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Counting and Tracking

Development and Use of New Methods for Detailed Analysis of Thrombus Formation

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Cover: Fluorescently labelled platelets in a flow chamber experiment

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During the course of the research underlying this thesis, Kjersti Tunströmer was enrolled in Forum Scientium, a multidisciplinary graduate school at Linköping University, Sweden.

Abstract

Blood platelets are a part of the complex system called haemostasis aimed at ensuring our blood's continuous transport of oxygen and nutrients throughout the body. The transport is ensured by limiting blood loss due to vessel injury and in this process, the platelets form a plug in the damaged area, reinforced by the formation of fibrin. Similar mechanisms may cause thrombus formation, often triggered by atherosclerotic plaque rupture, causing vessel occlusion, embolism or ischemia, which may cause irreversible damage to the heart or the brain.

Platelet research is crucial for improved prevention and treatment of thrombotic disorders. For such research, flow chambers are an interesting tool for studies of platelet adhesion, aggregation and thrombus formation under similar flow conditions as in the blood vessels, which is important, as the flow affects the mechanisms involved in both haemostasis and thrombosis. Flow chambers can be designed for specific purposes, such as for the study of haemostasis at specific flow conditions or to evaluate drugs or biomaterials. In this thesis, our aim has been to improve the usefulness of in-vitro flow chambers and develop a more robust and informative image analysis of such experiments.

Initially, we introduced an internal control within each flow chamber experiment, thereby reducing the experimental variance caused by unknown factors. Furthermore, control and sample were thus exposed to identical experimental settings. By using platelet count as quantification of thrombus formation we introduce a method of analysis with increased or similar sensitivity to today's standards. The platelet count method facilitated comparison of results obtained in different types of flow chambers by an absolute scale of measurement, independent of user settings. The platelet count method was further developed so that additional parameters could be analysed, providing more information about each individual platelet and the overall thrombus. The parameters analysed included platelet stability, height, movement and contraction. The method was used to evaluate how the pharmacokinetics of a reversible (ticagrelor) and irreversible (prasugrel) platelet ADP-receptor inhibitor affected the overall thrombus formation. Especially, how a non-inhibited platelet fraction, formed between drug administrations of irreversible inhibitors, affected thrombus formation. In addition, we sought to understand the regulation of the thrombin receptor, PAR1, expression in cancer cells. We found the microRNA miR20b to be anti-oncogenic through its downregulation of PAR1 expression.

This thesis contains numerous flow chamber experiments. However, for further use and full potential of the method increased standardisation is important. Our work regarding the quantification and analysis of flow chamber experiments will contribute to a more robust analysis and maybe even more important, provide new and detailed information on thrombus formation.

Populärvetenskaplig sammanfattning

Våra celler är i konstant behov av syre och näring för att kunna fungera. Transporten av syre och näringsämnen sköts av blodet som når ut i kroppen via våra blodkärl med hjälp av hjärtats slagkraft. Om dessa kärl går sönder eller täpps till kan stora skador uppkomma, så som stora blödningar, slaganfall eller hjärtinfarkter.

I vårt blod finns så kallade trombocyter (blodplättar) och deras huvudsyfte är att skydda kroppens blodtillförsel genom att täppa igen hål i blodkärlen. Trombocyterna rör sig längs kärlens vägar och om de stöter på en skada i kärlet så klumpar de ihop sig och sätter igång en kedja av reaktioner vars syfte är att skapa en propp i hålet för att stoppa blödningen. Denna process kallas haemostas och är livsviktig för oss.

I en frisk människa ska hemostasen endast ske då blodkärlet är skadat, men samma process kan på grund av flera omständigheter bilda en propp som täpper till våra kärl och därmed syretillförseln för de celler som finns nedströms från en sådan tilltäppning. Om en sådan propp uppstår eller fastnar i de mindre kärlen runt hjärtat eller i hjärnan kan stora skador uppkomma på grund av syrebrist.

Eftersom trombocyter både hindrar blödning och kan orsaka proppar är det svårt att hitta en behandling mot proppar som inte samtidigt ökar risken för blödningar. Genom att förstå mer om trombocyter och deras funktion ökar möjligheterna att kunna hitta lämpliga behandlingar och förstå mer om mekanismerna bakom några av våra vanliga folksjukdomar.

I denna avhandling har syftet varit att förbättra de metoder som används för att forska på trombocyter och hur trombocyterna fungerar när de träffar på en skada i våra kärl. Vi har använt så kallade flödeskammare för att titta på trombocyter med hjälp av mikroskopi. En flödeskammare är en smal kanal, som efterliknar ett blodkärl. I denna flödeskammare finns på en punkt samma proteiner som exponeras vid en kärlskada, detta göra att trombocyterna kan börja skapa en propp. Genom dessa flödeskammare drar vi sedan blod med en pump och genom att märka trombocyterna kan vi sedan se dem i mikroskopet när de fastnar i flödeskammaren. Med mikroskop kan man sedan ta bilder under hela skedet och även i höjdled så att man kan se vad som händer i hela kanalen.

Flödeskammare har funnits en tid och används av flera forskargrupper. Det jag har fokuserat på under avhandlingsarbete har varit att göra så att man kan få ut mer information och kunskap ur denna typ av experiment. Målet har också varit att utveckla en metod för att tolka de mikroskopibilder som tas under ett flödeskammarexperiment och att den metoden ska göra så att resultaten går att jämföra mellan olika forskargrupper.

Genom denna metod kan vi lära oss mer om de trombocyter som hindrar blödning och hur de fungerar när de klumpar ihop sig och bildar den propp som kan stoppa blödningar eller orsaka skada när de täpper igen våra kärl. Med hjälp av den metod som utvecklats under detta avhandlingsarbete finns nu möjligheten att studera detaljer som tidigare inte har kunnat undersökas. När ett experiment görs, fås efter analysen en stor mängd information, i det finns det information om enskilda trombocyter och deras plats i proppen.

Denna information kan sedan användas till exempelvis för att följa de rörelser som trombocyter gör, undersöka hur olika läkemedel påverkar hur kompakt och stabil en propp blir och hur nära trombocyterna är varandra i proppen. Med hjälp av den information som fås kan händelseförloppet också återskapas i detalj genom 3D-bilder och filmer eller genom att skapa bilder som motsvarar genomskärningar av den propp som bildats. Med hjälp av sådana bilder kan det vara lättare att förstå vad som händer.

Metoden som utvecklats i detta avhandlingsarbete kan hjälpa forskare runt om i världen att lära sig mer om hur våra trombocyter funkar och komma till användning när man vill testa hur nya läkemedel verkar och då kunna utvärdera dem på ett bra och informativt sätt. Med mer kunskap och bättre metoder för att få ny kunskap kan vi förhoppningsvis hitta nya och bättre behandlingar i framtiden.

List of Papers

Paper 1

An internal control method to reduce variability in platelet flow chamber experiments

Kjersti Claesson, Katarina Bengtsson, Lars Faxälv, Nathaniel D. Robinson, Tomas L. Lindahl

In manuscript

Paper 2

Counting the platelets: a robust and sensitive quantification method for thrombus formation

Kjersti Claesson, Tomas L. Lindahl, Lars Faxälv

Thrombosis and Haemostasis, 2016; 115: 1178–1190

Paper 3

Quantification of Platelet Contractile Movements during Thrombus Formation

Kjersti Tunströmer, Lars Faxälv, Niklas Boknäs, Tomas L. Lindahl

Thrombosis and Haemostasis, *in press*, 2018

DOI: 10.1055/s-0038-1668151

Paper 4

Effects of a drug-free platelet fraction during P2Y₁₂ inhibition during thrombus formation in flow chambers

Kjersti Tunströmer, Lars Faxälv, Tomas L. Lindahl

In manuscript

Paper 5

miR-20b regulates expression of proteinase-activated receptor- 1 (PAR- 1) thrombin receptor in melanoma cells

Amina Saleiban, Lars Faxälv, Kjersti Claesson, Jan-Ingvar Jönsson, Abdimajid Osman

Pigment Cell and Melanoma Research, 2014; 27: 431-441

Abbreviations

AC	Adenylyl Cyclase
ADP	Adenosine 5'-diphosphate
ASA	Acetylsalicylic Acid
ATP	Adenosine 5'-triphosphate
BSA	Bovine Serum Albumin
cAMP	cyclic Adenosine Monophosphate
CTI	Corn Trypsin Inhibitor
ECM	Extracellular matrix
GP	Glycoprotein
mRNA	messenger Ribonucleic Acid
NET	Neutrophil Extracellular Trap
NO	Nitric Oxide
OCS	Open Canicular System
PAR	Protease-Activated Receptor
PDMS	Polydimethylsiloxane
PLC	Phospholipase C
PGI ₂	Prostaglandin I ₂
PRP	Platelet Rich Plasma
PSGL-1	P-selectin glycoprotein ligand-1
ROCK	Rho-associated protein kinase
TF	Tissue Factor
TxA ₂	Thromboxane A ₂
VASP	Vasodilator-stimulated phosphoprotein
VWF	Von Willebrand Factor

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Introduction

The cells in our bloodstream transport oxygen, nutrients, remove waste products and defend the body against foreign microbes or substances. The constant flow of blood is crucial for other cells, organs and the whole individual. Haemostasis is the process that ensures that the blood stays within the blood circulation and that any blood loss due to injury is minimised. Haemostasis is generated by a complex interaction between blood platelets and the coagulation system.

The same interactions might instead cause injury when activated within the blood vessel and there form a plug that may constrain or block the blood flow, causing ischemia to areas downstream of the plug, the thrombus.

The delicate balance of the haemostatic system complicates the treatment of both bleeding and thrombosis as the treatments for either condition is likely to cause serious adverse events.

