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MICROPARTICLES IN HEMOPHILIA – FRIEND OR FOE? – TO IMPROVE HEMOSTASIS OR TO INDUCE ATHEROTHROMBOSIS?

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Microparticles in hemophilia - friend or foe? - to improve hemostasis or to induce atherothrombosis?

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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ABSTRACT

As one of the most well-known inherited bleeding disorders, hemophilia A (HA) is caused by the deficiency or dysfunction of coagulation factor VIII (FVIII). Patients with HA (PWHA) suffer from abnormal bleeding after injuries or surgeries, or even spontaneous bleeding in severe cases, particularly at joints and muscles. The main treatment for PWHA is FVIII replacement therapy. Patients with the same residual FVIII levels may present different clinical bleeding phenotypes, thus individualized adjustment of the treatment is necessary. Moreover, the risks of cardiovascular disease (CVD) are increased in middle-aged or elderly patients. The management of CVD in PHWA has become a new clinical challenge.

Circulating microparticles (MPs) are small membrane vesicles that originate mostly from platelets in healthy individuals. MPs play important roles in hemostasis and thrombosis and are reported to participate in the development of CVD. However, the role of MPs in hemostasis of HA as well as in the development of CVD in PWHA remains unclear.

The overall aim of this thesis was to investigate the role of MPs in HA. The first part focused on the effect of MPs on hemostasis of HA *in vitro* and *in vivo*. In the second part, the prevalence of CVD, the profiles of MPs, as well as their correlations in the middle-aged and elderly PWHA were investigated. In another study, fibrin formation for combinations of a new bispecific antibody and bypassing agents (aPCC or rFVIIa) was studied using *in vitro* approaches.

In **Paper I**, we characterized the fibrin formation and fibrin clot structure in a human plasma model of severe HA after the addition of sequence-identical analogue (SIA) of emicizumab, alone or in combinations with bypassing agents (aPCC or rFVIIa). The combination of SIA and aPCC exhibited hypercoagulable patterns, suggesting that this combination might introduce thrombotic risk when used to treat PWHA.

In **Papers II** and **IV**, the procoagulant effect of MPs in HA was studied using *in vitro* plasma models and *in vivo* mouse models of HA, respectively. MPs improved hemostasis and were found to be incorporated into the fibrin network in both *in vitro* and *in vivo* HA models.

In **Paper III**, the prevalence of subclinical CVD in a Swedish PWHA (≥40 years old) cohort was determined using the advanced electrocardiography (A-ECG) technique, retrospectively. PWHA were found to have higher probabilities of developing CVD

compared to the age-matched male control. In **Paper V**, a cross-sectional study with a Chinese cohort of PWHA treated on-demand (≥30 years old) was conducted. PWHA treated on-demand were not protected from developing subclinical CVD. No correlations between the CVD risks and MPs profiles were observed. In addition, the hypercoagulable state might lead to increased platelet activation in PWHA.

Taken together, this thesis has provided new insights to understand the procoagulant effect of MPs in HA and confirmed that PWHA are not protected from developing CVD. MPs may modify the clinical bleeding phenotypes in PWHA. Moreover, MPs might be a potential biomarker for individualized therapy in PWHA, particularly for aging patients, who have increased risk of CVD.

LIST OF SCIENTIFIC PAPERS

I. **Yanan Zong**, Aleksandra Antovic, Nida Mahmoud Hourani Soutari, Jovan Antovic, Iva Pruner.
Synergistic effect of bypassing agents and sequence identical analogue of emicizumab and fibrin clot structure in the in vitro model of hemophilia A.
TH Open, 2020,4(2):e94-e103

II. **Yanan Zong**, Iva Pruner, Aleksandra Antovic, Apostolos Taxiarchis, Zara Pons Vila, Nida Soutari, Fariborz Mobarrez, Roza Chaireti, Jerker Widengren, Joachim Piguet & Jovan P. Antovic
Phosphatidylserine positive microparticles improve hemostasis in *in-vitro* hemophilia A plasma models
Scientific Reports, 2020;10(1):7871

III. **Yanan Zong**, MarenMaanja, Roza Chaireti, Todd T. Schlegel, Martin Ugander, Jovan P. Antovic
Substantial prevalence of subclinical cardiovascular diseases in patients with hemophilia A evaluated by advanced electrocardiography
Journal of Electrocardiology, 2020;58:171–175

IV. **Yanan Zong**, Iva Pruner, Apostolos Taxiarchis, Jun Wan, Marisa Ninivaggi, Nida Soutari, Agnes Rasmuson, Bas de Laat, Jovan P. Antovic
Procoagulant microparticles improve hemostasis and accumulate at the injured site in hemophilia A mice.
Manuscript.

V. **Yanan Zong**, Hongfei Kang, Maren Maanja, Shengmei Chen, Todd Schelegel, Roza Chaireti, Marin Ugander, Jovan P. Antovic, Xiangdong Kong
Subclinical cardiac diseases and the role of microparticles (MPs) in patients with hemophilia A treated on-demand.
Manuscript.

CONTENTS

LIST OF ABBREVIATIONS

A-ECG	advanced-electrocardiography
aPCC	activated prothrombin complex concentrate
aPTT	activated partial thromboplastin time
CAD	coronary artery disease
CAT	calibrated automated thrombogram
CMVD	coronary microvascular disease
CT	computerized tomography
CTI	corn trypsin inhibitor
CVD	cardiovascular disease
Del-1	developmental endothelial locus-1
ECG	electrocardiography
EMPs	endothelial-cell derived MPs
ETP	endogenous thrombin potential
EVs	extracellular vesicles
FII	plasma coagulation factor II
FIX	plasma coagulation factor IX
FIXa	activated plasma coagulation factor IX
FV	plasma coagulation factor V
FVa	activated plasma coagulation factor V
FVII	plasma coagulation factor VII
FVIIa	activated plasma coagulation factor VII
FVIII	plasma coagulation factor VIII
FVIIIa	activated plasma coagulation factor VIII
FX	plasma coagulation factor X
FXa	activated plasma coagulation factor X
FXI	plasma coagulation factor XI
FXIa	activated plasma coagulation factor XI
FXII	plasma coagulation factor XII
HA	hemophilia A
HB	hemophilia B
IMT	intima-media thickness
LMPs	leukocyte-derived MPs

LPS	lipopolysaccharide
LVER	left ventricular electrical remodeling
LVH	left ventricular hypertrophy
LVSD	left ventricular systolic dysfunction
MPs	microparticles
OCP	overall coagulation potential
OFP	overall fibrinolysis potential
OHP	overall hemostasis potential
PAI 1	plasminogen activity inhibitor 1
PNP	pooled normal human plasma
PS	phosphatidylserine
PT	prothrombin time
PWHA	patients with HA
rFVIIa	recombinant activated factor VII
SEM	scanning electron microscopy
SIA	sequence-identical analogue (of emicizumab)
STED microscopy	stimulated emission depletion microscopy
TF	tissue factor
TNF-α	tumor necrosis factor-α
t-PA	tissue-type plasminogen activator
vWF	von Willebrand factor
WT	wild type

1 INTRODUCTION

1.1 HEMOPHILIA A (HA)

1.1.1 Introduction

Hemophilia A (HA) is a recessive X-linked bleeding disorder characterized by deficiency or dysfunction of the coagulation factor VIII (FVIII)[1]. HA is caused by a mutation in the *F8* gene, which affects 1 in 5000 male births[2]. Approximately 70% of patients with HA (PWHA) have a positive family history, while 30% are due to spontaneous mutations[1]. PWHA suffer from recurrent bleeding episodes in joints and muscles[3]. Lack of treatment leads to chronic arthropathy, limited movement of joints, and disability. Severe intracranial hemorrhages and bleedings in other organs can be life-threatening in PWHA[1].

HA should be suspected in males with an unexplained or excessive bleeding history. HA is diagnosed with blood tests including coagulation screening tests and FVIII activity tests. The coagulation screening tests typically show a normal prothrombin time (PT), but a prolonged activated partial thromboplastin time (aPTT)[4]. The residual FVIII activity in plasma can be measured using one-stage clotting or chromogenic assays[5]. PWHA are classified as three groups, according to predicted disease severity: severe (<1% of normal FVIII activity), moderate (1-5%), and mild (5-40%)[6]. The proportions of patients with severe, moderate, and mild HA are about 50%, 10%, and 40%, respectively. Patients with severe HA usually suffer from spontaneous bleeding or excessive bleeding even after a minor injury, while those with moderate or mild HA only bleed abnormally after trauma or surgery.

1.1.2 HA and cell-based coagulation model

There are two currently accepted coagulation models: 1) the classical coagulation cascade model; and 2) the cell-based coagulation model[7].

The classical coagulation cascade model is shown in **Figure 1.1** and suggests that there are two independent coagulation pathways in normal individuals: the intrinsic (measured by the aPTT test) and the extrinsic (evaluated by the PT test) coagulation pathways. These two coagulation pathways merged into the common pathway which generates thrombin, which then leads to the formation of stable fibrin clots[8]. Although the classical coagulation cascade model supports the laboratory evaluations of HA

(prolonged aPTT and normal PT), it does not explain why the extrinsic pathway fails to compensate for the deficiency in the intrinsic pathway (deficiency of FVIII) in HA.

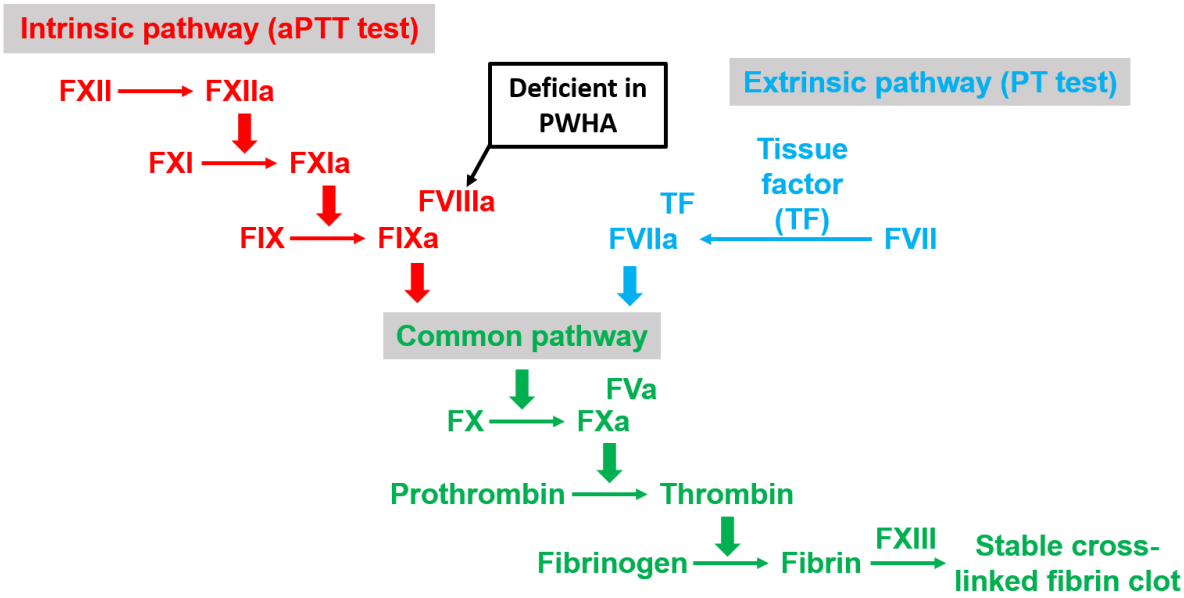

Figure 1.1 Classical coagulation cascade model, including the intrinsic pathway (Red), the extrinsic pathway (Blue), and the common pathway (Green). Coagulation (both the intrinsic and common pathways) is impaired in PWHA due to the deficiency of FVIII. PWHA exhibit prolonged aPTT and normal PT.

The cell-based coagulation model is shown in **Figure 1.2** and provides a better interpretation of the clotting mechanism defect seen in PWHA[7]. According to the cell-based model, coagulation occurs in three overlapping phases: a) initiation, b) amplification, and c) propagation[7].

a) Initiation When a blood vessel is injured, coagulation is immediately initiated and two important processes occur during this phase[10]: 1) coagulation FVII binds tightly to the surface of tissue factor (TF) bearing cells, forming FVIIa-TF complex, which activates both FX and FIX (**Figure 1.2**); and 2) platelet activation: platelets adhere to the injured site by binding to von Willebrand factor (vWF), which acts as a bridge to connect platelets and collagen outside of the vessel. Once bound to collagen, platelets are activated, thus surface receptors become modified and stored proteins, including activated FV (FVa), are released. This initiation stage is normal in PWHA.

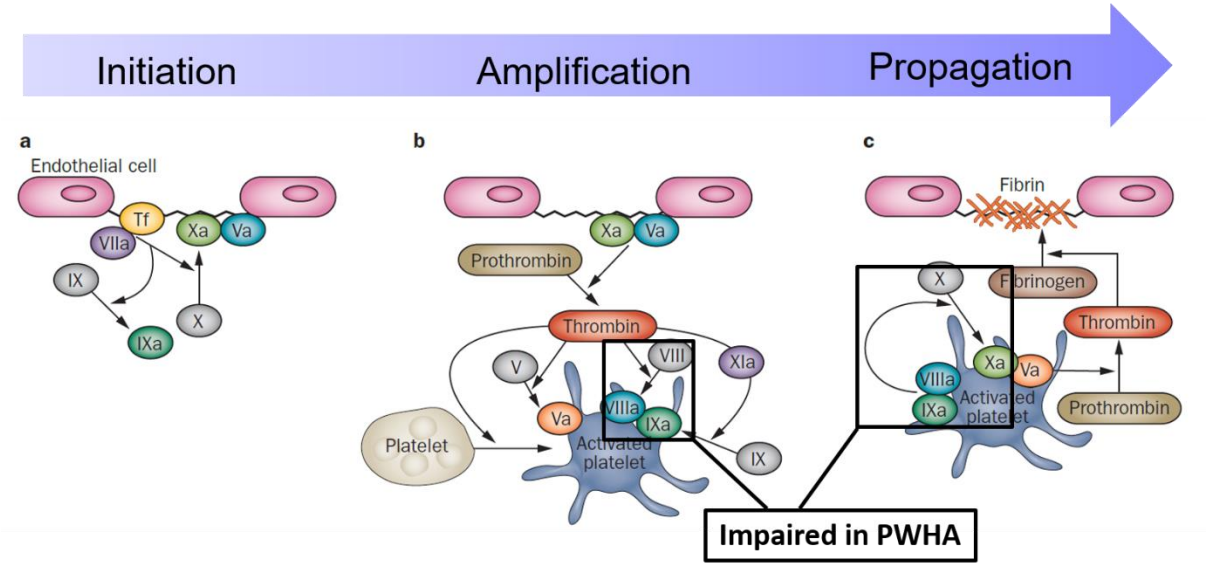

Figure 1.2 Cell-based coagulation model, which consists of a series of three overlapping steps: a), initiation phase; b), amplification phase; c), propagation phase. The amplification and propagation phases are impaired in PWHA due to the deficiency of FVIII. Modified with permission[9].

b) Amplification The FXa and FVa formed during the initiation phase leads to the generation of a small amount of thrombin, which transfers the initial hemostatic signal from the TF+ cells to the surface of platelets. Although thrombin in plasma can be rapidly inhibited by antithrombin (half-life time about 1 min), thrombin that binds to platelet surface receptors (e.g. glycoprotein Ib and protease-activated receptor-1) is protected from inhibition and has multiple important functions during this amplification phase (**Figure 1.2**)[10].

