Innovation and standardization of near-patient platelet function assays

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Innovation and Standardization of Near-patient Platelet Function Assays

Yaqiu Sang

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Innovation and Standardization of Near-patient Platelet Function Assays

Dissertation

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in accordance with the decision of the Board of Deans,
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Chapter 1

General introduction and outline

General introduction

1. Haemostasis and thrombosis

Haemostasis is a complex interplay between at least four major activating and inhibitory pathways: (1) blood vessels, (2) platelets, (3) coagulation factors, coagulation inhibitors and (4) fibrinolysis [1]. Vascular injury results in exposure of blood to the subendothelial matrix, which triggers haemostasis to stop bleeding via several steps: 1) platelets adhere to the subendothelium via von Willebrand factor (VWF) and collagen, followed by platelet activation and aggregation to form a plug; 2) coagulation, triggered by subendothelial TF, proceeds to form thrombin, which cleaves fibrinogen to fibrin and consolidates the platelet thrombus by fibrin mesh; 3) fibrinolytic system serves to break down the fibrin clot via plasmin generation, leading to fibrin fragments that are subsequently cleared from the circulation. Complex and highly organized interactions between these pathways are key in regulating haemostasis. In the following paragraphs, the role of platelets, coagulation and the interactions between them in haemostasis and their association with haemostatic disorders will be discussed concisely, while more detailed background information can be found in **chapter 4** (Review: Interplay between platelets and coagulation).

Platelets, coagulation and the interactions between them

In healthy blood vessels, nitric oxide (NO) and prostacyclin (PGI₂) released by endothelium inhibit platelet adhesion and aggregation to the intact endothelium and also lead to smooth muscle relaxation [1, 2]. Once the integrity of the blood vessel is perturbed, for instance by mechanical trauma, the platelet membrane glycoprotein (GP) Ib-V-IX complex will interact with VWF, which comes from the plasma or the extracellular matrix (ECM). Then platelets swiftly sense and respond to the exposed collagen via GPVI and $\alpha 2\beta 1$, fibronectin via $\alpha 1\text{Ib}\beta 3$, $\alpha \text{v}\beta 3$, $\alpha 5\beta 1$ and laminin via $\alpha 6\beta 1$ [3, 4]. Meanwhile, locally generated soluble agonists, e.g., thromboxane A₂ (TxA₂) and adenosine diphosphate (ADP) secreted by activated platelets and thrombin generated via coagulation, distribute unevenly around the injury site. These agonists will synergistically activate platelets to different extent via the G protein-coupled receptors (GPCRs, i.e., Protease-activated- receptor (PAR)-1, PAR4, P2X1, P2Y1, P2Y12, thromboxane receptor (TP) and prostacyclin receptor (IP), etc.) [4]. Platelet secretion not only contributes to further platelet activation, but also enriches coagulation related factors (Factor (F) V, FIX, FXIII, protein S, antithrombin III (AT III), etc.) [5]. Upon platelet adhesion, activation and aggregation, the platelet plug is formed to stop the primary blood loss. However, this plug is loose and vulnerable to disperse (especially at high flow rate) without

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the fibrin mesh, which forms via coagulation and helps to cover and tighten the platelet thrombus.

Fibrin is formed by thrombin catalyzed cleavage of fibrinogen during the coagulation process. Coagulation is triggered in parallel with platelet activation at site of injury. Minute amounts of FVIIa circulates in blood (100 pM) in a zymogen-like conformation [6, 7]. At physiological concentration, FVIIa without TF shows little activity. Once circulating zymogen-like FVIIa binds to its membrane-bound cofactor TF, catalytic activity of FVIIa will be greatly enhanced. In addition, more FVII will be activated via FVIIa autoactivation [6]. Upon formation of the FVIIa-TF complex, activation of FIX and FX (extrinsic pathway, initiation phase) is initiated. In the absence of the cofactor FVa, FXa can produce trace amounts of thrombin. This initial minute amount of thrombin can activate platelets, FV, FVIII, FXI, FXIII and catalyze fibrinogen to fibrinopeptide A and B. (amplification phase) [1, 8, 9]. FXIa further activates FIXa that forms intrinsic tenase complex with FVIIIa. The FIXa-FVIIIa-FX complex accelerates FX activation, leading to the formation of prothrombinase complex (FXa-FVa). A larger quantity of thrombin is formed by FXa-FVa-prothrombin complex on a negatively charged surface (propagation phase) provided by activated platelets. Under certain conditions (artificial or biologic surface), the intrinsic pathway is triggered via the FXIIa activation. FXIIa activates FXI to FXIa at the presence of high-molecular weight kininogen (HMWK). Extrinsic pathway and intrinsic pathway merge at the FX activation process, the downstream cascade (propagation phase) is also known as common pathway. When enough thrombin is generated, fibrin polymerization proceeds on the platelet plug and contributes to clot retraction, leading to a densely packed thrombus [10].

Healthy endothelial cells are protected against excessive platelet activation by local release of NO and PGI₂, while secondary platelet activation is inhibited by the hydrolysis of nucleosides (including ADP) by the ectonucleotidase CD39 on the endothelial cell surface. Furthermore, endothelial cells are covered with heparan sulfate, which support the function of antithrombin III (ATIII) to inhibit FXa, FXIa, FIXa and FXIIa. Moreover, endothelial cells express protein C receptor and thrombomodulin (TM). TM-bound thrombin can activate protein C to activated protein C (APC), which inhibit FVa and FVIIIa. Coagulation is also regulated by tissue factor pathway inhibitor (TFPI), which inhibits TF-FVIIa complex and FXa. TFPI is mainly present on the endothelial surface (in the microvasculature), with a smaller amount (~10%) being found in platelets and circulating in plasma [11-15]. However, plasma TFPI is bound to lipoprotein and has undergone variable amounts of proteolytic degradation within its C-terminal region, resulting in reduced anticoagulant activity [14]. The release of endothelial TFPI can be enhanced by infusion of heparin, leading to 2-4 fold increase of circulating TFPI level [16, 17].

Platelets and coagulation communicate with each other during the entire coagulation process. In healthy vessels, coagulation factors share (or compete for) membrane receptors (or binding sites) on resting platelets. Once platelets are activated, platelets will provide more binding sites with higher affinity to the activated coagulation factors than to the non-activated coagulation factors [18]. More details can be found in **Chapter 4**.

Effect of blood rheology on platelet-coagulation interaction

Blood flow rates are important modifiers of the platelet-coagulation interaction *in vivo*. Due to laminar flow in vessels (maximal flow velocity in the center part of the vessel, while maximal shear rate at the vessel wall) [19], red blood cells migrate towards the axis of vessel, pushing platelets and other plasma proteins towards the vessel wall [20]. Shear rate is inversely correlated with the diameter of the vessels [21, 22]. Under normal physiological flow conditions, wall shear rate increases from about 10 s^{-1} in veins to about 2000 s^{-1} in the smallest arteries, where maximal wall shear rate can rise up to $40,000 \text{ s}^{-1}$ in severe atherosclerotic arteries [19, 23-25]. The increase in shear rate both activates platelets and promotes their transport to the growing thrombus [26, 27], resulting in platelet-rich arterial thrombi. At arterial flow conditions, platelet deposition is the major contributor of the thrombus formation through GPIIb-V-IX interaction with VWF as well as the contribution of GPVI and P2Y₁₂ [28]. While the generation of thrombin and fibrin decrease at higher shear rates, as a consequence of thrombin dilution by blood flow [29, 30]. Coagulation plays a major role at low shear rate, as described by Virchow's triad [31], which allows the coagulation proteins to concentrate and react to form fibrin clot. At stenotic sites, shear rates increase during thrombus growth. While the small perturbations in the flow around a thrombus can create low flow regions that protect coagulation factors from dilution [32] and therefore the thrombin generation and fibrin formation proceed on the platelet thrombus.

Thrombin formed at the surface of the thrombus activates platelets via interaction with PAR receptors [29, 33]. However, thrombin-mediated PAR activation is dependent on the blood flow rate as the role of PARs in activating platelets diminish at pathologically high shear rates [34]. Circulating TF plays a critical role in promoting thrombus growth [35, 36], and the incorporation of TF into thrombi is shear dependent [37, 38]. Exposing platelets to increased levels of shear stress has been shown to induce procoagulant activity on the platelet surface [39]. To investigate the effect of blood flow on platelet activation, coagulation and the interaction between them, microfluidic devices have been developed recent years and reviewed elsewhere [40].

Bleeding disorders

The genetic molecular mechanisms of many inherited platelet disorders (IPDs) and inherited coagulation factor deficiencies have been identified with modern recombinant DNA techniques, mouse genetic models and high-throughput sequencing (HTS) [41-45].

Congenital platelet disorders can be divided into thrombocytopenia and thrombocytopathy [46]. Inherited thrombocytopenia is mostly an accompanied feature of a generalized bone marrow failure or metabolic diseases causing marrow infiltration, which is extremely rare. Furthermore, some thrombocytopenia may coexist with thrombocytopathy [46]. Non-inherited thrombocytopenia can be caused by drugs and chemical agents, isoimmune (placental transfer of platelet antibodies from mother to fetus), infiltration of bone marrow, infection and other causes (maternal autoimmune thrombocytopenia, tumor, disseminated intravascular coagulation (DIC)) [46, 47]. According to platelet function at different stage of haemostasis, abnormalities in thrombocytopathy include [48]: 1) platelet receptors for adhesive proteins; 2) platelet receptors for soluble agonists; 3) platelet granules; 4) signal transduction pathway; 5) procoagulant phospholipid; 6) miscellaneous disorders (primary secretion defects, Montreal platelet syndrome (MPS), etc.).

Among patients with IPDs, some present with severe bleeding phenotype, i.e. abnormalities of platelet adhesive receptors (Bernard-Soulier syndrome (BSS) and Glanzmann thrombasthenia (GT)), abnormalities of granule deficiency (Wiskott-Aldrich syndrome, Familial platelet disorder with predisposition to AML (FPD/AML), Quebec platelet disorder) and abnormalities of membrane phospholipids (Scott syndrome). Of note, these IPDs are closely linked to impaired platelet-based coagulation. Besides VWF, GPIb-IX-X complex binds multiple coagulation factors (thrombin, HMWK, FXIIa, APC, FXI and fibrin) directly or indirectly. $\alpha IIb\beta 3$ provides binding sites for fibrin, fibrinogen, VWF, prothrombin and FXIII(a). Platelet granules release rich coagulation factors and the binding receptors for them. More importantly, coagulation complexes, which bound to exposed platelet PS, accelerate thrombin generation and are prevented from the inhibition by anticoagulation factors.

Inherited coagulation factors defects include the common inherited bleeding disorders (hemophilia A, B and von Willebrand disease (VWD)) and the rare inherited coagulation factors deficiencies. Rare coagulation factor deficiency, also known as rare bleeding disorders (RBDs) includes the inherited deficiencies of coagulation factors fibrinogen, prothrombin, FV, combined FV and FVIII, FVII, FX, FXI, FXIII and of multiple deficiency of vitamin-K-dependent factors [44, 49]. The clinical features can be varying for the same coagulation factor deficiency based on the heterogeneous level of coagulation factors.

Platelets can supplement coagulation factors and contributes to improve the clinical features of some coagulation factor disorders (VWD3 and FV deficiency (Owren parahemophilia)).

Thrombotic disorders

According to the localization, thrombosis is referred to as venous or arterial thrombosis. The most clinically significant arterial thrombosis involves: (1) thrombosis in the coronary arteries of the heart, which may result in myocardial infarction (MI); (2) thrombosis in arteries in the brain, which might lead to cerebral ischemia and stroke; (3) thrombosis in the peripheral arteries, contributing to peripheral arterial occlusive disease (PAOD). Arterial thrombi usually form around ruptured atherosclerotic plaques. Clinically significant venous thrombosis includes: (1) thrombosis in peripheral veins – deep vein thrombosis (DVT), and (2) thrombosis in the pulmonary arteries – pulmonary embolism (PE), caused by the translocation of peripheral thrombi [50].

The heritable thrombophilia – deficiency of antithrombin, Protein C, Protein S and the mutations in FV (FV_{Leiden}) and prothrombin gene – is associated with at least twofold increased risk of venous thrombosis [51]. Increased FVII, FVIII, FIX, FXI levels are also linked to enhanced risk of venous thrombosis. Some patients with the abnormal production of fibrinogen (dysfibrinogenemia) have a higher risk of thrombosis [52]. Homocysteinemia has been reported to be an independent risk factor for both arterial and venous thrombosis [52]. Increased platelet activation and hyperreactivity result in the enhanced interaction of platelets with endothelial, platelet-derived inflammatory mediators and thrombo-inflammation, which contribute to atherothrombosis and to a lesser extent, venous thrombosis [53]. Updated risk factors for arterial or venous thrombosis have been summarized elsewhere [54, 55].

The traditional opinion about arterial and venous thrombosis is that arterial thrombi (defined as “white”) is mainly composed of platelet aggregates, whereas venous thrombi (defined as “red”) is enriched in fibrin and erythrocytes [56]. Hence, antiplatelet drugs are usually used for prevention and treatment of arterial thrombosis, and anticoagulant drugs are used to treat mostly venous thrombosis, but also for prevention of AF related ischemic stroke. Antithrombotic drugs are summarized in **Table 1**.

Although the percentage of platelets is relatively low in the venous thrombi compared to arterial thrombi, they still exist. Moreover, experimentally induced venous thrombosis showed early platelet accumulation at the “head” of the thrombus. As the thrombus grows,

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platelet recruitment slows down and the clots become “red” [57]. Clots formed with acquisition of platelets are more compact and more resistant to fibrinolysis [58, 59]. The antiplatelet drug, aspirin, can enlarge the clot pore and make it less compact and more vulnerable to fibrinolysis [50]. Indeed, increasing evidence indicates that aspirin can lower the risk of recurrent VTE, although not so effective as warfarin or direct oral anticoagulants (DOACs) [60-62].

Table 1. Antithrombotic agents [63-69].

Category	Subcategory	Name	Route	Indication	Metabolism
Anticoagulants	Direct thrombin inhibitors (DTI)	Argatroban	IV	Thrombosis in patients with HIT undergoing PCI	Hepatic
		Bivalirudin	IV	Patients with angina undergoing PCI, with HIT	Renal
		Dabigatran	Oral	Stroke Prophylaxis with AF, DVT or PE treatment or prophylaxis,	Renal
		Desirudin	SC	Prophylaxis of DVT for patients undergoing hip replacement surgery	Renal
		Hirudin	IV, SC	Treatment of DVT and for PCI	Renal
	Direct FXa inhibitors	Apixaban	Oral	Stroke prophylaxis with AF, postoperative prophylaxis DCT/PE, DVT or PE treatment	Hepatic
		Betrixaban	Oral	VTE prevention	Hepatic and renal
		Edoxaban	Oral	Stroke prophylaxis with AF, DVT or PE treatment	Hepatic and renal
		Rivaroxaban	Oral	DVT prophylaxis for hip or knee replacement surgery, nonvalvular AF, DVT or PE treatment, reduction of risk of major cardiovascular events	Hepatic and renal
	Indirect thrombin/FXa inhibitors	Fondaparinux	SC	Prevention of DVT in patients who have had orthopedic surgery, treatment of DVT and PE	Renal
		LMWH	IV, SC	Treatment of VTE or MI	Renal
		UFH	IV, SC	ACS (e.g., MI), AF, DVT and PE, CPB for heart surgery, ECMO circuit for extracorporeal life support, etc.	Hepatic
	Vitamin K antagonists (VKA)	Warfarin	Oral, IV	Treatment or prevention of DVT or PE, prevent stroke in people who have AF, valvular heart disease or artificial heart valves	Hepatic
	Cyclooxygenase (COX) inhibitors (NSAIDs)	Aspirin (ASA)	Oral	CAD, cerebrovascular disease, PAD, stents, CABG, CEA	Hepatic
Antiplatelets	Irreversible ADP-receptor (P2Y12) antagonists	Clopidogrel	Oral	Prior MI, ischemic stroke, or symptomatic PAD as monotherapy; ACS or coronary stenting, as part of dual antiplatelet therapy with aspirin	Hepatic
	Reversible ADP-receptor (P2Y12) antagonists	Prasugrel	Oral	Patients with ACS treated with stents, as part of dual antiplatelet therapy with aspirin	Hepatic
		Ticlopidine	Oral	Cerebrovascular disease, coronary stents (now rarely used)	Hepatic
		Cangrelor	IV	Adjunct to PCI to reduce the risk of periprocedural MI, repeat coronary revascularization and ST in patients who have not been yet treated by oral P2Y12 inhibitors	Not requiring metabolic conversion Not requiring metabolic conversion
		Elinogrel	Oral, IV	Treatment in cardiovascular disorders and MI	
		Ticagrelor	Oral	Patients with ACS, as part of dual antiplatelet therapy with aspirin	Hepatic
	PAR-1 inhibitors	Vorapaxar	Oral	Reduce thrombotic cardiovascular events in patients with a history of MI or with PAD	Hepatic
		Atopaxar	Oral	Treatment of CAD and ACS	Hepatic
		PZ-128	IV	Prevention of acute thrombotic complications of PCI	Hepatic
	α IIb β 3 inhibitors	Abciximab	IV	Prevention of cardiac ischemic complications in patients undergoing PCI, or with unstable angina with PCI, adjunctive therapy during thrombolysis	Hepatic
		Eptifibatide	IV	Decrease the rate of thrombotic cardiovascular events in patients with ACS, including patients undergoing PCI	Renal
		Tirofiban	IV	Decrease the rate of thrombotic cardiovascular events in patients with ACS, including patients undergoing PCI	Renal
	Phosphodiesterase inhibitors	Cilostazol	Oral	PAD	Hepatic
		Dipyridamol	Oral	Combine with aspirin for the secondary prevention of stroke or TIA	Hepatic

Category	Subcategory	Name	Route	Indication	Metabolism
Thrombolytic	Plasminogen activators	Reteplase	IV	Treatment of AMI	Hepatic and renal
		Streptokinase	IV, IC	For lysis of emboli in AMI, PE, DVT	Renal
		Tenecteplase	IV	Treatment of AMI and lysis of IC emboli	Hepatic
		Alteplase (t-PA)	IV, IC	Treatment of acute STE-MI, PE associated low blood pressure, AIS and blocked CVAD	Hepatic

ACS, acute coronary syndrome; AF, atrial fibrillation; AIS, acute ischemic stroke; AMI, acute myocardial infarction; ASA, acetylsalicylic acid; CABG, coronary artery bypass graft; CAD, coronary artery disease; CEA, carotid endarterectomy; CPB, cardiopulmonary bypass; CVAD, central venous access devices; DVT, deep venous thrombosis; HIT, heparin-induced thrombocytopenia; IC, intra coronary; IV, intravenous; LMWH, low molecular weight heparin; MI, Myocardial infarction; NSAIDs, nonsteroidal anti-inflammatory drugs; NSTEMI, non-ST elevation; PAD, peripheral arterial disease; PCI, percutaneous coronary interventions; PE, pulmonary embolism; SC, subcutaneous; ST, stent thrombosis; STE, ST elevation; TIA, transient ischemic attack; t-PA, tissue plasminogen activator; UFH, unfractionated heparin; VTE, venous thromboembolism

2. Platelet function test and clinical application

Platelet function analysis is an important attributor for identifying patients with a bleeding diathesis (e.g. platelet dysfunction) and in some cases for hyperreactivity, e.g. in case of unexplained cases of arterial thrombosis in young subjects. In addition, tests can be used to assess the degree of platelet suppression in monitoring of antiplatelet therapy [70]. The extended indication for use of platelet function tests has led to the development of new, simpler tests and point-of-care instruments designed for monitoring of antiplatelet therapy. However, the more traditional and complex tests for diagnosis of platelet disorders are still performed at specialized haemostasis laboratories [71, 72].

Platelet function testing began with the application of the *in vivo* bleeding time by Duke in 1910 [73]. Although it is still used on occasion, the bleeding time has failed to become a clinically reliable haemostasis test. Simple and robust platelet function assays, which are ideally suitable for routine clinical applications, are still limited after one century. Up until the late 1980s, the only clinical platelet function tests that were available were bleeding time, light transmission aggregometry (LTA) and various biochemical assays [74]. Even so, most of these tests were mainly performed within specialized research and clinical laboratories. Since flow cytometric analysis of platelet function was introduced in the 1980s, it has become an important tool for functional and phenotypic characterization of platelets. Similarly, flow cytometry-based tests are restricted to specialized laboratories.

Flow cytometry analysis of platelet function - versatile tool but unmet challenge

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Flow cytometry is capable of detecting multiple specific characteristics on cells in suspension, and is widely used for the functional and phenotypic characterization of platelets: determination of platelet activation (both activation state and reactivity of circulating platelet), diagnosis of specific disorders, monitoring of antiplatelet agents, monitoring of thrombopoiesis, blood bank application, platelet counting, etc. [75, 76]. Compared to other platelet function assays, whole blood flow cytometric platelet activation analysis uses much less sample volume (5-20 μ l), is closer to the physiological milieu, and limits artifactual *in vitro* activation by minimal manipulation before analysis.

Whole blood flow cytometry studies have demonstrated circulating activated platelets and increased leukocyte-platelet aggregates in patients with arterial or venous thrombosis [77-91]. Platelet activation-dependent biomarkers determined by flow cytometry may be useful for determination of optimal antiplatelet therapy in clinical settings e.g., in ACS patients [92, 93], or after coronary stenting [94, 95]. Platelet function tests measured by flow cytometry, independent of platelet count, are associated with bleeding severity in immune thrombocytopenia (ITP) [96]. Whole blood flow cytometric measurement of platelet hyperreactivity, circulating activated platelets and/or circulating leukocyte-platelet aggregates, may have a clinical role [75] in numerous clinical disorders, such as diabetes mellitus [97], metabolic syndrome [98], atrial fibrillation [99], sickle cell disease [100], etc. Whole blood flow cytometry may also be useful in evaluating platelet hypo-reactivity in premature neonates [101], patients with thrombocythemia [102], active myeloma [103] or as prognostic markers of haemorrhage in patients with acute myeloid leukemia [104]. Platelet surface P-selectin is a useful measure of quality control during sample processing, storage, and manipulation [105-107].

Flow cytometric analysis provides a rapid and simple means for diagnosis of the homozygous and heterozygous states of Bernard-Soulier syndrome (BSS) [108] or Glanzmann Thrombasthenia (GT) [109], Scott syndrome [110], inherited or acquired storage pool disease [111, 112] and heparin-induced thrombocytopenia (HIT) [113, 114] as well as the differentiation of platelet-type von Willebrand disease (VWD) from type 2B VWD [115]. Flow cytometric analysis of platelet function has been increasingly used for clinical trials and recommended by SSC of ISTH in diagnostic work-up of inherited platelet function disorders (IPFDs) [116]. According to the survey among more than 200 laboratories by ISTH, flow cytometer is available in 57.3% of the respondent laboratories (clinical, research or both) [117]. However, the poor standardization of laboratory tests used for IPFDs diagnosis and the large heterogeneity in diagnostic approaches represent serious limitation to the identification of IPFDs and treatment. A significant proportion of diagnosis failure among patients with IPFDs may result from the limitation of available methodologies [118, 119].

Currently there is no consensus or standardized approach to the diagnosis of IPFD and many laboratory techniques used for assessment are insufficiently standardized, technically challenging and poorly reproducible [116].

Despite the multiple utilities of flow cytometry in platelet function evaluation, the complicated operating procedures and the specific instrument needed make it less popular in the routine diagnosis of platelet function disorders. Moreover, current flow cytometric analysis of platelet function is poorly standardized, which makes the comparison of results between laboratories difficult. Although flow cytometric determination of platelet activation markers may be performed at any clinical site where samples are generated, in practice only a small number of specialized laboratories have the equipment and expertise to perform these tests [120]. Samples are often prepared (activation and fixation) at clinical site and then transferred to the central core laboratories for analysis. In some studies, clinical sites were supplied with pre-aliquoted kits (agonists/fluorophore conjugated monoclonal antibody cocktail) [92, 121-123]. At the experimental day, a small amount of diluted whole blood was added into the ready-to-use kit and eventually fixation solution was added after a certain reaction time. Technical variation in blood collection methods, delays in sample transport and processing and heterogeneity in subject features may confound the measurements and hamper the accurate comparisons between study groups and across centers. To minimize site-to-site, lot-to-lot, time-to-time variations of flow cytometric assay within or between laboratories, measures should be taken to standardize reagents, core instrumentation, operating procedures, sample processing time, etc. Obtaining reliable and reproducible data in multicenter clinical research requires design of optimal standard operating procedures.

Specific platelet GPVI receptor agonist

In the past decades, along with the development of various *in vivo* and *in vitro* techniques and knockout mice model, increasing evidence has shown that platelet immunoglobulin (Ig) superfamily GPVI is crucial for the formation of thrombosis *in vivo* [124, 125]. New antiplatelet drug, revacept (a soluble dimeric GPVI-Fc fusion protein), has been developed and is being assessed for the therapeutic efficacy in clinical trials [126, 127]. Therefore, not only can GPVI-specific agonist be used for investigating specific platelet activation pathway, but also helpful to monitoring of antiplatelet therapy.

Recent years fibrin has been found an interesting ligand of GPVI [128, 129], historically, collagen is considered as the major physiological GPVI ligand. Of all the types of collagen in the vasculature, only type I and III collagen are able to form large fibrils and bind GPVI with

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a high affinity [130, 131]. The binding site on collagen is figured out to be the short glycine-proline-hydroxyproline (GPO)_n tandem repeats, leading to the development of synthetic GPVI ligand – cross-linked collagen related peptide (CRP-XL) [132-134]. The synthetic CRP-XL allows evaluation of platelet activation response via GPVI pathway alone.

Collagen is considered the major GPVI ligands and used as a standard in LTA. However, collagen cannot be used in flow cytometry, because collagen fibers can form a surface for platelet aggregation, which obstructs the individual cell measurements in flow cytometry. Several GPVI agonists has been used, such as, convulxin and CRP-XL. Nevertheless, convulxin is unstable during its preanalytical storage[135]. Despite CRP-XL is stable and currently the first choice to quantify the GPVI-mediated platelet activation pathway in flow cytometry [134, 136], there is only one noncommercial laboratory that can produce CRP-XL [137]. Shortage of supply might occur if CRP-XL is used as a standard for GPVI-dependent platelet function test. Therefore, other GPVI-specific agonists should be developed as an alternative. Another problem with CRP-XL is that the randomized cross-linking in the final production procedure introduces inter-batch variation. New strategy should be adopted to solve this problem.

Clinical tests of platelet function

A summary of commonly used platelet function tests is listed in **Table 2** together with their clinical application. Application of platelet function test in clinical trials may: (1) suggest biological mechanisms contributing to clinical outcomes, (2) allow estimation of risk of specific clinical outcomes (e.g., severe bleeding or recurrence of thrombosis), (3) identify possible targets for intervention and (4) assess the efficacy of specific intervention (e.g., antiplatelet therapy) [120]. The decision about which test(s) to use in the clinical trials is primarily based on the research question in the study and greatly influenced by study logistics. The utilization of platelet function test is also limited by the availability of specialized equipment. For example, flow cytometry and liquid chromatography-mass spectrometry (LC-MS, for quantitation of platelet releasate) are only available in specialized laboratories which are remote from patients and sample collection site. Of note, the instability of platelets to varying environment indicates that many platelet tests must be performed within minutes to hours after blood collection at, or close to, the site of blood collection. The arrival of near-patient instruments such as PFA-100 and VerifyNow® system expanded the application of platelet function test within the limited time period [120]. Platelet function tests used for clinical trials should be less complex and more robust than tests used in research laboratories. The development of point-of-care instruments which can be used uniformly contributes to the consistency of platelet function data. Point-of-care

platelet function techniques can be applied not only to the diagnosis of platelet dysfunction, but also to monitoring of anti-platelet therapy [138] and to assess risk of bleeding or thrombosis [139, 140].

Table 2. Commonly used platelet function test and related clinical application [70, 72, 120, 141].

Test name	Principle	Sample (WB volume required)	Advantages	Disadvantages	Application
Bleeding time	<i>In vivo</i> cessation of blood flow	Native WB	<i>In vivo</i> test, physiological point-of-care	Insensitive, invasive, scarring, high coefficient of variation	Screening test (obsolete)
Flow cytometry	Detection of suspending fluorescent label platelets in a flowing solution	Citrate WB, PRP, washed platelets (30 µl)	Whole blood test, small blood volumes, wide variety of tests	Specialized operator, expensive, samples prone to artifact unless carefully prepared	Diagnosis of platelet glycoprotein defects, detection of platelet activation <i>in vivo</i> or <i>in response</i> to agonists, monitoring antiplatelet therapy
Global thrombosis test (GTT)	Measurement of time cessation of WB flow by high shear-dependent platelet plug formation	Native WB (4 ml)	WB assay, global platelet method	Lack of clinical studies, not widely available	Evaluation of platelet function and thrombolysis
Impact-Cone and platelet analyzer	Shear-induced platelet adhesion-aggregation upon specific surface	Citrate WB (150 µl)	WB assay, global platelet method, small sample volume	Expensive, experienced staff, lack of clinical studies, not widely available	Screening of primary hemostasis
Impedance platelet aggregation (WBA)	Measurement of electrical impedance increase in relation to agonist-induced platelet aggregation	Citrate WB (300 µl)	No sample manipulation, smaller sample volumes, sensitive to antiplatelet therapy, dose to POCT	Insensitive, variation higher than LTA, requires technical expertise	Screening test for bleeding tendency; Diagnosis of acquired and inherited platelet defects; Monitoring antiplatelet treatment effect
Light transmission platelet aggregation (LTA)	Optical measurement of light transmission increase in relation to agonist-induced platelet aggregation under low shear	Citrate PRP (3.5 ml)	Gold standard, flexible, different platelet pathway investigated, sensitive to anti-platelet therapy	Time-consuming, sample preparation, poorly standardized	Screening test for bleeding tendency; Diagnosis of acquired and inherited platelet defects; Monitoring antiplatelet treatment effect
LTA: 96-well plate	As above but in 96-well plate	Citrate PRP (1.3 ml)	Lower blood/PRP volumes than LTA. Many replicates and dose-response curves possible	Little widespread experience	Detection of defects in primary hemostasis
Lumiaggregometry	Combine WBA or LTA with nucleotide release	Citrate WB (500 µl)	Monitoring release reaction with secondary aggregation	Semiquantitative	Diagnosis of acquired and inherited platelet defects, diagnosis of storage and release defects
Microfluidic devices (e.g., T-TAS [®])	Miniaturized multichannel devices	Citrate WB (350 µl)	WB test, real-time thrombus formation, changeable shear	Little widespread, experience	Research only
PFA-100; Innovance PFA-200 [®]	High-shear platelet adhesion and aggregation during formation of a platelet plug	Citrate WB (800 µl)	WB test, high shear, small blood volumes, simple, rapid, POCT	Inflexible, platelet count and HCT-dependent, not sensitive to platelet secretion defects.	Detection of inherited and acquired defects in primary hemostasis, monitoring aspirin, monitoring DDAVP therapy
Platletworks	Platelet counting pre- and postactivation in whole blood	Citrate WB (1 ml)	POCT, minimal sample preparation, eas, rapid screening test	Indirect assay, required adjunctive platelet count, limited data	Monitoring of platelet response to antiplatelet agents
Platelet nucleotide assays	Measurement of total and released nucleotides by luminescence or HPIC	Citrate PRP or washed platelets (200 µl)	Sensitive, accurate and precise	Experienced operators, sample preparation, assay calibration, extra equipment	Diagnosis of storage and release defects
Radio-or enzyme-linked immune assays	Measurement of TxA2 metabolites (and B-TG, PFA, soluble P-selectin)	Serum, urine or citrated plasma (3 ml)	Dependent upon platelet COX-1 activity, relatively simple	Prono to artifact during blood collection and processing	Monitoring aspirin therapy, detection of thromboxane, production defects, detection of <i>in vivo</i> platelet activation
VerifyNow [®]	Fully automated platelet aggregometer to measure antiplatelet therapy	Citrate WB (2 ml)	Simple, point-of-care, three test cartridges (aspirin, P2Y12 and GPIIb/IIIa)	Inflexible, cartridges can only be used for single purpose	Monitoring of antiplatelet therapy
Viscoelastic assays (TEG [®] or ROTEM [®])	Monitoring of rate and quality of dot formation	Citrate WB (300-400 µl)	Global WB test, POCT	Measures clot properties only, largely platelet-independent unless platelet activators are used, relatively low throughput and expensive	Assessment of global hemostasis, prediction of surgical bleeding, aid to blood product usage, monitor antiplatelet therapy

β -TG, β -thromboglobulin; HCT, hematocrit; HPLC, high-performance liquid chromatography; PF4, platelet factor 4; POCT, point-of-care test; PRP, platelet rich plasma; T-TAS, Total thrombus-formation analysis system; TxA2, thromboxane A2; WB, whole blood; WBA, whole blood aggregometry

3. Coagulation test and clinical application

Unlike platelet function tests which need to be performed quickly after blood collection, coagulation tests can be performed not only in fresh blood or platelet rich plasma (PRP), but also in platelet poor plasma (PPP) that can be stored in -80 °C for further study. Citrated blood is usually collected for coagulation tests. Calcium is necessary for the assembly of coagulation factor complexes and is added along with other coagulation triggers to the sample before the coagulation assay is performed.

Abnormalities of coagulation can be caused by inherited deficiencies of coagulation factors (described in section 1) or acquired coagulation abnormalities (e.g., acquired inhibitory autoantibodies to coagulation factors, liver disease, vitamin K deficiency or disseminated intravascular coagulation (DIC)). Specific coagulation factor disturbance causes the decrease of single factor, whereas liver disease or vitamin K deficiency results in shortage of multiple factors.

Clinical tests of coagulation

Routine coagulation tests include the prothrombin time (PT), activated partial thromboplastin time (aPTT), thrombin time (TT, also called thrombin clotting time (TCT)) and Clauss fibrinogen level. These clot-based assays use fibrin clot formation as the end point. Usually, these screening tests are used for evaluation of patients with suspected bleeding diathesis or to monitor anticoagulant therapy [142]. The deficiency of HMWK, prekallikrein, FXII, FXI, FIX or FVIII will result in the prolongation of aPTT, while the deficiency of FX, FV or prothrombin prolongs both aPTT and PT; deficiency of FVII only prolongs PT, and fibrinogen deficiency results in the prolongation of aPTT, PT and TT. Anticoagulant therapy with therapeutic UFH increases the aPTT and TT, and to some extent the PT. Administration of LMWH prolongs the TT and to some extent aPTT (relying on reagent sensitivity). Generally, aPTT and TT are sensitive to direct thrombin inhibitors (hirudin, bivalirudin, dabigatran, etc.), whereas PT is relatively sensitive to direct FXa inhibitors. Warfarin therapy or vitamin K deficiency mainly increase the PT and to some extent the aPTT. Liver dysfunction and DIC usually prolong the PT, TT and to some extent the aPTT [142, 143]. Modified screening tests can help to distinguish factor deficiencies from autoimmune inhibitors. Although these routine tests are widely used for screening coagulation defects,

they only provide limited information and sometimes are not sensitive enough to therapeutic anticoagulant monitoring [144]. Accordingly, specific/dedicated coagulation tests are required for diagnosis of certain specific abnormality and monitoring anticoagulant therapy.

Other than the routine test, a number of clot-based coagulation assays have been developed for monitoring anticoagulant therapy [144]: (1) Activated clotting time (ACT) for high-dose heparin therapy in patients on cardio-pulmonary bypass, on extracorporeal membrane oxygenation (ECMO), undergoing percutaneous transluminal coronary angioplasty (PTCA), or on hemofiltration or hemodialysis; (2) Ecarin clotting time (ECT) for direct thrombin inhibitors (DTI, i.e., Argatroban and Dabigatran); (3) Prothrombinase-induced clotting time (PiCT) for heparins, DTI and direct FXa inhibitors; (4) Diluted thrombin time (dTT) for DTI; (5) Heptest for heparins, DTI and direct FXa inhibitors.

Another coagulation assay that is commonly used includes the chromogenic assay which evaluates the hydrolysis of chromogenic substrate by activated coagulation factors. Compared to the clot-based assay, the chromogenic assay is less affected by other factors and therefore often used to determine the accurate level of target coagulation factors. The plasma chromogenic anti-Xa assay is widely used for monitoring patients on LMWH, UFH and direct FXa inhibitors.