Further studies of both processes, thrombosis and haemostasis, are therefore crucial to investigate the mechanisms that might hinder the formation of thrombosis without hindering the role of the haemostasis. The research area has been studied for more than 100 years and there is a multitude of methods and experimental models that have been used in significant discoveries. However, many methods and models fail to consider and recreate the flow conditions that are present in the vasculature and the shear stress on the platelets and surrounding cells. The blood flow and shear stress affect several aspects of haemostasis and thrombosis; it influences the mechanisms for platelet adhesion and aggregation, it affects the transport of molecules to and from the platelet plug, it affects the platelets and endothelial cells themselves and in addition, it may also have an effect on the efficacy of specific medications.

In this thesis, we have primarily used so-called flow chambers. An experimental system where platelet adhesion, aggregation, and coagulation can be studied under different flow conditions. The flow chambers are highly versatile and can be adapted to answer a multitude of research questions. One issue with these methods is the lack of standardisation and it is therefore hard to compare and validate experiments performed at different laboratories. The difference between the flow chamber use concerns the design, quantification methods and experimental parameters such as microscope settings. In the work presented in this thesis, we have aimed at developing a robust quantification method independent of the user and experimental settings. By introducing an absolute unit of measurement, that is not defined by the user settings, the method enables easier comparison and validations and introduces a common scale of measurement.

The methods used to evaluate thrombus formation, in-vitro or in-vivo, are aimed at quantifying the thrombus size. Such a measurement does not reflect the dynamics, instability, contraction or movements within the thrombus. One

additional focus of this work has been to evolve the use of flow chambers so that the number of parameters and amount of information, that such experiments can generate, is increased.

New parameters of thrombus formation allow for the evaluation of thrombus stability, platelet movements, thrombus contraction. Quantifying and characterising thousands of platelets throughout the thrombus mass generates knowledge about the overall thrombus characteristics.

The methods presented herein are also suitable for clinical research questions and have been used to evaluate the difference in inhibition of thrombus formation, between irreversible and reversible P2Y₁₂ inhibitors.

Background

Haemostasis

Haemostasis ensures that our blood is maintained flowing within our blood vessels. There are three vital parts in haemostasis; to seal damaged vessels and thereby limit blood loss, to keep the blood as a fluid and to remove the blood clot at the resealed blood vessel (1).

Starting with the first task, to seal damaged vessels, there are two main actors, the blood platelets, which are nonnucleated cell fragments that are derived from megakaryocytes residing in the bone marrow, and the coagulation cascade. These are often described as two separate parts, with platelets as the primary haemostasis and the coagulation cascade as the secondary part of haemostasis. During the primary haemostasis platelets will adhere and aggregate at the site of damage and form a temporary plug limiting the blood loss. The secondary haemostasis is aimed at reinforcing the formed platelet plug with a fibrin mesh, which is achieved as the endpoint of the coagulation cascade, initiated by the exposure of tissue factor (TF) during a vascular injury (1). The cascade will by a number of clotting factors and cell surfaces, initiate and amplify the formation of thrombin, the enzyme capable of cleaving fibrinogen into fibrin and activating platelets. Platelets have an important role in the coagulation itself as a procoagulant surface required for efficient coagulation (2). The need for platelet surfaces contains the coagulation response to the site of the platelet plug (3).

The second task of haemostasis is to keep the blood in a fluid state, which means to contain platelet activation and coagulation to the site of vascular damage only. Here the interplay between the platelets and the vessel wall is crucial. An intact and healthy endothelium will keep the platelets in a quiescent state by the release of substances such as NO and PGI₂. In contrast, a damaged endothelium may activate the platelets (4). As previously mentioned it is important that platelet aggregation and coagulation are contained and do not spread downstream of the injury. The spreading is regulated by negative feedback signals and through the structure of the platelet plug (4, 5).

After the vessel repair, the blood clot is no longer needed and should be dissolved. The principal step in this process is to degrade the fibrin, and the process is referred to as fibrinolysis. This part of haemostasis is not a focus of this thesis and will therefore not be discussed further.

The primary haemostasis

Damage to the endothelium will expose extracellular matrix (ECM) proteins, such as collagen, VWF and fibronectins (6), to the bloodstream and especially to the platelets that circulate at the outermost part of the blood vessel (Figure 1).

Figure 1. Damages to the blood vessel causes exposure of extracellular matrix proteins to the passing platelets (green).

As the platelets come into contact with the exposed ECM, the platelets are activated and adhere to the substrate (Figure 2). This phase is the first step of the haemostatic response. The exposed ECM contains a variety of proteins that may interact more or less with platelets. Other proteins that are present in the bloodstream, such as fibrinogen and VWF may also be immobilized on the exposed surfaces (6). The main adhesive substrates are VWF and collagen, however, their relative function and importance to adhesion and activation depend on the shear rate. At shear rates above approximately $500\text{--}800\text{s}^{-1}$, VWF is essential for initiating adhesion, VWF is present in plasma and can be adsorbed to exposed collagen. The initial platelet tethering is mediated by the interaction of the VWF-A1 domain and the platelet receptor GPIb α . The adhesion has a fast off-rate and therefore platelets roll over the surface, the slower pace allows for additional platelet receptors to interact with the ECM. After the initial tethering, GPVI can bind to collagen. However, the binding is not enough for stable adhesion, but is a strong platelet activator, causing integrins to shift into a high-affinity state and the platelet to release secondary mediators, such as ADP.

Figure 2. Platelet adhesion and activation induced by the exposed ECM proteins. Collagen, fibronectin, laminin, et cetera are exposed during vessel injury and additional proteins such as VWF and fibrinogen are immobilized on the exposed surface. VWF and collagen are important for the initial tethering and activation of the platelets.

The activated integrins are the final step needed for firm adhesion where $\alpha_2\beta_1$ binds to the collagen substrate and causes additional activation. Additional integrins may also interact with fibronectin or laminin (7, 8). At lower shear rates the VWF-GPIb α binding may not be required, instead, GPVI and $\alpha_2\beta_1$ may bind collagen directly or $\alpha_{IIb}\beta_3$ (GPII/IIIa) may bind fibrinogen, even without prior activation.

Platelet activation induces a number of responses in the platelet through intracellular signalling. The activated platelets synthesis and release secondary mediators to recruit additional platelets after the initial adhesion. Thromboxane A₂ is synthesised and released while ADP is released from the granules, both of these secondary mediators are important for the recruitment and activation of more platelets. Platelet activation will also lead to the exposure of P-selectin on the platelet surface, this protein may interact with other cell types including leukocytes and endothelial cells through P-selectin glycoprotein ligand -1 (PSGL-1). This interaction leads to leukocyte recruitment to the injury site (9). The platelet integrins change their conformation to a high-affinity state following platelet activation. The high-affinity state is more prone to binding and by the activation of $\alpha_{IIb}\beta_3$, fibrinogen binding is facilitated, a key element in platelet aggregation. The platelet cytoskeleton is also rearranged upon platelet activation, causing shape change, spreading and the formation of pseudopodia. As a part of these processes, calcium is mobilized to increase the intracellular calcium concentration (4, 10).

During platelet aggregation, activated platelets start to adhere to each other (Figure 3). It has historically been seen as a bridging of the platelet integrin receptor $\alpha_{IIb}\beta_3$ and fibrinogen and that the binding is important to form stable platelet aggregation at a range of shear rates. However, similarly to the primary adhesion, increasing shear rates cause the need for other means of aggregation. At the lower shear rates ($< 1000s^{-1}$) the fibrinogen receptor ($\alpha_{IIb}\beta_3$) can bind to fibrinogen adsorbed to the surface of the forming platelet plug, this interaction may be stabilised by P-selectin (11). Stimulation of the outermost platelets by secondary mediators cause further activation and increased affinity for fibrinogen, thereby causing a stable adhesion to the surrounding platelets. At

increasing shear rates ($1000-10\ 000\text{s}^{-1}$) the aggregation becomes more dependent on VWF, at these shear rate both GPIb and Integrin $\alpha_{\text{IIb}}\beta_3$ are important to achieve sustained platelet-platelet adhesion. At the highest shear rates ($> 10\ 000\text{s}^{-1}$) the platelets do not require activation to aggregate and are independent of $\alpha_{\text{IIb}}\beta_3$. Instead, only the VWF-GPIb α bond is required for platelet aggregation (12).

Figure 3. The vessel injury is plugged by platelet aggregation. Additional platelets are recruited and aggregate at the site of injury. The platelet plug forms the basis for continued coagulation that may reinforce the plug.

The secondary haemostasis

In the classic textbook example of secondary haemostasis, coagulation is described as two converging cascades or waterfalls, where active coagulation factors will activate the coagulation factor in the subsequent step of the cascade. The cascades are initiated by TF bearing surfaces exposed during a vascular injury (extrinsic pathway) or by negatively charged surfaces (intrinsic pathway). Parts of the model originates from the 1960s and was described by two different groups in parallel (13, 14).

In figure 4 a simplified but more modern version of the coagulation cascade is presented with what is usually described as the intrinsic pathway at the left and the extrinsic pathway on the right. Coagulation should not be seen as a cascade of subsequent activations, instead, the steps are overlapping and depend on cell surfaces that protects and enhances the reactions. The two pathways are not separate but have specific and complementary roles in the coagulation process (2).

Figure 4. The coagulation cascade. The coagulation can be described as three parts; initiation, amplification and propagation that together leads to a burst of thrombin formation. The initiation takes place on TF bearing surfaces (yellow) while the propagation takes place on activated platelets (blue). The image has been based on the description by Monroe and Hoffman(2).