The binding of thrombin to platelet surface receptors leads to platelet activation, as characterized by the rearrangement of membrane phospholipids, conformation alteration of platelet surface receptors (glycoprotein IIb/IIIa, which binds to fibrinogen), and the release of partially activated FV, fibrinogen, and other stored proteins[10-13]. Both the platelet-derived partially activated FV and the plasma-derived FV can be completely activated by thrombin[10].

FVIII circulates in the blood as a complex with vWF[14]. Since both vWF and thrombin bind to the same platelet receptor (glycoprotein Ib), the FVIII/vWF complex is rapidly bound to thrombin, and FVIII gets activated to form FVIIIa[10]. Thrombin also activates FXI (FXIa), which in turn converts FIX to FIXa. The amplification phase is relatively normal in PWHA, except that the FVIII activation is impaired due to the deficiency of FVIII in the blood (**Figure 1.2**).

At the end of the amplification stage, platelets have the appropriate phospholipids and surface receptors and are packed with activated coagulation factors/cofactors (FVa, FIXa, and FVIIIa) on their membrane surface. These changes enable coagulation to move to the next phase, propagation.

c) Propagation The FIXa generated during the earlier stages binds to FVIIIa to form the FIXa/FVIIIa complex, which activates FX to FXa on the platelet surface[10]. Immediately, FXa combines with its cofactor FVa to form the FXa/FVa complex. This complex causes a burst of thrombin generation and stable fibrin clot formation[10]. Deficiency of FVIII in HA impairs the generation of FXa, so less FIXa/FVIIIa complex is formed, blocking propagation, resulting in reduced or absent thrombin generation, and impaired fibrin clot formation (**Figure 1.2**).

1.1.3 Bleeding modifiers of HA

Although the residual FVIII activity generally correlates well with bleeding phenotype, patients with similar FVIII activity may present heterogeneous clinical characteristics[15]. For example, 85-90% of patients whose FVIII activity <1% have indeed severe HA phenotype, and typically experience 15-35 spontaneous bleedings per year on average if not treated. However, the remaining 10-15% of patients whose FVIII activity <1% experience few bleeding episodes and consequently less arthropathy, even without treatment, thus are not severe HA clinical phenotype[16]. It is important for the physicians and patients to be able to predict which of these patients with low FVIII will have severe vs. mild HA clinical bleeding phenotype. Much research is being conducted to understand the coagulation pathway mechanisms that could be responsible for these different clinical observations in PWHA and several potential modifiers have been identified, as listed below.

1) Type of F8 gene mutation. HA is a monogenetic disease, and over 2000 different *F8* gene mutations have been identified[1], which can have widely different effects on the structure, function, and activity of the mutant FVIII proteins. Null mutations (including inversions, large deletions, and nonsense mutations, which lead to nonfunctional FVIII or no FVIII synthesis) in *F8* gene usually cause severe bleeding phenotype in PWHA. Santogostino et al. studied 72 patients with <1% FVIII activity and found that a non-null mutation is an independent predictor of a mild HA clinical phenotype[17]. Genetic testing of PWHA for their type of *F8* gene mutation is becoming

routine and provides important prognostic information on their clinical bleeding phenotype as well as response to treatement[1,18].

2) Compensating mutations (inherited thrombophilia). Inherited thrombophilia is a condition with an increased tendency to form clots in the blood, and is usually caused by abnormal levels or functions of coagulation factors (e.g. prothrombin and FV) or natural anticoagulants (e.g. antithrombin, protein C, and protein S). The influence of thrombophilic mutations in PWHA on their HA clinical phenotypes has been addressed in several cohort studies. Patients with FV Leiden or prothrombin G20210A mutations had lower FVIII concentrate consumption and less frequent bleeding[19,20], however, other studies did not reveal the protective effect of thrombophilia[21]. Many mild bleeders do not carry any thrombophilic mutations, indicating that other mechanisms must be responsible for the heterogeneous bleeding phenotype in severe PWHA.

3) Fibrinolysis. A few studies have investigated the role of fibrinolysis on the heterogeneous bleeding phenotype of PWHA[22,23]. Grunewald et al. found that PWHA who were severely hemorrhagic had elevated tissue-type plasminogen activator (t-PA) concentration and pro-thrombin-activatable fibrinolysis inhibitor activity, and reduced levels of plasminogen activator inhibitor 1 (PAI 1) activity and t-PA-PAI 1 complex[23].

4) Platelets counts and coagulant activity. Platelets are another plausible bleeding modifier in PWHA[24]. In 1973, Walsh et al. reported that PWHA with <1% FVIII and the mild clinical bleeding phenotype exhibited a higher level of platelet coagulant activity than those with severe bleeding phenotype[25]. Siegemund et al. studied platelet-rich plasma from patients with <1% FVIII and found that thrombin generation increased with platelet count, suggesting that individual differences in platelet function may modify the clinical bleeding phenotype of PWHA[26]. Several other studies on platelet function in hemophilia were reported[27]. Van Bladel et al. measured P-selectin on platelets and the level of soluble platelet activation markers in plasma and reported that platelet activation was increased in severe PWHA compared to those with mild-moderate HA[28]. In a subsequent study with a larger population of severe PWHA, however, the same group did not find any relevant differences in platelet activation or platelet reactivity to different agonists[29]. The discordance of the two studies could be due to differences in patient selection and the definition of bleeding phenotype.

Several other studies also reported conflicting results regarding the role of platelet activation in HA, which suggests that further and larger studies are needed to unravel the role of platelets in the clinical phenotypes of PWHA[30]. One direction of this research is the investigation of microparticles (MPs) released from platelets[31,32].

1.1.4 Global hemostasis assays for HA

The deficiency of coagulation FVIII in HA results in impaired thrombin generation, leading to ineffective clot formation. Assessment of the residual FVIII activity is used to diagnose HA and to monitor patients' response to treatment. However, this assay only evaluates the initiation of clotting and does not give the complete picture of hemostasis. The new generation of global hemostasis assays provide additional information about coagulation in HA, such as the calibrated automated thrombogram (CAT) and the overall hemostasis potential (OHP) assays[33,34].

CAT assay. As described in the cell-based model of coagulation, thrombin generation plays an essential role in hemostasis, and no coagulation pathways could bypass thrombin. The CAT assay is a simple and rapid method to monitor the dynamics of thrombin generation as developed by Hemker et al.[34]. Synthetic thrombin substrate coupled with a fluorophore (Z-Gly-Gly-Arg-7-amino-4methylcoumarin) is added to the mixture of coagulation trigger and plasma sample. The fluorescent signal is continuously measured by a thrombinoscope, which is proportional to the amount of thrombin that is generated during the reaction. The parameters of thrombin generation are calculated using the Thrombinoscope BV software, and the underlying mathematic methods used for data management are as described[35]. **Figure 1.3** shows representative thrombin generation curves of severe HA plasma and normal control plasma, and the impaired thrombin generation in HA can be characterized by the following parameters: a prolonged lag time and time to peak thrombin generation, reduced peak thrombin, and reduced endogenous thrombin potential (ETP)[36].

Several studies have established the correlations between the parameters of the CAT assay and the clinical bleeding phenotype of PWHA[17,37,38]. Patients with severe bleeding phenotype were reported to have ETP<50% of normal, regardless of their residual FVIII activities[38]. In patients with severe HA after a standard FVIII infusion, the plasma FVIII levels correlated with the ETP and peak thrombin values at the studied time points within 72 hours[39]. In addition, the CAT assay is also useful for

assessing treatment responses to bypassing agents in patients with FVIII inhibitor, as well as for evaluating the effect of novel treatments, such as emicizumab, and the therapeutics targeting the natural anticoagulant system[40-42]. Two major limitations have hindered the clinical use of the CAT assay, including the lack of pre-analytical standardizations and the difficulty to define the normal reference ranges for the parameters[42]. Nevertheless, the CAT assay could provide important information on the overall coagulation function for the research of HA.

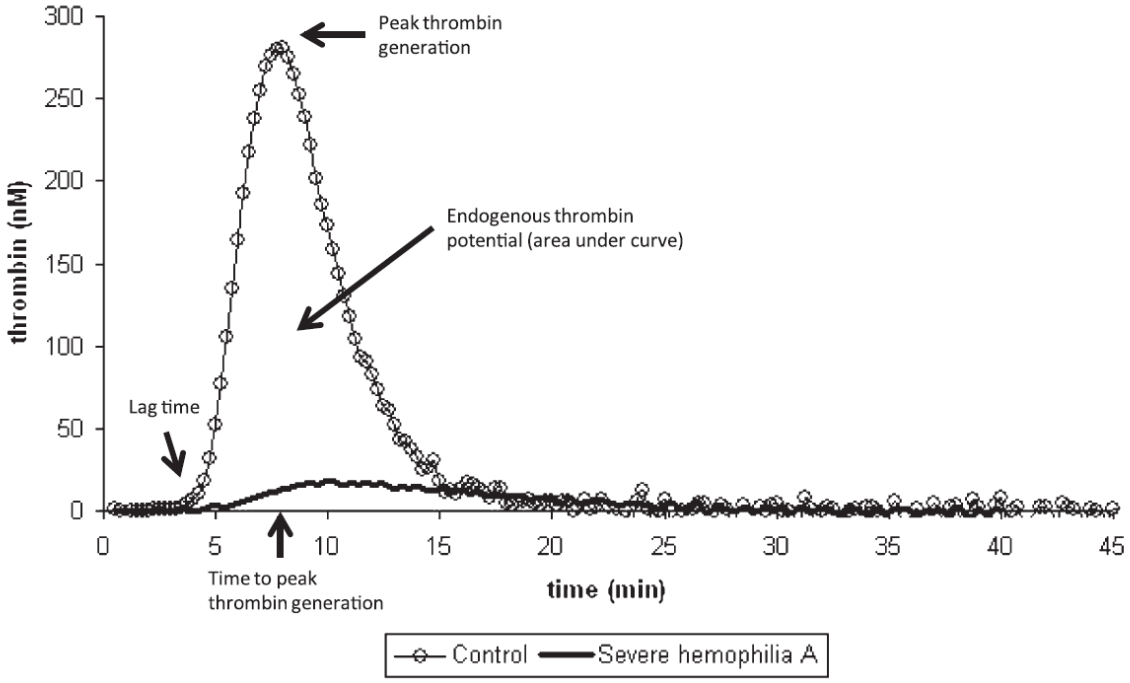

Figure 1.3 Examples of thrombin generation curves of normal control plasma and severe HA plasma as tested by the CAT assay. Reproduced with permission[36].

OHP assay Bleeding in PWHA is not only due to impaired thrombin generation, but also due to defective fibrin clot formation and premature fibrinolysis. To assess the fibrin formation and fibrinolysis, our lab developed a simple and high-throughput laboratory method, the OHP assay[43]. This assay is based on the continuous measurement of the absorbance values after adding coagulation trigger (small amount of thrombin and phospholipids) and calcium, with or without t-PA, to plasma. Each absorbance value corresponds to the amount of fibrin in the mixture at the particular time point, and the area under the curve of absorbance vs. time reflects the total fibrin formed throughout the monitored time period. Overall coagulation potential (OCP) was calculated as the area under the curve of fibrin formation, while the OHP was the area under the curve of fibrin formation and fibrinolysis, and the overall fibrinolytic potential (OFP) as OFP=([OCP-OHP]/OCP) x 100%.

Patients with severe HA may have various overall hemostasis profiles, and **Figure 1.4** shows typical OHP assay results, with an example of curves for the fibrin formation and fibrinolysis of three severe PWHA (all had FVIII levels <1%) as well as the normal control (NPP)[33]. We previously showed that the OHP values correlated with the residual FVIII levels in PWHA, moreover, the OHP values were significantly lower in the severe vs. the mild clinical bleeding phenotype groups of patients[43]. Furthermore, the OHP assay was also used to evaluate the hemostasis status in PWHA treated with rFVIIa[44].

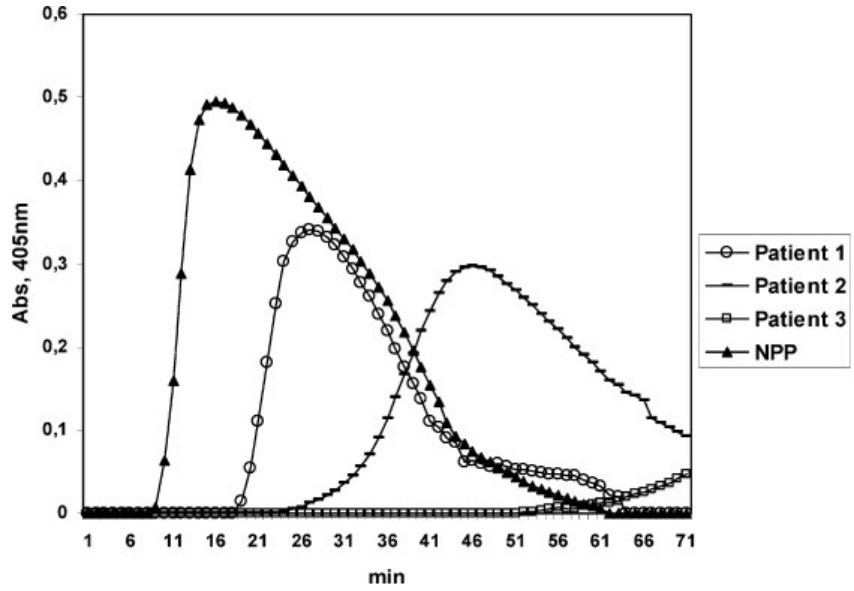

Figure 1.4 Example of fibrin formation-fibrinolysis curves of normal pooled plasma (NPP) and three different patients with severe HA (Patient 1, 2, and 3). Reproduced with permission[33].