Generally, routine coagulation tests only assess certain aspect of haemostasis using non-physiological activators and only give an endpoint evaluation, e.g., clot formation. These tests are not capable of predicting the risk of bleeding or thrombosis. Coagulation is a complex process that depends on the interactions of many cellular and plasma components. Therefore, global tests which measure the overall haemostatic profile have gained increasing interest and have been used to assess bleeding and thrombosis risk, as well as to guide blood product replacement in trauma and massive transfusion setting [145, 146]. Major global coagulation tests include [145, 147]: (1) Thrombin generation (TG) assay, which measures the amount of thrombin generated over time and may reflect an individual's coagulation potential; (2) thromboelastography (TE, Thromboelastography® (TEG®) or Rotational Thromboelastometry® (ROTEM®)), a point-of-care test that assesses the viscoelastic properties of whole blood under low shear conditions and reflects *in vivo* haemostasis; (3) overall haemostatic potential assay (OHP), a global assay of fibrin generation and fibrinolysis.

The primary clinical application of TEG®/ ROTEM® is to predict the surgical bleeding risk and guide the use of blood products. The turnover time for TEG®/ROTEM® is 15-20 min which

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enables real-time analysis of the viscoelastic properties of clot formation and dissolution in whole blood [148]. During the last decades, TEG®/ ROTEM® has been shown to be suitable for detecting and treating coagulopathy in trauma, cardiac surgery and liver transplantation, providing an important tool in guiding hemostatic therapy at bedside [149-152]. Recent evidence also indicates that the whole blood viscoelastic tests may have some use in the assessment of the risk of thromboembolic events [153]. However, TEG®/ROTEM® does not fully reflect the effects of LMWH or DOACs, nor the effects of inherited or drug-induced platelet dysfunction [149].

Thrombin generation assay (TGA)

Compared to the viscoelastic tests which assess the fibrin clot formation in general, the TGA gives more information on the overall haemostatic capacity [154]. TGA evaluates the overall balance between procoagulant and anticoagulant factors in PPP, PRP or WB. TG in PPP, also called calibrated thrombin generation (CAT) can be used to evaluate the coagulation process via different pathway (intrinsic or extrinsic) by changing the trigger (TF) concentration. TGA in PRP or WB can be used to determine the role of platelets in coagulation and the interactions between coagulation and platelet activation. Recent years, some WB-TGA have been developed but the clinical utility of WB-TG still awaits [155-157]. A number of potential clinical applications of TGA have been investigated, such as identifying hemorrhagic disease (hemophilia A and B, other factor deficiencies) [158-160], predicting venous or arterial thrombosis [161-163] and bleeding risk [164, 165], and guiding antithrombotic therapy [166, 167]. Although global haemostatic tests can provide more detailed information than routine tests, the lack of reference value and standardization, the specific instruments and skills needed for the global tests still limit their clinical application.

Urgent need of near-patient test for monitoring anticoagulation therapy

Tens of millions of patients worldwide are on short- or long-term anticoagulant therapy. A variety of fast-acting and extended prophylaxis agents are available, all of which may be associated with risk of thrombosis or bleeding. Each anticoagulant varies in its effect on routine and specific coagulation assays [143]. It is thus imperative that clinicians have the appropriate assay(s) to monitor therapy, determine drug concentration and further evaluate the risk of thrombophilia or bleeding in patients.

Since approved by European Medicines Agency (EMA) and Food and Drug Administration (FDA), DOACs have been increasingly used for prevention of VTE and stroke in non-valvular AF (NVAF) or recurrent atherothrombotic events [168]. Although it is indicated that there is

no need to monitoring of DOAC levels routinely because of the predictable bioavailability, pharmacokinetic and pharmacodynamic profiles, assessment of the anticoagulant effect of these drugs may be required under certain circumstances [169]. Currently, there is no simple near-patient test for rapid evaluation of DOACs effects in emergent situations. Some POCTs, such as ACT and TEG®/ROTEM®, have become available for use in the emergency department. A few studies have evaluated the ability of these POCTs to determine the effects of DOACs. Results showed that TEG®/ROTEM® can detect dabigatran-induced coagulopathy, while their accuracy is limited for apixaban and rivaroxaban in the cases of low drug plasma concentrations[170-175]. Additionally, the requirement of specific equipment and technician make the current POCTs only available in specific laboratories.

4. Point-of-care test (POCT) for platelet-coagulation interactions

Most of the available haemostatic assays either use PPP to study the coagulation process or use anticoagulant blood to investigate the platelet's function. Haemostasis assays using PRP or WB, get one step closer to the physiological situation and take the roles of platelets, coagulation and their interactions into consideration. Among the widely known global tests, only TEG®/ROTEM® is available for near-patient application in the clinical scenarios. As for the point of care platelet function tests [176], such as WBA, PFA-100, PlateletWorks and VerifyNow®, exclude the role of the coagulation.

POC WB-TG has been suggested with broader clinical benefit [177]. Recently a novel POC TG test has been developed and validated in some small clinical trials, and its implementation in clinical settings seems to be promising [178, 179]. Although POC TG can be finished within half an hour, the operating procedure is relatively complex and operators need to be well-trained to get reproducible results. A simple and reliable POC test is still in need for assessing the complete haemostatic profile of patients at bedside.

With the introduction of new anticoagulation drugs, new clinical challenges occur as well. Simple POCT and portable POC devices are in urgent need. Such POC system need to be sufficiently sensitive to detect relatively low levels of DOACs, for instance, to rule out remaining anticoagulant in patients prior to major surgery. In urgent situations, such as intra cranial bleeding (where one may want to give expensive antidote) or ischemic stroke (thrombolysis is considered), POCT result-based fast decision-making can avoid the medical complications due to delay of acute intervention.

Bridging platelet function assay and coagulation assay

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Thrombin, the central factor of coagulation process, activates human platelets via PAR1 and PAR4. TGA is the only test for monitoring the thrombin generation kinetics. The development of POC WB-TG largely shortens the measurement time for TG kinetics curves (till the peak thrombin is reached) to less than 10 minutes [178, 180]. Current POC WB-TG tests use fluorogenic thrombin substrates which occupy the active center of thrombin and influence the coagulation process [181, 182]. Moreover, as the small substrates are unable to differentiate free thrombin and α_2 M-thrombin, a second measurement (calibration curve) is required to calibrate the fluorescence signal [182].

It is well known that platelet activation can be triggered by trace amount of thrombin formed in the initiation of coagulation [8]. Platelet activation kinetics processes, such as aggregation and secretion, are usually investigated by LTA, WBA or lumi-aggregometry. Normally, typical platelet aggregation or secretion curve can be finished within 5 minutes [183]. The major platelet activation process precedes before bulk thrombin formation. Therefore, it will be ideal to monitor the coagulation-induced platelet activation kinetics, that not only integrates the platelet activation and coagulation process but also saves more time.

Luminometric assay of platelet activation in clotting blood has been reported almost two decades ago and hinted for quantifying coagulation and studying anticoagulants [184]. This luminometric assay has quite high sensitivity and hence has the potential to be adopted in microplate formats for large-scale screening study. In the present study, we focus on a more near-patient application of the luminometric assay, such as in the emergency room (ER). Meanwhile, we would like this assay to be as simple as possible so that it can be operated by clinical lab technicians after simple training.

Bioluminescent assay for assessing platelet secretion

Inherited platelet secretion disorders (IPSDs) are a group of common and heterogeneous platelet function disorders characterized by defective content or release of α -granules, dense (δ)-granules or both, during platelet activation [185]. Platelet granule release is regulated by multiple pathway: granule synthesis and multi-step secretion pathway (agonists interactions with platelet receptors, intracellular signaling, cytoskeletal remodeling, fusing of granule with platelet membrane). Any abnormality in the pathway may lead to defective platelet secretion. Platelet secretion defects constitute the majority of PFDs and its prevalence may be higher than VWD [186, 187]. However, normal results given by LTA, the currently gold standard test for PFD diagnosis, cannot exclude the platelet secretion abnormality [188].

Different methodologies have been described to measure the content and release of platelet granules [185], the most frequently used assay of δ -granule secretion is assessment of platelet nucleotides. Among the assays measuring nucleotides, lumi-aggregometry is the simplest and most prevalent one [72]. The release of platelet δ -granule contents can be monitored as part of WBA or LTA assay [189, 190] by the bioluminescence assay. This bioluminescence assay is performed by adding a luciferin-luciferase reagent along with an agonist to the sample, while stirring the sample at low shear, to promote platelet activation and aggregation. Upon activation, normal platelets release their δ -granule contents: ATP, ADP, serotonin, pyrophosphate, Ca^{2+} and Mg^{2+} . ATP released from platelets reacts with luciferin-luciferase reagents, resulting in light (luminescence) emission that is quantified by lumi-aggregometer with an ATP standard [190].

The bioluminescent assay was first introduced by co-evaluation with LTA since 1977 [191], however, after four decades, application of this test into clinical diagnostic practice remains inconsistent. The survey conducted by ISTH indicated that more than half of the responding laboratories did not evaluate platelet granule content or release, and only around 21% of the laboratories used lumi-aggregometry as the first screening tests [117]. An important reason might be the larger variability of dense granule release endpoints compared to aggregation, which limits their usefulness to detect impaired platelet function from a bleeding disorder [192-194]. For the commonly used lumi-aggregometry test, a certain concentration of standard ATP was added to quantify the amount of released ATP at the beginning or last step of assay [195-197]. Compared to LTA, this extra step might explain partly the larger variation mentioned above [198]. Moreover, protocols are far from standardization for ATP release analysis by lumi-aggregometry, though studies have indicated the need to modify the assay procedure [198]. In addition, evaluation of platelet secretion, by the current lumi-aggregometry assay, requires large amount of blood and cannot be used in thrombocytopenia samples.

Based on the same principle of lumi-aggregometry, we developed the bioluminescent analysis of platelet activation kinetics and evaluated platelet activation process via the kinetics curve of ATP release. Instead of measuring the endpoint – the amount of produced ATP, we focus on the whole ATP release kinetics curve. Not only can more information be delivered from the kinetic curve, but also the operating procedure is simplified by omitting the step of adding standard ATP, which prevents possible variability. Additionally, this new test is performed in recalcified blood so that coagulation process is involved as well.

This new bioluminescent platelet activation kinetics can be performed in any luminometer in which the kinetics measurement program is equipped. The luminometer used in our assay

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is quite small and affordable and can be implemented easily in various clinical settings. Furthermore, the amount of blood needed is quite low - 25 μ l per test, making the test also feasible for the pediatric patients. Because of the high sensitivity, this assay can also be used for determination of platelet activation in samples with low platelet count. With the preparation of ready-to-use kit (agonists/luciferin-luciferase cocktail) in advance, analysis can be initiated simply by one-step procedure. The activity of reagents is examined regularly to ensure the stability of reagents. Depending on different study aim, different triggers can be used to activate platelet. **Figure 1** shows the principle of whole blood platelet activation kinetics induced by different agonists.

Figure 1. Principle of whole blood platelet activation kinetics. Reaction is started by adding whole blood into the test tube, within which the agonist and luciferin-luciferase mixture are added in advance. After rapidly mixture, test tube is put in the luminometer for detection of the light signal. Platelet can be activated either directly by platelet agonists (thrombin, thrombin receptor activating peptide (TRAP), CRP, 2-Methylthioadenosine diphosphate (MeSADP, analog of ADP) and AA) or indirectly by coagulation triggers (TF and RVV-X) via thrombin generation. Upon activation, platelet secretion proceeds and ATP released from the δ -granule can be detected continuously by the luminometer.

Outline of this thesis

Platelets play versatile roles in haemostasis and thrombosis, however, there is inadequate tools for evaluating platelet function in routine clinical settings. This thesis describes the standardization and innovation of platelet function assays towards near-patient application.

Part I: Flow cytometric analysis of platelet function

Chapter 2 reports the standardization and optimization of the whole blood flow cytometric analysis of platelet function and the establishment of reference intervals in healthy population with the standardized assay. In **Chapter 3**, we describe the development of a new synthetic triple helical collagen peptide and its validation as a specific platelet GPVI receptor activator by comparing with the widely used CRP-XL in flow cytometry.

Part II: Platelet-coagulation interplay in point-of-care settings

In **Chapter 4**, we review the interplay between platelets and coagulation, their relationship with haemostasis abnormalities and indicate the importance of a global assay for studying the platelet-coagulation interplay in clinical settings. We adapt a bioluminescent assay to investigate the platelet activation kinetics in coagulant blood. When platelet agonists are used as triggers, **Chapter 5** shows the capability of this new assay to determine platelet secretion defects in multiple myeloma patients. When coagulation trigger is used, as depicted in **Chapter 6**, we apply this new assay to the rapid monitoring of LMWH efficacy in intensive care unit patients by investigating coagulation-induced platelet activation. Similarly, **Chapter 7** examines the fast detection of DOACs effects in spiked blood samples from healthy individuals via coagulation-induced platelet activation.

Finally, **Chapter 8-summary, general discussion and prospects** provides an overview of the most important findings of this thesis and imply future research directions.

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Part I

Flow cytometric analysis of platelet function

Chapter 2

Standardization and reference ranges for whole blood platelet function measurements using a flow cytometric platelet activation test

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Abstract

Introduction: Platelet function testing with flow cytometry has additional value to existing platelet function testing for diagnosing bleeding disorders, monitoring anti-platelet therapy, transfusion medicine and prediction of thrombosis. The major challenge is to use this technique as a diagnostic test. The aim of this study is to standardize preparation, optimization and validation of the test kit and to determine reference values in a population of 129 healthy individuals.

Methods: Platelet function tests with 3 agonists and antibodies against P-selectin, activated $\alpha\text{IIb}\beta\text{3}$ and glycoprotein Ib (GPIb), were prepared and stored at -20°C until used. Diluted whole blood was added and platelet activation was quantified by the density of activation markers, using flow cytometry. Anti-mouse Ig κ particles were included to validate stability of the test and to standardize results. Reference intervals were determined.

Results: Blood stored at room temperature (RT) for up to 4h after blood donation and preheated/ tested at 37°C resulted in stable results (%CV<10%), in contrast to measuring at RT. The intra-assay %CV was <5%. Incubation of anti-mouse Ig κ particles with antibodies stored for up to 12 months proved to give a stable fluorescence. The inter-individual variation measured in the 129 individuals varied between 23% and 37% for P-selectin expression and $\alpha\text{IIb}\beta\text{3}$ activation, respectively.

Conclusions: The current study contributes to the translation of flow cytometry-based platelet function testing from a scientific tool to a diagnostic test. Platelet function measurements, using prepared and stored platelet activation kits, are reproducible if executed at 37°C . The reference ranges can be validated in clinical laboratories and ongoing studies are investigating if reduced platelet reactivity in patients with bleeding complications can be detected.

Introduction

The assessment of platelet (dys)function is of crucial relevance in several clinical settings, including: monitoring the response to antiplatelet treatment, evaluation of perioperative haemostasis, transfusion medicine and identification of bleeding disorders [1].

The current gold standard of testing platelet function for the diagnosis of bleeding disorders is light transmission aggregometry (LTA) [2]. However, LTA is laborious, relatively insensitive to small changes in platelet function and the outcome is affected by haemolysis and an abnormal platelet count [3]. Additionally, the test is poorly standardized, consumes large blood volumes and requires skilled technicians in specialized laboratories [4, 5].

Platelet function testing by flow cytometry in whole blood was first described by Shattil et al. [6]. Under current guidelines, the use of flow cytometry is recommended as an integral component of the investigation of platelet function disorders [7, 8]. In mild bleeding disorders, flow cytometric platelet activation shows a good correlation with LTA [9]. In acute coronary syndromes and after coronary stenting, expression of platelet activation markers predicts major adverse cardiac events and, in patients with atrial fibrillation, increased expression is a risk factor for silent cerebral infarction [5, 10, 11].

Flow cytometric analysis of platelet function in whole blood is a technique that has many advantages. First of all, flow cytometry measures within limited time the specific characteristics of a large number (e.g. 5000 or 10,000) of individual platelets. Furthermore, platelets are analysed in their physiological milieu of whole blood, including erythrocytes and leukocytes, both of which affect platelet activation [12, 13], without any pretreatment of the blood. This allows for measurement of both the baseline activation state of circulating platelets and their reactivity in response to various agonists without the influence of artefactual in vitro activation by preconditioning of blood [6]. Also, this method permits the detection of two specific activation-dependent modifications in the platelet surface membrane. The amount of activated $\alpha\text{IIb}\beta\text{3}$ receptors and the expression of P-selectin on the cell membrane correspond to the aggregation potential and granule release capacity of platelets, respectively [14-16]. The test has been used for measuring platelet function in bleeding disorders, during surgery, in platelet concentrates, and in patients with thrombocytopenia [17, 18]. Because only minimal volumes (5 μl) of blood are required, the technique is also suitable for neonatal studies [19, 20]. Unfortunately, there are many different protocols circulating and flow cytometry detection of platelet function is still only applied in research settings, due to the absence of standardization between labs and instruments. In the past, the tests for the detection of platelet function by flow cytometry

required fresh preparation of reaction mixes. This was not only laborious but also detrimental for the reproducibility of the test. A simultaneous improvement of reproducibility and of the workload was achieved by preparing large batches of stable reaction mixes, that are aliquoted in small reaction tubes and stored in a freezer for several months. Another issue in flow cytometry-based platelet function testing is the temperature of the blood before and during the test. Currently, the majority of studies using this technique are performed at room temperature (RT) [21, 22], while it is known that RT by itself is a co-trigger of platelet activation [23, 24]. Finally, no consensus is reached on the optimal time frame post blood drawing in which the test should be performed to exclude ex vivo platelet activation. Some studies claim that blood samples should be processed within 30 minutes [6], while others advice to wait for a stable response, achieved 1 to 2 hours post blood collection [25].

The aims of this study are (1) to solve the discussion about the optimal temperature for measuring flow cytometric platelet function, (2) to standardize, optimize and validate the preparation of the test kits and (3) to determine reference values in a healthy population.

Materials and methods

Reagents

Platelet agonists used for measuring platelet function include the P2Y₁₂ agonists ADP (01897, Sigma-Aldrich, the Netherlands), the protease activated receptor (PAR)-1 agonist thrombin receptor activator peptide (TRAP-6 (SFLLRN), H-2936; Bachem, Germany) and the glycoprotein VI (GPVI) agonist collagen-related peptide (CRP, a generous gift by Professor Farndale, university of Cambridge, UK). Professor Farndale identified fragments of collagen, synthesized and assembled in active, triple-helical conformation and in this study, a single batch of cross-linked CRP (CRP-XL) was used. The antibodies used in this test were FITC-conjugated monoclonal antibody PAC1 directed against the activated α IIb β 3 receptor, PE-conjugated monoclonal antibody anti-P-selectin (CD62P, clone AK4) and APC-conjugated anti-CD42b (GPIb) monoclonal antibody (clone HIP1), all purchased from BD Pharmingen (NJ, USA). Anti-mouse Ig κ particles and negative control particles were also purchased from BD Pharmingen.

Preparation of the platelet activation tests

Optimal agonist concentrations were determined in 5 healthy donors of which the data are shown (S1 Fig). Test strips consisting of the four optimal test conditions (no agonist, 30 $\mu\text{mol/L}$ TRAP, 5 $\mu\text{g/ml}$ CRP and 30 $\mu\text{mol/L}$ ADP) were prepared in advance and stored at -20°C. Each reaction mixture with a total volume of 20 μl consists of 2 μl FITC-conjugated PAC1, 1.5 μl PE-conjugated anti-P-selectin and 0.5 μl APC-conjugated anti-CD42b with or without agonist in HEPES-buffered saline (HBS, 10 mmol/L HEPES, 150 mmol/L NaCl, 1 mmol/L MgSO_4 , 5 mmol/L KCL, pH 7.4).

Preparation of the reaction mixtures for the beads

In each platelet activation test, 3 additional conditions to detect binding of antibodies to anti-mouse Ig κ particles or negative control particles were included. The negative control condition (NC) contains 2 μl FITC-conjugated PAC1 and 1.5 μl PE-conjugated anti-P-selectin in HEPES-buffered saline (total volume of 20 μl). Two positive control (PC) conditions were prepared: PC1 contains 2 μl FITC-conjugated PAC1 and PC2 contains 1.5 μl PE-conjugated anti-P-selectin in HEPES-buffered saline (total volume of 20 μl).

Performing the platelet activation tests

Test strips were thawed and shortly centrifuged. All tests were performed at 37°C, unless stated otherwise. Whole blood was stored at RT for at least 30 min and maximum 4h, incubated at 37°C for 10 minutes and diluted 1:4 in pre-heated HEPES-buffered saline to minimize the formation of platelet aggregates. From this diluted blood, 5 μl was added to each reaction mixture (20 μl , final dilution 1:20) and the tests were incubated for exactly 20 minutes at 37°C. At the same time, 5 μl of negative control particles and 5 μl of anti-mouse Ig κ particles were added to the NC condition or to both PC1 and PC2, respectively.

Reactions were stopped by adding 250 μl fixation solution (137 mmol/L NaCl, 2.7 mmol/L KCl, 1.12 mmol/L NaH_2PO_4 , 1.15 mmol/L KH_2PO_4 , 10.2 mmol/L Na_2HPO_4 , 4 mmol/L EDTA, 0.5% formaldehyde). Upon fixation, platelet activation markers were stable from day 2 to day 8 (change in MFI <5%, S2 Fig), but most samples were analysed one day after the experiment (day 2).

Flow cytometry was used to discriminate platelets from other cells, using the forward and sideward scatter pattern and by gating on the CD42b positive cells. Fluorescent intensity in the FITC gate and PE gate was selected to determine activated $\alpha\text{IIb}\beta_3$ and P-selectin density, respectively, and results are expressed as median fluorescent intensity (MFI).

Study population

Our study protocol was evaluated by the local medical ethical board (Medical Ethical Committee of Maastricht University Medical Center). The study population consisted of 129 healthy adult individuals, aged 20-65 years. All participants did not take any oral anticoagulant or anti-platelet drugs for at least two weeks, did not have a history of thrombosis or bleeding and gave full written informed consent according to the Helsinki declaration. Experiments were conducted at the Synapse Research Institute in accordance with approved guidelines and regulations. Blood was collected aseptically by antecubital puncture via a 21-gauge needle (0.8x32 mm) into vacuum tubes (1 volume trisodium citrate 0.105M to 9 volumes blood) (BD Vacutainer System/Greiner). The blood was kept at RT and used within 4 hours from withdrawal. Cell counts in whole blood were performed with a Coulter Counter analyzer (Beckman Coulter, Woerden, The Netherlands).

Statistics

Beads were used to normalize the flow cytometric data with the following equation:

$$100 \times (\text{MFI}_a - \text{MFI}_c) / (\text{MFI}_{PC} - \text{MFI}_{NC})$$

Where MFI_a was the MFI of the activated platelets, MFI_c the MFI of the unstimulated platelets, MFI_{PC} the MFI measured on the positive control beads and MFI_{NC} the MFI of the negative control beads.

For the reference values, outlier analysis was performed using the D/R method (Dixon). One outlier was detected and excluded (P-selectin expression in response to ADP measured on the Accuri as well as the Canto). Normality was tested using the Shapiro Wilk test and except for $\alpha\text{IIb}\beta 3$ receptor activation in response to CRP, data were not normal distributed. Continuous variables were expressed as median and interquartile range (25%-75%). Also, mean and standard deviation (SD) were shown and inter-individual variability (%CV) was calculated for each variable as $100 \times (\text{SD}/\text{mean})$. Reference intervals were obtained using non-parametric calculation methods, in accordance with the recommendation in the latest CLSI guidelines ^[26]. More precisely, the lower reference interval limit was estimated as the 2.5th percentile, and the upper limit as the 97.5th percentile of the distribution. Groups were compared using the Mann-Whitney U test for independent samples.

Results

Optimisation of the platelet function test

Determination of the optimal temperature conditions

To study the effects of the storage temperature on platelet function, blood was drawn and stored at 37°C or RT. After 30 minutes, whole blood was diluted and added to reaction mixtures containing the agonists TRAP, CRP or ADP, or a control buffer. In the control condition without agonist, no $\alpha\text{IIb}\beta 3$ receptor activation or P-selectin expression was detectable, regardless of the storage temperature (S3 Fig). Incubation of whole blood with platelet agonists resulted in a strong increase in the amount of $\alpha\text{IIb}\beta 3$ receptor activation and in the expression of P-selectin. Activation of the $\alpha\text{IIb}\beta 3$ receptor in response to TRAP, CRP or ADP as well as P-selectin expression in response to ADP was significantly lower at 37°C compared to RT. On the other hand, the temperature did not significantly affect the P-selectin expression in response to TRAP or CRP.

When measuring at RT, standardization of the time from blood collection to the start of platelet function testing has been advised to reduce the variation in results [27]. We investigated this in more detail and blood was drawn from 3-5 donors and stored at 37°C or RT. At different time points (immediately ($t=0$) and after 10 min, 20 min, 30 min, 45 min, 60 min and 90 min), blood was diluted and added to the platelet function reaction mixtures. In clinical setting, constant incubation at 37°C is challenging because the collected blood needs to be transported to the laboratory. Therefore, we included a RT/37°C condition, in which the blood stored at RT (from 10 min up till 80 min) was incubated at 37°C for 10 min before the platelet activation test was initiated. Subsequently, reactions were incubated for 20 min at 37°C or at RT followed by fixation of the cells. The MFI for $\alpha\text{IIb}\beta 3$ receptor activation and P-selectin expression were recorded and the percentages compared to the 37°C condition measured 30 minutes after blood donation are shown in Fig 1. Pre-analytical storage of blood at RT showed a time-dependent increase in platelet reactivity. Blood samples that were stored pre-analytically for up to 20 min at RT after blood collection had a significant lower $\alpha\text{IIb}\beta 3$ receptor activation in response to TRAP, CRP or ADP than blood that was pre-analytically stored at RT for longer periods (Fig 1). The same pattern was observed for P-selectin expression if platelets were activated with ADP. In contrast to RT, pre-analytical storage of blood at 37°C hardly influenced platelet reactivity from 20 min up to 90 min after blood collection. Interestingly, the responses of platelets stored at RT but preheated for 10 min at 37°C before performing the platelet function test were comparable to those of platelets stored at 37°C immediately after blood collection (Fig 1). These findings indicate that blood should be preheated to 37°C, at least 10 min before platelet function analysis.

Fig 1. Effect of temperature on platelet function testing by flow cytometry. Immediately after blood donation, blood was stored at RT or at 37°C. At each time point (X-axis), platelet activation in response to TRAP (panel a), CRP (panel b) or ADP (panel c) was tested. For the 37°C condition, blood was immediately placed at 37° and platelet function tests were performed at 37°C. For the RT condition, blood was kept at RT and the tests were performed at RT. For the RT/37°C condition, blood was kept at RT and 10 min before performing the platelet function test, blood was placed at 37°C and tests were performed at 37°C. The median fluorescence intensity (MFI) of αIIbβ3 receptor activation (left panels) and P-selectin expression (right panels) was recorded and expressed as the percentage compared to the MFI of the 37°C condition measured after 30 min at 37°C. Data are presented as mean ± SD (n=3 to 5).

Additionally, the variability introduced by pre-analytical storage of the blood up to 4 hours at RT was tested in 4 donors (Table 1). Prior to the experiment, blood was stored for at least 30 minutes and maximally 4 hours at RT, followed by a 10 minutes incubation at 37°C.

Acceptable variability was found when platelets were activated with TRAP and CRP (coefficient of variation less than 10%; Table 1), while platelet activation by ADP showed more variability due to pre-analytical storage of blood. Given the enormous practical advantage of blood storage at RT without the need to standardize the time between blood collection and test initiation, we selected the RT/37°C condition for further experiments.

Table 1. Assay variation when blood is stored at RT up till 240 min followed by 10 min preincubation at 37°C prior to performing the platelet function test

		Variability 30 min - 240 min (%CV)				
		Donor 1	Donor 2	Donor 3	Donor 4	MEAN %CV
αIIbβ3 activation	TRAP	9.3	7.9	7.3	6.3	7.7
	CRP	3.1	6.4	2.5	7.2	4.8
	ADP	18.3	5.5	7.3	10.6	10.4
P-selectin expression	TRAP	5.8	6.1	10.1	5.5	6.9
	CRP	6.2	6.2	8.4	7	7
	ADP	24.4	7.9	24.2	16.8	18.3

Blood was stored for 30, 60, 120, 180 and 240 min before it was preincubated for 10 min at 37°C.

Stability of platelet function tests stored at -20°C

In this platelet function assay, reaction mixtures containing the complete package of conjugated antibodies and agonists, were stored at -20°C. To test the stability of the antibodies present in the platelet function tests, we included anti-mouse Ig κ particles, which bind the FITC conjugated PAC-1 as well as the PE conjugated anti-P-selectin antibodies in two separate conditions (PC1 and PC2). The beads, with approximately the same size as platelets, provided distinct positive stained populations which can also be used to determine the compensation levels of the flow cytometer. Tests consisting of two positive control solutions and one negative control, were stored for up to 12 months. Every month, anti-mouse Ig κ particles or negative control beads were added to four tests accordingly. Binding of the FITC-conjugated PAC-1 antibody as well as the PE-conjugated anti-P-selectin antibody was recorded as MFI measured on the PC beads minus MFI of the NC beads (Fig 2). After 6 months and 12 months a new batch of beads was used. The binding of the anti-P-selectin antibody to the PC beads resulted in a higher fluorescence than the binding of the PAC-1 antibody. Importantly, fluorescence measured was constant in time for both antibodies (slope of the linear regression line not significantly different from zero with P-values of 0.5 and 0.4 for PAC-1 and anti-P-selectin, respectively).

Fig 2. Determination of the stability of the antibodies stored at -20°C. Tests consisting of two positive control solutions (containing either the PAC-1 antibody (PC1) or the anti-P-selectin antibody (PC2)) and one negative control (containing both the PAC-1 and the anti-P-selectin antibodies), were stored at -20°C for up to 12 months. Every month, four tests of each condition were thawed. Anti-mouse Ig κ particles or negative control beads were added accordingly and incubated for 20 min at 37°C. Binding of the FITC-conjugated PAC-1 as well as the PE-conjugated anti-P-selectin was recorded as MFI measured on the PC beads minus MFI of the NC beads. After 6 months and 12 months a new batch of beads was used (indicated by the dashed lines). Data are presented as mean $\pm$ SD (n=4) and linear regression lines are shown. Every month, anti-mouse Ig κ particles or negative control beads were added to four tests accordingly.

Determination of the precision of the platelet activation test

To obtain intra-assay precision data of our test, we performed replicate experiments (n=10) in 4 healthy donors. For each donor, these experiments were carried out in parallel after preheating the blood for 10 minutes at 37°C, using the same batch of platelet function tests. Platelets were activated with TRAP, CRP and ADP and incubated with FITC-conjugated PAC-1 and PE-conjugated anti-P-selectin, and MFI were measured to determine α IIb β 3 receptor activation and P-selectin expression on the platelet membrane, respectively. At the same time, the binding of these antibodies to positive control beads was measured. For each donor, the mean values with the SD and %CV of the MFI are depicted in Table 2. For each condition, the mean intra-assay variation was found to be below 5% with the exception of the platelet activation in response to ADP.

Table 2. Intra assay variability of the platelet function test and beads

		Intra-assay variability								MEAN %CV
		Donor 1 (n=10)		Donor 2 (n=10)		Donor 3 (n=10)		Donor 4 (n=10)		
		Mean±SD	%CV	Mean±SD	%CV	Mean±SD	%CV	Mean±SD	%CV	
<i>αIIbβ3 activation</i>										
TRAP	1050±38	3.6	1738±71	4.1	972±51	5.2	1196±84	7	5	
CRP	8475±100	1.2	10384±568	5.5	7162±195	2.7	8297±374	4.5	3.5	
ADP	8077±984	12.2	6243±280	4.5	4351±144	3.3	3912±161	4.1	6	
<i>P-selectin expression</i>										
TRAP	8804±340	3.9	9621±232	2.4	7965±377	4.7	7603±363	4.8	3.9	
CRP	8995±227	2.5	10205±244	2.4	8246±382	4.6	8308±190	2.3	3	
ADP	5219±1560	29.9	1048±155	14.8	923±272	29.4	599±46	7.7	20.4	
<i>Positive control beads</i>										
PAC-1-FITC	18209±134	0.7	21047±573	2.7	19795±468	2.4	19584±331	1.7	1.9	
aCD62P-PE	39510±692	1.8	46287±1833	4	43513±732	1.7	40992±1361	3.3	2.7	

Due to the need of fresh blood, a true inter-assay CV determined on consecutive days could not be assessed.

Usage of beads to reduce the lot-to-lot variability and comparison of data obtained by different flow cytometers

In a next set of experiments, we investigated if normalisation of the results of the platelet activation test by results obtained on beads reduces variability between lots of tests and/or allows the comparison of data obtained by different flow cytometers.

The lot-to-lot variability was determined by measuring platelet activation in 4 donors using 5 different test lots per donor. The tests, prepared and stored at -20°C for 1, 4, 7, 8 or 9 months, consisted of a control condition without agonist, three conditions with agonists and three conditions for NC and PC beads (materials and methods). Data were normalized as described in materials and methods. For each donor, the lot-to-lot variability was determined by calculating the %CV of the raw data as well as the normalized data for each condition (mean %CVs are shown in Fig 3). Normalization did not affect the lot-to-lot variability for αIIbβ3 receptor activation, however, the lot-to-lot variability did increase for P-selectin when data were normalized (Fig 3). With the exception of P-selectin expression in response to ADP, the lot-to-lot variability for all conditions was below 15%.

Fig 3. Influence of normalization on the lot-to-lot variability of the platelet activity test. Five lots of platelet function tests were prepared and stored at -20°C for 1, 4, 7, 8 and 9 months. Whole blood of 4 donors was used to study the variability between the lots. For each condition MFI values were recorded and normalized using the fluorescence measured on the NC and PC beads. Coefficient of variations for both the raw data and normalized data are shown with their corresponding SD.

Measuring the same sample on different flow cytometers often results in different absolute MFI values due to different lasers and fluorescent detectors in the systems. To investigate if normalization of the data allows the comparison of results obtained with different flow cytometers, blood was collected from 126 donors and the platelet function assay was performed. The tests were analysed on a BD Accuri and a BD FACS Canto, and for each donor, the MFI values as well as the normalized values of the two cytometers were compared. To create a clear general picture, values of the first 50 donors are shown in the S4 Fig. Mean MFI values measured on the FACS Canto/Accuri were 509/1105 AU, 2909/6398 AU and 1661/3733 AU for α IIb β 3 receptor activation and 5141/5102 AU, 5169/5206 AU and 421/323 AU for P-selectin expression in response to TRAP, CRP and ADP, respectively. Normalization of the MFI values resulted in mean normalized values measured on FACS Canto/Accuri (%CV) of 7.3/6.8 (7%), 41.9/39.4 (6%) and 23.9/22.9 (6%) for α IIb β 3 receptor activation and 18.0/12.6 (26%), 18.1/12.8 (25%) and 1.5/0.8 (48%) for P-selectin expression in response to TRAP, CRP and ADP, respectively.

Additionally, platelet function tests of 2 donors were analysed on an Accuri and a Navios flow cytometer of Beckman Coulter. Mean MFI values were measured on Navios/Accuri and were 3.9/739.5, 31.1/6039.3, 13.5/2543 for α IIb β 3 receptor activation and 22.2/8320.5, 22.6/8618.8 and 1.6/477.5 for P-selectin expression in response to TRAP, CRP and ADP, respectively. Mean normalized values measured on Navios/Accuri (%CV) of 3.9/3.7 (2%),

31.3/30.5 (3%) and 13.5/12.7 (4%) for $\alpha\text{IIb}\beta 3$ receptor activation and 13.2/16.3 (15%), 13.4/16.9 (17%) and 0.9/0.9 (6%) for P-selectin expression in response to TRAP, CRP and ADP, respectively.

Measuring platelet function in a healthy population

Determination of inter-individual variability and reference intervals of platelet function

Demographic data of 129 healthy individuals (63 females and 66 males, median 32.0 years) together with blood counts are summarized in table 3. Concerning the blood counts, the haemoglobin levels, haematocrit and red blood cell counts were significantly increased and platelet counts significantly reduced in male compared to female samples.

Table 3. Baseline data of 129 healthy individuals.