Coagulation can be described in three parts, initiation, amplification and propagation, which are highlighted in figure 4 and presented below. 1) The initiation starts by the exposure of tissue factor (TF) bearing cells and all steps

in the initiation take place on TF exposing cells surfaces. TF together with FVIIIa can activate small amounts of FIX and FX. The activated FX forms a complex with TF and FVa (small amounts may come from activated platelets) and thereby generate a small amount of thrombin from prothrombin. 2) The amplification, this is a booster mechanism that activates the cofactors that will enhance the rate of coagulation. Located on platelet surfaces FV and FVII are activated and in addition, FXI is also activated. 3) The propagation takes place on activated, procoagulant platelets. These are platelets exposing phosphatidylserine (PS) in their cell membrane, which is essential for an efficient coagulation. On the procoagulant cell surfaces, FIXa will bind FVIIIa, FIX is activated on the platelet surface by FIXa. FX is activated by the FIXa/FVIIIa complex and associates with FVa. The FXa/FVa complex is a highly efficient producer of thrombin from prothrombin (2).

The TF exposing surface and the procoagulant platelet surfaces are important for the assembly of complexes but also to protect the factors from inhibition. FXa for example is inhibited when not bound to a surface. The activation is therefore limited to the surface on which it is needed.

FXII is not included in this description of coagulation. FXII is activated by negatively charged surfaces and is mainly seen *in vitro* (1). However, there have been recent studies indicating that FXII may be activated by several physiological pathways and thereby be a highly important part of haemostasis and thrombosis. Three interesting possibilities have been discussed but not fully validated. Fibrillar collagen is thought to activate FXII in addition to activating platelets (15), neutrophil extracellular traps (NETs) are negatively charged and could thereby trigger FXII activation (16) and activated platelets may induce coagulation through FXII by the release of polyphosphates. However the platelet-induced activation has been debated, it was originally postulated by Müller et al (17) and has thereafter been contradicted by our research group, Faxälv et al (18).

Thrombosis

The process of haemostasis, aimed at stopping bleeding, may at pathological conditions, instead cause vessel occlusion, embolization and ischemia. The process of unwanted platelet activation and aggregation is referred to as thrombosis.

Thrombosis can occur in both arteries and veins but with distinctly different mechanisms and underlying pathological causes. Platelets are highly significant in the arterial thrombosis, the focus here.

The arterial thrombosis is often initiated at the site of atherosclerotic plaques, and especially at the site of plaque rupture. These areas have a high thrombotic potential as compared to healthy blood vessels. Atherosclerotic plaques cause a narrowing of the vessel, a stenosis, thereby increasing the local shear stress on the platelets, which may cause platelet activation and aggregation (19). High shear stress may also cause VWF to self-associate, forming a multimeric structure that may have enhanced platelet reactivity (20). There is also an accumulation of tissue factor in the atherosclerotic plaque, thereby adding potential activation of the coagulation process, forming thrombin, that may activate the platelets further and make fibrin strands that can reinforce the platelet plug. Other platelet activators, such as collagen, are also present in the atherosclerotic plaque (19).

Platelets themselves may also be a contributing factor to the formation of atherosclerotic plaques. The adhesive interaction between platelets, endothelium and leukocytes may have a significant impact on the formation and development of plaques. The interaction can cause an inflammation at the site, increasing the rate of plaque formation (21).

The endothelium at the atherosclerotic plaque may induce thrombus formation, without prior rupture, as the endothelium is often damaged during the formation of the atherosclerotic plaque (22). The damaged endothelium may thereby lose its anti-thrombotic properties and have a supporting role in platelet adhesion through the interaction of P-selectin and PSGL-1 (23).

The platelet

In 1881 Bizzozzer described the platelet and its role in both haemostasis and thrombosis. He described the platelet as coming from precursor cells (megakaryocytes) in the bone marrow. In the same decade, Osler found platelets in white thrombi (24).

Platelets are the smallest cell in the bloodstream, in a resting state the platelet is discoid with a diameter of approximately 2-5µm and a thickness of around 0.5µm. The mean cell volume of a platelet is 6-10 femtoliter, in comparison, the red blood cells have a volume of 80-100 femtoliter. The circulating platelets amounts to 150 -400 x 10⁹ platelets /L blood (25, 26). The discoid shape is maintained through an internal cytoskeleton, during platelet shape change this cytoskeleton is restructured.

About 100 billion platelets are produced per day in the human body, the platelets are produced and released from megakaryocytes primarily found in the bone marrow and the production is controlled by the hormone thrombopoietin (25, 27). The platelets survive approximately 10 days in the circulation, however certain factors such as disease or major bleedings may increase the platelet turnover (28). Platelets are removed from the circulation by senescent mechanisms and are cleared in the liver and spleen.

Platelets have a relatively smooth surface with membrane infoldings, entrances into the open canicular system (OCS). This increases the overall surface area of the platelet and allows for uptake and release of proteins and molecules to the platelet. Internally the platelets contain platelet-specific granules (α-granules and dense granules) and organelles also found in other cells such as mitochondria and lysosomes, however, there is no cell nucleus in the platelets (29, 30).

Platelets are essential for haemostasis and thrombosis but are also involved in inflammation (26) and host defence (31). Activated platelets and coagulation are in addition important for metastasis of tumour cells, with a specific role in aiding tumour survival and spreading (32, 33).

Platelet receptors

The most important platelet receptors for platelet activation, adhesion and aggregation are presented in figure 5. Platelet adhesion and aggregation receptors have previously been mentioned, the remainder is described in this section.

Figure 5. Schematic illustration of the platelet receptors most important for platelet adhesion, aggregation and activation during thrombus formation.

ADP/P2-receptors

Despite being a weak agonist by itself, ADP is a highly important mediator of haemostasis. The small molecule is stored in the dense granule at a high concentration and released during platelet activation. The addition of small amounts of ADP to a platelet suspension causes platelet shape change and reversible aggregation (34). But in vivo ADP amplifies the response of most other platelet agonist and thereby contribute to thrombus stabilisation(35).

There are two G-protein-coupled ADP receptors on the platelets P2Y₁ and P2Y₁₂, the latter one is expressed in higher numbers on the platelet surface. The P2Y₁ receptor, coupled to G_{αq}, initiates the ADP induced platelet aggregation response by triggering calcium mobilization, causing shape change and an instable platelet aggregation. The P2Y₁₂ receptor, coupled to G_{αi2} stabilizes the platelet aggregation caused by P2Y₁. The P2Y₁₂ response includes among others the inhibition of cyclic AMP production through adenylyl cyclase (AC) and VASP dephosphorylation. That ADP is a weak agonist by itself is possibly related to

the low number of P2Y₁ receptors on the platelet and that it is therefore a need for additional agonist (34, 36).

TP-receptor

Thromboxane A₂ is an important second mediator alongside ADP (37). The molecule is synthesised and secreted upon platelet activation and can diffuse out of the platelet. The TxA₂ receptor (TP-receptor) is a G-protein coupled receptor with seven transmembrane regions. Similar to ADP the platelet response is transient, but the TxA₂ stimulation amplifies the response of other platelet agonists (38). The TxA₂ molecule has a short half-life, approximately 30s, thereby limiting the impact that the molecule might have down-stream of the thrombus. Knock-out mice lacking the receptor have longer bleeding times and slower platelet aggregation on collagen, indicating the importance of the receptor (39).

P2X₁

P2X₁ is a ligand-gated ion channel that belongs to the same family of receptors as P2Y₁ and P2Y₁₂. However, the P2X₁ receptor interacts with ATP instead of ADP. The interaction with ATP induces a rapid entry of Ca²⁺ from the extracellular space. This activation leads to a temporary shape-change. The receptor is desensitized within milliseconds of sustained ATP exposure, presumably to contain the platelet activation to the injury site. The receptor and its function are most essential under high shear conditions. Mice deficient in the receptor do not have a prolonged bleeding time but are protected against induced thrombus formation. On the contrary, induced thrombosis increased with receptor overexpression (36).

PAR1 and PAR4

Thrombin is a highly potent platelet activator, the two main thrombin receptors on the human platelet are the Protease-Activated Receptors 1 and 4 (PAR1 and PAR4). The PAR receptors are G-protein coupled receptors (GPCRs) with seven transmembrane regions. Thrombin cleaves both receptors at their N-terminal extracellular domains, thereby creating a new N-terminus, a tethered ligand, which activates the receptor, presumably by binding to the extracellular loop II, and thereby inducing a conformation shift (40, 41). The receptors are coupled to the G-proteins G_{12/13}α and Gqα which through downstream signalling via ROCK activation and via PLC, respectively, leads to shape change, granule secretion and integrin activation (38).

There are distinct differences in the platelet response induced by activation depending on the receptor. The PAR1 receptor has a higher affinity for thrombin and is therefore responsible for the first activation response, a spike in the intracellular calcium concentration. The activation of the PAR4 receptor is slower but gives a more prolonged calcium signal (41) and is in addition important for clot lysis resistance (42). The two receptors may also form heterodimers (43). The PARs are found on other cell types than platelets, including endothelial cells, vascular smooth muscle cells and also tumour cells(44) and participate in wound healing and inflammation in addition to the haemostatic functions. PAR1 is in addition involved in the spreading of several cancer types such as breast cancer, colon cancer, prostate cancer and melanoma, and the thrombin activated receptor may release adhesive, invasive and angiogenic factors (45).

Calcium mobilization

Platelet activation through the mentioned receptors has different signalling pathways. However, the activation will cause an increased intracellular calcium concentration and Ca^{2+} is an important second messenger in almost all cells (46). The change in calcium concentration is critical for among others; integrin $\alpha_{\text{IIb}}\beta_3$ activation, platelet aggregation, platelet shape change and also granule secretion in all cell types (4). The increased calcium concentration stems from two main sources; release of intracellular calcium stores or the influx of calcium through the plasma membrane (46).