1.1.5 Treatment of HA

The current major treatment for HA is FVIII replacement therapy, either prophylactically or on-demand[1]. Patients with prophylaxis are treated with FVIII concentrates regularly, thus preventing spontaneous bleeding and chronic arthropathy. Patients with on-demand therapy are infused with FVIII concentrates only when a bleeding episode occurs or prior to invasive procedures. In general, patients with severe HA and some patients with moderate HA require regular prophylaxis to prevent severe debilitating arthropathy[45]. However, treatment of HA is unaffordable for most patients in many countries, and >75% of the patients worldwide are not regularly, if at all, treated[46].

FVIII concentrate was first purified from human blood in 1964 and has been used to treat PWHA ever since[47]. This treatment unfortunately led to the epidemics caused by

transfusion-related viral infections, such as human immunodeficiency virus and hepatitis B and C viruses, in PWHA in the late 1970s to 1980s. The epidemics were controlled by blood-borne virus inactivation and strict donor selection[48]. The introduction of recombinant FVIII in 1989 provided a safer and more convenient drug for both prophylaxis and on-demand treatment of PWHA[49].

The biggest challenge for the treatment with FVIII concentrate is the development of FVIII inhibitors. About 30% of patients with severe HA develop inhibitors during FVIII replacement therapy, mostly during the first 30 exposure days[1]. The most effective therapies for patients with FVIII inhibitors are the bypassing agents, which could restore thrombin generation independent of FVIII. There are two bypassing agents available, activated prothrombin complex concentrate (aPCC; also known as FEIBA) and recombinant activated factor VII (rFVIIa; also known as NovoSeven), however, bypassing agents are not always effective[50]. A novel bispecific antibody, emicizumab, offers an alternative approach for patients with or without inhibitors[40].

Other novel therapeutic approaches for HA have also been available. Novel FVIII concentrates with extended half-lives are being developed which could decrease the frequency of infusions[51]. In addition, non-factor products that targeted natural anticoagulants, such as TF pathway inhibitor and antithrombin, have also shown promising results in clinical studies[45,52]. Gene therapy is a promising method to clinical cure HA in the near future[53]. In particular, 6 of 7 PWHA treated with an adeno-associated virus 5 vector carrying the normal *F8* gene with a deleted B-domain, achieved sustained normal FVIII activity and level over a one-year period[54].

1.1.6 Cardiovascular disease (CVD) in patients with HA (PWHA)

With improved treatment, especially the early initiation of prophylaxis, PWHA can now expect a normal lifespan[55]. According to the latest annual global survey of the World Federation of Hemophilia, 19% and 31% of PWHA are over 45 years old in the Americas and Europe, respectively[56]. Since the risk of developing cardiovascular disease (CVD) increases with age, middle-aged or elderly PWHA are considered to have higher risk for CVD events[57].

CVD risk factors of PWHA. The CVD risk factors are common among PWHA. In a cohort of 709 patients with hemophilia (≥30 years, including HA and hemophilia B,

HB), although the prevalence of obesity and hypercholesterolemia were lower among patients compared to the general age-matched male population, those of diabetes and smoking were similar[58]. Moreover, hypertension was more common among patients than in the general population[59,60], for unclear reasons. One plausible explanation is the impaired renal function in patients due to renal bleeding or virus infection[61]. Additional reasons could be: 1) increased diagnosis in PWHA vs. normal individuals, as hypertension is more easily diagnosed in PWHA during their regular follow-up visits; and 2) the lack of physical activity among patients because of hemarthrosis or being discouraged due to bleeding risk.

Atherosclerosis in PWHA. PWHA are not protected from developing atherosclerosis[62]. Sramek et al. reported that the carotid or femoral artery intima-media thickness (IMT) were similar between patients with hemophilia and healthy controls[63]. Several studies also confirmed that the IMTs of PWHA and controls did not differ[64-66]. Tuinenburg et al. reported that coronary artery calcium score was slightly higher in PWHA compared with controls evaluated by computerized tomography (CT)[62].

CVD mortality in PWHA. Several cohort studies reported that PWHA have decreased mortality from CVD than the general population[67,68]. One potential explanation is that PWHA suffer from less atherosclerotic plaque rupture or their plaque ruptures are “silent”, which do not lead to atherothrombosis or arterial occlusion[69]. The cardiovascular mortality in PWHA was also suggested to be comparable to (instead of higher than) that of the general controls[57]. Nevertheless, there is growing evidence indicating that it is important to prevent and manage the risk of CVD in aging PWHA.

1.2 MICROPARTICLES (MPS)

1.2.1 Introduction

MPs, also called microvesicles or ectosomes, are small membrane vesicles (0.1-1 μm in diameter) originating from various types of cells[70]. MPs are released from the cell membranes upon cell activation or apoptosis[71]. The bilayer membrane structure of MPs resembles that of their parental cells, consisting of lipids and cell-specific surface protein markers. The internal components of MPs are consist of proteins, DNA, mRNA, and other cytoplasmic components derived from their parental cells[71].

One of the earliest studies of MPs was conducted by Chargaff and West in 1946, where they found that platelet-derived particles in normal plasma were procoagulant[72]. In 1967, Wolf named these "platelet dust"[73]. In the past decades, growing evidence has shown that MPs have heterogeneous cell origins, and exhibit diverse biological functions in different processes (e.g. coagulation, inflammation, immune response, tissue repair)[70,71].

The release of extracellular vesicles (EVs) by cells is an evolutionally conserved process for intercellular communication, which exists in both lower organisms like bacteria and parasites, and higher organisms as humans[70]. Apart from MPs, there are two other major subgroups of EVs, apoptotic bodies, and exosomes (**Figure 1.5**)[70]. Apoptotic bodies are released by cells upon apoptosis and are usually rapidly recognized and cleared by macrophages[74]. Apoptotic bodies have a larger size (1-5 μm in diameter) compared to MPs and exosomes. Exosomes (30-100 in diameter) are derived from multivesicular bodies, which can fuse with the cell membrane to release the exosomes inside[70]. Apoptotic bodies and exosomes are not studied in this thesis, since we focused on the role of MPs.

Circulating MPs in the blood-steam are derived from the blood cells, including platelets, lymphocytes, monocytes, and so on, as well as from the endothelial cells[70]. In healthy individuals, the majority of circulating MPs originate from platelets (PMPs) which represents usually 70%-80% of total blood MPs[75,76], although a much lower percentage of PMPs was also reported[77]. In several pathological conditions, the levels of total and specific subtypes of MPs, as well as the protein and RNA content, were found to be altered[70]. The potential use of MPs as biomarkers for clinical diagnosis and treatment of diseases remains to be investigated.

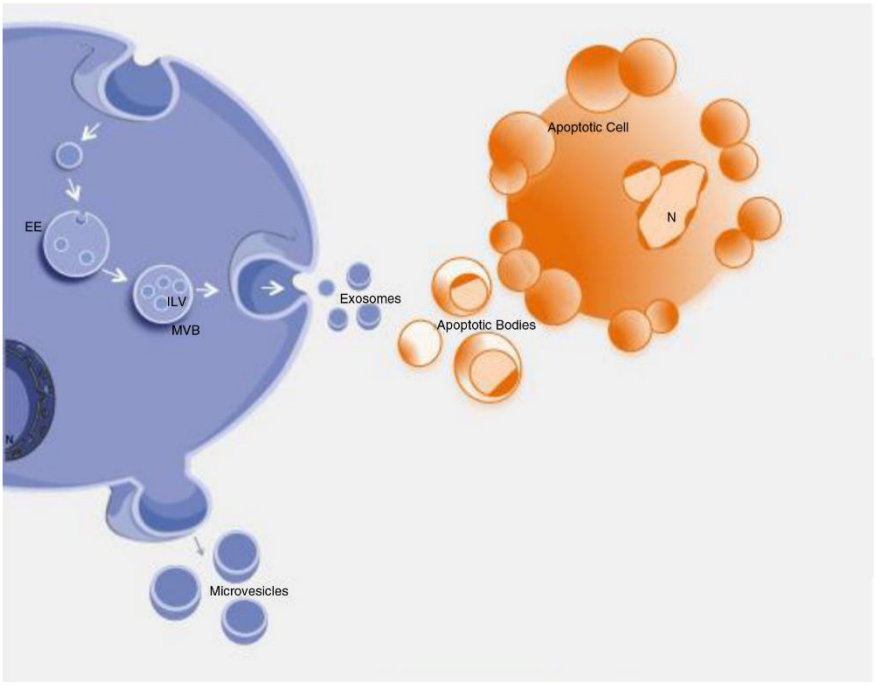

Figure 1.5 The release of MPs (also called microvesicles), exosomes, and apoptotic bodies by cells. Modified with permission[70].

1.2.2 Generation of MPs

Although the detailed mechanisms of MP biogenesis have only started to be understood, rearrangement of cell membrane phospholipids asymmetry and cytoskeletal remodeling are essential in this process (**Figure 1.6**). Three Ca^{2+}-dependent enzymes, scramblase, flippase, and floppase, regulate the cell membrane phospholipids asymmetry[78]. Rearrangement of the phospholipid asymmetry (externalization of phosphatidylserine, PS) causes membrane curvature, which is important for MP formation[71]. Calpain, a Ca^{2+}-dependent protease that can cleave cytoskeletal proteins, is involved in MP formation[79]. Similarly, caspase 2 and transglutaminase 2, which can regulate cytoskeletal dynamics, have also been suggested to participate in MP formation[80,81]. Additionally, lipid-rich microdomains are enriched in MPs and are associated with enhanced MP formation[82].

The generation and release of MPs can be viewed as a receptor-mediated event. When the coagulation mediators, such as collagen and thrombin, bind to their corresponding surface receptor(s) of platelets, the production of MPs is stimulated[84]. Other *in vivo* or *in vitro* stimuli for PMPs formation have also been reported, including lipopolysaccharide (LPS), tumor necrosis factor-α (TNF-α), interleukin-6, thrombin-receptor-activating peptide, calcium ionophore A23187[71]. Similarly, LPS and TNF-α

could also promote MPs generation in monocytes, lymphocytes, and endothelial cells[71].

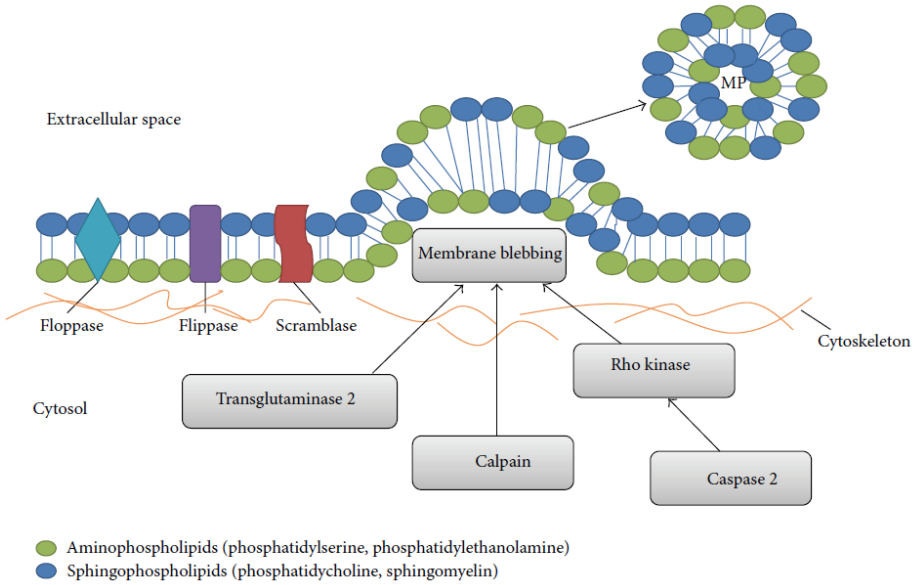

Figure 1.6 Possible mechanisms involved in the biogenesis of MPs. Reproduced with permission [83].

1.2.3 Clearance of MPs

The clearance of MPs *in vivo* remains unclear. Most studies were performed by infusing external MPs into animal models and then detecting the levels of the infused-MPs in circulation. The reported half-lives of MPs in the circulation differ widely. Rank et al. monitored the MPs levels in patients after platelet transfusion and found that the half-lives of MPs were approx. 5 hours[85]. However, a much shorter half-life time has been reported in animal studies[86-88]. For example, the infused MPs were cleared from the blood of rabbits in less than 10 min[88]. Interestingly, the infused MPs that were incorporated into the thrombus in mice were found to remain for a longer period compared to those that were not incorporated into the thrombus[86].

Phagocytosis of MPs by the endothelial cells is considered as the primary mechanism for the elimination of MPs *in vivo*[71]. This phagocytosis is mediated by the PS-exposure of MPs, which are recognized by the scavenger receptors on endothelial cells[87]. Del-1 (developmental endothelial locus-1), a surface protein of endothelial cells, is essential for the clearance of MPs *in vivo*[89]. Macrophages were also found to participate in the clearance of apoptotic MPs[90].

1.2.4 Isolation and characterization of MPs

Standardized isolation, identification, and quantification of MPs are essential for studying MPs[91]. Pre-analytical procedures including sample collection, handling, and storage are important sources of artifacts, which may affect the concentration, composition, and function of MPs. For example, platelet activation during sampling will lead to the release of MPs, and therefore should be avoid[92]. Similarly, plasma should be depleted of platelets before freezing, since a freeze-thaw cycle will induce residue platelets to release MPs and fragments[92]. The protocol used for the isolation of MPs may also affect their concentration, purity, and function. Several methods have been established to isolate MPs from blood, and the principle behind these different techniques are based on either their physical properties such as size and density, or particular surface ligands present on MPs[92].

Due to the small size of MPs, the detection and identification of MPs, especially the small MPs, remains a challenge. Transmission electron microscopy is the golden standard method for imaging MPs and can visualize the smallest MPs[92]. While this method can measure the size distribution of MPs, it cannot measure the concentration of MPs due to the sample preparation procedure. Flow cytometry, a widely available technique, has the highest throughput for characterizing the profiles of MPs in normal individuals and patients with different diseases[92]. Using flow cytometry, MPs pass one by one through a laser beam, so that the light scattered (which is related to the size and density of MPs), as well as the emitted fluorescence signals (specific for specific markers), can be detected.

1.2.5 Biological functions of MPs

MPs have been proposed to play roles in coagulation, inflammation, and the development of cancer, etc[70].

