD, although genotype-guided de-escalation of P2Y12 inhibition has a class IIb guideline recommendation and can be considered for ACS patients deemed unsuitable for potent platelet inhibition, i.e., with a high bleeding risk.[181]

Based on the above-presented evidence, some centers have implemented a genotype-guided strategy for P2Y12 inhibition.[182] Their results are in line with previous meta-analyses and thus promising.

Most evidence for genotype-guided antiplatelet treatment was obtained in patients with CAD. Nevertheless, other vascular patients sharing the same pathophysiology may also benefit from genotyping. A meta-analysis in patients with IS or TIA demonstrated that intermediate and poor metabolizers of clopidogrel have a higher risk of recurrent stroke.[183] These results are supported by the RCT CHANCE-2, demonstrating in 6,412 patients with acute IS or TIA, who were intermediate or poor metabolizers of clopidogrel, that ticagrelor reduced thrombotic risk as compared with clopidogrel.[184]

Clinical Rationale for Antagonizing Antithrombotic Agents in Bleeding Patients

Novel Reversal Agents

Although the DOACs have considerably improved anticoagulant treatment, the risk of bleeding is still present. Importantly, all bleeds are multifactorial in nature depending on an interaction of modifiable and nonmodifiable risk factors.[185,186] Furthermore, ethnic differences may play a role, as recently discussed for Asian populations and antithrombotic medication.[187] This implies that the presence of an anticoagulant, whether a vitamin K antagonist (VKA) or a DOAC, is merely a contributing factor, rather than a causal one.

Rapid reversal of the anticoagulant effect of DOACs may therefore be required in the case of life-threatening bleeding, emergency surgery, or severe trauma. Prothrombin complex concentrates (PCCs) and recombinant FVIIa (rFVIIa) have the ability to overcome the anticoagulant effects of DOACs. More recently, specific reversal agents have been developed that act as a decoy and scavenge the thrombin and FXa inhibitors. Idarucizumab is a monoclonal antibody fragment that tightly binds to and effectively counteracts the anticoagulant action of dabigatran.[188] For the FXa inhibitors, andexanet alfa was developed, a modified FXa molecule that lacks the phospholipid-binding Gla domain, and has its active site mutated to prevent enzymatic activity.[189] Both idarucizumab and andexanet alfa have been registered, although not everywhere in the world. Since both idarucizumab and andexanet

Table 1 Overview of the different CYP2C19 phenotypes with the coherent CYP2C19 diplotypes and the antiplatelet therapy recommendations when considering clopidogrel for cardiovascular indications

Phenotype	CYP2C19 diplotypes	Response to clopidogrel	Therapeutic recommendation
Ultra-rapid metabolizer (UM)	*17/*17	Normal or increased antiplatelet response to clopidogrel	If considering clopidogrel, use at standard dose
Rapid metabolizer (RM)	*1/*17	Normal or increased antiplatelet response to clopidogrel	If considering clopidogrel, use at standard dose
Extensive metabolizer (EM)	*1/*1	Normal antiplatelet response to clopidogrel	If considering clopidogrel, use at standard dose
Intermediate metabolizer (IM)	*1/*2, *1/*3, *2/*17 or *3/*17	Reduced antiplatelet response to clopidogrel	Avoid standard-dose clopidogrel. Use prasugrel or ticagrelor at standard dose if no contraindication
Poor metabolizer (PM)	*2/*2, *2/*3 or *3/*3	Significantly reduced antiplatelet response to clopidogrel	Avoid clopidogrel. Use prasugrel or ticagrelor at standard dose if no contraindication

alfa have to bind to their target, they have to be in excess of the circulating anticoagulant and consequently large quantities have to be administered, which is one of the reasons that their use is associated with high costs. Also, these agents are specific for their target and knowledge about DOAC intake has to be available before reversal can be initiated. The search for novel reversal agents for anticoagulant drugs is therefore continuing.

►**Table 2** summarizes the available reversal agents and several novel reversal agents that are currently under development. Scavenging proteins such as gamma-thrombin-S195A (for dabigatran or potentially other antithrombin anticoagulants)[190] and Gla-domainless FXa-α2-macroglobulin (for anti-FXa anticoagulants),[191] interact with the small-molecule anticoagulants and have been shown to be effective in vitro and in animal models. Alternatively, several hemostasis-enhancing proteins have been identified, characterized, and tested in vitro and in vivo. Examples for this approach are modified FX(a) molecules, such as FXa-I16L, FX-C and FX-Phe174, and so-called superFVa.

FXa-I16L is a FXa molecule that is zymogen-like and therefore resistant to active-site inhibitors.[192] Its activity is restored after binding to FVa and is thereby more potent than decoy FX molecules. Because of its potent hemostatic-enhancing effect, it not only counteracts FXa inhibitors, but also thrombin inhibitors. This variant has been tested in a phase 1 clinical trial, appeared safe and well-tolerated,[193] and demonstrated a dose-dependent procoagulant effect.

FX-C is a chimera of human FX with an inserted 99 loop of snake FX from *Pseudonaja textilis*.[194] This makes the molecule insensitive to FXa DOACs. Functionality has been proven in vitro and in vivo, and the molecule is currently undergoing phase 1 testing (source: VarmX Web site).

SuperFVa is an aPC-resistant FVa variant with three mutations: Arg306/506/679Gln.[195] In addition, a disulfide bond has been inserted between the A2 and A3 domains to enhance stability. SuperFVa improved thrombin generation in plasma and reversed bleeding by both FXa and thrombin inhibitors in mice.[196]

Apart from protein approaches, there are currently two small molecules in development as reversal agents.

Table 2 Overview of reversal agents. The agents are categorized in reversal of dabigatran (anti-IIa), anti-Xa anticoagulants or with universal action. Furthermore, the agents were divided in proteins or small molecules and by mechanism of action (decoy or non-decoy). (●): protein-based, decoy; (●): protein-based, non-decoy; (●): small molecule, decoy; (●): small molecule, non-decoy.