Platelet inhibition

The aim of platelet inhibition is to be able to control unwanted thrombus formation, mainly to inhibit unwanted platelet activation and aggregation without affecting the haemostatic function of the same platelet. If the haemostatic function is preserved, the risk of bleeding is minimized. The processes that cause recurrent atherothrombotic episodes are to a high degree driven by platelet activation, adhesion and aggregation. Therefore, there is a need for an effective pharmacological treatment that can inhibit the platelets.

The standard treatment in acute coronary syndromes for almost two decades has been dual antiplatelet therapy, combining inhibition of TxA_2 production (acetylic salicylic acid) and ADP induced activation (P2Y_{12} inhibitors) (21, 47). In some patient groups, triple therapy is becoming more used, these are patients with multiple cardiac pathologies. The triple therapy includes some sort of anticoagulant, such as warfarin, on top of the dual antiplatelet treatment, but the bleeding risks are high (21).

There are a few main classes of platelet inhibitors that are approved for clinical use (47);

- COX inhibitors, blocking TxA_2 production (aspirin, i.e. acetylic salicylic acid)
- P2Y_{12} inhibitors (clopidogrel, prasugrel, ticagrelor)
- $\alpha_{\text{IIb}\beta_3}$ inhibitors (abciximab, tirofiban)
- PAR_1 inhibitor (vorapaxar)

In addition, limiting the formation of thrombin through anticoagulants or lysing the formed fibrin through fibrinolytic treatments, are also important for the therapeutic management of atherothrombotic disorders (21).

Specific inhibitors

Acetylsalicylic acid (ASA/aspirin)

ASA reduces the production of TxA_2 through inhibition of COX-1. ASA thereby hinders one of the most important secondary mediators of platelet activation (4). The inhibition is irreversible and will thereby block the platelets TxA_2 synthesis during its lifespan (48).

Aspirin was introduced already 1899 as an anti-inflammatory drug. About 70 years later it was recognized for its function as a platelet inhibitor and was the first available antiplatelet agent (49). The drug is widely used and about one-third of all adults in the US are taking aspirin in the prevention of cardiovascular disease (CVD) (50).

P2Y_{12} inhibitors (clopidogrel, prasugrel, ticagrelor)

Together with aspirin, P2Y_{12} inhibition is the standard treatment for secondary prevention of acute coronary syndrome or after PCI. The P2Y_{12} inhibitors are

selective and inhibit further platelet activation and aggregation by blocking ADP binding. The first type of P2Y₁₂ inhibitors were the thienopyridines, there has been an ongoing development of this class of drugs. The first generation (ticlopidine) had a number of adverse effects that prompted the development of an alternative. The second generation was clopidogrel. Unfortunately, this drug had a high variability in the pharmacokinetics and pharmacodynamics between patients (49). The third generation, prasugrel was more reliable, with higher bioavailability in patients and less adverse effects in comparison to ticlopidine(47) and was found to decrease the risk of ischemic events in comparison to clopidogrel(51). All thienopyridines are irreversible inhibitors and are prodrugs that require metabolism in the liver before becoming active metabolites.

Ticagrelor belongs to a new class of oral P2Y₁₂ inhibitors (cyclopentyltriazolopyrimidines) that reversibly binds the receptor. The receptor binding is non-competitive, presumably to a separate binding site than that of ADP (52). To maintain inhibition there is a need for twice-daily dosing of the drug due to a short half-life (47). Ticagrelor was evaluated compared to clopidogrel in the PLATO trial, the main outcome of the study was a significantly lower mortality in the ticagrelor group without an increase in major bleedings (53).

Cangrelor is an intravenous inhibitor that similarly to ticagrelor does not require metabolism. The drug has a very rapid onset and offset, and is therefore suitable in acute scenarios where the inhibition is needed directly and for a shorter period (47, 54).

$\alpha_{IIb}\beta_3$ inhibitors (abciximab, tirofiban)

The blocking of fibrinogen binding to $\alpha_{IIb}\beta_3$ will inhibit platelet aggregation. Drugs inhibiting $\alpha_{IIb}\beta_3$ are approved for use in the clinic, but there is a limited use of the drug (47). The drugs are only administered intravenously during in-hospital treatment. However, as the receptor is highly important for any aggregatory response, the use of the drugs has led to bleeding complications, especially in women (38).

PAR1 inhibitors (vorapaxar)

PAR1 has been an emerging target for anti-thrombotic inhibition with new potential drug candidates (55). The idea behind inhibiting the PAR receptors is that it would reduce the thrombin-induced platelet activation without inhibiting the other roles of thrombin, mainly coagulation (21).

Vorapaxar is a PAR1 inhibitor that is approved for specific patient groups in the US. The drug has not been approved in Europe. Vorapaxar was evaluated on top of dual antiplatelet therapy and for some patient groups led to an increased risk of serious bleeding, while it had a significant positive effect in the secondary prevention in others (21, 47, 55).

Flow and shear

General

In the previous section, “shear” was mentioned as an important factor for the mechanisms of platelet adhesion and aggregation. Shear rate can be described as the relative velocity gradient between two parallel layers of fluid (19). In the vasculature, the blood does not flow with a uniform velocity; instead, the flow can be described as parabolic with a maximum velocity in the middle and a minimum velocity at the vessel wall (Figure 6). This generates parallel fluid layers with differing velocity and thereby a gradient, shear rate, between them, with a maximum in this gradient close to the vessel wall and zero at the centre (Figure 6). Shear rate is measured in s^{-1} , the unit is derived from velocity over distance. Shear stress is the force, measured in mPa, exerted between the fluid layers. The flow rate is proportional to the shear stress (19).

Figure 6. Illustration of velocity and shear profiles within a blood vessel.

In blood circulation, the platelets are mainly found in proximity to the vessel wall and the red blood cells concentrated at the centre (Figure 6)(19, 56). This process, margination, subjects the platelets to the highest shear stress. In the vasculature, the platelets are exposed to a wide variety of wall shear rates, from as low as 20 s^{-1} in the largest veins up to 5000 s^{-1} in small arterioles. In stenosed arteries, the shear rate can reach $40\,000 \text{ s}^{-1}$ (12, 57). The shear rates described are estimated at the vessel wall, as this is where the highest concentration of platelets is. As previously mentioned, the platelet adhesion and aggregation are to some extent dependent on different mechanisms so that haemostasis may function independently of the prevailing shear rate (12, 19, 56, 58, 59).

There are other aspects of flow that needs to be considered when studying haemostasis and thrombosis. 1) Flow itself, and especially non-laminar flow, may have a specific impact on the formation of atherosclerotic plaques. These typically forms at vessel branch points or where the vessel is curved, the geometries may lower the shear rate and cause a non-uniform flow (19). 2) An increase in flow transports platelets, agonists and coagulation factors to and

from the site of injury or the atherosclerotic lesion. Such mass transfer may regulate the haemostatic response by limiting the possibility for the coagulation cascade to work properly and may also dilute such activators as thrombin and deliver inhibitors such as antithrombin, thereby restricting the coagulation response to the area of the platelet plug (56). The mass transfer thereby affects not only thrombus growth but also the stability, composition and risk of embolic or occlusive thrombotic events (60). 3) Depending on the flow rate, the transport of molecules may be dominated by either diffusion or convection (transport caused by flow). The Peclet number (N_{PE}) (ratio of convection to diffusion) describes which dominates under the specific flow conditions; at a small N_{PE} diffusion dominates, while convection dominates at larger numbers (61, 62). For time periods of a few seconds or shorter, diffusion can only transport small solutes over tens of microns and is the dominating transport inside the thrombus (60, 63). 4) A haemostatic plug is mainly initiated inside the vessel wall and the extravascular space and will not cause a large protrusion into the vessel lumen; the flow profile around such an area will therefore differ distinctly from that near a thrombus forming exclusively in the vessel lumen, which may even cause vessel occlusion. 5) A sufficiently high shear rate may in itself cause platelet aggregation.

Shear stress may induce platelet aggregation at pathological shear rates ($>5000\text{ s}^{-1}$). The high shear rates that might cause such an aggregation can be found at the site of stenosed arteries. Shear-induced aggregation depends highly on the binding of GPIb to VWF, and VWF, a multimer, by itself is affected by the present shear stress. At a low shear rate, the protein assumes a globular conformation that occludes the binding sites for GPIb. In solution, a shear rate over approximately 1500 s^{-1} causes the molecule to extend to a more linear conformation, exposing the binding sites. However, during interaction with the endothelium, the shear rate needed for string formation is around 100 s^{-1} (20, 64, 65).

Over a certain shear rate ($>10\,000\text{ s}^{-1}$), platelet aggregation can occur even without platelet activation(56). Stable platelet aggregation induced by shear may depend on the sequential acceleration and deceleration of the platelet before and after a stenosis and that the elevated shear itself is not the cause of the aggregation. The platelet aggregation then takes place downstream of the stenosis apex, in the deceleration zone (66–68). Immobilized and soluble VWF may also impact this process, where dissolved VWF may attach to any immobilized VWF and thus increase the local concentration of GPIb binding domains and thereby enhance the platelet adhesion. Dissolved VWF can also bind to adhered platelets, encouraging additional platelet to attach to the surface of the forming thrombus. High shear rates may even elongate the adhered platelets, and thus support additional VWF binding to a larger surface area (69).

Studying platelet adhesion, aggregation and thrombus formation

To understand fundamental molecular mechanisms, identify agonists, antagonists, and surface receptors, further understand intracellular signalling and find new potential drug there is a need for suitable assays for analysis of platelet function and thrombus formation. With the right method, it is possible to learn more about platelet-platelet interactions, platelet adhesion and the mechanism behind these functions (12).