Coagulation. The procoagulant properties of MPs depend on the exposure of PS on their surface. Some subtypes of MPs, such as leukocyte-derived MPs (LMPs) and endothelial-cell derived MPs (EMPs), also express TF and thus have a higher level of procoagulant activity[93]. PS is an anionic phospholipid, which is located in the inner layer of the cell membrane under resting conditions, and is flipped out upon activation or apoptosis[94]. The anionic PS on MPs is available to interact with positively charged coagulation factors, including factors VII, IX, X, and II[31]. The PS+ PMPs can be

considered a smaller version of activated platelets, which express receptors for collagen, vWF, as well as fibrinogen/fibrin (**Figure 1.7**)[31].

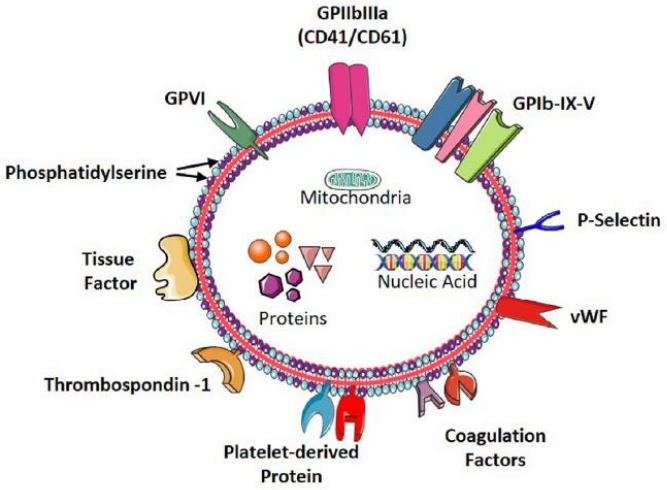

Figure 1.7 PMPs can be viewed as a smaller version of activated platelets. Reproduced with permission[95].

Inflammation. Increased levels of MPs have been found in patients with various inflammatory diseases[71,96]. MPs play a role in intercellular communication during inflammation. For example, the macrophage-derived MPs present in human atherosclerotic plaques were found to stimulate the proliferation of endothelial cells and to promote angiogenesis via CD40 ligation[97]. In another *in vitro* experiment, LMPs activated endothelial cells to release cytokines and express TF[98].

Cancer. Elevated levels of MPs, particularly TF+ MPs, have been reported in patients with several types of cancer[99,100]. Interestingly, TF+ MPs were shown to be associated with venous thrombosis in patients with pancreatic cancer[101], but not in other types of cancer[102]. In a mouse thrombus formation model, the MPs derived from the Panc02 pancreatic tumor cell line were injected into the mouse and those MPs were found to be accumulated in the developing thrombus[86]. PMPs can induce angiogenesis, and thus may be involved in tumor metastasis[103].

1.3 THE ROLE OF MPS IN HA

1.3.1 MPs and hemostasis in HA

MPs play an important role in hemostasis. Berckmans et al. found that MPs isolated from healthy individuals were mostly PMPs and they generate thrombin via a TF-independent pathway[76]. Zubairova et al. compared fibrin clot formation and structure in normal plasma and MPs-depleted plasma and found that MPs in healthy individuals both accelerated thrombin generation and improve the structure and stability of fibrin clots by becoming incorporated into the fibrin network[104].

MPs are involved in several bleeding disorders. Patients with Castamans's defect, a mild bleeding disorder, have prolonged bleeding time and a deficiency of PMPs generation[105]. This mild bleeding disorder suggests a potential contribution of PMPs on coagulation. Similarly, Scott syndrome, a rare congenital bleeding disorder, is characterized by reduced PS exposure on platelets and impaired MPs shedding[106].

Few studies have been published regarding MPs in HA. In one early study, Proulle et al. used a MP capture assay to study 79 hemophilic patients and 62 controls and found that the levels of MPs were similar in the two groups[107]. Recently, Jardim et al. compared the MPs profiles in 32 previously untreated PWHA and 47 healthy controls using flow cytometry and found that the level of several subtypes of MPs was elevated in the patient group[108].

MPs levels in PWHA are altered after treatment. Proulle et al. reported that the procoagulant activity of MPs increased after FVIIa infusion in 6 out of 15 patients[109]. Their study suggests that FVIIa could activate platelets to release PMPs, which could account for the hemostatic efficacy of FVIIa. Our previous study showed that MPs decreased in PWHA treated on-demand, which suggested that MPs were incorporated into the hemostatic plug formed at the injured site[110].

In a HA mice model, Hrachovinova et al. reported that an increased level of MPs, which was induced by soluble P-selectin, could normalize the tail vein bleeding time and shorten the aPTT time in HA mice[111]. These results suggest that procoagulant MPs could provide a novel potential therapeutic approach, however, the corresponding hemostatic effects of TF and PS upon MPs need to be further clarified. Kim et al. reported that MPs from aged red blood cells shortened tail bleeding time *in*

vivo and improved clot formation *ex vivo* in wild type (WT) mice[112]. Their data suggests that MPs may play an independent role to accelerate hemostasis, which could be beneficial for hemorrhaging patients. More detailed information is needed about how MPs contribute to hemostasis in HA.

1.3.2 MPs and atherothrombosis in HA

Research data suggest that MPs may play an important role in the development and manifestation of CVD[113]. Significant higher levels of circulating EMPs and PMPs were detected in patients with hypertension, diabetes mellitus, and metabolic syndrome compared to normal age-matched controls[114-116]. Elevation levels of MPs have also been observed in patients with atherosclerosis. An increased level of PMPs was correlated with the degree of atherosclerosis, as measured by the IMT of the carotid artery and the plaque burden[117,118]. An increased level of EMPs, which is associated with dysfunction of endothelial cells, has also been reported in various CVD[119].

Although PWHA were previously considered as being protected from CVD, there are increasing reports of atherothrombotic events among PWHA[57-60,67-68]. However, the role of MPs in atherothrombosis and/or CVD in PWHA has not been studied.

2 AIMS

The overall aim of this thesis was to investigate the effect of MPs on hemostasis of HA *in vitro* and *in vivo*; and to study the prevalence of CVD, the profiles of MPs, as well as their correlations in middle-aged and elderly PWHA.

The specific aims for each study were:

Paper I:

I: To investigate the *in vitro* fibrin formation and fibrin clot structure for combinations of aPCC, rFVIIa, and a sequence-identical analogue of emicizumab (SIA) in a severe HA plasma model.

Paper II and IV:

II: To study the procoagulant effect of MPs in *in vitro* HA plasma models, and to confirm that MPs are incorporated into the fibrin network of HA plasma.

IV: To evaluate the procoagulant effect of MPs in HA mouse, and to examine the incorporation of MPs into the thrombus formed at the injured site *in vivo*.

Paper III and V:

III: To determine the presence of subclinical CVD in asymptomatic PWHA using the advanced-electrocardiography (A-ECG) technique.

V: To characterize the risks of CVD, the global hemostatic status, the MPs profiles, and their correlations in PWHA treated on-demand.

3 RESEARCH APPROACH

The studies included in this thesis were designed to address specific research questions regarding MPs and their roles in HA. Three different approaches were used: *in vitro* assays, mouse model study approaches, and observational clinical study approaches. The methods used are described in this section, and further details can be found in the individual papers included in this thesis.

3.1 METHODS FOR *IN VITRO* STUDIES (PAPERS I AND II)

In vitro global hemostasis assays are increasingly used in coagulation research. Using the OHP assay and scanning electron microscopy (SEM) analysis, the potential hypercoagulability of co-administration of emicizumab and bypassing agents was investigated (in Paper I). Methods to study MPs are in the process of being developed and standardized. A combination of these methods and global hemostasis assays was used to study the procoagulant effect of MPs in *in vitro* HA plasma models (in Paper II), as described below.

3.1.1 Isolation of MPs from PNP

MPs were isolated from pooled normal human plasma (PNP). PNP-derived MPs were used in our studies because they exist under physiological conditions and thus are considered more natural, compared to the MPs derived from other sources (e.g. plasma of patients with different diseases, cultured cells). MPs were isolated from PNP via differential centrifugation, as described[120]. Platelets and other blood cells were removed from the blood sample by centrifugation twice at 3000 g for 15 min at 15°C. PNP was prepared by pooling the platelet-free plasma from the healthy individuals (n≥10 individuals for each batch of PNP) and the mixture was stored at −80°C in 1-2 mL aliquots. To isolate MPs from PNP, PNP was thawed at 37°C for 4 min and centrifuged at 20,800 g for 30 min at 10°C. Subsequently, the pellet containing the MPs was washed with PBS buffer by centrifugation three times to remove any residual FVIII. MPs prepared for each experiment was characterized as described in Section 3.1.2 below.

3.1.2 Quantification of the isolated MPs

The isolated MPs (as described in Section 3.1.1) were quantified using flow cytometry, which is a fast, convenient, and well-standardized method to characterize MPs[120]. The isolated MPs was incubated with lactadherin-FITC, and mouse anti-human antibodies

to two platelet surface markers, CD42a-PE and CD61-APC. After incubation, samples were tested using a FACs Canto I flow cytometer (BD). The size gate for MPs was defined by standard beads (Megamix Plus SSC beads)[121]. To discriminate between MPs and negative events (e.g. protein aggregates), a background control (triton X-100 lysed MPs) was tested by flow cytometry[122].

Since there is no universal marker for total MPs and the majority (approx.80%) of MPs isolated from PNP are PMPs[76], the number of PMPs was used to represent the concentration of the isolated MPs used in the *in vitro* coagulation tests. The concentration of PMPs in the isolated MPs sample was calculated as follows: Concentration (/μL) = (Events reading×4*550)/(44×20), and typically ranged from 0.9-6.5 × 10^5/μL: 4 is the dilution of isolated MPs sample, 550 is the total volume of sample in the tube analyzed, 44 is the volume of the analyzed sample, and 20 is the volume of the diluted isolated MPs sample added in the tube. The concentrations of isolated MPs added to plasma in the *in vitro* coagulation tests were presented as "amount of PMPs/μL plasma" as described in Paper II.

3.1.3 Calibrated automated thrombogram (CAT) assay

The CAT assay is a global hemostasis assay, which measures the rate and the amount of thrombin generated in the plasma[34]. This method was used to study the effect of MPs at different concentrations (2, 3, and 7×10^4 PMPs/μL plasma) on thrombin generation in *in vitro* human HA plasma models, in the presence or absence of coagulation trigger. PPP-Reagent LOW was used as the coagulation trigger since it contains a low concentration of TF (1 pM) and it demonstrates high sensitivity to FVIII-deficiency. Coagulation was initiated by spiking calcium and fluorescent substrate to the plasma sample. The parameters including lag-time, peak thrombin generation, and ETP were determined using the thrombinoscope software[34].

3.1.4 Overall hemostasis potential (OHP) assay

The OHP assay is another global hemostasis assay, which was used in the *in vitro* studies (Papers I and II) as well as in the mouse study (Paper IV) and observational clinical study (Paper V). It evaluates the overall fibrin formation and fibrinolysis in plasma[33]. Coagulation was initiated in the OHP assay by adding a small amount of exogenous thrombin (0.04 U/mL), phospholipids, and calcium to plasma, with or without exogenous t-PA (300 ng/mL). In Paper II, this method was used to study the

effect of MPs at different concentrations (2, 3, and 7×10^4 PMPs/µL plasma) on fibrin formation and fibrinolysis in *in vitro* human HA plasma models. The change of absorbance was monitored by a spectrophotometer. The fibrin aggregation curve (i.e. absorbance vs. time) represents the process of fibrin formation and polymerization, which is driven by the thrombin generated. The fibrinolysis curve represents the digestion of fibrin by plasmin, which is produced from plasminogen by tPA. The area under the curve and the turbidity parameters were calculated as described in the individual papers. As indicated in the papers, the fibrin gels produced in the OHP assay were further analyzed by SEM (see Section 3.1.5) to characterize the fibrin structure in more detail.

3.1.5 Scanning electron microscopy (SEM)

To study the fibrin network density and fibrin fiber thickness, the gels collected from the OHP assay were analyzed by SEM. The gels were washed with PBS, fixed with 2.5% glutaraldehyde, and prepared for SEM imaging as described in Paper I. SEM images were acquired using an emission scanning electron microscope (Zeiss, Germany), and analyzed with Fiji software.

3.1.6 Confocal microscopy

Fibrin gels were formed on the coverslips by 20 µL mixture as described in the OHP assay (Section 3.1.4), with the addition of Alexa Fluor 594-fibrinogen. To visualize the structure of the fibrin gel in different sections, a Leica SP8 laser scanning confocal microscope with a 63x/1.4 oil lens (Leica Microsystems) was used. In each sample, z-stacks were collected at 8 randomly chosen areas with 5 at the periphery and 3 in the center. Z-stacks, consisting of multiple layers taken at 0.3-µm intervals, were acquired, and the fibrin clot density was analyzed using Fiji software.

3.1.7 Stimulated emission depletion (STED) microscopy

Since the diameters of the majority of MPs and fibrin fibers were below the resolution limit of standard confocal microscopy (200-300 nm), we used stimulated emission depletion (STED) microscopy, which has a lateral resolution of 70 nm, to better resolve the location of MPs in the fibrin network. Fibrin gels prepared in Section 3.1.6 were incubated with Abberior STAR-635-conjugated anti-CD61 antibodies. Fibrin fibers containing Alexa Fluor 594-fibrinogen were visualized in the orange channel, while MPs were detected in the red channel. Images were acquired using an Abberior

Instruments setup (Abberior Instruments, Germany) with a Leica 100x, NA 1.4 objective. Images were analyzed using Imspector and Fiji software.

3.2 METHODS FOR MOUSE STUDIES (PAPER IV)

Hemostasis is the process that stops bleeding at an injured blood vessel, which depends on the interactions of coagulation factors, platelets, blood vessels, and blood flow rate. Only the first two components can be measured in *in vitro* laboratory tests. To take into consideration the other coagulation components, it is necessary to perform *in vivo* studies, which enables us to investigate the effect of MPs in living conditions. Mice have similar coagulation factors and function as human beings. HA mouse models (having <1% FVIII activity) have been available since the 1990s and are important tools employed in the development of therapeutics for HA[123].