	All	Women (n=63)	Men (n=66)	P-values
Age, years	32 (27-44)	32 (27-44)	31 (26-44)	ns
Haemoglobin, mmol/L	9.0 (8.3-9.6)	8.36 (8.0-8.9)	9.5 (9.1-9.8)	<0.0001
Haematocrit, %	42 (39-44)	39 (38-42)	44 (42-45)	<0.0001
Red blood cell count, $\times 10^{12}/\text{L}$	4.9 (4.5-5.1)	4.6 (4.4-4.9)	5.0 (4.8-5.2)	<0.0001
Platelet count, $\times 10^9/\text{L}$	316 (273-370)	332 (289-391)	295 (264-359)	0.004
White blood cell count, $\times 10^9/\text{L}$	5.7 (5.0-6.7)	5.9 (5.1-7.0)	5.4 (4.8-6.5)	ns

Medians and interquartile ranges (25%-75%) are indicated.

The inter-individual variability (%CV) as well as reference intervals (2.5 percentile- 97.5 percentile) for P-selectin expression and $\alpha\text{IIb}\beta 3$ receptor activation in response to the agonists TRAP, CRP and ADP were determined in these 129 healthy volunteers (Table 4). Samples were measured on an Accuri flow cytometer and a FACSCanto. For CRP, two relatives with a previously unknown GPVI deficiency were excluded. Apart from the reference values, medians, interquartile ranges (IQRs), minimum and maximum values of each test parameter are indicated. Looking at the inter-individual variation, for TRAP and CRP the %CV for P-selectin expression and $\alpha\text{IIb}\beta 3$ receptor activation proved to be acceptable and in the range of 23 to 37%.

Chapter 2

Table 4. Inter-individual variation and reference intervals of the platelet function test

	N	Mean (% of beads)	Stdev	%CV	Median	IQR (25%-75%)	Reference Interval (2.5%-97.5%)	Min	Max
Accuri									
<i>αIIbβ3 activation</i>									
TRAP	129	6.8	2.5	36.5	6.5	5.1-8.2	2.3-12.8	2.2	13.2
CRP	127	40	9.8	24.4	40.7	34.0-47.3	18.1-60.6	10.7	65.4
ADP	129	22.8	8.7	38	22.6	16.9-27.0	7.7-43.8	4.6	45.4
<i>P-selectin expression</i>									
TRAP	129	12.7	4	31	12.5	9.5-14.9	6.6-22.4	5.4	26
CRP	127	13.1	4.1	31.3	12.5	9.9-15.4	7.2-23.8	6.4	26.2
ADP	128	0.8	0.7	86.4	0.6	0.2-1.1	0.09-2.5	0.1	3.7
Canto									
<i>αIIbβ3 activation</i>									
TRAP	126	7.3	2.6	35.9	7.1	5.4-9.0	2.7-13.8	2.4	14.5
CRP	124	42.4	9.7	22.9	41.9	36.3-48.7	20.9-63.1	11	68.8
ADP	126	23.9	8.8	36.8	23.3	17.3-28.4	8.5-45.0	5.9	46.6
<i>P-selectin expression</i>									
TRAP	126	18	4.5	24.7	17.8	14.4-20.5	10.8-29.4	10	34.1
CRP	124	18.3	4.4	24.3	17.6	14.9-20.8	11.7-28.1	9	34.7
ADP	125	1.4	1.1	81.3	1.1	0.6-1.8	0.3-5.0	0.1	5.4

Effect of sex and age on platelet activation

The effect of sex on platelet activation was investigated for all agonists. P-selectin expression and αIIbβ3 receptor activation in response to ADP were significantly higher in females compared to males (S5 Fig). The same trend was observed for the agonist TRAP and CRP, although the differences did not reach statistical significance.

Looking at the effect of age of the healthy volunteers on platelet activation, weak but significant correlations were found for αIIbβ3 receptor activation in response to TRAP and ADP (Spearman correlation coefficient $r=0.19$ ($p=0.03$), $r=0.22$ ($p=0.01$), respectively) and for P-selectin expression in response to ADP ($r=0.18$ ($p=0.04$)).

Discussion

This study contributes to the development of an *in vitro* diagnostic test that measures platelet activation by flow cytometry. We investigated the effect of pre-analytical temperature and storage conditions on platelet function and showed that blood can be

stored at RT after blood collection if the blood is preheated at 37°C, 10 min before platelet function is measured at 37°C. Results obtained by three types of flow cytometers (BD Accuri, BD FACS Canto, Beckman Navios) were found to be not comparable, unless the tests are calibrated by calibrator beads. Another important qualification of diagnostic tests is the reproducibility, which is acceptable for our test as shown by the low %CV (with the exception of P-selectin expression in response to ADP). Finally, we have determined platelet function in 129 healthy volunteers to obtain the normal ranges, inter-subject variation and to study the effects of age and sex on platelet function.

Our goal was to develop a user-friendly, reliable and quick platelet function test using three platelet agonists (TRAP, CRP and ADP) and two platelet activation markers (α IIb β 3 receptor activation and P-selectin expression). This reveals a limitation of our study because adding additional agonists (PAR4-selective agonist, arachidonic acid, ristocetin) and activation markers (CD40L, CD63, annexin-V, mepacrine) would result in a more detailed picture on platelet activation, however, this was beyond the scope of this study.

Currently, important discrepancies exist in the published flow cytometric platelet function protocols. Despite the evidence of spontaneous platelet activation at RT, most tests are performed at RT and no consensus is reached on the ideal time interval between blood collection and initiation of the test [21, 22, 25, 27-30]. As shown by our results, platelet function at RT is affected by storage time between blood collection and analysis, while pre-analytical storage of blood at 37°C will not affect platelet function over time, if the time frame is from 20 min up to 4 hours after blood collection. Interestingly, the effect of low temperature on platelet function was reversible, as platelets stored at RT and subsequently incubated for 10 minutes at 37°C were equally responsive as platelets stored continuously at 37°C (irrespective of the time after blood donation). Taking into account its advantages in a clinical setting (allowing blood transport at RT from the clinics to the laboratory), the RT/37°C condition was preferred for further experiments.

Incubation at RT resulted in higher signals for α IIb β 3 activation in response to TRAP, CRP and ADP and P-selectin expression in response to ADP in comparison with incubation at 37°C. This is in agreement with previous studies reporting that *in vitro* hypothermia enhances platelet α IIb β 3 activation and P-selectin expression in response to platelet agonists [23, 24, 27]. This also resulted in a higher optimal concentration of agonist (determined by the shoulder of the dose-effect curve) when measuring at 37°C, in comparison with other studies testing platelet activation at RT [25, 31, 32]. Further studies on patient populations will determine if this increase in agonist concentration influences the sensitivity of the test. The difference in MFI (RT versus 37°C) regarding α IIb β 3 activation

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seems to be more distinct for TRAP than for the other agonists, likely reflecting a fundamental difference in their mechanisms of platelet activation. Of note, the temperature-dependent binding kinetics of the PAC-1 antibody contributes to the higher signals for $\alpha\text{IIb}\beta 3$ activation at RT [33]. In our view, measuring a stable platelet function response over time is more important than achieving extremely high fluorescent signals.

It has been suggested that flow cytometry-based platelet function measurements are laborious. This impression is based on old school test set-ups, which rely on freshly prepared reaction mixes and depend on skilled staff to ensure reproducible results. In this report, we show that reaction mixtures (containing both agonists and antibodies) can be stored stably at -20°C for at least one year. Using anti-mouse Ig κ particles to capture the antibodies of a reaction mixture, a strong stable fluorescent signal was detected. The stability of the agonists in the reaction mixtures stored at -20°C is also shown by the seasonal variation study started in February 2016 in which no consecutive decline of platelet function was measured over time. The advantage of the stable storage of reaction mixtures is that the test becomes less time consuming and more user friendly. Additionally, one batch of tests can be prepared per clinical study to avoid lot-to-lot variability.

Another issue in platelet function testing is the standardisation within and between laboratories. The current study describes a new approach, which uses anti-mouse Ig κ particles. We observed comparable $\alpha\text{IIb}\beta 3$ activation (FITC signal) between different flow cytometers while the raw data differed enormously. In contrast, P-selectin expression (PE-signal) measured on different flow cytometers was more complex to calibrate. The much higher fluorescent signal measured on the positive control beads for the PE-conjugated anti-P-selectin may provide an explanation for this discrepancy. This reasoning may also explain why the usage of beads increases the lot to lot variability for P-selection expression. In the future, other beads with a lower amount of antibody binding sites will be developed to deal with this problem.

For the identification of patients with platelet dysfunction, it is essential to establish appropriate reference intervals. We have determined reference values for platelet function by flow cytometry in more than 120 individuals, as recommended by the latest CLSI guidelines [26].

In our healthy population, platelet activation in response to ADP was higher in women than in men and for this parameter, gender-specific reference values can be found in S5 Fig. Previously, it was demonstrated that platelets of women were significantly more active [34, 35] due to the conversion of a greater proportion of $\alpha\text{IIb}\beta 3$ receptors into the active

confirmation after agonist stimulation [36]. In contrast, a number of small studies failed to detect any differences in the reactivity of platelets between men and women [37-40]. Since we aimed to determine reference values in a broad normal population presenting at the clinic, we did not aim to determine gender-specific reference ranges. Furthermore, we found a low but significant correlation between platelet function and age. This is in agreement with a recent review on the effects of ageing on platelet function concluding that platelet function increases during middle age [41].

The reference intervals will serve as guidance to identify patients at risk for bleeding and thrombosis. We were able to reduce pre-analytical and analytical variables that affect platelet function. However, the measurement variation between different flow cytometers may still have an influence on platelet function results. Therefore, we emphasize that each laboratory should validate these reference intervals using their specific experimental conditions. Validation is typically performed in 20 normal individuals, and if no more than two results are outside our proposed reference intervals a laboratory may adopt our proposed reference intervals [26].

Studies investigating normal ranges for platelet function in healthy donors have shown an inter-individual variation of 16%-32%, especially at low and medium doses of agonists, with a considerable overlap in responses between individuals with and without reported bleeding problems [42-44]. These data correspond with our data showing an inter-individual variability of around 30% (apart from P-selectin expression in response to ADP).

Comparing the different agonists in our study, in most individuals ADP induces a low expression of P-selectin on the platelet outer membrane. This can be explained by the fact that ADP is a weak agonist for granule release. P-selectin expression is therefore in the noise range, which results in poor reproducibility. This in contrast with a strong agonist, such as thrombin or collagen, that can trigger granule secretion even when aggregation is prevented [45]. Therefore, we do not recommend to use P-selectin expression in response to ADP as a biomarker to predict bleeding, but the test may be useful in the prediction of thrombotic events.

Conclusions

We optimized the flow cytometric platelet function assay by determining pre-analytical conditions and by preparing ready to use kits including calibrator beads. This resulted in stable user-friendly platelet function tests with acceptable intra-assay variability. Furthermore, this is the first study determining reference intervals in a large healthy cohort.

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These reference intervals can be validated in clinical laboratories and ongoing studies are investigating if we can detect reduced platelet activation in patients with bleeding complications.

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Supporting information

Supplemental figure 1. Determination optimal agonist concentration. Whole blood of 5 donors was incubated for 10 min at 37°C and subsequently, platelets were activated for 20 min at 37°C with TRAP (panel A), CRP (panel B) and ADP (panel C). Dose-dependent activation curves were created for both α IIb β 3 receptor activation (left panels) and P-selectin expression (right panels). The dotted line represents the concentration of the agonists used in the subsequent experiments.

Supplemental figure 2. Stability of the platelet activation markers after fixation. At day 1, blood from 1 donor was added to two reaction mixtures consisting of an antibody mixture with the agonist CRP. After incubation for 20 minutes at 37°C, samples were fixated and analysed on the flow cytometer on different days. The change in median fluorescence intensity (MFI) over time is shown. The change was calculated as percentage of the MFI at day 2 (n=2).

Supplemental figure 3. Effect of temperature on platelet function testing by flow cytometry. Immediately after blood collection, blood was stored at 37°C (panel A) or RT (panel B) for 30 minutes. Platelet activation was tested in a control condition (grey) and after activation with TRAP (blue), CRP (green) or ADP (red) for 20 minutes at 37°C (panel A) or RT (panel B). The fluorescence histograms of α IIb β 3 receptor activation (left panels) and P-selectin expression (right panels) of one representative experiment are shown.

Supplemental figure 4. Normalisation of data to compare results measured on different flow cytometers. Samples of 126 donors were measured on an Accuri and on a FACSCanto flow cytometer. Both $\alpha_{IIb}\beta_3$ receptor activation (panels A-B) and P-selectin expression (panels C-D) of the first 50 donors are presented as MFI values (panels A and C) and normalized data (panels B and D).

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Supplemental figure 5. Effect of sex on platelet activation. Both $\alpha\text{IIb}\beta_3$ receptor activation and P-selectin expression in response to TRAP, CRP and ADP was determined in blood of 129 healthy volunteers. Normalized data were calculated for males (n=66) and females (n=63). Median and IQR are indicated. The grey areas delineated by the dotted lines represent the reference intervals of the total population (2.5 percentile – 97.5 percentile). Because platelet activation in response to ADP was significantly higher in females, reference intervals for males (blue) and females (red) were indicated. ** p < 0.01; *** p < 0.001 using the Mann-Whitney u test

Chapter 3

A synthetic triple helical collagen peptide as a new agonist for flow cytometric measurement of GPVI specific platelet activation

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Abstract

Synthetic cross-linked synthetic collagen-related peptide (CRP-XL) is a glycoprotein VI receptor activator for platelet activation. This triple helical peptide, widely used in platelet function tests, is synthesized and cross-linked through cysteine residues at its N-terminus and C-terminus. Currently, there is only one laboratory, which is capable to produce this valuable peptide for clinical applications. In an attempt to provide a standardized alternative for CRP-XL, we developed a synthetic triple helical collagen peptide (STH-CP) with the same primary sequence as CRP-XL (GPC-(GPO)₁₀-GPCG-amide)₃, which was both on the C-terminus and on the N-terminus fixed on a scaffold with a binding side for each of the three peptides. The performance of STH-CP on platelet function was studied using flow cytometry and compared with CRP-XL. We found that platelet activation pattern in response to STH-CP and CRP-XL is similar, although the STH-CP requires 6-fold higher concentrations to activate platelets to the same state. The intra-assay %CV of STH-CP and CRP-XL were both <5% and the inter-individual variation measured in 118 individuals for both peptides was around 23% and 21% for α Ib β 3 activation and P-selectin expression, respectively. The STH-CP in ready-to-use reaction mix has lower variation than CRP-XL over one-year storage. In reference values and seasonal variation study, the platelet activation response showed a strong correlation between STH-CP and CRP-XL.

Our findings show that this new STH-CP is a stable and potent platelet GPVI agonist which can induce the same reproducible platelet activation as CRP-XL and that STH-CP can be considered as a good alternative for CRP-XL.

Introduction

Under physiological conditions, platelets circulate in a resting state in blood. Upon vessel wall injury, platelets rapidly respond by adhering to the exposed subendothelial collagen, followed by platelet–platelet interaction and platelet activation to form a plug that effectively seals the injured vessel wall to prevent excessive blood loss [1]. Thus, one of the crucial initial steps in haemostasis is the interaction between platelets and collagen which results in platelet activation.

Despite decades of research, there is still no adequate and generally accepted platelet function test platform in routine diagnostic laboratories that covers the needs for platelet function diagnostics. Light transmission aggregometry (LTA) has been considered as the gold standard for many decades [2]. In brief, LTA measures the changes in light transmission of platelet rich plasma upon stimulation with different platelet agonists. One of the standard agonists used is collagen, native type I fibrils isolated from Achilles tendon of horses. This type of collagen only partly represents the collagen properties of collagen in the vessel wall, which makes the relevance of this agonist disputable. Also, the extraction process of collagen from its biological source may lead to loss of its fibrillar structure, which may result in unstable reactivity [3]. Several other techniques have been proposed to replace LTA as gold standard for bleeding diagnostics, including multiplate®, PFA-100®, TEG® or ROTEM®. Validation studies have however shown that these techniques lack the specificity to replace LTA in bleeding diagnostics [4]. Still, they may be of additional value to LTA, because they give a more global overview of platelet function. In addition to LTA, flow cytometry is another platelet function measurement that can be used for specific detection of platelet function disorders [5, 6].

The collagen fibers, which are the standard in LTA cannot be used in flow cytometry, because they form a surface for platelet aggregation, which obstructs the individual cell measurements in flow cytometry. To increase the specificity of the collagen specific pathways, the flow cytometry-based platelet activation requires receptor specific agonists.

Efforts have been made to develop specific agonists to glycoprotein (GP)VI, which is considered as the most important collagen activation receptor on the platelet surface [7, 8]. Many studies have used convulxin, a snake venom toxin, to stimulate GPVI, but a major disadvantage of convulxin is the instability during its pre-analytical storage [9]. This excludes convulxin for clinical diagnostic use, unless the agonist can be modified to a stable compound. Other GPVI specific agonists are synthetic collagen related peptides (CRPs). Collagen contains large domains comprised Gly-X-Y repeats where X is often a proline (P)

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and Y a 4-hydroxyproline (O) and Richard Farndale and coworkers discovered that the GPO repeats are important for the activation of GPVI. Monomeric CRP (mCRP), a partial agonist of GPVI, stimulates the tyrosine phosphorylation of the tyrosine kinase Syk and phospholipase C gamma2 (PLCgamma2) in platelets and induces the weak secretion of [3H]5-hydroxytryptamine and a weak aggregation [10].

Because collagen is folded into a unique triple-helical structure which is necessary for its activity [11], a triple-helical, cross-linked CRP (CRP-XL), was synthesized containing GPO repeats cross-linked through cysteine residues at its N-terminus and C-terminus. CRP-XL is a powerful stimulus of tyrosine phosphorylation and platelet activation (approximately 100-fold more potent than collagen on a weight-for-weight basis) but is unable to bind to $\alpha 2\beta 1$ [12-14]. CRP-XL is currently the first choice to quantify the most important collagen dependent platelet activation pathway in flow cytometry because it is a stable and specific GPVI agonist [15-17]. Despite the excellent intra-batch variation and the high stability in pre-analytical conditions, CRP-XL has some variation between batches, which requires an extensive calibration before a new batch can be used in a diagnostic setting [18, 19]. This variability makes the comparison between functional platelet tests from different laboratories difficult. A further threat of using CRP-XL as standard for GPVI dependent platelet function is that there is only one (non-commercial) laboratory, which can produce the agonist. Many laboratories depend on the supply of the CRP-XL by only one group. If there is any problem in the production or the supply of then many of those laboratories will run out of GPVI agonists.

We have designed a peptide with a primary sequence of the trimeric peptides identical to CRP-XL (GPC-(GPO)₁₀-GPCG-amide)₃ with three helices connected by 1,3,5-tribromomesitylene (**T3**) Clips on both termini of the triple helical peptides.

Materials and methods

GPVI specific agonists

CRP-XL was purchased from the department of Biochemistry of the University of Cambridge, UK. The activity and specificity of the peptide has been extensively evaluated by many groups, including ourselves [15, 20, 21]. We have designed and developed a novel peptide synthesis procedure based on our knowledge of the three-dimensional structure and sequence of CRP-XL (GPC-(GPO)₁₀-GPCG-amide)₃ (Figure 1A). The peptide was synthesized at Pepscan (Lelystad, The Netherlands) following a stepwise procedure: peptide 1

[C_{SH}G(POG)₁₀PC_S(Acm)], where 'O' = *L*-hydroxyproline, molecular weight (MW)=3163.7 Da] was dissolved in a 1:1:1-mixture of 8M urea/acetonitrile (ACN)/N,N-dimethylformamide (DMF) to a concentration of 5.0 mM (i.e. 15.8 mg/mL). Subsequently, 0.25 equiv. of 1,3,5-tribromomesitylene (T3) in ACN (125 µL of a 10 mM solution/mL of peptide-solution) was added, followed by 0.25 mL of a 200 mM ammonium bicarbonate solution. The mixture was homogenized, incubated for 15-30 minutes at room temperature, diluted in Milli-Q water (5-10x the original volume), and purified by preparative HPLC. The purified trimeric peptide T3-[1-(Acm)]₃ was then Acm-deprotected by dissolving the peptide in 8M guanidine-HCl to a concentration of 1.0 mM (i.e. 9.7 mg/mL), and 1.0 mL of this solution was mixed with 100 µL of a 34 mg/mL solution of I2 in methanol. The reaction was stirred at room temperature for 30-60 minutes, quenched with 50 µL of a 1.0 M solution of 1,4-DTT followed by the addition of 260 µL of an 0.2 M sodium carbonate solution (until pH>7), and again stirred for an additional 5-10 minutes at room temperature. The reaction mixture was then quenched with 10% of TFA until pH<3 and diluted with 5-10 volumes of Milli-Q water (peptide product should not precipitate!) and purified by preparative HPLC.

The purified Acm-deprotected trimeric peptide T3-[1-(SH)]₃ was dissolved in a 1:1:1-mixture of 8M urea/acetonitrile (ACN)/N,N-dimethylformamide (DMF) to a concentration of 0.1 mM (i.e. ~1.0 mg/mL), and to 1.0 mL of this solution, 10 µL of a 10 mM 1,3,5-tribromomesitylene (T3, ~1.1 equiv.) in ACN was added followed by the addition of 25 µL of 200 mM ammonium bicarbonate solution. The mixture was again homogenized and incubated for 15-30 minutes at room temperature and checked by analytical UPLC/ESI-MS to confirm completion of the reaction. Finally, the mixture was diluted in Milli-Q water (5-10x the original volume) and purified by preparative HPLC to obtain the final double-CLIPS constrained trimeric peptide T3-[1]₃-T3 in an overall yield of 1-2% [22]. The mass spec product characterization data were: Retention time (UPLC): 1.11 min (=32.75% ACN); [5-55% gradient of eluent-B in eluent-A; eluent-A: 0.05% TFA in Milli-Q water; eluent B: 0.05% TFA in CAN]. Characterization of high-MW compounds by ESI-MS goes via detection of multiple-charged ions in the *m/z* range of 500 to 2,000. The single-charged molecular ion is usually not observed, because ESI-MS machines typically do not measure ion currents at >2,000 Da. Thereby, it is not predictable which multicharged species is observed and in what amount; that is simply a characteristic of the molecule itself. As shown in Figure 1B, the molecule is mainly present as 5+ -charged species in the gas phase together with approximately 20% of the 6+ -charged species, while higher charged species are not observed (quite normal if there are not sufficient protonation sites available). On the other hand, any lower charged species (4+, 3+, 2+) may be present as well, but have a *m/z* ratio of >2,000 Da and are therefore not observed. ESI-MS: MW_{exp}: 9502.2±1.5Da; MW_{calc}: 9503.1Da (Figure 1B).

Figure 1. Synthesis of STH-CP triple helical peptide. (A) Peptide 1 [$C_{SH}G(POG)_{10}PC_{S(Acm)}$] was mixed with 0.25 equivalent of 1,3,5-tribromomesitylene (T3) and purified by preparative HPLC. The purified trimeric peptide $T3-[1-(Acm)]_3$ was Acm-deprotected to trimeric peptide $T3-[1-(SH)]_3$, which was mixed with 1,1 equivalents of T3. The mixture was checked by analytical UPLC/ESI-MS to confirm completion of the reaction. Finally, the mixture was diluted in Milli-Q water (5-10x the original volume) and purified by preparative HPLC to obtain the final double-CLIPS constrained trimeric peptide $T3-[1]_3-T3$. (B) Electron-spray Ionization Mass Spectrometry. ESI-MS spectrum of the UPLC-peak at $t_R = 1.11$ min., showing the $[M+5H]5+$ signal at $m/z = 1901.34$ as the main peak (100%) and the $[M+6H]6+$ signal at $m/z = 1584.71$ in 25% intensity. From these signals, an $MW_{exp} = 9502.2 \pm 1.5$ Da was calculated, which compares well with the theoretical MW of 9503.1 Da that was calculated for this compound.

Reagents

Platelet activation leads to expression of activation markers on the outer membrane of platelets, which can be quantified by the binding of antibodies, using flow cytometry. The antibodies used in the platelet activation test were FITC-conjugated monoclonal antibody PAC1 directed against the activated $\alpha\text{IIb}\beta_3$ receptor, PE-conjugated monoclonal antibody anti-P-selectin (CD62P, clone AK4) and APC-conjugated anti-CD42b (GPIb) monoclonal antibody (clone HIP1), all purchased from BD Pharmingen (NJ, USA). Anti-mouse Ig κ particles and negative control particles were also purchased from BD Pharmingen.

Preparation of the platelet activation tests

Platelet reaction mixture, with a volume of 20 μl , consisted of 2 μl FITC-conjugated PAC1, 1.5 μl PE-conjugated anti-P-selectin and 0.5 μl APC-conjugated anti-CD42b with or without CRP-XL or STH-CP in HEPES-buffered saline (HBS, 10 mmol/L HEPES, 150 mmol/L NaCl, 1 mmol/L MgSO_4 , 5 mmol/L KCL, pH 7.4). Optimal peptide concentrations were determined in 5 healthy donors by testing a serial dilution of STH-CP and CRP-XL. Further tests were prepared with 30 $\mu\text{g}/\text{ml}$ and 5 $\mu\text{g}/\text{ml}$ for STH-CP CRP-XL, respectively. In each platelet activation test, 3 additional conditions were included to detect binding of antibodies to anti-mouse Ig κ particles or negative control particles. The negative control condition (NC) contained 2 μl FITC-conjugated PAC1 and 1.5 μl PE-conjugated anti-P-selectin in HEPES-buffered saline (total volume of 20 μl). Two positive control (PC) conditions were prepared: PC1 contained 2 μl FITC-conjugated PAC1 and PC2 contained 1.5 μl PE-conjugated anti-P-selectin in HEPES-buffered saline (total volume of 20 μl). Test strips were prepared in advance and stored at -20°C .

Performing the platelet activation tests

Test strips were thawed and briefly centrifuged. All tests were performed at 37°C . Whole blood was stored at RT for at least 30 min and a maximum of 4h, incubated at 37°C for 10 minutes and diluted 1:4 in pre-heated HEPES-buffered saline to minimize the formation of platelet aggregates. From this diluted blood, 5 μl were added to each reaction mixture (20 μl , final dilution 1:20) and the tests were incubated for exactly 20 minutes. Simultaneously, 5 μl of negative control particles were added to the NC condition, and 5 μl of anti-mouse Ig κ particles were added to both PC1 and PC2. Reactions were stopped by adding 250 μl fixation solution (137 mmol/L NaCl, 2.7 mmol/L KCl, 1.12 mmol/L NaH_2PO_4 , 1.15 mmol/L KH_2PO_4 , 10.2 mmol/L Na_2HPO_4 , 4 mmol/L EDTA, 0.5% formaldehyde).

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Flow cytometry was used to discriminate platelets from other cells, using the forward and sideward scatter pattern and by gating on the CD42b positive cells. Fluorescent intensity in the FITC gate and PE gate was selected to determine activated $\alpha\text{IIb}\beta\text{3}$ and P-selectin density, respectively, and results are expressed as median fluorescent intensity (MFI).

In addition to flow cytometry, we investigated platelet response to CRP-XL (5 $\mu\text{g/mL}$), Horm collagen (2 $\mu\text{g/mL}$ and 5 $\mu\text{g/mL}$) and STH-CP (30 $\mu\text{g/mL}$) in platelet rich plasma, using Light transmission aggregometry.

Study population

Our study protocol was evaluated by the local medical ethical board (Medical Ethical Committee of Maastricht University Medical Center). The study population consisted of 118 healthy adult individuals, aged 20-65 years. All participants did not take any oral anticoagulant or anti-platelet drugs for at least two weeks, did not have a history of thrombosis or bleeding and gave full informed consent according to the Helsinki declaration. Experiments were conducted at the Synapse Research Institute in accordance with approved guidelines and regulations. Blood was collected aseptically by antecubital puncture via a 21-gauge needle (0.8x32 mm) into vacuum tubes (1 volume trisodium citrate 0.105M to 9 volumes blood) (BD Vacutainer System/Greiner). The blood was kept at RT and used within 4 hours after collection. Cell counts in whole blood were performed with a Coulter Counter analyzer (Beckman Coulter, Woerden, the Netherlands).

Statistics

Beads were used to normalize the flow cytometric data with the following equation:

$$100 \times (\text{MFI}_a - \text{MFI}_c) / (\text{MFI}_{\text{PC}} - \text{MFI}_{\text{NC}})$$

Where MFI_a was the MFI of the activated platelets, MFI_c the MFI of the unstimulated platelets, MFI_{PC} the MFI measured on the positive control beads and MFI_{NC} the MFI of the negative control beads.

For the reference values, outlier analysis was performed using the D/R method (Dixon). Three outliers were excluded (P-selectin expression in response to STH-CP and CRP-XL) by visual inspection. Normality was tested using the Shapiro Wilk test and data were normally distributed after excluding the outliers. Mean and standard deviation (SD) were shown and inter-individual variability (%CV) was calculated for each variable as $100 \times (\text{SD}/\text{mean})$.

Reference intervals were obtained using non-parametric calculation methods, in accordance with the recommendation in the latest CLSI guidelines [23]. More precisely, the lower reference interval limit was estimated as the 2.5th percentile, and the upper limit as the 97.5th percentile of the distribution. Groups were compared using the Mann-Whitney U test for independent samples.

Data of the repeated measurements over a 1-year period were expressed as mean $\pm$ SD and statistical significance was tested using Tukey's multiple comparisons.

Results

Dose dependent response of platelets on STH-CP

An optimal concentration of STH-CP and CRP-XL to activate platelets was determined by testing a serial dilution of STH-CP and CRP-XL in fresh blood from 5 healthy donors in triplicate. For each donor, these experiments were executed on a single day, using the same batch of platelet function tests. As shown in Figure 2, the activation of α IIb β 3 receptor activation and the expression of P-selectin increased in a dose-dependent manner and optimal concentration of 30 μ g/ml and 5 μ g/ml were chosen for STH-CP and CRP-XL, respectively. To confirm the potency of STH-CP, STH-CP (30 μ g/ml) and CRP-XL (5 μ g/ml) were used in LTA to activate platelets and comparable platelet aggregation was observed (Supplemental figure S1).

Figure 2. Determining optimal concentrations for STH-CP and CRP-XL. Whole blood of 5 donors was incubated for 10 min at 37°C and subsequently, platelets were activated for 20 min at 37°C with STH-CP (red) and CRP-XL (blue). Dose-dependent activation curves were created for both α IIb β 3 receptor activation (left panel) and P-selectin expression (right panel). The dotted line represents the concentration of the agonists used in the subsequent experiments.

The precision of platelet activation induced by STH-CP

To obtain intra-assay precision data of platelet activation tests, we performed replicate experiments (n=10) in 3 healthy donors. For each donor, these experiments were executed on a single day, using the same batch of platelet function tests. Platelets were activated with CRP-XL (5 µg/ml) or STH-CP (30 µg/ml), incubated with FITC-conjugated PAC-1 and PE-conjugated anti-P-selectin, and MFI were measured to determine $\alpha\text{IIb}\beta 3$ receptor activation and P-selectin expression on the platelet membrane, respectively. Comparable results were found for both STH-CP and CRP-XL and the intra assay variability was well below 5% for both $\alpha\text{IIb}\beta 3$ receptor activation and P-selectin expression (Table 1).

Table 1. Intra assay variability of the platelet function test

	Intra-assay variability(%CV)			
	Donor 1	Donor 2	Donor 3	Mean
	(n=10)	(n=10)	(n=10)	%CV
<i>αIIbβ3 activation</i>				
STH-CP	2.5	4	2.8	3.1
CRP-XL	2.7	2.5	2.1	2.4
<i>P-selectin expression</i>				
STH-CP	3	4	2.6	3.2
CRP-XL	2.6	1.8	1.9	2.1

STH-CP induced platelet activation in a healthy population

In total, platelet activation in response to STH-CP was measured in 118 healthy individuals. Demographic data of 115 healthy individuals (53 females and 62 males, median 33 years) together with blood counts are summarized in table 2 (data of 3 donors were missing). In our population, there were significantly more men than women. The hemoglobin levels, hematocrit and red blood cell counts were significantly higher while the platelet count was significantly lower in men compared to women.

Table 2. Baseline data of 115 healthy individuals.

	All	Women (n=53)	Men (n=62)	P-values
Age, years	33 (27-49)	41 (30-51)	31 (26-44)	0.012
Hemoglobin, mmol/L	9.1 (8.3-9.8)	8.5 (8.0-8.9)	9.8 (9.5-10.2)	<0.0001
Hematocrit, %	43 (39-47)	40 (38-42)	47 (45-49)	<0.0001
Red blood cell count, *10 ¹² /L	5.1 (4.6-5.4)	4.7 (4.4-5.0)	5.3 (5.1-5.7)	<0.0001
Platelet count, *10 ⁹ /L	283 (248-344)	304 (268-368)	276 (240-331)	0.035
White blood cell count, *10 ⁹ /L	5.8 (5.0-7.0)	5.9 (5.2-7.0)	5.8 (5.0-7.0)	ns

Medians and interquartile ranges (25%-75%) are indicated.

The inter-individual variability (%CV) as well as reference intervals (2.5 percentile- 97.5 percentile) were determined for P-selectin expression and α Ib β 3 receptor activation in response to STH-CP and CRP-XL in 118 healthy volunteers (Table 3). For P-selectin expression, data of 3 donors were determined as outliers. Apart from the reference values, medians, interquartile ranges (IQRs), minimum and maximum values of each test parameter are indicated. The inter-individual variation of platelet function in response to STH-CP and CRP-XL was comparable and within acceptable ranges with mean %CV of 23.1% and 20.9% for α Ib β 3 receptor activation and P-selectin expression, respectively.

Table 3. Inter-individual variation and reference intervals of the platelet function test

	N	Mean (% of beads)	SD	%CV	Reference Interval (2.5% - 97.5%)	Min	Max
<i>αIbβ3 activation</i>							
STH-CP	118	29.3	6.9	23.5	17.1 - 47.7	12	49.8
CRP-XL	118	30.9	7	22.7	17.5 - 49.1	14.2	51.7
<i>P-selectin expression</i>							
STH-CP	115	14.8	3.1	20.9	7.3 - 22.0	4.6	23.8
CRP-XL	115	15.7	3.3	21	9.0 - 23.5	5.7	24.7

Correlation between platelet activation responses to STH-CP and CRP-XL

Platelet activation induced by STH-CP and CRP-XL was strongly correlated with correlation coefficients of $r=0.93$ ($p<0.0001$) and $r=0.97$ ($p<0.0001$) for α Ib β 3 activation and P-selectin expression, respectively (Figure 3).

Figure 3. Correlation of STH-CP and CRP-XL for determining platelet function. Platelet function was measured as α IIb β 3 activation (left panel) and P-selectin expression (right panel). The Pearson coefficient is depicted.

Stability of peptides during freezer storage

To investigate the stability of the stored peptides in time, platelet function was measured every month in 10 donors over a one-year period. Upon collection of blood, platelets were activated with STH-CP or CRP-XL, activated markers were measured and normalized data calculated (Figure 4). α IIb β 3 receptor activation in response to STH-CP was stable over the whole year. For CRP-XL, α IIb β 3 receptor activation was the lowest with mean value of 18.7 in May and highest in July with mean value 27.4. Concerning P-selectin expression, the lowest and highest value in response to STH-CP /CRP-XL were detected in February (mean of 19.5/19.6) and August (mean of 23.9/25.5), respectively. Significant differences between the months are shown in detail in Figure 4.

Figure 4. Seasonal variation in platelet activation in response to STH-CP and CRP-XL. Every month during a one-year period, platelet activation in response to STH-CP and CRP-XL were measured in 10 healthy donors and the data for both α IIb β 3 receptor activation (left panel) and P-selectin expression (right panel) were normalized using

the NC and PC beads. Data were expressed as mean $\pm$ SD. Differences between the groups were analyzed by Friedman test, followed by Dunn's multiple comparison test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

The seasonal variation in response to the two GPVI-specific peptides is also shown in Table 4. Compared to CRP-XL, the variation in a one-year period was lower when platelet activation was induced by STH-CP with %CV of 5.3% and 6.6% for α Ib β 3 receptor activation and P-selectin expression, respectively. Between STH-CP and CRP-XL, strong correlations were found with correlation coefficients of $r=0.84$ ($p=0.0003$) and $r=0.91$ ($p<0.0001$) for α Ib β 3 activation and P-selectin expression when measured every month during a year in 10 donors, respectively (Figure 5).