The earliest test of platelet function, *in vivo* bleeding time, was performed by Duke in the early 1900s (70). The test has been continually used but is highly affected by both the patient and the laboratory personnel, and later studies have shown that the test is less suitable for diagnostic purposes (71). Since then several methods have been developed and there are a number of methods that are currently used in research but also in the clinic to assess the response to antiplatelet treatment (72).

One instrumental and early technique to was the platelet aggregometer, constructed in 1962 by both Born (73) and O'Brien (74) independently. The method measures the increase in light transmission taking place during platelet aggregation in a suspension (platelet-rich plasma or washed platelets in a buffer). The suspension is continuously stirred and the addition of an activator will induce platelet aggregation (12). There are also other types, evaluating aggregation by monitoring the change in impedance. Both described methods are performed under continuous stirring, but the resulting flow rate is very low and non-uniform. Therefore the results from an aggregometry assay may differ considerably from an *in-vivo* situation (12, 75).

Platelet adhesion and aggregation under the influence of shear can be evaluated with a cone and plate device. In the device, shear stress is induced between blood and a surface (the plate) by a rotating cone. The surface may be coated to evaluate shear-induced platelet adhesion and aggregation on different substrates, such as coated proteins (72, 76). The result from such experiments is evaluated by the use of platelet staining and fluorescent microscopy.

Both the described methods are far from the *in vivo* milieu in many regards. One important aspect is that both methods utilise a "closed system" of sample and activator, no material is added or removed from the sample during the experiment. During platelet adhesion and aggregation, in the vasculature, the injured area is an "open system" where "new" blood can continuously flow over the area. In such an open system, platelets can rapidly accumulate to concentrations that will exceed that of whole blood. The flow will deliver, dilute and remove coagulation factors or platelet activators such as ADP and thrombin. This will together have important effects on any antiplatelet or anti-coagulants used (75).

A more physiological alternative is to use *in-vivo* animal models to study platelet aggregation and specifically thrombus, and it is mainly mice models that are used (77). By the use of animals, there is the possibility to evaluate the physiological effect of knock-in or knock-out specific genes. Thrombus

formation is generated by injuring the endothelium mechanically or chemically (78). There are some distinct differences between human and mice concerning haemostasis, the platelet count, platelet volume and there are also differences in the receptors (PARs) (79). The experimental setting may also be somewhat difficult to control as compared to an in-vitro setting.

The combination of open system, controllable shear rates and human blood can be achieved through the use of in-vitro flow chambers, described in details in the next section.

In vitro flow chambers

In-vitro flow chambers allow for the study of thrombus formation in an open system, with human blood and controllable shear rates. The first flow device was described by Baumgartner in the 1970s. His device was an annular chamber with an activating surface from a rabbit artery. About a decade later a parallel plate flow chamber made of glass coverslips was developed by Sakariassen (70, 80).

The flow chambers have since the 1980s been developed further and is used by many laboratories today. In a flow chamber blood can be pushed or pulled by the help of a pump at any chosen flow rate. However, the designs and implementation are widely different between research groups. There are possibilities to design and construct the flow chambers in-house at a relatively low cost, and in addition, there are commercial flow chambers on the market (81). Adhesive and thrombogenic surfaces are achieved through coating of the chamber (82) and the shear rate can be set to mimic conditions in veins, arteries or stenosed vessels. Many flow chambers are around a few hundred micrometres in width, thereby, the blood volume needed is relatively low, below the millilitre.

The wide variety of design options makes it possible to construct advanced flow chambers adapted to specific research questions (70). Such designs may include obstructions to simulate a stenosis (66, 67), microarrays of protein spots to evaluate adhesion and concentration thresholds (83, 84) or coupled parallel channels for simplified dose-response and inhibitor evaluations (85, 86).

Platelet adhesion and aggregation in flow chambers are mainly evaluated by microscopy, both bright field (87) and fluorescence (82). By the use of specific fluorescent probes, it is also possible to distinguish for example fibrin (88), procoagulant platelets (89, 90) and thrombin(91). The extent of variation in construction, usage and analysis results in a high degree of variation and complicates comparison and verification of the results (81). However, there are recommendations for design and use aimed at standardizing the method (81, 92, 93).

Standard methods for quantifying platelet adhesion and thrombus formation in-vitro or in animal models are based on either fluorescent intensity, thrombus surface area or thrombus volume (70, 75, 81, 82). The result from all of these techniques are affected by the settings during image acquisition, may be operator dependent and require manual assessments, and thereby increase the variability of the results, hinder standardisation and comparison between different laboratories. The information obtained from such analysis is also one-sided, as the outcome is primarily an end-point quantification of the adhesion or aggregation.

Aims of the thesis

The overall aim of this thesis was to develop the use and analysis of in-vitro flow chamber experiments for thrombus formation. By improved image processing it is also possible to promote standardisation, create a more robust analysis and thereby gain a more useful research tool.

- Develop methods to accurately and reproducibly quantify parameters of platelet adhesion, aggregation and thrombus formation in-vitro
- Increase the number of parameters that can be quantified from one individual experiment
- Further characterize the dynamics of thrombus formation
- Evaluate and use the developed methods for research questions related to clinical issues

Methods and materials

Flow chamber

Flow chambers are an attractive method for studying platelet physiology, haemostasis and thrombosis. This due to the possibility of studying the blood and especially platelets in a milieu that in aspects of flow and shear rate is similar to the human vessel, without the use of animal models. A flow chamber is an open system where the experiment is conducted with a continuous addition of new blood, and with this platelets, proteins and small molecules. In most other laboratory equipment for platelet testing there is no new addition of blood during the experiment, i.e. they are closed systems.

Flow chambers are versatile and can be constructed to meet specific research questions and to resemble specific in-vivo milieus, like an obstructed atherosclerotic vessel. However, there might be constraints on the geometry based on the used construction materials or the geometry needed to achieve a uniform shear rate distribution within the flow chamber (70). In its simplest form, the flow chamber is a rectangular channel through which blood is pulled or pushed to achieve a specific shear rate. To initiate a thrombotic event the flow chamber is coated with some sort of platelet activator and the platelet response is studied through light or fluorescent microscopy.

Set-up

A common method for flow chamber fabrication is to use photolithography to produce a reusable mould on a silica wafer on which a softer material such as PDMS is cast in exact replicas. The photolithography is a labour-intensive and somewhat costly procedure but is only needed once as the mould is reusable (94). PDMS is a biocompatible and gas permeable silicone material that is a liquid until polymerised by the addition of a curing agent and exposure to heat. It is, therefore, possible to mould in any desired shape. However, the polymerized material is not rigid and there are constraints on the geometries that can be used in a flow chamber regarding the width and height ratio, this is not an issue with harder materials. However, with PDMS it is possible to construct channels in the micrometer scale at a low cost and with fast reproduction. A microscope glass slide is used as the base of the flow chamber, onto this a platelet activating or adhesive substrate can be coated.

PDMS flow chambers have been used in a majority of the experiments within this thesis and in paper 1 and 2 commercial systems from Ibidi (Martinsreid, Germany) was used as a compliment.

The flow chambers were coated with proteins to induce adhesion or thrombus formation. In paper 2-4 fibrillary Horm collagen (Takeda, Linz, Austria) was used to induce thrombus formation and in paper 1 fibrinogen and VWF was coated separately to induce adhesion. The collagen was coated as a strip on the

glass slide perpendicular to the direction of flow using a separate PDMS channel to achieve reproducible coatings. Only a small part of the flow chamber was thereby coated with collagen (Figure 7) and the remainder was blocked with BSA to hinder unspecific binding of plasma proteins to the PDMS and glass. In paper 1 the Ibidi flow chambers were coated by filling the entire chamber with a protein solution that was thereafter rinsed and blocked with BSA.

Figure 7. Sketch of the flow chamber with the collagen coating perpendicular to the flow.

The PDMS adheres firmly to glass, but leakage may appear when fluids are pressed through the channel. PDMS and glass surfaces may be irreversibly bonded together to form a tight seal with the use of plasma oxidation (94), however, this might affect any proteins that have been coated on the glass surface. If the flow chamber is bonded to the glass it is therefore not possible to coat only a part of the channel. To avoid leakage, minimize the presence of gas bubbles in the channel and to be able to coat the glass prior to assembling the flow chamber, we introduced air-channels in the PDMS (as seen in figure 8 and 9). From these channels, air could be pumped out and thereby creating a negative pressure within the channels, forming a reversible seal between the PDMS and glass. The reversibility of the seal can also be beneficial as the PDMS can be removed to aid further analysis or fluorescent staining of the platelets on the glass surface.

Figure 8. Flow chamber with air channels. The white areas are PDMS throughout, the light blue areas are channel/hollow structures and the darker blue areas are punched holes through the PDMS. 1) Blood reservoir, the blood is added here in the start of the experiment, 2) the flow chamber channel containing adhesive proteins, 3) Hole connected to the syringe that pulls the blood from the reservoir through the channel, 4) Hole connected to the pump pulling air from the flow chamber device.

During the experiments, there are two syringe pumps connected to the flow chamber through an in-house constructed holder. One pump continuously holds the negative air pressure, sealing the flow chamber, and one pump that draws the blood through the flow chamber (as seen in figure 9). To avoid air bubbles the flow chamber and syringe was filled with buffer before the experiment and checked for air bubbles.

Figure 9. The flow chamber setup during an ordinary experiment. The two tubings are used to pump blood and to create a reversible seal between the PDMS and glass.