3.2.1 Saphenous vein bleeding model

The saphenous vein bleeding model has been used to evaluate the effect of pro-coagulant compounds in HA mouse in recent years[124]. The saphenous vein has relatively low blood pressure, and the injury in this model is inflicted at a specific anatomical site of the vein. This model has low variations and high sensitivity to evaluate procoagulant compounds compared to other bleeding models (e.g. tail bleeding model)[124]. Mice were administered with different drugs, and the saphenous vein bleeding model experiments were performed as described with minor modifications (**Figure 3.1**)[124,125]. After the primary bleeding stopped, we dislodged the primary clot to determine the secondary bleeding time[125]. This dislodging procedure was repeated each time after a new clot was formed at the injured site, over a 10-min period. The number of hemostasis events was recorded, and the bleeding time and blood loss were measured as described in Paper IV. Immediate after the bleeding model experiments, blood samples were taken from the mice.

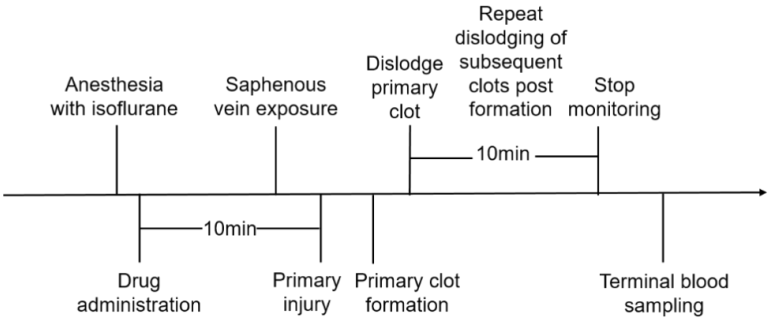

Figure 3.1 Timeline for the saphenous vein bleeding model. See Methods in Paper IV for details.

3.2.2 Whole blood thrombin generation assay

The whole blood thrombin generation assay requires only a small volume of the blood sample, which is ideal for murine studies[126]. Moreover, this assay is more

physiologically relevant compared to the plasma-based CAT assay, since it reflects the influence of platelets and other blood cells on thrombin generation. Fluorescent thrombin substrate was added to mouse whole blood, and coagulation was initiated after the addition of TF (final concentration 1 pM) and calcium. Thrombin generation was measured by monitoring the fluorescent signals using a Fluoroskan Ascent microplate fluorometer with Fluoroskan Ascent software (Thermolabsystem). A dedicated programmed spreadsheet template allowed the following parameters to be calculated: the peak of TG, thrombin activity/ETP before the peak (ETPp), lag-time, time to peak (tt peak), and velocity[127].

3.2.3 Cremaster muscle thrombus formation model

To visualize the incorporation of MPs into the thrombus at the site of blood vessel injury, thrombus formation was monitored using the HA mice cremaster muscle model by intravital microscopy. MPs were isolated from human PNP as described and kept at −80°C after the addition of 50 mM trehalose (to protect the membrane structure and activities of MPs from freezing)[128]. After exposure of the cremaster muscle, blood vessels were examined using transmitted light with an Apo LWD 25 x/1.10 NA objective and Eclipse FN1 intravital microscope (Nikon). After testing in some pilot experiments, we summarized the following principles to choose the optimal site to induce the injury and to visualize the thrombus formation in HA mouse. Firstly, venules are preferred over arterioles, because vasoconstriction in arterioles upon injury usually interferes with the observation of thrombus formation. Secondly, the proximal part of the major venule is desired, since the blood flow is sufficient in this area. We found that in the periphery venules with a low flow rate, even minor injuries could terminate the blood flow immediately, therefore, the dynamics of thrombus formation could not be monitored. Lastly, we found that the optimal site for our study was along the major venule of the cremaster muscle, where a branch venule merged into it. Since we needed to induce the injury manually under the stereomicroscope, and then put the mouse back under the objective of the intravital microscope, an anatomical “landmark” near the injured site of the blood vessel makes it much easier to fetch on the thrombus formation promptly.

Calcein-AM labeled human PNP-derived MPs were injected into HA mouse intravenously. An injury was induced by placing a 5% $FeCl_3$ soaked filter paper (1 x 1 mm) above the selected site of the venule for 20 s. The mouse was placed under the

intravital microscope to check whether a thrombus was formed and the injury process was repeated until a thrombus could be visualized. Time-lapse images were acquired using the microscope with a multiband filter cube (DAPI/FITC/TRITC). The calcein AM-labeled MPs were imaged via the FITC-channel.

3.2.4 Clearance of MPs by mouse organs

To study whether the infused human PNP-derived MPs were cleared by the mouse organs via endocytosis, mouse lung, liver, and spleen were collected after the cremaster muscle experiments. The organ samples were incubated with collagenase, filtered with 40 µm-cell strainers. After lysing red blood cells, the single-cell suspensions were resuspended in PBS buffer and examined using a Canto I flow cytometer. Single cells were identified along the diagonal of the SSC-H by SSC-A graph, and dead cells (which showed positive staining with propidium iodide) were excluded from the analysis. The cells which had endocytosed the calcein-AM-labeled MPs were identified by their fluorescent signals in the FITC channel.

3.3 METHODS FOR CLINICAL STUDIES (PAPERS III AND V)

To study the prevalence of CVD in adult PWHA, we conducted observation clinical studies. To investigate the presence of clinical and/or subclinical CVD, the A-ECG technique was used. A-ECG can be performed rapidly and inexpensively in clinical practice, moreover, it shows higher accuracy for particular disease conditions than conventional ECG[129].

3.3.1 Study design and subject population

Paper III describes a pilot retrospective of Swedish PWHA who had a digital ECG file available at the Karolinska University Hospital, Stockholm, Sweden. Paper V describes a cross-sectional study of Chinese PWHA treated on-demand conducted at the First Affiliated Hospital of Zhengzhou University, Zhengzhou, China.

Paper III study subjects The patients in this study were identified retrospectively by reviewing the medical journals of PWHA (regardless of the HA severity) followed up at the Coagulation Unit, Department of Hematology, Karolinska University Hospital from 2005 to 2018. We included patients ≥40 years and had at least one digital ECG file available. Patients who were diagnosed with CVD were excluded. A total of twenty-nine PWHA were included in this study, as shown in **Figure 3.2**. Twenty-nine age-matched male controls were also included in the study. Further details regarding this study population are given in the Methods of Paper III.

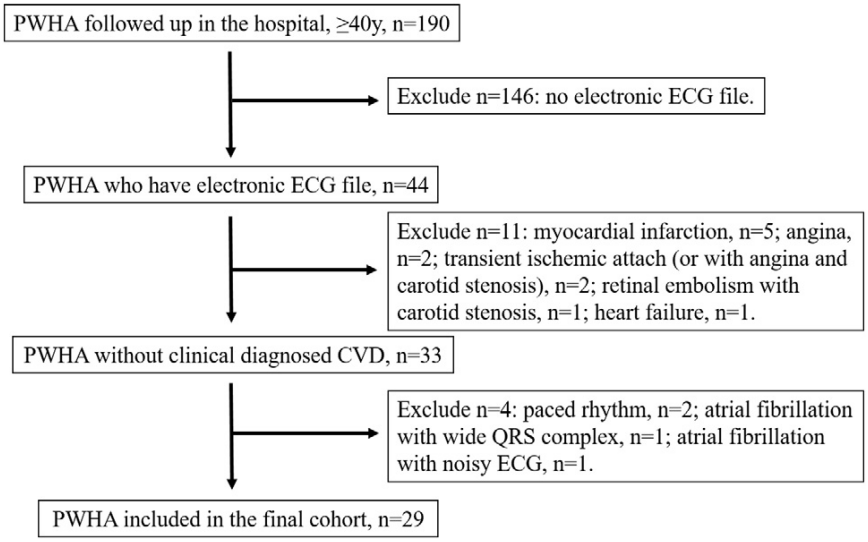

Figure 3.2 Flow chart showing the inclusion and exclusion of patient cases in Paper III[130].

Paper V study subjects The patients in this study were recruited and examined at the Center of Genetics and Prenatal Diagnosis, the First Affiliated Hospital of Zhengzhou University, China. Male patients ≥30 years of age, diagnosed with HA, irrespective of the severity, and treated with factor concentrates on-demand were included in the study. Age-matched male controls from the general public were recruited in the same clinic by placing a local advertisement. The age cut-off in this study is younger than that used in the retrospective study in Paper III, since we aimed to be able to include more patients. A total of forty-two patients and thirty-seven controls were included in this study. Further details regarding this study population are given in the Methods of Paper V.

3.3.2 Collection of clinical data and ECG files

In Paper III, clinical data were retrospectively extracted from digital medical records in TakeCare®. Standard 12-leads ECG files, which were recorded under clinical conditions were exported from the digital database (MUSE® Cardiology Information System).

In Paper V, clinical data and ECG files of the participants were obtained during their visit to the clinic. Clinical information was obtained by interview, and a 5-min ECG was recorded for each participant by a trained medical doctor.

3.3.3 Advanced-electrocardiography (A-ECG) analysis

ECG or A-ECG files were analyzed using the semi-automatic A-ECG software CardioSoft® (NASA, Johnson Space Center, TX, USA). In both Paper III and V, conventional ECG parameters such as the QRT and T wave durations, axes, and voltages amplitudes, and A-ECG parameters including the derived vector cardiographic ECG parameters and QRS and T waveform complexity measures were obtained. In addition, in Paper V, parameters for the beat-to-beat variability of the R-to-R and QT intervals were also used.

Previously validated A-ECG scores to determine the probability of having CVD, left ventricular systolic dysfunction (LVSD), coronary artery disease/coronary microvascular disease (CAD/CMVD), and left ventricular hypertrophy/left ventricular electrical remodeling (LVH/LVER) were calculated[129-130].

3.3.4 Analysis of circulating MPs by flow cytometry

Blood was collected from the participants and centrifuged twice at 3,000 g for 10 min to obtain platelet-free plasma. The plasma sample was incubated with Annexin-V-FITC (PS+ MPs), mouse anti-human antibodies against CD61-PE (PMPs), CD62P-APC (P-selectin+ MPs), CD51/61-FITC (EMPs), CD142-PE (TF+ MPs), or CD45-APC (LMPs) for the identification and measurement of different subtypes of MPs. The samples were tested using the BD Canto II flow cytometer and analyzed using the Flowjo software.

3.4 METHODS FOR STATISTICAL ANALYSIS

The IBM SPSS statistics (version 25.0) and Graph Pad Prism software were used to carry out the statistical analysis. To compare continuous variables, the Student t-test (for normally distributed data) or the Man-Whitney test (if data is not normally distributed or the sample size is relatively small) was used for comparison of two groups, and the Kruskal-Wallis test for comparison of more than two groups. To compare categorical variables, the Pearson Chi-square test was used. Correlation analysis was performed using Spearman's rank correlation test. Sample sizes were determined empirically, and no statistical tests were used to predetermine the sample size. A p-value <0.05 was considered as statistically significant.

3.5 ETHICAL CONSIDERATIONS

The study in Paper I did not involve any human participation, or collection of personal data, or usage of research animals. Therefore, an ethical permit was not required.

The study in Paper II was part of a larger study, which was approved by the regional ethics review board in Stockholm (Dnr 01–0003;2006/778–32, completed with 2013/1045–32, 2015/275–32 and 2018/1480–32).

The study in Paper III was approved by the regional ethics review board in Stockholm (Dnr: 2018/636-31/2). The studies were conducted following the Declaration of Helsinki.

The study in Paper IV was approved by the local ethics committee for the animal experiment in Sweden (Dnr 5708-2018). The “KI-mallen” was used to assess animal welfare. Mice were sacrificed if the humane endpoint was observed.

The study in Paper V was approved by the ethical committee of the First Affiliated Hospital of Zhengzhou University, Zhengzhou, China (2020-KY-041). The studies were conducted following the Declaration of Helsinki, and informed consent was obtained from the participants.

4 RESULTS AND DISCUSSION

This section focuses on the key findings generated by the research work that was conducted for this thesis, categorized by sub-studies.

4.1 THE EFFECT OF COMBINATIONS OF APCC, RFVIIA, AND SIA ON FIBRIN FORMATION *IN VITRO* IN A HUMAN PLASMA MODEL OF SEVERE HA (PAPER I)

This part of the thesis focuses on the standardization of the coagulation assays (the OHP assay and SEM analysis) using *in vitro* HA plasma models, which were then used in the following studies included in this thesis.

FVIII replacement therapy causes the development of FVIII inhibitors in approximately 30% of PWHA and interferes with treatment efficacy[1]. To treat patients with FVIII inhibitors, bypassing agents (aPCC and rFVIIa) are used, however, they are not always effective[1,50]. Emicizumab (trade name Hemlibra) is a novel therapy for PWHA and has recently been approved to treat patients with or without inhibitors[40,131]. This drug is a humanized bispecific antibody that mimics the function of FVIII to connect FIXa and FX, thus activating FX and restoring coagulation in HA[40]. A potential concern with emicizumab treatment for PWHA is that when additional bypassing agents are used to control breakthrough bleeding, thrombotic events were reported[131]. The HAVEN study observed thrombotic microangiopathy (3 cases) and thrombosis (2 cases: cavernous sinus thrombosis or skin necrosis-superficial thrombosis) in patients treated with a high cumulative dose of aPCC for breakthrough bleeding during the prophylactic administration of emicizumab; no thrombotic events were reported with the co-administration of emicizumab and rFVIIa[131].

In Paper I, the effect of SIA (synthesized based on the amino acid sequence of emicizumab), alone and in combination with bypass agents (aPCC or rFVIIa) was studied. One previous study investigated the effects of these drugs and drug combinations using the thrombin generation assay and the whole blood based rotational thromboelastometry assay[132]. In this study, we used the OHP assay and SEM analysis to study fibrin formation and fibrin clot structure.

The combination of SIA with aPCC gave a higher OHP value and clotting rate compared to SIA alone (**Figure 4.1**). Although the OHP value did not reach the normal

range, the fibrin clotting rate surpassed the normal range dramatically (**Figure 4.1**). The results suggested that the combination of SIA and aPCC may lead to a hypercoagulable state in HA. This would be highly undesirable in the clinic because it would be expected to increase the risk of thrombotic events[33]. Our results confirmed the results in the previous study, which found that the peak thrombin value of the combination of SIA and aPCC exceeded the reference range[132]. The mechanism of the synergistic effect of aPCC-SIA combination is likely that aPCC contains FIX and FIXa, which enables SIA to form the FX activation complex more rapidly[132].

The combination of SIA and rFVIIa only slightly increased the OHP value and fibrin clotting rate compared to SIA alone, and the values were all below the normal range (**Figure 4.1**). No obvious synergistic effect was observed with this drug combination. These results suggest that the combination of SIA and rFVIIa could improve hemostasis slightly without increasing the thrombotic risks in PWHA, which is consistent with another *in vitro* study[132].

