UiT

THE ARCTIC UNIVERSITY OF NORWAY

Role of Extracellular Vesicles in the Pathogenesis of Venous Thromboembolism

Simin Jamaly

June 2018

A dissertation for the degree of Philosophiae Doctor

Faculty of Health Sciences, Department of Clinical Medicine

List of Errata

(Update 29.05.2018)

- Pages 10 and 30:

ALL poly-P words were corrected to *polyP*.

- Page 34. Tabel 1.

Row 1, column 4: No difference were corrected to ↑ *(increased).*

Row 3, column 2: History of VTE were corrected to *History of recurrent VTE.*

Row 6, column 2: (n=148) were corrected to *(n=418).*

- Page 39

Paragraph 3, line 5. Exportin- 5- RNA-GTP were corrected to *Exportin-5- Ran-GTP*.

Paragraph 3, line 6. RNAase III were corrected to *RNase III*.

Contents

Acknowledgement…….....5
Summary…….....7
List of paper……9
Abbreviation…….....10
1 General introduction……12
1.1 Venous thromboembolism……..12
1.2 Pathophysiology of venous thromboembolism…….....14
1.3 The coagulation system……...17
1.4 Risk factors of Venous thromboembolism……..22
1.4.1 Inherited risk facots…….....22
1.4.1.1 Loss-of-function mechanisms……..22
1.4.1.2 Gain-of-function mutation of procoagulant factors……23
1.4.1.3 Other inherited risk factors…….…….24
1.4.2 Environmental risk facotrs……...24
1.5 Extracellular Vesicles……..25
1.5.1 Formation and uptake of extracellular vesicles……26
1.5.2 Procoagulant functions of extracellular vesicles……..30
1.5.3 Plasma levels of extracellular vesicles and risk of Venous thromboembolism……..31
1.5.4 Isolation and measurements of extracellular vesicles in plasma.……..35
1.6 microRNA……...39
1.6.1 Structure and biogenesis of miRNA……..39
1.6.2 Association between miRNA and venous thromboembolism……42
2 Aim of the study……...43
3 Methods……...44
3.1 Isolation of Extracellular vesicles and leukocytes……...44
3.2 Plasma concentrations and cellular origin of extracellular vesicles by flow cytometry……..45
3.3 Analysis of concentration and size distribution of extracellular vesicles by nanoparticle tracking analysis…….....46
3.4 Visualizing the three-dimensional and ultrastructure of extracellular vesicles by electron microscopy……..47
3.4.1 Negative staining……..48
3.4.2 TEM ultrathin section……...48

3.4.3 Scanning electron microscopy……..50

3.5 Measuring the level of negatively charged phospholipid in extracellular vesicles…….50

4 Main results……52

Paper-I…….....52

Paper-II…….....53

Paper-III…….....54

Paper-IV…….....55

5 Discussion…….56

6 Conclusions……...66

7 References…….67

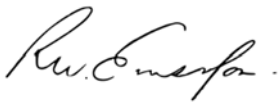
Paper I, II, III, IV……78

Dedicated to my mother, and my family Mehrdad and Nora.

“Do not go where the path may lead,

go instead where there is no path and leave a trail”.

Ralph Waldo Emerson



Acknowledgements

The present work was carried out at the Hematological Research group (HERG), Department of Clinical Medicine at the Arctic University of Tromso from January 2013 to June 2014, and at the K.G.Jebsen Thrombosis Research Center (TREC) from June 2014 to June 2018. TREC is financed by Stiftelsen Kristian Gerhard Jebsen, the Arctic University of Norway and Northern Norway Regional Health Authority. My Ph.D. position has been funded by a grant from UiT-the Arctic University of Norway.

First, I want to express my deepest gratitude to my primary supervisor, Professor John-Bjarne Hansen. Your tremendous scientific knowledge combined with endless enthusiasm are inspiring. Second, I want to thank my co-supervisor, associate Professor Sigrid Kufaas Brækkan. Your writing skills, optimism, and capacity are impressive.

I would like to express my special appreciation and thank to Randi Olsen and Tom-Ivar Eilertsen, who believed in me and encouraged me every day. I also want to thank laboratory technicians Nadezhda Latysheva and Line Wilsgård and my co-authors, Irina Starikova, Cathrine Ramberg, Mikhail Sovershaev, Timofey Sovershaev, Manjunath G. Basavaraj, and Paul Webster working and cooperating with you was my pleasure. I would like to direct special thanks to Professor Bjarne Østerud, Professor Ruth Paulsen, Mona Nystad, Dr.Åse Lillian Vårtun, Gry Wiik and Erik Nordhagen and Rozmana Ahmadi. I have benefited immensely from your support and kindness.

I express my gratitude to my mother and siblings Nader, Mahin and Shahin and for continuous support. Nothing is better than knowing I always have an unconditional, consistent relationship in life. You are my go-to when I am experiencing not only my high moments, but my low moments. You know exactly what to say to make me feel like I have the world in my hands.

You also know what to do and say to pick me up, when I've been pushed and pushed. Special thanks to my nephews Alireza and Parsa. You are my escape. Being a Ph.D. student is stressful, but when I see your face, you remind me that my stress doesn't matter. You may not be my own child, but I sure do love you like you are.

Finally, and most important, I would like to thank my husband, Mehrdad, I appreciate your never-ending love and for all of the sacrifices. Only a few lucky ones like me have life partners who help heal the wounds of yesterday and give wings for a happier tomorrow. I love that I get to do life with you. And last, but not certainly not least, I want to thank my little girl, Nora and my dog, Toby. My princess with her smiles remind me springs in the polar zone and I feel summer warmness in my heart and Toby with hilarious French Bulldog's voice greets me when I went home tired after hard working days. I have needed them for motivation and encouragement during PhD journey.

Summary

Venous thromboembolism (VTE) is a common disease with an incidence of 1–2 per 1000 individuals. VTE is a collective term for deep vein thrombosis and pulmonary embolism. Extracellular vesicles (EVs) are lipid-membrane-bound spherical vesicles that bud off from the cells upon activation and apoptosis. Their size varies between 30 nm and 1 μm. Recent advances in experimental and observational studies suggest a pivotal role of EVs in VTE.

The main goals of the present thesis were to assess plasma levels, the cellular origin and the morphological characteristics of P-selectin Glycoprotein Ligand-1 (PSGL-1) positive microvesicles (MVs) in patients with unprovoked VTE. We investigated the potential of microRNA (miRNA) plasma levels as biomarkers for unprovoked VTE, as well as the impact of pre-analyses handling and fasting status on plasma EVs. We also developed a cost-effective modified assay to measure procoagulant phospholipid (PPL) activity.

In the study described in Paper I, we evaluated plasma levels and parental origins of PSGL-1^+ MVs in patients with unprovoked VTE and controls. The results showed that high plasma PSGL-1^+ MV levels were associated with an increased risk of unprovoked VTE. In Paper II, we analyzed the effect of anticoagulant choice on EVs, and found no difference between anticoagulants in terms of total EVs plasma concentrations and size distribution. Postprandial lipidemia was not accompanied by apparent changes in plasma EVs concentration, but an increase in the mean size of VLDL particles interfered with EV measurements in the postprandial phase. The results of the study described in Paper III revealed that plasma miRNA profiling could provide novel biomarkers for unprovoked VTE. In Paper IV, we showed that a modified PPL assay performed as well as a commercial competitor (STA-Procoag-PPL assay), with advantages of being insensitive to postprandial lipoproteins and tissue factor positive (TF^+) EVs and lower cost, making it suitable for large-scale studies.

List of Papers

Paper I

Simin Jamaly, Manjunath G. Basavaraj, Irina Starikova, Randi Olsen, Sigrid K. Brækkan, and John-Bjarne Hansen. Elevated Plasma Levels of P-Selectin Glycoprotein Ligand-1 (PSGL-1) Positive Microvesicles in Patients with Unprovoked Venous Thromboembolism

Journal of Thrombosis and Hemostasis. Accepted, (May 2018).

Paper II

Simin Jamaly, Cathrine Ramberg, Randi Olsen, Nadezhda Latysheva, Paul Webster, Timofey Sovershaev, Sigrid K. Brækkan, John-Bjarne Hansen. Impact of preanalytical on plasma concentration and size distribution of extra cellular vesicles using Nanoparticle Tracking Analysis. Submitted.

Paper III

Irina Starikova, Simin Jamaly, Antonio Sorrentino, Thorarinn Blondal, Nadezhda Latysheva, Mikhail Sovershaev, John-Bjarne Hansen. Differential expression of plasma miRNAs in patients with unprovoked venous thromboembolism and healthy control individuals. Thrombosis Research, 2015;136(3):566-72.

Paper IV

Cathrine Ramberg , Simin Jamlay , Nadezhda Latysheva , Tom Wilsgaard , Manjunath G. Basavaraj, Timofey Sovershaev , John-Bjarne Hansen. A simple, inexpensive and robust assay to measure levels of negatively charged phospholipids-a modified PPL assay. Manuscript.

Abbreviation

(Ago2)- Argonaute-2

(APC)- Activation of protein C

(CTAD)- Citrate –theophylline- Adenosine-Dipyridamole

(CV)- Coefficient variation

(DVT)- Deep vein thrombosis

(EPCR)-Endothelial protein C receptor

(EV)- Extracellular Vesicle

(EVDP)- Extracellular vesicle-depleted plasma

(FC)- Flow cytometry

(miRNA)- microRNA

(MV)- Microvesicles

(NTA)- Nanoparticle tracking analysis

(PAG)- Protein A gold particles

(PBS)- Phosphate buffer saline

(PE)- Pulmonary Embolism

(PFP)- Platelet free plasma

(PolyP)- Polyphosphate

(PPL)- Procoagulant Phospholipids

(PPP)- Platelet poor plasma

(PS)- Phosphatidylserine

(PSGL-1)- P-Selectin Glycoprotein Ligand-1

(SEM)- Scanning Electron Microscopy

(TEM)- Transmission Electron Microscopy

(TF)- Tissue factor

(TFPI)- Tissue factor pathway inhibitor

(VLDL)- Very Low Density Lipoproteins

(VTE)- Venous Thromboembolism

1. General introduction

1.1 Venous thromboembolism

Venous thromboembolism (VTE) is a common term for pulmonary embolism (PE) and deep vein thrombosis (DVT). DVT involves the generation of a thrombus inside a deep vein, commonly in the deep veins of the lower extremities that can obstruct venous blood flow. PE occurs when a fragment of the blood clot dislodges from the place it was formed, and is transported by the bloodstream to the arteries of the lungs. The origin of PE remains undetected in up to 50% of PE-patients [1], and recent findings suggest that approximately 20% of the PEs may originate from right atrium thrombi due to atrial fibrillation [2].

VTEs can be classified into provoked and unprovoked (idiopathic) events depending on the presence of predisposing factors at the time of the VTE. Provoked events may occur due to transient or persistent risk factors such as major general surgery or orthopedic surgery, spinal cord injury, fracture of the pelvis, hip or long bones, myocardial infraction, stroke, congestive heart or respiratory failure and cancer [3]. Unprovoked VTE occurs in the absence of malignancy or other predisposing factors.

VTE is the third most common cardiovascular disorder after myocardial infarction and stroke , with an annual incidence of 1–2 per 1000 inhabitants in developed countries [4]. Cohen *et al.* reported that the estimated annual incidence of symptomatic VTE events across Europe, based on extrapolation of the six modeled countries, exceeded 1.7 million [5]. The incidence of DVT is higher than PE and the incidence of VTE has slightly increased during the last two decades [6]. Few studies display trends over time in the incidence of VTE. However, Wiener *et al.* reported an 81% increase in the incidence of PE after the introduction of CT pulmonary angiography (CTPA) [7]. Huang *et al.* stated that incidence rates of VTE in the USA increased

by 82% between 1985 and 2009 [8], principally because of an increase in the incidence of PE. The overall age-adjusted incidence of VTE increased from 158 per 100, 000 in 1996/1997 to 201 per 100, 000 in 2010/2011 in a large cohort study in Norway (Tromsø) [6].

The long-term complications of VTE are post-thrombotic syndrome (PTS) and chronic thromboembolic pulmonary hypertension (CTPH). PTS is the most common complication of DVT, affecting 20–50% of patients with a lower limb DVT [9]. Classical symptoms of PTS include chronic pain and swelling of the affected extremity [10]. Severe PTS develops in approximately 10% of subjects with PTS, and they may develop venous leg ulcer, which can be very difficult to treat and impairs patient mobility [10]. The presence of PTS has a significant impact on disease-specific quality of life [11]. Braekkan *et al.* reported that DVT was associated with a 60% increased risk of disability pension [12]. Risk factors such as proximal DVT, male gender, and high D-dimer levels are independently associated with development of PTS after first DVT [13] and ipsilateral recurrence of DVT increased the risk of PTS six-fold [14].

The hallmark of CTPH, affecting 1-4% of the patients with PE [15], is fibrotic transformation of pulmonary arterial thrombus, leading to mechanical obstruction of the major pulmonary arteries. CTPH is characterized by dyspnea and pleuritic-type chest pain [16]. In advanced stages, CTPH can cause right heart failure symptoms and ultimately death [17].

VTE is a complex disease with severe complications, including recurrence and death. The cumulative incidence of recurrent VTE is reported to be 11 % after one year, 20% after 3 years and 30% after 5 years and 40% after 10 years [18]. Independent clinical predictors of VTE recurrence include male sex, increasing age and body mass index, active cancer and neurologic disease with extremity paresis [19]. A recent Norwegian study reported an overall cumulative mortality rate of 19.4% at 30 days and 62% at one year in VTE related cancer patients, and 9% and 16.6% in VTE-cancer-free patients, respectively [20]. Up to 10% of acute PE patients die

suddenly [21]. The mortality rate is higher in subjects with provoked VTE than in those with unprovoked VTE. This may relate to older age and the presence of comorbidities, such as cancer.

1.2 Pathophysiology of venous thromboembolism

The terms ‘thrombosis’ and ‘embolism’ were first named and defined for VTE by the German physician Rudolf Virchow. He proposed a mechanistic link between DVT and pulmonary embolism in 1856 [22], and a triad involving hypercoagulability, altered blood flow (stasis and turbulence) and vessels walls changes as key elements in the pathogenesis of venous thrombosis (Figure 1).

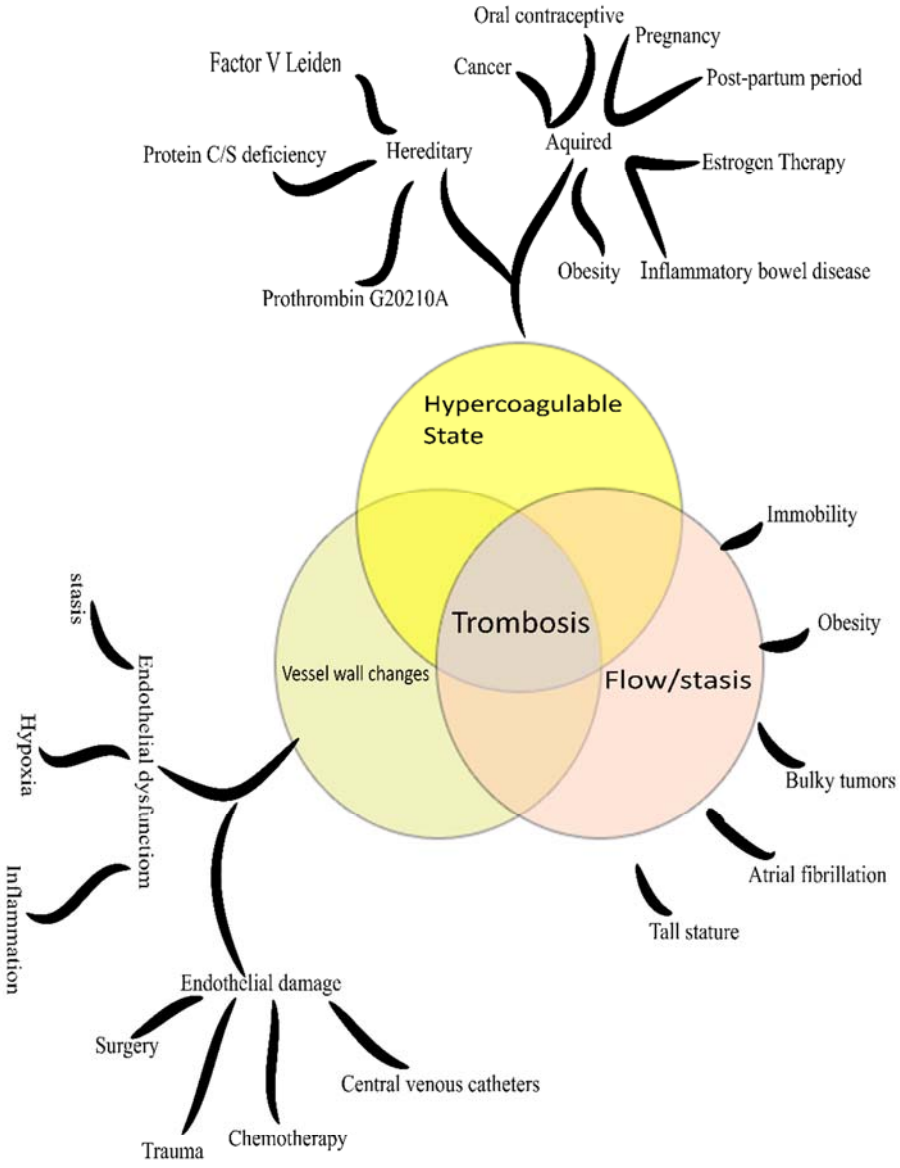


Figure 1. Virchow's Triad.

Most recognized risk factors for VTE apply to one or more of the elements proposed by Virchow

Venous thrombi orginate in the slow-flowing venous systems, often in the valvular sinuses of the veins of the lower legs [23]. The thrombi mainly consists of red blood cells and fibrin [24]. The valvular sinuses are susceptible to haemoglobin desaturation and hypoxia when blood flow ceases because blood is trapped in a vortex [25]. In the healthy vasculature, the endothelium has an antithrombotic and profibrinolytic surface that prevents clot formation [23]. Under normal physiological conditions the endothelial lining expresses high levels of anticoagulant proteins such as thrombomodulin, tissue factor pathway inhibitor (TFPI) and endothelial protein C receptor (EPCR) [23]. Furthermore, endothelial cells inhibit coagulation activation by the proteoglycans heparan sulfate and dermatan sulfate which bind and activate the anticoagulants anti thrombin and heparin cofactor activity [26]. Hypoxia and stasis activate the endothelial cells and induce a prothrombotic state [27]. The endothelial cells are activated by hypoxia whereby the transcription nuclear factor-κB (NF- κB) is activated, and this induces the adhesiveness of endothelium and regulates the expression of tissue factor (TF) [28, 29]. The activated endothelial cells translocate P-selectin from Weibel–Palade bodies to the outside of cell membranes. P-selectin is a membrane glycoprotein stored within the secretory granules of platelets and endothelial cells [30]. Activated endothelial cells capture leukocytes (mainly monocytes), platelets and leukocyte-derived microvesicles (small plasma membrane vesicles that are shed from many cell types) to the active sites by a P-selectin and PSGL-1-dependent mechanism [31]. Subsequently, there is recruitment of TF and negatively charged phospholipids-positive extracellular vesicles [32, 33]. These events lead to activation of the coagulation pathway (Figure 2).