Anti-IIa	Anti-Xa	Hemostasis enhancing	Universal
Idarucizumab (●)	Andexanet alfa (●)	FVIIa (●)	Ciraparantag (●)
Gamma-thrombin-S195A (●)	GladomainlessFXa-alfa2M (●)	(a)PCC (●)	OKL-1111 (●)
		FXa-I16L (●)	
		FX-C (●)	
		FX-Phe174 (●)	
		SuperVa (●)	

Ciraparantag, a small molecule that specifically binds to the DOACs and heparin, acts rapidly and reduces bleeding induced by these anticoagulants in animals.[197] In humans, it is well tolerated.[198] Major disadvantage of the (clinical) use of ciraparantag is that it can only be monitored with a whole blood clotting time, since it binds to citrate in collection tubes and to clotting reagents that are normally used in the coagulation laboratories.

Another small molecule under development is OKL-1111. This is a cyclodextrin that does not initiate coagulation, but enhances thrombin formation in both the absence and presence of anticoagulants. In bleeding models in animals, it could be demonstrated that reversal was obtained toward DOACs, low-molecular-weight heparin, VKAs and clopidogrel (Meijers, unpublished observations) making it a truly universal reversal agent. Phase 1 studies are planned for 2023.

Potential areas for future investigation:

- Determine which of the characteristics of the novel reversal agents (specific or universal, small molecule or protein, decoy or nondecoy) will be leading in the choice for the best reversal agent.
- The next hurdle will be the demonstration of improved clinical outcome of novel reversal agents compared with PCC, idarucizumab, or andexanet alfa in patients presenting with serious bleeding or requiring urgent intervention or surgery.

Theme 4: Hemostasis in Extracorporeal Systems: The Value and Limitations of In Vitro Models

Assessing Thrombosis and Hemostasis Ex Vivo

Evaluation of the hemostatic process in preclinical as well as clinical settings becomes increasingly important in the assessment of the thrombotic or bleeding risk in patients. The routine hemostasis assays in the clinical diagnostic laboratory are imperative for the screening and diagnosis of hemostatic abnormalities and for monitoring the effectiveness of antithrombotic therapies, especially in high-risk patients. Although most widely used point-of-care assays like whole blood aggregometry and coagulation tests (prothrombin time, activated partial thromboplastin time [aPTT]) can detect severe hemostatic defects and effects of pro- and antithrombotic drugs, these assays lack sensitivity and fail to measure the interdependency of hemostatic pathways, i.e., platelet activation, coagulation, fibrin formation, and fibrinolysis, in clot formation[199–201] (►**Table 3**). In an effort to include as many components of the hemostatic system as possible, more robust and global assays were developed such as thrombin generation assays, viscoelastic assays (thromboelastography/-metry), and microfluidic models.[199,202,203] Some of the global assays, like thrombin generation and thromboelastometry, have demonstrated potential to improve the identification of patients on antithrombotic drugs who are at risk of bleeding.[204,205] Still, clinical applicability of these global assays is difficult due to (pre-)analytical variables, duration of test procedure, and interpretation of test results.

Microfluidic flow devices have been used for decades mainly in research, enabling the simultaneous assessment of platelet and coagulation activation under flow conditions.[206] In addition, endothelialized models allow to study effects of endothelial barrier function and endothelial activation on hemostatic processes, providing a more physiological approach to assess the risk of bleeding or thrombosis ex vivo (►**Fig. 6**).[207,208] This has improved patient diagnostics and our understanding of inherited or acquired hemostatic abnormalities tremendously. However, standardization of such assays and their (routine) use in clinical diagnostics

Table 3 Overview of hemostatic parameters and the corresponding clinical tests

Hemostatic factor/process	Corresponding test
Platelet adhesion	Platelet function analyzer (PFA)
Platelet secretion	Lumiaggregometry (ATP release) Flow cytometry (P-selectin)
Platelet aggregation	Aggregometry (e.g., light transmission aggregometry, multiple electrode impedance aggregometry) Platelet function analyzer (PFA) Flow cytometry
Coagulation	PT, aPTT, thrombin generation Viscoelastic methods (e.g., ROTEM, TEG) Coagulation factor determination
vWF	Platelet function analyzer (PFA) Platelet agglutination assay vWF antigen and activity assay
Hematocrit	Hematology analyzer
Shear-dependent platelet function	Platelet function analyzer (PFA)
Vasoconstriction	No test available, bleeding time is obsolete

Abbreviation: aPTT, activated partial thromboplastin time; PT, prothrombin time; ROTEM, rotational thromboelastography; TEG, thromboelastography; vWF, von Willebrand factor.

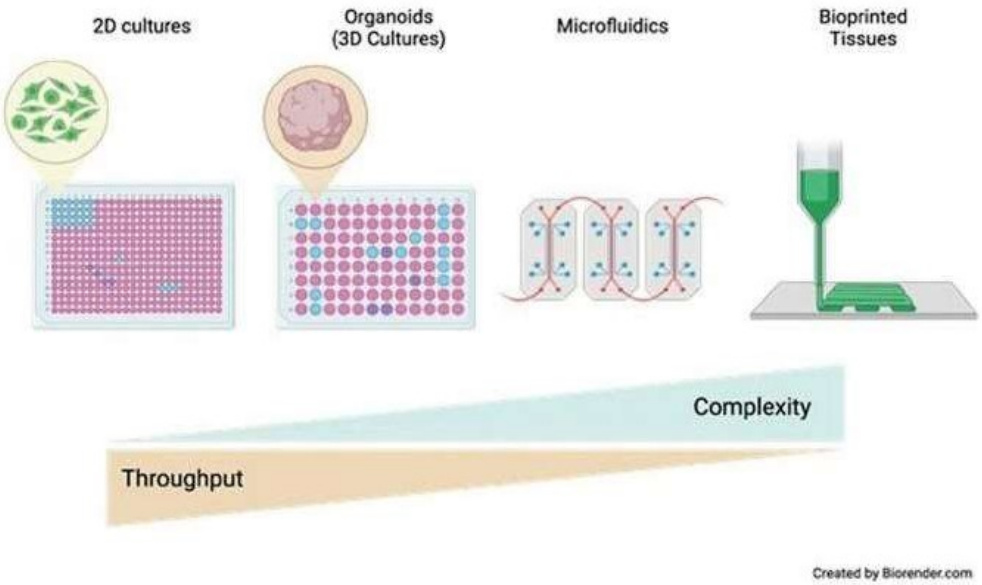


Fig. 6 Balancing system complexity with throughput to meaningfully address biological and translational questions. With the development of progressively more physiologically relevant and complex in vitro models, there is a concurrent decrease in throughput which has significant implications for addressing biological questions. A key consideration will be maintaining scalability, both experimentally and in terms of cost, as improved 3D cultures, microfluidic platforms, and bioprinted models are developed.