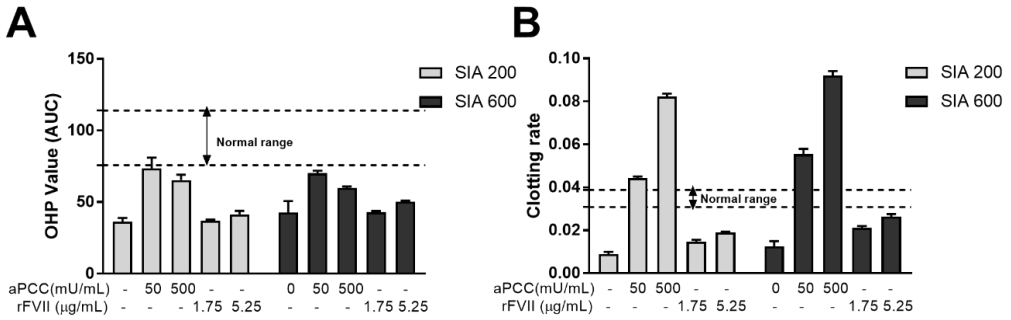

Figure 4.1 The OHP value and the clotting rate in the severe HA plasma model after the addition of SIA (200 or 600 nM) in combination with aPCC or rFVII, at different concentrations. Normal range: reference range presented as a minimum and maximum value measured in PNP. Reproduced from Paper I with permission.

In summary, results in this study support conclusions of the previous studies, that additional caution should be taken when combinations of emicizumab and bypassing agents are used to treat PWHA. Treatment with a combination of emicizumab and rFVIIa is likely a safer approach vs. treatment with emicizumab and aPCC to control breakthrough bleeding in PWHA treated with emicizumab, in order to reduce the risk of thrombotic events.

We did not investigate the role of MPs in PWHA treated with emicizumab in this thesis, and this is of further interest. Procoagulant MPs might be a potential biomarker for

bleeding or thrombosis risks for patients treated with emicizumab. *Potential future work:* To study the levels of MPs in PWHA treated with emicizumab, and; to investigate if the addition of MPs could enhance the procoagulant effect of emicizumab in *in vitro* HA plasma models and/or in HA mouse model.

4.2 THE EFFECT OF MPS ON HEMOSTASIS IN HA *IN VITRO* AND *IN VIVO* (PAPERS II, AND IV)

MPs play important roles in coagulation, however, their procoagulant effect in the bleeding disorder, HA, is not understood[31,32]. Our lab previously reported a study in PWHA treated on-demand, the levels of MPs decreased after administration of FVIII concentrates. The hypothesis that was generated from this study was that MPs are incorporated into the hemostatic plugs formed at the site of injury in PWHA[110]. In the following thesis project, the aim was to better understand the procoagulant effect of MPs in HA, using the *in vitro* human HA plasma models (Paper II) and the HA mouse model (Paper IV).

4.2.1 MPs improve hemostasis *in vitro* in human plasma models of HA (Paper II)

Previous work by Berckmans et al. indicates that circulating MPs in healthy individuals support thrombin generation via TF-independent pathways[76]. *We hypothesized that PNP-derived MPs could improve thrombin generation and fibrin formation in in vitro HA plasma models.*

It was previously (in 2014 and earlier) observed in our lab that interesting particle-like structures (around 50-200 nm in diameter) attached to fibrin fibers using SEM imaging (revealed in the studies aimed to investigate the fibrin clot structure in plasma sample from normal controls or patients with, eg. hemophilia or systematic lupus erythematosus patients, personal communication). They hypothesized that these particles were some type of MPs. Interestingly, another research group (Zubairova et al.) in 2015 reported a similar observation of particles attached to fibrin fibers *in vitro*, in clots of normal plasma, and they confirmed that these were MPs of platelet origin (PMPs) using confocal microscopy[104]. *We hypothesized that MPs are incorporated into the fibrin network of HA formed in vitro, thus improving hemostasis in vitro.*

MPs improve thrombin and fibrin formation in HA plasma models via PS exposure on their membrane surface

MPs increased peak thrombin generation dose-dependently in CAT assay using the *in vitro* severe HA plasma model, both with and without the addition of PPP-Reagent LOW (**Figure 4.2**). MPs (2 x 10^4 MPs/µL plasma) also increased peak thrombin

generation in the moderate and mild HA plasma models as well as in PNP (**Figure 4.2**).

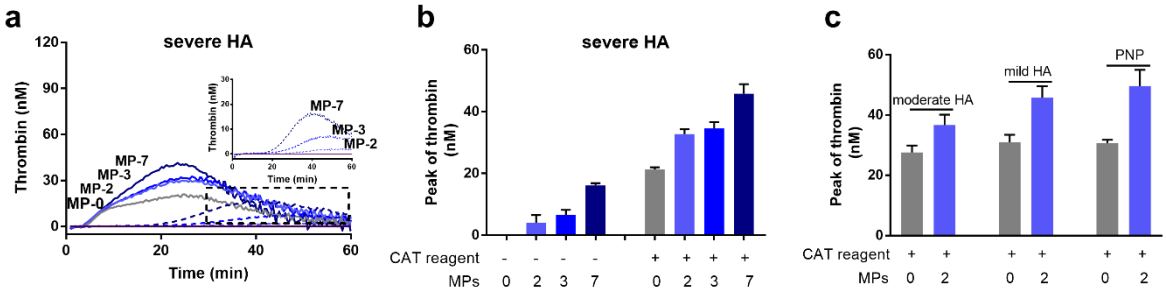

Figure 4.2 PNP-derived MPs improve thrombin generation in HA plasma models as well as in PNP. (a) Thrombin generation in the severe HA plasma model with different concentrations of MPs (MP-0, 2, 3 and 7: 0, 2, 3 and 7 × 10^4 MPs/µL plasma), in the presence (solid lines) and absence (dashed lines) of PPP Reagent LOW (CAT reagent). The inset shows thrombin generation curves (with an adjusted y-axis scale) in the absence of CAT reagent. (b) Peak thrombin value in the severe HA plasma model. (c) Peak thrombin value in the moderate HA (2.5% FVIII), mild HA (20% FVIII) plasma models, and in PNP (100% FVIII) with MPs (2 × 10^4 MPs/µL plasma) in the presence of CAT reagent. Data shown are mean ± SEM values, n = 9 replicates. Reproduced from Paper II with permission.

MPs increased the OHP value dose-dependently in the severe HA plasma model, in the absence of OHP reagent (which contains thrombin and phospholipids) (**Figure 4.3**). The procoagulant effect of MPs was decreased dramatically after lysing the MPs (7 x 10^4 MPs/µL plasma) with 0.25% TritonX-100 (**Figure 4.3**), suggesting that the intact membrane surface of MPs is of crucial importance for the hemostatic response. MPs (2 x 10^4 MPs/µL plasma) increased the OHP value in all HA plasma models as well as in PNP, in the presence of OHP reagent (**Figure 4.3**).

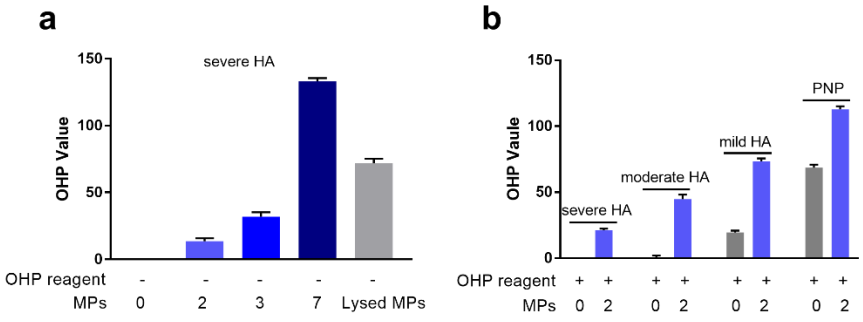

Figure 4.3 The effect of MPs on fibrin clot formation and clot stability in different HA plasma models. (a) OHP values in the severe HA plasma model with the addition of different concentrations of MPs (2, 3, and 7 × 10^4 MPs/µL plasma) in the absence of OHP reagent. Lysed MPs: MPs (7 × 10^4 MPs/µL plasma) were treated with TritonX-100; (b) OHP values in different HA plasma models and PNP, without and with MPs (2 × 10^4 MPs/µL plasma) in the presence of OHP reagent. OHP value: area under the curve

(absorbance vs. 2 h) of clot formation and fibrinolysis (with t-PA). Data are means ± SEM values, n = 9 replicates. Reproduced from Paper II with permission.

Consistent with the OHP assay results, in the severe HA plasma model, MPs (2 x 10^4 MPs/µL plasma) improved fibrin clot structure, forming a denser fibrin network compared to the typical loose fibrin structure with large pores seen without MPs, (**Figure 4.4**).

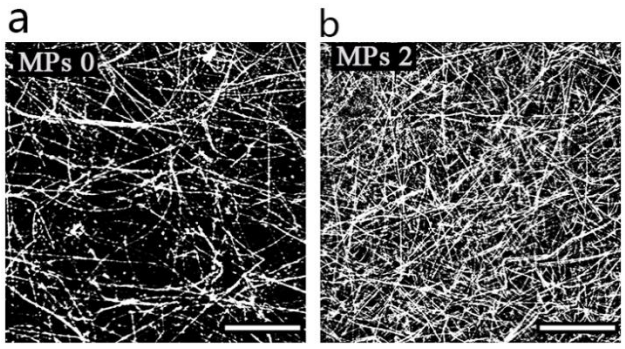

Figure 4.4 Standard confocal microscopy showed that MPs increased fibrin clot density in the severe HA plasma model. Representative images are shown of fibrin clot structures: (a), without the addition of MPs (MPs 0); (b) with the addition of MPs (2 × 10^4 MPs/µL plasma, MPs 2). Scale bar = 25 µm. Reproduced from Paper II with permission.

To study the mechanism of this procoagulant effect, MPs were first incubated with lactadherin (which blocks PS on MPs), anti-TF antibodies (which blocks TF on MPs), or corn trypsin inhibitor (CTI, which blocks contact activation) were added to the plasma, before testing in the CAT and OHP assays. After targeting PS with lactadherin, both thrombin generation and fibrin formation were inhibited in the mild HA plasma model (**Figure 4.5**). In contrast, anti-TF antibodies did not affect the CAT or OHP assay results (**Figure 4.5**). Blocking the contact activation pathway using CTI slightly prolonged the lag-phase of thrombin and fibrin formation, however, the total thrombin generation (ETP) and fibrin formation (OHP) were not reduced (**Figure 4.5**).

These results suggest that the mechanism by which MPs improve global hemostasis in HA plasma models depends mainly on the presence of PS molecules exposed on intact MP membranes (PS+ MPs). This is the first study to investigate the procoagulant effect of MPs in HA plasma models. Several studies investigated the procoagulant effect of MPs in normal plasma models[76,104,133]. Tripisciano et al. showed that platelet concentrate-derived MPs improved thrombin generation in normal vesicle-free plasma dose-dependently, which was inhibited by blocking PS but not by blocking TF[133]. They

found that the procoagulant effect of monocytic cells-derived MPs (after LPS stimulation) was inhibited by blocking TF[133]. Since we focused on MPs derived from human PNP, which were characterized by flow cytometry as mainly being platelet-derived (PMPs), the contribution of TF to the procoagulant effect of MPs in our study was negligible.

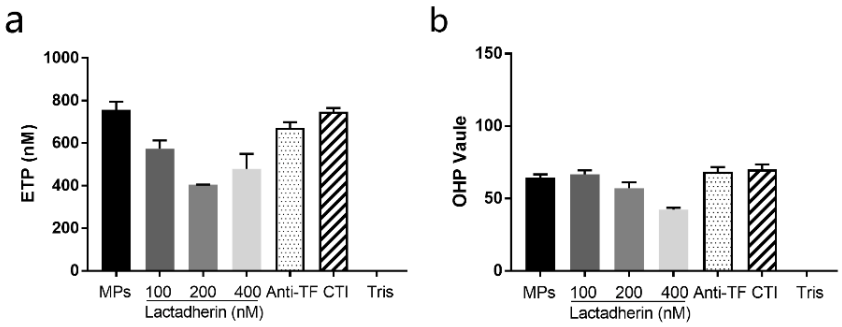

Figure 4.5 The procoagulant effect of MPs after blocking PS, TF, or contact pathway activation in the mild HA plasma model and the absence of CAT or OHP reagent. (a) ETP detected by the CAT assay, (b) OHP values detected by the OHP assay. MPs were incubated with lactadherin (MPs + lac 100 nM, 200 nM, and 400 nM) to block PS; anti-TF antibodies (50 µg/mL) to block TF; and plasma was incubated with CTI before the addition of MPs (20 µg/mL) to block contact activation. Tris buffer was used as a negative control. Data are means ± SEM, n = 9 replicates. Reproduced from Paper II with permission.

Our data showed that the procoagulant effect of MPs in HA plasma models is mostly independent of contact activation via FXII. Different findings have been reported in studies using normal plasma[133-135]. For example, Tripisciano et al. found that the addition of CTI to plasma reduced thrombin generation induced by platelet concentrate-derived MPs significantly, although not completely[133]. The inhibition of contact activation is more potent when CTI is directly added to the blood sampling tube [133-135], however, in our study, CTI was added to the HA plasma model right after thawing the plasma tube. It is possible that contact activation was not totally blocked in our study. Nevertheless, we found that blocking PS inhibited thrombin and fibrin formation dose-dependently, suggesting that the PS exposure on intact MP membranes is essential for the procoagulant effect of MPs in the HA plasma model.

According to the cell-based coagulation model, the PS+ membrane is an essential component in the amplification and propagation phases, which provides the surface for the formation of coagulation complexes (including FVIIIa/FIXa and FVa/FXa), as shown in **Figure 1.2**. These two coagulation phases are impaired in HA due to the deficiency of FVIII. Our data in this study showed that MPs improved hemostasis in

HA plasma models, we suggest the underlying mechanism is that the PS+ membrane on MPs could accelerate the biding of FIXa and FX, and increase the formation of FXa, thus improve the following thrombin and fibrin formation.

MPs are incorporated into the fibrin network in the HA plasma model

Using SEM imaging, we observed “MP-like structures”, which were attached to fibrin fibers formed in the HA plasma model (**Figure 4.6**). Few attempts were made using immune-gold labeling SEM, however, I was not able to confirm that the particles bound to fibrin fibers were MPs using *in vitro* clots from normal plasma.