Table 4. Seasonal variation of the platelet function test.

	N	Mean (% of beads)	SD	%CV
<i>αIbβ3 activation</i>				
STH-CP	10	24.7	1.3	5.3
CRP-XL	10	23.6	2.1	8.9
<i>P-selectin expression</i>				
STH-CP	10	21.3	1.4	6.6
CRP-XL	10	22.1	1.8	8.1

Figure 5. Correlation of STH-CP and CRP-XL for determining platelet function. Platelet function was measured in response to STH-CP and CRP-XL in 10 healthy donors every month during a one-year period. The Spearman coefficient is depicted for α Ib β 3 activation (left panel) and P-selectin expression (right panel).

The conclusion of comparison between STH-CP and CRP-XL is summarized in Table 5.

Table 5. Comparison between STH-CP and CRP

Item	STH-CP	CRP-XL
Potency	**	***
Precision	***	***
Stability	***	**
GPVI activation	***	***

Reactivity -- Dose-dependent response curve; Precision -- Intra-assay variability and inter-individual variation; Stability-- Seasonal variation; GPVI activation -- Reference intervals for α Ib β 3 activation and P-selectin expression

Discussion

We studied the performance of a novel synthetic Triple Helical Synthesized Collagen Peptide (STH-CP) on platelet function [15], and compared it with the performance of widely used cross-linked triple helical collagen related peptides (CRP-XL). The unique property of STH-CP is that peptides are first coupled to a T3-scaffold and then coupled to a second T3-staple which benefits from a proximity effect since all three peptides to be connected, reside within one molecule.

Many in vitro diagnostic platelet function tests, including LTA, use collagen fibrils, extracted from equine tendon, to measure platelet function. This type of collagen is poorly defined, has a bad inter-batch reproducibility and most probably, it does not represent the human collagens in the blood vessels, because the effects of fibrillar collagen on platelet activation depends on secondary structure of the collagen fibers [24]. Despite the fact that the evidence for the specificity and the generalizability for human collagens are debatable, horse tendon collagen has developed as the gold standard for collagen function testing in diagnostic settings. This may have led to a systematic biased diagnosis of collagen dependent platelet dysfunction for many decades. In our view, collagen mimetic peptides, specific for binding and activation of the most important platelet collagen receptors integrin α 2 β 1 and GPVI, may be a more specific and better reproducible alternative for the collagen variant from equine tendon extracts.

When comparing the efficacy of STH-CP and CRP-XL to platelet activation response, we found that the activity pattern was identical, although the CRP-XL required 6-fold lower concentrations than STH-CP to come to the same platelet activation state. This difference may be caused by the tight binding configuration between the two T3-Clips, which may influence the binding of the peptide to GPVI. Although 6-fold higher concentrations of STH-CP are required for complete activation of platelets, the performance on platelet function

measurements with flow cytometry and LTA show a reproducible agreement between STH-CP and CRP-XL. Therefore, we are convinced that our data show that STH-CP is not inferior to CRP-XL with regard to platelet activation research.

We determined reference values for platelet response to CRP-XL and STH-CP in 118 healthy volunteers. The platelet activation response to both triggers showed a strong correlation (Pearson correlation of 0.93 for $\alpha\text{IIb}\beta\text{3}$ -activation and of 0.97 for P-selectin expression). Furthermore, we showed that both STH-CP and CRP-XL have very high precision (both intra-assay %CV's are below 5%). Furthermore, both tests distinguish between individuals (inter-individual %CV's for both tests between 20-24%). These observations were in agreement with a previous study on CRP-XL performance in 120 healthy volunteers [15].

The stability of STH-CP during storage at -20°C in ready to use reaction mixtures was tested every month over a whole year. The correlations for platelet activation between both peptides were very high and this indicates that STH-CP and CRP-XL are most likely directed against the same platelet receptor, which has been established as GPVI for CRP-XL. The performance of both peptides is very good: CRP-XL seems a little bit more efficient, as indicated by the lower concentrations required to trigger platelet activation, while STH-CP seems a little bit more stable, as indicated by the lower variation over twelve months.

In summary, we have shown that STH-CP is stable and that the agonist gives reproducible platelet activation patterns, similar to CRP-XL. At present, the production of STH-CP is not yet optimized for large quantities, while production of different batches of STH-CP is required for validation studies to investigate the variation between different batches of STH-CP. These issues need to be solved before STH-CP can be used as an alternative for CRP-XL as trigger for platelet activation in in vitro diagnostic devices.

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Supplemental information

Supplemental figure 1. Platelet aggregation induced by STH-CP. LTA measurements showing platelet aggregation in response to 30 µg/ml STH-CP (red), 5 µg/ml CRP-XL (black) and 2 µg/ml and 5 µg/ml Horm collagen (green and blue, respectively)

Part II

Platelet-coagulation interplay in point-of-care settings

Chapter 4

Interplay between platelets and coagulation

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Abstract

Haemostasis stops bleeding at the site of vascular injury and maintains the integrity of blood vessels through clot formation. This regulated physiological process consists of complex interactions between endothelial cells, platelets, von Willebrand factor and coagulation factors. Haemostasis is initiated by a damaged vessel wall, followed with a rapid adhesion, activation and aggregation of platelets to the exposed subendothelial extracellular matrix. At the same time, coagulation factors aggregate on the procoagulant surface of activated platelets to consolidate the platelet plug by forming a mesh of cross-linked fibrin. Platelets and coagulation mutually influence each other and there are strong indications that, thanks to the interplay between platelets and coagulation, haemostasis is far more effective than the two processes separately. Clinically this is relevant because impaired interaction between platelets and coagulation may result in bleeding complications, while excessive platelet-coagulation interaction induces a high thrombotic risk. In this review, platelets, coagulation factors and the complex interaction between them will be discussed in detail.

The role of platelets in haemostasis

Resting circulating platelets have a discoid shape and do not interact with the intact vessel wall. They are present in high numbers (150-400 billion per liter blood) and continuously assess their environment using a wide array of cell surface receptors and adhesion molecules. Of these, the most abundant are the adhesion and signaling integrin molecules, such as α IIb β 3, α V β 3 and α 5 β 1, α 6 β 1 and α 2 β 1. Furthermore, leucine-rich glycoproteins such as the GPIb-IX-V complex, G protein-coupled receptors such as PAR-1, PAR-4, P2X1, P2Y1 and P2Y12 and prostaglandin receptors such as Thromboxane receptor (TP) and prostacyclin receptor (IP), and immunoreceptors such as GPVI and CLEC-2 play important roles in platelet activation and aggregation. Platelets also contain two unique organelles, α -granules and dense bodies, that contribute to these processes [1]. The α -granules contain membrane bound receptors (α IIb β 3, GPVI, GPIb-IX-V complex, P-selectin and others), coagulation related factors (including factor (F)V, FVIII, protein S, antithrombin (AT), plasminogen/plasmin and protein inhibitors such as C1-inhibitor, protease nexin 1 and 2 (PN1 and PN2)), cytokines and chemokines, growth factors, microbicidal proteins and immune mediators which will be released upon activation [2]. Dense granules, with CD63, LAMP-2, GPIb and α IIb β 3 on their membrane [3], contain high concentrations of adenine nucleotides (ADP/ATP ratio > 1.7), serotonin, histamine, Ca^{2+} , Mg^{2+} , K^{+} , pyrophosphate and polyphosphate (PolyP) [4].

Platelet adhesion to the exposed subendothelial matrix is a multistep process depending on the local shear rate of blood [5]. At venous flow (low shear rates, 100-1000 s^{-1}), platelets can interact with collagen (via GPVI and α 2 β 1), fibronectin (via integrin α IIb β 3, α V β 3 and α 5 β 1) and laminin (via α 6 β 1) present in the extracellular matrix [6-8]. At arterial flow (high shear rates, 1000-4000 s^{-1}), the initial reversible adhesion absolutely depends on the interaction between platelet GPIb α and VWF [9]. Exposed collagen captures VWF from circulating blood, and subsequently, VWF unfolds to expose the A1 domain which is a binding site for the platelet GPIb-IX-V complex [10]. Although VWF-GPIb interactions can resist high shear, the binding is transient and, as a result, fast-flowing platelets will slow down and roll over the vessel wall, allowing interaction of other platelet receptors with matrix proteins leading to stable platelet adhesion (Figure 1 part 1) [11].

Initial platelet activation by VWF-GPIb α interaction is enhanced by binding of collagen to platelet GPVI receptors [12]. This interaction induces strong signaling via FcR γ , immunoreceptor tyrosine-based activation motif (ITAM), Src kinases (Fyn and Lyn) and Syk tyrosine kinase resulting in activated phospholipase C γ (PLC γ) [13-15]. Subsequently, PLC γ hydrolyzes phosphatidylinositol-4,5-bisphosphate (PIP2) in inositol trisphosphate (IP3) and

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1,2-diacylglycerol (DAG). IP₃ binds to the dense tubular system (DTS) and allows the efflux of Ca²⁺ from the DTS to the cytoplasm. Membrane bound DAG, together with Ca²⁺, activates the protein kinase C (PKC) resulting in integrin activation, platelet spreading and granules secretion [11]. Although GPVI plays a crucial role in collagen-dependent thrombus formation [14, 16], it shows a low affinity for collagen. The adhesion of platelets on collagen can be considerably enhanced by interactions with another collagen receptor, integrin $\alpha 2\beta 1$. Weak signals through GPIb, GPVI or via interactions of positive feedback mediators ADP and thromboxane A₂ (TxA₂) with P2Y₁/P2Y₁₂ and TP, respectively, change $\alpha 2\beta 1$ from a low-affinity to a high affinity receptor for collagen [17-20]. As a result, $\alpha 2\beta 1$ activation supports firm adhesion of activated platelets to collagen and triggers a weak outside-in signal transduction [21, 22]. Under high shear conditions, this $\alpha 2\beta 1$ is essential for the compact thrombus formation and its resistance to shear [23, 24]. Together, $\alpha 2\beta 1$ and GPVI synergistically stimulate Ca²⁺ signaling, phosphatidylserine (PS) exposure, granule secretion and aggregation [14, 20]. However, deficiencies of one of these receptors results only in a mild bleeding disorder, suggesting that these receptors can replace each other (Figure 1 part 2).

On resting platelets, the most abundant platelet membrane receptor $\alpha IIb\beta 3$ is in a so-called closed conformation with a low affinity for its ligands VWF, fibronectin and fibrinogen. Upon platelet activation, inside-out signaling drives a conformational change in $\alpha IIb\beta 3$ resulting in a high affinity, 'open' conformation [25-27]. Subsequently, because of the multiple binding sites for $\alpha IIb\beta 3$, fibrinogen and VWF can form bridges between platelets. Binding to $\alpha IIb\beta 3$ results in outside-in signaling and, through the involvement of Src family kinases and Syk, in irreversible platelet aggregation and clot retraction [28-32]. Furthermore, granule secretion following platelet activation increases the number of $\alpha IIb\beta 3$ on the platelet membrane which further increases the platelet-platelet interactions [1, 33] (Figure 1 part 2).

Furthermore, to prevent unwanted activation of platelets under normal circumstances and to limit the hemostatic response at a site of vascular injury it is essential to have inhibitory mechanisms. Nitric oxide (NO), released from endothelial cells inhibits platelet aggregation through activation of soluble guanylyl cyclase (sGC) and a consequent upregulation of cGMP and activation of protein kinase G (PKG), resulting in phosphorylation of downstream proteins, reduction of Ca²⁺ levels, inhibition of integrins activation and of granule secretion [34, 35]. In addition, prostacyclin (PGI₂), synthesized in endothelial cells from arachidonic acid by COX-1 or COX-2 and prostacyclin synthase, binds to IP and stimulates membrane bound adenylyl cyclase which results in the increase of cAMP and activation of protein kinase A (PKA) [36-38]. Activated PKA phosphorylates many signal regulatory proteins,

leading to the inhibition of cytoskeleton reorganization and of cytosolic Ca^{2+} elevation [39, 40]. Also, endothelial CD39 (a ectonucleoside triphosphate diphosphohydrolase) prevents the further activation and aggregation of platelets by hydrolyzing ADP, released from activated platelets, to AMP [41]. Mice deficient in CD39 have an increased thrombotic risk [42] (Figure 1 part 3).

The role of coagulation in haemostasis

After a rupture in a blood vessel, blood is exposed to tissue factor (TF)-expressing cells present in the subendothelial tissue (such as smooth muscle cells) or the extracellular matrix (such as fibroblasts). Circulating activated FVIIa binds to TF to form the FVIIa-TF complex (extrinsic coagulation, initiation phase) that activates FIX and FX. FXa can activate more FVII to FVIIa, accelerating the start of coagulation. In the absence of cofactor FVa, FXa alone can produce trace amounts of thrombin from prothrombin that can : 1) activate FV and FVIII; 2) activate FXI which cleaves FIX to FIXa; 3) activate platelets via PAR-1 and PAR-4 (amplification phase) [43, 44]. Furthermore, it was recently reported that besides thrombin, FXa-mediated FV activation is of vital importance in facilitating rapid thrombin generation in the initiation phase of coagulation (Figure 1 part 1) [45]. After FIX and FVIII activation, the formed tenase complex (FIXa-FVIIIa) converts FX to FXa. Subsequently, the formation of the prothrombinase complex (FXa-FVa) leads to a burst of thrombin which can cleave fibrinogen to fibrin, which stabilizes the thrombus (propagation phase). Ca^{2+} is essential for assembly of the tenase and prothrombinase complexes to anionic phospholipids (PS) exposed on activated platelets and recently, it was reported that these complexes concentrate in cap regions of balloon-like procoagulant platelets [46] (Figure 1 part 2).

In the intrinsic pathway, FXII is autoactivated following binding to artificial or biologic surfaces. High-molecular-weight kininogen (HMWK) and prekallikrein facilitate the FXIIa activation which in turn activates FXI. Of note, binding of FXI to polyP appears to be integral to the mechanism for enhancement of FXI activation [47, 48]. Also, FVa was described as a cofactor for thrombin induced FXI activation by Maas et al. [49], however, this could not be confirmed by Matafonov et al. [50]. It was assumed that the intrinsic pathway is much less important to haemostasis under normal physiological conditions than the extrinsic pathway. However, there is renewed interest in FXII as (1) collagen, nucleic acids and PolyP were introduced as triggers for FXII autoactivation [51-53], (2) FXII deficiency is not associated with bleeding but reduces thrombosis risk [54, 55], and (3) pharmacologic targeting FXII or FXIIa provides protection from thrombosis without increased incidence of bleeding [56, 57] (Figure 1 part 1).

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Furthermore, it is also essential to localize clot formation at the site of injury and prevent the formation of clots in healthy vessels. Antithrombin (AT), an important SERPIN present in human plasma, forms stable complexes with predominantly thrombin and FXa, but also to some extent with FIXa, FXIa, FXIIa and Kallikrein, and subsequently the inactivated enzyme-serpin complex will be cleared [43]. Heparin can accelerate this inactivation of coagulation factors by AT [58]. Similar to AT, Heparin cofactor II inhibits thrombin (but no other serine proteases) in the presence of polyanionic molecules such as heparins and dermatan sulfate [59]. In addition, alpha-2-Macroglobulin (α 2M) entraps thrombin and prevents activation of other coagulation factors. Another important anticoagulant system is the Protein C axis. Protein C circulating in blood is activated by thrombin bound to thrombomodulin (TM) on the endothelial surface. Activated protein C (APC), associated with its cofactor protein S that is bound to the surface of activated platelets, inactivates FVa and FVIIIa. In large blood vessels, the endothelial protein C receptor (EPCR) supports protein C activation by localizing protein C to the endothelial cell surface and presenting it to the nearby thrombin-TM complex. Furthermore, tissue factor pathway inhibitors (TFPI), a kunitz-type inhibitor that is present in platelets, on the endothelial cell surface and in plasma, binds to the active site of FXa and inhibits its activity. Subsequently the TFPI-FXa complex interacts with the TF-FVIIa complex thereby inhibiting its activity. Protein S, which exists both in plasma and platelet α -granules [60], is a cofactor of TFPI by promoting the interaction between full-length TFPI and FXa [61]. Moreover, a short form of FV prolongs the half-life of TFPI in the circulation and is important as a chaperone for the most active forms of TFPI [62, 63] (Figure 1 part 3).

Figure 1. Interplay between platelets and coagulation. Part 1 Coagulation initiation and platelet activation. Part 2 Platelet-based amplification and propagation of coagulation and platelet activation and aggregation. Part 3 Inhibition of coagulation and platelet activation.

The complexity in platelet-based coagulation

Although platelet plug formation and coagulation are also called primary and secondary haemostasis, respectively, these two processes are initiated simultaneously when blood vessel injury occurs, which means that these two processes are communicating with each other mutually during the whole coagulation process. In the beginning, coagulation factors share (or compete for) membrane receptors (or binding sites) on resting platelets. Once platelets are activated, platelets will provide more binding sites with higher affinity to the activated coagulation factors than to the not-activated coagulation factors [64-68].

Heterogeneity in thrombus composition is promoted by extrinsic (environmental factors such as blood flow dynamics, vascular environment and local availability of platelet agonists), and by intrinsic (platelet size, volume and age, platelet levels of membrane receptors, and levels of cytoplasmic, granular and cytoskeletal proteins and platelet) platelet-specific factors [69]. Procoagulant platelets, exposed to collagen and strongly expressing PS on their surface, serve to sustain the procoagulant response by concentrating coagulation factors and protecting them from inactivation/inhibition [70]. In the initial phase of coagulation, it is now believed that trace amounts of FIXa and FXIa formed play very important roles by diffusing from one surface to another. The initial FIXa formed by TF/FVIIa complex can diffuse to the platelet surface, because FIXa is not rapidly inhibited by AT or other plasma protease inhibitors [71]. The only relevant binding site known for FIX and FIXa on the platelet membrane is PS, and they share 300 low-affinity binding sites on thrombin-activated platelets which can be replaced partly by prothrombin and FX [66]. However, in the presence of FVIII and FX, FIXa binds to ~250 additional high affinity binding sites and the affinity increases 5-fold [66]. Coated platelets, formed after combined stimulation with collagen and thrombin, also express PS on their surface and they retain α -granule derived factors like FV, fibrinogen and thrombospondin on their surface [69, 72]. In the alpha granules, FV is stored in complex with the protein multimerin and this platelet derived FV accounts for approximately 20% of the total body pool of FV. In contrast to plasma FV, platelet FV, secreted upon platelet stimulation, is partially activated, exhibiting substantial cofactor activity that is increased two-to-three-fold following activation by thrombin or FXa. Moreover, platelet derived FVa is thought to be (GPI)-anchored and is two to threefold more resistant to APC-catalyzed inactivation [73-75]. Interestingly, platelets also contain FIX, both in alpha granules and diffusely in the platelet cytoplasm and

membrane-bounded vesicles, which can be released upon activation [76]. Although the physiological importance of this small amount of FIX is unknown, it may be significant since only a few percent of normal FIX levels are required to support haemostasis. Furthermore, whether or not platelets contain or express FXI is relatively uncertain [77-80], however, recently Zucker et al. reported the presence of FXI in platelet granules and FXI pre-mRNA that is spliced upon platelet activation [81]. Coated platelets may also retain larger amounts of fibrin and FVII, FIX and FX [69, 72]. However, recent data suggest that FVIII also binds to less-activated platelets (stimulated with thrombin alone, PS exposure below threshold), and this binding is mediated by fibrin bound to α Ib β 3 [82-84]. Aggregated platelets with active α Ib β 3 on their surface, are proposed to be responsible for contracting and retracting the clot by interacting with fibrin [85, 86].

The macrocirculation differs from the microcirculation in vessel wall structure and local hemodynamics [87, 88]. However, in both cases there was heterogeneity in the gradient of platelet activation (with a shell of activated, but still P-selectin negative, platelets overlying a core of P-selectin positive platelets), close packaging of the platelets, and the asymmetric distribution of fibrin towards the extravascular side of the plug. Thrombi formed in femoral artery were considerably larger than those required to achieve hemostasis in the arterioles, but a smaller proportion became P-selectin positive. Furthermore, in the microcirculation where flow rates are slower and the vessel wall thinner, hemostatic thrombi tend to project into the vessel lumen. As the vessel wall grows thicker, not only is TF farther away from the lumen, but any thrombin formed has a greater distance to diffuse in the tortuous path produced by the narrowing gaps between adjoining platelets. Platelets in the core of the thrombus contact with each other more closely and also release more active content from their granules. This is important because when a thrombus tears apart due to high shear forces the released polyP of the platelets comes into contact with the circulating blood. Platelet-derived polyP accelerates FV activation, abrogates TFPI activity, enhances fibrin clot structure, and promotes FXI back-activation by thrombin [89] (Figure 1). It was also proposed that FXII activation is caused by polyP from platelets [90], however, because medium-chain platelet-derived polyP is thousands of times less potent than very long-chain polyP in triggering the contact pathway the physiological relevance is questioned [89, 91]. Interestingly, the membrane-associated polyP largely exceeds the polymer size of platelet-derived polyP, and may play a role in FXII activation [92].

In the later stage of coagulation, anticoagulants proteins released from platelets contribute to restrain excessive coagulation. TFPI α can be produced by megakaryocytes and the platelet TFPI α pool exclusively consist of full-length TFPI α that might be localized in multivesicular bodies or exosomes of platelets instead of α -granules or lysosomes [93].

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Platelet TFPI α is slowly released upon activation and can be secreted as a soluble protein [94] or it can bind to the membrane of coated platelets [95]. At the site of vascular injury, local TFPI α concentrations might increase through the release of TFPI α from accumulating platelets within the thrombus and it was speculated that platelet TFPI α is important to prevent systemic coagulation and thrombosis and restrict thrombus formation to the site of the growing platelet plug [96]. Furthermore, platelet protein S can directly bind to stimulated platelets and decrease thrombin and FXa generation in an APC /TFPI-independent way [97] (Figure 1 part 3).

Platelets also harbor a major inhibitor of the contact activation system, C1-inhibitor (C1-INH) as well as fibrinolytic proteases in their α -granules [98-101]. Although platelet-derived C1-INH accounts for only 0.08% of the total circulation pool, this does not preclude an important inhibitory role, as local concentrations within thrombi may be high and plasma-derived C1-INH may not have the capacity to penetrate to the thrombus core [102]. However, at arterial thrombi filtration velocities, the released C1-INH may be rapidly washed out from platelet aggregates which results in the increase of FXIIa prothrombotic activity. This might explain the more important role of FXIIa in arterial thrombosis than in haemostasis [103] (Figure 1 part 3). Finally, α -granules of platelets also contain SERPINS protease nexin I and II (PN1 and PN2), both released during platelet activation [104, 105]. PN1 negatively regulates coagulation and fibrinolysis by inactivating thrombin and fibrinolytic proteases [105, 106]. PN2 inhibits FXIa via its kunitz protease inhibitor domain [107], and heparin can accelerate this inhibition [108]. However, FXIa bound to its receptors on activated platelets is completely protected from inactivation by PN2 [67, 109] (Figure 1 part 3).

Interaction between coagulation factors and platelet receptors

Coagulation factors interact with platelets by binding to platelet receptors directly or indirectly or by cleaving of the platelet receptors (Figure 2). Thrombin (α -thrombin) is one of the most potent physiological agonists of platelets and activates platelets by either proteolytic (cleavage of PAR-1 and PAR-4 [110, 111] or GPV [112]) or non-proteolytic (signaling via binding to GPIb α [113]) mechanisms. PAR-1 (~ 2500 copies per platelet) is the high-affinity thrombin receptor responding to nanomolar concentrations of thrombin which results in a transient calcium signal. In contrast, PAR-4 has a low-affinity for thrombin, however, activation of PAR-4 results in a more sustained Ca²⁺ response. Sub-nanomolar levels of thrombin can also bind a high-affinity binding site (residues 269-287 region) of GPIb α [114-117]. The number of GPIb-complexes with high-affinity binding sites for thrombin is about 1000 [118], which is less than 5% of the total GPIb copies (~25,000) on

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the platelet membrane [119]. The role of GPIb-IX-V in thrombin-induced platelet activation remains poorly understood. Whereas some proposed that GPIb-IX-V serves as a dock that facilitates thrombin cleavage of PAR receptors [117], others suggested that binding induces platelet activation independent of PARs [113]. Recent research, however, indicated that the mutual cooperativity between thrombin-induced GPIb-IX-V signaling and PAR signaling is required for optimal platelet response to low concentrations of thrombin [120]. Furthermore, prothrombin binds to non-activated $\alpha\text{IIb}\beta 3$ on resting platelets [121], and this binding accelerates activation of prothrombin by FXa or FXa-FVa, which might be pivotal for the initial platelet-based thrombin generation.

Figure 2. Interaction of (anti)coagulation factors with key platelet membrane receptors. Coagulation factors interact with platelets by cleaving (red lines) the receptor directly (solid line) or indirectly (dashed line), or by binding (green line) to their respective receptors.

Once the first layer of platelets forms at the vascular injury site, the recruitment of more platelets to the growing thrombus relies on the formation of fibrinogen bridges. Initially, the integrin $\alpha\text{IIb}\beta 3$ resides in a low-affinity state, however, inside-out signaling drives a conformational change resulting in binding of fibrinogen, fibrin and other proteins. Bridging of fibrinogen between $\alpha\text{IIb}\beta 3$ on adjacent platelets results in platelet aggregation. Individual activated $\alpha\text{IIb}\beta 3$ molecules also attach to fibrin fibers such that aggregated platelets are major components of haemostatic clots. $\alpha\text{IIb}\beta 3$ binds to different sites on fibrin and fibrinogen [122, 123], and the mechanical stability is different for the $\alpha\text{IIb}\beta 3$ -ligand complexes (fibrin polymer > fibrin monomer > fibrinogen) [124]. Furthermore, platelet adhesion and spreading on FXIIIa occur through fibrinogen independent binding of $\alpha\text{IIb}\beta 3$ and $\alpha\text{V}\beta 3$ [125]. More recently it was also demonstrated that the zymogen FXIII interacts

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with platelets, however, strong platelet stimulation, fibrinogen and $\alpha\text{IIb}\beta 3$ play essential roles in this interaction [126].

Besides $\alpha\text{IIb}\beta 3$, GPVI has been identified as a receptor for fibrinogen and fibrin and this interaction induces signaling that supports thrombus formation and stabilization [127, 128]. However, contrasting observations have been reported on whether fibrin binds to monomeric or dimeric GPVI or to neither form [129-131]. In addition, polymerized fibrin interacts, not directly but with VWF as a linker, with GPIb [132-134]. In this way, fibrin fibers formed on the thrombus surface serve as a scaffold for binding of coagulation factors and stimulate thrombus formation [135-137].

HMWK can bind GPIb-IX-V on unstimulated platelets in a Zn^{2+} -dependent manner [138, 139], however, precise binding sites remain to be defined since both anti-GPIb and anti-GPIX antibodies block HMWK binding [140]. HMWK and FXIIa compete with thrombin for binding to GPIb-IX-V and in this way they can inhibit thrombin-induced platelet aggregation [140, 141]. PAR-4 is also involved in FXIIa inhibition of thrombin activation of platelets but only at high concentrations [141]. Another coagulation factor interacting with GPIb α in Zn^{2+} -dependent manner is the homodimer FXI that circulates in plasma in a complex with HMWK [142]. More in detail, the apple-3 (A3) domain of FXI interacts with the leucine-rich repeats of GPIb α [143, 144], leaving the other FXI monomer free for activation by thrombin [145-147]. Although HMWK is required for optimal FXI binding to GPIb α on activated platelets in suspension, FXI binding to platelets under flow is not enhanced by HMWK [146, 148]. Furthermore, FXI binding to platelets is also mediated in part by an interaction with apolipoprotein E receptor 2 (ApoER2, or LRP8) [146, 147], and since ApoER2 colocalizes with GPIb α it appears that one FXI homodimer binds simultaneously to both receptors to mediate shear-dependent interactions [146].

Immobilized protein C or APC also mediate platelet binding and activation signaling through ApoER2 and GPIb α under shear conditions [149]. The ability of platelets to bind APC might imply a dual role in haemostasis: stimulating platelet activation and limiting thrombus growth by localizing the anticoagulant role of the protein C system.

Another example of cross-talk between platelet receptors and coagulation is the induction of platelet GPVI shedding by FXa in a metalloproteinase-dependent mechanism in the absence of GPVI ligands and this results in down-regulation of GPVI under procoagulant conditions [150].

Bleeding and the interplay between platelets and coagulation

Balanced platelet function and coagulation are crucial for stable blood circulation. If one of the factors involved is not functioning, this will lead to impaired haemostasis, which can clinically express as bleeding complications. Abnormalities in GPIb (Bernard-Soulier syndrome (BSS) caused by mutations within *GPIBA*, *GPIBB* and *GP9*) and $\alpha\text{IIb}\beta 3$ (Glanzmann thrombasthenia (GT) caused by mutations in *ITGA2B* and *ITGB3*) expression on the platelet surface are associated with moderate to severe bleeding symptoms [151]. The impaired prothrombin consumption in BSS patient can be corrected by the addition of human FVIII or FVIII-VWF, which might indicate the essential role of FVIII/VWF-GPIb interaction in the activation of coagulation [152]. In GT, abnormal $\alpha\text{IIb}\beta 3$ expression results in defective clot retraction due to decreased fibrinogen endocytosis and subsequently decreased binding of fibrinogen to platelets. Both patients with BSS and GT receive good clinical efficacy with recombinant FVIIa (rFVIIa) treatment [151]. The exact working mechanism of rFVIIa is still under debate and the enhancement of thrombin generation was explained by (1) a TF-dependent mechanism where rFVIIa competes with circulation FVII zymogen for TF binding and (2) a TF-independent mechanism where rFVIIa binds to anionic phospholipids, GPIb α or EPCR expressed on activated platelets to localize rFVIIa to the surface of activated platelets [153-158]. rFVIIa can also be used to treat patients with hemophilia. Furthermore, there is a long list of Familial thrombocytopathies (reviewed by Nurden et al. [159, 160]), including genetic variation affecting platelet adhesion (platelet-type von Willebrand disease (*GP1BA*) and GPVI deficiency (*GP6*)), the secondary platelet activation response (P2Y₁₂ ADP receptor deficiency (*P2YR12*) and thromboxane A₂ receptor deficiency (*TBXA2R*)), signaling pathways (thromboxane A synthase (*TBXAS1*) and cytosolic phospholipase A₂ (*PLA2G4A*)) and the procoagulant activity of platelets (Scott Syndrome (*ANO6*)). Other inherited defects of platelet function are caused by genetic variants affecting granule secretion, such as Hermanski-Pudlack (*HPS1*, *AP3B1*, *HPS3-6*, *DTNBP1*, *BLOC1S3*, *BLOC1S6*), Chediak-Higashi syndrome (*LYST*), Familial haemophagocytic lymphohistiocytosis types 3-5, Grey platelet syndrome (*UNC13D*, *STX11*, *STXBP2*), Arthrogryposis-renal dysfunction-cholestasis syndrome (*VPS33B*, *VIPAS39*) and Quebec platelet syndrome (*PLAU*). A genetic defect in the *NBEAL2* gene is responsible for lack of alpha-granule synthesis, which is known as Gray-platelet syndrome. In addition to inherited thrombocytopathies, there are also several familial thrombocytopenias (FT), including defects in transcription factor defects (*GATA1* and *FOG1*) [161] or variants in genes giving reduced expression of proteins (*MYL9*, *PKC* and *ALOX12*) [162]. Furthermore, variation in the *MYH9* and in the *FLNA* gene leads to cytoskeleton defects and hence macro-thrombocytopenia [163, 164]. Other forms of familial thrombocytopenia are X-linked Wiskott-Aldrich syndrome [165], in which the actin polymerization is affected or a genetic variant in *ANKRD26*, affecting the mitochondrial metabolism [166]. The most obvious platelet defect affecting coagulation is the Scott

syndrome. This is a rare inherited bleeding disorder of Ca^{2+} -induced membrane phospholipid scrambling resulting in impaired prothrombin and FX activation due to decreased binding sites for FVa, FVIIIa, FIXa and FXa [167-169]. The disease is caused by mutations in *ANO6* (anoctamin 6, also known as *TMEM16F*) that encodes transmembrane protein 16F (TMEM16F), a phospholipid scramblase that is vital for Ca^{2+} -dependent PS exposure on cell surfaces, and this could explain part of the molecular mechanism in Scott patients [170]. However, recently, a TMEM16F-independent pathway which results in collagen/thrombin-induced PS exposure was discovered [168, 171, 172]. Patients with Scott syndrome are treated with platelet transfusions. Another platelet defect that affects coagulation is the autosomal dominant Quebec platelet disorder (QPD). QPD is caused by a tandem duplication of a 78 kb genomic segment that includes the *PLAU* gene, leading to overexpression of urokinase-type plasminogen activator (uPA) [173]. Bleeding is the result of hyperfibrinolysis in the vicinity of platelets caused by increased activation of plasminogen (which is also present within the α -granules) to plasmin. Interestingly, the formed plasmin can also degrade intraplatelet stores of FV, multimerin 1, thrombospondin 1, VWF, fibrinogen, fibronectin, osteonectin and P-selectin [173, 174].

Well known coagulation disorders are hemophilia A (FVIII deficiency), hemophilia B (FIX deficiency) and von Willebrand disease. The significant role of platelets in coagulation was shown when measuring thrombin generation (TG) in plasma of VWD3 patients (absent VWF in plasma, platelet and endothelial cells) [175]. Although TG was markedly impaired in the plasma of VWD3 patients, it was close to normal in the presence of platelets [176]. The importance of platelets in coagulation is also clear in congenital FV deficiency (Owren parahemophilia), a rare bleeding disorder (the incidence is 1:1.000.000) inherited in an autosomal recessive trait [177]. In contrast to hemophilia A/B which is usually associated with mild to severe bleeding, people with severe FV deficiency only experience mild to moderate bleeding [178]. This might partly be explained by the existence of functional platelet FV that supports enough thrombin generation to rescue patients with undetectable plasma FV from fatal hemorrhage [179]. Moreover, patients with a FV deficiency have low circulating levels of TFPI α because FV short prolongs the lifetime of TFPI in the circulation.

Despite of these new insights, there is still a paradox that the gold standard platelet function tests and plasma coagulation tests fail to give sufficient insight, and many bleeding complications remain undiagnosed.

Thrombosis and the interplay between platelets and coagulation

Arterial thrombosis, manifesting as myocardial infarction or ischaemic stroke, arises from an atherosclerotic plaque disruption (high shear) that triggers platelet aggregation and activation of coagulation and is characterized by platelet rich thrombi that obstruct blood flow. The causal relationship between platelet hyper reactivity and arterial thrombosis has been established in large clinical trials, and four main classes of drugs are currently used clinically, either alone or in combination: P2Y₁₂ antagonists, COX-1-inhibitors (aspirin), PAR-1 antagonists and $\alpha 2\beta 3$ inhibitors [180, 181]. However, this strategy also results in an increased risk of hemorrhagic complications. New antiplatelet drug such as inhibitors of phosphatidylinositol 3-kinase- β , protein disulfide-isomerase, activated $\alpha 2\beta 3$, $\alpha 2\beta 3$ outside-in signaling, protease-activated receptors and GPVI-mediated adhesion pathways may pave the way to safer therapies causing minimal perturbation of haemostasis [181, 182]. Also recently, targeting components of the intrinsic coagulation pathway (FXI, FXII and PKK) are considered as possible strategies to reduce arterial thrombosis without increasing the bleeding risk [183].

Venous thromboembolism (VTE), including deep vein thrombosis (DVT) and pulmonary lung embolism (PE), arises where shear is low and venous thrombi contain fewer platelets and more fibrin than arterial thrombi. In general, the concentration and function of hemostatic proteins are considered as the main determinants of venous thrombotic risk. Multiple inherited thrombophilic defects, including the factor V Leiden (FVL) and prothrombin G20210A (PT20210A) mutations, along with deficiencies of AT, PC, and PS were discovered that can increase the thrombotic risk. Conclusive evidence for causal involvement of hyper coagulation in venous thrombosis has been delivered by large clinical trials which showed that inhibition of the coagulation pathways with heparins, vitamin K antagonists or with direct anti-coagulants (DOACs) will prevent many venous thrombotic incidents [184]. Despite this conclusive evidence, there is still a missing link that needs to be solved: none of the gold standard coagulation tests gives sufficient insight in the haemostasis to predict a high thrombosis risk in healthy subjects or in clinical populations. Indeed, in the most current view, stasis of blood and the accompanying low oxygen tension (in particular downstream of a venous valve), activation of the endothelium, activation of innate immunity (involving monocytes and neutrophils and platelets), activation of platelets, concentration and nature of microparticles also play a role in the formation of a venous thrombotic complication [44].