Image acquisition

For the main part of our studies we have used a wide-field fluorescence microscope, Zeiss Axio Observer Z1 equipped with a 20x objective (NA 0.8) and a Colibri led Module all from Carl Zeiss Microscopy (Jena, Germany) equipped with a Neo 5.5 sCMOS camera (Andor, Belfast, UK). The image acquisition was

controlled by the μ Manager software (Vale Lab, University of California, San Francisco, CA, USA). The combination of the LED-modules and the fast camera allows for fast image acquisition. During most experiments, two fluorophores were used and images were taken as z-stacks and in time-lapse thereby capturing the entire thrombus structure during its entire development. In such experiments one z-stack was acquired completely before the switching excitation wavelength, this was the most time efficient approach as compared to switching excitation wavelength between each frame in the z-stack.

Confocal microscopy is considered the golden standard for determining thrombus size and volume. This was therefore used in paper 2 for validation of and comparison with our platelet count method. Confocal microscopy will give a more detailed microscopy image of the studied specimen by reducing the out of focus light otherwise present in a wide-field image. However, confocal microscopy is a slower technique that often requires the specimen to be scanned rather than just photographed. However, new techniques are emerging that reduces the time needed to capture the image. Confocal microscopy is therefore highly useful for image acquisition where the image resolution is more important than the time resolution, while wide-field microscopy might be more suitable for time-lapse experiments where dynamic changes should be captured.

Shear rate settings

As the aim of flow chamber experiments is to mimic the flow conditions of the human vessels it is important to know what flow and shear rates are reached within the system. As described in the background, mechanisms behind platelet adhesion and aggregation are highly dependent on the shear rates. As our main interest is in the area close to the coated glass surface, it is in this area we want to determine the shear rate. The wall shear rate can be described as a function of flow, width and height of the channel as shown in eqn.1.

$$\gamma = \frac{100 Q}{h^2 w} \quad (1)$$

In the equation γ is the shear rate (s^{-1}), Q is the volumetric flow rate (mL/min), h is the chamber height (mm) and w is the chamber width (mm). The equation is based on a number of assumptions; the flow is parabolic, the fluid is Newtonian and in addition that the width of the channel is greater than the height (92). When doing these calculations blood is assumed to be Newtonian, i.e. the viscosity is not affected by the shear rate. There is also a parameter denoted shear stress (τ) which is based on the shear rate (γ) and viscosity (η) of the fluid (eqn (2)).

$$\tau = \eta * \gamma \quad (2)$$

The flow rate was set on the syringe pump. To validate the flow rate inside the flow chambers, particle image velocimetry (PIV) was performed in paper 1.

Image processing and analysis

In this section, the image processing and analysis developed in paper 2 and 3 are described, and the same analysis was used in paper 4.

During each experiment, images were acquired in z-stack and time-lapse (20 or 30s for each time frame). Each experiment was conducted for about 15-20 minutes and one or two fluorophores were used. To evaluate the large number of images obtained we needed an automated system for image processing and analysis.

The image processing and analysis of the acquired images aimed at reducing the background noise in the images, to distinguish individual platelets and determine their individual position. The computer script for processing and analysis was developed using the open source programming language Python. The language has been developed for scientific use and there is an abundance of libraries for e.g. statistics, data visualisation and data handling.

The python script was based on the following steps and described in greater detail in the supplemental material for paper 2. All image metadata are handled and sorted in a data structure, with the filename, time-point, z-stack level and fluorescence channel, to aid further analysis. Functions were written to work with this data structure and the image library to facilitate automatic loading, image processing and analysis of the captured image data.

1. Reduction of background noise (pixel-sized background fluorescence) and the in-focus platelets is emphasized by applying a “difference of Gaussian” filter.
2. By thresholding, the platelets are distinguished from the background.
3. The platelets are detected in the z-stack volume and each platelet position in x, y and z-axis is determined along with the total platelet count.
4. Additional analysis is performed based on platelet positions during the time-lapse; such as tracking, stability, contraction, height and depth.
5. Visualization of the data regarding thrombus formation is done as graphs, heat maps and 3D images.

The steps 2-3 were repeated over increasing threshold levels and the platelet count was determined for each threshold level. These values were then used to set the optimal threshold.

The output from the image analysis is a large data-frame with information about the position of each single detected platelet at each time point (step 3) and to this additional information is provided depending on which secondary analyse steps were performed (step 5). Such a data frame may contain several millions of rows. However, Python has tools and packages that aids to filter out and aggregate data of interest and also to visualize the results using different graphs. The packages include; NumPy (95), SciPy (96), pandas (97), Matplotlib (98) and

seaborn (99). The data can be exported and analysed using additional software to produce graphs, 3D visualisation, and statistical analyses.

A large part of the work with this thesis has gone to analysis and visualisation of the large amounts of data generated from the flow chamber experiments. The python script has been developed in-house, in some parts based on existing packages, however, with much-needed adaptation for our research. With the acquired data, it has been important to visualise it in different forms. The thrombus formation is a dynamic process happening in 3D and over time, visualisation of the data from such a process is therefore complicated and requires careful consideration.

Blood collection, anticoagulation and labelling

Hirudin or heparin was used as anticoagulants in all samples. In literature hirudin is stated as a preferable option as the effect on the calcium balance is low and therefore the anticoagulation itself has a low impact on platelet adhesion and aggregation under flow (92). Another advantage is that the anticoagulant is in powder form and will not dilute or change the haematocrit of the sample (red blood cell – plasma ratio). Blood was drawn directly into hirudin or heparin-containing blood collection tubes.

Sodium citrate is a reversible mild calcium chelator. Citrate was not used despite the possibility to reverse the anti-coagulative effect prior to infusion by the addition of calcium. In preliminary experiments performed with recalcified citrated blood, we experienced the platelet accumulation to be substantially reduced compared to the use of CTI (inhibitor of contact activation, where coagulation can be induced with TF) or hirudin. Citrate in combination with CTI and CTI alone was compared by Neeves et al. (100) and without citrate the platelet accumulation was doubled, showing that the sodium citrate has a notable effect on the platelets that cannot be reversed by the addition of calcium.

In the main part of our studies (paper 2-4), we have used a labelling technique where 5% of the platelets (5 % of the total platelet volume) are labelled with a fluorophore-conjugated platelet-specific antibody. By labelling a small number of platelets, the labelled platelets are distinguishable from each other in the acquired micrographs, which facilitates detection and counting, and reduces the risk of conjoint objects in the images. A smaller part of the blood is incubated with the fluorescent antibody, and the labelled and unlabelled fractions are mixed prior to each experiment. In experiments performed with inhibitors, the labelling is performed after inhibition of the full sample, avoiding any differences in concentration between the labelled and unlabelled fraction.

The dilution of the antibody after mixing and that it is done shortly before the experiments hinders the unlabelled platelets from binding detectable quantities of the antibody. The antibodies used for platelet labelling were either CD41, clone PM6/248 or CD42a, clone GRP-P (Bio-Rad Antibodies, Oxford, UK) conjugated with fluorescent probes. Other fluorescent markers such as annexin

V and fluo-4 (Invitrogen Molecular Probes, Eugene, OR, USA) have been used but these have been added to the entire blood volume. By labelling smaller portions of platelets it is also possible to use several fluorophores, this was done in paper 4, where each fluorophore was used to indicate a specific fraction of platelets. In the paper, two platelet fractions with different inhibitory treatments were labelled with CD41 (AF488 or AF647) and could thereby be distinguished and analysed separately, within the same flow chamber experiment.

In paper 1, DiOC6 (Invitrogen Molecular Probes, Eugene, OR, USA) and rhodamine 6G (Sigma-Aldrich, St. Louis, MO, USA) were used for labelling. Labelling with these fluorophores require the separation of platelets into PRP prior to labelling. After incubation with the dye for approx. 15 min the PRP was added back to the red blood cell fraction. These dyes label the platelet itself instead of receptors on its surface and are more sensitive to bleaching or heat exposure.

Changes in intracellular calcium can be detected by the use of the calcium indicator Fluo-4. The probe can be detected with fluorescent microscopy or a plate reader that can excite at 488nm. The indicator is used to detect platelet activation and in paper 5 the probe was used to detect the calcium signal in melanoma cells activated with PAR1 or PAR4.

Inhibitor concentration

In paper 4, in particular, we sought the lowest inhibitor concentrations that achieved full inhibition of the platelets. Such evaluations of concentrations were performed by whole blood impedance aggregometry (Multiplate, Roche Diagnostics, Rotkreuz, Switzerland). This allowed to test several concentrations and measure the aggregation response. However, as the test is not performed under physiological shear rates the effects might differ in the flow chamber setting. Therefore, the concentrations were also controlled to be in a similar range as found in the literature.

Results

Paper 1

An internal control method to reduce variability in platelet flow chamber experiments

Kjersti Claesson, Katarina Bengtsson, Lars Faxälv, Nathaniel D. Robinson, Tomas L. Lindahl

The purpose of this paper was to introduce a method that could reduce the variability that is often associated with in-vitro flow models due to their single-use, individual fabrication and overall variability during platelet adhesion and aggregation studies.

By combining a control sample and a treated/activated sample in the same flow chamber, all physical parameters, such as shear rate, geometry and protein surface coatings will be identical, which would increase the reliability and robustness of flow chamber experiments, and also aid the interpretation due to a direct visual comparison. Platelet adhesion (at 1500s^{-1}) was evaluated on a fibrinogen or VWF coated surface during inhibition of the integrin $\alpha_{\text{IIb}}\beta_3$ or GPIIb respectively. The introduction of an internal control reduced the standard deviation by 44 - 53% for 8-10 repetitions as compared to using an external control. By using ANOVA statistics, the source of variation was evaluated and for the internal control, the main source contributing to the overall variance was the different blood donors. In comparison, experiments utilising an external control, had an increased variance due to undefined sources. The internal control was introduced during platelet aggregation in a flow-chamber with constricted flow. The increased shear and flow rate around the stenosis was calculated by mathematical modelling based on velocity measurements from streak imaging of fluorescent particles.