Figure 4.6 Representative SEM image showing the “MP-like structures” (indicated by arrows) attached on fibrin fibers. Fibrin clots shown are from the severe HA model with the addition of PMPs (2×10^4 PMPs/μL plasma) isolated from platelet concentrate without TRAP6 treatment. Bar = 1 μm. Reproduced from Paper II with permission.

Confocal microscopy confirmed the platelet origin of the MPs, however, the resolution limit (laterally 200-300 nm) of this technique was not ideal to address the precise location of MPs in the fibrin network. Therefore, we used super-high-resolution STED imaging and showed for the first time that MPs (platelet origin) are attached to fibrin fibers, particularly at branch points and junctions (**Figure 4.7**). The specific locations where the MPs are incorporated into the fibrin network suggest that MPs may participate in the formation of fibrin monomers as well as mediate the density and 3D structure of the fibrin network, in the HA plasma model.

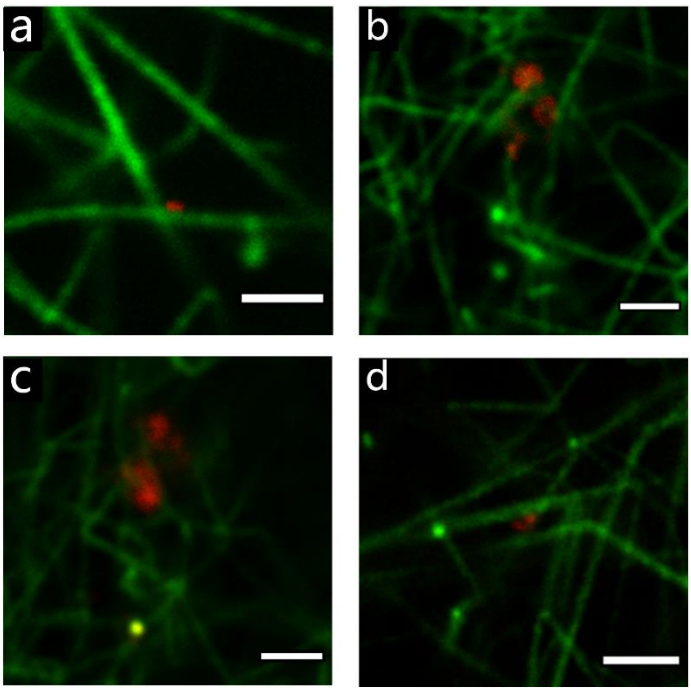

Figure 4.7 Different typical patterns showing the location of PMPs incorporated into the fibrin network detected by STED microscopy. (a) PMPs located at the cross point of two fibers; (b) Fiber curved towards the attached PMPs; (c) PMPs embedded in a mesh of fibrin fibers; (d) PMPs act as a bridge connecting two fibers. Images are all from the severe HA plasma model with the addition of MPs. Representative images from two independent experiments are shown; duplicate samples/experiment. Bar = 2 µm. Green: fiber (Alexa fluor 594), Red: PMPs (Aberrior STAR 635). Reproduced from Paper II with permission.

In summary, we found that MPs improved hemostasis via the exposure of PS, and were incorporated at specific locations (at joints) in the growing fibrin network in *in vitro* HA plasma models, which confirmed our hypotheses of this study. Attachment of MPs to fibrin fibers opens opportunities to investigate the potential modulatory role of MPs during the fibrin formation. Our data also suggest that MPs could potentially shift the impaired hemostasis seen in the severe HA clinical profile, towards the moderate or mild HA clinical profiles. MPs might be a potential adjunctive therapy to improve replacement therapy in PWHA. Therefore, we continued to investigate the *in vivo* procoagulant effect of MPs in HA mice.

4.2.2 MPs improve hemostasis *in vivo* in the HA mouse model (Paper IV)

Based on the data from the *in vitro* studies, *we hypothesized that treatment with MPs could improve hemostasis in vivo in HA mouse; and the administered MPs might be incorporated into the hemostatic plugs formed at the site of injury in vivo in HA mouse.*

In addition, we hypothesized that the injected MPs might be eliminated from mice circulation rapidly.

Administration of MPs improve hemostasis in vivo in HA mouse

To evaluate the hemostatic effect of MPs *in vivo* in the HA mouse model, the saphenous vein bleeding model was used as described with minor modifications[124]. HA mice were treated with control saline, MPs (3.8 x 10^4 MPs/g mouse), rhFVIII (5 IU/kg mouse; rFVIII-5), rhFVIII (25 IU/kg mouse; rFVIII-25) or combinations of these (rFVIII-5 + MPs, rFVIII-25 + MPs).

Administration of 25 IU/kg rhFVIII to the HA mice revealed a similar number of hemostatic events as the control WT mice, and the average bleeding time was almost normalized (**Figure 4.8**), consistent with a previous study[124]. Treatment with MPs alone had little effect, and only slightly increased the number of hemostatic events and decreased the average bleeding time, compared to the saline-treated group of HA mice (**Figure 4.8**). Co-administration of MPs and rhFVIII did not reveal any additional hemostatic effect compared to treatment with rhFVIII alone (**Figure 4.8**). This result suggested that while MPs may improve hemostasis *in vivo* in HA mice, and their procoagulant effect *in vivo* in the HA mouse model is not potent compared to rhFVIII.

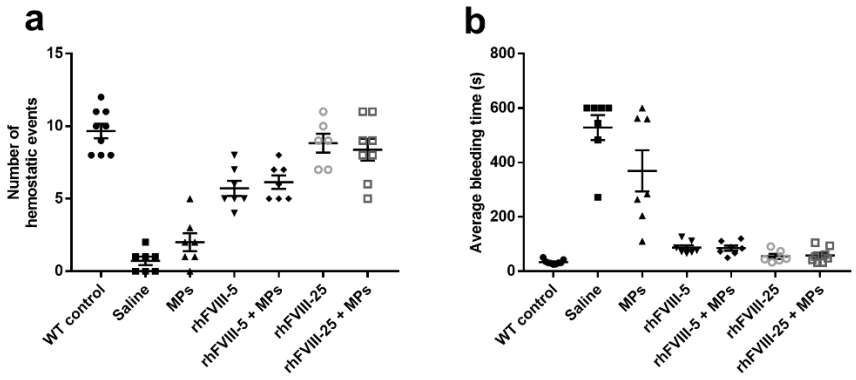

Figure 4.8 Bleeding parameters of the saphenous vein bleeding model in WT mice and HA mice receiving different treatments. (a) the number of hemostatic events, (b) average bleeding time. n=6-9 mice/group. Adapted from Paper IV.

Results of whole blood thrombin generation assay

Blood samples collected from mice after the bleeding model experiment (approx. 30 min post-injection) were tested using the whole blood thrombin generation assay. HA mice treated with MPs prior to the saphenous vein bleeding model showed similar low

levels of thrombin generation compared to those treated with saline. Administration of rhFVIII increased thrombin generation, however, the combination of MPs and rhFVIII did not improve thrombin generation even further. These results suggest that no *ex vivo* improvement of thrombin generation was detected for the treatment with MPs.

The contradictory findings of the *in vivo* and *ex vivo* experiments might be explained by the fact that the infused MPs were rapidly eliminated from mice circulation. We found that the injected MPs (human PNP-derived MPs) were not detectable in mice plasma approx. 30 min post-injection as tested using flow cytometry. Taken together, these results suggest that the injected MPs might be rapidly consumed by incorporation into the hemostatic plugs formed at the site of saphenous vein injury, or be rapidly eliminated from the circulation by mice organs.

MPs accumulate in the thrombus formed at the site of injury in HA mice

In order to study the incorporation of MPs at the site of injury *in vivo* in HA mice, we performed the intravital imaging of thrombus formation in the cremaster muscle of HA mice. Fluorescent (calcein-AM) labeled human-PNP-derived MPs were injected into HA mice, injury with $FeCl_3$ was induced to the blood vessel in the cremaster muscle, and thrombus formation was monitored by intravital microscopy. Fluorescent signals corresponding to the injected MPs were detected in the thrombus formed at the site of injury in the HA mice (**Figure 4.9**). The thrombi were found to be unstable and washed away by blood flow, however, new thrombi were formed rapidly (within 1 minute) again at the site of injury.

Our results confirmed the hypothesis that MPs participate in the formation of thrombi *in vivo*. Furthermore, this appears to occur independently of the presence or absence of FVIII, as it was seen in both WT and HA mice by our group and others[86,111]. The origins of MPs used differed from our study. The first study used MPs isolated from blood samples from a gene-modified mouse strain (ΔCT mice[111]), and the second study used MPs from an *in vitro* cancer cell line (Panc02) [86]. Both studies found that the injected MPs accumulated at the site of injury in WT mice, and the latter suggested that the MPs accelerated the kinetics of thrombus formation *in vivo*.

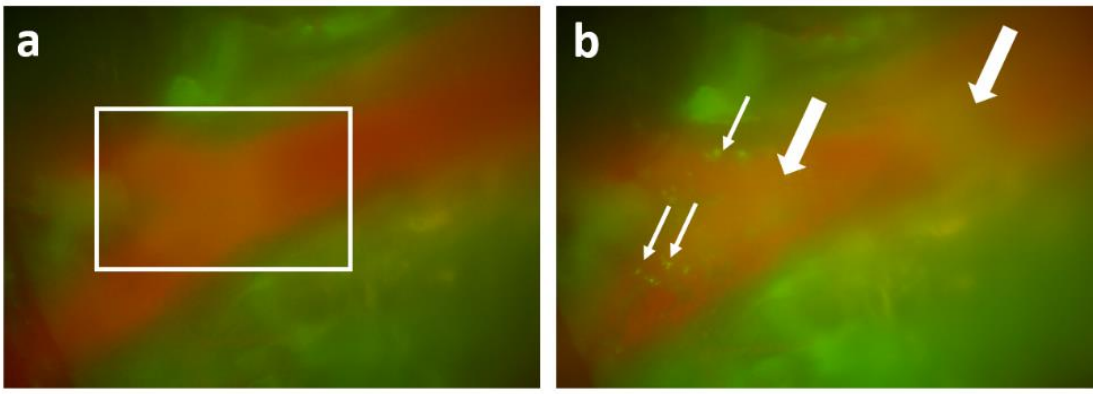

Figure 4.9 Recruitment of calcein-AM-labeled MPs into the thrombus at the site of injury in HA mice cremaster muscle model. Composites images are shown: a merge of the FITC channel (for calcein-AM-labeled MPs, Green) and the TRITC channel (Red). (a) image before the injury, and the rectangle indicate the site of injury, where a branch venule merged with a major venule (see Methods); (b) image after injury, the thick arrows indicate the thrombus inside the vessel, the thin arrows indicate where MPs were incorporated into the thrombus. Representative images are shown n=3-5 mice/group. Adapted from Paper IV.

In the current study, we for the first time observed the incorporation of MPs in the thrombus formed at the injured site *in vivo* in HA mice. We suggest the potential mechanism is that MPs mimic activated platelets and bind to collagen exposed after injury of endothelial cells in the circulatory system and/or to fibrin via exposed glycoprotein IIb/IIIa[135], thus participate in the formation of thrombi or hemostatic plug at the site of injury.

Clearance of the administered MPs in mouse organs

We showed that calcein-AM labeled MPs were endocytosed by the lung and liver but not the spleen of HA mice (**Figure 4.10**). This result partially explained our observation that the injected MPs were not detectable in mice circulation approx. 30 min post-injection. Similar results were reported by one previous study, MPs were found to be cleared rapidly (within 5 min) by the liver and lung of WT mice *in vivo*[89].

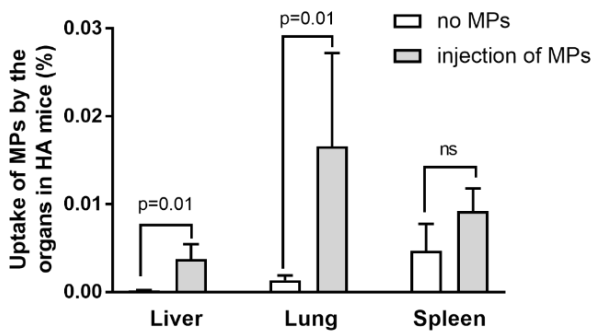

Fig 4.10 Clearance of MPs by the organs in HA mice. In HA mice injected with calcein-AM-labeled MPs, the levels of calcein-AM+ cells in liver and lung were significantly higher compared to mice without injection of MPs. p values were from 4-6 separate experiments, based on the Mann-Whitney U test. ns, no significant difference. Adapted from Paper IV.

In summary, data from this study confirmed our hypothesis that the administration of human-PNP-derived MPs could improve hemostasis in HA mice *in vivo*, however, this improvement is not as potent as the standard treatment with rhFVIII. Moreover, the injected MPs were partly incorporated into the thrombus formed at the site of the vessel injury and partly endocytosed by the liver and lung of mice rapidly. Future study to investigate the underlying mechanism that MPs participate in the thrombus formation *in vivo* in HA mice is of interested.

Clinical perspectives of Paper II and IV

In Paper II and IV of this thesis, I showed that human MPs (mainly platelet-derived) improved hemostasis both *in vitro* (in human plasma models of HA) and *in vivo* (in HA mice, in saphenous vein bleeding model and cremaster muscle thrombus formation model). I showed that MPs are incorporated into fibrin fibers and fibrin clots both *in vitro* and *in vivo*. The significance of my work for clinical treatment of PWHA is that it has generated several new hypotheses which can be tested in the future:

1. A natural increment of MPs might be an additional bleeding modifier in some severe PWHA, and could transform the bleeding phenotype from a severe to a non-severe type, as seen in the 10–15% of “mild bleeders”, regardless of the FVIII concentration. MPs may be used as a potential biomarker to predict bleeding phenotype severity in PWHA.
2. Theoretically, MPs could provide a potential adjunctive therapy for PWHA. The use of autologous MPs may shift the bleeding phenotype from severe to non-severe, which may decrease the consumption of FVIII concentrates, and thereby the cost of FVIII replacement therapy.
3. In PWHA who have increased levels of procoagulant MPs in circulation, the FVIII replacement therapy might need to be adjusted to reduce the potential risk of thrombosis, especially in middle-aged or elderly PWHA, who may have an increased risk of developing CVD.