remains challenging, in spite of previous efforts from international scientific committees and the general consensus on the need for standardization.[209] Reasons for the lack of standardization include the complicated and time-consuming (pre)analytical handling of the (endothelialized) assays, alongside with high costs currently associated with available assays. Important aspects to enable translation of flow-based assays into clinical diagnostics or treatment monitoring include:

- The full automation in (pre)analytical handling.
- Easy-to-use software applications (development using artificial intelligence-based algorithms, integration of bioinformatics).
- Fast, user-independent output.
- Cost-effectiveness.
- Manufacturability and implementation of quality control measures.
- Clinical validation of microfluidic assays.

Multicenter Studies—Committees

Applying global assays in multicenter studies will reveal the clinical value and applicability of (a combination of) these assays for risk prediction, diagnosis, and treatment. These multicenter studies could accelerate the standardization of novel flow-based tests by providing access to large datasets and thereby allowing assessment of test variation between centers. In addition to achieving standardization at the level of manufacturing, sample preparation, data extraction, and analysis, such studies can define patient populations that benefit from novel assays. In line with these goals, large multicenter studies come with swift recruitment of the appropriate patient population and adequate power. Thereby, setting of reference ranges can be established relatively easy for the general population and specific disease states. When implementing global assays, evaluation of the net clinical benefit will be an important aspect to support the coverage of health care costs by health insurance companies. Moreover, funding for such efforts could be provided and/or supported by pharmaceutical companies, as the developed and tested global assays can also be used to test potential novel antithrombotic, pro-hemostatic drugs and antidotes in earlier phases. Approval processes of novel tests come with inherent challenges, but these can be tackled by involving expert committees that participate in clarifying and streamlining the process. Therefore, international scientific committees can initiate and oversee studies and publish results in standardized, internally validated ways (e.g., Scientific and Standardization Committee of International Society of Thrombosis and Hemostasis) along with consensus statements so that petitioners for approval can follow a more efficient process.

Potential areas for future investigation:

- To further develop global hemostasis assays that encompass all aspects of hemostasis and to bring these from a research setting toward a clinical setting.
- To standardize global hemostasis assays and their corresponding analyses for the screening and diagnosis of hemostatic abnormalities.
- To define the optimal combinations of global and routine hemostasis tests for specific clinical questions or settings.

Extracorporeal Circuits and Hemostatic Challenges

Extracorporeal membrane oxygenation (ECMO) is a form of temporary life support for patients with severe but potentially reversible lung and/or heart failure, unresponsive to optimal conventional care. The ECMO machine provides blood oxygenation (veno-venous, V-V ECMO) or both oxygenation and circulatory support (veno-arterial, V-A ECMO) with an artificial circuit and membrane, thereby taking over

the circulatory and respiratory functions.[210] Thus, ECMO secures support while the health care team works on treating the underlying disease or until organs for transplant become available.

Although ECMO represents a potentially lifesaving therapy and its increase in clinical practice has mirrored a rapid expansion of research on this technology, it still retains intrinsic side effects and complications due to the artificial materials required and its effects on the circulatory, endothelial, hematologic, inflammatory, and immune systems.

Complications in patients receiving ECMO therapy are common and can be associated with worse outcomes.[211] In particular, current rates of bleeding events are unacceptably high and reported to occur in approximately 30% of patients,[212] with a 10% risk of major bleeding and 4 to 10% risk of intracranial hemorrhage.[213,214] Bleeding events independently impact patient prognosis, including mortality.[215–217]

Patients undergoing ECMO support are predisposed to bleeding through various mechanisms, and these can be classified into patient, treatments, and circuit-related. Many factors that may place patients undergoing ECMO at higher risk of bleeding have been identified,[218] including underlying critical conditions prompting ECMO initiation, comorbidities, multiorgan dysfunction, and the technology itself.[218–220] The contact between the patient's blood with the ECMO circuit and the SIRS lead to activation of the coagulation cascade, with effects on fibrinolysis, thrombin formation, and platelet function.[218,221,222] These changes to hemostatic balance result in the coexistence of both thrombotic and hemorrhagic risks, and the final effects may be difficult to predict. Moreover, although anticoagulation remains a standard practice in patients undergoing ECMO,[223,224] thrombotic events have been identified in approximately 15% of ECMO courses[225] and might complicate ECMO therapy with significant morbidity and mortality.

Despite the increasing clinical experience and research data available, much is still unknown about best practices and risk minimization in patients receiving ECMO therapy. In addition, our current knowledge and understanding of what predisposes patients on extracorporeal circuits to bleeding or thrombosis are poor. Therefore, advancement in prevention and early recognition of hemostatic complications, both hemorrhage and thrombosis, is essential to improve the management and outcomes of patients undergoing ECMO. A genetic predisposition to coagulation disorders in these settings, where blood and body are exposed to artificial surfaces, is already well-known but still poorly investigated and might represent an additional target for future research.

Unfortunately, there is a lack of consensus regarding the most suitable approach to best identify risk factors, especially in very sick patients, and genetic screening, while attractive, may not be proven fruitful.