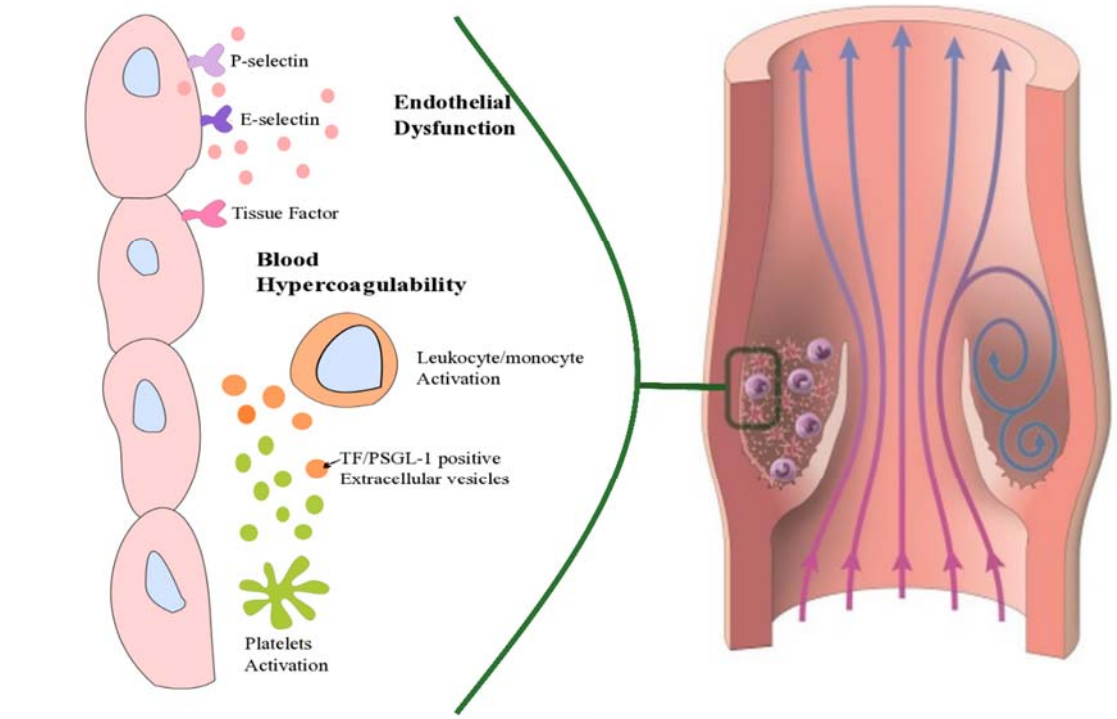


Figure 2.

Intermittent hypoxia and stasis are key elements in the pathophysiological mechanisms of venous thromboembolism: According to Virchow's Triad, hypercoagulability, hemodynamic changes, and endothelial dysfunction predispose for thrombus formation. Blood is caught in a vortex flow within the valve pockets and becomes desaturated. Hypoxia promotes prothrombotic and proinflammatory processes in endothelial cells and expression of cell adhesion molecules such as P-selectin and E-selectin accompanied by recruitment, and activation of leukocytes, especially monocytes, and platelets. Activated monocytes and leukocytes bud-off procoagulant (TF positive) extracellular vesicles, which can activate the coagulation cascade.

1.3 The Coagulation System

In 1964, two groups simultaneously proposed a "cascade" or "waterfall" model of blood coagulation. This cascade comprises a series activation of serine proteases and cofactors that culminate in thrombin generation and conversion of fibrinogen to fibrin (Figure 3). The cascade can be triggered by the TF–VIIa complex (extrinsic pathway) [34] or by factor XIIa (intrinsic/contact pathway) (Figure 3). However, both pathways result in the formation of factor Xa. In the presence of co-factor Va, Phospholipids (PS) exposed on activated cells, especially

activated platelets, and calcium ions, FXa forms a prothrombinase complex with prothrombin (FII). This complex converts FII to thrombin (FIIa). Thrombin cleaves the plasma-soluble protein fibrinogen into Aα and Bβ polypeptide chains, which release fibrinopeptide A and fibrinopeptide B, respectively. The release of these fibrinopeptides results in the formation of fibrin monomers, which are still soluble. Fibrin monomers, comprising α, β and γ chains, polymerize spontaneously to form a polymer gel that traps blood cells. Thrombin also triggers conversion of FXIII to FXIIIa, which in turn mediates covalent binding between the fibrin polymers to form a stable fibrin clot [35, 36].

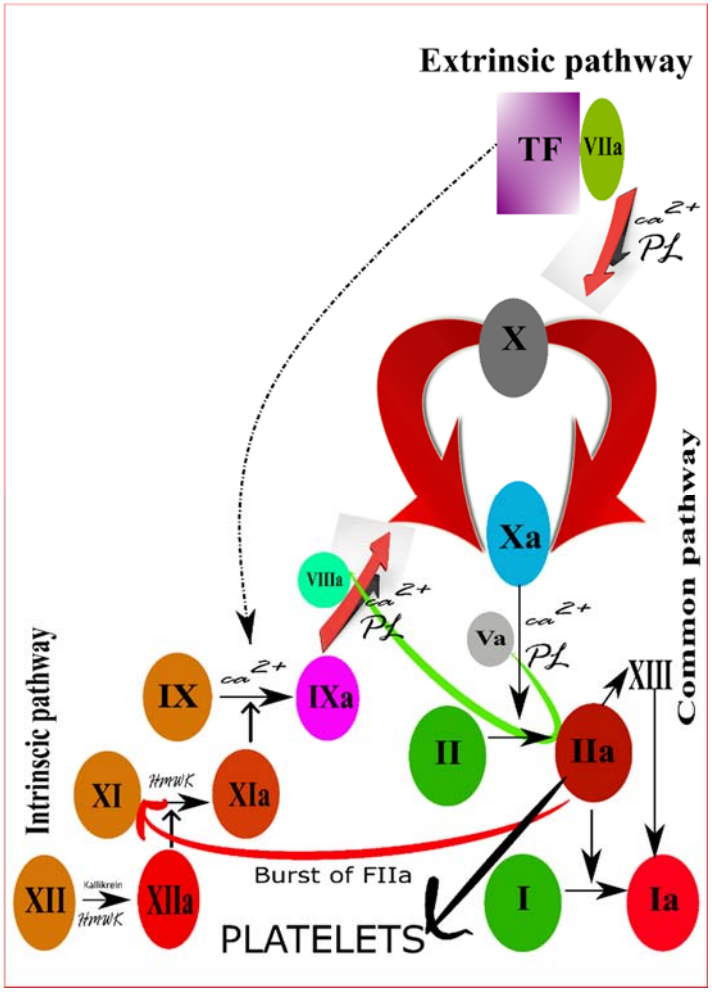


Figure 3. Outline of coagulation cascade.

The extrinsic pathway is activated at the site of tissue injury, due to exposure of TF on extravascular cells. It involves TF (FIII), FVII, FX and Ca^{2+} (FIV). In vessel damage, plasma FVII and FVIIa bind to TF expressed at perivascular cells such as fibroblasts, pericytes and smooth muscle cells forming a TF-FVII/FVIIa complex that activates FX and FIX [37, 38]. Under physiologic conditions, TF exists in a "hemostatic envelope" within the vasculature, and is therefore inaccessible to flowing blood [34]. According to previous studies, activated monocytes serve as the primary (predominant) source of TF in whole blood [39]. Although TF expression has also been reported in endothelium [40], platelets [41], neutrophils [42], eosinophils [43] and T-cells [44], but TF expression on these cells are controversial due to non-specific assays [45].

The intrinsic pathway (contact activation) is activated by negatively charged surfaces *in vitro* (such as glass) and involves factors XII, XI, IX, VIII and X, as well as prekallikrein (PK), high-molecular weight kininogen (HMWK), Ca^{2+} and phospholipids. During the initial contact phase, factors PK, HMWK, XII and XI are exposed to a negatively charged activating surface. FXII is activated to FXIIa at a limited rate by autoactivation, with HMWK as a cofactor. When a small amount of FXIIa accumulates, it converts PK to kallikrein and catalyzed by HMWK as cofactor. This newly produced kallikrein accelerates the conversion of FXII to FXIIa through a positive feedback loop. FXIIa (along with HMWK as cofactor) converts FXI to FXIa then FXIa activates FIX to FIXa. FIXa in the presence of thrombin activated FVIII, and negatively charged phospholipids (phosphatidylserine: PS) and Ca^{2+} (tenase complex) converts FX to FXa [46].

In short, the hemostatic mechanism can be outlined as follow: TF exposed at the vessel wall injury binds FVII/FVIIa whereby more FVII is converted to FVIIa. This complex activates FX and FIX, and formed FXa interacts with FVa and PS, forming the prothrombinase complex that converts prothrombin to thrombin. The first traces of thrombin activates plasma FV and FVIII

and platelets whereby FIXa and FVIIIa generates more FXa that interacts with FVa and PS on more activated platelets and a burst of thrombin which is generated now activates FXI, and resultant FXIa activates more FIX. The initial formation of FIXa formed by TF-FVIIa complex is thought to be important for the amplification of thrombin generation, as FIXa is slowly inactivated by antithrombin, and the TF is rapidly inactivated in the quaternary complex of TF-FVIIa-TFPI-FXa. A threshold of thrombin appears to be important for the activation of FXI on activated platelets. In addition to form the final fibrin net, thrombin is also activating FXIII which stabilize the fibrin clot. Therefore, thrombin generation in the hemostatic mechanism is independent of FXII, prekallikrein and HMWK.

In the thrombotic mechanism, most of the activation steps are similar to hemostasis, but the contact activation through FXII, prekallikrein and their co-factor HMWK, plays an important role in the thrombin formation. Recently, focus on using inhibitors to block FXII or FXI in VTE has been discussed. This could reduce the risk of venous thrombosis and at the same time avoid adverse bleeding [47].

The coagulation system in vertebrates is regulated at several levels by three major anticoagulant pathways. Inactivation or loss of any of the three genes involved are lethal to embryos [48-51]. TFPI is a single-chain polypeptide produced by endothelial cells, that inhibits the extrinsic pathway by forming the inactive complex of TF-FVIIa-TFPI-FXa whereby TF is blocked and TFPI also reversibly inhibits FXa [52]. Antithrombin is the main inhibitor of thrombin. Antithrombin is a serine protease inhibitor that binds to and inactivates the activated coagulation proteases FIXa, FXa, FXIa, FXIIa and FVIIa in complex with TF [53]. The protein C anticoagulant pathway inhibits the propagation phase of the coagulation cascade. Binding of thrombin to the endothelial surface protein thrombomodulin (TM) alters the substrate specificities of the enzyme, leading to loss of its procoagulant activities. Protein C binds to

EPCR, which presents it to the thrombin–TM complex. The thrombin-TM complex acts as an anticoagulant through the activation of protein C (APC), which leads to the dissociation of APC from EPCR. APC binds to its cofactor protein S and inactivates FVa and FVIIIa [54](Figure 4).

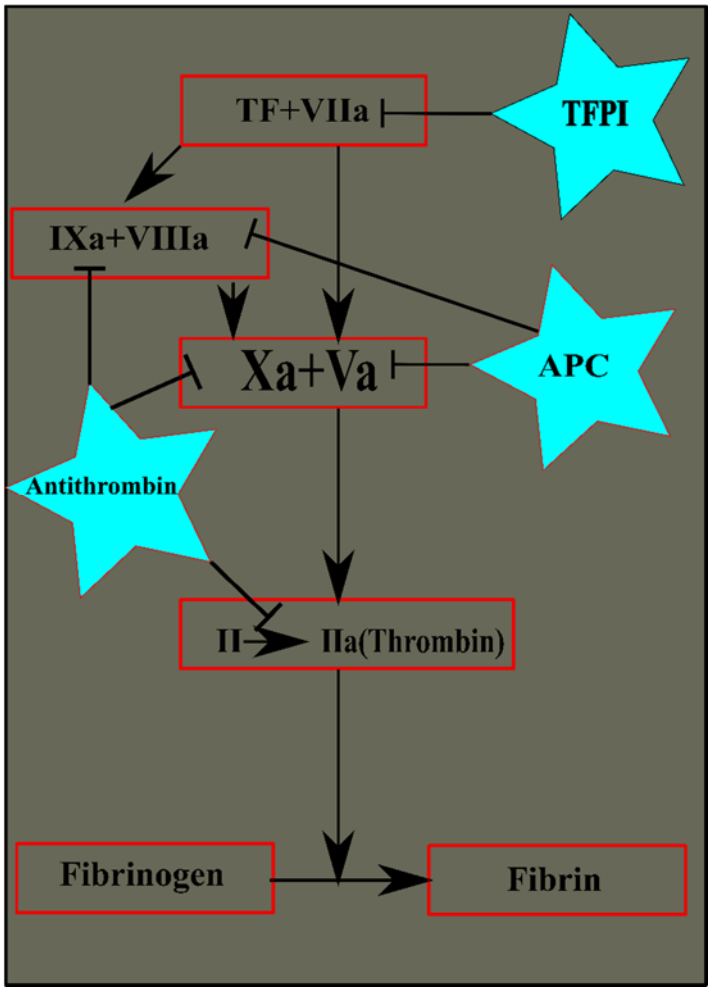


Figure 4:
The three major anticoagulant mechanisms regulating the coagulation system.

APC: Activated Protein C. TFPI: Tissue Factor Pathway Inhibitor.

1.4 Risk factors for VTE

VTE is a complex disease with both inherited and environmental risk factors. Family and twin studies indicate that 50–60% of the VTE events are due to inherited risk factors [55, 56]. Two main mechanisms are involved: (i) loss-of-function of antithrombotic pathway agents (involving impaired functioning of antithrombin, protein C, or protein S); or (ii) gain-of-function of procoagulant factors (e.g. increased synthesis of proteins such as prothrombin G20210A) [57].

Many environmental factors increase the risk of VTE. The classic environmental risk factors for VTE include hospitalization, surgery and trauma, immobility, estrogen therapy, oral contraceptives, and pregnancy/post-partum. Moreover, comorbidities such as congestive heart failure, myocardial infraction, stroke, chronic kidney disease, acute infections, chronic inflammatory disease, exacerbation of chronic obstructive pulmonary disease, and cancer are associated with increased risk of VTE. Advancing age, body height and obesity are clinical risk factors for VTE [3, 58-61].

1.4.1. Inherited risk factors

1.4.1.1. Loss-of-function mechanisms

Antithrombin deficiency: Antithrombin is the main inhibitor of thrombin (factor IIa) and additional target proteases such as factors Xa, IXa, XIa, XIIa and FVIIa in complex with TF. Its effect is facilitated by the presence of heparin or glycosaminoglycans (GAGs) at the endothelial surface. However, antithrombin deficiency is rare with a prevalence of 5–17 per

10,000 in the general population. These subjects are susceptible to a 10-50-fold increased risk of VTE due to inefficient inhibition of activated coagulation factors [62, 63].

Protein C deficiency: Protein C is a natural anticoagulant protein, activated by the thrombin–thrombomodulin complex. Activated protein C inactivates factors Va and VIIIa and stimulates fibrinolysis by inhibiting plasminogen activator inhibitor (PAI-1). Mutations resulting in protein C deficiency are rare, occurring in less than 1% of the general population. There is a 4-8 fold higher risk of VTE in subjects with protein C deficiency [62, 63].

Protein S deficiency: Mutations in the *PROS1* gene results in reduced levels of protein S, which is a cofactor of APC. The APC-Protein S complex inactivates FVa and FVIIIa. Protein S deficiency is also rare, occurring in only 1–5 per 1000 of the population. It is associated with a 10-fold increased risk of VTE [62, 63].

1.4.1.2. Gain-of-function mutation of procoagulant factors

Prothrombin G20210A: This mutation was discovered in 1996. It involves a single-nucleotide mutation in the 3′-untranslated region of the prothrombin gene, which results in high plasma levels of prothrombin. This variant is present in 1–2% of the population and is associated with 2–3-fold increased risk of VTE [64, 65].

Factor V Leiden: A SNP missense mutation (arginine to glutamine) of factor V Leiden causes resistance of factor V to the anticoagulant action of APC. Factor V Leiden occurs in approximately 5% of the general population of European descent origin [66] and heterozygous carriers have a 2-3-fold increased risk of VTE. The prevalence of homozygous carriers in the white population is 1 in 5000 and is associated with 80-fold increased risk of VTE [67].

1.4.1.3 Other inherited risk factors

Several novel single-nucleotide polymorphism (SNP) associated with VTE have been identified through genome-wide association studies (GWAS). GWAS seeks to identify the association of huge number of SNPs with a phenotype in studies with large sample size [68]. VTE-associated SNPs have mainly been found in or near genes encoding for proteins in the coagulation or fibrinolytic pathways that have a role in modifying the function and plasma levels of proteins [69]. Most of these SNPs have a modest effect on the VTE risk, and alone they may have limited clinical utility. However, multiple SNP testing might improve the predictive ability of risk prediction models for VTE [70].

1.4.2. Environmental risk factors

Age: There is extensive evidence that the incidence of VTE increases steeply with age. The annual rate of VTE increases from less than 5 cases per 100,000 among children under 15 years, to 500 cases per 100,000 adults aged 80 years [71]. In the Tromsø study, subjects aged 70+ years had an 11-fold higher risk of VTE than those below the age of 50 [72]. The reasons for this increased risk with *age* are *not* fully understood, but may relate to an accumulation of classic environmental risk factors such as immobility, surgery and malignant diseases. In addition, overall muscle strength declines with aging, which affects the calf-muscle pump. There are also age-related vascular changes, such as dilation of the venous wall and valve dysfunction, leading to blood stasis and elevation of distal venous blood pressure [73-76]. Changes in the proteins of the coagulation system, prothrombotic markers, anticoagulant proteins and platelet function might also be involved [77, 78].

Obesity: Obesity is defined as a body mass index (BMI) >30 kg/m^2. It is associated with a 2–3-fold higher risk of VTE compared to normal weight subjects [79]. Comparison of obese and non-obese subjects shows differences in blood-flow dynamics of the deep veins in the lower extremities [80], as the femoral vein diameter was significantly increased in obese subjects. This could be explained by elevated intra-abdominal pressure leading to vein wall distension, increased stasis and reduced forward flow velocity in obese subjects [81]. Adipose tissue synthesizes various proinflammatory and antifibrinolytic substances, such as plasminogen activator inhibitor-1 (PAI-1)(primary inhibitor of circulating plasminogen activators) [82], leading to inhibition of fibrinolysis and a prothrombotic state [83]. Obesity increases the probability of high-level platelet aggregation and TF-mediated coagulation [84], and higher levels of fibrinogen and certain coagulation factors [85].

1.5 Extracellular Vesicles

In 1967, Peter Wolf identified extracellular vesicles (EVs) during his coagulation research as a product of platelets and he termed them “platelet dust” [86]. Initially, EVs were considered to be cell debris, generated by cell-handling procedures [87]. Later studies have highlighted EVs as a potential biomarker in various diseases, especially in cardiovascular, bleeding and thrombotic disorders [88]. Notably, EVs is a general term involving exosomes (40-120 nm) and microvesicles (100-1000 nm) released from blood cells and different tissues upon activation or apoptosis [89]. EVs can be sorted according to size, quantities, cellular origin and composition in different body fluids such as plasma, ascites fluid, urine and saliva [90]. They are characterized by a bilayer phospholipid membrane without nucleus, and a membrane skeleton, and possess cytosolic components, such as enzymes, transcription factors, and RNA or DNA

molecules derived from their parental cells [91]. Pathological mechanisms that may trigger EVs shedding include inflammation, activation of the coagulation or complement systems, inducers of apoptosis, and shear stress in the circulation [92].

1.5.1 Formation and uptake of extracellular vesicles

The cytoplasmic membrane of eukaryotic cells is characterized by an asymmetric distribution of phospholipids. Negatively charged phospholipids, such as PS, are located in the inner leaflet of the membrane. The asymmetry is maintained by three enzyme systems: flippase, floppase and scramblase. Activation of floppase and the increased calcium dependent activity of scramblase in activated or apoptotic cells, leading to transfer of phospholipids from the inner to the outer membrane leaflet [93]. Flopping (externalization) of negatively charge phospholipids to the outer surface of the membrane is the key event in EV shedding [94]. The high calcium concentration stimulates intracellular signaling molecules, such as calpain and gelsolin. Calpain hydrolyzes and disassociates actin in the membrane, and gelsolin is involved in the cleavage of actin-capping proteins (in platelets only) [95](Figure 5).