Thus, although platelets are widely accepted to play a crucial role in arterial thrombosis, there is increasing evidence that platelets also have a role in the formation of venous thrombi [185, 186]. In contrast to arterial thrombosis, where platelets form large aggregates [187], in DVT, platelets are mainly recruited as single cells and adhere either directly to the

activated endothelium or to adherent leukocytes forming small heterotypic aggregates [185]. Platelet recruitment to the venous thrombus depends on the interaction between GPIb α and endothelial surface exposed VWF and deficiency in either GPIb α or VWF prevents experimental DVT [185, 188]. Additionally, recruitment also depends on binding of platelet CLEC-2 to podoplanin, a mucin-type transmembrane protein expressed in the middle and external layers of the venous wall [189, 190]. Interestingly, it has been proposed that hypoxia-induced activation of the endothelial cells renders endothelial cell-cell junctions looser, allowing for platelet penetration into subendothelial spaces where the interaction between CLEC-2 and podoplanin may take place [189]. Furthermore, platelets recruited to the venous wall may release high-mobility group box 1 (HMGB1) that can induce NETosis (formation of neutrophil extracellular traps), resulting in a scaffold for adhering platelets and red blood cells and promoting thrombin generation and fibrin deposition [190-192]. Not surprisingly, recent studies found that aspirin reduces DVT in mice and VTE in patients undergoing orthopedic surgery [193-195].

Impact of platelet-coagulation interplay in clinical disorders

There are several clinical conditions with a high prevalence of thrombosis, including cancer [196, 197], systemic inflammation (including sepsis) [198], antiphospholipid syndrome (APS) [199], immune thrombocytopenia (ITP) [200], trauma [201], stent implantation [202], blood transfusion [203], liver disease [204], thrombotic microangiopathies (TTP, HUS, HELLP) [205], heparin induced thrombocytopenia (HIT) [206], malaria [207], SARS-CoV-2 (COVID-19) induced infection [208] and many other diseases. All these disorders have a typical thrombosis, which are not clearly definable with the available coagulation tests or with the available platelet function tests. The interplay between platelets and coagulation could be an important factor because many of these disorders combine thrombocytopenia with prolonged coagulation times but they still have an increased thrombotic risk.

Many functional and structural properties of platelets in combination with the evolution of these cells indicate that platelets belong to the family of innate immune cells [209, 210]. Platelets contain many cytokines and chemokines which they secrete after activation. Moreover, they express a number of toll-like receptors (TLR2, TLR3, TLR4, TLR7 and TLR9), GPIb and GPIX are also members of the TLR superfamily. Through these receptors, platelets can recognize different pathogens such as bacteria, viruses, parasites and fungi. Thrombocytopenia is therefore common in infections and the cause of these drop in numbers is partly explained by increased platelet consumption. Although, the real cause of the high thrombosis risk in many inflammatory diseases with thrombocytopenia remain to be established, it can be speculated that the platelets of these patients are chronically

exposed to (auto)inflammatory triggers, leading to a chronic pre-activation state of the platelets. This chronic activation state of platelets may lead to a reduced lifespan because the platelets may lose their granule contents and become more pro-coagulant than platelets of healthy subjects. High numbers of pro-coagulant platelets predispose to a high risk of thrombosis and to thrombocytopenia disorders, because pro-coagulant platelets are more rapidly removed from the body mainly by macrophages in the spleen. Moreover, the endothelial cells of the vessel wall will also respond on the released cytokines and will lose some of their antithrombotic functions such as a drop in thrombomodulin expression. A major complexity of this hypothesis is that there are no reliable tests in the clinical diagnostic settings to measure the interplay between platelets and coagulation since validated coagulation tests (e.g. PT, APTT and thrombin generation) are done in plasma in the absence of platelets, while platelet function testing is done in anti-coagulated blood. Global tests such as TEG and ROTEM have too many shortcomings to be a serious candidate for the measurement of the interplay between platelets and coagulation [211]. Recently, a novel whole blood thrombin generation test (WB-TG) was developed that theoretically should give a precise insight in the interplay between platelets and coagulation, but the test needs to be clinically validated before real studies on the interplay between platelets and coagulation can be initiated [212].

Patients with malignancies have a high incidence of venous thrombosis with a 7-fold and 28-fold elevated risk of venous thromboembolism (VTE) with the highest incidence in pancreatic cancers and lung cancers [196, 197]. As a result, venous thrombo-embolic complications are the second most important cause of death in cancer. Despite many speculations, the definitive answer to the cause of the high incidence of venous thrombosis in cancer has not been established. Tumor cells possess the capacity to interact with the haemostatic system in multiple ways, including the production of haemostatic proteins (e.g. TF, thrombin), activation of platelets and the direct adhesion of tumor cells to normal cells, including platelets, endothelial cells and monocytes [213, 214]. The interplay between plasma coagulants and blood cells are crucial in this process for the formation of thrombi and future investigation studying the interaction between platelets and coagulation may give further insight in the pathophysiology of thrombosis in cancer.

Similar to cancers, VTE is a common complication in infectious and inflammatory disorders, including sepsis [198]. Systemic inflammation is a potent prothrombotic stimulus because it upregulates procoagulant factors, downregulates natural anticoagulants and inhibits fibrinolytic activity [215]. In addition to modulating plasma coagulation mechanisms, inflammatory mediators increase platelet reactivity. The far majority of studies on the procoagulant state and on platelet hyper-responsiveness have been performed in separate

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test models and did not measure the effects of inflammation on the interplay between platelets and coagulation and this may result in an underestimation of the thrombotic risk.

Both in cancer and in inflammatory disorders the venous thrombosis risk is high, despite a high prevalence of thrombocytopenia. Initially, this seems paradoxical, because there are less platelets available to support the coagulation. However, there is a common link between thrombocytopenia and thrombosis pathophysiology in patients with cancer and in inflammatory disorders. Both processes depend on the activated surface of platelets. During cancer progression or inflammatory triggers, platelets become activated, release part of their granule contents and express P-selectin and eventually negatively charged phosphatidyl serine on their surface. These platelets are procoagulant, however, they are also rapidly removed from the circulation by monocytes in the spleen. The exposure to procoagulant surfaces and the rapid removal of platelets explains the relation between thrombosis and thrombocytopenia related disorders. This theory explains the high incidence of cancer and inflammatory related thrombosis due to pathophysiological modifications of the blood cells and not by modifications of coagulation factors in the blood plasma.

Several global haemostasis tests have been proposed to study the interplay between platelets and coagulation, including bleeding time (obsolete), Global Thrombosis Test (GTT), Thromboelastography (TEG), platelet mapping system and Rotational Thromboelastometry (ROTEM) [216] and clot waveform analysis (CWA) [217]. Although these global tests provide more information than assays measuring platelet function and coagulation separately, a major limitation of these tests is that they lack the precision for a reliable haemostasis test, as they are poorly associated with coagulation deficiencies and platelet function defects. The capacity to detect coagulation defects or platelet function defects is a minimal requirement for a haemostasis test. This may also explain the disappointing relationship between TEG/ROTEM with thrombotic disorders. Although it is still in its infancy, thrombin generation in platelet rich plasma or in whole blood seems to be a more promising approach to study the interplay between coagulation and platelet function, although the real validation of this test in relation to thrombotic disorders remains to be performed [218].

Conclusion

The complex interplay between platelets and the coagulation system has been underestimated for many decades. Although the awareness that many steps in platelet thrombus formation are closely connected to the different stages of thrombin formation and physiological coagulation is completely dependent on the expression of procoagulant

surfaces, the expression or activation of specific receptors on platelets and the delivery of FV, there are still no accessible tools to study the interplay between platelets and coagulation in clinical research. Many disease- and therapy-related thrombotic events are induced by damaged or affected blood cells rather than by changes in coagulation factors. This may explain the lack of associations between plasma coagulation tests and thrombotic incidents. There are no dedicated tools to study blood cell-mediated thrombotic risk in clinical studies, despite viscoelastic tests, such as TEG and ROTEM. The disadvantage of viscoelastic tests is that they are too a-specific to be a serious alternative for plasma coagulation tests or platelet function tests. Our group recently developed a whole blood thrombin generation test that shows good correlations with plasma thrombin generation tests, with platelet numbers and with the use of different platelet inhibitors [212]. Although the WB-TG seems to be the first serious method to study involvement of blood cells in thrombosis, the technique is in its infancy. Many clinical validation studies are required before serious conclusions can be drawn regarding the additional value for WB-TG as tool to measure the interplay between blood cells and coagulation.

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Chapter 4

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Chapter 5

High prevalence of acquired platelet secretion defects in multiple myeloma

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Abstract

Introduction: Thrombocytopenia at admission predicts mortality in multiple myeloma and might also occur during disease progression or treatment. Little is known about the effects of a reduced physical condition of platelets in MM.

Methods: We conducted a case-control study and compared platelet function of MM patients with age-matched controls via flow cytometry and bioluminescent assays.

Results: MM patients had impaired $\alpha\text{IIb}\beta 3$ activation and reduced δ -granule secretion (ATP). Categorization of patients according to their platelet count and platelet secretion capacity indicated that 29% of patients had thrombocytopenia, while 57% had reduced platelet granule secretion capacity. Only a small group of patients (14%) had both thrombocytopenia and low platelet secretion capacity, while some patients (29%) had both normal platelet count and normal platelet secretion capacity. We found no support for a role of medication in causing the low release capacity in MM patients.

Conclusion: Our data suggest that most MM patients have a reduced platelet secretion capacity, which reflects a poor condition of platelets. We surmise that the combined measurement of platelet count and platelet secretion capacity gives a more complete view on platelet phenotype than platelet count alone. Large prospective follow-up studies are needed to confirm the importance of acquired platelet secretion defect on the prognosis of MM patients.

Introduction

Multiple myeloma (MM) is a malignant proliferation of plasma cells that produce a monoclonal immunoglobulin (M protein, also called paraprotein). Although platelets are not directly involved in the disease pathophysiology, they may give insight in the prognosis of the patient [1-3]. Thrombocytopenia is a common complication in MM that may cause serious bleeding with minor scrapes, cuts or bruises. Remarkably, thrombocytopenia at the start of MM is an independent predictor of mortality [1-3]. Thrombocytopenia in MM might be a result of reduced platelet formation by infiltration of neoplastic plasmocytes in the bone marrow, which inhibit megakaryocytopoiesis [4]. Chemotherapy is another important reason for thrombocytopenia [5]. Alternatively, thrombocytopenia can be caused by reduced platelet lifespan due to platelet exhaustion. A major characteristic of platelet exhaustion is a decreased granule secretion capacity, which has been observed in clinical conditions with continuous platelet triggering, such as during cardiopulmonary bypass surgery, disseminated intravascular coagulation, thrombotic thrombocytopenic purpura or hemolytic uremic syndrome [6, 7]. Platelet hyperactivation is a common feature in newly diagnosed MM patients [8]. Thrombosis is a frequent complication of MM. The high thrombosis risk in MM can be caused by disease-related complications or treatment side-effects[9]. Furthermore, thalidomide-dexamethasone (TD) combination therapy may further enhance platelet and coagulation activation[10].

Thrombocytopenia is a side effect of many malignant disorders, including MM. The prevalence of impaired platelet activity in MM is less well described. The combination of platelet count and platelet secretion capacity measurement may give better insights in the state of platelets than measuring platelet count alone. Low platelet number in combination with impaired platelet granule secretion may indicate a more severe disease state. The current study investigates the platelet secretion capacity of patients with multiple myeloma, with regard to closely related controls without MM.

Material and methods

Materials

We used the following commercial chemicals: 2-methylthio-adenosine-5'-diphosphate (2-MeSADP, Tocris), ADP (Sigma, USA), TRAP-6 peptide SFLLRN (TRAP, H-2936; Bachem, Germany) and cross-linked collagen-related peptide (CRP-XL, purchased from Professor Farndale, university of Cambridge, UK) [11]. Recombinant luciferase was from Synapse B.V.

(Maastricht, The Netherlands). Luciferin was purchased from Synchem UG & Co KG (Felsberg, Germany). The nanobodies to detect P-selectin expression, fibrinogen binding and GP1b density were developed in house [12].

Patients

In total, 21 patients who had been diagnosed with MM, were receiving regular therapy and had a stable condition, were recruited at the Meander Medical Center, Amersfoort, the Netherlands. The medication use of MM patients is described in Supplemental table S1. Among these patients, 11 (52.4%) received antiplatelet drugs (aspirin), 14 (66.7%) received immunomodulatory imide drugs (IMiDs, including lenalidomide or thalidomide), 5 (23.8%) received proteasome inhibitors (PIs, bortezomib), 4 (19%) received anticoagulants (Acenocoumarol (vitamin K antagonist (VKA)), Xarelto (Rivaroxaban: direct factor Xa inhibitor) or Fraxiparine (nadroparin: low molecular weight heparin (LMWH))), 11 (52.4%) received corticosteroids (dexamethasone, prednisolone or triamcinolone acetonide) and 12 (57.1%) received antibiotics. As representative control group (n=21), we invited a closely related person (a, family member or friend with a comparable age) of each patient to donate blood at the same time and location. Two controls received aspirin and two controls received VKA. The study was approved by the Medical Ethical Committee of Maastricht University Medical Center, and patients and volunteers gave full informed consent according to the Helsinki declaration (2013).

Blood collection and cell count

Peripheral venous blood from patients and controls was collected aseptically by antecubital puncture via a 21-gauge needle into two 3.2% (109 mM) trisodium citrate vacuum tubes and one K2EDTA (7.2 mg) tube (BD Vacutainer System/Greiner). Citrate blood was used for whole blood experiments and EDTA blood was used for measuring cell count (Roche Cedex HiRes Analyzer). All blood samples were kept at room temperature (RT) and used within 4 hours after collection. Age and medication use of patients and controls were registered.

Platelet function analysis by whole blood flow cytometry

Platelet function was investigated with a flow cytometry-based assay. Citrated blood was kept at RT for at least 60 min and maximally 4 hours after collection. Whole blood (5 μ l) was diluted 1:10 (v:v) in HEPES buffered saline (HBS; 10 mM HEPES, 150 mM NaCl, 1 mM MgSO₄, 5 mM KCl, pH 7.4), containing a platelet agonist (TRAP, 6.25 μ M and 25 μ M; ADP, 2.5 μ M and 62.5 μ M) and in-house developed fluorophore-conjugated nanobodies against

glycoprotein Iba (GPIba), fibrinogen, P-selectin, or the isotype control nanobody R2. Baseline platelet activation was assessed in the absence of a platelet agonist. After 10 minutes at 37°C, samples were fixed with 0.148% (v:v) formaldehyde, 137 mM NaCl, 2.7 mM KCl, 1.12 mM NaH₂PO₄, 10.2 mM Na₂HPO₄, 1.15 mM KH₂PO₄, 4 mM EDTA, pH 6.8 for 20 minutes at RT and then stored at 4°C until analysis on a BD FACS Canto II (BD Biosciences) [13]. Platelets were identified based on forward and sideward scatter pattern, as well as GPIba surface expression. Platelet fibrinogen binding and surface expression of P-selectin were used as markers of integrin α IIb β 3 activation and platelet granule secretion, respectively. Data are expressed as median fluorescent intensity (MFI).

Platelet secretion kinetics

Citrated whole blood was kept at RT and processed between 30 minutes and 4 hours after collection. Whole blood (25 μ l) was added to a prepared reaction mixture (25 μ l) that consisted of luciferin, luciferase and CaCl₂ at final concentrations of 1.3 mg/ml, 25.4 μ g/ml and 16.7 mM, respectively and an agonist (5 μ g/ml CRP-XL, 2.5 μ M TRAP and 2.5 μ M MeSADP) in HEPES buffer (10 mM HEPES, 150 mM NaCl, pH 7.4 with 5 mg/mL BSA (BSA5)) [14]. The luminescent signal was measured every second and monitored within 5 min on a Glomax®20/20 luminometer (Promega, Madison, Wisconsin, United States). The initiation and potential of platelet secretion were quantified by defining two parameters of the raw luminescence curve, i.e. lag time (LT), and area under the curve (AUC) (Supplemental method and supplemental figure S1). Another parameter – time to peak (TtP) - was quantified from the first derivative curve and reflects the rate of platelet secretion. Peak was the maximum value of the first derivative curve. The performance of this test is evaluated in the supplemental text and in Supplemental tables S2-5.

Data analysis

Continuous variables are expressed as median with interquartile ranges (IQR; 25%-75%). Normality was tested using the Shapiro Wilk test. Mann-Whitney test was used to compare the difference between patients and controls. Pearson or Spearman correlation was used after testing the normality.

Results

Multiple myeloma patient characteristics

Chapter 5

In total, 21 multiple myeloma patients of the Meander Medical Center and 21 controls (patients' relatives, partners or friends) were included in this study. Demographic data of this study population together with blood counts are summarized (Table 1). In MM patients, the white blood cells and red blood cell counts, hemoglobin, hematocrit and platelet count were lower than in the controls, while the mean platelet volume was higher. In total, 29% (6 in 21) of MM patients were defined as thrombocytopenic (platelet count $<100 \times 10^9/L$). M protein level was lower than 10 g/L for most patients (19 of 21), and lower than 15 g/L in all MM patients.

Table 1. Baseline characteristics of multiple myeloma patients and related controls

	All (n=42)	Patient (n=21)	Control (n=21)	P-values
Age, years	67 (53-74)	66 (57-75)	67 (51-73)	ns
Female (%)	43	38	48	ns
Cell count				
White blood cell count, $\times 10^9/L$	5.8 (4.2-7.2)	4.5 (4.0-6.9)	6.2 (5.3-7.5)	0.029
Red blood cell count, $\times 10^{12}/L$	4.5 (3.9-5)	4.0 (3.4-4.5)	4.8 (4.4-5.4)	<0.001
Haemoglobin, mmol/L	8.8 (7.9-9.4)	8.1 (6.7-9.1)	9 (8.4-9.5)	0.013
Haematocrit, %	41.9 (38.7-44.9)	39.6 (32.6-44.3)	43 (41-46.3)	0.013
Platelet count, $\times 10^9/L$	222 (156-285)	178 (76-222)	250 (222-292)	<0.001
Mean platelet volume, fL	10.1 (9.6-11)	10.3 (9.8-11.4)	9.8 (9.5-10.5)	0.041
Platelet distribution width, fL	11.2 (10.2-12.7)	11.6 (10-13.6)	10.7 (10.1-11.9)	ns
Medication (n (%))				
Platelet inhibitor	13 (31%)	11 (52.4%)	2 (9.5%)	
Anticoagulants	6 (14.3%)	4 (19%)	2 (9.5%)	
Immunomodulatory imide drugs	14 (33.3%)	14 (66.7%)	0	
Corticosteroid	11 (26.2%)	11 (52.4%)	0	
Proteasome inhibitor	5 (11.9%)	5 (23.8%)	0	
Antibiotics	11 (26.2%)	11 (52.4%)	0	
Laxation	8 (19%)	8 (38.1%)	0	
Gastric acid inhibitor	9 (21.4%)	9 (42.9%)	0	
Ca+Vit D3	5 (11.9%)	4 (19%)	1 (4.8%)	
Antivirus	7 (16.7%)	7 (33.3%)	0	
Antihypertension	10 (23.8%)	7 (33.3%)	3 (14.3%)	
Asthma medication	4 (9.5%)	4 (19%)	0	
Uric acid inhibitor	5 (11.9%)	5 (23.8%)	0	
Cholesterol lowering drug	6 (14.3%)	4 (19%)	2 (9.5%)	

Median and Interquartile ranges (IQR, 25%-75%) are indicated. Mann-Whitney test was used for comparing the difference between patient and control group.

Platelet activation phenotype in MM patients determined with flow cytometry

To study the impact of MM on platelet function we measured the response to different platelet activation triggers in our study. Platelet activation was determined after stimulation with low or high concentrations of TRAP (6.25 μ M and 25 μ M) and ADP (2.5 μ M and 62.5 μ M), using flow cytometry (Table 2). The amount of activated α IIb β 3 molecules, detected by fibrinogen binding density on the platelet surface, was lower in MM patients compared to controls in response to both agonists. P-selectin expression in response to low or high concentrations of TRAP was also lower in MM patients than in controls, however, this was not the case after stimulation with ADP. The circulating platelet baseline state, no agonist used to stimulate the platelets, showed no difference between patients and controls.

Table 2. Agonist induced platelet activation in patients with MM and controls.

	Control (n=21)	Patients (n=21)	P-values
<i>Fibrinogen binding</i>			
No agonist	812 (640-1119)	715 (403-1154)	ns
6.25 μ M TRAP	1376 (994-1520)	649 (383-815)	<0.001
25 μ M TRAP	2138 (1764-2545)	1299 (1097-1624)	<0.001
2.5 μ M ADP	1065 (803-1282)	733 (584-1001)	0.026
62.5 μ M ADP	1511 (1270-2111)	1270 (937-1704)	0.034
<i>P-selectin expression</i>			
No agonists	177 (148-223)	192 (148-237)	ns
6.25 μ M TRAP	619 (405-744)	253 (217-413)	<0.001
25 μ M TRAP	861 (706-951)	646 (467-778)	0.001
2.5 μ M ADP	244 (221-286)	243 (212-283)	ns
62.5 μ M ADP	394 (350-471)	367 (293-419)	ns

Median and IQR (25%-75%) are indicated. Mann-Whitney test was used for comparing the difference between patient and control group.

The platelet secretion capacity of MM patients

The platelet secretion capacity was determined by the kinetic ATP release parameters in response to platelet agonists (TRAP, CRP-XL and MeSADP) in whole blood of MM patients and related controls. AUC in response to all agonists was lower in patients than in controls (Table 3). There was a strong correlation between platelet count and total platelet granule release for all the agonists (Spearman $r=0.78$, $P<0.001$ for TRAP, Pearson $r=0.74$, $P<0.001$

for CRP-XL and Spearman $r=0.73$, $P<0.001$ for MeSADP), indicating that the platelet number contributed to the total granule release capacity. However, after adjusting for platelet count, mean granule release per platelet (AUC/PLT) was no longer correlated with platelet count. Interestingly, AUC/PLT of patient group was still lower than that of control group for all the agonists. As TRAP is the most potent (highest AUC) agonist to trigger platelet δ -granule secretion (Table 3), we further performed subgroup analysis for TRAP-induced platelet secretion (Figure 1a).

Table 3. Platelet secretion parameters in patients and controls.

		Patients (n=21)		Controls (n=21)		
		Median	IQR (25%-75%)	Median	IQR (25%-75%)	p-value
<i>LT(s)</i>						
	TRAP	16	13-18	14	11-16	ns
	CRP-XL	32	24-41	31	28-35	ns
	MeSADP	9	0-13	12	9-14	0.045
<i>AUC (kRLU*s)</i>						
	TRAP	930	568-2333	3952	3172-6000	<0.001
	CRP-XL	1354	628-1772	2411	1645-2983	<0.001
	MeSADP	487	210-1217	1610	762-2198	<0.001
<i>AUC/PLT (RLU*s)</i>						
	TRAP	0.25	0.16-0.47	0.66	0.55-0.93	<0.001
	CRP-XL	0.28	0.23-0.35	0.39	0.30-0.47	0.028
	MeSADP	0.13	0.08-0.23	0.26	0.13-0.35	0.05
<i>TtP (s)</i>						
	TRAP	36	25-41	24	18-26	<0.001
	CRP-XL	54	46-63	63	44-80	ns
	MeSADP	20	10-26	21	14-29	ns

Median and IQR (25%-75%) are indicated. Mann-Whitney test was used for comparing the difference between the patient and control group. Abbreviations: LT, lag time; AUC, area under the curve; PLT, platelet; TtP: time to Peak.

Effects of medication on platelet secretion capacity in response to TRAP

Patients were classified in groups according to their medication usage (platelet inhibitor, anticoagulants, IMiDs or corticosteroid) and then compared with the control group (Figure 1b-1e). For both AUC and AUC/PLT, no differences were observed between patient groups. Both patient groups (with or without use of medication) had a reduced AUC and AUC/PLT compared to the control group. Patients who did not receive IMiDs therapy also tended to a reduced platelet secretion capacity, but the difference was not statistically significant.

Figure 1. Platelet secretion capacity in response to TRAP and effects of medication. Platelet secretion in response to 2.5 μ M TRAP was determined (a) for all controls (n=21) and patients (n=21); (b) with the effect of platelet inhibitor; (c) with the effect of anticoagulants; (d) with the effect of IMiDs; (e) with the effect of corticosteroid. AUC (left Y axis) - total platelet ATP release, AUC/PLT (right Y axis) - mean platelet ATP release. Median with IQR are shown. Red triangles: controls (n=2) who received platelet inhibitors; blue triangles: controls (n=2) who received anticoagulant therapy. Patient (-): patients who did not take the related drug; patient (+): patients who received the related drug. Difference between three groups were analyzed by Kruskal-Wallis test, followed by Dunn's multiple comparison test. Abbreviations: IMiDs: immunomodulatory imide drugs; AUC, area under the curve; PLT, platelet.

Combined prevalence of individuals with low platelet count and low release capacity

To explore whether platelet granule secretion capacity gave additional insights to platelet count measurements, we categorized the variables in quadrants of patients with low platelet count and low secretion capacity (low AUC/PLT), low platelet count and normal secretion capacity, normal platelet count and low secretion capacity and patients with normal platelet count and normal secretion capacity (Figure 3). Cut-off values for low platelet count and platelet vitality were the clinical cut-off point of 100×10^9 platelets/L [15-17] and the lower limit of the 95% reference interval of the controls mean (AUC/PLT = 0.29), respectively. In total, 29% (6/21) of MM patients had low platelet count and 57% had a low secretion capacity (12/21). Further categorization showed that there were three patients with both low platelet number and low platelet secretion capacity. In contrast, all controls had normal platelet count and none of the controls had a reduced platelet secretion capacity.

Figure 3. Subgroups of patients and controls based on mean platelet secretion capacity (AUC/PLT) and platelet count (PLT). Whole blood platelet vitality in response to TRAP for all participants (n=42) were classified into 4 subgroups according to their platelet count (PLT) and mean ATP release (AUC/PLT): upper left - "Low PLT/Normal secretion", lower left - "Low PLT/Low secretion", lower right - "Normal PLT/Low secretion" and upper right - "Normal PLT/Normal secretion". Grid line is depicted as cutoff value for platelet count (100×10^9 /L) and for AUC/PLT (0.29 RLU*s).

Discussion

Many patients with MM develop an acquired thrombocytopenia. We observed a prevalence of thrombocytopenia in 29% (6 in 21) of the MM patients, which is higher than a previously described prevalence of 10% in newly diagnosed MM patients [18], this might be partly

explained by the chemotherapy or disease progression [19-21]. Furthermore, we showed that several MM patients had a reduced platelet secretion capacity (low ATP release/PLT), which was mainly independent of thrombocytopenia. Platelet ATP secretion capacity could reflect the overall content of δ -granule components of platelets, but it may also indicate a signal transduction effect. In line with the reduced granule contents, we show that platelets of MM patients had a reduced α IIb β 3 activation and P-selectin expression response to PAR1-mediated platelet activation by TRAP, indicating a reduced secondary platelet activation response via ADP release from δ -granules [12]. The observation of impaired integrin α IIb β 3 activation and reduced δ -granule content (ATP) secretion in MM patients, may refer to a reduced vitality of circulating platelets. Some MM patients acquired both a reduced PLT number and a reduced platelets secretion capacity. Although it was suggested that many treatments can affect platelet count and function [20, 22], we did not observe a significant effect of a single medication on platelet secretion capacity.

Although there was a correlation between platelet count and total secretion capacity, there were also MM patients with low platelet count and normal secretion capacity and patients with normal platelet count and impaired secretion capacity. This may be important, because the combined phenotype of thrombocytopenia and impaired platelet secretion capacity will have the highest bleeding risk. Our case-control study does not allow to confirm this hypothesis, but it will be our aim in future studies to investigate this. The findings that the presence of reduced platelet count and reduced platelet secretion capacity in MM are often independent of each other illustrates the importance of a dual measurement of both platelet number and of platelet secretion capacity [23, 24]. More importantly, the combined measurement of platelet number and condition may be a better predictor of high mortality in MM patients than thrombocytopenia alone.

It is not expected that low platelet count in MM patients is the only explanation for bleeding complications. In general, severe spontaneous bleeding mainly occurs if platelet count drops below $10 \times 10^9/L$ [25-27], which is extremely rare in MM patients [17]. Still, many MM patients show bleeding symptoms if PLT counts are $>100 \times 10^9/L$ [28]. Therefore, we recommend to test the combination of platelet number and platelet function, to get a better insight in platelet phenotype. Defects in platelet function can lead to hemorrhagic complications especially in combination with thrombocytopenia. It has been shown that platelet aggregation measured by LTA was reduced in some MM patients with severe life-threatening bleeding [29, 30]. This acquired platelet dysfunction has been linked to the elevated M protein in patients, which bind to platelet receptor/surface specifically or non-specifically [23, 31-37]. We could not study the relation between platelet phenotype and

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M-protein levels, because all MM patients in our study were treated for high M protein levels.

Our findings of a reduced platelet secretion capacity in most MM patients is in line with two previous studies evaluating whole blood platelet aggregation and secretion simultaneously [28, 38]. It has been shown that MM disease progression is characterized by platelet hypo-reactivity, because patients with active MM show reduced aggregation and P-selectin expression compared to both monoclonal gammopathy of undetermined significance (MGUS) patients and MM patients post treatment [39]. Increased platelet activation is a common characteristic of newly diagnosed MM patients [8, 40]. In our study, patients were on regular MM treatment, which explains that we did not measure increased platelet reactivity with flow cytometry in our study. Our findings are in accordance with a recently published study, which suggested that platelet mitochondria dysfunction might be an important reason for the impaired platelet activity during chemotherapy [41].

A major strength of our study is the patient and control recruitment. We invited the family member or a friend of each patient as control, to guarantee an equal distribution for lifestyle between patients and controls. An additional advantage is the age and gender distribution, which was also comparable between patients and controls with this approach. Furthermore, patients and their related controls were invited together, to donate blood in the same room by the same technician, with maximally 5 min time difference. This strategy guarantees an identical pre-conditioning and conditioning of blood during the whole chain from blood collection to final data analysis. Testing of platelet secretion capacity in whole blood, as used in the present study, requires maximally 100 µl whole blood, which is more efficient than LTA, which requires PRP, prepared from 10 mL whole blood.

Our study has some major limitations. The total amount of 21 patients and 21 controls was adequate to distinguish the variation in platelet release capacity between cases and controls. However, these numbers do not allow an in-depth subgroup analysis to link reduced platelet vitality to multiple medication therapy within the patient cohort. The case control design makes a clear distinction between cases and controls, but it cannot be used for prediction disease outcome in MM patients, because we do not have follow-up data. Based on different platelet phenotypes of patients, it would be interesting to determine the association of platelet secretion defects with bleeding events or prognosis in future research.

In summary, our data show that most MM patients have a reduced platelet secretion capacity, which reflects a poor condition of platelets. It is our hypothesis that the combined

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measurement of platelet number and platelet secretion capacity gives a more complete view on platelet phenotype than platelet number alone. Our study does not allow to draw firm conclusions about the importance of a reduced platelet storage pool on the prognosis of MM patients, but it opens the door for large prospective follow-up studies to follow MM patients from diagnosis to disease outcome on platelet number and platelet condition.

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Supplemental information

Supplemental figures

Platelet secretion kinetics parameters

LT was defined as the time between the start of the measurement and the timepoint when the relative light units (RLUs) were higher than 3000 RLU. AUC in $\text{kRLU}\cdot\text{s}$ is the area under the curve within 1 min after the LT. Mean platelet ATP release per platelet (AUC/PLT) in $\text{RLU}\cdot\text{s}$ was calculated for each variable as $(\text{AUC}\cdot 1000)/\text{platelet count}$ (Supplemental figure S1). The maximum rate of ATP release (Peak) and the time from the beginning to the maximum rate (time to Peak (TtP)) were quantified from the first derivative curve (Supplemental figure S1).

Supplemental figure 1. Representative ATP release kinetics raw curve and first derivative curve. Based on the platelet activation curve, the initiation and potential of platelet secretion are quantified by defining two parameters of the raw curve (left panel), i.e. lag time (LT) and area under the curve (AUC). The maximum rate of ATP release (Peak) and the time from the beginning to the maximum rate (time to Peak (TtP)) are quantified by defining two parameters of the first derivative curve of the raw curve (right panel).

The optimal concentration of each agonist for platelet secretion kinetics

The optimal concentration of each agonist was determined by measuring platelet ATP release in time in response to 0.625, 1.25, 2.5, 5 and 10 μM TRAP, 1.25, 2.5, 5, 10 and 20 $\mu\text{g/mL}$ CRP-XL, 0.625, 1.25, 2.5, 5 and 10 μM MeSADP (Supplemental figure S2). Using these curves, 2.5 μM TRAP, 2.5 μM MeSADP and 5 $\mu\text{g/mL}$ CRP-XL were selected as optimal concentrations for normal values and clinical studies.

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Supplemental figure 2. Dose-dependent platelet secretion in response to direct and indirect platelet agonists. Whole blood was triggered with the platelet agonists TRAP (panel A), CRP-XL (panel B) and MeSADP (panel C). Platelet ATP release was assessed by measuring the increase in luminescence in time.

Supplemental Tables

Supplemental table 1. Medication history of all MM patients (n=21)

Medication	1	2	3	4	5	6	7
Platelet inhibitor	Acetylsalicylic acid	Acetylsalicylic acid	Acetylsalicylic acid			Acenocoumarol (VKA)	
Anticoagulants							
Corticosteroid	Dexamethason			Dexamethason	Dexamethason	Dexamethason	Prednisolone
IMiD	Thalidomide		Lenalidomide		Lenalidomide		
Proteasome Inhibitor	Bortezomib			Bortezomib			
Antibiotics	Amoxiclav	Amoxiclav	Amoxiclav, Azitromycin	Azithromycin/Doxycycline/Chloramphenicol/Cotrimoxazole	Cotrimoxazole	Cotrimoxazole	Cotrimoxazole
Laxation	Macrogol + electrolytes					Macrogol	
Gastric acid inhibitor	Pantoprazole	Pantoprazole				Pantoprazole	
Ca + vit D3	Tacal d3		Cholecalciferol				
Anti virus	Valaciclovir		Valaciclovir			Valaciclovir	Valaciclovir
Blood pressure lowering	Amlodipine					Metoprolol Succinate	
Asthma medication	salmeterol/fluticasone/salbutamol				Fluticasone propionate / Salmeterol		
Uric acid inhibitor						Allopurinol	
Cholesterol lowering			Rosuvastatin		Simvastatin		
Nausea inhibitor						Metoclopramide	
Anti depression	Bromazepam/ Oxazepam						Tenazepam
Muscle relaxation	Tamulosine						
Sleeping/relaxation	Zolpidem Tartrate		Metamucil				
Nutrition supplement							
Erection				Sildenafil			
Diarrhea inhibitor				Loperamide			
Urination							Furosemide
K suppletion							
Anti allergy							
Pain reduction							
Female contraception							

Medication	8	9	10	11	12	13	14
Platelet inhibitor	Acetylsalicylic acid	Acetylsalicylic acid	Acetylsalicylic acid	Ascal			
Anticoagulants						Xarelto	
Corticosteroid	Dexamethason		Dexamethason	Dexamethason			Dexamethason
IMiD		Lenalidomide	Lenalidomide	Thalidomide		Lenalidomide	
Proteasome Inhibitor				Bortezomib	Bortezomib		
Antibiotics	Cotrimoxazole					Azithromycin	Cotrimoxazole
Laxation			Movicolon	Macrogol	Movicolon, Microlax, Senosides	Movicolon	
Gastric acid inhibitor	Pantoprazole	Pantoprazole				Pantoprazole	
Ca + vit D3						Cholecalciferol	
Anti virus					Valaciclovir		Valaciclovir
Blood pressure lowering	Metoprolol Succinate/Perindopril			Perindopril/Metoprolol Succinate		Carvedilol	nifedipine
Asthma medication							
Uric acid inhibitor	Allopurinol		Allopurinol			Allopurinol	
Cholesterol lowering		Questran			Metoclopramide		
Nausea inhibitor							
Anti depression							
Muscle relaxation							
Sleeping/relaxation			Dormicum				
Nutrition supplement							
Erection							
Diarrhea inhibitor							
Urination	Furosemide					Furosemide	
K suppletion	Spironolactone						
Anti allergy			Clemastine/Fluticasone furoate			Desloratadine	
Pain reduction					Oxycodone/Fentanyl	Codeine Phosphate	
Female contraception							

Medication	15	16	17	18	19	20	21
Platelet inhibitor	Acetylsalicylic acid	Acetylsalicylic acid					
Anticoagulants		Fraxiparine		Xarelto			
	Dexamethason, triamcinolone acetanide	triamcinolone acetanide					
Corticosteroid							
IMiD	Thalidomide	Lenalidomide	Lenalodimide	Lenalidomide	Lenalidomide	Lenalidomide	Lenalidomide
Proteasome Inhibitor	Bortezomib						
Antibiotics		Amoxicillin	Amoxi/Clav				
			Bisacodyl, Moviprep				
Laxation	Macrogol, Sennosides						
Gastric acid inhibitor		Pantoprazole		Pantoprazole			Pantoprazole
Ca + vit D3				Alendronic acid			
Anti virus							
			Valaciclovir				
Blood pressure lowering	Perindopril/Verapamil						Amlodipine/Metoprolol
Asthma medication		Foster aerosol/ Salbutamol					
Uric acid inhibitor		Salbutamol					
Cholesterol lowering		Allopurinol			Questran		
Nausea inhibitor	Granisetron	Metoclopramide					
Anti depression							
Muscle relaxation							
Sleeping/relaxation							
Nutrition supplement							
Erection							
Diarrhea inhibitor							
Urination							
K suppletion		Didophenac					
Anti allergy	Cetirizine						
Pain reduction						Codeine phosphate	
Female contraception					Evra		

Performance and reference values of the platelet granule secretion test

To obtain intra-assay precision data of our test, replicate experiments (n=10) in 3 healthy donors were performed (Supplemental table S2). Platelet granule secretion was tested in response to 2.5 μ M TRAP, 5 μ g/ml CRP-XL or 2.5 μ M MeSADP, and the activation kinetics were measured for 5 min. A good reproducibility with mean intra-assay variability (%CV) of less than 10% for LT and TtP and less than 13% for AUC was measured in response to CRP-XL, TRAP and MeSADP.

