Platelet activation and aggregation: Clinical and experimental studies

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Cover illustration: Schematic illustration of a platelet with a number of receptors, activation pathways and targets of the antiplatelet agents acetylsalicylic acid (ASA), ticagrelor and clopidogrel done by the author.

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To my family

Platelet activation and aggregation: Clinical and experimental studies

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ABSTRACT

Background: Dual antiplatelet therapy with acetylsalicylic acid (ASA) and the adenosine diphosphate (ADP)-receptor antagonist ticagrelor increases the risk of bleeding complications during cardiac surgery. The overall aim of this thesis was to identify and evaluate current and potential methods to reduce and prevent bleeding complications in patients with ongoing antiplatelet therapy.

Methods: In Study I, three types of platelet concentrates were sampled on day 1, 4 and 7 after donation. In Study II, adrenaline and platelet concentrate were added to blood samples from acute coronary syndrome patients on ASA and ticagrelor. In Study III, blood samples from healthy volunteers were collected after ticagrelor, adrenaline and metoprolol administration. In Study IV, blood samples were collected before and after anesthesia induction from cardiac surgery patients randomized to standard treatment or maintenance of preoperative mean arterial pressure using noradrenaline. Platelet aggregation was assessed with impedance aggregometry (Studies I–IV) while platelet activation was assessed with flow cytometry (Studies I–III). Clot formation was assessed with thromboelastometry (Studies III and IV).

Results: Platelets in interim platelet unit (IPU) concentrates maintained a lower activation state and better aggregation response to the end of storage compared to buffy-coat concentrates (I). More platelets in IPU concentrates were activated and had a lower aggregation response throughout storage compared to apheresis concentrates (I). Adrenaline, but not platelet concentrate, improved ADP-induced platelet aggregation and activation in the presence of ticagrelor *in vitro* (II). Adrenaline infusion improved ADP-induced platelet aggregation, activation and clot formation in healthy volunteers treated with ticagrelor (III). Intraoperative noradrenaline infusion improved ADP-induced platelet aggregation and clot formation in cardiac surgery patients (IV).

Conclusions: The quality of IPU concentrates is at least comparable to buffy-coat concentrates. Adrenergic agents improve platelet reactivity and may thus potentially be used to prevent excessive bleeding during surgery in patients with ongoing antiplatelet therapy.

Keywords: Platelet concentrate, adrenaline, noradrenaline, ticagrelor, platelet aggregation, platelet activation

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SAMMANFATTNING PÅ SVENSKA

Allvarlig blödning under och efter hjärtoperationer är en farlig men relativt vanlig händelse som påtagligt ökar risken för död och andra allvarliga komplikationer. Orsaken till blödning kan exempelvis vara att patienten har ätit ett eller flera läkemedel som hämmar trombocyters (blodplättars) förmåga att aktiveras och aggregera (klumpa ihop sig med varandra), såsom acetylsalicylsyra ensamt eller i kombination med ticagrelor, tätt inpå operationen. Helst vill man att patienten ska ha slutat äta ticagrelor flera dagar innan operation men ibland går det inte p.g.a. patientens tillstånd. Trombocythämmande läkemedel används vid behandling av akut koronart syndrom (AKS). AKS uppstår då blodproppar förhindrar blodflödet genom artärerna i hjärtat vilket kan leda till hjärtinfarkt eller hjärtstopp. För att förhindra bildandet av blodproppar behandlas AKS-patienter med acetylsalicylsyra och ticagrelor vilket är effektivt men tyvärr ökar risken för blödning om patienter behöver opereras akut. Idag ges ofta transfusioner av trombocytkoncentrat från blodgivare för att stoppa blödningarna. Trombocytkoncentraten kan förvaras i maximalt 7 dagar efter blodgivning men trombocyternas kvalitet försämras tyvärr under förvaringstiden. Det har tidigare visats att trombocyttransfusioner förbättrar den signalväg i trombocyten som acetylsalicylsyra hämmar men att de endast har en liten effekt på den ticagrelor-hämmade signalvägen. När patienter vars trombocyter är hämmade av ticagrelor hjärtopereras så är risken för allvarlig blödning kopplad till trombocyters förmåga att aggregera via den ticagrelor-hämmade vägen. Målet med denna avhandling var att utvärdera nuvarande samt nya strategier för behandling och förebyggande av blödning hos patienter behandlade med trombocythämmare.

I delarbetena mättes trombocyternas förmåga att bilda koagel (aggregation) med impedansaggregometri. Med denna metod mäts impedansen (förändring i motstånd) mellan två elektroder i en testcell. Då trombocyter aggregerar på elektroderna ökar impedansen vilket mäts i instrumentet och man får ett mått på trombocyteras aggregationsförmåga. Trombocyternas aktiveringsgrad och förmåga att aktiveras mättes med flödescytometri. I denna metod används antikroppar (en typ av proteiner) som endast binder till aktiverade trombocyter vilket kan mätas i instrumentet när trombocyterna utsätts för laserstrålar. Instrumentet räknar ett antal trombocyter och resultatet visar hur stor procent av trombocyterna som är aktiverade. Slutligen mättes koagelbildning (levringsförmåga) med tromboelastometri där en roterande pinne i en testcell hindras i sin rörelse när ett koagel bildas. Parametrar som

man kan få ut är exempelvis tiden det tar för koagelbildning och koaglets stabilitet.