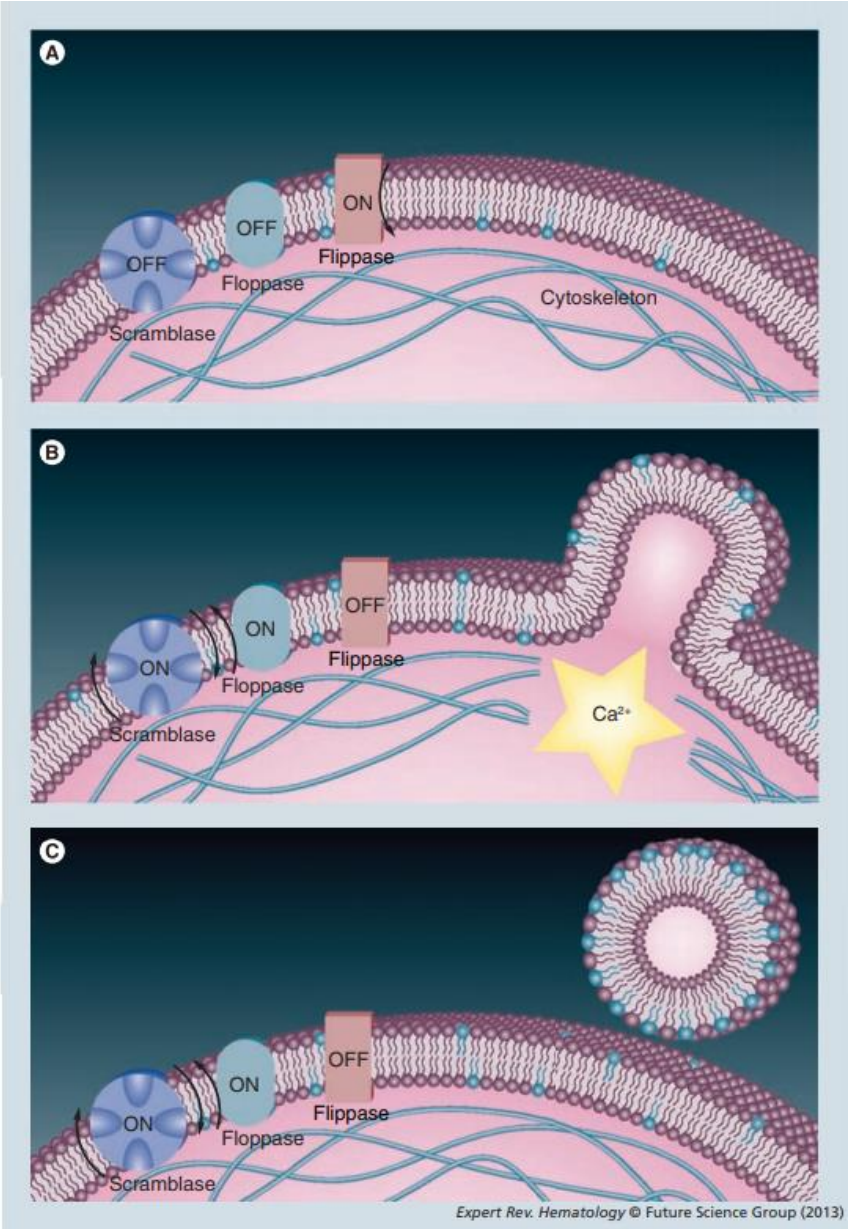


Figure 5: The formation of extracellular vesicles.
(A) In resting cells, phospholipids in the plasma membranes showed an asymmetrical distribution. In particular, negatively charged phospholipids such as phosphatidylserine are located in the inner leaflet. This distribution of the membrane lipids is the result of an active process under the dependence of complementary phospholipid transporters governing either the inward (flip) or the outward (flop) translocation. (B) Cell undergoing activation or apoptosis. Two key events occurred. First, the disruption of phospholipid membrane asymmetry leads to exposure of phosphatidylserine (blue circles) on the outer leaflet. This is a consequence of the activation of floppase, the increased calcium dependent activity of scramblase and the inhibition of translocase/flippase. Second, cytoskeletal reorganization takes place. (C) These events eventually lead to microvesicle release into the extracellular space. (Reproduced with permission from Taylor & Francis) [96].

Apparently, several mechanisms are involved in cellular uptake of EVs and they may enter to cells via more than one route depending on the recipient cell types and EVs concentration [97]. EVs are usually taken up into endosomal compartments via endocytic pathways including endocytosis (clathrin-dependent, caveolin-dependent, lipid-raft-mediated), macropinocytosis, and phagocytosis. In addition, EVs can be taken up by the cells through the direct membrane fusion (figure 6).

Several experimental studies suggested that EVs are mostly taken up into endosomal compartments via endocytosis [98, 99]. Clathrin-mediated endocytosis involves engulfment of receptors associated with their ligands. Dynamin2 is a GTPase protein that it is required in membrane binding in the clathrin-dependent process. In phagocytic cells, blocking of dynamin2 prevented uptake of EVs [97]. It seems that clathrin-independent pathway (caveolin-dependent) exist in eukaryotic cells. Caveolae are cup-shaped invaginations of plasma membrane that are rich in cholesterol, sphingolipids and caveolins. Caveolin-1 is a protein that is essential for the formation of caveolae [100]. Lipid rafts are distinct membrane microdomins which are rich in protein receptors, cholesterol and sphingolipids. It has been reported that annexin II and annexin VI may have roles in anchoring of EVs to these microdomains, and in trafficking of EVs to the late endosomal compartment, respectively [101]. Macropinocytosis is a kind of endocytic uptake pathway that involves the formation of membrane ruffles that then pinch off into the intracellular compartment [100]. This mechanism is dependent on Rac1, actin and cholesterol, and requires Na/H exchanger activity [102]. Phagocytosis pathway involves the internalization of opsonized vesicles by specialized cells (e.g. macrophages) [100]. PS can facilitate the entry of EVs during phagocytosis, and Phosphatidylinositol-3-kinase (PI3Ks) has a key regulatory role in phagocytic processes, especially in the formation of phagosomes [97].

The other possible mechanism in which EVs populations gain entry into cells is direct fusion of the EV membrane with the cell plasma membrane [103]. In this process two distinct lipid bilayer membrane are merged in an aqueous environment and the acidic PH condition presented in the endosomes is fundamental for fusion [103].

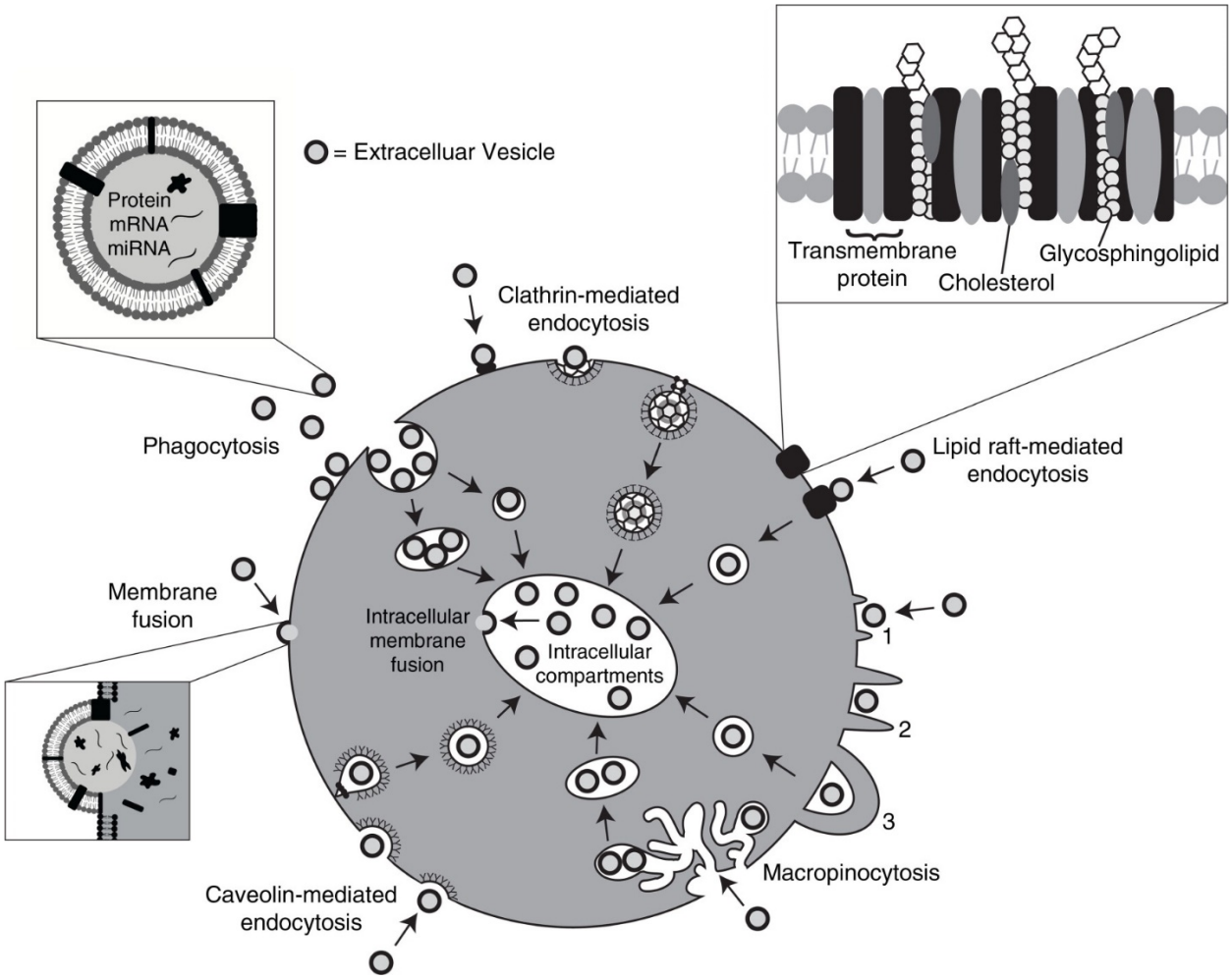


Figure 6: Mechanisms of EV uptake

Pathways shown to participate in EV uptake by target cells. EVs transport signals between cells. EVs have been shown to be internalized by cells through phagocytosis, clathrin- and caveolin-mediated endocytosis. There is also evidence to support their interaction with lipid rafts resulting in EV uptake. Lipid rafts are involved in both clathrin- and caveolin-mediated endocytosis. EVs can be internalized by macropinocytosis where membrane protrusions or blebs extend from the cell, fold backwards around the EVs and enclose them into the lumen of a macropinosome; alternatively EVs are macropinocytosed after becoming caught in membrane ruffles. EVs may also deliver their protein, mRNA and miRNA cargo by fusion with the plasma membrane. Alternatively, intraluminal EVs may fuse with the endosomal limiting membrane following endocytosis to enable their EV contents to elicit a phenotypic response

(Reproduced from Journal of Extracellular Vesicles, Volume 3, 2014-Issue 1 [97]).

1.5.2 Procoagulant functions of extracellular vesicles

EVs are highly procoagulant due to the large quantity of surface-exposed negatively charged phospholipids (e.g. PS), TF and polyphosphate (polyP) [104]. The procoagulant activity and function of EVs were first described by Sandberg et al. in 1982 [105]. EVs are present under both normal and pathophysiological conditions, and plasma levels of TF+ EVs are very low, or undetectable, in healthy individuals [106]. The presence of TF on EV membranes increases their procoagulant activity, as exposed TF readily binds to FVII/FVIIa. TF/FVIIa complexes on EVs are inhibited by TFPI, which is synthesized in endothelial cells and prevents inappropriate activation of the coagulation cascade [107]. Cancer patients have an increased risk of VTE [108], and a role for TF+ EVs in the pathogenesis and prognosis of cancer-associated VTE has been suggested by several studies [109, 110].

PolyP is an anionic linear polymer of inorganic phosphate derived from ATP (adenosine triphosphate) [111]. Mammalian platelets contain short-chain polyP polymers (60–100 phosphate units) while bacteria produce long-chain polymers (several thousand phosphate units). Human platelets accumulate a high concentration of polyP in dense granules [112]. Platelet-derived polyP have different functions in the coagulation cascade [113]. Platelet-derived polyP is associated with accelerating the activation rate of FXI by thrombin, elevate FV activation by FXIa, enhance the inhibition of TFPI by FXIa and enhance fibrin clot structure [113]. In a study on mice, Muller et al. reported that platelet-derived polyP induces fatal PE, increases vascular permeability, and induces fluid extravasation [114].

PS facilitates the assembly of protease complexes in the clotting cascade [115], as it has a pivotal role in the binding of the Gla domains in vitamin K-dependent proteins [116]. There is an electrostatic interaction between the EV membrane PS and the γ-carboxyglutamic acid

domain of proteins in the coagulation cascade. Factors VII, IX, X and prothrombin contain a carboxyglutamic acid domain. PS enhances the catalytic efficiency of prothrombinase (FVa/FXa) and tenase (FVIIIa/FIXa) complexes by a factor of 1000- and 200-fold, respectively [117]. Generally, an increase in total EV concentration is associated with shorter whole-blood clotting times [118, 119].These findings suggest that the procoagulant function of negatively charged phospholipids on the EV surface may serve as a biomarker for identifying patients at increased risk of VTE. Therefore, assessing the phospholipid-dependent procoagulant activity of isolated EVs may be a reliable method for evaluating their procoagulant function, and for determining the role of EVs in the pathogenesis of thrombosis.

1.5.3 Plasma level of extracellular vesicles and risk of venous thromboembolism

Plasma concentrations, cellular origin and the composition of circulating EVs may provide valuable information relating to the diagnosis and prognosis of various diseases and monitoring responses to treatment. Previous studies have reported significant associations between elevated plasma EVs, especially MVs, in patients with sickle cell anemia [120], antiphospholipid antibody syndrome [121], heparin-induced thrombocytopenia [122], cardiovascular disease (e.g. atherosclerosis [123], hypertension [124], myocardial infarction [125], stroke [126]) and infectious diseases (e.g. sepsis [127] and HIV infection [128]).

Although some studies have indicated a role of EVs in cancer-related VTE [109, 110, 129], the association between EVs and VTE in patients without cancer is less clear. Previous reports have demonstrated that plasma levels of total EVs, platelet-derived EVs (PEV), and annexin V^+ EVs are associated with an increased risk of VTE [130-133]. However other studies found no

relation between total EVs, annexin V^+ EVs and PEV levels with VTE risk [134, 135] (Table 1).

Growing evidence from observational studies [136, 137] and experiments [32, 138, 139] suggests that EVs in general, and EV receptor—ligand interactions in particular, play a fundamental role in the pathogenesis of VTE. Cell adhesion molecules (CAMs) are located on EV membranes, and are involved in cell—cell or cell—EV interactions.

Among the well-known transmembrane CAMs is the selectin family [140]. There are three subsets of selectins: E-selectin (endothelial cells), P-selectin (platelets and endothelial cells), and L-selectin (lymphocytes) [141]. Selectins are distinguished from each other by their variable numbers of consensus repeats [141]. P-selectin is the longest selectin with nine motifs; E-selectin contains six motifs; and L-selectin has just two consensus repeats. Their common structure consists of an *N*-terminal lectin-type domain (the main ligand-binding sites), a transmembrane region, and a short cytoplasmic *C*-terminal [141].

The role of P-selectin in thrombus formation was discovered in 1992 [142]. P-selectin is stored in granule membranes of unstimulated platelets (α granules) and endothelial cells (Weibel—Palade bodies) [118]. It initiates the leukocyte—endothelial cell interactions and facilitates extravasation of neutrophils in response to inflammation of blood vessel walls or VTE [143, 144]. Additionally, it plays a role in fibrin generation and propagation during VTE [145]. P-selectin antagonists have similar efficacy to enoxaparin in term of re-opening venous occlusion in animal models [139]. Soluble (circulating) P-selectin is significantly elevated in DVT patients with or without PE [137]. High circulating levels of P-selectin are associated with recurrent VTE [136] and may be a suitable marker for diagnosing of VTE [137].

PSGL-1 is the high affinity counter-receptor for P-selectin. It has a lower affinity for L-selectin and E-selectin [147]. Its expression on platelets can be 25—100-fold lower than on leukocytes,

and on the EVs derived from these cells [148, 149]. The mucin-like domain and sulphated tyrosine residue of PSGL-1 have an impact on ligand-binding affinity. The interaction of PSGL-1 and P-selectin has a significant role in leukocyte–platelet interactions during inflammation and thrombus formation [139].

An *in vivo* study in mice showed that the interaction between leukocyte-derived PSGL-1^+ EVs (particularly monocyte-derived EVs) and P-selectin-positive activated cells (platelets and endothelium) lead to development of a thrombi [32]. In a P-selectin overexpressed mouse model, elevated plasma levels of TF^+ EVs correlated with reduced clotting time and larger thrombi, which disappeared after intervention with a PSGL-1 inhibitor [138]. These findings suggest an important role for a P-selectin—PSGL-1 axis in thrombus formation. Until now, no study has conducted to evaluate the role of PSGL-1^+ EVs as a risk factor for unprovoked VTE in humans.

Table 1. Association between plasma levels of EVs with a particular origin and venous thromboembolism (case-control studies).

Author Year	Study Population	Method	Main Results	Ref.
Martinez *et al.* (2007)	Behcet's disease (n=72)→20 with and 52 without history of VTE; Healthy controls (n=25)	FC	↑ PEV between subjects with and without VTE	[130]
Flores-Nascimento *et al.* (2009)	DVT at diagnosis (n=9) 1-3 years after warfarin withdrawal (n=10);associated with antiphopholipid syndrome (n=11) asymptomatic Factor V Leiden (n=7);healthy controls (n=30)	FC	↑Total number of EVs 1-3 years after withdrawal of warfarin therapy vs healthy controls. No difference of the EVs cellular origin comparing patients to controls.	[133]
Ay *et al.* (2009)	History of recurrent VTE without cancer (n=116); healthy controls (n=129)	P.A.	No difference in EV PS activity between VTE patients and controls.	[134]
Bal *et al.* (2010)	Acute PE (n=45) →3 cancers Healthy control subjects with (n=45) and without (n=45) various risk factors.	FC	↑Annexin V^+ EVs and PEVs in acute PE vs controls without risk factor, but no association vs controls with risk factors.	[132]
Owen *et al.* (2011)	History of VTE without cancer (n=36);healthy controls (n=51)	CAT FC	No difference in PEV/ annexin V^+ EVs levels between subjects and controls.	[135]
Bucciarelli *et al.* (2012)	VTE without cancer (n=186), healthy controls (n=418).	FC	↑Annexin V^+ and $CD41^+$ EVs in VTE patients vs healthy subjects.	[131]
Ye *et al.* (2012)	Initial DVT without cancer (n=25), Recurrent DVT without cancer (n=25); healthy controls (n=25).	TF# FC	↑platelets derived TF^+EVs in recurrent DVT vs initial DVT patients. ↑Monocyte derived TF^+EVs in recurrent DVT vs healthy subjects. ↑Endothelial cells TF^+EVs and total EVs in recurrent DVT vs initial DVT patients.	[150]

Abbreviations: PEV (platelets derived extracellular vesicles); VTE (venous thromboembolism); DVT (deep vein thrombosis); PS^+ EVs (phosphatidylserine positive extracellular vesicles); CAT (Calibrated Automated Thrombogram). FC (Flow cytometry) TF# (TF Chromogenic Activity Assay Kit); P.A. (Prothrombinase assay)

1.5.4 Isolation and measurements of extracellular vesicles in plasma

Pre-analysis handling parameters include blood collection, plasma isolation, type of anticoagulant, storage and EV isolation procedures. The effect of these parameters has not been extensively studied [151], and there is not a consistent handling protocol in published studies (Table 2). Pre-analysis conditions are a major source of variability, with the potential to introduce artifacts in EVs analysis [151]. Standardized pre-analysis protocols are necessary for obtaining accurate and comparable results in EV research.

Transportation of samples is an important pre-analysis parameter because it may promote *in vitro* production of EVs [151]. Ideally, blood samples should be collected and analysed in the same laboratory room to prevent *ex vivo* activation of blood cells and release of EVs. Samples should be collected using a large-diameter needle (e.g. 19-gauge) to allow appropriate flow into the collection tubes and contribute to minimization of pre-analysis variability [152]. Furthermore, the first 1-2 mL of blood should be discarded to avoid contamination with endothelial EVs that arise from vascular injury [152]. Different anticoagulants are used for studying EVs. It is essential that the chosen anticoagulant should inhibit *in-vitro* production of EVs. It was reported that calcium chelators' anticoagulants facilitate rapid bindings between EVs and platelets, thus the platelet and endothelial derived EVs in blood treated with sodium citrate, ethylenediaminetetraacetic acid (EDTA) and acid citrate dextrose (ACD) decrease significantly [153]. They suggested heparin preserve EV concentration in plasma samples [153]. Although a previous study suggested that heparin might stimulate the release of platelet- and erythrocyte-derived EVs [154].