Supplemental table 2. Intra-assay variability of platelet activation.

	Intra-assay variability (%CV)			
	Donor 1	Donor 2	Donor 3	Mean
	(n=10)	(n=10)	(n=10)	%CV
<i>LT (s)</i>				
CRP-XL	5.5	11.3	6.9	7.9
TRAP	7	11.2	7.9	8.7
MeSADP	8.7	7.4	10.9	9
<i>AUC (kRLU*s)</i>				
CRP-XL	15.5	10	12.1	12.5
TRAP	9.4	11.5	8.6	9.8
MeSADP	16	9	7.6	10.8
<i>TtP(s)</i>				
CRP-XL	4.2	5.3	11.3	6.9
TRAP	4.2	8.8	15.3	9.4
MeSADP	7.9	6.5	12.3	8.9

Platelet secretion capacity in a healthy population

Whole blood was collected from 76 healthy individuals (32 females and 44 males) with a mean age of 29 years, to determine the normal distribution of platelet secretion kinetics in response to CRP-XL and TRAP (Supplemental table S3). This healthy population was part of the healthy population which has been depicted in a previous publication [1].

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Supplemental table 3. Platelet secretion in a healthy population

	All (n=76)	Women (n=32)	Men (n=44)	P-values
LT (s)				
TRAP	19 (16-21)	19 (16-22)	18 (16-21)	ns
CRP-XL	28 (24-32)	29 (25-33)	27 (24-31)	ns
AUC (kRLU*s)				
TRAP	3934 (2478-5404)	4008 (2848-5880)	3934 (2367-4770)	ns
CRP-XL	3572 (2654-4655)	3692 (2686-4550)	3474 (2637-4683)	ns
AUC/PLT (RLU*s)				
TRAP	0.81 (0.60-1.17)	0.89 (0.64-1.30)	0.77 (0.56-1.04)	ns
CRP-XL	0.48 (0.31-0.63)	0.49 (0.28-0.58)	0.48 (0.31-0.70)	ns
TtP (s)				
TRAP	43 (31-55)	44 (31-56)	41 (30-54)	ns
CRP-XL	60 (49-81)	67 (49-83)	57 (49-79)	ns

Median and Interquartile ranges (25%-75%) are indicated. 100 µl total reaction system (50 µl reaction mixture + 50 µl whole blood) was used in the healthy population.

Inter-individual variation of platelet secretion capacity in healthy subjects

The inter-individual variation is between 19-25% for LT, 41%-48% for AUC, 52%-77% for AUC/PLT and 34-46% for TtP (Supplemental table S4). Overall, platelet secretion seems gender independent, and upon stimulation with TRAP, a Spearman correlation coefficient of $r=0.32$ ($p=0.005$) was found between the platelet number and total ATP released (AUC) (data not shown).

Supplemental table 4. Inter-individual variation of platelet secretion capacity in healthy subjects

	Inter-individual variation (%) (n=76)			
	LT	AUC	AUC/PLT	TtP
CRP-XL	19.3	41.2	52	45.3
TRAP	24.2	47.9	76.4	34.4

Inter-individual variation of platelet secretion capacity in patients and controls

In the control group, the inter-individual variation for LT, AUC, AUC/PLT and TtP was between 24-47%, 43-67%, 30-53% and 24-42%, respectively, and was comparable to variations of the reference group (Supplemental table 5). In MM patients, the variation was

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much higher with %CV between 32-72%, 71-86%, 46-113% and 34-94% for LT, AUC, AUC/PLT and TtP, respectively (Supplemental Table S5).

Supplemental table 5. Inter-individual variation in patients and controls.

	Inter-individual variation (%) (n=21)							
	LT		AUC		AUC/PLT		TtP	
	Patients	Controls	Patients	Controls	Patients	Controls	Patients	Controls
TRAP	32.9	24.6	85.2	46.6	90.4	30.5	34.6	24.3
CRP-XL	33.7	26.2	71.2	43.2	46.2	37.5	46.3	32.4
MeSADP	71.9	46.2	85.3	66.4	112.6	52.8	93.2	41.3

Reference

1. Wan, J., et al., *A novel assay for studying the involvement of blood cells in whole blood thrombin generation*. Journal of Thrombosis and Haemostasis, 2020. n/a(n/a).

Chapter 6

Rapid monitoring of nadroparin efficacy in patients with therapeutic treatment in intensive care unit

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Manuscript in preparation

Abstract

Critically ill patients in intensive care unit (ICU) have higher risk of thrombosis than normal hospitalized patients, and they are usually treated with low molecular weight heparin (LMWH). It is recommended to monitor LMWH treatment in ICU patients to sustain the targeted therapeutic range, and therefore prevent thrombotic or bleeding event caused by underdose or overdose. Anti-factor Xa assay and activated partial thromboplastin time (aPTT) are currently two commonly used coagulation assays to monitoring heparins, however, they both have limitations. Thrombin generation (TG) assay, a global coagulation test, has been suggested a superior tool to evaluate the overall coagulation process and monitor anticoagulants.

We developed a whole blood bioluminescent assay based on coagulation (tissue factor, TF)-induced platelet coagulation, which is sensitive to the effect of LMWH. We validated this new assay in ICU patients with nadroparin treatment and compared with anti-Xa assay, aPTT and calibrated automated thrombinography (CAT) assay. Anti-Xa activity of patients was within the therapeutic ranges, while aPTT was not sensitive enough to detect the LMWH effect. All TG parameters were significantly inhibited by nadroparin. Moreover, LT of TG was correlated with aPTT, while TTP, Peak and ETP of TG were correlated with anti-Xa activity. Surprisingly, all parameters of the whole blood coagulation-induced platelet activation kinetics were correlated with all TG parameters, indicating the capability of this new test to investigate both anti-Xa and anti-IIa activity, and therefore to monitor the general anticoagulation effect of LMWH. Our new test is fast, user-friendly and requires rather small amount of sample, it shows great potential for monitoring LMWH treatment at bedside.

Introduction

Thromboembolic complications frequently occur in critically ill patients [1, 2]. Besides the general risk factors for thromboembolism, these patients are also exposed to risk factors specifically related to intensive care unit (ICU) admittance like immobilization, sepsis, vasopressor use, respiratory or cardiac failure, pharmacologic sedation, mechanical ventilation, central venous catheter and end-stage renal failure, all of which have been associated with increased occurrence of venous thromboembolism [3]. Thromboembolism, as in non-ICU patients, requires therapeutic anticoagulation. In ICU patients this is generally achieved with use of heparins, either unfractionated heparin (UFH) or low molecular weight heparin (LMWH). Due to the superior bioavailability, less interaction with other blood contents and more predictable pharmacokinetic and pharmacodynamic properties, LMWH has gradually replaced the UFH in the treatment of thromboembolism [4, 5]. However, in ICU patients, lower levels of plasma LMWH activity after subcutaneous administration have been observed [6]. Apart from increased thrombotic risks, bleeding tendency is also increased in ICU patients due to a myriad of factors and the occurrence of significant bleeding and subsequent administration negatively affects outcome [7, 8]. The combination of increased thrombotic and bleeding tendencies makes monitoring of therapeutic anticoagulation very pertinent in these patients and is recommended in current guidelines [6, 9]. Though their antithrombotic effects are similar, LMWHs are heterogeneous: produced by different process and have distinct biochemical and pharmacological properties [10].

aPTT, as a routine coagulation test, is widely available but is generally considered suboptimal for monitoring agents other than UFH since it has low and varying sensitivity to anticoagulation caused by LMWHs [11-13]. The most commonly used test for monitoring LMWH is a chromogenic anti-Xa assay which measures the ability of patient's plasma to inhibit exogenous factor Xa (FXa) [14]. However, anti-Xa activity is poorly correlated with LMWH efficacy as the test measures pharmacokinetics (concentration) but not pharmacodynamics (effects) [15, 16]. Furthermore, an anti-Xa assay does not measure anti-IIa activity of LMWH or UFH. This is an important limitation of this test, because some LMWHs are more effective FIIa inhibitors than others [17, 18]. Anti-FXa measurements are not suitable to distinguish between the effectivity of different LMWH types, because FXa measurements overestimate anti-Xa activity and underestimate anti-IIa activity [15, 16, 19]. Calibrated thrombin generation (CAT), a global coagulation test on the interactions of pro- and anti-coagulant factors, predicts LMWH anticoagulant function, rather than levels. Until recently, TG was the only available method to measure both anti-Xa and anti-IIa activity of

heparins, although, the physiological contribution of blood cells is still missing [17, 20, 21]. Global tests, such as CAT and thromboelastography (TEG), provide a broader assessment of hemostatic function, and may give some insight in the haemostatic phenotype of patients upon treatment. These assays require experienced operators for the use in routine clinical laboratories [22-24].

We have developed a novel assay to determine the effect of LMWH on tissue factor (TF) initiated whole blood coagulation. The formed thrombin initiates platelet activation and platelet granule release, and subsequently the kinetic ATP released from platelets can be measured with a bioluminescent method. This whole blood method is extremely sensitive to study the physiological effect of heparin on coagulation, provides a global evaluation of the coagulation system. The test is portable, requires minimal handling and results are available within a few minutes at the site of blood collection. Furthermore, the test principle is based on platelet activation by thrombin formation, which detects both FXa and FIIa inhibition of LMWH. The aim of this study is to validate the new rapid assay in ICU patients with therapeutic nadroparin administration and compare this technique with anti-Xa assay, aPTT and with CAT assay.

Material and Method

Reagents

Recombinant human tissue factor (TF, Innovin®; Dade-Behring, Germany) was used to initiate thrombin generation and platelet activation. The protease activated receptor (PAR)-1 agonist thrombin receptor activator peptide (TRAP-6 (SFLLRN), H-2936; Bachem, Germany) was used to initiate platelet activation. Reagents were dissolved in HEPES buffer saline (HBS; 10 mM HEPES, 150 mM NaCl, 1 mM MgSO₄, 5 mM KCl, pH 7.4), containing 5 mg/ml bovine serum albumin (BSA5) [25]. Recombinant Luciferase was produced for Synapse research institute by U-protein expression (Utrecht, Netherlands). Luciferin was purchased from Synchem UG & Co KG (Felsberg, Germany). Nadroparin (Fraxiparine® (2850 IU/0.3 ml), Aspen, Netherlands) was aliquoted and stored in -80°C, diluted in BSA5 buffer when using for spiking experiment.

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Study group

In total, 19 ICU patients were recruited at the Maastricht University Medical Center (MUMC), Maastricht, Netherlands. These patients, with high risk of venous thromboembolism (VTE) or myocardial infarction (MI), received therapeutic doses of nadroparin according to the body weight (twice daily). The study was approved by the Medical Ethical Committee of Maastricht University Medical Center (MUMC), and patients gave full informed consent according to the Helsinki declaration.

Blood collection

Four hours after the morning injection of nadroparin, blood was collected via an existing arterial line into 2 trisodium citrate vacuum tubes (1 volume trisodium citrate 0.105M to 9 volumes blood, BD Vacutainer System/Greiner). From 1 tube, a 0.5 ml blood was separated and aliquoted for platelet activation kinetics testing, and the rest blood was centrifuged twice (2821·g, 10 minutes) for PPP preparation to use for aPTT and CAT assay. PPP was stored at -80°C until further use. Furthermore, the other tube was sent to the central diagnostic laboratory of MUMC+ to measure anti-Xa activity.

In the central diagnostic laboratory of the MUMC+, routine laboratory monitoring for hematology (white blood cell count, hemoglobin, platelet count), inflammation (c-reactive protein (CRP)), liver function (aspartate aminotransferase (ASAT), alanine aminotransferase (ALAT), bilirubin) and renal function (creatinine and estimated glomerular filtration rate (eGFR)) was performed on daily basis. The standard laboratory data from the patient group was obtained from the hospital information system on the corresponding days of the blood collection for CAT assay, coagulation-induced platelet activation and anti-Xa assay.

Coagulation induced platelet activation kinetics

Together 14 patient blood samples were used for the coagulation induced platelet activation kinetics test, as a control, fresh blood from 15 healthy volunteers (collected aseptically by antecubital puncture via a 21-gauge needle (0.8x32 mm) into vacuum tubes (mentioned above) was also tested. Meanwhile, whole blood samples from 5 donors were spiked with serial dilution of nadroparin to yield activities of 0, 0.2, 0.4, 0.8 IU anti-Xa/ml. Blood spiked with nadroparin was used for determining the *in vitro* effect of nadroparin on platelet activation kinetics. All blood samples were kept still at room temperature (RT) for at least half an hour and used to measure platelet activation kinetics within 4 hours after collection.

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Whole blood (25 μ l) with or without nadroparin was added to a reaction mixture (25 μ l) consisting of luciferin, luciferase and CaCl_2 at final concentrations of 1.3 mg/ml, 25.4 μ g/ml and 16.7 mM, respectively and an agonist (TRAP or TF) in BSA5. The luminescent signal (Relative Light Units, RLU) was measured every second for 5 minutes (control group) or 20 minutes (nadroparin group) on a Glomax[®]20/20 luminometer (Promega, Madison, Wisconsin, United States). Based on the platelet activation curve, the initiation and potential of platelet activation were quantified by defining two parameters, i.e. lag time (LT) and area under the curve (AUC). Furthermore, the maximum rate of ATP release and the time from the beginning to the maximum rate were quantified by defining two parameters of the first derivative curve of the raw curve, i.e. Peak and time to Peak (TtP). LT was defined when RLU was higher than 3000 RLU. AUC in kRLU*s is the area under the curve within 1 min after the LT. Peak is the highest value of the first derivative and TtP is time from beginning to the peak.

Anti-Xa analysis

The ICU physician ordered an anti-Xa analysis as routine care for therapeutic nadroparin dosage monitoring. Platelet free plasma (PFP) was obtained by centrifugation of citrated blood at 2500 g for 5 min and subsequently at 10 000 g for 10 min at 18°C. PFP was prepared within 4 hours of blood collection and stored at -80°C until analysis. The anti-Xa activity was measured in PFP on a Sysmex CS2100i (Sysmex Corporation, Kobe, Hyogo, Japan) using the Biophen Heparin LRT reagent (Hyphen Biomed, Neuville-sur-Oise, France) according to the manufacturer's instruction. Samples for the anti-Xa test were first diluted 2x with pooled plasma containing approximately 100% antithrombin and were subsequently determined using a specific LMWH calibrator (Biophen Heparine Calibrator; Hyphen Biomed).

Calibrated automated thrombinography (CAT) assay

CAT assay was performed as previously described [26]. In PPP, TG was determined in duplicate at 37°C in response to 5 pM TF plus 4 μ M phospholipids (PPP-reagents, Thrombinoscope, the Netherlands), according to the manufacturer's instructions. As a calibrator, α 2-macroglobulin-thrombin complex (α 2M-T, 750 nM thrombin activity, Thrombinoscope, the Netherlands) was used Fluorogenic substrate Z-GlyGly-Arg-AMC (FluCa kit, Thrombinoscope, the Netherlands) was added. When measuring TG in patients PPP, also normal pool plasma (NPP) and plasma of 15 healthy donors (Sanquin, the Netherlands) were measured under the same testing conditions. The thrombograms were measured in a 96-well plate fluorometer equipped with a 390/460 nm filter set (excitation/emission). Immulon 2HB, flat-bottom 96-well plates (Thermo Fisher Scientific,

USA) were used. TG parameters (lagtime (LT), time to Peak (TTP), Peak and endogenous thrombin potential (ETP)) were calculated with Thrombinoscope software version 5.0. When comparing TG parameters of patients with that of healthy controls, Peak and ETP were normalized as a percentage of NPP.

Activated partial thromboplastin time (aPTT)

Patient PPP sample was taken out from -80°C and thaw to RM for aPTT measurement. Determination of aPTT was performed with STA-C.K. Prest® 5 kit (kaolin activator) on STA-R® Max (STAGO, France) according to the manufacturer's instruction.

Data analysis

Continuous variables were expressed as median with interquartile ranges (IQR: 25%-75%). Also, mean and standard deviation (SD) were shown and inter-individual variability (%CV) was calculated for each variable as $100 \times (\text{SD}/\text{mean})$. Normality was tested using the Shapiro Wilk test. Independent groups were compared using the Mann-Whitney test. Pearson or Spearman correlation was used after testing the normality.

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Results

Coagulation induced platelet activation kinetics

In whole blood, initiation of coagulation by TF (2.5, 5, 10, 20 and 40 pM TF) resulted in a dose-dependent increase of platelet activation (Figure 1). To test reproducibility, replicate experiments (n=10) were performed in 3 donors with 10 pM TF as the trigger, and LT, AUC and TtP were calculated. For the time dependent parameter, the mean %CV was below 10% and for AUC below 15% (Supplemental table 1).

Figure 1. Dose-dependent response to a trigger of coagulation. Whole blood was triggered with serial dilutions of TF and luminescence was measured in time.

Platelet activation kinetics in response to 2.5 μM TRAP and 2.5 pM TF was measured in 8 and 15 healthy individuals, respectively (Table 1). The inter-individual variation, comparable for TRAP and TF, was around 25% for the time dependent parameters and around 45% for AUC.

Table 1. Inter-individual variation (CV%) of platelet activation kinetics parameters in healthy population

	n	Inter-individual variation (%)					
		LT (s)		AUC (kRLU*s)		TtP (s)	
		MEAN $\pm$ SD	CV%	MEAN $\pm$ SD	CV%	MEAN $\pm$ SD	CV%
TRAP (2.5 μM)	8	15 $\pm$ 4	27	6978 $\pm$ 3389	49	27 $\pm$ 8	29
TF (2.5 pM)	15	53 $\pm$ 11	20	5219 $\pm$ 2144	41	85 $\pm$ 17	20

***In vitro* effect of nadroparin spiked in whole blood from healthy donors**

To investigate the effect of nadroparin on coagulation, we tested blood samples of 5 healthy individuals and spiked them with a serial dilution of nadroparin (0.2, 0.4, 0.8 IU anti-Xa/ml), corresponding to the therapeutic plasma anti-Xa level (0.5-1.0 IU/ml) [20]. Subsequently, platelet activation was triggered with 2.5 pM TF. There was a clear dose-dependent inhibition of platelet activation in nadroparin spiked whole blood (Figure 2) resulting in a prolongation of LT and TtP and a decrease in the AUC compared to the reference (no nadroparin spiking). However, the prolongation of LT and TtP was only observed in higher concentrations of nadroparin, while AUC showed the obvious linear dose-dependent effect for all nadroparin concentrations.

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Figure 2. Dose-dependent effects of nadroparin on TF-induced platelet activation kinetics. Whole blood for 5 healthy donors spiked with or without nadroparin was triggered by 2.5 pM TF and LT (% of control) (panel A), TtP (panel B) and AUC (panel C) are shown. Median and IQR are depicted. No nadroparin spiking group was set as the reference.

Rapid determination of nadroparin effects in ICU patients receiving therapeutic nadroparin

Demographic and clinical characteristics of patient group Clinical characteristics of these patients were shown in Table 2. ICU patients have relatively high BMI, indicating most of them are overweight or obese. Compared to the reference range, the white blood cell count was significantly higher and hemoglobin was significantly lower. The number of platelets was normal compared to reference range. The decreased estimated glomerular filtration rate (eGFR) and enhanced creatine implies the impaired renal function may exist in these patients. C-reactive protein (CRP) was significantly increased indicates possible inflammation in these ICU patients. Median anti-Xa activity was 6.5 IU/ml (IQR: 0.52-0.79 IU/ml) and is within the aimed therapeutic range. Median aPTT was 31.6s (IQR: 30.2-33.9s), which is similar to the reference range (29.6 ± 2.4 s) provided by the manufacturer or the normal reference range (30-40 s).

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Table 2. Demographic and clinical characteristics of ICU patients

	Patients (n=19) (Median $\pm$ IQR)	Normal Range
Age, year	69 (58-75)	
Height, m	1.75 (1.7-1.8)	
Weight, kg	90 (79-104)	
Body mass index, kg/m²	29.4 (24.9-35.6)	18.5-24.9
White blood cell count, *10⁹/L	12.5 (7.4-16.2)	3.5-11
Hemoglobin, mmol/L	5.6 (5-6.1)	Male 8.2-11 Female 7.3-9.3
Platelet count, *10⁹/L	264 (181-298)	150-400
Creatinine, μmol/L	101 (77-142)	Male 45-100 Female 45-80
Estimated Glomerular Filtration Rate, mg/mol	54.3 (41.7-72.6)	>90
C-reactive protein, mg/L	107 (45-150)	<10
Aspartate aminotransferase, U/L	37 (26-75)	Male <35 Female <31
Alanine aminotransferase, U/L	32 (26-89)	Male <45 Female <34
Bilirubin	6.7 (4.9-12.4)	<20
anti-Xa activity (IU/ml)	6.5 (0.52-0.79)	0.5-1
aPTT (s)	32 (30-34)	30-40

Median and IQR (25%-75%) are indicated.

Comparison among TG, anti-Xa activity, aPTT and coagulation-induced platelet activation

Correlation of TG with anti-Xa activity, coagulation-induced platelet activation and aPTT

TG reflects the global coagulation profile of ICU patients on nadroparin treatment. There is a strong correlation between plasma anti-Xa activity with ETP, Peak and TTP of TG ($r=-0.83$ ($p<0.001$), $r=-0.72$ ($p=0.002$) and $r=0.77$ ($p=0.002$), respectively; table 3). Remarkably plasma anti-Xa activity did not correlate with the LT of TG ($r=0.2$; ns; table 3). We found exactly the opposite if the aPTT was compared with the TG parameters. aPTT values significantly correlated with the LT of TG ($r=0.63$, $p=0.008$), while there was no correlation between aPTT and the ETP, Peak and TTP of TG. The coagulation induced platelet activation

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kinetics correlated with all TG parameters, indicating that it gives a more complete picture of coagulation than the aPTT and the anti-FXa measurement.

Table 3. Correlation of TG parameters with platelet activation kinetics parameters, plasma anti-Xa activity and aPTT

	PPP TG							
	LT (min)		ETP (% of NPP)		Peak (% of NPP)		TTP (min)	
	r	p	r	p	r	p	r	p
<i>Platelet activation kinetics parameters</i>								
LT (s)	0.5	ns	-0.75	0.001	-0.67	0.007	0.7	0.007
AUC (kRLU*s)	-0.75	0.002	0.81	0.001	0.85	<0.001	-0.82	<0.001
TtP (s)	0.45	ns	-0.7	0.004	-0.69	0.006	0.68	0.01
<i>Plasma anti-Xa activity (IU/ml)</i>								
	0.2	ns	-0.83	<0.001	-0.72	0.002	0.77	0.002
<i>aPTT (s)</i>								
	0.63	0.008	-0.36	ns	-0.47	ns	0.47	ns

Spearman correlation coefficients are shown.

Correlation between anti-Xa activity and coagulation-induced platelet activation Anti-Xa activity correlated significantly with LT (Spearman $r=0.7$, $p=0.014$) and TtP (Spearman $r=0.71$, $p=0.012$) of TF-induced platelet activation. After a logarithmic transformation from AUC to $\log(AUC)$, a significant negative correlation was found with anti-Xa activity (Pearson $r=-0.71$, $p=0.01$).

Effect of nadroparin on TG and coagulation-induced platelet activation

TG profiles of patients ($n=17$) and controls are presented in Figure 4 (also supplemental table 2). TG parameters of six patients fell below the detection limits, because it passed the maximum measuring time. If LT and TtP of TG measurements exceeded the maximum time then they were given a value of 100 min, while the missing Peak and ETP were set as zero. Compared to control plasma ($n=15$), LT and TTP were significantly longer and ETP and Peak were significantly lower in patients, with $p<0.001$ for all the parameter. TG profiles of controls are comparable to that of NPP except for the Peak. One patient whose ETP is 110% of NPP, has comparable TG curve as the healthy controls. Accordingly, this patient showed longer LT but much higher Peak (170% of NPP) than controls. ETP of ten patients were lower than 10% of NPP.

Figure 4. Effects of nadroparin on TG profile in ICU patients PPP. PPP of ICU patients (n=17) and healthy controls plasma (n=15) were tested with CAT assay at 5 pM TF. All TG parameters were normalized as the percentage of that of the normal pooled plasma (NPP) in the sample run. TG parameters of six patients (with anti-Xa activity > 0.65 IU/ml) were adjusted due to a flat or unfinished TG curve. The missing LT and TtP were set as 100 min and the missing Peak and ETP were set as zero. The Mann-Whitney test was used to compare between groups. ***, p<0.001. Median and IQR were shown.

Whole blood platelet activation kinetics in response to 2.5 μ M TRAP and 2.5 pM TF was measured in these ICU patients (n=14) who received subcutaneous injection of nadroparin according to their body weight. TF-induced platelet activation was significantly inhibited in ICU patients compared to controls, while TRAP-induced platelet activation was comparable in both populations (representative curves shown in Supplemental figure 1). For TF-induced platelet activation, ICU patients had significantly prolonged LT and TtP and significantly reduced AUC compared to controls (Figure 5). TRAP-induced platelet activation was not influenced by nadroparin and no significantly difference for LT, AUC or TtP between patients and controls were measured (Supplemental table 3).

Figure 5. Effects of nadroparin on coagulation-induced platelet activation kinetics. Whole blood platelet activation in response to 2.5 pM TF was measured in ICU patients (n=14) who received subcutaneous injection of nadroparin and in healthy individuals (n=15 for TF and n=8 for TRAP) without anticoagulant administration. LT (panel A), AUC (panel B) and TtP (panel C) of TF-induced platelet activation kinetics parameters are shown. Data are presented as median with IQR and the Mann-Whitney test was used to compare between groups. ***, $p < 0.001$. Median and IQR were shown.

Discussion

The current study describes the initial validation of a novel approach to measure the effect of LMWH on TF initiated whole blood platelet activation kinetics. The measurement is extremely sensitive to inhibition of FXa and FIIa, because thrombin formation has a direct impact on platelet activation kinetics. Our data shows that TF induced platelet activation kinetics detects the physiological impact of heparin on coagulation, via both FXa inhibition and FIIa inhibition. The aim of this study is to validate the new rapid assay in ICU patients with therapeutic nadroparin administration and compare this technique with anti-Xa activity and with CAT assay.

Anti-Xa measurement is currently the most used method for quantifying coagulation inhibition by heparins and LMWHs in the clinics. Plasma anti-Xa activity was well within the targeted therapeutic range (0.5-1.0 IU/ml) in ICU patients [20]. Although anti-Xa measurements give comparable insight between patients who are on the same heparin or LMWH type, this measurement is not recommended for the comparison between different LMWH brands or full-length heparins. Because FXa measurement does not give insight in the inhibition of thrombin [19, 20]. This is of crucial importance, because different brands of heparin/LMWH react differently on FIIa/FXa [17]. It was indicated that the capacity to inhibit the coagulation process in plasma is primarily determined by the concentration of C-domain, which carries the anti-FIIa activity [27, 28]. Different LMWHs with the same given anti-Xa activity showed quite different anti-FIIa activity and therefore prolonged aPTT differently [18, 29]. This illustrates the need for a test to measure both FXa and FIIa

inhibition. TG is the first choice for testing global coagulation. The main advantage of TG testing is that it gives insight in both FXa inhibition and FIIa inhibition. A major restriction of TG is that it is not (yet) available as point-of-care test, to determine the effectivity of heparins at the side of treatment. In the current study, we show in table 3 that TG correlates with both FXa inhibition (anti Xa measurement) and with FIIa inhibition (APTT). TF-induced platelet activation covers the effects of nadroparin on the whole TG curve for all the TG parameters, indicating that it also reflects the combination of FXa inhibition and FIIa inhibition.

Each anticoagulant varies in its effect on routine and specific coagulation assays, therefore it is crucial to choose appropriate assay(s) to monitor specific anticoagulant therapy [30]. aPPT was not sensitive enough to detect the LMWH effects in this study. It was suggested that aPTT was not accurate enough to monitoring heparin treatment in critically ill patients [31], because abnormal elevated levels of acute phase proteins (such as FVIII) may lead to shortened or prolonged aPTT [32-34]. Despite anti-Xa assay reflects the heparin activity more directly than aPTT and shows less variability from the presence of biological factors, anti-Xa does not reflect the anticoagulant effects of LMWH. Studies focusing on comparing the LMWH monitoring roles of the established aPTT, anti-Xa and CAT assay have shown that CAT is superior to other tests in the LMWH monitoring[18, 35-37], however, there is still a big step to take before CAT can be used as anti-coagulant monitoring test. Our study introduced a newly developed whole blood coagulation induced platelet activation kinetics test which can be easily operated, requires no pre-analysis procedures and very small amount of sample volume. By detecting the platelet activation process, thrombin formation was quantified simultaneously. We validated this new test in ICU patients with therapeutic LMWH treatment and compared with other widely used test. Our study provides alternative insight in the new-generation anticoagulant monitoring test and is of important clinical interest.

A major limitation of this study is the number of patients in our study group, which was too low for in-depth subgroup analysis on, for example, patients with or without renal dysfunction. Furthermore, we did not collect blood at baseline to test the TG or coagulation-induced platelet activation profile in patients before nadroparin injection, to explain the relative change caused by nadroparin. We compared the effects of some heparin antidotes (protamine and heparinase) to neutralize the effects of nadroaprin in blood sample, however, neutralizing agents could only neutralize the effects of heparins in spiked samples of healthy individuals (data not shown) and did not normalize the TG or TF-induced platelet activation in ICU patients. The haemostatic status of patients is more complicated than

healthy individuals and heparins also undergo transformation after entering the circulating system.

Summary and conclusion

In conclusion, we developed and validated a coagulation-induced platelet activation test to monitor nadroparin treatment. The test was compared with standard aPTT test, anti-Xa activity and CAT assay. In the present study, aPTT has low sensitivity to the anticoagulant effect of nadroparin, and TG parameters correlated strongly with anti-Xa activity. New parameters from our test strongly correlates with both anti-Xa activity and TG parameters, indicating that it is a promising point-of-care technique for determination of combined anti-Xa and anti-IIa effects of nadroparin.

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Supplemental information

Supplemental figure

Supplemental figure 1. Platelet activation in an ICU patient and a healthy control. Whole blood platelet activation in response to 2.5 pM TF (panel A) or 2.5 μM TRAP (panel B) measured and a representative curve was shown for one ICU patient and one healthy control without nadroaprin administration.

Supplemental tables

Supplemental table 1. Intra assay variability of platelet activation.

Intra-assay variability(%CV)							
	Donor 1 (n=10)		Donor 2 (n=10)		Donor 3 (n=10)		Mean
10 pM TF	Mean±SD	%CV	Mean±SD	%CV	Mean±SD	%CV	%CV
LT (s)	35±3	8.6	44±3	5.9	53±4	6.7	7,1
AUC (kRLU*s)	3788±536	14.2	3471±702	20.2	2638±265	10	14,8
TtP (s)	46±5	11	63±5	7.8	75±4	4.8	7,9

Supplemental table 2. TG parameters of patients and compared with healthy control

	Patients (n=17)		Controls (n=15)		p-value
	Median	IQR	Median	IQR	
5 pM TF					
LT (min)	10.2	4.7-100	2.7	2.3-3.2	<0.001
ETP (% of NPP)	7	0-27	91.5	81.6-97.4	<0.001
Peak (% of NPP)	0.9	0-13.8	66.1	59.2-76.9	<0.001
TTP (min)	41.2	18.4-100	7.6	9-Jul	<0.001

Rapid monitoring of nadroparin efficacy in patients with therapeutic treatment in intensive care unit

Supplemental table 3. Platelet activation parameters

		Patients		Controls		
		n	Median (IQR)	n	Median (IQR)	p-value
<i>LT (s)</i>						
	2.5 μM TRAP	14	13 (10-15)	8	14 (11-18)	ns
	2.5 pM TF	14	103 (85-199)	15	51 (48-55)	<0.001
<i>AUC (kRLU*s)</i>						
	2.5 μM TRAP	14	3426 (2492-7937)	8	6327 (4561-9625)	ns
	2.5 pM TF	14	378 (129-949)	15	4421 (3952-6341)	<0.001
<i>TtP (s)</i>						
	2.5 μM TRAP	14	24 (20-27)	8	29 (19-35)	ns
	2.5 pM TF	14	272 (203-450)	15	83 (75-99)	<0.001

Chapter 7

Rapid monitoring of direct oral anticoagulants

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Manuscript in preparation

Abstract

Direct oral anticoagulants (DOACs) are increasingly used for prevention and treatment of venous thromboembolism and prevention of ischemic stroke in atrial fibrillation. Routine monitoring is not necessary, because DOACs have a wide therapeutic range and predictable pharmacokinetics. However, in urgent and emergent situations, severe bleeding complications may be prevented if the medical doctors are aware of DOACs use.

There is currently no point-of-care test (POCT) for the rapid assessment of DOACs in emergent settings. We developed a method to measure DOAC effects in whole blood, within a few minutes, at the site of blood collection. Russel viper venom was used to initiate coagulation and thrombin formation was quantified by platelet ATP release, using bioluminescence. This method has the advantage that there was no external substrate added that interferes with the detection.

DOACs were spiked to whole blood in different concentrations to determine dose-dependency. The area under the curve (AUC) of coagulation-induced platelet activation kinetics was the most representative parameter to quantify the effects of different DOAC concentrations. Clinical studies are needed to determine if DOAC measurements with this new POCT device has additional value for acute surgery care.

Introduction

The direct oral anticoagulants (DOACs) apixaban, edoxaban, dabigatran and rivaroxaban are recommended for prevention and treatment of venous thromboembolism and the prevention of ischemic stroke in atrial fibrillation (AF) [1, 2]. DOACs have a wide therapeutic range and predictable pharmacokinetics. Compared with vitamin K antagonists (VKAs), DOACs have a favorable benefit-risk profile and fewer food-drug and drug-drug interactions [3-10]. Therefore, in general patients can be treated with a fixed dose DOAC without the need for routine therapeutic coagulation monitoring or dose adjustment for body weight. Testing of DOACs might be helpful for clinicians to better interpret acute situations such as bleeding or thrombosis in selected patients. Particularly, in elder patients with deteriorated renal function, normal DOAC therapy might result in high drug accumulation and thus unwanted bleeding [11].