Truth be told, since both ECMO patients and ECMO technology imply the involvement of multiple variables and biological pathways, our current clinical practice may suffer from a compartmentalized approach. Therefore, cooperation between basic scientists and clinicians is very much needed to bridge the gap, tackle the challenges, and reply to the compelling questions that are still waiting to be answered. While historically the well-known strategy "divide et impera" has been used by empires to succeed in expanding their territories, the scientific community should come together and share our knowledge and resources to thrive.

Potential areas for future investigation:

- Current rates of bleeding in patients treated on extracorporeal circuits are unacceptably high.
- Our current knowledge and understanding of what predisposes patients on extracorporeal circuits to bleeding or thrombosis are poor.
- Cooperation between basic scientists and clinicians is needed to bridge the gap to enable the difficult questions that need to be answered regarding the use of extracorporeal circuits.
- Lack of consensus on prioritizing those studies that would best identify risk factors, especially in patients who are very sick and with multiple biological pathways involved.
- Genetic screening, while attractive, may not be proven fruitful.

Generating Novel Vascularized Organoids for Disease Modeling and Drug Development

The advent of organoids, bioprinting, and organ-on-a-chip technologies has at long last offered viable alternatives to simplistic in vitro models and nonhuman in vivo approaches.[226–228] Species and tissue-specific three-dimensional (3D) cultures which mimic the architectural, molecular, and cellular complexity of human organs (to varying degrees) offer many of the benefits of in vitro systems (scalability, manipulability, etc.). They have demonstrated remarkable utility in drug screening, the generation of patient-specific and precision medicine models, and are allowing for unique insights into how cells interact with one another in complex 3D structures.[226]

During the SARS-CoV-2 pandemic, organoid models demonstrated their utility in investigating poorly understood aspects of disease pathology. Our own work using microvascular organoid models demonstrated an important role of pericyte-mediated viral uptake in the loss of vascular integrity contributing to thrombosis in severe COVID-19 infection.[229] More recently, a vascularized BM organoid was developed and validated which faithfully recapitulates key features of the myelopoietic central BM. It was demonstrated that this system allows for drug screening in the context of myelofibrosis, but more importantly supports the engraftment of primary patient cells from several cancers which have been classically difficult to study ex vivo (primary myelofibrosis, multiple myeloma, acute lymphocytic leukemia).[230]

With the promise of these approaches in mind, they are not without their limitations. Self-arranging organoids, particularly those derived from human-induced pluripotent stem cells, remain relatively fetal in their development, and engineering more "adult" versions of these systems remains a key area of study and improvement. While "organ-on-a-chip" and bioprinting strategies offer the promise

of mimicking more adult tissue, they do so at the cost of the scalability and accessibility of these models. Moreover, cost remains a significant factor in the generation of certain organoid systems.

As the tissue engineering field continues to grow and expand, a key consideration is interpreting data derived from these models. While most researchers would balk at the notion of completely replacing animal systems with 3D human models, this is ultimately the end goal of many who are working in the field.

Key questions remain: how to reconcile conflicting human and murine data? How to meaningfully interpret mechanistic information in a (still) artificial system? These and other considerations are, and should be, part of the ongoing dialogue between basic scientists, engineers, and clinicians about meaningfully exploiting what promise to be revolutionary approaches to how to model disease and develop therapies.

Potential areas for future investigation:

- Organoids are an important advance that will enrich the drug discovery process, alongside the use of current assays/mouse models.
- Organoids could be used as part of an iterative approach, with simpler organoid models used in screening before moving on to more complex systems.
- Use of organoids to instruct choice of drug in personalized medicine approaches is challenging and currently unproven, but with future developments could be feasible and valuable.

Theme 5: Clinical Dilemmas in Thrombosis and Antithrombotic Management

New Insights into Inherited Thrombophilia

The association between inherited thrombophilia and the occurrence of (recurrent) VTE has been demonstrated in the past focusing only on a few genetic defects including antithrombin (AT), protein C, protein S deficiencies and two polymorphisms, factor V Leiden (FVL) and prothrombin G20210A mutations.[231] Surprisingly, the vast majority of information clinicians daily use for the management of thrombophilic patients is based on the results of previous studies only dealing with thrombophilia mechanisms discovered in the second half of the last century. In contrast, it is commonly seen that in a large number (almost 50%) of families symptomatic for thrombophilia, none of these defects can be identified. The logical consequence is that other still unknown inherited thrombophilia may exist. Recently, new genetic defects responsible for severe thrombophilia have been identified, namely, pseudo-homozygosity for aPC resistance, the hyperfunctional FIX and FVIII, and the resistance to AT.[231]

FVL is responsible for approximately 95% of cases of APC resistance. However, several point mutations in the F5 gene causing APC resistance have been identified in different populations.[231] Recently, severe thrombophilia in a factor V-deficient patient homozygous for the Ala2086Asp mutation (FV Besançon) has been described that affects anticoagulant pathways more strongly than the prothrombinase activity of FVa.[232] It can also occur that heterozygous FVL carriers present with a concomitant heterozygous F5 gene mutation responsible for FV deficiency, resulting in the 50% of FV plasma levels being all FVL. In these pseudo-homozygotes the thrombotic risk is as high as that observed in homozygous individuals.[233]

Factor IX Padua is a gain-of-function mutation in the F9 gene (R338L) discovered in 2009 detected in a family symptomatic for VTE and exhibiting extremely high plasma factor IX activity (eight times the normal) with concomitant normal antigen levels.[234] Very recently, another hyper-functional FIX variant (R338Q, Factor IX Shanghai) was identified in a 13-year old boy referred for recurrent deep vein thrombosis (DVT).[235] In 2021 the first thrombophilic defect in the F8 gene (FVIII Padua) associated with markedly elevated FVIII levels and severe thrombophilia was described in two Italian families.[236] Genetic analysis revealed a 23.4-kb tandem duplication of the proximal portion of the F8 gene (promoter, exon 1, and a large part of intron 1), which co-segregated with high FVIII levels in the family. Finally, in 2012 a novel gain-of-function polymorphism leading to resistance to AT has been identified.[237] The molecular basis is a missense mutation of the prothrombin Arg596 residue (exon 14) resulting in impaired thrombin–AT binding and defective inhibition of the mutated thrombin by AT. Other similar cases were subsequently described in Serbia, India, and Italy. The symptomatic five families show three different mutations of the Arg596, and namely: prothrombin Yukuhashi Arg596Leu,[237] prothrombin Belgrade and Amrita Arg596Gln,[238,239] and prothrombin Padua 2 Arg596Trp.[240] Although all these hereditary thrombophilias are rare, clinicians ought to keep in mind these novel mutations when dealing with patients or families with unexplained history of recurrent VTE. Nonetheless, the large number of newly discovered inherited defects in the last decades seems to justify why one should not abandon testing for thrombophilia patients belonging to families with VTE.