Table 2: Pre-analytical variables to detect and characterize EVs.

Blood Specimen Collection		Plasma Preparation			Storage	Extracellular Vesicles Isolation			Ref.
Needle Size(G)	Type of Anticoagulant	Speed (g)	Time (min)	Tem	Tem	Speed(g)	Time (min)	Tem	
19	EDTA	1st:1200 2nd:12000	15 12	20 20	-70	2x100,000	60	20	[155]
n.s.	Citrate	1550	20	20	-80	18,890	30	20	[110]
19	Citrate	3000	15	n.s.	-40	20,000	30	n.s.	[156]
21	Citrate	2x2000	2x10	20	n.s.	18,890	30	20	[157]
n.s.	Citrate	1550	20	20	n.s.	17570	30	20	[158]
n.s.	Citrate	2x2000	2x30	n.s.	-70	20800	45	10	[159]
n.s.	Citrate	1550	20	20	-80	17570	30	20	[160]
19	Citrate	1550	20	20	-80	18890	30	20	[161]

n.s.: not specified, Tem: Temperature.

Figure 7 represents an overview of various technical approaches for isolating and quantifying plasma EVs. Accurate measurement is challenging due to the low refractive index of EVs and their great heterogeneity. Quantification by conventional flow cytometry (FC) was clearly affected by EV size and the choice of probe. The older versions of commercial FC systems (e.g. Becton-Dickinson FACScan) recognized the degree of light scattering and was exclusively used for particles with diameters in excess of 500 nm [162]. More recent technologies and fluorescence probes significantly improve the signal-to-noise ratio, and are more reliable and valid than light-scatter models [163].

Direct imaging is the preferred method for exploring EV nanostructure. Conventional optical microscopes use visible light, which has wavelengths (390-700 nm) that exceed the nanometric size of EVs. In contrast, electron microscopes use electron beams with a resolution of less than 1 nm. The two main types of electron microscopy (EM) are transmission electron microscopy (TEM) and the scanning electron microscopy (SEM). TEM produces a beam of electrons that pass through thin specimens, and is the most commonly used technique in this field. SEM detects secondary electrons emitted by atoms that are excited by an electron beam. The resolution of SEM is lower than that of TEM, but SEM can provide three-dimensional images of the EV surface [164, 165].

Nanoparticle tracking analysis (NTA) is a useful alternative for measuring EV concentration and size distribution in plasma. It detects Brownian motion of individual particles in the sample, from which particle size can be calculated using the Stokes-Einstein equation [166]. The majority of plasma EVs are less than 200 nm and EVs with diameters between 50 nm and 1 μm can be analyzed by NTA [167]. However, current experimental protocols for NTA are unable to differentiate EVs from protein aggregates and large very-low-density lipoproteins (VLDL). Therefore, more studies are warranted to improve EV isolation, characterization and downstream analysis, and to evaluate pre-analytical parameters for measuring EVs by NTA.

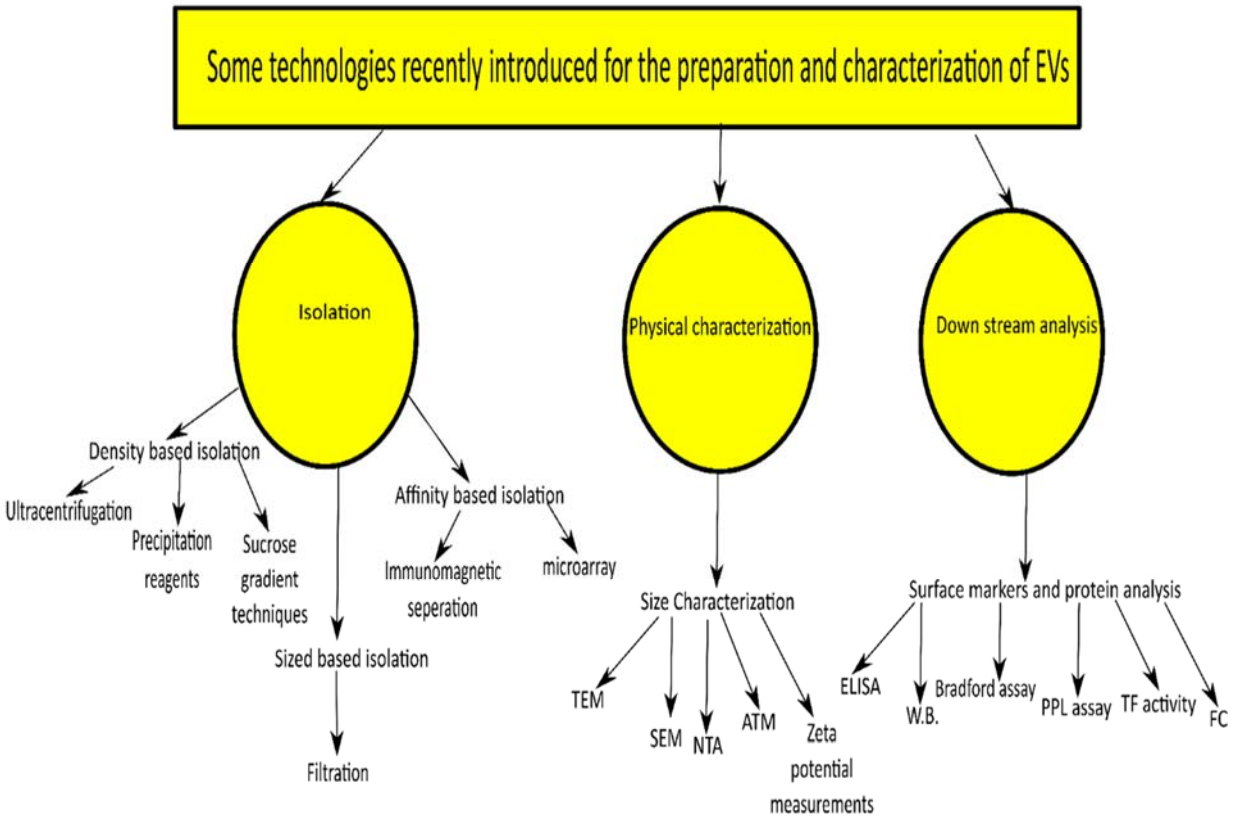


Figure 7: Technologies recently introduced for the preparation and characterization of EVs

Abbreviations: Transmission electron microscopy (TEM), Scanning electron microscopy (SEM), Nanoparticle tracking analysis(NTA), Atomic force microscopy(ATM), Enzyme-linked immunosorbent assay(ELISA), Flow cytometry (FC), Procoaagulant phospholipid activity (PPL assay), Tissue factor activity (TF activity), Physical characterization (Size and concentration analysis methods), Downstream analysis (EVs molecular content, biomarkers and functional analysis methods), W.B. (western blotting).

1.6 microRNA

1.6.1 Structure and biogenesis of miRNA

Ambors and colleagues discovered a single-stand, regulatory RNA molecule in *Caenorhabditis elegans* in 1993 [168]. The first microRNA in humans was discovered sometime later in 2000 [169]. miRNAs are non-coding small RNAs that impact on messenger RNAs (mRNAs) deadenylation, translational repression and degradation [170]. A single miRNA can influence hundreds of different mRNA transcripts, which is why they are implicated in a wide range of biological and pathological processes [171, 172]. They inhibit the target genes by pairing to 3´ untranslated regions (3´UTRs) and regulate the protein expression [173]. 3´UTRs perform a critical regulatory role by increasing poly (A) site efficiency [174] and by pairing with miRNA [175].

MiRNAs are transported in the extracellular environment by different carriers, such as MVs (microvesicles), exosomes, lipoproteins, and other ribonucleoprotein complexes [176-178]. In plasma, miRNAs are remarkably stable and protected from endogenous RNase activity when they are associated with carriers [179].

It has been shown that miRNA genes may be located within the exons or introns of protein-coding genes or non-coding regions [180]. RNA polymerase II/III transcribes miRNA gene or intron within the nucleus to a primary miRNA (pri-miRNA), after which Dorsha and DGCR8 (Pasha) cleave pri-miRNA to precursor miRNA (pre-miRNA), with a stem-loop structure of around 70 nucleotides. Exportin-5-Ran-GTP transports pre-miRNA from the nucleus to the cytoplasm, where RNase III enzyme (Dicer), in complex with the double-stranded RNA-binding protein TRBP, cleaves pre-miRNA to its mature length (of around 22 nucleotides). Generally, one strand is retained as the mature miRNA, while the other is degraded. The

functional strand together with argonaute 2 (Ago2) proteins, interacts with the RNA-induced silencing complex (RISC) and guides RISC to silence target mRNAs [181]. miRNAs interact with target mRNAs through a 'seed' region (a common motif with a 2-7 nucleotide sequence). When the binding of the 'seed' region is not sufficiently strong for stabilizing the whole interaction, a bond forms between residues 13-16 of miRNA and target mRNA [170] (Figure 8). New evidence suggests that the interaction between miRNA and the target gene may take place at different binding sites, such as 5′UTR [182], and the promoter regions [183]. Any modification in the sequence of miRNA alters miRNA expression and/or maturation. Recent studies have shown that SNPs at miRNA sequences, especially at the seed region or mRNA target site, may eliminate or generate a new target gene. Such alterations lead to an increase or decrease in protein products [184].

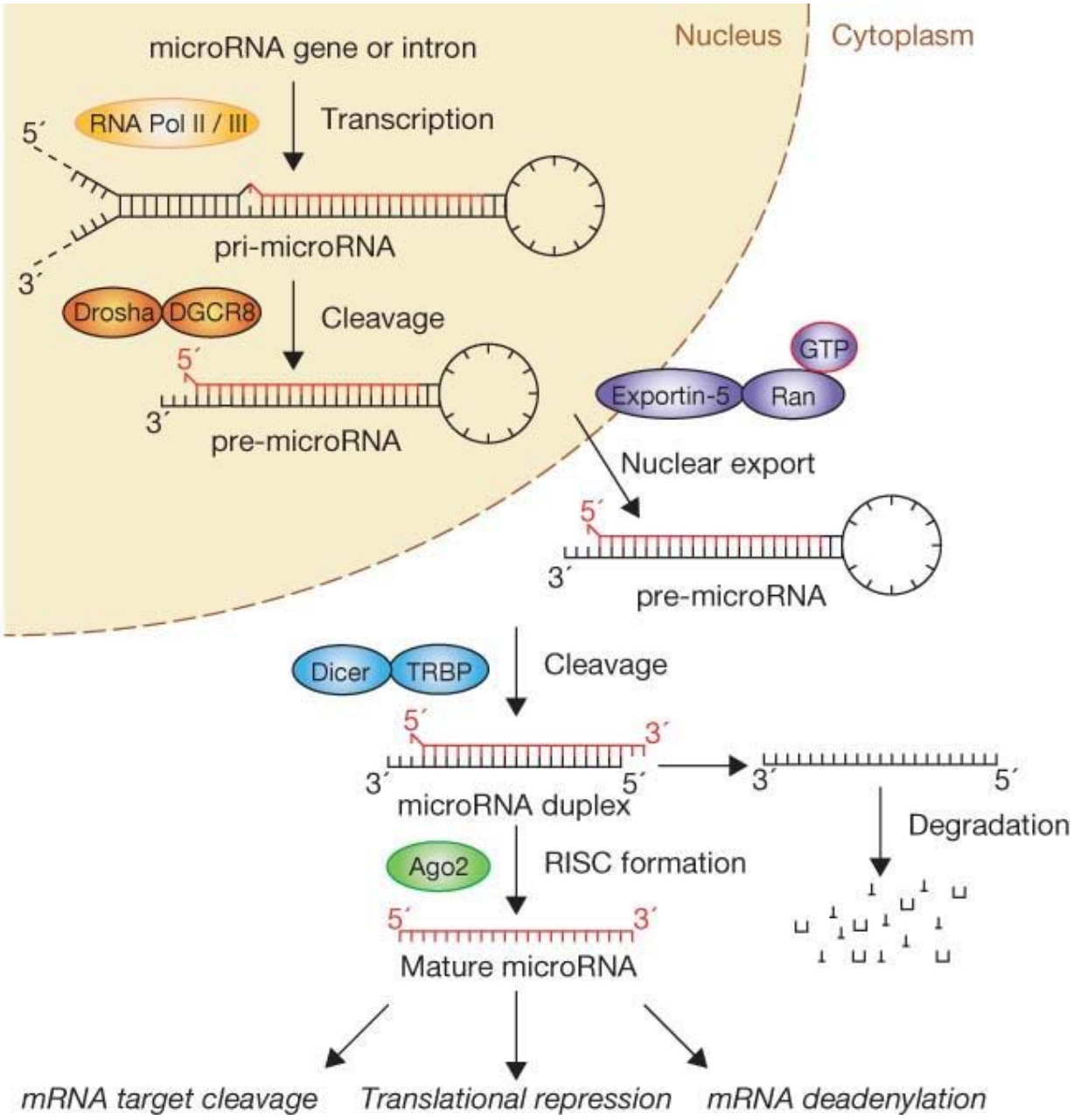


Figure 8: The miRNA processing pathway

The miRNA processing pathway has long been viewed as linear and universal to all mammalian miRNAs. This canonical maturation includes the production of the primary miRNA transcript (pri-miRNA) by RNA polymerase II or III and cleavage of the pri-miRNA by the microprocessor complex Drosha–DGCR8 (Pasha) in the nucleus. The resulting precursor hairpin, the pre-miRNA, is exported from the nucleus by Exportin-5–Ran-GTP. In the cytoplasm, the RNase Dicer in complex with the double-stranded RNA-binding protein TRBP cleaves the pre-miRNA hairpin to its mature length. The functional strand of the mature miRNA is loaded together with Ago2 proteins into the RNA-induced silencing complex (RISC), where it guides RISC to silence target mRNAs through mRNA cleavage, translational repression or deadenylation, whereas the passenger strand (black) is degraded.

Reproduced with permission from copyright clearance center's rights Link service. [185]

1.6.2 Association between miRNA and venous thromboembolism

Recently, circulating miRNAs have attracted attention because of their predictive value in disease states, emerging as a promising class of biomarkers. Earlier studies reported that many coagulation factors are regulated by miRNAs, such as FVIII (miR-1246) [186], FXI (miR-181a-5p) [172], fibrinogen (the miR-409-3P, -29 family) [187], and TF (miR-17-92 cluster, -223) [188, 189]. Similarly, miRNAs also regulates inhibitors of the coagulation pathway including antithrombin (miR-18a) [190], protein-S (miR-492) [191], TFPI (miR-27a/b, -494) [192], and plasminogen-activator inhibitor-1 (miR-421, -30c, -143/-145, -181b, -449a/b) [193, 194].

Many studies have highlighted the role of circulating miRNAs as predictors of diseases, but there is limited evidence on altered miRNAs in VTE patients. Wang *et al*. reported that DVT patients had high plasma level of miR-424-5p and a low level of miR-136-5p. The miR-424-5p level is associated with hypercoagulability, increased production of cell adhesion molecules, metalloprotease enzymes, platelet-activating factor, and cell division regulators [195]. Kong *et al*. established an *in vivo* vein thrombosis rat model in which overexpression of miR-let-7e-5p in endothelial progenitor cells caused enhanced ability of homing and thrombus revascularization [196], while overexpression of miR-483-3p decrease ability of endothelial progenitor cells homing and thrombus resolution in a rat model [197].

The VTE-related miRNAs identified to date may potentially provide novel predictive biomarkers that could optimize current risk assessment models for VTE. However, due to heterogeneity in designs, populations and methodologies of previous studies, as well as the limited quantities of screened miRNA, there is still no consensus on the definition of VTE-related miRNA clusters.

2. Aim of the study

Current knowledge about risk factors and mechanisms involved in VTE do not adequately predict the majority of future VTE events. It is therefore necessary to identify new risk factors and expand our knowledge about the pathogenesis of VTE in order to improve risk stratification, and facilitate targeted prevention and treatment, thereby reducing the burden of VTE.

The specific aims of the project were:

1. To assess and compare the plasma levels, cellular origin and the morphological characteristics of PSGL-1$^+$ MVs in patients with unprovoked VTE.
2. To investigate the impact of pre-analytical conditions, such as the choice of anticoagulants, plasma preparation and fasting status, on plasma concentration and size distribution of EVs determined by NTA.
3. To determine the plasma expression profile of miRNAs in patients with unprovoked VTE.
4. To develop a cost-effective modified clot-based assay for measuring procoagulant phospholipid (PPL) activity.

3. Methods

3.1 Isolation of extracellular vesicles and leukocytes

The most widely used method for isolating EVs from plasma or other body fluids is differential centrifugation, in which the vesicles are separated according to their physical properties (such as molecular weight), and their movement through the suspending medium [198]. Thery *et al*. proposed a protocol for sequential centrifugation, and this is still widely used with minor modifications [199].

In all studies described in these four papers, we used a low speed centrifugation (3000 x g for 10 minutes at 22°C) to obtain platelet-poor plasma (PPP) followed by 13,500 x g for 2 minutes to separate the remaining cells and debris to obtain platelet-free plasma (PFP). It is recommended that high-viscosity biofluids should be diluted with buffer prior to EV isolation [200]. In order to isolate plasma EVs, we used PFP diluted with phosphate-buffered saline (PBS) without Ca^{2+} and Mg^{2+} (1:20 dilution) and centrifuged samples at 20,000 x g for 30 minutes at 22°C. However, previous studies used high-speed ultracentrifugation (100,000 x g) to pellet the EVs [201], which may have produced pellet exosomes along with MVs [202]. With differential centrifugation methods, EVs pellets may co-precipitate with lipoprotein contaminants such as HDL, LDL, VLDL, and Ago2. Lipoproteins [203, 204] and Ago2 carry a specific subset of miRNAs that are independent of EVs [205].

No consensus has been reached on a standard methodology for isolating EVs. One recommendation is to increase the EV purity using sucrose gradient-density floatation together with ultracentrifugation [206].

In order to isolate the peripheral blood mononuclear cell (PBMCs) for confocal microscopy in

the study described in Paper I, we used a density-gradient medium (Lymphoprep, Axis-Shield PoC AS, Oslo, Norway). Granulocytes and erythrocytes have a higher density than PBMCs, thus they separate during centrifugation on Lymphoprep. EDTA-anticoagulanted whole blood samples were used in this study, as EDTA results in minimal monocyte-platelet aggregation, and is less cytotoxic [207]. The whole-blood samples with Lymphoprep were centrifuged at 800 x g for 20 minutes and the PMBCs were retained at the interphase between the plasma and the Lymphoprep, washed with endotoxin-free PBS twice at 150 x g and once at 250 x g at room temperature. These low-speed centrifugation steps serve to eliminate the majority of contaminated platelets. To avoid platelets activation and monocyte aggregation, maintaining a constant room temperature was essential during the experiments [208, 209]. PBMCs were then resuspended in RPMI-1640 medium.

3.2 Plasma concentrations and cellular origin of extracellular vesicles assessed by flow cytometry

FC is an optimal and high-throughput method for quantification and multi-parameter characterization of EVs [210]. The principle of FC is to measure forward- and side-scattered light, as the ratio of particle size to laser wavelength alters this scattering behavior. Side-scatter signals provide information about the internal structure and composition of EVs, and forward-scatter signals detect particle diameter. The antibody concentration, clonality and the use of proper isotype control has a notable effect on the FC output [211].

In the studies described in Paper I and Paper IV, we used annexin V to measure the concentration of EVs in plasma by FC. Annexin V is a collagen-binding protein initially isolated from chondrocytes. It appears to be a suitable marker for PS-positive EVs, and dead and apoptotic cells. However only a small proportion of circulating EVs bind to annexin V and

lactadherin (PS-binding proteins). This may be due to a lack of, or insufficient, expression of PS on EV membranes [212]. Therefore, some of the previous studies identified the EVs populations by staining for the interested biomarker without considering annexin V expression [213, 214]. However, it appears that these studies reported imprecise total EV plasma concentration due to false-positive signals of protein complexes, especially insoluble immune complexes [215]. In this context, we highly recommend for labelling EVs by annexin V in order to a reliable differentiate between EVs and cell debris or precipitates.