Although in many patients there is no need for routine monitoring of DOAC levels, DOAC-treated patients that require acute intervention or in emergent situations, such as bleeding, acute stroke, trauma, or surgery, may require assessment of their coagulation status to assure appropriate management [12]. In these patients, evidence-based decisions need to be made within a few minutes and there is no time to wait for delayed laboratory outcomes of conventional coagulation testing or quantitative DOAC test results.

Several laboratory tests have been described for the measurement of DOAC levels and/or activity in patients [12-18]. Most tests are adequate and sensitive for the detection of physiological DOAC concentrations in plasma in central clinical chemistry laboratories. A disadvantage of this logistics is the long processing time between blood collection and test outcome. This is a major drawback if acute interventions are needed, e.g. thrombolysis in a patient with ischemic stroke, in whom the presence of relevant amounts of anticoagulant is a risk factor for bleeding.

The current manuscript describes the development of an innovative approach to study the impact of DOACs on whole blood platelet response to coagulation initiation via Russell viper venom factor X activator (RVV-X). Thrombin formation is quantified with ATP release kinetics from thrombin activated platelets. Coagulation and platelet activation are integrated in a one-step procedure of mixing blood with ready-to-use reaction mixture. We postulate that the effects of DOACs can be monitored by the dose-dependent impaired platelet response to coagulation trigger.

Material and method

Reagents

Russell's viper venom factor X activator (RVV-X, Synapse Research Institute, Maastricht, the Netherlands) was used for initiation of thrombin generation and platelet activation. The majority of the reagents were dissolved in HEPES buffer saline (HBS, 10 mM HEPES, 150 mM NaCl, 1 mM MgSO_4 and 5 mM KCl, PH 7.4) containing 5 mg/ml bovine serum albumin (BSA5, prepared as described previously [19]). Recombinant Luciferase was produced for Synapse research institute by U-protein expression (Utrecht, Netherlands). Luciferin was purchased from Synchem UG & Co KG (Felsberg, Germany). The direct oral anticoagulants (DOACs) dabigatran, rivaroxaban and apixaban were purchased from Alsachim (Illkirch Graffenstaden, France) and diluted first in dimethyl sulfoxide (DMSO) and then in BSA5 buffer.

In vitro effect of DOACs spiked in whole blood from healthy volunteers

To test the effects of DOACs on RVV-X induced platelet activation, venous blood was collected from 6 healthy individuals into BD vacuum tubes (1 volume trisodium citrate 0.105M to 9 volumes blood) by antecubital puncture via a 21-gauge needle. The blood was spiked with different concentrations of DOACs (or BSA5 buffer for the blank group). The concentrations of DOACs were determined based on the plasma range [14], after taking into account the hematocrit (HCT). All blood samples were kept still at room temperature (RT) and processed between 30 minutes and 4 hours after collection.

Coagulation induced platelet activation kinetics

Whole blood (25 μl) with or without DOAC was added to a reaction mixture (25 μl) consisting of luciferin, luciferase and CaCl_2 at final concentrations of 1.3 mg/ml, 25.4 $\mu\text{g}/\text{ml}$ and 16.7 mM, respectively and different concentrations of RVV-X in BSA5. The luminescent signal (Relative Light Units; RLU) was measured every second and monitored for 5 min (without DOACs) to 20 min (with DOACs) on a Glomax®20/20 luminometer (Promega, Madison, Wisconsin, United States). Based on the platelet activation curve, the initiation and potential of platelet activation were quantified by defining two parameters from the raw curve, i.e. LT and AUC. LT was defined as the time needed to reach a luminescent signal higher than 3000 RLU. AUC in $\text{kRLU} \cdot \text{s}$ is the area under the curve within 1 min after the LT (Supplemental figure 1). Furthermore, the maximum rate of ATP release and the time from

the beginning to the maximum rate are quantified by defining two parameters of the first derivative of the raw curve. Peak is the highest value of the first derivative and time to Peak (TtP) is the time from the beginning until the Peak. LT, AUC and TtP are used for analysis.

Statistics

Mean and standard deviation (SD) were shown and intra-assay variation and inter-individual variability (%CV) was calculated as $100 \times (\text{SD}/\text{mean})$. Paired t tests were used for comparing difference between different concentration of DOAC on the same individual. After spiking blood with DOACs, the prolongation of LT and TtP were calculated as follows: (LT (or TtP) after addition of a DOAC/LT (or TtP) before addition of a DOAC) *100-100. The inhibition of AUC was calculated as follows: $100 - (\text{AUC after addition of a DOAC}/\text{AUC before addition of a DOAC}) * 100$.

Results

Coagulation induced platelet activation kinetics

RVV-X triggers thrombin generation induced platelet activation in a dose-dependent manner in blood of a healthy donor (Figure 1). To study the reproducibility of the test, 1:5000 RVV-X was chosen, and granule release was measured for 5 minutes. Replicate experiments (n=10) were performed with blood of 3 healthy donors, resulting in an intra-assay variation between 10-13% for the LT, TtP and AUC (Table 1).

Figure 1. Representative coagulation induced platelet activation curve from one healthy individual. Whole blood was triggered with five dilutions of RVV-X (1:50, 1:500, 1:5000, 1:50000, 1:500000) and the luminescent signal was measured in time.

Table 1. Intra assay variability of platelet activation.

1:5000 RVV-X	Intra-assay variability			Mean %CV
	Donor 1 (n=10) Mean $\pm$ SD	Donor 2 (n=10) Mean $\pm$ SD	Donor 3 (n=10) Mean $\pm$ SD	
<i>LT (s)</i>	59 $\pm$ 4	62 $\pm$ 5	35 $\pm$ 5	10.3
<i>AUC (kRLU*s)</i>	2699 $\pm$ 324	1783 $\pm$ 256	5080 $\pm$ 623	12.9
<i>TtP (s)</i>	90 $\pm$ 10	97 $\pm$ 9	48 $\pm$ 7	11.5

Inter-individual variability. Platelet activation in response to 1:5000 RVV-X was measured in healthy individuals (n=13) to determine the variation among healthy subjects for this test (Table 2). This inter-individual variation is 20%, 29.6% and 25.6% for LT, AUC and TtP, respectively.

Table 2. Variation of thrombin generation induced platelet activation among healthy population

1:5000 RVV-X	N	Inter-individual variation		%CV
		Mean	SD	
<i>LT</i>	13	46	9	20
<i>AUC</i>	13	4860	1437	29.6
<i>TtP</i>	13	80	20	25.6

Dose-dependent effect of spiked DOACs on RVV-X induced platelet activation

Dose-dependent curves. To quantify the effect of spiked DOACs in whole blood, we initiated thrombin formation with 1:20000 RVV-X and monitored thrombin-induced platelet activation curve in 6 healthy volunteers. Blood was collected and spiked with a serial dilution of dabigatran (5.6, 16.7, 50, 150, 450 nM), rivaroxaban (7.4, 22.2, 66.7, 200, 600 nM) or apixaban (5.6, 16.7, 50, 150, 450 nM), to determine the effects of DOACs on RVV-X induced platelet activation. DOACs were able to inhibit RVV-induced platelet activation dose-dependently (Figure 2). The measurement is sensitive for various concentrations of DOACs.

Figure 2. Dose-dependent effect of spiked DOACs on RVV-X induced platelet activation. Whole blood spiked with DOACs was triggered with 1:20000 RVV-X. Representative platelet activation curves in DOACs spiked whole blood from one healthy donor: (A) Dabigatran; (B) Rivaroxaban; (C) Apixaban.

Dose-dependent effects of DOACs on parameters of RVV-X induced platelet activation.

When concentrations of DOACs increase, LT and TtP were significantly prolonged, while AUC was significantly reduced (Figure 3). LT, TtP and AUC of platelet activation were significantly different from those of blank for 5.4 nM dabigatran ($p=0.002$ for LT, $p=0.005$ for TtP, $p=0.02$ for AUC), 66.7 nM rivaroxaban ($p=0.005$, $p=0.018$ and $p=0.011$) and 16.7 nM apixaban ($p=0.016$, $p=0.023$ and $p=0.046$, respectively). Difference between adjacent concentrations of DOAC was also significant for most of the parameters, but not for LT and TtP between 16.7 nM and 50 nM apixaban, neither for TtP and AUC between 7.4 nM and 22.2 nM rivaroxaban.

Figure 3. Dose-dependent effects of DOACs on coagulation induced platelet activation. Platelet activation (n=6) in response to 1:20000 RVV-X was monitored as LT, TtP and AUC in the presence or absence of different DOACs. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Varying responses among individuals to different DOACs

Large variation was found among healthy individuals for the same DOAC. When DOACs concentrations increased, the variation among individuals also increased for LT and TtP, but not for AUC (Figure 4). Furthermore, the same individual responded differently to different DOACs. For example, donor 2 was sensitive to dabigatran, as shown by the longest LT and TtP and the lowest AUC. However, for rivaroxaban, donor 2 had the least prolonged LT and TtP, and relatively less decrease of AUC. On the contrary, donor 6 is relatively more sensitive to both rivaroxaban and apixaban as shown by the lowest AUC.

Figure 4. Varying response among individuals to different DOACs. The percentage change compared to the blank group was shown individually ($n=6$) for each parameter after DOACs spiking. Different individual is displayed as dot with different color.

DOACs effect as shown by coagulation induced platelet activation parameters

Considering the results of all individuals ($n=6$), a linear relation was found between DOACs concentrations and mean LT, between DOACs concentrations and mean TtP, but not between DOACs concentration and AUC (more like exponential curve, data not shown). After a logarithmic transformation of the concentrations of DOACs to $\log(\text{Concentration})$, a linear association was also found between $\log(\text{concentration})$ and mean AUC (Figure 5). AUC of platelet activation was largely reduced at low concentrations of DOACs. However,

Chapter 7

the prolongation of LT and TtP was not obvious for low concentrations, though the difference between these concentrations was statistically significant.

Figure 5. Linear relation between DOACs concentrations and parameters of coagulation-induced platelet activation. Mean and SD of each platelet activation parameter was shown for each concentration of DOACs. (A) LT; (B) TtP; (C): AUC. Red: dabigatran, blue: rivaroxaban, green: apixaban.

Discussion

The current manuscript describes the development of a whole blood approach to study the impact of DOACs on coagulation initiation. Thrombin formation is quantified indirectly through ATP release of thrombin activated platelets. Using this method, DOAC activity can be determined within 10-15 minutes in fresh whole blood at the site of blood collection with a small portable luminometer. The test can be done by any user, after a short instruction.

Studies in the real-world setting revealed that testing is useful if immediately available in urgent clinical situations where assessment of drug presence is judged to be needed [13]. Examples of those urgent and emergent situations are trauma, stroke, hemorrhage, or need for emergent surgery or procedures [20, 21]. Patients with an acute ischemic stroke benefit from intravenous thrombolysis in conjunction with thrombectomy, however this treatment carries a high risk for bleeding complications, especially if patients are on anti-coagulants. The proposed cut-off values for plasma DOAC concentrations to allow intravenous (i.v.) thrombolysis with r-tPA in patients with acute ischemic stroke after an individual risk–benefit assessment are 50 (dabigatran), 100 (rivaroxaban) and 10 (apixaban) ng/mL [22]. Hence any clinically useful assay should be sufficiently sensitive to detect such low concentrations.

In recent years several commercial dedicated tests, e.g., ECT, anti-Xa assay with specific calibrators, have been introduced to predict the concentration of DOACs in plasma [18]. A disadvantage of those conventional tests is that they are not applicable for urgent or

emergent settings. Likewise, semi- point-of-care (POC) coagulation tests, such as rotational thromboelastometry (ROTEM) or thromboelastography (TEG) and the activated clotting time (ACT) [23] have been proposed for emergent settings, but, *in vitro* studies indicate that the sensitivity of those tests for DOACs is too low [24-27]. We introduced the first step to a whole blood test that fulfils the need for POC testing of DOACs in acute and emergent care settings.

Low concentrations of DOACs had a strong effect on the AUC, while it had only a small effect on the LT or TtP, therefore, the AUC seems the most sensitive parameter to measure low concentrations of DOACs. Although we showed that we can detect low DOAC concentrations in whole blood, we need to be careful with drawing firm conclusions from our data. Our study is based on spiked DOACs to blood of healthy volunteers. This is just the first step in the development of a point-of-care DOAC test. The finding of a clear dose-dependent inhibition of thrombin induced platelet activation is an encouraging step to initiate clinical studies in urgent and emergent settings.

Our findings of a low intra-assay variation and a high variation between individuals, indicates that there are good and bad responders to DOACs, which can be defined in the low concentration ranges of DOACs. Interestingly, high responders for thrombin-inhibitors were not by definition high responders to FIXa inhibitors. This may indicate that some patients will have more benefit from a thrombin inhibitor, while others respond better to a FXa inhibitor. It would be interesting to further investigate why there seems to be a specific DOAC preference: is it related to the levels of coagulation factors?

In conclusion, there is a need for POC testing of DOACs in acute care and in emergent care settings. Currently no adequate POC test is on the market for DOACs measurement. Our present study describes the first evaluation of a potential POC test to measure DOAC activity in fresh whole blood. The test can be completed in a few minutes and is therefore suitable for acute and emergent care. Further studies are now needed to validate whether results from this POC test are correlated with the gold standard liquid chromatography-mass spectrometry (LC-MS/MS) or dedicated quantitative assays (calibrated anti-Xa assay or anti-IIa assay) for measurement of DOACs [28].

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Supplemental information

Supplemental figure 1. Representative ATP release kinetics raw curve and first derivative curve. Based on the platelet activation curve, the initiation and potential of platelet activation are quantified by defining two parameters of the raw curve, i.e. lag time (LT) and area under the curve (AUC). LT was defined when the relative light units (RLUs) was higher than 3000 RLU. AUC in kRLU*s is the area under the curve within 1 min after the LT. AUC per platelet (AUC/PLT) in RLU*s was calculated for each variable as $(AUC \times 1000) / \text{platelet count (PLT)}$. Furthermore, the maximum rate of ATP release and the time from the beginning to the maximum rate are quantified by defining two parameters of the first derivative curve of the raw curve, i.e. Peak and time to Peak (TtP). Peak is the highest value of the first derivative and TtP is time from beginning to the Peak.

Chapter 8

Summary, discussion and prospects

Summary, discussion and prospects

This thesis focuses on innovation and standardization of platelet function assays in order to optimize patient diagnosis and treatment with respect to haemostasis. The thesis is divided into two parts: standardizing the existing whole blood flow cytometric analysis of platelet activation towards a diagnostic tool (**Part I**) and innovating the bioluminescent platelet secretion analysis by integrating with coagulation to evaluate platelet-coagulation interaction in point-of-care settings (**Part II**).

In **Part I**, the flow cytometric analysis of platelet function was optimized regarding the following aspects: pre-analysis temperature, preparation and validation of ready-to-use kit and normalization of data derived from different flow cytometers. As a result, we standardized the flow cytometric analysis of platelet function with acceptable intra-assay variability and established reference intervals in a healthy population (**Chapter 2**). Using this optimized flow cytometric assay, we validated a newly designed synthetic triple helical collagen peptide (STH-CP) as a stable platelet glycoprotein VI (GPVI) agonist and provided a good alternative for the commonly used triple-helical cross-linked collagen related peptide (CRP-XL) (**Chapter 3**). In summary, the optimized flow cytometric analysis of platelet function helps to translate flow cytometry from a research tool to a diagnostic tool.

In **Part II**, we first investigated the different aspects of platelet-coagulation interactions in (ab)normal haemostasis in **Chapter 4**, and thereby stressed the importance of appropriate tools to investigate the interactions. However, there is currently no adequate strategy to evaluate the interplay between platelets and coagulation, thus we aimed to develop a simple and rapid bioluminescent method to investigate this. Furthermore the multiple clinical utilities of this method were studied by (1) platelet function analysis in patients with haemostasis related disorders (**Chapter 5**); (2) rapid monitoring the anticoagulant effects of low molecular weight heparin (LMWH, **Chapter 6**) and direct oral anticoagulants (DOACs, **Chapter 7**) in order to guide patient management.

A schematic overview of the studies performed in this thesis is shown in figure 1. In this chapter, we summarize and discuss the importance of our findings in updating platelet function analysis and anticoagulant monitoring and suggest future research directions.

Figure 1. Schematic overview of the studies depicted in this thesis. Chapter 2 and 3 describe the flow cytometric analysis of platelet function; Chapter 4 describes the already existing literature regarding the interplay between platelets and coagulation; Chapter 5-7 determine the multiple utilities of bioluminescent analysis of platelet activation kinetics. GPII, glycoprotein II; LMWH, low molecular weight heparin; DOACs, direct oral anticoagulants.

Part I: Flow cytometric analysis of platelet function

Flow cytometry is a very powerful laboratory technique for assessing platelet disorders [1]. Since Shattil et al. introduced the whole blood flow cytometric platelet function assay in 1987 [2], the technique has evolved continuously during the last three decades. Some disadvantages of flow cytometric analysis to measure platelet function include the need for expensive instruments and specialized technicians, complicated sample preparation and requirement of fast processing after blood collection. We studied the effects of pre-analytic conditions (temperature and storage) on platelet function, stability of ready-to-use kits (agonists/antibody cocktail) and we established reference intervals in a local healthy population in **Chapter 2**. This standardized flow cytometric platelet function assay is more consistent and efficient than traditional methods and shows great potential for clinical diagnosis.

Standardization and optimization of whole blood platelet activation measured by flow cytometry

Whole blood flow cytometry assay has important advantages over the traditional platelet function tests, such as, requiring smaller sample volumes, closer to the physiological milieu, showing qualitative results. Flow cytometry has been increasingly used for the assessment of platelet (dys)function, platelet reactivity in clinical disorders, etc. [3-5]. Unfortunately, there are too many different protocols on flow cytometric analysis of platelet function. Due to the poor standardization between laboratories and instruments, this technique is still only applied in a research setting.

The need for standardization in sample processing and data analysis is well recognized, and some measures to minimize the influence of artifacts on platelet activation *in vitro* are generally known and adopted: discard the first few milliliters of blood, use trisodium citrate as anticoagulant and dilute whole blood to prevent aggregation [6]. Still these precautions remain underestimated. Despite the evidence of spontaneous platelet activation at room temperature (RT), platelet function tests are generally performed at RT [6-9]. In addition, there is no consensus on the ideal time scale between blood collection and initiation of the reaction. For example, when measured at RT, significant changes in platelet activation can be seen 10 minutes after blood collection [10, 11]. Hence most published flow cytometry protocols for research stated that testing was started “immediately after blood collection”, while some others started testing after 1 hour [8, 9, 12, 13]. To guarantee stable $\alpha\text{IIb}\beta\text{3}$ activation and P-selectin expression, we investigated pre-analytical conditions (temperature and time) and showed that, after blood collection, blood can be stored at room temperature (RT) for 30 minutes up to 4 hours, if it is preheated and tested at 37 °C. More in detail, we found that platelet function measured at RT is indeed affected by storage time of blood, however, the effect of RT on platelet function is reversible if blood is heated at 37 °C before and during analysis. Although these conditions improved the stability of measuring $\alpha\text{IIb}\beta\text{3}$ activation and P-selectin expression in response to platelet agonists, other platelet activation markers may require other sample handling conditions. For example, activated platelets form transient aggregates with monocytes, and these platelet-monocyte aggregates (PMA) increase along with the time after blood withdrawal [14]. Therefore, measuring $\alpha\text{IIb}\beta\text{3}$ activation and P-selectin using our practical sample storage/processing condition (RT/37°C: transport and storage at RT, heating at 37 °C before and during analysis) may be superior in situations where facilities for flow cytometry are not in close proximity to the clinical site and delay in processing is inevitable. Moreover, the relatively wide time frame makes it possible for high through output processing.

Another optimization of the procedure to measure platelet function is the storage of ready-to-use kits (agonist/antibody mixtures) at -20°C. Old school flow cytometry-based platelet function tests are laborious and time-consuming, requiring freshly prepared reaction kit and

well-trained staff to ensure reproducible results. By demonstrating that these prepared ready-to-use kits are stable during one-year storage at -20°C, the previously mentioned challenges can be overcome.

We established reference intervals with the standardized flow cytometric assay in a healthy population with more than 120 individuals. Each laboratory should set up such reference intervals using their own instrument with the standardized assay and this reference intervals can be used to recognize patients with the suspected platelet function disorders (PFDs) locally [4, 5]. Using this standardized assay, we demonstrated that flow cytometric analysis of platelet activation has added value for the routine diagnostic work-up in patients with suspected bleeding diathesis in another laboratory [15]. However, the reference intervals established in our own laboratory cannot be used in other research laboratories, because analysis result from different flow cytometry is not comparable. To realize the interchangeable data between laboratories, more efforts need to be made regarding to the normalization of data from different flow cytometry in the future studies.

New alternative platelet GPVI agonist

Platelet GPVI plays an important role in the formation of thrombosis *in vivo* and the antiplatelet drug (Revacept) has been developed to inhibit the specific receptor and shows promising results in clinical trials [16-18]. Determination of GPVI-mediated platelet activation is not only helpful to diagnose specific platelet disorders but also to monitor antiplatelet therapy by revacept in the future. The finding of a specific binding site of collagen for platelet GPVI - the short glycine-proline-hydroxyproline (GPO)_n tandem repeats- leads to the development of synthetic GPVI ligand, cross-linked collagen related peptide (CRP-XL) [19-21]. However, this CRP-XL is only produced by one noncommercial laboratory [22]. In **Chapter 3** we developed a synthetic triple helical collagen peptide (STH-CP), sharing the same primary sequence as CRP-XL (GPC-(GPO)₁₀-GPCG-amide)₃. Instead of cross-linking GPO repeats at the last procedure of CRP-XL production [22], STH-CP fixed both the C-terminus and N-terminus of peptides on a scaffold (1,3,5-tribromomesitylene (T3)-clips). We validated this STH-CP in the flow cytometric analysis of platelet function and compared its performance with CRP-XL. Although our novel STH-CP was not as potent as the CRP-XL, STH-CP proved to be a stable agonist and gives reproducible platelet activation results. The observed slightly lower efficiency of STH-CP might be caused by the tight binding configuration between two T3-clips. Another explanation for the higher potency of CRP-XL might be that CRP-XL at high concentrations can also bind to activated integrin $\alpha 2\beta 1$ [23]. Further studies are important to determine whether STH-CP binds to $\alpha 2\beta 1$ or not. On the other hand, the adoption of T3-clips to fix the peptides helps to standardize the

production of STH-CP and might contribute to less variation between batches. Further studies still need to be performed to validate our assumption.

The strong correlations between STH-CP and CRP-XL and the similar activation pattern for platelet activation demonstrated the usefulness of STH-CP as a specific GPVI agonist. Moreover, compared to CRP-XL, this new STH-CP showed more stable results during one-year storage at -20°C, which makes the latter a potent applicant for long-term multicenter studies. Revacept has been shown to improve efficacy of antithrombotic treatment without increasing bleeding [18, 24, 25]. Once the Revacept is approved for antithrombotic treatment, monitoring of its efficacy might be of vital significance in certain patients, although it was claimed to have a wide therapeutic window. The effect of Revacept can be estimated, or even quantified by flow cytometric analysis of platelet activation in response to STH-CP, in patients with antiplatelet drug therapy.

Part II Platelet-coagulation interplay in point-of-care settings

Platelet function assessment in anticoagulated blood contributes to the detection and characterization of multiple specific platelet disorders. However, to evaluate the different functions of platelets in haemostasis, coagulation should also be integrated in the platelet function assay.

Platelet-coagulation interplay in (ab)normal haemostasis

As described in **Chapter 4**, platelets and coagulation mutually influence each other under physiological circumstances and an unbalanced platelet-coagulation interaction would result in bleeding or thrombosis. Physiological haemostasis consists of complex interactions between endothelial cells, platelets, von Willebrand factor and coagulation factors. However, platelet plug formation and the coagulation process are usually mentioned as primary and secondary haemostasis, respectively [26]. Platelet function assays usually only focus on platelet thrombus formation, while most coagulation tests investigate coagulation in the absence of platelets. A cell-based model of haemostasis has been mentioned almost two decades ago [27], which proposed that coagulation takes place on different cell surfaces in three overlapping steps: initiation, amplification and propagation. This cell-based model allows a more thorough understanding of how haemostasis works *in vivo* and sheds light on the pathophysiological mechanisms causing certain coagulation disorders [28]. Once coagulation is initiated by tissue factor (TF)-bearing cells, coagulation will be transferred to the membrane of activated platelets, and the amplification and propagation phase of coagulation proceed on the activated platelet surface [29]. Other than providing

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procoagulant phosphatidylserine (PS) and specific binding receptors, activated platelets also facilitate the coagulation reaction by the secretion of various (pro)coagulation factors [30]. In a later stage, anticoagulant factors (such as tissue factor pathway inhibitors (TFPI), protein S, protease nexin-2 (PN2), etc.) released by platelets contributes to modulate coagulation locally.

Patients with most PFDs normally show mild to moderate bleeding [31]. However, coagulation-related thrombocytopathies, such as Scott syndrome (SS) and Quebec platelet disorder (QPD), are associated with relatively severe bleeding diathesis. Despite the fact that venous thrombi are generally accepted to consist of fibrin and erythrocytes, platelets are thought to play a role [32]. Antiplatelet drug aspirin, often used to treat arterial thrombosis, can also lower the risk of recurrent venous thromboembolism (VTE) [33, 34].

Despite the awareness that many steps in platelet thrombus formation are closely connected to different stages of thrombin formation, there are still no accessible tools to study the interplay between platelets and coagulation in a clinical research setting. Whole blood thrombin generation (WB-TG) seems to be a promising tool to study the blood cells in bleeding and thrombosis, though this technique is still in its initial stage [35].

With the development of various novel antithrombotic drugs, new challenges come along with the management of these drugs. It is necessary for the clinicians to have appropriate tools to monitor the therapy and evaluate the risk of thrombophilia or bleeding. Therefore, new and simpler global tests for exploring platelet-coagulation interaction are in urgent need, together with easy-to-operate point-of-care instruments, for routine or emergent clinical settings. Hence, in **Part II**, we aimed to rapidly assess platelet activation in the integrated system of platelets and coagulation. This new system determines the kinetics of ATP released from platelet granules in whole blood using portable luminometers. Platelet activation can be either induced by platelet agonists or by coagulation triggers in recalcified blood. In **chapter 5**, we focused on determining platelet dysfunction (storage pool defect) and identifying heterogenous platelet state in patients using the new method as well as the flow cytometric assay. Herein platelet activation was induced by different platelet agonists. Instead, if coagulation-activating triggers (tissue factor (TF) or Russell's viper venom factor X activator (RVV-X)) are used to activate platelet via thrombin generation, both the coagulation pathway and the platelet activation pathway are evaluated simultaneously. Thus, the anticoagulants effects can also be monitored using this newmethod in **Chapter 6 and 7**.

Potential clinical utility of bioluminescent analysis of platelet activation kinetics

Based on the currently available knowledge and techniques, we modified the bioluminescent platelet activation assay and integrated the coagulation process. The potential clinical purposes of this integrated system include but are not limited to: (1) studying platelet function via different pathways with different platelet agonists; (2) detecting platelet secretion defects; (3) monitoring platelet inhibitors; (4) monitoring coagulation inhibitors.

Platelet secretion defects

Chapter 5 presented the results of a cross-sectional case control study in patients with multiple myeloma (MM). Thrombosis is a common complication of patients, which can be caused by disease-related complications or treatment side-effects [36]. Platelet hyperactivation at baseline and hypo-reactivity in response to agonists exist in newly diagnosed MM patients [37]. Aside from antimyeloma treatment, MM patients with risk of thrombosis also received antithrombotic therapy. Accordingly, platelet function might be altered in MM patients during disease progression and treatment. Although severe bleeding is relatively rare in MM patients, thrombocytopenia is a common complication in MM that may cause bleeding [38]. Interestingly, thrombocytopenia at the start of MM is an independent predictor of mortality [39-41], however, little is known about the platelet function phenotype during the disease progress of MM. We observed reduced platelet activation and secretion by using flow cytometric and bioluminescent assays in MM patients. The proportion of patients with defective platelet secretion (57%) is much larger than that of patients with thrombocytopenia. Only a small group of patients (14%) had both thrombocytopenia and low platelet secretion, while some patients (29%) had both normal platelet count and normal platelet secretion. The high prevalence of acquired platelet secretion defects might indicate the continuous platelet triggering in MM patients, which results in platelet exhaustion [42]. Though patient treatment has been suggested to affect platelet count and function, we did not find the role of medication in low platelet secretion capacity of MM patients.

Severe spontaneous bleeding occurs if platelet count drops below $10 \times 10^9/L$ [43, 44], which is extremely rare in MM patients [45]. Still many MM patients show bleeding symptoms even when their platelet counts $>100 \times 10^9/L$ [46]. Impaired platelet function is clinically important in patients with no or mild thrombocytopenia [46-48]. As an addition to the present platelet function tests, such as flow cytometry and light transmission aggregometry

(LTA), measuring platelet secretion capacity may be another indicator of reduced platelet function [46]. *In vitro* assessment of platelet function in MM patients would aid clinical management by predicting the risk of bleeding and thrombosis [46]. On the other hand, platelet hypo-reactivity might be an indicator of disease progression in MM patients [49]. Based on the heterogenous phenotype of patients in our present study, it might be interesting to perform prospective studies on the association between platelet secretion defects and clinically relevant events.

Anticoagulants monitoring

Platelets are physiological substrates for thrombin, which makes platelet activation a potential read-out for a coagulation process. **Chapter 6 and 7** described studies on ultra-rapid measurement of anticoagulant drugs. Coagulation triggers, e.g., tissue factor (TF) or Russell's viper venom factor X activator (RVV-X), were used to initiate thrombin generation, and subsequently platelet secretion kinetics (ATP release) was measured in response to thrombin-mediated platelet activation.

LWMH monitoring in intensive care unit (ICU) patients

In **Chapter 6** we described the validation of a coagulation-induced platelet activation for monitoring the nadroparin efficacy in ICU patients. Results were compared with the results of standard tests to monitor heparin (activated partial thromboplastin time (aPTT) and chromogenic anti-Xa assay) and with thrombin generation assay (TGA). Parameters of our new test correlated strongly with all the parameters of TGA, which was recommended a superior tool for evaluating the overall effects of anticoagulants on coagulation [50-52]. In contrast, results measured with aPTT and anti-Xa assays only showed partial agreement with the TGA.

The anti-Xa assay is the most commonly used method to measure LMWH and fondaparinux therapy and it gives a clear indication of the *in vivo* pharmacokinetic features of LMWH [53, 54]. However, anti-Xa activity does not give insight in the residual coagulation state of patients (pharmacodynamics). Moreover, the addition of exogenous antithrombin during anti-Xa analysis may result in the overestimation of *in vivo* anticoagulation activity [54]. Although routine aPTT tests can measure the anticoagulant effect of LMWH with sensitive reagents [55, 56], the anticoagulant effect of nadroparin was only marginally detected by aPTT in the present study and aPTT results of patients were within the normal range. Taken together, neither the anti-Xa assay nor the aPTT test were ideal methods for evaluating the overall anticoagulation effect of LMWH.

TG is the preferred method to measure LMWH efficacy because it measures both the anti-Xa and anti-IIa activity of heparins [51]. TG results of patients suggested that this test is extremely sensitive for nadroparin effects in ICU patients. Most patients showed largely, if not completely, inhibited TG curves. Only a few patients showed a normal TG curve, which may indicate low plasma levels of nadroparin or a poor response to nadroparin. Though TGA is a useful tool to assess the overall coagulation capacity and anticoagulant therapy, it is only available in special laboratories, has a long turnaround time and needs trained technicians. These disadvantages limit the application of TGA in clinical settings.

Our newly developed coagulation-induced platelet activation was sensitive to the nadroparin effect. Both the initiation phase and the potential of platelet activation were significantly inhibited. As recalcified whole blood was triggered with TF, the initiation phase and speed of platelet activation also reflect the onset and rate of thrombin generation [35, 57]. A strong correlation between TF-induced platelet activation kinetics parameters and the TG parameters indicated that our new method can also be used to assess the overall anticoagulant effect of LMWH. In addition, the simple one-step procedure and fast turnaround time makes it possible for routine point-of care clinical applications.

As the sample size is relatively small in the present study, it does not allow for subgroup analysis of patients according to their clinical characteristics. Moreover, the unknown coagulation profile of patients before treatment does not allow to evaluate whether the anticoagulant effect is enough. A large study is required to investigate if results of our test can guide the anticoagulant therapy in ICU patients and reduce complications.

Rapid monitoring of DOACs -potential application in urgent situations

Compared to warfarin, DOACs have better risk-benefit profiles and therefore have become more prevalent in recent years [58-60]. However, there are only a few laboratory assays available to measure drug levels in patients. Furthermore, there are concerns amongst physicians about reversing these agents in the event of bleeding or in a patient requiring emergency surgery [61]. The risk of uncontrolled bleeding may also delay urgent surgery or invasive procedures until coagulation is restored [62]. Routine tests are not accurate enough and specific tests are only available in specific laboratories [63, 64]. Thus simple, sensitive and rapid tests are in urgent need to determine the effect of DOACs. **Chapter 7** describes the capability of the new whole blood platelet secretion test to study the effect of spiked DOACs within 10-15 minutes in blood from healthy individuals. Our test might be helpful for quickly detection of DOACs and therefore allow for the immediate intervention. Each anticoagulant varies in its effect on routine and specific coagulation assays [63, 65].

Similarly, different DOACs show different inhibition on coagulation-induced platelet activation in the present study. This coagulation-induced platelet activation test was sensitive enough to measure the effects of both direct thrombin inhibitor (DTI) and direct FXa inhibitors. Moreover, one parameter (AUC) of the platelet activation kinetics, which reflects the platelet activation potential, is extremely sensitive to the low to medium concentrations of DOACs.

Additionally, besides the high inter-individual variation for each DOAC [66], we found that each individual responds differently to DTI or direct FXa inhibitors. This might indicate that some patients may benefit more from a specific DOAC. Individualized treatment with a certain DOAC may help to reduce the risk of thrombotic or bleeding complications [67]. Our findings are the first step into the development of a point-of-care test (POCT) for rapid DOACs detection. The next step is to investigate the value of this new coagulation-induced platelet activation strategy in the detection of DOACs in patients and compare these results with the current golden standard. Here we provided a potential useful tool for both routine management of patient therapy and fast decision-making in emergency clinical settings.

Future validation of coagulation-induced platelet activation in anticoagulant monitoring

The initial validation of coagulation-induced platelet activation is promising to quantify the effectivity of anticoagulants (LMWHs and DOACs) in blood. Our new strategy uses platelet activation as a read-out of thrombin generation, which is more accessible and faster than TG. More validation is needed to establish the clinical applications for this POC strategy.

Specificity for different anticoagulants from the same category

LMWHs from different brands have a different specificity for FXa and FIIa (anti-Xa/anti-IIa activity ratio). LMWHs with the same anti-Xa activity, showed variable inhibition on aPTT and TG [51, 68], stressing the importance of global test in evaluating the overall anticoagulant capacity of LMWH. There was a strong correlation between our new test and TG test for nadroparin function, a similar validation is needed for other LMWHs (enoxaparin, dalteparin, tinzaparin, bemiparin, reviparin, parnaparin, certoparin). If Coagulation-induced platelet activation can differentiate different LMWH which has comparable anti-Xa activity, it may have added value for the individualization of LMWH treatment among patients.

A major advantage of using platelet activation as substrate for thrombin generation is that it is sensitive for detecting the effects of low concentrations of DOACs. An application to

differentiate between DTI and direct Xa inhibitors is in development. This extra option is important in acute or emergency situations, if specific DOAC reversal (Praxbind[®] (idarucizumab) for dabigatran and Andexxa[®] (andexanet alfa) for rivaroxaban and apixaban) is required [69]. Currently the most accurate method for detecting the levels of both DTI and direct FXa inhibitors is the liquid chromatography/tandem mass spectrometry (LC-MS) [70]. Further validation comparing with LC-MS would contribute to the establishment of the specificity and sensitivity of the new method.

Selected patient group with larger sample size

In both the LMWH and DOAC study, we have limited patient validation data. The promising result from our small sample size of patients encourage launching larger study in ICU patients. These studies should include, effect modification by other medication, coagulation profiles and clinical history of patients (bleeding or thrombotic complications). Larger sample size also allows to account for clinical condition of the patient, including renal dysfunction and infection in LMWHs treatment. This is of major importance because LMWHs are cleared mainly by kidney [71] and a high proportion of ICU patients have acute or chronic renal dysfunction [72]. Besides ICU patients, other specific patient group with rising risk of thrombosis or bleeding (such as pregnancy, cancer, liver and/or renal dysfunction) can also be further studied.