In fact, previous epidemiological studies and recommendations are based on limited knowledge of inherited thrombophilic conditions. Advanced diagnostic tools including NGS are now adding important information on the etiology of thrombosis. Thus, new clinical studies are needed to redefine the role of inherited thrombophilia in the management of patients with thrombosis.

Managing Atrial Fibrillation in Hemophilia

In the community of patients with hemophilia (PWH), cardiovascular disease is an emerging medical issue as the lifespan of these individuals continues to approach that of the general population.[241] A specific topic concerns patients with AF, where anticoagulants are widely used for the prevention of IS and systemic embolism.

The overall prevalence of AF in PWH in Europe is 0.84% and increases to 3.4% in patients >60 years and is therefore not different from that in the general population.[242] In a patient with a congenital bleeding disorder such as hemophilia, the decision to start antithrombotic therapy is even

more challenging as the balance between thrombosis and hemorrhage is quite delicate.

In PWH with AF, there are many uncertainties to deal with by clinicians in clinical-decision making. First, the minimum clotting level to be able to start anticoagulation therapy is unknown. Several experts and consensus statements suggest that a minimum factor VIII/IX level of 20 to 30 IU/dL is needed for oral anticoagulation[241–245] and this is somewhat confirmed by a clinical registry.[246] On the other hand, PWH with factor levels <20 IU/dL might be considered naturally anticoagulated, as depicted by lower endogenous thrombin potential levels.[247]

In the general population with AF, a risk score, such as the CHA_2DS_2-VASc score, is used to identify patients at risk for IS and therefore in need for anticoagulation therapy. In addition, the HAS-BLED score has been used to predict bleeding events on oral anticoagulation therapy. Balancing these two scores helps the clinician to decide whether the downside of oral anticoagulation outweighs the prevention of thrombotic events. However, in PWH these scores have not been and probably never will be prospectively validated due to the low number of adverse events in this specific population. Therefore, due to lack of evidence, treatment of PWH with AF should always be individualized taking into account the bleeding and thrombotic risk. As a general thought, PWHs with factor levels <20 IU/dL probably do not need additional antithrombotic therapy. In patients with mild hemophilia (>20 IU/dL), oral anticoagulation therapy is probably feasible. In that case, a DOAC has the preference over VKAs due to their favorable safety profile.[248]

There is a strong need for more clinical data on anticoagulation therapy in PWH. Ideally, a registry is started to document the efficacy and safety of different types of antithrombotic treatment in PWH. However, due to the low event rates this will be a difficult task. Furthermore, there is a need for clinical validation of global hemostatic assays or thrombin generation tests to adapt individualized treatments. Especially, with the rapid adaptation of nonfactor replacement therapies (i.e., emicizumab), our long-lasting experience with factor levels will be challenged and the need for these hemostatic tests will be increasing.

The Elusive Safe Antiplatelet Agent

Platelets are activated by two major groups of receptors, G protein-coupled receptors, which are the targets for current antiplatelet drugs, and tyrosine kinase-linked receptors, which are targets for a new class of antiplatelet agents. All of the current antiplatelet drugs increase the risk of bleeding and this can give rise to nuisance bleeds that may influence compliance and, in a minority of patients, life-threatening bleeds. Furthermore, over 50% of patients on antiplatelet medication experience further thrombotic episodes. Thus, there is an urgent need for drugs with improved efficacy that spare hemostasis.

The last major, widely prescribed new class of antiplatelet drugs introduced into the clinic was that of the $P2Y_{12}$ receptor antagonists over 20 years ago, with the thienopyridine, clopidogrel, being the first in class. Several other $P2Y_{12}$ receptor antagonists have since been introduced of which ticagrelor is the most notable because of its reversible action and greater efficacy. This offers an advantage over the irreversible thienopyridines but at the risk of increased bleeding. A PAR1 thrombin receptor antagonist, vorapaxar, has also been introduced but has not been widely described due to the increase in risk of bleeding.

The major tyrosine kinase-linked receptors in terms of signal strength are those with a motif in their cytosolic tail known as an immunoreceptor tyrosine-based activation motif (ITAM). Human platelets express three ITAM receptors, CLEC-2, GPVI and FcγRIIA, and all three signal through Src, Syk, and Btk tyrosine kinases. However, within this group, only the collagen and fibrin(ogen) receptor GPVI has been shown to play a role in hemostasis, although the importance of this appears to have been overestimated. This is shown by clinical data on patients in Chile with an insertion mutation that introduces a stop codon prior to the transmembrane sequence of GPVI and thus prevents surface expression. It is estimated that over 4,000 individuals are homozygous for loss of GPVI in Chile and yet only 12 cases from 11 unrelated families have been found.[249] The majority of these have a mild bleeding diathesis which in some cases has diminished/disappeared on reaching adulthood. Furthermore, only two patients with an inherited deficiency in GPVI have been reported outside of Chile. Given that collagen is a standard agonist in the clinic for the study of patients with a suspected platelet disorder, these data suggest that loss of GPVI does not give rise to a major bleeding diathesis.