In Paper I, we analyzed PSGL-1^{+} MVs and their cellular origin by immunostaining the total MV fraction. We incubated 50µl of isolated MVs in PBS with antibodies at room temperature for 30 minutes. After incubation, 450 µl of PBS was added and samples were analyzed by a FACSAria-I flow cytometer. A size-specific gate for MVs (<1 µm) was set using megamix beads (mix of 0.5, 0.9, and 3 µm). Data were collected in logarithmic scale, and a minimum of 10,000 MVs gate events were recorded.

3.3 Analysis of concentration and size distribution of extracellular vesicles by nanoparticle tracking analysis

NTA is a new and commonly used technique with a resolution suitable for detecting small vesicles, especially EVs. NTA is capable of measuring EVs concentration and size distribution in a short processing time. The lowest detection threshold of NTA for measuring the size is 50 nm, which could cover broad rage size distribution of EVs. This technique is suitable to assess the quality of EVs isolation process by tracking EVs aggregation or co-precipitation of cells in the sample. However, accurate measures rely on the diameter and refractive index of EVs [163]. When assessing EVs in polydisperse specimens (such as blood plasma) larger-sized particles are overestimated as they scatter more light which may mask the presence of smaller particles

[216]. It is therefore necessary to dilute the samples to a particle concentration $<10^9$ per ml until a clear image of the particle population (at least 100 particles in the scattering volume) is obtained. Other parameters, such as the camera level and settings, may also effect the accuracy of size and concentration measurements [163].

In Paper II, we used the NanoSight NS300 system to quantify EV concentration and size distribution. Samples were thawed in water at 37°C [217] immediately before analysis, and diluted with PBS. To obtain a clear image of the EVs, we prepared three separate dilutions for each sample, and each dilution was video-recorded three times for 60 seconds. The nine videos were analysed by NTA software version 3.0 with a detection threshold of 5. The mean results were obtained for EV plasma concentration and size distributions.

3.4 Visualizing the three-dimensional and ultrastructure of EVs by electron microscopy

The main limitation of FC is its inability to detect EV populations with diameters of 30-150 nm that can be easily detected by TEM and SEM techniques. SEM is suitable for analyzing EV topography pattern and morphology. TEM or immune-electron microscopy (IEM) techniques are used to assess bilayer phospholipid membrane, surface markers and cell or vesicles size. Both size and surface markers may be used to differentiate exosomes and MVs from lipoproteins. However, specimen preparation, labeling and pre-analysis prior to imaging with TEM and SEM are time-consuming and may lead to EV dehydration, thus giving imprecise data on EV size and morphology. Nonetheless, TEM and SEM remain the “gold standard” for studying EVs [218].

3.4.1 Negative staining

This technique was performed on isolated and fixed EVs according to Harris' recommendations [219]. Grids were floated on sample drops for 30 minutes and blocked with 1% Fish Skin Gelatin (FSG, Sigma-Aldrich, Cat: G7756) and 1.5% BSA buffer for 20 minutes. In Papers I and II, the grids were immunolabeled for target markers. Immunolabeled EVs were conjugated with protein-A gold particles (PAG) for 15 minutes and stained/dried by adding 1.8% methyl cellulose and 0.3 % uranyl acetate.

3.4.2 TEM ultrathin section

EVs have nanometric diameters and form invisible pellets, which makes it difficult to find EV particles in EPON resin. The grids were embedded in a very thin layer of EPON**,** between two layers of *aclar***,** and polymerized at 60 °C for 48 hours. The EVs were adsorbed to the EPON-coated EM grids in a manner similar to that with negative staining. The grids were fixated in glutaraldehyde, post-fixated in osmium tetroxide (OsO_4) on ice for 5 minutes and stained with uranyl acetate, then dehydrated in a graded series of ethanol and infiltrated in EPON equivalents. Finally, grids were embedded in EPON and polymerized at 60 °C for 48 hours and ultrathin sectioned using a Leica Ultracut-S (Vienna, Austria) microtome (Figure 9).

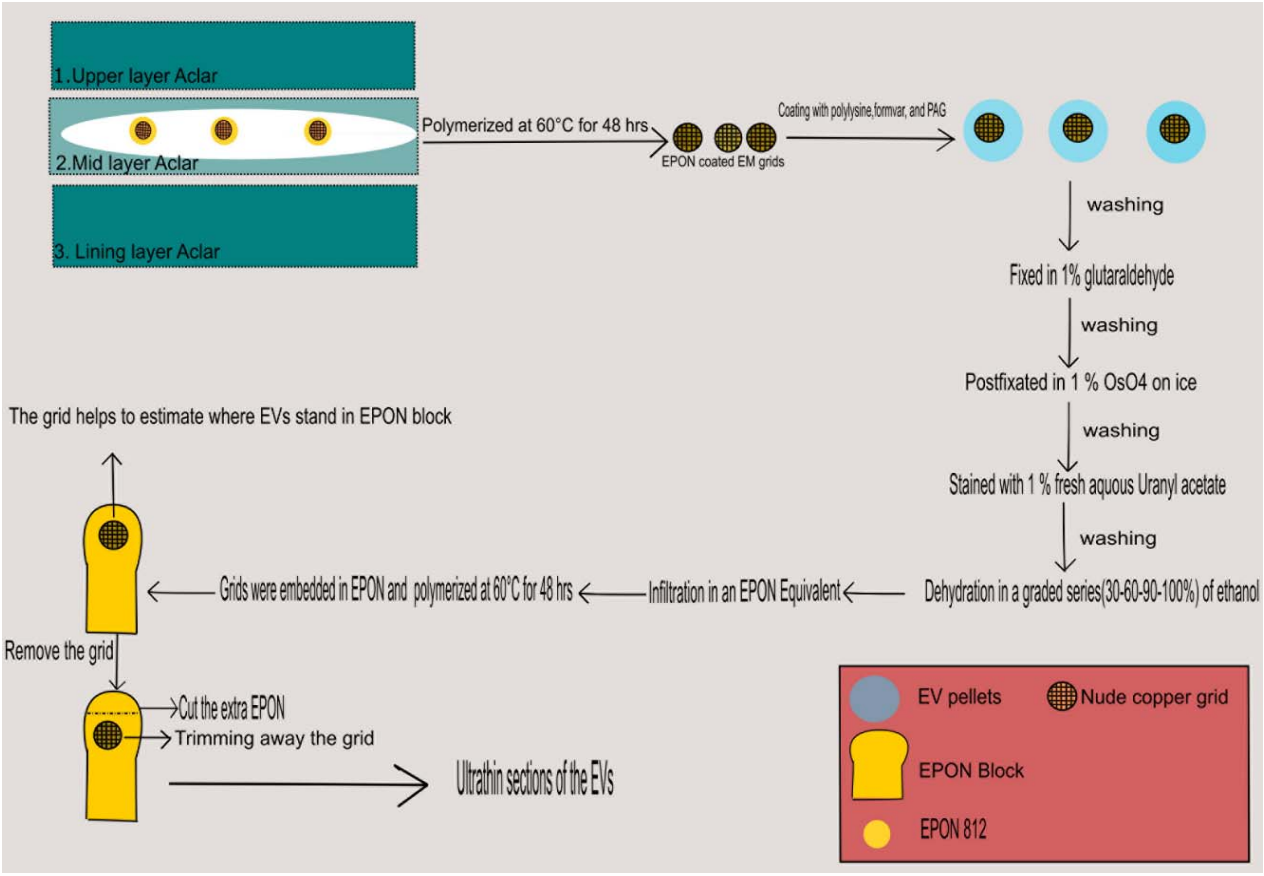


Figure 9: Schematic illustration of ultrathin section method in transmission electron microscopy.

Grids embedded in a very thin layer of EPON between two layers of *aclar* and polymerized at 60°C for 48 hours. Afterwards, they labeled with PAG and coated with polylysine and Formvar. EPON coated grids incubated with EVs similar to negative staining method. Then, they washed, fixed and stained; followed by dehydration in a graded series of ethanol and infiltrated in Epon equivalents. Grids were embedded in EPON, polymerized and finalized by cutting.

EV: Extracellular vesicles, PAG: Protein A-gold.

3.4.3 Scanning Electron Microscopy (SEM)

To validate the impact of isolation and handling processes on EV morphology and biomarker expression, we immunolabeled samples according to established standard protocols [219]. Formvar coated nickel grids were floated on fixed EVs, grid on drop, for 30 minutes. After the washing and blocking steps, samples were incubated with mouse primary antibody for 20 minutes at room temperature. The corresponding secondary antibody was then loaded for 15 minutes. Immunolabeled EVs were conjugated with PAG and treated with 1% glutaraldehyde, and postfixated in 1% OsO4 in double distilled water (DDW). They were dehydrated using ethanol gradients of 60-100% before being dried in HMDS (Hexamethyldisilazane). Images were taken by Zeiss Merlin VP Compact microscope (GmBH, Jena, Germany).

3.5 Measuring the level of negatively charged phospholipid in extracellular vesicles

Recent studies suggest that EV procoagulant activity may be associated with a higher risk of VTE [135, 220]. Currently, the STA-Procoag-PPL commercial assay is available for studying EV procoagulant activity. This assay is quick (<4 minutes) and easy with excellent reproducibility. The assay detects PPL activity in platelet-poor plasma, platelet-rich plasma and whole blood using a clot-based assay. Phospholipase-treated (PPL-depleted) plasma must be used, which is an expensive step within the protocol.

In Paper IV, we developed a cost-effective modified assay to measure PPL activity in FXa-dependent clotting assay. We substituted phospholipase-treated plasma with EV-depleted plasma (EVDP) to obtain phospholipid-depleted plasma. To obtain EVDP, citrated PFP was

centrifuged at 100,000 x g for 60 minutes at 4 °C. Duplicate clotting tests were performed on a StarT4 analyzer (Diagnostica Stago, Mount Olive, New Jersey, USA). Equal amounts of the specimen and EVDP were mixed in a start cuvette containing a steel ball, and pre-warmed for 2 minutes at 37 °C. The reaction was initiated by the addition of 100 µl of pre-warmed assay buffer, using a cabled pipette that automatically starts the timer upon pipetting, to measure clotting time. The assay buffer contained bovine FXa (0.01 U/ml) in calcium chloride (15 mM), sodium chloride (100 mM) and HEPES buffer (20 mM). The STA-Procoag-PPL assay was performed according to the manufacturer's protocol.

4. Main Results

Paper I: Elevated Plasma Levels of P-Selectin Glycoprotein Ligand-1 (PSGL-1) Positive Microvesicles in Patients with Unprovoked Venous Thromboembolism

MVs express antigens from their parental cells and have a highly procoagulant surface. Animal studies suggest that PSGL-1^+ MVs play a role in the pathogenesis of VTE.

The aim of this study was to determine the plasma levels, the cellular origin and the morphological characteristics of PSGL-1^+ MVs in patients with unprovoked VTE.

We conducted a population based case-control study in 20 patients with a history of unprovoked VTE and 20 age- and sex-matched healthy controls recruited from the general population. Plasma levels, the cellular origin, and the morphological characteristics of PSGL-1^+ MVs was evaluated using flow cytometry, electron microscopy and confocal microscopy.

Plasma levels of PSGL-1^+ MVs were associated with increased risk of VTE. The odds ratio (OR) per one standard deviation increase in PSGL-1^+ MVs was 3.11 (95% CI: 1.41-6.88) after adjustment for age and sex, and 2.88 (95% CI: 1.29-6.41) after further adjustment for BMI. The PSGL-1^+ MVs originated mainly from monocytes and endothelial cells determined by double-staining with markers of parental cells using FC and TEM. SEM of PSGL-1 labeled plasma-derived MVs displayed dominantly spherical vesicles that varied between 50 nm and 300 nm in diameter.

In conclusion, increased plasma levels of PSGL-1^+ MVs were associated with the risk of unprovoked VTE. Large population-based prospective studies are warranted to validate our findings.

Paper II: Impact of preanalytical conditions on plasma concentration and size distribution of extracellular vesicles using Nanoparticle Tracking Analysis

Optimal pre-analytical handling is essential for valid and reproducible measurements of plasma concentration and size distribution of EVs. NTA is able to measure small EVs (50-200 nm in diameter), but the method has methodological concerns. We aimed to investigated the impact of plasma preparation, various anticoagulants in commercial blood collecting tubes (Citrate, EDTA, Sodium Citrate-Theophylline-Adenosine-Dipyridamole (CTAD), and Heparin), and fasting status on plasma concentration and size distribution of EVs measured by NTA.

Blood was drawn from 10 healthy individuals from the research staff to investigate the impact of various anticoagulants, and forty individuals from the Tromsø study to investigate the impact of VLDL particles during postprandial lipidemia. Plasma concentration of EVs were measured by NTA after isolation from plasma by high speed centrifugation and size distribution of EVs assessed by both NTA and SEM. TEM was used to confirm the presence of bilayer membranes. Proton nuclear magnetic resonance (NMR) spectroscopy was used to determine the concentration and mean particle sizes of VLDL.

The plasma concentrations, determined by NTA, and size distributions, determined by NTA and SEM, were essential similar in plasma anticoagulated by various anticoagulants. The EVs retained a round-shaped morphology after isolation from plasma by high speed centrifugation evaluated by TEM and SEM. Postprandial lipidemia was not accompanied by apparent changes in plasma EVs, but increased the mean sizes of VLDL particles ($p<0.001$) which interfered with EV measurements and explained 66% of the variation in plasma EVs in the postprandial phase. In conclusion, we found similar concentrations and size distribution of plasma EVs detected by NTA across the different anticoagulants. Optimization of procedures for separating VLDL particles and EVs in plasma is needed in order to use EVs detected by NTA.

Paper III: Differential expression of plasma miRNAs in patients with unprovoked VTE and healthy control individuals

Venous thromboembolism (VTE) remains the third most common cardiovascular disease with a vague pathogenesis. Circulating miRNAs are small regulatory RNAs found in plasma, serum and other body fluids in an apparently stable form. Although circulating miRNAs, a novel family of regulatory molecules, emerge as a promising class of biomarkers in many cardiovascular diseases and malignancies, knowledge on plasma miRNA levels in VTE remains sparse. The present work was conducted, as a pilot study, to estimate the plasma expression profile of miRNAs in patients with unprovoked VTE.

Twenty patients with a history of unprovoked VTE 1–5 years prior to inclusion in the study and twenty age- and sex-matched healthy control participants were enrolled in a case–control study (Tromsø IV). Plasma levels of 742 miRNAs were assessed after RNA extraction and reverse transcription. Profiling of miRNA was conducted on the Universal RT microRNA PCR Human panels I and II (Exiqon, Denmark). For normalization of the data, the average of the assays detected in all samples (n= 40 samples) was applied.

Ninety-seven miRNAs were detected throughout all samples. Of these, miR-10b-5p, −320a, −320b, −424-5p, and −423-5p were upregulated, whereas miR-103a-3p, −191-5p, −301a-3p, and 199b-3p were downregulated in plasmas of VTE patients versus controls ($P \leq 0.05$). These miRNAs were confined to the extracellular vesicles-depleted plasma fraction, and yielded clear clustering distinguishing samples from the VTE and control groups.

The results of this pilot study may suggest that plasma miRNAs profiling can provide novel biomarkers of unprovoked VTE.

Paper IV: A readily accessible and robust assay to measure levels of procoagulant (P) activity of negatively charged phospholipids (PL) – a modified clot-based PPL assay

Growing evidence support a role of extracellular vesicles (EVs) in haemostasis and thrombosis partly due to exposure of negatively charged procoagulant phospholipids (PPL) which facilitates coagulation activation. Current clotting assays uses resource-demanding chemically phospholipid-depleted plasma to measure PPL activity in a FXa-dependent clotting assay.

We aimed to modify the FXa-dependent clotting assay to measure PPL activity by substituting phospholipid-depleted plasma by EV-depleted plasma obtained by ultracentrifugation and the introduction of a standardized PPL reagent to allow for comparisons of results between laboratories.

Blood was collected from healthy volunteers. Assay properties of our modified assay were compared to a commercial PPL assay. The two PPL assays displayed essentially similar sensitivity to exogenously added standardized phospholipids and the PPL activity measured by the two assays in plasma samples from healthy volunteers (n=10) were highly correlated (R^2=0.95, $p<0.0001$). The intraday- and between-days coefficients of variation (CV) varied between 2-4% depending on the PPL activity. The modified PPL activity was insensitive to increased postprandial lipoproteins in plasma after a standard fat tolerance test and to tissue factor-positive EVs isolated from whole blood exposed to Lipopolysaccharide (LPS)-stimulation (5 ng/ml) and PMA (30 ng/ml) for 4 hrs. The PPL activity in the modified assay was positively associated with annexin V^+ EVs determined by flow cytometry (R^2=0.55). The modified PPL assay performed equally to the comparator, was insensitive to postprandial lipoproteins and TF^+ EVs, and had low assay costs making it suitable for large-scale applications.

5. Discussion

Previous mouse model studies have shown that thrombus development is strongly dependent on the interaction between circulating PSGL-1$^+$ MVs and cellular expression of P-selectin [32, 138, 139, 221]. In Paper I, we explored the plasma concentration, cellular origin and morphological characteristics of PSGL-1$^+$ MVs in patients with unprovoked VTE compared with healthy controls. We found that plasma concentrations of PSGL-1$^+$ MVs in VTE patients was higher than in controls. PSGL-1$^+$ MVs were mainly derived from monocyte and endothelial cells and displayed a round-shaped morphology with a diameter of 50–300 nm. Our findings support the concept that PSGL-1$^+$ MVs may have an important role in the pathogenesis of VTE.

To date, there have only been a handful of studies exploring the association between plasma MV concentration and the risk of unprovoked VTE [96]. A case–control study in DVT patients quantified the total plasma concentration of MVs, showing higher levels of circulating MVs 1–3 years after warfarin withdrawal [133]. Bucciarelli *et al*. reported that patients with elevated annexin V$^+$ MV levels (above the 95% percentile), had a 2.2-fold increased risk of VTE compared to cases with MV concentrations below the 95% percentile [131]. A study on acute PE patients revealed that levels of annexin V$^+$ EVs were higher in patients than in controls subjects with no history of VTE or vascular risk factors [132]. We found that the risk of unprovoked VTE increased by 1.6-fold per one standard deviation (SD) increase in plasma concentration of annexin V$^+$ MVs. However, another study found no significant difference between plasma MV concentration in VTE patients and controls [135]. The diverging results of these studies could potentially be explained by differences in time between VTE and blood sampling.

Our case–control study is, to the best of our knowledge, the first to explore plasma concentrations and cellular origins of PSGL-1$^+$ MVs in patients with unprovoked VTE. The

elevated plasma levels of PSGL-1$^+$ MVs that we observed in unprovoked VTE patients may facilitate accumulation of MVs at the site of activated endothelial cells and platelets expressing P-selectin. Genetically engineered mice that over-express P-selectin showed increased levels of circulating TF$^+$ MVs in association with shorter clotting times, which were abolished after injection of inhibitory anti-PSGL-1 antibodies [138, 221]. In addition, P-selectin blockers were associated with a dose-dependent decrease in thrombosis and spontaneous recanalization in animal models [139]. These findings provide evidence for a role of the PSGL-1—P-selectin axis in thrombosis. Del Conde *et al.* [222] reported that monocyte-derived MVs are enriched with TF and PSGL-1 proteins (80% and 120%, respectively) compared to monocytes. These MVs subset bud off from lipid-rich raft regions of monocytes and they are selectively enriched in both TF and PSGL-1 [222]. Furthermore, the circulating MVs accumulate at the site of injury via the interaction of P-selectin with PSGL-1 and may participate in the thrombin generation through the expression of active TF at their surface as shown in animal study [33]. Similar animal studies have shown that plasma MVs that co-express TF and PSGL-1 [223] have a role in thrombus formation via the interaction of P-selectin with PSGL-1 [32].