Laboratory DOACs measurement before planned surgery is the preferable strategy to avoid bleeding complications [73], though the general stopping rules for elective procedures are also recommended [74]. Therefore, the next step for validating our POC strategy can be performed in patients who are planned for elective procedures, as these patients are more accessible than patients who need urgent or emergency surgery. There is a large quantity of patients who receive different DOACs and require various procedures. Besides routine coagulation assays (aPTT, PT/INR), DOACs levels in these patients are also detected by the gold standard – LC-MS [75]. Implementation of our new strategy to the peri-operative management is easy and enables comparison with these conventional or specific assays.

Conclusion and prospects

In summary, research performed in this thesis contributes to the standardization and innovation of platelet function assays.

The flow cytometric platelet function assay was optimized to a user-friendly platelet function test with acceptable precision. By determining reference intervals in a large

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healthy cohort, flow cytometry becomes more practical in investigating impaired platelet function in patients with bleeding complications. Furthermore, a new collagen receptor agonist with great potency for specific platelet GPVI receptor activation was designed and validated.

In the second part of this thesis, a bioluminescent assay was developed that allows rapid assessment of platelet activation, which brings laboratory tools to study platelet activity closer to the patient. With this assay, we demonstrated that platelet secretion defects are a common complication in MM patients. Furthermore, measuring coagulation-induced platelet activation allows fast heparin monitoring in ICU patients and has great potential for detecting the presence of DOACs in emergency clinical settings.

Our pilot studies with small sample sizes only show the initial near-patient utilities of this new assay. Studies with larger sample size are needed in the future and will provide more profound information. Currently our innovation of the platelet function analysis is just the first step towards the development of a simple global POCT. More clinical research is needed to realize its clinical utility by (1) comparing this new assay with the standard methods in the diagnosis of platelet function defects or monitoring of anticoagulants in patients; (2) standardizing and optimizing the protocol to obtain stable and reproducible results during long-term study; (3) determining whether results of this new assay are associated with clinically relevant events.

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Chapter 9

Nederlandse samenvatting

Nederlandse samenvatting

In dit proefschrift beschrijven we onze strategie om het onderzoeken van de functie van bloedplaatjes dichter bij de patiënt te brengen. In de eerste hoofdstukken beschrijven we verbeteringen en validaties voor het klinisch gebruik van bloedplaatjesfunctie metingen met behulp van flowcytometrie. In het tweede deel van het proefschrift introduceren en onderzoeken we een nieuw testplatform om antistollingsmedicatie, bloedplaatjesremmers en bloedplaatjesfunctie te onderzoeken.

Hoofdstuk 1 van dit proefschrift is een algemene inleiding en geeft een overzicht van de hemostase en van verschillende testen voor het evalueren van bloedplaatjesfunctie of bloedstolling. De nauwe interacties tussen bloedplaatjes- en stollingsactivatie spelen een belangrijke rol bij fysiologische hemostase. Momenteel worden bloedplaatjesfunctie en bloedstolling als afzonderlijke processen onderzocht, waarbij de activering van bloedplaatjes wordt geëvalueerd in bloed met anticoagulantia, terwijl stoltesten worden uitgevoerd in bloedplaatjesarm plasma (PPP). Aangezien trombose wereldwijd veel voorkomt [1], worden antistollingsmedicatie frequent gebruikt om primaire of secundaire trombotische gebeurtenissen te voorkomen. Een probleem van deze antitrombotische therapie is dat het bloedingen veroorzaakt bij een groot deel van de patiënten. Het is dus van cruciaal belang dat klinici over de juiste testen beschikken om de therapie te kunnen instellen op de juiste dosis om zo een optimale effectiviteit en veiligheid te bereiken.

Deel I. Bepaling van bloedplaatjesfunctie met behulp van flowcytometrie.

Flowcytometrie heeft vele voordelen ten opzichte van traditionele bloedplaatjesfunctie testen. Helaas blijft het gebruik van flowcytometrie beperkt tot laboratoria die toegang hebben tot de apparatuur en kennis hebben van de gespecialiseerde technieken.

Hoofdstuk 2 beschrijft de standaardisatie van bloedplaatjesfunctiemetingen met behulp van flowcytometrie met als doel het toegankelijk maken van de techniek voor klinische toepassingen [2]. Preanalyse en analysecondities werden geoptimaliseerd en dit resulteerde in een test met een lage intra-assay variatie (<5%). Verder bepaalden we dat gebruiksklare mixen met bloedplaatjesactivatiemarkers en agonisten stabiel bewaard kunnen worden gedurende 1 jaar bij -20°C. Met deze gestandaardiseerde methode werden normaalwaarden bepaald in een populatie van meer dan 120 gezonde vrijwilligers die in de

toekomst gebruikt kunnen worden voor de diagnose van patiënten met mogelijk een bloedplaatjesfunctiestoornis (PFD).

Hoofdstuk 3 beschrijft de ontwikkeling en validatie van een nieuw peptide om GPVI-gemedieerde bloedplaatjesactivering te onderzoeken. “Cross-linked” collageen-gerelateerd peptide (CRP-XL) is de beste GPVI-agonist die momenteel beschikbaar is maar kan maar door één laboratorium geproduceerd worden. Wij beschrijven een synthetisch triple-helix collageen peptide (STH-CP) met dezelfde primaire sequentie als CRP-XL. De prestaties van dit STH-CP werden gevalideerd in een flowcytometrisch assay en vergeleken met CRP-XL op de volgende vier aspecten: de potentie om bloedplaatjes te activeren, de intra-assay precisie, de stabiliteit tijdens langdurige opslag en de specificiteit voor GPVI-activering [3]. Hoewel de concentratie STH-CP hoger is dan die van CRP-XL om maximale bloedplaatjesactivering te induceren, vertonen beide peptides een vergelijkbaar activeringspatroon. Een sterke correlatie werd gevonden tussen STH-CP en CRP-XL voor zowel $\alpha\text{IIb}\beta\text{3}$ -activering als P-selectine-expressie. STH-CP was stabiel wanneer opgeslaan gedurende 1 jaar in een gebruiksklare mix wat resulteerde in een lagere variatie in bloedplaatjesactivatie tussen de verschillende seizoenen in vergelijking met CRP-XL. Samenvattend kan STH-CP in de toekomst worden gebruikt als alternatief voor CRP-XL bij het onderzoeken van GPVI-specifieke bloedplaatjesactivering of het monitoren van GPVI-specifieke plaatjesaggregatieremmers.

Deel II. Samenspel tussen bloedplaatjes en stolling

Flowcytometrie is een aantrekkelijke techniek om bloedplaatjesfunctie te onderzoeken, maar kan niet worden gebruikt om het samenspel tussen bloedplaatjes en stolling in kaart te brengen. In een uitvoerig literatuuronderzoek (**hoofdstuk 4** [4]) hebben we aangetoond dat de communicatie tussen bloedplaatjes en stolling onmisbaar is voor een goedwerkende hemostase [5] en beschreven we de interacties tussen stolfactoren en bloedplaatjesmembraanreceptoren. Een onevenwichtige interactie tussen bloedplaatjes en stolling kan leiden tot bloedingen of trombotische gebeurtenissen bij verschillende hematologische of andere klinische aandoeningen. Daarom is het essentieel om het geïntegreerde bloedplaatjesactivatie- en stollingsproces te bepalen bij de evaluatie van het hemostaseprofiel van patiënten.

Wij hebben een snelle en gebruiksvriendelijke strategie ontworpen om stolling en/of agonist geïnduceerde bloedplaatjesactivatie te meten in volbloed. De snelheid van de activatie van de stolling als ook de bloedplaatjesfunctie kunnen op hetzelfde platform gemeten worden. Dit platform is buitengewoon handig voor klinisch onderzoek en voor de

ontwikkeling van toekomstige klinische testen, omdat het snel en gebruiksvriendelijk is, het slechts kleine hoeveelheden onbewerkt volbloed vereist en betaalbaar is. In **Hoofdstuk 5** hebben we dit platform gebruikt om de relatie tussen verminderde granule inhoud van bloedplaatjes en multipel myeloom (MM) te bestuderen. Bloedplaatjes zijn niet betrokken bij de pathofysiologie van MM, maar ze kunnen wel inzicht geven in de prognose van de patiënten. Trombocytopenie is een veel voorkomende complicatie en een onafhankelijke voorspeller van mortaliteit bij MM-patiënten. In een case-control studie met 21 MM-patiënten en 21 gerelateerde controles hebben wij laten zien dat MM-patiënten een sterk verminderde δ -granule (ATP) secretie capaciteit hebben. Verdere categorisering van patiënten op basis van hun aantal bloedplaatjes en secretie capaciteit duidde op de heterogene toestand van bloedplaatjes bij deze MM-patiënten: 29% van de patiënten had trombocytopenie, meer dan de helft van de patiënten (57%) vertoonde defecten in de δ -granule secretie en 14% van de patiënten met trombocytopenie vertoonde een normale bloedplaatjessecretiecapaciteit. De hoge prevalentie van defecten in de bloedplaatjessecretie bij MM-patiënten geeft aan dat een gecombineerde meting van het aantal bloedplaatjes en de capaciteit van de bloedplaatjessecretie een vollediger beeld van het bloedplaatjesfenotype geeft dan alleen het aantal bloedplaatjes.

In **Hoofdstuk 6** beschrijven we de stolling-geïnduceerde bloedplaatjesactivatie om de behandelingsintensiteit met laagmoleculairgewicht heparine (LMWH) te bepalen bij patiënten op de intensive care (IC). In vergelijking met normale ziekenhuispatiënten hebben IC-patiënten een hoger risico op veneuze trombo-embolie (VTE) en worden ze meestal behandeld met LMWH. Een anti-factor Xa test en de geactiveerde partiële tromboplastinetijd (aPTT) zijn momenteel twee veelgebruikte testen om IC-patiënten te monitoren op therapeutische dosering van LMWH. Deze testen geven echter een vertekend beeld van de werkelijke heparine functie. Trombinegeneratie (TG) is een globale test om het volledige verloop van de stolling in kaart te brengen en geeft daardoor een meer representatief beeld van de antistollende werking van heparines [6]. Helaas is TG een arbeidsintensieve methode die bovendien relatief veel tijd in beslag neemt. Vandaar dat wij een nieuwe (volbloed) test hebben ontwikkeld die een representatief beeld geeft van de antistollende werking van heparine, maar die ook snel en gemakkelijk uit te voeren is. In deze studie hebben we de nieuwe test gevalideerd voor het bepalen van de efficiëntie van LMWH bij IC-patiënten en vergeleken met een anti-Xa-test, een aPTT en een gekalibreerde TG (CAT). Er was een goede correlatie tussen de LMWH-metingen van onze nieuwe test en TG-metingen. Er was ook een partiële correlatie tussen onze nieuwe test en factor Xa metingen en de aPTT. De partiële correlatie ligt geheel in lijn met onze verwachtingen doordat aPTT en factor Xa metingen slechts een selectief onderdeel van de stolling meten en hierdoor essentiële informatie kunnen missen.

In **hoofdstuk 7** beschrijven we het initiële onderzoek naar de functie van direct werkende orale anticoagulantia (DOAC) in volbloed met behulp van stollingsgeïnduceerde bloedplaatjesactivatie. Volbloed werd verrijkt met verschillende concentraties DOAC's om de dosisafhankelijke inhibitie van de stolling aan te tonen. De initiële data laten zien dat dit platform zowel de inhibitie door factor Xa remmers (rivaroxaban en apixaban) en factor IIa remmers (dabigatran) kan bepalen, variërend van extreem lage DOAC-concentraties tot extreem hoge concentraties. Verdere validatie van deze nieuwe test zal gedaan worden bij patiënten op DOAC in de acute zorg en resultaten zullen vergeleken worden met de standaardmethoden om zo de klinische bruikbaarheid van de test te bepalen.

Hoofdstuk 8 is een samenvatting met de belangrijkste conclusies, implicaties van onze bevindingen en met suggesties voor toekomstige studies. Flowcytometrische analyse van de plaatjesfunctie is gestandaardiseerd en vereenvoudigd tot een meer praktische techniek voor de diagnose van patiënten met een PFD of voor het volgen van patiënten die behandeld worden met bloedplaatjesaggregatieremmers. Volbloed metingen van agonist en/of stolling geïnduceerde bloedplaatjesactivatie biedt een betrouwbare, gebruiksvriendelijke, snelle en kosteneffectieve oplossing om plaatjesfunctie samen met stolling te meten. Toekomstig klinisch onderzoek met grotere steekproefomvang zijn nodig om de toegevoegde waarde te bepalen voor de diagnostiek van PFD's, voor de prognose van hematologische aandoeningen en voor het monitoren van effectiviteit en veiligheid van antistollingsmedicatie en van bloedplaatjesremmers.

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Appendices

Appendix I

中文总结

中文总结

本论文主要描述了我们将血小板功能分析进一步推向病人的研究。在第一部分，我们致力于优化以及验证基于流式细胞术的分析策略以将其推向临床血小板功能检测。在第二部分，我们介绍了一种方便迅捷的平台，用于研究血小板功能以及凝血抑制。

第一章是整篇论文的前言，介绍了关于止血的背景知识，各种评价血小板功能以及凝血的分析方法以及本论文背后的原理。血小板与凝血活化之间紧密的相互作用在维持正常生理止血中起着重要的作用。现阶段对血小板功能分析和凝血过程的研究通常是分离的：抗凝的血液被用于研究血小板活化，而无血小板血浆用于凝血分析。虽然目前也有一些全面的测试方法用于同时研究血小板功能及凝血，但这些方法需要的技术相对复杂。

由于全球范围内血栓的高发生率 [1]，抗凝药物被广泛地应用于预防初发或继发性血栓，而抗凝治疗的副作用-出血-也伴随着而来。因而，采用合适的方法来监测及指导治疗对临床工作者来说非常重要。

第一部分: 流式细胞术分析血小板功能

流式细胞术分析血小板功能具有其他传统的血小板功能分析方法所不具备的多种优势，然而由于对特定技术和设备的需要以及标准化检测方案的欠缺，流式细胞术的应用主要局限在特殊实验室。在论文的**第二章**，我们致力于标准化流式细胞术分析血小板功能的方案 [2]，使其临床应用更高效可行。我们研究了预分析及检测条件对血小板活化标记物的影响并确定了能得到稳定结果的最佳的温度组合（室温+37°C）。优化的方法具有较低的分析批次间变异（<5%）。我们还证明抗体在配制好的即用型试剂中，在-20°C 能稳定保存一年。同时我们还用这一标准化的方法在当地健康人群中（超过 120 人）建立了标准参考范围，为将来诊断本地的疑似血小板功能障碍（PFDs）的患者提供参考。

为了研究血小板膜表面糖蛋白 VI (GPVI) 受体介导的血小板活化，需要选择合适可靠的 GPVI 激动剂用于流式细胞术分析。交联的胶原相关肽 (CRP-XL) 是目前最佳的 GPVI 激动剂，尽管 CRP-XL 具有极低的批次内差异，由于交联步骤的存在其可能存在批次间变异，因而每当使用新一批的 CRP-XL 时需要进行校正。另外 CRP-XL 只由一家实验室生产，可能存在短缺的威胁。在**第三章**中，基于 CRP-XL 的一级结构，我们研发了一

种可替代 CRP-XL 的 GPVI 激动剂-合成的三螺旋状胶原肽 (STH-CP)。我们利用流式细胞术分析来验证了 STH-CP 对血小板的激活能力, 并并与 CRP-XL 进行了比较, 主要涉及到以下四个方面: 激活血小板的能力, 重复实验中的可重复性, 长期储存中的稳定性以及对血小板 GPVI 活化的特异性 [3]。虽然与 CRP-XL 相比, 需要使用更高浓度的 STH-CP 来引起最大程度的血小板活化, 然而 STH-CP 活化血小板的模式与 CRP-XL 相似。我们测试 STH-CP 或 CRP-XL 的稳定性发现, 在配制好的即用型试剂中保存一年, STH-CP 引起的血小板活化具有更低季节性变异。无论是在为期一年的稳定性重复测定实验中还是大样本健康人群的单次测定实验中, STH-CP 和 CRP-XL 激发的血小板活化都具有极强的相关性。总体来说, 我们开发的 STH-CP 是一个合格的 CRP-XL 替代品, 能被用于研究 GPVI 特异性的血小板活化或者在未来用于监控 GPVI 特异性抗血小板药物。

第二部分: 即时检测中的血小板-凝血相互作用

尽管流式细胞术能用于独立评估血小板功能, 但是它要求血液处于非凝状态。血小板和凝血之间的相互作用在维持正常止血功能中不可或缺, 我们在第四章中进行了详细的文献综述 [4]。在差不多二十年前, 基于细胞的凝血概念就已经被提出来 [5], 从那以后, 越来越多的凝血因子和血小板膜受体之间的相互作用被发现。在一个栓子中存在着异质的血小板亚群, 他们在凝血过程扮演着不同的作用: 促凝血血小板调节凝血酶的生成, 包被血小板结合释放的凝血因子 (凝血因子 V, 纤维蛋白原, 凝血因子 XIII 等等), 聚集血小板支持纤维蛋白的形成以及血块回缩 [6]。在血液学疾病或是其他临床疾病中, 血小板-凝血相互作用失衡会导致出血或是血栓。因此, 全面地测定血小板活化和凝血过程对于评价病人的止血概况至关重要。

由于现有的研究血小板和凝血相互作用的技术十分有限, 我们基于生物荧光分析法设计了一个快速简便的研究平台, 用于检测血小板活化时致密颗粒内容物三磷酸腺苷 (ATP) 的动态释放过程。该平台既可用于血小板功能检测, 也能用于分析凝血系统是否处于抑制状态。因其快速, 样品用量小以及可负担等优势, 这一平台可方便地用于临床研究以及开发未来的临床测试方法。在第五章中, 我们利用这一平台研究获得性血小板储存池缺陷和多发性骨髓瘤 (MM) 之间的联系。尽管目前所知血小板在 MM 的发病机理中不起作用, 但是血小板可能对研究病人预后有所帮助。血小板减少症是 MM 病人中的一种常见并发症, 同时它还是 MM 死亡率的独立预测因子。我们展开了一项病例-对照实验来研究 21 个 MM 病人和 21 个相应的对照的血小板功能。与对照相比, MM 病人的整合素 $\alpha\text{IIb}\beta 3$ 活化以及致密颗粒释放均受损。根据血小板

数目以及血小板释放能力对 MM 病人进行进一步分类发现这些病人具有异质的血小板状态: 29%的病人具有血小板减少症, 然而有超过一半的病人 (57%) 具有血小板释放缺陷, 有趣地是, 还有 14%的病人虽然有血小板缺少症但是他们的血小板释放功能正常。MM 病人中血小板释放缺陷的高发生率表明联合检测血小板数目以及血小板释放能力与仅检测血小板数目相比, 能提供关于疾病更全面的理解。

使用组织因子 (TF) 作为触发剂时, 凝血系统被激活, 继而导致血小板激活和储存池内容物释放, 所以此方法能间接指示凝血系统的凝血潜能。在第六章中, 我们利用凝血诱导血小板活化的方法来测定重症监护室 (ICU) 病人体内的低分子量肝素 (LMWH) 水平。与普通住院病人相比, ICU 病人因为长期卧床以及相关并发症有更高的静脉血栓风险, 因而需要接受 LMWH 治疗。抗凝血因子 Xa (anti-Xa) 分析法以及活化部分凝血活酶时间 (aPTT) 是两种常用的监测 LMWH 的方法, 然而这两种方法都有一定的局限性。我们的新平台检测凝血诱导的血小板活化反映了凝血酶的生成过程。凝血酶生成 (TG) 分析法是一种整体测试法, 这一方法被认为是一种出色的抗凝治疗检测方法 [7]。在这一章研究中, 我们验证了新平台检测 ICU 病人体内的 LMWH (nadroparin) 作用并与 anti-Xa、aPTT 以及校正的凝血酶生成分析法 (CAT) 作比较。anti-Xa 分析法结果表明 ICU 病人体内的血浆 anti-Xa 活性都在目标治疗范围内, 然而 aPTT 结果与正常参考范围相比并没有延长。TG 分析法结果表明该方法对 nadroparin 十分敏感, 并且能评估整体凝血能力, 但是 TG 通常只在实验室才能进行。我们的新分析方法同样对 LMWH 敏感: 凝血诱导的血小板活化的触发以及潜力均被抑制。凝血诱导的血小板活化实验中的所有参数与 TG 各参数间有强烈的相关性, 这表明这一新平台能用于测定 LMWH 的整体抗凝作用。由于操作简单, 所需样品量少以及周转时间短, 这一新方法在未来床旁检测 LMWH 治疗上有很大的潜力。

凝血诱导的血小板活化的另一种可能的用途是快速检测直接口服抗凝药 (DOACs)。DOACs 因其优越的风险收益特征被越来越多地用于预防初发或继发性血栓。DOACs 有着较宽的治疗窗以及可预测的药代动力学, 因对 DOACs 的常规检测通常不是必须的, 然而在某些紧急情况下, 如果医护人员了解病人的 DOACs 使用情况, 可以避免围手术期严重的出血并发症。目前并没有在急救条件下快速评估 DOACs 的即使检测方法, 在第七章中, 我们测试了凝血诱导的血小板活化这一方法检测 DOACs 作用的能力。我们利用罗素毒蛇毒素因子 Xa 激活剂 (RVV-X) 来触发凝血过程, 凝血酶的形成通过血小板 (ATP) 释放来间接量化。我们将不同浓度的 DOACs 加入到全血中来测定剂量依赖性。在凝血诱导的血小板活化的所有参数中, 曲线下面积 (AUC, 这一参数反应血小板在特定的时间内活化的潜力), 最具代表性地定量不同浓度的 DOACs 的作用。

未来需要进一步在病人中验证这一新方法并与标准方法进行比较，以确立该新方法的临床用途。

最后, **第八章**总结了主要的结论, 这些结论可能的启发以及未来研究的方向。我们将流式细胞术分析血小板功能这一方法进行标准化与简化, 使其成为更实用的技术, 以便用于检测 PFDs 病人或是监控抗血小板治疗。我们基于生物荧光分析法开发的新测试平台提供了一种简单快速的方式来评估血小板功能以及凝血过程。这一新方法的初步成果表明其能用于检测血小板释放缺陷以及监控抗凝治疗。尽管如此, 为了验证该新平台对 PFDs 的常规诊断检查的附加值, 同时建立血小板释放缺陷和血液疾病诊断及分层之间的联系, 未来的大样本临床研究必不可少。另外, 在管理抗凝治疗的过程中, 还需要进一步地研究凝血诱导的血小板活化的测试结果与临床相关表征之间的联系。

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Appendix II

Valorisation

Valorisation

This thesis aims to bring platelet function analysis closer to patients to optimize the patient diagnosis and treatment. Due to the limited tools for routine evaluation of platelet function in patients with bleeding diathesis, there is underestimated prevalence of platelet function disorders (PFDs). On the other hand, with the development and worldwide use of new antithrombotic drugs, new challenges come along with the clinical management of patients receiving antithrombotic therapy. Our research focuses on improving our knowledge of platelets in clinical diseases by the adoption of standardized and simplified strategies. Valorisation is an official name used by the Dutch government since 2009, to describe the process of creating value from knowledge by making knowledge suitable and/or available for economic and/or societal use and translating that knowledge into products, services, processes and entrepreneurial activity [1]. As our new strategies for platelet function assessment is still in its infancy, it takes time to realize its societal and economic benefit. In this chapter, we will introduce the scientific and societal background regarding our studies and discuss the potential contribution of scientific knowledge obtained from our studies to the development of research strategy and improvement of clinical practice.

Platelet function disorders (PFDs)

Underdiagnosis and burden

The advances from research on platelet molecular and cell biology and (patho)physiology over the last few decades have not been adequately translated to clinical laboratory diagnosis [2]. The prevalence of inherited PFDs (IPFDs) among general population has not been established, likely because testing is time consuming and requires rapid processing of freshly collected blood samples [3]. The diagnosis of IPFDs is challenging due to the lack of defined diagnostic criterion. Prospective studies evaluating patients with bleeding disorders identified that IPFDs are as common as, or even greater than, von Willebrand disease (VWD) [4-8]. Among various IPFDs, platelet secretion and signal transduction defects are the most frequent [9, 10]. It was speculated that prevalence of some mild platelet secretion defects can be up to 1/100 [11]. The laboratory diagnosis of IPFDs places a significant burden on hematology laboratories [12].

Some IPFD-associated bleeding symptoms such as, excessive bruising and prolonged menses, are also quite common in the general population [13]. IPFDs are more commonly diagnosed in women, likely because the added haemostatic challenges of menstruation and

childbirth affect the burden of living with a congenital platelet disorder and the likelihood of been referred for evaluation [14, 15]. A bleeding tendency in patients with a suspected or confirmed congenital PFDs impacts healthy status-related quality of life (HR-QoL), such as, decreased physical functioning, limitations in daily activities and social activities, decreased energy levels and fatigue, etc [16]. Even after extensive laboratory testing, as recommended by the International Society on Thrombosis and Haemostasis (ISTH) [17], many patients remain unrecognized. Accurate diagnosis can address prompt and appropriate treatment of patients with suspected IPFDs and minimize the associated bleeding risk, and therefore improve HR-QoL of these patients [18].

Added values of our strategies to the diagnostic work-up of PFDs

A half century after light transmission aggregometry (LTA) was developed [19], it remains the most commonly used method for evaluating platelet function, and its main use is to assess bleeding disorders. As LTA is a time-consuming and technically challenging technique that is affected by many pre-analytical and analytical variables, the methodological standardization of LTA is necessary [20]. The guidelines for standardization of LTA was provided by ISTH in 2013 [21], followed by the international guidelines on the diagnostic approach to inherited PFDs [17]. In the first-step screening of inherited PFDs, flow cytometry plays the complementary role for LTA in the assessment of platelet granule release and major platelet surface glycoproteins. Compared to LTA, Flow cytometry has multiple advantages in platelet function analysis: higher sensitivity, less sample volume needed and applicable to low platelet count samples. Therefore, it is quite useful for thrombocytopenia or pediatric samples [22]. Platelet flow cytometry has versatile utilities in diagnostic and therapeutic hematology [23], however, the complexed old-school sample processing and unstandardized technique hamper its application in wider clinical practice. As depicted in **Chapter 2**, our standardized protocol pushes flow cytometry towards the diagnostic routine for patients with PFDs. The sample processing and analysis can be performed in routine clinical scenarios by clinical lab technicians after simple training. Flow cytometry has shown added values in diagnostics of suspected IPFDs [24, 25]. The pre-prepared test kits also make high throughput analysis of platelet function possible.

Patient with inherited platelet GPVI defect have a mild to moderate bleeding phenotype [4]. The deficiency of platelet GPVI can be homozygous (GP6^{hom}, an adenine insertion in exon 6 of the GP6 gene) [26] or heterogenous (GP6^{het}) [27-29]. GP6^{hom} platelets completely lack GPVI surface expression, however, GP6^{het} might have incomplete deficiency. GPVI quantification by flow cytometry can first screening the possible GPVI defects patients for further gene detection. The GPVI-specific agonist is critical for assessment of GPVI

deficiency by flow cytometry. The current widely used GPVI agonists (CRP-XL) was only produced by one lab and has variation between batches because of the final unstandardized cross-link step. The synthetic triple-helical collagen peptide (STH-CP) designed by us, as described in **Chapter 3**, provides a good alternative for CRP-XL by standardization of the synthesis procedures, though further studies are still needed to determine the inter-batch variation of STH-CP. CRP-XL is instable after dilution. The relatively low seasonal variation of our STH-CP compared to CRP-XL implies that our new peptide might be more suitable for the long-term clinical trials. Flow cytometric analysis of platelet GPVI-mediated function facilitates the investigation of GPVI-associated defects in research and diagnostic laboratories.

Flow cytometry provides a superior way to evaluate platelet function in anticoagulant blood. We developed another new strategy, which is sensitive, simple, fast and need minimum sample volume, to investigate platelet secretion kinetics in coagulant blood. When coagulation triggers are used, coagulation is initiated first and then platelets are activated by generated thrombin. Thus, coagulation can be evaluated together with platelet activation. However, if direct platelet agonists are used, platelet dysfunction can be assessed directly. The initial validation in a specific patient population – multiple myeloma (MM) (as presented in **Chapter 5**) - demonstrated the usefulness of this new strategy in determining acquired platelet secretion defects in patients with hematologic disorders as well as thrombocytopenia. Platelet dysfunction might be an important indicator of disease progress or prognosis. The analysis platform is portable, affordable and can be easily implemented to any clinical laboratory. Such strategy would contribute to further understanding of platelet function in various clinical diseases.

Antithrombotic therapy

Deadly burden of thrombosis

Thrombosis can be divided into arterial and venous thrombosis. Ischemic heart disease and stroke, comprise the major arterial thrombosis, account for 1 in 4 deaths worldwide in 2010 and are one of the leading causes of years of life lost due to premature mortality [30, 31]. Data about the worldwide burden of venous thromboembolism (VTE) is limited, probably due to the multifactorial nature of death attributable to VTE [31]. In six EU countries, the estimated attack rate was 148 per 100,000 person-years and 95 per 100,000 person-years for deep vein thrombosis (DVT) and pulmonary embolism (PE), respectively, [32]. Retrospective data showed that around 5%-10% of in-hospital death are a direct result of

PE [33, 34]. In addition to the risk of sudden death, VTE is associated with long-term morbidity and reduced survival [35-37].

Management of antithrombotic therapy

Treatment paradigms have been changing with the evolving knowledge about the roles of platelets in venous thrombosis and thrombin in arterial thrombosis [38]. Despite new antithrombotic drugs, such as direct oral anticoagulants (DOACs) and newer antiplatelet drugs, show better benefit-risk profiles compared to traditional antithrombotic agents, there remains an unmet clinical need for appropriate strategy that can achieve a balance between the benefit of treatment and the risk of thrombosis and bleeding [38, 39]. The standard dose of antithrombotic agents, which suits normal patients, might be underdose or overdose for specific patients. Monitoring of antithrombotic therapy is helpful for certain patients to sustain the efficacy without excessive bleeding. Indication for determining antithrombotic drug levels can be considered in 2 categories: (1) determine the optimal dose for some patient populations, such as those with weight extremes, renal insufficiency, etc.; (2) critical clinical situations, such as major bleeding or need for emergent or elective surgery [40].

Monitoring of low molecular weight heparins (LMWHs) in specific patients

Due to the high risk of thromboembolic disease for hospitalized patients, LMWHs are commonly used in clinical practice. Critically ill patients in intensive care unit (ICU) have a greater risk of thrombotic events and are usually treated with LMWH [41]. Because ICU patients have altered pharmacokinetic and pharmacodynamic variability, this can lead to unpredictable drug effects and increased potential for adverse drug effects [42]. Therefore, monitoring of antithrombotic therapy is necessary for these patients. Although anti-Xa is widely available for LMWH monitoring in clinical laboratories, it has important limitations, which might affect the interpretation of results [43]. The development of our simple and cost-effective strategy contributes to more efficient and safer clinical anticoagulation management. The findings presented in **Chapter 6** indicated that our new strategy (coagulation-induced platelet activation) has great potential for determining the overall anticoagulation effects of LMWH as it strongly correlated with the results of TG test. This new strategy may be used to differentiate the effects of different LMWH and therefore screen the most effective for individual patients in the future. Furthermore, this strategy may be easily implemented to routine clinical anticoagulation management as it is technically simple and fast. Compared to anti-Xa and TG assay, our new strategy uses whole blood which is closer to physiological conditions and might provide more comprehensive

information. However, it is unknown whether results of this new strategy are clinically relevant and further large studies are needed to investigate whether there is any association between experimental results and bleeding or thrombotic events.

Fast clinical decision-making based on results of haemostasis assays

For patients who receive DOAC for long-term prevention of VTE recurrence or stroke in AF, urgent surgery or intervention is necessary under certain conditions, such as life-threatening haemorrhage, thrombotic events or trauma. It is important to determine the presence of DOACs as quickly as possible in these patients. The availability of rapid turnaround DOAC tests could help clinicians with fast decision-making: proceed with surgery or use costly reversal agents. Although DOAC-specific assays are reliable and reproducible in different clinical laboratories [44], these assays use plasma instead of whole blood and have relatively long turnaround time (approximately 35 minutes) [45]. Point-of-care tests, which use small quantities of whole blood to assess the presence of DOACs, are under various stages of development. Our new strategy was sensitive to detect the effects of low-to-medium concentrations of DOACs (as described in **Chapter 7**). It is promising to launch clinical trial to validate the utility of this new strategy in monitoring of DOACs and compared with the commonly used method, high-performance liquid chromatography-coupled tandem mass spectrometry (HPLC-MS/MS), which is expensive, complex and time-consuming.

Conclusion

Research performed in this thesis aims to bring platelet function assessment to the patients via standardization and innovation of the existed technologies. Our findings here is just the first step to the final goal: fulfill the clinical values (diagnostic, monitoring, etc.) of our optimized tests and improve the management of patients.

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Appendix III

Curriculum vitae

About the author

Yaqiu Sang was born on September 8th, 1990 in Jingzhou, China. She completed her pre-university education in her hometown. In 2009, She was admitted by Hainan University (Haikou, China) to study Food Quality and Safety in the School of Food Science and Engineering. After completing her bachelor's degree, she became a master student of Jiangnan University (Wuxi, China) in 2013. During her master (2013-2016), she studied cytotoxicity of mycotoxin and the protective roles of phytochemicals in the School of Food Science and Technology. After she obtained her master's degree in 2016, she came to the Netherlands with the State Scholarship (China Scholarship Council) and became a PhD candidate at the department of Biochemistry of Maastricht University and at Synapse Research institute. During four years' PhD training, she worked on the topic "Innovation and standardization of new-patient platelet function assays" under the supervision of prof. dr. H. ten Cate, dr. B. de Laat and dr. M. Roest.

Appendix IV

List of publications

List of Publication

Publications

Huskens D, **Sang Y**, Konings J, van der Vorm L, de Laat B, Kelchtermans H, Roest M. Standardization and reference ranges for whole blood platelet function measurements using a flow cytometric platelet activation test. *PLoS ONE* 2018; 13(2): e0192079.

Sang Y, Huskens D, Wichapong D, de Laat B, Nicolaes GAF, Roest M. A synthetic triple helical collagen peptide as a new agonist for flow cytometric measurement of GPVI-specific platelet activation. *Thromb Haemost* 2019; 119: 2005–2013.

Sang Y, Roest M, de Laat B, de Groot PG and Huskens D. Interplay between platelets and coagulation. *Blood Reviews*: Available online 12 July 2020, 100733.

Sang Y, Remijn JA, Fijnheer R, Smit K, Korporaal SJA, Urbanus RT, Huskens D, Konings J, Li L, de Laat B, Roest M. High prevalence of acquired platelet secretion defects in multiple myeloma. *Submitted*.

Sang Y, Mulder M, Huskens D, de Laat B, Sels JW, Roest M. Rapid monitoring of nadroparin efficacy in patients with therapeutic treatment in intensive care unit. *Manuscript in preparation*.

Sang Y, Huskens D, ten Cate H, de Laat B, Roest M. Rapid monitoring of direct oral anticoagulants. *Manuscript in preparation*.

Poster presentations

Sang Y, Wan J, Roberts L, Huskens D, Hendrix W, Konings J, Ow T, Rabinowich L, Barbouti O, de Laat B, Arya R, Patel V, Roest M, Lisman T, Bernal W. The synergy between coagulation and platelet function in chronic liver disease patients. International Symposium on Coagulation and liver disease (ISCL), Groningen, the Netherlands, 2019.

Sang Y, Huskens D, Wichapong K, de Laat B, Nicolaes G, Roest M. A synthetic triple helical collagen peptide as a new agonist for flow cytometric measurement of GPVI specific platelet activation. International Society on Thrombosis and Haemostasis (ISTH), Melbourne, Australia, 2019.

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Sang Y, Huskens D, Wichapong K, de Laat B, Nicolaes G, Roest M. A synthetic triple helical collagen peptide as a new agonist for flow cytometric measurement of GPVI specific platelet activation. Nederlandse Vereniging voor Trombose en Hemostase (NVTH), the Netherlands, 2019

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