This conclusion is also supported by a phase I safety trial on a GPVI-blocking Fab, now known as glenzocimab.[250] A press release in February 2022 on a Phase Ib and IIa trial on glenzocimab reported a tendency to a reduction in bleeding and improvement in cognitive symptoms in patients with acute IS when given in combination with standard treatment (thrombolysis or thrombectomy). This study was powered for safety rather than efficacy but the observation of an encouraging therapeutic effect provides a basis for a phase III trial and reinforces GPVI as a target for a new class of antiplatelet drugs.

This safe targeting of GPVI in terms of bleeding is further supported by clinical data on the use of Btk and Syk kinase inhibitors in the treatment of B cell malignancies and immune thrombocytopenia (ITP). In both cases, the bleeding symptoms reduce over time showing that ITAM-based signaling pathways can be safely targeted (in terms of bleeding) with kinase inhibitors even when the starting platelet count is thrombocytopenic. Inhibitors of Src, Syk, and Btk tyrosine kinases have been introduced into the clinic for treatment of these disorders and have been shown to be well tolerated for up to several years. Moreover, these inhibitors target activation of platelets by all their ITAM receptors. The first-generation inhibitor of the Tec family kinase Btk, ibrutinib, was shown to cause excessive bleeding raising concerns about its use as an antiplatelet drug, but this is now recognized to be due to one or more off-target effects, most likely on other kinases. The second- and third-generation inhibitors of Btk, such as acalabrutinib, and the Syk inhibitor, fostamatinib,

have been shown to be well tolerated in patients, with bleeding symptoms reducing over time as patients respond to treatment. This is particularly notable for fostamatinib which is used in patients with refractory ITP and who therefore have a low platelet count.[251]

The C-type lectin-like receptor CLEC-2 appears to have little or no role in hemostasis in humans and an uncertain role in arterial thrombosis. In contrast, CLEC-2 has been shown to drive thrombosis at sites of inflammation in the venous system in mouse thrombo-inflammatory models, namely DVT and bacterial infection.[252] Platelet activation in these models is mediated by inflammation-driven up-regulation of the ligand for CLEC-2 in the vessel wall podoplanin. Patients treated with ibrutinib show a reduction in DVT suggesting that CLEC-2 may also drive thrombosis in thrombo-inflammatory disease in humans.[253]

The low affinity immune receptor FcγRIIA is the only Fc receptor on platelets and has no known role in hemostasis. Activation of FcγRIIA underlies heparin-induced thrombocytopenia (HIT) which is associated with a marked reduction in platelet count and in some patients life-threatening thrombosis. The molecular basis of this disorder is the formation of antibodies that bind to the positively charged chemokine PF4 which forms an immune complex with the negatively charged heparin. A related, but much rarer condition, with less than 50 cases world-wide, autoimmune HIT, is also mediated by anti-PF4 antibodies but is independent of heparin. In February 2021, the first cases of a new syndrome, now known as vaccine-induced immune thrombocytopenia and thrombosis (VITT), were identified in patients who had received a first dose of the Oxford-AZ adenovirus vaccine to SARS-COV-2 in the previous 5 to 20 days. The frequency of VITT is extremely low, in the order of 1:50,000 to 100,000. VITT is also mediated by antibodies to platelet factor 4 (PF4), with the binding of PF4 to the adenovirus vector driving antibody production.[254] Platelet activation by sera from patients with VITT can be prevented by treatment with a Src, Syk, or Btk inhibitor, although the low frequency of the syndrome, cost of the kinase inhibitors, and potential side-effects prevent this being translated to a clinical trial.[255]

In summary, platelet tyrosine kinase-linked receptors, notably glycoprotein VI (GPVI), represent targets for a new class of antiplatelet drugs that may be more powerful against arterial thrombotic disorders such as ACSs and IS than current drugs with a reduce risk of bleeding. In addition, they are targets in both thrombo-inflammatory disorders and immune complex-driven thrombosis, two groups which are not currently treated with antiplatelets. Receptor blockade can be achieved using protein-based inhibitors such as the GPVI Fab glenzocimab or small-molecule inhibitors targeted to Src, Syk, or Btk tyrosine kinases. Potent small-molecule inhibitors of GPVI, CLEC-2, and FcγRIIA have not been identified. The kinase inhibitors have the advantage of being orally available and blocking activation by all three ITAM receptors but with the concern of off-target effects on myeloid cells and lymphocytes leading to an increase in susceptibility to infection. The irreversible nature of the second- and third-generation Tec family kinase inhibitors such as rilzabrutinib may enable them to be used at a much lower concentration thus reducing off-target effects.[256]

Will FXIa Inhibition Fulfill a Promise?

FXI Deficiency (Hemophilia C or Rosenthal Disease)

In 1953 Rosenthal et al described this autosomal disorder in a family with bleeding events during surgery or dental procedures. The prevalence of severe FXI deficiency is about ≈1/million, and more frequent in certain populations. Clinically, the prolongation of the aPTT may lead to the diagnosis, rather than bleeding complications, which are generally mild, even in severe deficiency. Bleeding may be provoked by surgery, particularly in tissues with high fibrinolytic activity like urogenital or oropharyngeal, but may also include epistaxis, heavy menstrual bleeding, or postinjury, while unprovoked bleeding into muscle or soft tissue or hemarthrosis is not frequent.

Bleeding may also occur in heterozygous subjects with mild deficiency (20–60%) and does not correlate with FXI level. Bleeding can be corrected by FXI (blood product or recombinant clotting factor). Pronounced FXI deficiency lowers risk for IS and venous thrombosis.

FXI(a) Inhibition

FXI(a) is therefore an interesting target for antithrombotic therapy as upstream inhibition of the intrinsic cascade may be effective, yet potentially safer with regard to bleeding as FXI-deficient patients rarely have spontaneous bleeding, suggesting that FXI may have a limited role in hemostasis. $FXI^{-/-}$ mice have normal tail bleeding times but show decreased clot formation at injury sites of arterial or venous.[257] Likewise, treatment of rodent or rabbit models with FXI antisense oligonucleotides (FXI-ASO) or anti-FXI antibodies has shown resistance to experimentally induced thrombosis and a low risk of bleeding complications.[258,259] Different strategies targeting FXI/FXIa for antithrombotic therapy are under development in clinical trials. Novel FXI inhibitor agents include inhibitors of biosynthesis, antibodies, and small molecules (►**Table 4**).