The PSGL-1 expression varies significantly between various leukocytes, with the highest expression on monocytes, followed by T cells and natural killer cells, as well as neutrophils (to a lesser extent) [224]. PSGL-1 expression on platelets is 25-100-fold lower than on leukocytes [148]. PSGL-1 expression on endothelial cells has a functional role in facilitating tethering and firm adhesion of monocytes and platelets to activated endothelium [225]. The MVs derived from these cells are expected to express PSGL-1 on their membranes. In our study, we aimed to identify the main parental origin of PSGL-1$^+$ MVs in unprovoked VTE patients. Using FC with MVs co-labelled with PSGL-1 and specific markers of potential parent cells, we found that monocytes and endothelial cells were the main source, whereas platelet-derived MVs were only slightly positive for PSGL-1. The FC findings were verified by IEM and confocal

microscopy. Confocal microscopy with immunofluorescent-labelled targets showed that monocytes display scattered signals of PSGL-1 expression on the cell membranes.

Previous studies have reported that MVs have a short half-life in circulating blood compared to parent cells [226]. The enhanced levels of MVs even long time after an unprovoked VTE event suggest that ongoing activation of the monocytes/endothelial cells and to lesser extent platelets still take place. However, the >1-year gap between the VTE event and the blood sampling was performed to minimize the impact of the VTE event itself on the status of cellular activation in blood samples. Even though a case-control study design is unable to establish the temporal sequence between PSGL-1^+ MV and VTE risk, the >1-year gap between the event and blood sampling may suggest that high levels of PSGL-1^+ MV are associated with risk of VTE. However, further research using a prospective design is warranted to investigate whether high plasma PSGL-1^+ MV concentrations are associated with future risk of VTE.

In Paper II, we focused on the impact of pre-analytical conditions of blood samples, such as fasting status, centrifugation methods, and the choice of anticoagulant, on plasma concentration and size distribution of EVs assessed by NTA. We found that plasma concentrations and size distributions of EVs were similar with all four anticoagulants (Sodium citrate, Sodium heparin, EDTA, and CTAD). Two different TEM methods (negative staining and ultra-thin sectioning) confirmed the presence of EV bilayer membranes with all four anticoagulants. EV size distribution, detected by NTA, was validated by SEM. Using SEM, a similar morphology was observed for all four anticoagulants.

Direct measurement of concentration and size distributions of EVs without centrifugation and washing would have been optimal to avoid morphological changes. However, with these methods other plasma particles (e.g. triglyceride-rich lipoproteins) can interfere with the accuracy of EV measures, as they have similar sizes and light-scattering properties [227]. In

our study, we used high-speed centrifugation followed by re-suspension of EV pellets in particle-free buffer, to maximize isolation and to minimize interference from triglyceride-rich particles. The triglyceride concentrations were unchanged after high-speed centrifugation in plasma supernatants, while serum triglyceride and concentrations of VLDL particles in the plasma of fasting participants was moderately correlated with EV concentration (13–19% of the variation in plasma EVs). We used SEM to confirm the size distribution that was determined with NTA. We found that the size distribution of EVs isolated from plasma was essentially similar when measured by NTA and SEM. The mean EV diameter was 80–90 nm. Using NTA, we showed that only 3.5–5% of EVs had a diameter above 200 nm, and only 0.7–1.3% had a diameter above 300 nm. The EV plasma concentration varied between 1.6 x 10^{10} /mL and 2.0 x 10^{10} /mL, with an interquartile range of 1.3–2.7 x 10^{10} /mL. These findings were consistent with the results of Dragovic *et al.* [166] who evaluated EV concentrations by directly labeling EVs in plasma using a cell tracker peptide. They reported that the vesicle size ranged from 50 nm to 300 nm with a peak size around 80 nm. The total concentration of unlabeled vesicles (1.49 x 10^{12}) differed greatly from the labeled concentration (1.2 x 10^{10}) [166]. This may be due to the presence of other plasma particles, such as chylomicrons and VLDL, in the samples.

An oral fat-tolerance test was conducted to assess the impact of postprandial lipemia on plasma concentration of EVs, as measured by NTA. After eating a standardized fat-rich meal, the plasma samples showed an increase in triglyceride that peaked after 4 hours and returned to baseline levels after 8 hours. VLDL concentrations did not change significantly, but the mean VLDL size was significantly higher in postprandial samples than in fasting samples (55 ± 9 nm to 42 ± 6 nm, respectively). NTA is capable of measuring particles with a lower detection limit of around 50 nm [166], suggesting that even partial sedimentation of VLDL particles during high-speed centrifugation influences EV levels, especially under postprandial conditions. In our study, plasma concentrations of EVs and VLDL particles correlated strongly 4 hours post-

ingestion of the meal, and explained 59–66% of the variation in plasma EVs. Our present result is in the line with the findings of a previous study [228], which investigated the impact of pre-prandial and post-prandial lipidemia on plasma levels of EV assessed by FC. They reported that the majority of circulating particles within the size range of EVs, lacked common EVs markers and most of these particles were lipoproteins.

As well as high-speed centrifugation, magnetic beads have been used for separating VLDL particles from EVs [229]. However, antibody-mediated removal of ApoB-expressing lipoproteins from plasma using magnetic beads has some limitations, such as removing EVs, most probably due to the formation of LDL-EV complexes [228], and causing a shift in particle size due to aggregation of antibodies and lipoproteins [229]. Future studies should optimize centrifugation procedures, antibody-mediated removal or combined procedures in order to separate EVs and VLDL particles so that NTA could accurately and reliably be applied to measure plasma concentrations of EVs.

We established a unique SEM/TEM protocol for capturing high-resolution SEM images and for clear visualisation of EV bilayer phospholipid membranes, in order to distinguish EVs from lipoproteins. In our study, the majority of the extracted particles were EVs as they had a visible bilayer membrane and a lower electron density than lipoproteins.

Some studies using FC have investigated the impact of various anticoagulants on plasma EV concentrations, showing that EV levels were higher in heparin-treated tubes than in samples treated with sodium citrate, ACD or CTAD as anticoagulants [151, 230]. Using NTA, we found that levels of large EVs (>300 nm), which are detectable by FC, were twice as high in heparin samples than in samples exposed to other anticoagulants. Citrated plasma samples showed the lowest median plasma EV concentrations in all size categories, compared with heparin, EDTA and CTAD.

In Paper III, our aim was to detect circulating miRNAs as potential biomarkers for predicting the risk of unprovoked VTE. Dysregulation of a single miRNA or a panel of miRNAs is closely associated with various diseases, including cancer [231] and cardiovascular disease [232]. No previous investigation has profiled miRNA expression in unprovoked VTE. Plasma levels of 742 miRNAs were assessed after RNA extraction and reverse transcription and 97 miRNAs were detected throughout all samples, of which nine showed levels that were significantly different between patient and control groups. We observed that five miRNAs involving miR (-10 b-5p, -320a, -320b, -424-5p and -423-5p) were upregulated in VTE patients, and four (miR-103a-3p, -191-5p, -301a-3p, and 199b-3p) were downregulated.

Prior to our investigation, only a few studies profiled miRNA expression in VTE patients. Xiao *et al.* analyzed miRNAs in plasma samples of 32 acute PE patients and 32 healthy controls with the Human TaqMan miRNA microarray panel. Among 30 differentially expressed miRNAs across PE patients and controls (miR-134, -410, -520g, -485-3p, -362-5p, -382, -548b-5p, -139-3p, -197, -574-3p, -190, -489, -146a and -320) were over-expressed in patients, and miRNA-134 was stated to be a potential marker for diagnosing PE [233]. Interestingly, higher expression of miRNA-320a and -320b was present in this study, which was confirmed in our study. Platelet activation has been related to the pathogenesis of arterial thrombosis, and in myocardial infarction, a couple of miRNAs have been associated with platelet activation, including miR-320a, -320b, -423-5p, -185 [234]. This finding corresponds with our own results, as we observed over-expression of miR-320a, -320b, and -432-5p in the VTE patient group. Several experimental and observational studies supports the role of platelets in VTE [235, 236]. An experimental study showed early accumulation of platelets at the head of the thrombus, but as the thrombus aged, acquisition of platelets slowed and the thrombus became predominantly composed of fibrin and erythrocytes [234]. In addition, a large population-based cohort study reported that subjects with mean platelet volume (MPV) ≥9.5 fL was associated with a 1.5-fold

higher risk of unprovoked VTE [236], and a randomized controlled trial showed that platelet inhibition with aspirin reduced the risk of recurrent VTE (34% risk reduction) [237].

Another study reported the over-expression of miR-582, -195 and -532 in 18 patients with orthopedic postoperative DVT, compared to 20 controls [238]. None of these miRNA differences were significant in the report by Xiao *et al.* [233], or in our study. The variation in miRNA expression may relate to the type of VTE (acute PE, postoperative DVT) or the time since the initial thrombotic event, (long-term after a single VTE event), existing comorbidities, or the type of biofluid (plasma or serum) used in the various studies.

According to previous reports, miRNAs are localized within membrane-derived vesicles including MVs and exosomes, lipoproteins (HDL and LDL) and ribonucleoprotein complexes (e.g. AGO-2), which protects them from degradation [176, 178]. It was reported that the vast majority of plasma miRNAs are localized and packed in MVs [177]. However, when we pelleted EVs by high-speed centrifugation (20,000 x g for 30 minutes) in a subsample of the study subjects the expression of EVs in EV-depleted plasma remained essentially similar. However, we could not rule out a role of exosomes, as they would still be present in EVDP samples. Another plausible explanation might be the influence of ultracentrifugation during isolation, which elevates the amount of multilayer MVs. During high-speed centrifugation, multilayer MVs can burst and release their contents into the plasma [239]. Our findings in this regard were in line with the data of Arroyo *et al.* [205], suggesting that miRNAs -424, -320a and -423-5p were not expressed in MV plasma fractions, while miR-193a-5p, -103 and -191 were present at same level in both MVs and plasma proteins.

We investigated the possible targets genes regulated by the miRNAs that were differentially expressed among cases and controls in our study. The possible targets were signaling and adhesion proteins, growth factors and their corresponding receptors, and several proteases with

roles in the activation of blood clotting. In conclusion, we identified a substantial miRNA cluster in unprovoked VTE patients, and this may give new insights into the molecular mechanisms underlying development of VTE. Further experimental studies are warranted to provide novel insight in the role of miRNA in the pathophysiology of VTE. A prospective study is required for validating our results.

Several assays are developed to measure the PPL activity in human plasma, and we can classify them into two main groups: (i) assays that are based on the ability of annexin V to bind PS in the presence of Ca^{2+} [240, 241], and (ii) clotting assays which utilizes the ability of PPL to accelerate the conversion of prothrombin to thrombin. Currently, there are two commercially available assays, the STA-Procoag-PPL assay from Diagnosis Stago (Mount Olive, New Jersey, USA) and XACT assay from Haematex (Hornsby, NSW, Australia) which are both clotting assays.

As explained in Paper IV, we modified and validated a FXa-dependent PPL clotting assay for both plasma and isolated EVs samples. In this procedure, sequential centrifugation was used rather than an enzyme-based treatment to deplete PPL from plasma. To determine whether the assay data were comparable, our results were fully validated using a STA-Procoag-PPL assay (Stago Company) under various analytical conditions. Statistical analysis showed a strong correlation ($R^2 = 0.95$) between the output of our assay and the STA-Procoag-PPL. Our modified assay costs less than the commercially available assay, and yielded high sensitivity and reproducibility.

The high cost of commercial STA-Procoag-PPL assay relates to the use of lipase to remove PPL from plasma samples. We found that sequential centrifugation, rather than enzyme-dependent depletion, resulted in depletion of the majority of PPLs from pooled plasma. In addition, we decided to use a physiological source of PPL that has a stable phospholipid

concentration (UPTT reagent). UPTT consists of rabbit brain cephalin extract, and appears to be a reliable choice for the general calibration. Today, there are no common calibration solutions for use with the commercial assays, and the reference range for clotting time is not well-defined. For instance, the XACT assay has a synthetic PPL calibrator and the STA-Procoag-PPL assay leaves it to the operator to define a reference range for clotting time [242]. The similarity between the modified PPL assay and the STA-Procoag-PPL was quantified by using a UPTT reagent, showing a high degree of similarity. The consistency of the modified PPL assay was also calculated using within-day and between-day variation of results, and these were within acceptable limits [243], with a CV of 2.8–4.1%. Another study showed similar results using the XACT assay with intra-assay CVs of 3.3% and 3.1% for normal pooled plasma and patient plasma, respectively, and inter-assay CVs of 3.9% and 4.2% [244]. Stago reports intra-assay CVs of 0.3-0.6% and inter-assay CVs of 1.3-2.1% for the STA Procoag-PPL assay in the assay manual.

We analyzed the sensitivity of the assay using EVDP samples and since there is a possibility that the test results were affected by the presence of activated clotting factors. We examined the effect of different concentrations of coagulation factors (such as TF, FVIIa and FVa) in the modified PPL assay. Interestingly, physiological concentrations of these coagulation factors did not affect clotting times in the PPL assay. Taken together, these findings suggest that the modified PPL assay is a rather specific assay where preanalytical variation of these factors do not influence the assay.

As expected the PPL activity in plasma mediated by EVs led to a shortening of the clotting time. This was confirmed by blocking PS activity using lactadherin, which resulted in prolonged clotting times. Moreover, PPL activity was correlated with annexin V^+ EVs, but not with total EV count ($R^2 = 0.13$), which highlights the role of PS^+ EVs in the coagulation cascade.

In the next validation step, we aimed to determine whether the lipid profile of the blood samples taken under fasting and non-fasting conditions had any effect on the results of the modified assay [245]. We found no significant change in clotting times between fasting and postprandial samples (51.1±12.4 vs 48.9±9.3 sec) so fasting status of individuals are not a requirement. Biobanked plasma samples from large population studies, where pre-analytical conditions may vary, can therefore be used with confidence. Further, we observed a reliable output from isolated EVs at different concentrations, thus confirming the assay's reliability for measuring PPL activity on isolated EVs. Overall, the modified assay proved to be sensitive and reliable for measuring negatively charged phospholipids in plasma and isolated systems.

6. Conclusions

1. The findings of our pilot population-based case–control study suggest that increased plasma levels of PSGL-1^+ MVs are associated with the risk of unprovoked VTE. PSGL-1^+ MVs ranged in size between 50 nm and 300 nm, had a round shape structure, and originated mainly from monocytes and endothelial cells (and from platelets, to a lesser extent).

2. Using NTA to investigate the impact of pre-analysis handling variables, we found no significant change in plasma concentrations or size distribution of EVs using four different anticoagulants. Postprandial lipemia was not associated with significant changes in plasma EV concentration, but was associated with an increase in the mean size of VLDL particles, which interfered with EV measurements particularly in postprandial samples.

3. Our pilot study of 97 expressed miRNAs in plasma showed that miR-10b-5p, -320a, -320b, -424-5p and -423-5p were upregulated, whereas miR-103a-3p, -191-5p, -301a-3p and 199b-3p were downregulated in the plasma of VTE patients versus controls. The results of this pilot study indicate that plasma miRNAs profiling can provide novel biomarkers of unprovoked VTE.

4. Our results revealed that sequential centrifugation to precipitate and deplete procoagulant phospholipids is an appropriate alternative to treating plasma with phospholipase. The modified assay significantly reduces the cost of such a resource-demanding experiment, and is more practical for large-scale investigations. Our results were validated with a commercial PPL assay and showed high reproducibility. Interestingly, the developed assay was insensitive to postprandial lipoproteins and TF^+ EVs.

7. References

1. van Langevelde, K., et al., *Finding the origin of pulmonary emboli with a total-body magnetic resonance direct thrombus imaging technique.* Haematologica, 2013. **98**(2): p. 309-15.
2. Enga, K.F., et al., *Atrial fibrillation and future risk of venous thromboembolism:the Tromso study.* J Thromb Haemost, 2015. **13**(1): p. 10-6.
3. Geerts, W.H., et al., *Prevention of venous thromboembolism.* Chest, 2001. **119**(1 Suppl): p. 132S-175S.
4. Adelborg, K., J. Sundboll, and H.T. Sorensen, *Arterial cardiovascular events and mortality following venous thromboembolism.* Ann Transl Med, 2015. **3**(9): p. 117.
5. Cohen, A.T., et al., *Venous thromboembolism (VTE) in Europe. The number of VTE events and associated morbidity and mortality.* Thromb Haemost, 2007. **98**(4): p. 756-64.
6. Arshad, N., et al., *Time trends in incidence rates of venous thromboembolism in a large cohort recruited from the general population.* Eur J Epidemiol, 2017. **32**(4): p. 299-305.
7. Wiener, R.S., L.M. Schwartz, and S. Woloshin, *Time trends in pulmonary embolism in the United States: evidence of overdiagnosis.* Arch Intern Med, 2011. **171**(9): p. 831-7.
8. Huang, W., et al., *Secular trends in occurrence of acute venous thromboembolism: the Worcester VTE study (1985-2009).* Am J Med, 2014. **127**(9): p. 829-39 e5.
9. Farrell, J.J., et al., *Incidence and interventions for post-thrombotic syndrome.* Cardiovasc Diagn Ther, 2016. **6**(6): p. 623-631.
10. Ashrani, A.A. and J.A. Heit, *Incidence and cost burden of post-thrombotic syndrome.* J Thromb Thrombolysis, 2009. **28**(4): p. 465-76.
11. Kahn, S.R., A. Hirsch, and I. Shrier, *Effect of postthrombotic syndrome on health-related quality of life after deep venous thrombosis.* Arch Intern Med, 2002. **162**(10): p. 1144-8.
12. Braekkan, S.K., et al., *Venous thromboembolism and subsequent permanent work-related disability.* J Thromb Haemost, 2016. **14**(10): p. 1978-1987.
13. Stain, M., et al., *The post-thrombotic syndrome: risk factors and impact on the course of thrombotic disease.* J Thromb Haemost, 2005. **3**(12): p. 2671-6.
14. Prandoni, P., et al., *The long-term clinical course of acute deep venous thrombosis.* Ann Intern Med, 1996. **125**(1): p. 1-7.
15. Pengo, V., et al., *Incidence of chronic thromboembolic pulmonary hypertension after pulmonary embolism.* N Engl J Med, 2004. **350**(22): p. 2257-64.
16. Palla, A., et al., *From not detected pulmonary embolism to diagnosis of chronic thromboembolic pulmonary hypertension: a retrospective study.* Respiration, 1993. **60**(1): p. 9-14.
17. Delcroix, M., et al., *Long-Term Outcome of Patients With Chronic Thromboembolic Pulmonary Hypertension: Results From an International Prospective Registry.* Circulation, 2016. **133**(9): p. 859-71.
18. Prandoni, P., et al., *The risk of recurrent venous thromboembolism after discontinuing anticoagulation in patients with acute proximal deep vein thrombosis or pulmonary embolism. A prospective cohort study in 1,626 patients.* Haematologica, 2007. **92**(2): p. 199-205.
19. Heit, J.A., et al., *Predictors of recurrence after deep vein thrombosis and pulmonary embolism: a population-based cohort study.* Arch Intern Med, 2000. **160**(6): p. 761-8.
20. Arshad, N., et al., *Recurrence and mortality after first venous thromboembolism in a large population-based cohort.* J Thromb Haemost, 2017. **15**(2): p. 295-303.
21. Belohlavek, J., V. Dytrych, and A. Linhart, *Pulmonary embolism, part I: Epidemiology, risk factors and risk stratification, pathophysiology, clinical presentation, diagnosis and nonthrombotic pulmonary embolism.* Exp Clin Cardiol, 2013. **18**(2): p. 129-38.
22. Kumar, D.R., et al., *Virchow's contribution to the understanding of thrombosis and cellular biology.* Clin Med Res, 2010. **8**(3-4): p. 168-72.
23. Brooks, E.G., et al., *Valves of the deep venous system: an overlooked risk factor.* Blood, 2009. **114**(6): p. 1276-9.