In conclusion, by incorporating an internal control during flow chamber experiments, it is possible to decrease the variability. Thereby also increasing the overall robustness for this type of experimental models. By the introduction of the internal control, preliminary results can be observed already in the microscope and reliable results can be achieved with fewer experiments, saving time and resources.

Paper 2

Counting the platelets: a robust and sensitive quantification method for thrombus formation

Kjersti Claesson, Tomas L. Lindahl, Lars Faxälv

The aim of this paper was to describe and evaluate a new method for quantification and analysis of in-vitro thrombus formation that could aid the comparison of such experiments between different research groups. Thrombus formation during in-vitro flow chamber experiments is usually quantified by such parameters as surface coverage, fluorescent intensity or thrombus volume. These methods may be influenced by user setting or may underestimate the thrombus build up.

By the use of platelet count, the thrombus formation could be quantified in a more robust and reliable manner. To be able to distinguish individual platelet and to count them a low fraction (5% of the platelets) were labelled. Image processing and analysis were performed with the use of an in-house developed python script. The script ensured a reproducible and unbiased analysis with no adjustments needed to be set by the operator and that a suitable threshold was determined by the script. The method sensitivity was compared with quantification through fluorescent intensity and the gold standard thrombus volume estimated from confocal images. The method was found to be superior to fluorescent intensity and similar to volume estimations. The platelet count was not affected by microscope settings. In addition, the analysis also provided information on the position of the counted platelets in x-, y- and z- position. The positional information was used for 3D-visualization of the thrombus and to further characterise the thrombus dynamics, including platelet stability within the thrombus. The method was also compared in different flow chambers with comparable results.

In conclusion; the method was found to be sensitive, robust and unbiased. In addition to quantification of thrombus formation, it also provided additional information stemming from the positional information. As we quantify the platelet count, the measurement was less sensitive to microscope configuration as compared with standard techniques.

Paper 3

Quantification of Platelet Contractile Movements during Thrombus Formation

Kjersti Tunströmer, Lars Faxälv, Niklas Boknäs, Tomas L. Lindahl

In this study, we further developed the computational protocols presented in the previous paper. The focus was to develop and evaluate an analysis of platelet movements and contraction during thrombus formation.

Platelet movement and contraction were analysed, based on the positional information of individual platelets, in a thrombus formed on collagen at 400s⁻¹. Two separate platelet subgroups were analysed, CD42a labelled platelets (5% of the total volume) and Annexin V+ platelets, with or without platelet receptor inhibition. The method could thereby be evaluated for different scenarios and platelet subgroups.

The platelet number and thrombus height were quantified according to the protocol in paper 1. For the CD42a labelled platelets, all inhibitors caused a significant decrease in platelet accumulation, 95% decrease in platelet accumulation with the addition of abciximab. The inhibitors had minor effects on the accumulation of AV+ platelets.

The platelet movements were visualized by platelet trajectories and movements were reduced upon addition of inhibitors. Without inhibition, the movement seemed directed towards the centre of the thrombus, with increased movement in the outer areas of the thrombus. The movement along each axis was quantified and a regression analysis was performed to evaluate the direction of the movement in all regions of the thrombus.

The contractile component of the movement was determined as the part of the movement directed towards the centre of mass. In addition, the total overall movement and platelet displacement length were quantified. A considerable fraction of the overall movement was contractile, a maximum of 38% for the CD42a labelled platelets and 25% for the Annexin V+ platelets. The addition of inhibitors abolished the contractile movements while having minor effects on the overall movement.

The information obtained about platelet positions was used to simultaneously track the movement of thousands of platelets during thrombus formation. In conclusion, this generates a detailed and systematic approach to analysing platelet intrathrombus movements and how such movements will affect the overall thrombus structure.

Paper 4

Effects of a drug-free platelet fraction during P2Y₁₂ inhibition during thrombus formation in flow chambers

Kjersti Tunströmer, Lars Faxälv, Tomas L. Lindahl

The aim of the paper was to investigate how a non-inhibited platelet fraction, arising between administrations of thienopyridines, may impact thrombus formation under flow and we explored how the different mechanisms of action, between the irreversible inhibitor prasugrel and the reversible inhibitor ticagrelor, may impact thrombus formation and the potential impact of increased platelet turnover or poor compliance.

The P2Y₁₂ receptor is a common target for antiplatelet therapy. Two of the most used inhibitors against this receptor are prasugrel and ticagrelor, where prasugrel is an irreversible pro-drug requiring metabolization to become active while ticagrelor is reversible and direct acting. Due to the pharmacokinetics of irreversible P2Y₁₂ inhibitors, there will be new platelets forming between administrations that do not have adequate inhibition (approx. 10% of the platelet volume). Thrombus formation on collagen at 400s⁻¹ was evaluated for both drugs, at decreasing concentrations of ticagrelor and increasing fractions of non-inhibited platelets together with the prasugrel treated platelets.

The addition of 20% non-inhibited platelets caused an increased platelet accumulation, the increase in accumulation was comparable to a lower concentration of ticagrelor (0.4μM.) The addition of non-inhibited platelets also caused a shift in the relation between the number of inhibited and non-inhibited platelets within the thrombus, with an increased number of inhibited platelets.

Figure 10. Platelet accumulation on collagen with and without P2Y₁₂ inhibition by either prasugrel active metabolite (PAM) or ticagrelor.

In a thrombus containing both inhibited and non-inhibited platelets, the inhibited platelets were found higher up in the thrombus structure as compared to the non-inhibited fraction (mean platelet height of the two fractions was compared). The inhibited platelet fraction was, in addition, found more downstream of the centre than the non-inhibited fraction.

In conclusion, the addition of non-inhibited platelets caused an increased platelet accumulation, this increase was comparable to that of a low concentration of ticagrelor. However, within the forming thrombus, the relation

between the two platelet fractions was affected by the added non-inhibited platelets, the difference was increasingly pronounced by larger fractions of non-inhibited platelets.

miR-20b regulates expression of proteinase-activated receptor-1 (PAR-1) thrombin receptor in melanoma cells

Amina Saleiban, Lars Faxälv, Kjersti Claesson, Jan-Ingvär Jönsson and Abdimajid Osman

This paper aimed at elucidating the impact of microRNAs on the post-transcriptional regulation of PAR1 in malignant melanoma cells.

Procoagulant factors are overexpressed in many cancer forms and are believed to take part in cancer metastasis. In this, the PAR1 receptor may function as a key player. Activation of this receptor in cancer cells leads to the expression of adhesion molecules, increased intracellular calcium and tumour invasion and spreading.

PAR1 protein and mRNA expression were evaluated in three cell lines, the primary CRL-1675, and the metastatic CRL-1676 and Sk-mel-28. Western blot analysis showed PAR1 protein expression in the metastatic cells compared to no notable amounts in the primary cells while the mRNA expression was inversely, indicating post-transcriptional regulation of PAR1 expression. Stimulation of melanoma cells with PAR1 and PAR4 activating peptide resulted in a rapid calcium response in the CRL-1676, while there was no response in the CRL-1675.

By microRNA expression profiling, the microRNA miR-20b, targeting PAR1 mRNA, was found to be differentially expressed in melanoma. MiR-20b was abundantly expressed in the primary CRL-1675 cells, weakly expressed in CRL-1676, and barely detectable in Sk-mel-28. The miR-20b expression inversely correlated with PAR1 protein expression in the cell lines investigated, indicating a repressive function of miR20b on PAR1 protein expression in the primary cells. The repressive function of miR-20b on PAR1 expression was further confirmed by transduction of a lentivirus containing PAR1 mRNA coupled to a luciferase reporter gene. The same viral vector without cloned PAR1 mRNA was also separately transduced into the same cells as control. In addition, miR-20b mimic was transfected in melanoma cells and the luciferase activity was measured. These experiments revealed that miR-20b bound and downregulated PAR1 mRNA. Knockdown of the miR20b in the primary cell lines led to the expression of PAR1 protein as compared to no expression in the controls.

Together this provides new information on the mechanisms that regulate PAR1 expression in melanoma cells and that may be important for the development of melanoma metastasis. miR-20b appears to be anti-oncogenic (tumour suppressor) and appears to be a down-regulator of PAR1 expression and thereby in part explain the increased expression of PAR1 in metastatic cells.

General discussion

Issues with flow chambers in general and in the lab

A large part of the work behind the papers in this thesis has been related to the development and evaluation of flow chambers. As the project started we were using commercial and PDMS systems that were bonded to glass (in paper 1). With these it was not possible to coat parts of the chamber, air bubbles were trapped in the commercial systems, thereby affecting the overall flow and leakage appeared in the PDMS chambers, resulting in several failed experiments and increased frustration. Therefore, the development and evaluation of both the flow chambers construction and handling has been a time-consuming priority throughout the project. With the set-up used today, previous problems have been resolved and there is a good reproducibility between individual experiments. However, to handle the set-up requires training of new users and also require considerable time for preparation of the flow chambers and glass surfaces.

Is there a need for extensive image analysis?

During the work with paper 1, we introduced two differently labelled platelet fractions in the same channel. The analysis of platelet adhesion on fibrinogen and VWF was manageable as it included a few images and single platelets spread over a surface. The platelets of each label could thereby be counted with the help of a custom Matlab script, after some initial image processing (image thresholding to reduce background noise).

However, in the same study, we visualized that the dual labelling also was beneficial in evaluations of platelet aggregation under pathological shear conditions. We found that GPIb-antibodies had an inhibitory effect on the shear-induced platelet aggregation at the stenosis while inhibiting fibrinogen binding had no effect on the aggregation, which was in accordance with other studies performed on shear-induced aggregation showing that fibrinogen has a minor role and that VWF is highly important (12, 67, 68). However, we did not quantify the platelet aggregation as we did not feel that we had a good method alternative for doing so at that time.