The Clinical Trials of Targeting FXI

Four FXI(a) inhibitors have been tested in patients undergoing total knee arthroplasty (TKA). FXI ASO IONIS-FXIRX that inhibits FXI biosynthesis in liver and abelacimab (MAA868) that inhibits FXI by binding the catalytic domain of both FXI (zymogen) and FXIa were compared with enoxaparin (40 mg) for prevention of VTE in TKA patients. In the FXI ASO trial, the study showed that the higher dose (300 mg) regimen (4%) was superior to enoxaparin (30%) for the prevention of VTE and had a lower rate of bleeding events than with enoxaparin.[260] Similar to the FXI ASO result, the trial of abelacimab showed that the incidence of VTE in the 30 mg abelacimab regimen was noninferior to enoxaparin, and the 75 and 150 mg abelacimab regimens were superior to enoxaparin ($p < 0.001$).[261]

Osocimab (BAY 1213790), a monoclonal antibody that can inhibit FXIa, was tested in 813 adult TKA patients (FOXTROT).

Table 4 Factor XI(a) inhibition

	Type of FXI inhibition	Administration	Frequency	Onset of action	Offset of action	Renal excretion
ASOs	Block biosynthesis	Parenteral	Weekly to monthly	Slow (weeks)	Slow (weeks)	No
Antibodies	Bind target protein	Parenteral	Monthly	Rapid (hours to days)	Slow (weeks)	No
Small molecules	Bind target protein	Oral (or parenteral)	Daily	Rapid (minutes to hours)	Fast	Yes
Natural inhibitors	Bind target protein	Parenteral	Daily	Rapid (minutes)	Fast	Uncertain
Aptamers	Bind target protein	Parenteral	Daily	Rapid (minutes to hours)	Fast	No

Osocimab (0.6, 1.2, and 1.8 mg/kg) was compared with enoxaparin and apixaban for thromboprophylaxis, and was noninferior with respect to efficacy, while it caused less bleeding.[262] Likewise, milvexian, a small molecule that inhibits FXIa activity, was effective for the prevention of VTE and was associated with a low risk of bleeding when compared with enoxaparin at five different dosing regimens . Hence, these trials demonstrated that FXI contributes to postoperative VTE and that lowering FXI levels or inhibiting its activity provides an effective and possibly safe method for its prevention.

For the patient with AF, abelacimab (120 mg, 180 mg) (NCT04213807) and the small molecule asundexian (BAY 2433334) (PACIFIC-AF, NCT04218266) are compared with placebo or apixaban. The first phase 2b trial data of PACIFIC-AF have been already published. Compared with apixaban in patients with AF at risk of stroke, the bleeding rate for the primary endpoint (ISTH major and clinically relevant nonmajor bleeding) was reduced by 67% in patients receiving asundexian.[49,263] However, PACIFIC-AF was not powered to test differences in rates of thrombotic events between groups. There are another two different phase II clinical trials in which asundexian was tested: PACIFIC AMI and PACIFIC Stroke, which were both recently published. In patients with ACS (NSTEMI and STEMI), asundexian on top of DAPT (ASS plus any P2Y12 inhibitor) resulted in dose-dependent, near-complete inhibition of FXIa activity without a significant increase in bleeding and a low rate of ischemic events when compared with DAPT alone.[264] In patients with noncardioembolic IS, asundexian on top of single antiplatelet therapy did not increase the risk of major bleeding, but did also not reduce the composite of covert brain infarction or IS.[265] In the Axiomatic trial, the safety of milvexian, another direct FXIa inhibitor, was tested in noncardioembolic stroke compared with placebo. Similarly, this trial did not show significantly increased bleeding compared with placebo, without having the power to assess efficacy. Taken together, these findings warrant further investigation in phase III clinical trials. The OCEANIC AF (NCT02168829) is the first of its kind to test efficacy of asundexian as compared with apixaban in AF.

Bleeding Management of FXI Deficiency and FXI Inhibition

For the clinical use of FXI inhibitors—not only those with the long half-life—the management of bleeding or peri-procedural management is crucial. Bleeding management in patients with FXI deficiency includes fresh frozen plasma, FXI concentrates (half-life 50–70 hours), which may be administered every 48 to 72 hours, also low-dose rFVIIa (e.g., lower doses of rFVIIa [15–20 µg/kg]), and antifibrinolytic agents, such as tranexamic acid. Antithrombotic agents, such as anticoagulants and antiplatelet medications, should generally be avoided.

Reversal studies of FXI inhibitors are being performed in healthy volunteers using PCC and rFVIIa, and fully human antibody Fab fragments with very high affinity for FXIa inhibitors are being explored for their potential to neutralize their anticoagulant effects.

Outlook for FXI Inhibition

The pathophysiologic concept of FXI inhibition with separating thrombosis from bleeding is very promising and supported by the clinical presentation of FXI deficiency patients and animal models. In addition, FXI inhibition also links to inflammatory pathways and with the contact pathway may also be an effective antithrombotic treatment for foreign surfaces.

However, the benchmark of today's anticoagulant treatment achieved with DOACs is not easily surpassed. Therefore, identifying the important medical needs, selecting the appropriate indications, and choosing the optimal trial design will determine the future success of FXI inhibition. Potential other areas of interest are patients with cancer and thrombosis and patients with severe renal insufficiency or other factors that are associated with high risks for bleeding (and thrombosis).