24. Sevitt, S., *The structure and growth of valve-pocket thrombi in femoral veins.* J Clin Pathol, 1974. **27**(7): p. 517-28.
25. Bovill, E.G. and A. van der Vliet, *Venous valvular stasis-associated hypoxia and thrombosis: what is the link?* Annu Rev Physiol, 2011. **73**: p. 527-45.
26. Wakefield, T.W., D.D. Myers, and P.K. Henke, *Mechanisms of venous thrombosis and resolution.* Arterioscler Thromb Vasc Biol, 2008. **28**(3): p. 387-91.
27. Mackman, N., *New insights into the mechanisms of venous thrombosis.* J Clin Invest, 2012. **122**(7): p. 2331-6.
28. Li, Y.D., et al., *NF-kappaB transcription factor p50 critically regulates tissue factor in deep vein thrombosis.* J Biol Chem, 2009. **284**(7): p. 4473-83.
29. Michiels, C., T. Arnould, and J. Remacle, *Endothelial cell responses to hypoxia: initiation of a cascade of cellular interactions.* Biochim Biophys Acta, 2000. **1497**(1): p. 1-10.
30. Koedam, J.A., et al., *P-selectin, a granule membrane protein of platelets and endothelial cells, follows the regulated secretory pathway in AtT-20 cells.* J Cell Biol, 1992. **116**(3): p. 617-25.
31. von Bruhl, M.L., et al., *Monocytes, neutrophils, and platelets cooperate to initiate and propagate venous thrombosis in mice in vivo.* J Exp Med, 2012. **209**(4): p. 819-35.
32. Falati, S., et al., *Accumulation of tissue factor into developing thrombi in vivo is dependent upon microparticle P-selectin glycoprotein ligand 1 and platelet P-selectin.* J Exp Med, 2003. **197**(11): p. 1585-98.
33. Thomas, G.M., et al., *Cancer cell-derived microparticles bearing P-selectin glycoprotein ligand 1 accelerate thrombus formation in vivo.* J Exp Med, 2009. **206**(9): p. 1913-27.
34. Mackman, N., *The role of tissue factor and factor VIIa in hemostasis.* Anesth Analg, 2009. **108**(5): p. 1447-52.
35. Hoffman, M. and D.M. Monroe, 3rd, *A cell-based model of hemostasis.* Thromb Haemost, 2001. **85**(6): p. 958-65.
36. Weisel, J.W. and R.I. Litvinov, *Mechanisms of fibrin polymerization and clinical implications.* Blood, 2013. **121**(10): p. 1712-9.
37. Mackman, N., R.E. Tilley, and N.S. Key, *Role of the extrinsic pathway of blood coagulation in hemostasis and thrombosis.* Arterioscler Thromb Vasc Biol, 2007. **27**(8): p. 1687-93.
38. Osterud, B. and S.I. Rapaport, *Activation of factor IX by the reaction product of tissue factor and factor VII: additional pathway for initiating blood coagulation.* Proc Natl Acad Sci U S A, 1977. **74**(12): p. 5260-4.
39. Gregory, S.A., J.H. Morrissey, and T.S. Edgington, *Regulation of tissue factor gene expression in the monocyte procoagulant response to endotoxin.* Mol Cell Biol, 1989. **9**(6): p. 2752-5.
40. Parry, G.C. and N. Mackman, *Transcriptional regulation of tissue factor expression in human endothelial cells.* Arterioscler Thromb Vasc Biol, 1995. **15**(5): p. 612-21.
41. Brambilla, M., et al., *Do methodological differences account for the current controversy on tissue factor expression in platelets?* Platelets, 2017: p. 1-9.
42. Ritis, K., et al., *A novel C5a receptor-tissue factor cross-talk in neutrophils links innate immunity to coagulation pathways.* J Immunol, 2006. **177**(7): p. 4794-802.
43. Moosbauer, C., et al., *Eosinophils are a major intravascular location for tissue factor storage and exposure.* Blood, 2007. **109**(3): p. 995-1002.
44. De Palma, R., et al., *Expression of functional tissue factor in activated T-lymphocytes in vitro and in vivo: A possible contribution of immunity to thrombosis?* Int J Cardiol, 2016. **218**: p. 188-95.
45. Grover, S.P. and N. Mackman, *Tissue Factor: An Essential Mediator of Hemostasis and Trigger of Thrombosis.* Arterioscler Thromb Vasc Biol, 2018.
46. Wu, Y., *Contact pathway of coagulation and inflammation.* Thromb J, 2015. **13**: p. 17.
47. Weitz, J.I. and J.C. Fredenburgh, *Factors XI and XII as Targets for New Anticoagulants.* Front Med (Lausanne), 2017. **4**: p. 19.
48. Huang, Z.F., et al., *Tissue factor pathway inhibitor gene disruption produces intrauterine lethality in mice.* Blood, 1997. **90**(3): p. 944-51.

49. Jalbert, L.R., et al., *Inactivation of the gene for anticoagulant protein C causes lethal perinatal consumptive coagulopathy in mice.* J Clin Invest, 1998. **102**(8): p. 1481-8.
50. Ishiguro, K., et al., *Complete antithrombin deficiency in mice results in embryonic lethality.* J Clin Invest, 2000. **106**(7): p. 873-8.
51. Healy, A.M., et al., *Absence of the blood-clotting regulator thrombomodulin causes embryonic lethality in mice before development of a functional cardiovascular system.* Proc Natl Acad Sci U S A, 1995. **92**(3): p. 850-4.
52. Lwaleed, B.A. and P.S. Bass, *Tissue factor pathway inhibitor: structure, biology and involvement in disease.* J Pathol, 2006. **208**(3): p. 327-39.
53. Opal, S.M., et al., *Antithrombin, heparin, and heparan sulfate.* Crit Care Med, 2002. **30**(5 Suppl): p. S325-31.
54. Esmon, C.T., *The protein C pathway.* Chest, 2003. **124**(3 Suppl): p. 26S-32S.
55. Larsen, T.B., et al., *Major genetic susceptibility for venous thromboembolism in men: a study of Danish twins.* Epidemiology, 2003. **14**(3): p. 328-32.
56. Souto, J.C., et al., *Genetic susceptibility to thrombosis and its relationship to physiological risk factors: the GAIT study. Genetic Analysis of Idiopathic Thrombophilia.* Am J Hum Genet, 2000. **67**(6): p. 1452-9.
57. Rosendaal, F.R., *Venous thrombosis: a multicausal disease.* Lancet, 1999. **353**(9159): p. 1167-73.
58. Schmidt, M., et al., *Acute infections and venous thromboembolism.* J Intern Med, 2012. **271**(6): p. 608-18.
59. Heit, J.A., et al., *Risk factors for deep vein thrombosis and pulmonary embolism: a population-based case-control study.* Arch Intern Med, 2000. **160**(6): p. 809-15.
60. Braekkan, S.K., et al., *Body height and risk of venous thromboembolism: The Tromso Study.* Am J Epidemiol, 2010. **171**(10): p. 1109-15.
61. Geerts, W., et al., *Prevention of venous thromboembolism.* Chest, 2005. **128**(5): p. 3775-6.
62. Khan, S. and J.D. Dickerman, *Hereditary thrombophilia.* Thromb J, 2006. **4**: p. 15.
63. Mahmoodi, B.K., et al., *A prospective cohort study on the absolute risks of venous thromboembolism and predictive value of screening asymptomatic relatives of patients with hereditary deficiencies of protein S, protein C or antithrombin.* J Thromb Haemost, 2010. **8**(6): p. 1193-200.
64. Poort, S.R., et al., *A common genetic variation in the 3'-untranslated region of the prothrombin gene is associated with elevated plasma prothrombin levels and an increase in venous thrombosis.* Blood, 1996. **88**(10): p. 3698-703.
65. Coppens, M., et al., *A prospective cohort study on the absolute incidence of venous thromboembolism and arterial cardiovascular disease in asymptomatic carriers of the prothrombin 20210A mutation.* Blood, 2006. **108**(8): p. 2604-7.
66. Morange, P.E., P. Suchon, and D.A. Tregouet, *Genetics of Venous Thrombosis: update in 2015.* Thromb Haemost, 2015. **114**(5): p. 910-9.
67. Rees, D.C., M. Cox, and J.B. Clegg, *World distribution of factor V Leiden.* Lancet, 1995. **346**(8983): p. 1133-4.
68. Hirschhorn, J.N. and M.J. Daly, *Genome-wide association studies for common diseases and complex traits.* Nat Rev Genet, 2005. **6**(2): p. 95-108.
69. Morange, P.E. and D.A. Tregouet, *Lessons from genome-wide association studies in venous thrombosis.* J Thromb Haemost, 2011. **9 Suppl 1**: p. 258-64.
70. Morange, P.E. and D.A. Tregouet, *Current knowledge on the genetics of incident venous thrombosis.* J Thromb Haemost, 2013. **11 Suppl 1**: p. 111-21.
71. White, R.H., *The epidemiology of venous thromboembolism.* Circulation, 2003. **107**(23 Suppl 1): p. I4-8.
72. Braekkan, S.K., et al., *Family history of myocardial infarction is an independent risk factor for venous thromboembolism: the Tromso study.* J Thromb Haemost, 2008. **6**(11): p. 1851-7.
73. van Langevelde, K., A. Sramek, and F.R. Rosendaal, *The effect of aging on venous valves.* Arterioscler Thromb Vasc Biol, 2010. **30**(10): p. 2075-80.

74. Larsson, L., G. Grimby, and J. Karlsson, *Muscle strength and speed of movement in relation to age and muscle morphology.* J Appl Physiol Respir Environ Exerc Physiol, 1979. **46**(3): p. 451-6.
75. Olsen, H. and T. Lanne, *Reduced venous compliance in lower limbs of aging humans and its importance for capacitance function.* Am J Physiol, 1998. **275**(3 Pt 2): p. H878-86.
76. Young, C.N., et al., *Venous smooth muscle tone and responsiveness in older adults.* J Appl Physiol (1985), 2006. **101**(5): p. 1362-7.
77. Engbers, M.J., A. van Hylckama Vlieg, and F.R. Rosendaal, *Venous thrombosis in the elderly: incidence, risk factors and risk groups.* J Thromb Haemost, 2010. **8**(10): p. 2105-12.
78. Wilkerson, W.R. and D.C. Sane, *Aging and thrombosis.* Semin Thromb Hemost, 2002. **28**(6): p. 555-68.
79. Tsai, A.W., et al., *Cardiovascular risk factors and venous thromboembolism incidence: the longitudinal investigation of thromboembolism etiology.* Arch Intern Med, 2002. **162**(10): p. 1182-9.
80. Sugerman, H., et al., *Intra-abdominal pressure, sagittal abdominal diameter and obesity comorbidity.* J Intern Med, 1997. **241**(1): p. 71-9.
81. Willenberg, T., et al., *Impact of obesity on venous hemodynamics of the lower limbs.* J Vasc Surg, 2010. **52**(3): p. 664-8.
82. Skurk, T. and H. Hauner, *Obesity and impaired fibrinolysis: role of adipose production of plasminogen activator inhibitor-1.* Int J Obes Relat Metab Disord, 2004. **28**(11): p. 1357-64.
83. Darvall, K.A., et al., *Obesity and thrombosis.* Eur J Vasc Endovasc Surg, 2007. **33**(2): p. 223-33.
84. Schafer, K. and S. Konstantinides, *Adipokines and thrombosis.* Clin Exp Pharmacol Physiol, 2011. **38**(12): p. 864-71.
85. Allman-Farinelli, M.A., *Obesity and venous thrombosis: a review.* Semin Thromb Hemost, 2011. **37**(8): p. 903-7.
86. Wolf, P., *The nature and significance of platelet products in human plasma.* Br J Haematol, 1967. **13**(3): p. 269-88.
87. Hargett, L.A. and N.N. Bauer, *On the origin of microparticles: From "platelet dust" to mediators of intercellular communication.* Pulm Circ, 2013. **3**(2): p. 329-40.
88. van der Pol, E., et al., *Classification, functions, and clinical relevance of extracellular vesicles.* Pharmacol Rev, 2012. **64**(3): p. 676-705.
89. van der Pol, E., et al., *Recent developments in the nomenclature, presence, isolation, detection and clinical impact of extracellular vesicles.* J Thromb Haemost, 2016. **14**(1): p. 48-56.
90. Chandler, W.L., W. Yeung, and J.F. Tait, *A new microparticle size calibration standard for use in measuring smaller microparticles using a new flow cytometer.* J Thromb Haemost, 2011. **9**(6): p. 1216-24.
91. Yanez-Mo, M., et al., *Biological properties of extracellular vesicles and their physiological functions.* J Extracell Vesicles, 2015. **4**: p. 27066.
92. Ayers, L., et al., *Dynamic microvesicle release and clearance within the cardiovascular system: triggers and mechanisms.* Clin Sci (Lond), 2015. **129**(11): p. 915-31.
93. Burnier, L., et al., *Cell-derived microparticles in haemostasis and vascular medicine.* Thromb Haemost, 2009. **101**(3): p. 439-51.
94. Fox, J.E., et al., *Role of the membrane skeleton in preventing the shedding of procoagulant-rich microvesicles from the platelet plasma membrane.* J Cell Biol, 1990. **111**(2): p. 483-93.
95. Fox, J.E., et al., *Evidence that agonist-induced activation of calpain causes the shedding of procoagulant-containing microvesicles from the membrane of aggregating platelets.* J Biol Chem, 1991. **266**(20): p. 13289-95.
96. Rautou, P.E. and N. Mackman, *Microvesicles as risk markers for venous thrombosis.* Expert Rev Hematol, 2013. **6**(1): p. 91-101.
97. Mulcahy, L.A., R.C. Pink, and D.R. Carter, *Routes and mechanisms of extracellular vesicle uptake.* J Extracell Vesicles, 2014. **3**.
98. Montecalvo, A., et al., *Mechanism of transfer of functional microRNAs between mouse dendritic cells via exosomes.* Blood, 2012. **119**(3): p. 756-66.

99. Escrevente, C., et al., *Interaction and uptake of exosomes by ovarian cancer cells.* BMC Cancer, 2011. **11**: p. 108.
100. Doherty, G.J. and H.T. McMahon, *Mechanisms of endocytosis.* Annu Rev Biochem, 2009. **78**: p. 857-902.
101. Koumangoye, R.B., et al., *Detachment of breast tumor cells induces rapid secretion of exosomes which subsequently mediate cellular adhesion and spreading.* PLoS One, 2011. **6**(9): p. e24234.
102. Kerr, M.C. and R.D. Teasdale, *Defining macropinocytosis.* Traffic, 2009. **10**(4): p. 364-71.
103. Parolini, I., et al., *Microenvironmental pH is a key factor for exosome traffic in tumor cells.* J Biol Chem, 2009. **284**(49): p. 34211-22.
104. Geddings, J.E. and N. Mackman, *New players in haemostasis and thrombosis.* Thromb Haemost, 2014. **111**(4): p. 570-4.
105. Sandberg, H., L.O. Andersson, and S. Hoglund, *Isolation and characterization of lipid-protein particles containing platelet factor 3 released from human platelets.* Biochem J, 1982. **203**(1): p. 303-11.
106. Key, N.S. and N. Mackman, *Tissue factor and its measurement in whole blood, plasma, and microparticles.* Semin Thromb Hemost, 2010. **36**(8): p. 865-75.
107. Broze, G.J., Jr., *Tissue factor pathway inhibitor.* Thromb Haemost, 1995. **74**(1): p. 90-3.
108. Khalil, J., et al., *Venous thromboembolism in cancer patients: an underestimated major health problem.* World J Surg Oncol, 2015. **13**: p. 204.
109. Hron, G., et al., *Tissue factor-positive microparticles: cellular origin and association with coagulation activation in patients with colorectal cancer.* Thromb Haemost, 2007. **97**(1): p. 119-23.
110. Tesselaar, M.E., et al., *Microparticle-associated tissue factor activity: a link between cancer and thrombosis?* J Thromb Haemost, 2007. **5**(3): p. 520-7.
111. Morrissey, J.H., S.H. Choi, and S.A. Smith, *Polyphosphate: an ancient molecule that links platelets, coagulation, and inflammation.* Blood, 2012. **119**(25): p. 5972-9.
112. Ruiz, F.A., et al., *Human platelet dense granules contain polyphosphate and are similar to acidocalcisomes of bacteria and unicellular eukaryotes.* J Biol Chem, 2004. **279**(43): p. 44250-7.
113. Travers, R.J., S.A. Smith, and J.H. Morrissey, *Polyphosphate, platelets, and coagulation.* Int J Lab Hematol, 2015. **37 Suppl 1**: p. 31-5.
114. Muller, F., et al., *Platelet polyphosphates are proinflammatory and procoagulant mediators in vivo.* Cell, 2009. **139**(6): p. 1143-56.
115. Lentz, B.R., *Exposure of platelet membrane phosphatidylserine regulates blood coagulation.* Prog Lipid Res, 2003. **42**(5): p. 423-38.
116. Huang, M., et al., *Structural basis of membrane binding by Gla domains of vitamin K-dependent proteins.* Nat Struct Biol, 2003. **10**(9): p. 751-6.
117. Morel, O., et al., *Procoagulant microparticles: disrupting the vascular homeostasis equation?* Arterioscler Thromb Vasc Biol, 2006. **26**(12): p. 2594-604.
118. Cambien, B. and D.D. Wagner, *A new role in hemostasis for the adhesion receptor P-selectin.* Trends Mol Med, 2004. **10**(4): p. 179-86.
119. Tripisciano, C., et al., *Different Potential of Extracellular Vesicles to Support Thrombin Generation: Contributions of Phosphatidylserine, Tissue Factor, and Cellular Origin.* Sci Rep, 2017. **7**(1): p. 6522.
120. Adams, G.T., et al., *Genetic risk factors for cerebrovascular disease in children with sickle cell disease: design of a case-control association study and genomewide screen.* BMC Med Genet, 2003. **4**: p. 6.
121. Combes, V., et al., *In vitro generation of endothelial microparticles and possible prothrombotic activity in patients with lupus anticoagulant.* J Clin Invest, 1999. **104**(1): p. 93-102.
122. Kelton, J.G., et al., *Heparin-induced thrombocytopenia: laboratory studies.* Blood, 1988. **72**(3): p. 925-30.

123. Kockx, M.M., *Apoptosis in the atherosclerotic plaque: quantitative and qualitative aspects.* Arterioscler Thromb Vasc Biol, 1998. **18**(10): p. 1519-22.
124. Preston, R.A., et al., *Effects of severe hypertension on endothelial and platelet microparticles.* Hypertension, 2003. **41**(2): p. 211-7.
125. Zhang, Y., et al., *Circulating endothelial microparticles and miR-92a in acute myocardial infarction.* Biosci Rep, 2017. **37**(2).
126. Chen, Y., et al., *The Role of Circulating Platelets Microparticles and Platelet Parameters in Acute Ischemic Stroke Patients.* J Stroke Cerebrovasc Dis, 2015. **24**(10): p. 2313-20.
127. Perez-Casal, M., et al., *Activated protein C induces the release of microparticle-associated endothelial protein C receptor.* Blood, 2005. **105**(4): p. 1515-22.
128. Gris, J.C., et al., *The relationship between plasma microparticles, protein S and anticardiolipin antibodies in patients with human immunodeficiency virus infection.* Thromb Haemost, 1996. **76**(1): p. 38-45.
129. Tesselaar, M.E., et al., *Microparticle-associated tissue factor activity in cancer patients with and without thrombosis.* J Thromb Haemost, 2009. **7**(8): p. 1421-3.
130. Martinez, M., et al., *Platelet activation and red blood cell phosphatidylserine exposure evaluated by flow cytometry in patients with Behcet's disease: are they related to thrombotic events?* Pathophysiol Haemost Thromb, 2007. **36**(1): p. 18-22.
131. Bucciarelli, P., et al., *Circulating microparticles and risk of venous thromboembolism.* Thromb Res, 2012. **129**(5): p. 591-7.
132. Bal, L., et al., *Factors influencing the level of circulating procoagulant microparticles in acute pulmonary embolism.* Arch Cardiovasc Dis, 2010. **103**(6-7): p. 394-403.
133. Flores-Nascimento, M.C., et al., *Microparticles in deep venous thrombosis, antiphospholipid syndrome and Factor V Leiden.* Platelets, 2009. **20**(6): p. 367-75.
134. Ay, C., et al., *Circulating procoagulant microparticles in patients with venous thromboembolism.* Thromb Res, 2009. **123**(5): p. 724-6.
135. Owen, B.A., et al., *Procoagulant activity, but not number, of microparticles increases with age and in individuals after a single venous thromboembolism.* Thromb Res, 2011. **127**(1): p. 39-46.
136. Kyrle, P.A., et al., *Circulating P-selectin and the risk of recurrent venous thromboembolism.* Thromb Haemost, 2007. **97**(6): p. 880-3.
137. Antonopoulos, C.N., et al., *The role of soluble P selectin in the diagnosis of venous thromboembolism.* Thrombosis Research, 2014. **133**(1): p. 17-24.
138. Andre, P., et al., *Pro-coagulant state resulting from high levels of soluble P-selectin in blood.* Proc Natl Acad Sci U S A, 2000. **97**(25): p. 13835-40.
139. Myers, D.D., Jr., et al., *P-selectin antagonism causes dose-dependent venous thrombosis inhibition.* Thromb Haemost, 2001. **85**(3): p. 423-9.
140. Kappelmayer, J. and B. Nagy, Jr., *The Interaction of Selectins and PSGL-1 as a Key Component in Thrombus Formation and Cancer Progression.* Biomed Res Int, 2017. **2017**: p. 6138145.
141. Cummings, R.D. and D.F. Smith, *The selectin family of carbohydrate-binding proteins: structure and importance of carbohydrate ligands for cell adhesion.* Bioessays, 1992. **14**(12): p. 849-56.
142. Palabrica, T., et al., *Leukocyte accumulation promoting fibrin deposition is mediated in vivo by P-selectin on adherent platelets.* Nature, 1992. **359**(6398): p. 848-51.
143. Pfeiler, S., et al., *Propagation of thrombosis by neutrophils and extracellular nucleosome networks.* Haematologica, 2017. **102**(2): p. 206-213.
144. Mayadas, T.N., et al., *Leukocyte rolling and extravasation are severely compromised in P selectin-deficient mice.* Cell, 1993. **74**(3): p. 541-54.
145. Wakefield, T.W., et al., *P-selectin and TNF inhibition reduce venous thrombosis inflammation.* J Surg Res, 1996. **64**(1): p. 26-31.
146. Monreal, M., et al., *Comparative study on the antithrombotic efficacy of four low-molecular-weight heparins in three different models of experimental venous thrombosis.* Haemostasis, 1991. **21**(2): p. 91-7.