The available methods for quantification were in our opinion limiting in the experimental settings of interest. Fluorescent intensity was possibly affected by the intermingled platelets of differently labelled subgroups. Using confocal microscopy, for volume measurements was possible but limiting as the equipment was often occupied and our custom setup required pumps and risk of spill (why it was preferable to have it close to the laboratory). Furthermore, measuring surface coverage was not an alternative as we were interested in aggregation in this case. In addition, there are several issues with these methods that have been mentioned in previous sections.

However, adding another method for quantification, would that not hinder standardization and increase the possible variants of flow chamber use. In addition, a new method could be counterproductive compared to the original aim of paper 1, to reduce variation. With this in mind we aimed at developing a model based on a number of ideas; 1) The method should not require any user adjustments, thereby creating an unbiased analysis. 2) The method should be possible to use independent of the flow chamber type, microscope and initial settings that are used. 3) The method should expand and contribute with new information about the thrombus.

As described in paper 2, 3 and paper 4, we think that we have managed to fulfil these ideas and thereby contribute with a robust method that gives more information about every single experiment and the individual platelets within the thrombus. We think that there are further possibilities to extend on the present analysis so that more platelet characteristics can be analysed and evaluated. Fluorescent probes against specific receptors or substances can be used simultaneously as platelet-specific probes and thereby provide information about, for example, the platelets activation (calcium probes) and status (juvenile (reticulated) or mature platelet) (101).

Automated image analysis, in general, is time efficient. In paper 5 an analysis was done to evaluate the fluorescent intensity of melanoma cells incubated with a fluorescent calcium probe. Fluorescent intensity over time was evaluated for a number of cells. The manual handling limited the number of cells that were analysed. An automated processing would lead to less manual work and in addition a better mean value due to a higher number of analysed cells.

The heterogeneous thrombus

In many methods used for assessing platelet function including flow chamber experiments, the results do not reflect the heterogeneity that may exist in the sample. As previously mentioned the flow chamber experiments are often evaluated by one parameter reflecting the size of platelet aggregates or the covered surface area. However, a formed thrombus is not homogenous, neither in its structure or the platelets therein. During a haemostatic response, the formed platelet plug consists of two layers. A densely packed core, that contains thrombin and fibrin, where the platelets are P-selectin+, surrounded by a loosely packed shell with less activated platelets (5). There may also be differences in the platelets themselves due to their environment, such as what substrate they adhere to, the agonist and antagonist in the surrounding and also the local rheological conditions. In addition, the actual age of the platelets may affect their responsiveness (102). The platelets role in the coagulation related to their surface properties is also contributing to the heterogeneous content (103).

The analysis developed in this thesis quantifies parameters for thousands of platelets spread throughout the thrombus. Combined with the information about their location within the thrombus, this provides an opportunity to study the local conditions in that specific area. It is also possible to divide the

thrombus into regions of interests, as was done in paper 3, to evaluate those platelets located there. This could, for example, be used to further analyse the core and shell if thrombin is present.

Shear rate and anticoagulants

Several different shear rates were used in this thesis, in the papers 3 and 4, we used a shear rate of 400s^{-1} . At a low, venous shear rate, the overall pressure, in the flow chamber, during thrombus formation is low, a higher pressure can cause instabilities and embolization. The use of higher shear rates are clearly favourable from a physiological perspective but requires techniques that can adjust the pressure as it builds up in the channel, this issue has been discussed and studied by others (85, 86, 88).

Another experimental detailed to consider is the use of anticoagulation. Throughout paper 1-4 we have used heparin or hirudin as the anticoagulant and thereby not allowed coagulation in our experiments. These anticoagulants are suitable as they do not affect the calcium concentration in plasma or the cells. The downside is that with anticoagulation there will be no fibrin formation, thrombin production, platelet activation through PAR1 or 4, and, according to recent findings the addition of anticoagulants may affect the thrombus architecture (5, 104).

To not use anticoagulants would give a more physiological response in the flow chambers. However, eliminating anticoagulants would lead to coagulation already in the test tube before the start of the experiment, especially as time is required for platelet labelling and the addition of inhibitors. There is the possibility of using citrate as a reversible anticoagulant, but as mentioned in the material and methods section the platelets are affected by this treatment (105). We have also made preliminary experiments with CTI, a well-known inhibitor of FXIIa, which inhibits contact activation. However, it may only inhibit coagulation for about two hours, presumably due to slow activation due to activated factors released from platelets, and therefore needs to be used within that time interval and hence reduced the number of experiments that can be conducted. When using CTI, coagulation and platelet activation are initiated by collagen and TF.

Some probes are not suitable to use in combination with coagulation during in-vitro thrombus formation. In paper 3 we described the movement of two platelet subgroups, platelet labelled with a platelet-specific antibody and platelets that were positive for Annexin V, platelets that expose phosphatidylserine (PS). Platelets exposing PS is essential for a full coagulation response, Annexin V will hinder the coagulation factors from binding to the surface and thereby hinder coagulation (103). It was, therefore, the reason not to introduce coagulation in that setting of paper 3.

Future perspectives

Introduce coagulation

In the flow chamber experiments in this thesis anticoagulated samples have been used, it simplifies the experimental procedures and the blood can be used for experiments during a longer time then without anticoagulation, allowing for multiple experiments with blood from the same donor. As described earlier there are also some constraints concerning the choice of reversible anticoagulants that can be used. Initially, it would be interesting to do an overview of the available alternatives and evaluate which that could be used in our setup and thereby perhaps allow for longer experiments than with CTI alone.

If allowing coagulation to proceed, it would be beneficial to extend the labelling and analysis to detect thrombin or fibrin. An interesting alternative here is to utilize the thrombin-sensitive antibody introduced by Welsh et al. (91) or fluorescently conjugated fibrinogen that can be incorporated into the fibrin mesh. The thrombin-sensitive antibody allows for the detection of thrombin around the platelets in the forming thrombus.

It would be interesting to repeat some of the presented experiments under the influence of coagulation. Fibrin formation would possibly affect platelet movement and contraction and thereby the overall thrombus structure. To perform clinically relevant research, it is also of importance to be able to have coagulation and thereby get a more physiological situation.

The python scripts

An important future is to have our analysis method easily available for other researchers. To achieve this there are a number of steps that need to be taken to ensure that others can understand, utilize and have access to the python scripts. As of now, the script has been optimized to handle the type of data files that are generated by the image acquisition software in combination with our microscope (in regards to filetype, size, etc.). It won't be possible for us to generate a generic code that functions for all data types and all experimental setups. However, by careful code annotation in combination with the manuscripts describing the workflow, it is possible for potential users to adapt the code for their specific use. The script should after the annotation either be uploaded online on our research webpage or arranged so that it can be sent out on demand, with information regarding that on the webpage. By making the code available for others, there is the potential for further optimization and development by the research community. It would be valuable to have an open dialogue with those using and adapting the code, thereby keeping the code in use and evolving.

More advanced flow chambers

The main flow chambers we have used so far are straight channels with a collagen coating initiating thrombus formation. The setting can be adapted further to accommodate specific research questions and to be better fitted for higher shear rates.

Pressure relief

With the flow chambers that we have used throughout this project, the flow rate through the channel is determined by a syringe pump. It can be set to the desired flow rate, generating a distinct shear rate at the flow chamber walls. However, with a constant flow, the pressure increases with the formation of platelet aggregates, causing embolization at higher shear rates as mentioned in the discussion. To avoid embolization and allow vessel occlusion as the natural endpoint of the experiment the pressure should not be allowed to increase with increasing shear rate. One interesting alternative is to construct a flow chamber where the pressure is maintained by having two diverging channels, where thrombus formation is only initiated in one, and the other will thereby function as a pressure relief during thrombus build up. This has previously been reported (88), but would be an advantage to have available in our laboratory as well to allow for research over a wider variety of shear rates. Another alternative is to monitor and regulate the pressure over the channel but that would require additional resources, knowledge and time to implement.

Other geometries

Flow chambers with other geometries could be used to evaluate for example increasing or decreasing shear rates and turbulent flow.

PDMS in combination with photolithography can be used but have several limitations concerning the geometries that are possible to make with this combination. To go from an idea to a finished flow chamber is relatively time-consuming, taking a minimum of two days. For a design that will be used multiple times to generate identical flow chambers, this is not an issue. However, to go from idea to finished flow chamber within a few hours would be favourable for prototyping and testing.

3D printing would be an alternative in two different ways. One alternative would be to use a 3D printer for making the flow chamber itself thereby constructing a finished flow chamber from a computer model directly. Another alternative is to construct a template mould that PDMS can be cast upon, either for repeated or one-time use (106).

Alternative to animal models and use in drug discovery

With the combination of user-friendly image processing and more advanced flow chambers, the method is gaining potential as an alternative to animal models. Specific scenarios can be built and controlled in vitro and in addition, the analysis offers the possibility to see parameters that cannot be detected with animal models today. The method is therefore suitable during drug discovery and could be an alternative to animal models during the early stages. An important part in this would be to build collaboration with other research groups so that the methods are spread and used by a wider community and thereby reduce the use of research animals.

Additional parameters

The image analysis and processing generate data about individual platelets. The positions of all detected platelets are obtained and the position of the individual platelet can be tracked over multiple timeframes. The positional information has been used to evaluate additional parameters such as stability, thrombus height, velocity and length of movements and contraction.

Other parameters, linked to platelet position, that could be of interest are:

- To evaluate the clustering of platelets. Such analysis would be of value if different platelet subgroups are present.
- Volume estimation could be rendered based on the platelet positions.
- Platelet density throughout the thrombus or in specific zones, based on the volume estimation or based on the distance to the nearest neighbours.

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