4.3 PREVALENCE OF CVD AND THE ROLE OF MPS IN PWHA (PAPERS III AND V)

The wide availability of effective and safe FVIII concentrates has improved the life expectancy of PWHA significantly[67]. A UK study of 6018 patients (n=4874 HA and n=1144 HB) reported that the median survivals for patients with the severe and non-severe disease were 63 and 75 years, respectively[67]. In middle-aged and elderly PWHA, the incidence of age-related illnesses, e.g. CVD, has become a growing concern[55]. Although PWHA seems to have a lower cardiovascular mortality[67,68], they have at least an equal prevalence of CVD risk factors and a similar degree of atherosclerosis as the general control[136]. In Paper III of this thesis, we conducted a retrospective study of the prevalence of subclinical CVD in asymptomatic Swedish PWHA (n=29, ages 37-91 years) using the A-ECG technique. In Paper V, we conducted a cross-sectional study and investigated the prevalence of subclinical CVD, global hemostasis, and MPs profiles of Chinese PWHA treated on-demand (n= 42, ages 30-70 years).

4.3.1 Subclinical CVD in PWHA evaluated by A-ECG (Paper III)

We hypothesized that the CVD risk factors and subclinical CVD might be common in aging PWHA. ECG files of the participants were analyzed retrospectively using the A-ECG technique in a cohort of n=29 Swedish patients.

Our data showed that hypertension was modestly more prevalent in PWHA compared to age-matched male controls (41% vs. 21%, p=0.09). This result is consistent with the findings of a Sweden nation-wide study of hemophilic patients and reported that the prevalence of hypertension was 19.7% vs. 11.2%, in patients vs. controls[137]. The frequency of hypertension in our patient cohort was much higher compared to that of the national-wide study, which is probably due to our specific patient inclusion criteria (only those who had digital ECG file in the electronic medical record system could be included in our study), and these might tend to be older.

Subclinical CVD risks detected by A-ECG scores

Conventional resting ECG provides important information to diagnose several types of CVD, e.g. acute coronary syndromes. In the past two decades, several advanced (A-ECG) techniques, have been developed to detect CVD (e.g. CAD, LVH, LVSD) with higher sensitivity[129]. Using previously validated A-ECG scores, we found that

PWHA had a higher probability of CVD, particularly CAD/CMVD, compared to age-matched male controls (**Figure 4.11**). The predicted risks of LVSH and LVH/LVER were also higher in PWHA vs. controls (**Figure 4.11**), although risks for these diseases were much lower than that of CAD/CMVD.

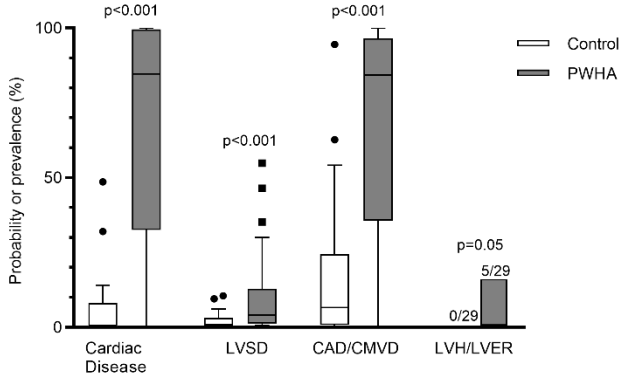

Figure 4.11 Subclinical CVD in PWHA and controls evaluated using A-ECG. Validated A-ECG scores were used to determine the probability of having CVD, LVSD, and CAD/CMVD, as well as the prevalence of LVH/LVER. Reproduced from Paper III with permission.

These results suggest that subclinical CVD is common in asymptomatic PWHA, who have access to adequate replacement therapy. Therefore, it is important to monitor the development of CVD in PWHA ≥40 years old. Furthermore, this study indicates that the A-ECG technique may be useful in detecting early CVD changes in PWHA.

4.3.2 Subclinical CVD and the role of MPs in PWHA treated on-demand (Paper V)

PWHA have lower cardiovascular mortality due to their hypocoagulable state[69]. However, this protective effect might be reduced by regular prophylaxis with FVIII concentrates. To better understand the protective effect of FVIII deficiency on the development of CVD, we investigated the prevalence of CVD risk factors and subclinical CVD in Chinese PWHA treated on-demand (n=42, ages 30-70 years). We also evaluated in these patients their MPs profiles by flow cytometry and correlated these with their risk of CVD.

We hypothesized that PWHA treated on-demand might have a reduced risk of CVD (cardiac disease) compared to normal individuals due to their hypocoagulation status, and this is correlated with their MPs profiles.

Clinical characteristics of PWHA treated on-demand As expected, PWHA treated on-demand, especially those with severe HA, had frequent spontaneous bleeding and insufficient treatment, which led to a relatively high prevalence of hemophilic arthropathy (**Table 4.1**).

We found that the prevalence of hypertension in this group of PWHA tended to be higher compared to the age-matched male control group (19% vs. 14.8%, p= 0.651). The other CVD risk factors were also relatively common in PWHA treated on-demand.

Table 4.1. Clinical characteristics of PWHA treated on-demand. Adapted from Paper V.

Patient characteristics, NO. (%)	**Total Patients (n=42)**	**Severe (n=24, 57.1%)**	**Moderate-mild (n=18, 42.9%)**
Age (years, mean ± SD)	42.3 ± 10.7	38.8 ± 8.6	46.9 ± 11.6
Frequency of spontaneous bleeding			
No	7 (16.7%)	0	7 (38.9%)
1-3 times/year	9 (21.4%)	4 (16.7%)	5 (27.8%)
3-10 times/year	6 (14.3%)	4 (16.7%)	2 (11.1%)
>10 times/year	20 (47.6%)	16 (66.7%)	4 (22.2%)
Frequency of the replacement therapy			
No	2 (4.8%)	0	2 (11.1%)
1-3 times/year	9 (21.4%)	3 (12.5%)	6 (33.3%)
3-10 times/year	8 (19%)	7 (29.2%)	1 (5.6%)
>10 times/year	23 (54.8%)	14 (58.3%)	9 (50.0%)
chronic arthropathy with limited joint movement			
No	6 (14.3%)	1 (4.2%)	5 (27.8%)
Yes	36 (85.7%)	23 (95.8%)	13 (72.2%)

Subclinical CVD risks in PWHA treated on-demand

Our data showed that PWHA had a similar probability of developing CVD as the age-matched male controls (median [interquartile range] 27.04 [4.87-92.61]% vs. 22.89 [1.63-43.06]%, p=0.204, **Figure 4.12**), which did not confirm our hypothesis. Compared to the patients with non-severe HA, patients with severe HA trended towards a lower predicted CVD risk (20.58 [3.58-86.16]% vs. 38.80 [5.92-96.58]%, p=0.294, **Figure 4.12**). This result suggests that PWHA treated on-demand are not protected from developing subclinical CVD due to their hypocoagulation status caused by chronically low levels of FVIII.

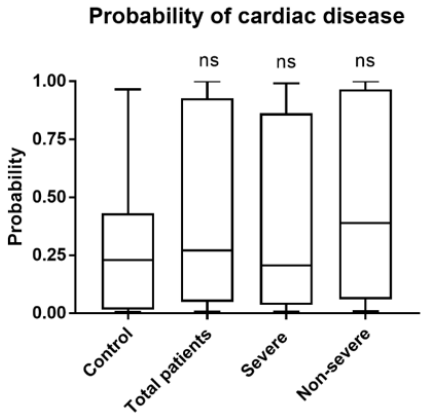

Figure 4.12 Probability of having CVD as evaluated using the A-ECG technique in PWHA and controls. Controls, n=21; and patients with HA, n=37 (21 have severe and 16 have non-severe HA). ns, no significant difference compared to the control group. Adapted from Paper V.

In the Chinese study, the predicted CVD risk of PWHA treated only on-demand was much lower than that in the Swedish study of PWHA treated prophylactically (as described in the Results of Paper III). This might due to the difference in the age of patients included in the two studies, since the Chinese cohort is younger than the Swedish cohort, their CVD risks are expected to be lower. The difference between the A-ECG scores used in the two studies should also be noted. In both studies, the A-ECG scores involved both conventional ECG parameters, as well as A-ECG parameters (i.e. the derived vector cardiographic ECG parameters and QRS and T waveform complexity measures). However, in the Chinese study (Paper V), A-ECG parameters for the beat-to-beat variability of the R-to-R and QT intervals were also included in the A-ECG analysis, because a 5-min ECG file was collected from each participant.

Swedish PWHA have access to adequate treatment, and prophylaxis is the standard treatment for patients with FVIII levels below 1-2%[137]. We assume that patients with prophylaxis may have higher risks of developing CVD compared to those with on-demand therapy. To test this hypothesis, a further study designed to compare the prevalence of subclinical CVD in age-matched patients with different treatment regimens (prophylaxis vs. on-demand) would be of interest.

MPs profiles in Chinese PWHA treated on-demand vs. normal controls

PWHA had significantly higher levels of PMPs (656.5 [472.0 - 826.8] vs. 494.0 [310.0 – 823.5], p=0.047) and CD62+ MPs (136.5 [117.8 – 164.8 vs. 107.0 [93.0 – 128.5],

p<0.001] compared to the normal controls **(Figure 4.13**). This result suggests a higher level of platelet activation in the PWHA compared to controls. One earlier study reported increase CD62P exposure in platelets of PWHA at baseline and suggested that platelets are pre-activated in PWHA[28]. The level of baseline activated platelets, however, did not correlate with the clinical bleeding severity[29]. No differences were found in the levels of different types of MPs among patients with severe vs. non-severe HA **(Figure 4.13**). Interestingly, higher counts of CD62P+ MPs were correlated with reduced capacity for fibrin clot formation, suggesting that impaired coagulation may be the reason for the increased platelet activation in PWHA.

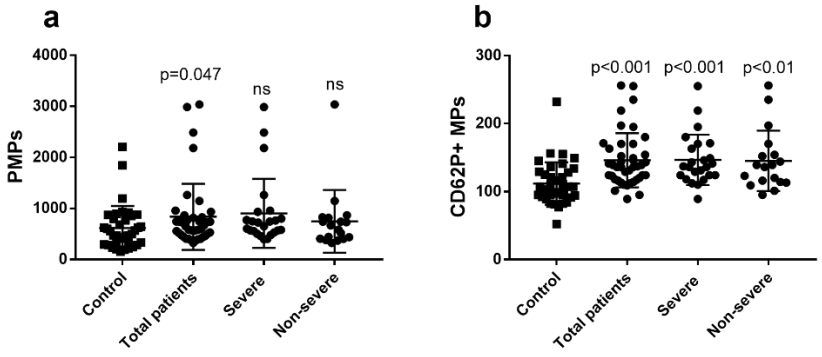

Figure 4.13 MPs profiles in PWHA and age-matched male controls. The graphs show the level of PMPs and P-selectin (CD62P+) MPs as measured by flow cytometry in the controls (n=37) and PWHA (n=42, with 24 have severe and 18 have non-severe HA). ns, no significant difference as compared to the control group. Adapted from Paper V.

Correlations between MPs levels and CVD risks

Several studies have reported elevated MPs, particularly EMPs in patients with CVD[138]. EMPs are released from endothelial cells upon activation and apoptosis. EMPs serve as a biomarker for endothelial dysfunction, which could lead to the development of CVD[113]. In the current study, we did not find any correlation between the MPs profiles (level of different types of MPs) and the risk of subclinical CVD, as evaluated by flow cytometry and A-ECG (**Table 4.2**).

Increased endothelial cell dysfunction has been reported in PWHA in other clinical studies, including increased levels of soluble intercellular adhesion molecule-1 and interleukin-6 in serum[139], and impaired flow-mediated dilation measured by ultrasound[64]. These results suggest that other molecules in PWHA might be more sensitive biomarkers for the detection of impaired endothelial cell function compared to the level of EMPs.

Table 4.2 Analysis showing no significant correlations between CVD risk, and MPs profiles in the Chinese study PWHA treated on-demand. Adapted from Paper V.

Subtype of MPs	The probability of having cardiac disease, p (r)
PS+ MPs	0.503 (0.090)
PMP+	0.742 (0.044)
EMP+	0.473 (0.096)
LMP+	0.599 (0.071)
TF+ MPs	0.693 (-0.053)
CD62P+	0.280 (0.144)

r, Spearman's correlation coefficient.

In summary, our results show that PWHA (≥30 years old) treated on-demand are not protected from developing subclinical CVD, thus our hypothesis that PWHA treated on-demand have lower CVD risk is not confirmed by this study. Moreover, no correlations between CVD risks and MPs profiles were found. Interestingly, the increased levels of PMPs and CD62+ MPs correlated with the impaired fibrin formation in PWHA, suggesting that the hypocoagulable state in PWHA might result in increased platelet activation.

Clinical perspectives of Paper III and V

In Paper III and V of this thesis, I showed that subclinical CVD risks are at least equally common (Paper V) or even more prevalent (Paper III) among PWHA ≥30 years old as compared to age-matched male controls. Therefore, the presence of CVD risk factors, as well as subclinical CVD, should be monitored in those patients. A-ECG as a rapid, convenient, and accurate method, could be used to detect CVD in PWHA, even when no overt clinical indications of CVD are present.

Although we did not find any correlation between the MPs profiles and CVD risks predicted by A-ECG, we assume the level of MPs might be a potential biomarker for the risk of atherothrombosis in aging PWHA. Elevated levels of PMPs and CD62+ MPs were found in PWHA. Based on our *in vitro* and *in vivo* results in Paper II and IV, we hypothesize that these subtypes of MPs may participate in the thrombus formation at the site of injured endothelial cells, by mimicking the role of platelets in hemostasis and thrombosis.

Since middle-aged and elderly PWHA generally have few physical activities compared to younger patients, and their CVD risks, especially risks of hypertension, are increased, adjustment for the FVIII replacement therapy (decrease dose and/or frequency) might be beneficial. Further studies to investigate MPs as a biomarker to justify the adjustment of the treatment in older PWHA is of interest.

5 CONCLUSIONS

The key findings of the studies included in this thesis can be summarized as below:

1. Paper I:

SIA in combination with high concentrations of aPCC induces hypercoagulable changes in fibrin clot formation and structure in *in vitro* HA plasma model, suggesting that this combination might be prothrombotic when used in the clinics to treat PWHA.

2. Paper II and IV:

MPs partially restore hemostasis in HA and are incorporated into the fibrin network of HA, both *in vitro* and *in vivo*.

3. Paper III and V:

In Paper III, we show that in the Swedish PWHA ≥40 years old, A-ECG exhibits increased risk of subclinical CVD compared to the age-matched male controls.

In Paper V, we demonstrate that the Chinese PWHA treated on-demand ≥30 years old are not protected from developing subclinical CVD, and the hypercoagulable state might contribute to increased platelet activation in these patients.

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