147. Symon, F.A. and A.J. Wardlaw, *Selectins and their counter receptors: a bitter sweet attraction.* Thorax, 1996. **51**(11): p. 1155-7.
148. Frenette, P.S., et al., *P-Selectin glycoprotein ligand 1 (PSGL-1) is expressed on platelets and can mediate platelet-endothelial interactions in vivo.* J Exp Med, 2000. **191**(8): p. 1413-22.
149. Davizon, P., A.D. Munday, and J.A. Lopez, *Tissue factor, lipid rafts, and microparticles.* Semin Thromb Hemost, 2010. **36**(8): p. 857-64.
150. Ye, R., et al., *Circulating tissue factor positive microparticles in patients with acute recurrent deep venous thrombosis.* Thromb Res, 2012. **130**(2): p. 253-8.
151. Lacroix, R., et al., *Impact of pre-analytical parameters on the measurement of circulating microparticles: towards standardization of protocol.* J Thromb Haemost, 2012. **10**(3): p. 437-46.
152. Witwer, K.W., et al., *Standardization of sample collection, isolation and analysis methods in extracellular vesicle research.* J Extracell Vesicles, 2013. **2**.
153. Jayachandran, M., et al., *Methodology for isolation, identification and characterization of microvesicles in peripheral blood.* J Immunol Methods, 2012. **375**(1-2): p. 207-14.
154. Shah, M.D., et al., *Flow cytometric measurement of microparticles: pitfalls and protocol modifications.* Platelets, 2008. **19**(5): p. 365-72.
155. Aras, O., et al., *Induction of microparticle- and cell-associated intravascular tissue factor in human endotoxemia.* Blood, 2004. **103**(12): p. 4545-53.
156. Jayachandran, M., et al., *Characterization of blood borne microparticles as markers of premature coronary calcification in newly menopausal women.* Am J Physiol Heart Circ Physiol, 2008. **295**(3): p. H931-H938.
157. Yuana, Y., et al., *Atomic force microscopy: a novel approach to the detection of nanosized blood microparticles.* J Thromb Haemost, 2010. **8**(2): p. 315-23.
158. Berckmans, R.J., et al., *Cell-derived microparticles circulate in healthy humans and support low grade thrombin generation.* Thromb Haemost, 2001. **85**(4): p. 639-46.
159. Pereira, J., et al., *Circulating platelet-derived microparticles in systemic lupus erythematosus. Association with increased thrombin generation and procoagulant state.* Thromb Haemost, 2006. **95**(1): p. 94-9.
160. van der Zee, P.M., et al., *P-selectin- and CD63-exposing platelet microparticles reflect platelet activation in peripheral arterial disease and myocardial infarction.* Clin Chem, 2006. **52**(4): p. 657-64.
161. van Beers, E.J., et al., *Circulating erythrocyte-derived microparticles are associated with coagulation activation in sickle cell disease.* Haematologica, 2009. **94**(11): p. 1513-9.
162. Bode, A.P. and D.H. Hickerson, *Characterization and quantitation by flow cytometry of membranous microparticles formed during activation of platelet suspensions with ionophore or thrombin.* Platelets, 2000. **11**(5): p. 259-71.
163. van der Pol, E., et al., *Innovation in detection of microparticles and exosomes.* J Thromb Haemost, 2013. **11 Suppl 1**: p. 36-45.
164. Sustar, V., et al., *Nanoparticles isolated from blood: a reflection of vesiculability of blood cells during the isolation process.* Int J Nanomedicine, 2011. **6**: p. 2737-48.
165. Linares, R., et al., *Imaging and Quantification of Extracellular Vesicles by Transmission Electron Microscopy.* Methods Mol Biol, 2017. **1545**: p. 43-54.
166. Dragovic, R.A., et al., *Sizing and phenotyping of cellular vesicles using Nanoparticle Tracking Analysis.* Nanomedicine, 2011. **7**(6): p. 780-8.
167. Huang, F., et al., *Quantitative nanoparticle tracking: applications to nanomedicine.* Nanomedicine (Lond), 2011. **6**(4): p. 693-700.
168. Lee, R.C., R.L. Feinbaum, and V. Ambros, *The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14.* Cell, 1993. **75**(5): p. 843-54.
169. Pasquinelli, A.E., et al., *Conservation of the sequence and temporal expression of let-7 heterochronic regulatory RNA.* Nature, 2000. **408**(6808): p. 86-9.
170. Filipowicz, W., S.N. Bhattacharyya, and N. Sonenberg, *Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight?* Nat Rev Genet, 2008. **9**(2): p. 102-14.

171. Wang, H.J., et al., *Serum miR-122 levels are related to coagulation disorders in sepsis patients.* Clinical Chemistry and Laboratory Medicine, 2014. **52**(6): p. 927-933.
172. Salloum-Asfar, S., et al., *Regulation of Coagulation Factor XI Expression by MicroRNAs in the Human Liver.* Plos One, 2014. **9**(11).
173. Yekta, S., I.H. Shih, and D.P. Bartel, *MicroRNA-directed cleavage of HOXB8 mRNA.* Science, 2004. **304**(5670): p. 594-6.
174. Colgan, D.F. and J.L. Manley, *Mechanism and regulation of mRNA polyadenylation.* Genes Dev, 1997. **11**(21): p. 2755-66.
175. Bartel, D.P., *MicroRNAs: target recognition and regulatory functions.* Cell, 2009. **136**(2): p. 215-33.
176. Vickers, K.C., et al., *MicroRNAs are transported in plasma and delivered to recipient cells by high-density lipoproteins.* Nat Cell Biol, 2011. **13**(4): p. 423-33.
177. Hunter, M.P., et al., *Detection of microRNA expression in human peripheral blood microvesicles.* PLoS One, 2008. **3**(11): p. e3694.
178. Valadi, H., et al., *Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells.* Nature Cell Biology, 2007. **9**(6): p. 654-U72.
179. Mitchell, P.S., et al., *Circulating microRNAs as stable blood-based markers for cancer detection.* Proceedings of the National Academy of Sciences of the United States of America, 2008. **105**(30): p. 10513-10518.
180. Rodriguez, A., et al., *Identification of mammalian microRNA host genes and transcription units.* Genome Res, 2004. **14**(10A): p. 1902-10.
181. Guil, S. and M. Esteller, *RNA-RNA interactions in gene regulation: the coding and noncoding players.* Trends Biochem Sci, 2015. **40**(5): p. 248-56.
182. Lee, I., et al., *New class of microRNA targets containing simultaneous 5'-UTR and 3'-UTR interaction sites.* Genome Res, 2009. **19**(7): p. 1175-83.
183. Place, R.F., et al., *MicroRNA-373 induces expression of genes with complementary promoter sequences.* Proc Natl Acad Sci U S A, 2008. **105**(5): p. 1608-13.
184. Landi, D., F. Gemignani, and S. Landi, *Role of variations within microRNA-binding sites in cancer.* Mutagenesis, 2012. **27**(2): p. 205-10.
185. Winter, J., et al., *Many roads to maturity: microRNA biogenesis pathways and their regulation.* Nat Cell Biol, 2009. **11**(3): p. 228-34.
186. Sarachana, T., et al., *Small ncRNA Expression-Profiling of Blood from Hemophilia A Patients Identifies miR-1246 as a Potential Regulator of Factor 8 Gene.* Plos One, 2015. **10**(7).
187. Fort, A., et al., *Regulation of fibrinogen production by microRNAs.* Blood, 2010. **116**(14): p. 2608-15.
188. Li, S., et al., *MicroRNA-223 inhibits tissue factor expression in vascular endothelial cells.* Atherosclerosis, 2014. **237**(2): p. 514-20.
189. Eisenreich, A. and U. Leppert, *The impact of microRNAs on the regulation of tissue factor biology.* Trends Cardiovasc Med, 2014. **24**(3): p. 128-32.
190. Teruel, R., et al., *Potential role of miRNAs in developmental haemostasis.* PLoS One, 2011. **6**(3): p. e17648.
191. Tay, J.W., et al., *Micro-ribonucleic Acid 494 regulation of protein S expression.* J Thromb Haemost, 2013. **11**(8): p. 1547-55.
192. Ali, H.O., et al., *The role of microRNA-27a/b and microRNA-494 in estrogen-mediated downregulation of tissue factor pathway inhibitor alpha.* J Thromb Haemost, 2016. **14**(6): p. 1226-37.
193. Villadsen, S.B., et al., *The miR-143/-145 cluster regulates plasminogen activator inhibitor-1 in bladder cancer.* Br J Cancer, 2012. **106**(2): p. 366-74.
194. Chen, Y.S., et al., *Levels of microRNA-181b and plasminogen activator inhibitor-1 are associated with hypertensive disorders complicating pregnancy.* Exp Ther Med, 2014. **8**(5): p. 1523-1527.
195. Wang, X., et al., *Diagnostic potential of plasma microRNA signatures in patients with deep-vein thrombosis.* Thromb Haemost, 2016. **116**(2): p. 328-36.

196. Kong, L., et al., *Downregulation of let-7e-5p contributes to endothelial progenitor cell dysfunction in deep vein thrombosis via targeting FASLG.* Thromb Res, 2016. **138**: p. 30-36.
197. Kong, L., et al., *Upregulation of miR-483-3p contributes to endothelial progenitor cells dysfunction in deep vein thrombosis patients via SRF.* J Transl Med, 2016. **14**: p. 23.
198. Li, J.M., K.D. Caldwell, and W. Machtle, *Particle characterization in centrifugal fields. Comparison between ultracentrifugation and sedimentation field-flow fractionation.* J Chromatogr, 1990. **517**: p. 361-76.
199. Thery, C., et al., *Isolation and characterization of exosomes from cell culture supernatants and biological fluids.* Curr Protoc Cell Biol, 2006. **Chapter 3**: p. Unit 3 22.
200. Momen-Heravi, F., et al., *Impact of biofluid viscosity on size and sedimentation efficiency of the isolated microvesicles.* Front Physiol, 2012. **3**: p. 162.
201. Shet, A.S., et al., *Sickle blood contains tissue factor-positive microparticles derived from endothelial cells and monocytes.* Blood, 2003. **102**(7): p. 2678-83.
202. Shet, A.S., *Characterizing blood microparticles: technical aspects and challenges.* Vasc Health Risk Manag, 2008. **4**(4): p. 769-74.
203. Zhang, J., et al., *Circulating TNFR1 exosome-like vesicles partition with the LDL fraction of human plasma.* Biochem Biophys Res Commun, 2008. **366**(2): p. 579-84.
204. Yuana, Y., et al., *Co-isolation of extracellular vesicles and high-density lipoproteins using density gradient ultracentrifugation.* J Extracell Vesicles, 2014. **3**.
205. Arroyo, J.D., et al., *Argonaute2 complexes carry a population of circulating microRNAs independent of vesicles in human plasma.* Proc Natl Acad Sci U S A, 2011. **108**(12): p. 5003-8.
206. Chiou, N.-T. and K.M. Ansel, *Improved exosome isolation by sucrose gradient fractionation of ultracentrifuged crude exosome pellets.* 2016.
207. Basavaraj, M.G., B. Osterud, and J.B. Hansen, *Influence of different anticoagulants on monocyte procoagulant functions and monocyte-platelet aggregates formation.* J Thromb Haemost, 2012. **10**(8): p. 1698-702.
208. Oliver, A.E., et al., *The internal calcium concentration of human platelets increases during chilling.* Biochim Biophys Acta, 1999. **1416**(1-2): p. 349-60.
209. Mentzer, S.J., et al., *Spontaneous aggregation as a mechanism for human monocyte purification.* Cell Immunol, 1986. **101**(2): p. 312-9.
210. van der Vlist, E.J., et al., *Fluorescent labeling of nano-sized vesicles released by cells and subsequent quantitative and qualitative analysis by high-resolution flow cytometry.* Nat Protoc, 2012. **7**(7): p. 1311-26.
211. Trummer, A., et al., *Isotype controls in phenotyping and quantification of microparticles: a major source of error and how to evade it.* Thromb Res, 2008. **122**(5): p. 691-700.
212. Connor, D.E., et al., *The majority of circulating platelet-derived microparticles fail to bind annexin V, lack phospholipid-dependent procoagulant activity and demonstrate greater expression of glycoprotein Ib.* Thromb Haemost, 2010. **103**(5): p. 1044-52.
213. Boulanger, C.M., N. Amabile, and A. Tedgui, *Circulating microparticles: a potential prognostic marker for atherosclerotic vascular disease.* Hypertension, 2006. **48**(2): p. 180-6.
214. Daniel, L., et al., *Increase of circulating neutrophil and platelet microparticles during acute vasculitis and hemodialysis.* Kidney Int, 2006. **69**(8): p. 1416-23.
215. Gyorgy, B., et al., *Detection and isolation of cell-derived microparticles are compromised by protein complexes resulting from shared biophysical parameters.* Blood, 2011. **117**(4): p. e39-48.
216. Gardiner, C., et al., *Extracellular vesicle sizing and enumeration by nanoparticle tracking analysis.* J Extracell Vesicles, 2013. **2**.
217. Trummer, A., et al., *Recovery and composition of microparticles after snap-freezing depends on thawing temperature.* Blood Coagul Fibrinolysis, 2009. **20**(1): p. 52-6.
218. Coumans, F.A.W., et al., *Methodological Guidelines to Study Extracellular Vesicles.* Circ Res, 2017. **120**(10): p. 1632-1648.
219. Harris, J.R., *Negative staining of thinly spread biological samples.* Methods Mol Biol, 2007. **369**: p. 107-42.

220. Gheldof, D., et al., *Procoagulant activity of extracellular vesicles as a potential biomarker for risk of thrombosis and DIC in patients with acute leukaemia.* J Thromb Thrombolysis, 2017. **43**(2): p. 224-232.
221. Chou, J., et al., *Hematopoietic cell-derived microparticle tissue factor contributes to fibrin formation during thrombus propagation.* Blood, 2004. **104**(10): p. 3190-7.
222. Del Conde, I., et al., *Tissue-factor-bearing microvesicles arise from lipid rafts and fuse with activated platelets to initiate coagulation.* Blood, 2005. **106**(5): p. 1604-11.
223. Lupu, C., et al., *Tissue factor-dependent coagulation is preferentially up-regulated within arterial branching areas in a baboon model of Escherichia coli sepsis.* Am J Pathol, 2005. **167**(4): p. 1161-72.
224. Kappelmayer, J., et al., *Identification of P-selectin glycoprotein ligand-1 as a useful marker in acute myeloid leukaemias.* Br J Haematol, 2001. **115**(4): p. 903-9.
225. da Costa Martins, P., et al., *P-selectin glycoprotein ligand-1 is expressed on endothelial cells and mediates monocyte adhesion to activated endothelium.* Arterioscler Thromb Vasc Biol, 2007. **27**(5): p. 1023-9.
226. Dasgupta, S.K., et al., *Lactadherin and clearance of platelet-derived microvesicles.* Blood, 2009. **113**(6): p. 1332-9.
227. Colhoun, H.M., et al., *Lipoprotein subclasses and particle sizes and their relationship with coronary artery calcification in men and women with and without type 1 diabetes.* Diabetes, 2002. **51**(6): p. 1949-56.
228. Sodar, B.W., et al., *Low-density lipoprotein mimics blood plasma-derived exosomes and microvesicles during isolation and detection.* Sci Rep, 2016. **6**: p. 24316.
229. Mork, M., et al., *Prospects and limitations of antibody-mediated clearing of lipoproteins from blood plasma prior to nanoparticle tracking analysis of extracellular vesicles.* J Extracell Vesicles, 2017. **6**(1): p. 1308779.
230. Gyorgy, B., et al., *Improved circulating microparticle analysis in acid-citrate dextrose (ACD) anticoagulant tube.* Thromb Res, 2014. **133**(2): p. 285-92.
231. Reddy, K.B., *MicroRNA (miRNA) in cancer.* Cancer Cell International, 2015. **15**.
232. Samanta, S., et al., *MicroRNA: A new therapeutic strategy for cardiovascular diseases.* Trends Cardiovasc Med, 2016. **26**(5): p. 407-19.
233. Xiao, J., et al., *MicroRNA-134 as a potential plasma biomarker for the diagnosis of acute pulmonary embolism.* J Transl Med, 2011. **9**: p. 159.
234. Gidlof, O., et al., *Platelets activated during myocardial infarction release functional miRNA, which can be taken up by endothelial cells and regulate ICAM1 expression.* Blood, 2013. **121**(19): p. 3908-17, S1-26.
235. Grossman, Z.D., et al., *Platelets labeled with oxine complexes of Tc-99m and In-111. Part 2. Localization of experimentally induced vascular lesions.* J Nucl Med, 1978. **19**(5): p. 488-91.
236. Braekkan, S.K., et al., *Mean platelet volume is a risk factor for venous thromboembolism: the Tromso Study, Tromso, Norway.* J Thromb Haemost, 2010. **8**(1): p. 157-62.
237. Brighton, T.A., et al., *Low-dose aspirin for preventing recurrent venous thromboembolism.* N Engl J Med, 2012. **367**(21): p. 1979-87.
238. Qin, J., et al., *A panel of microRNAs as a new biomarkers for the detection of deep vein thrombosis.* J Thromb Thrombolysis, 2015. **39**(2): p. 215-21.
239. Issman, L., et al., *Cryogenic transmission electron microscopy nanostructural study of shed microparticles.* PLoS One, 2013. **8**(12): p. e83680.
240. Connor, D.E., et al., *Detection of the procoagulant activity of microparticle-associated phosphatidylserine using XACT.* Blood Coagul Fibrinolysis, 2009. **20**(7): p. 558-64.
241. Nieuwland, R., et al., *Cell-derived microparticles generated in patients during cardiopulmonary bypass are highly procoagulant.* Circulation, 1997. **96**(10): p. 3534-41.
242. Exner, T., et al., *A new activated factor X-based clotting method with improved specificity for procoagulant phospholipid.* Blood Coagul Fibrinolysis, 2003. **14**(8): p. 773-9.
243. Andreasson, U., et al., *A Practical Guide to Immunoassay Method Validation.* Front Neurol, 2015. **6**: p. 179.

244. van Dreden, P., et al., *Clinical evaluation of a new functional test for detection of plasma procoagulant phospholipids.* Blood Coagul Fibrinolysis, 2009. **20**(7): p. 494-502.
245. Hald, E.M., et al., *Postprandial lipemia is not increased in patients with previous unprovoked venous thromboembolism.* J Clin Lipidol, 2013. **7**(1): p. 48-55.