How to Prevent Thrombosis in the Next Corona Pandemic; Lessons Learned

COVID-19 brought the clinical and research world into widespread recognition of the problem of coagulopathy in infections. Very early identification of thrombosis in patients with COVID-19 first reported from China paved the way for

the publication of a flurry of guidelines focused on the antithrombotic management of these patients.[266] Soon after, the research world started turning their attention to the mechanisms of thrombosis in COVID-19 and how the different pathways may be involved in the thrombotic complication.[267] In the Maastricht discussion, several clinical pointers were presented to assist in future management of hemostatic and thrombotic complications associated with infections. In COVID-19, the preponderance of thrombosis is in the pulmonary circulation.[268] This should ideally be termed as pulmonary thrombosis rather than pulmonary emboli. The rationale for this consideration is the activation of localized bronchoalveolar coagulation by the SARS-CoV-2 virus and the hosts' immune system (widely known as immunothrombosis) in the causation of these clots. These are different to the emboli from lower limbs or other parts of the circulatory beds, which are commonly recognized as pulmonary emboli. The presence of localized coagulation systems may occur in the gastrointestinal tract and possibly the integumental barrier or at sites where pathogen entry is likely. Moving on to the laboratory aspects, D-dimer elevation is a characteristic aspect of COVID-19.[269] This is predominantly due to alveolar fibrinolysis rather than clot breakdown and hence correlated with prognosis in these patients rather than with thrombotic risks. A useful future study would be to look at how extravascular fibrinolysis may correlate with disease outcomes in different pathological states. Severe thrombocytopenia is rare in COVID-19 although mild to moderate drop in platelet counts can be common.[270] There are also reports of markedly elevated vWF levels in these patients too, which in some reports were correlating strongly with poor outcomes. Can these two be linked? Possibly, the thrombocytopenia is caused by the release of large amounts of vWF from endothelial activation which means a decrease of platelet counts can be suggestive of microthrombus formation. This leads to under-recognition of microvascular thrombi from a clinical point of view. In the absence of other clear explanations, a drop in the platelet counts or fibrinogen levels in the setting of sepsis or inflammatory states may mean formation of microthrombi and the need for intervention to limit this process. But the timing of intervention is important too. Coagulation systems including platelets and fibrinogen are anti-infective and as such are commonly activated in different infections. There is a fine line between these beneficial effects of the host's hemostatic system turning to the harmful state of micro- and macrovascular thrombosis. The ideal time for intervention is that period when the shift to harm from a beneficial period occurs.[271] Monitoring trends in the common tests may be the way forward in this regard but future research should also focus on the different pathways and the correct timing for intervention targeting the coagulation system in infections.

Areas of potential research with the lessons learnt from the corona pandemic include: (1) the importance of differentiating localized thrombosis from systemic coagulation activation and how we can target site-specific thrombosis and thus minimize bleeding from systemic antithrombotic therapy; (2) examination of how extravascular fibrinolysis may correlate with disease outcomes in different pathological states; (3) how the trends in laboratory markers may guide treatment decisions to escalate or withdraw antithrombotic agents, and (4) what may be the best time and pathways to target the activated platelets and coagulation system for host benefit.

TICARDIO Translational Lecture: Ambivalent Role of Leukocyte-Derived Microvesicles in Hemostasis

Microvesicles, resulting from vascular and blood cell activation, are now recognized as new protagonists in cellular crosstalk involved in thrombo-inflammation.

Initially described as catalytic surfaces able to activate TF-dependent procoagulant pathways, leukocyte-derived microvesicles (LMVs) were more recently ascribed a fibrinolytic activity.[272,273] Using first whole blood stimulated with LPS (LPS-MV) to mimic inflammatory conditions, granulocyte MVs were found to lyse a thrombus in vitro, according to their plasmin generation capacity (MV-PGC), in a uPA/uPAR-dependent manner.[274] Second, defining MV coagulolytic balance (MV-CLB) as the ratio between MV procoagulant and fibrinolytic activity, the impact of MV with distinct CLB profile was investigated on the dynamics of thrombus formation in vivo, using a laser injury model of mice arterial thrombosis and intravital microscopy. Interestingly, plasminogen accumulation reflecting fibrinolysis initiation was higher in mice receiving fibrinolytic EV-BCL compared with procoagulant EV-CLB profile.

Accumulated knowledge on the role of LMV has not only revisited their role as ambivalent catalytic surfaces able to tune a coagulolytic balance[275–277] but have also driven technological advances, resulting in the development of sensitive and specific assays allowing the measurement of MV-driven TF procoagulant and plasmin fibrinolytic activity.[278–280]

According to TICARDIO objectives on new pathways and targets involving LMV in immuno-thrombotic responses and their translation into novel diagnostic and therapeutic strategies, sepsis-induced coagulopathy was chosen as a typical thrombo-inflammatory clinical situation associating coagulation activation and abnormal fibrinolysis. While converging animal and clinical studies emphasized the deleterious role of procoagulant MV in sepsis and septic shock, the hypothesis was that MVs have a protective effect supported by their capacity to lyse a thrombus. Granulocyte MVs from sepsis patients were found to display a heterogeneous pattern of PGC, driven by uPA-uPAR expression, and were able to lyse a thrombus according to their MV-PGC level. Injection of granulocyte MV with a high PGC level reduced clot formation and improved survival in a mouse model of septic shock, demonstrating a protective effect of these granulocytic subpopulations,[274] opening perspectives for a potential antithrombotic strategy. In a cohort of 225 patients with septic shock enrolled in a multicenter prospective study, the MV-CLB predicted mortality in septic shock patients with better performances than the procoagulant and profibrinolytic activities taken individually, and allowed stratifying the severity of septic shock. This new functional signature

of MV opens unexplored avenues for the guidance of individualized therapy targeting coagulopathy in septic shock.

Data presented in the SEPSIS context illustrate the view of granulocyte MV-CLB as an ambivalent microsystem tuning thrombo-inflammation.

Potential areas for future investigation:

- From one side, a deeper understanding of what determines the MV-CLB, including the role of the distinct triggers and subsets of MV and the impact of pharmacological modulations, is required.
- From another side, the definition of the true value of MV as biomarkers of thrombotic risk, through multicenter prospective clinical studies thanks to methodological innovation and standardization, to measure MV in a more automatized way and integrate them into scoring systems with other biomarkers and clinical variables. These perspectives are included in ongoing research programs.[125]

Conflict of Interest
None declared.

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