I första delarbetet jämfördes kvaliteten av olika trombocytkoncentrat. De nya interim platelet unit (IPU)-koncentraten jämfördes med buffy-coat och aferes-trombocytkoncentrat under förvaringstiden avseende metaboliska parameterar samt trombocytfunktion (trombocyternas aktiveringsgrad och aggregationsförmåga). Prover togs 1, 4 och 7 dagar efter givning. Resultaten visade att trombocyter i IPU-koncentraten hade en högre aktiveringsgrad och sämre aggregationsförmåga än buffy-coat koncentrat i början av förvaringen men behöll en bättre trombocytfunktion vid slutet av förvaringstiden. Aferes-trombocytkoncentraten hade en bättre trombocytfunktion under hela förvaringstiden jämfört med IPU-koncentraten.

I andra delarbetet tillsattes adrenalin och trombocytkoncentrat till blodprover från AKS-patienter med pågående behandling med acetylsalicylsyra och ticagrelor. För att utvärdera effekten på trombocytfunktionen mättes trombocyternas förmåga att aktiveras och aggregera. Resultaten visade att adrenalin förbättrade både den ticagrelor-hämmade och acetylsalicylsyra-hämmade signalvägen (aggregation och aktivering). Trombocytkoncentrat hade ingen effekt på den ticagrelor-hämmade vägen men förbättrade den acetylsalicylsyra-hämmade vägen (aggregation). En kombination av adrenalin och trombocytkoncentrat förbättrade aggregation via båda aktiveringsvägarna.

I det tredje delarbetet gavs adrenalin i form av en infusion till friska frivilliga. Prover togs efter att ticagrelor, adrenalin samt en betablockerare givits. Vi utvärderade effekten på trombocytfunktion (aktivering och aggregation) och koagelbildning. Resultaten visade att adrenalininfusion förbättrade den ticagrelor-hämmade signalvägen (aggregation och aktivering) och även signalvägen som hämmas av acetylsalicylsyra (aggregation). Dessutom förbättrades koagelbildningen. Att ge en betablockerare tillsammans med adrenalin påverkade inte adrenalinets effekt på trombocytaggregationen negativt.

I fjärde delarbetet fick patienter som genomgick hjärtoperation antingen sedvanlig behandling eller noradrenalininfusion för att behålla samma blodtryck som innan sövning. Prover togs innan samt 50 minuter efter patienten sövts. Noradrenalin förbättrade både signalvägen som hämmas av ticagrelor och den acetylsalicylsyra-hämmade signalvägen, jämfört med patienter som fick sedvanlig behandling. Dessutom förbättrades koagelbildningen.

För att sammanfatta, kvaliteten av IPU-koncentrat är åtminstone lika bra som buffy-coat koncentrat. Adrenalin och noradrenalin skulle kunna användas för att förbättra trombocytfunktion och koagelbildning hos patienter behandlade med trombocythämmare i syfte att förebygga allvarliga blödningar.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. Singh S, Shams Hakimi C, Jeppsson A, Hesse C. Platelet storage lesion in interim platelet unit concentrates: A comparison with buffy-coat and apheresis concentrates. *Transfus Apher Sci*. 2017; 56(6): 870-874.

II. Singh S, Malm CJ, Ramström S, Hesse C, Jeppsson A. Adrenaline enhances in vitro platelet activation and aggregation in blood samples from ticagrelor-treated patients. *Res Pract Thromb Haemost.* 2018; 2(4): 718-725.

III. Singh S, Damén T, Nygren A, Shams Hakimi C, Ramström S, Dellborg M, Lindahl TL, Hesse C, Jeppsson A. Adrenaline improves platelet reactivity in ticagrelor-treated healthy volunteers: A proof of concept study. Submitted.

IV. Singh S, Damén T, Dellborg M, Jeppsson A, Nygren A. Intraoperative infusion of noradrenaline improves platelet aggregation in patients undergoing coronary artery bypass grafting: A randomized controlled trial. Submitted.

CONTENT

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ABBREVIATIONS

AA	Arachidonic acid
ACS	Acute coronary syndrome
ADP	Adenosine diphosphate
APTT	Activated partial thromboplastin time
ASA	Acetylsalicylic acid
AUC	Area under curve
cAMP	Cyclic adenosine monophosphate
COX	Cyclooxygenase
CPB	Cardiopulmonary bypass
DAPT	Dual antiplatelet therapy
EDTA	Ethylenediaminetetraacetic acid
GP IIb/IIIa	Glycoprotein IIb/IIIa
INR	International normalized ratio
IPU	Interim platelet unit
MAP	Mean arterial pressure
PAR	Protease-activated receptor
PAS	Platelet additive solution
PBS	Phosphate-buffered saline
PC	Platelet concentrate
PI3K	Phosphoinositide 3-kinase

POC	Point-of-care
PT	Prothrombin time
TRAP	Thrombin receptor-activating peptide-6
TXA_2	Thromboxane A_2
vWF	von Willebrand factor

1 INTRODUCTION

1.1 Hemostasis

When a vascular injury occurs, the human body responds to stop the bleeding. This response is named hemostasis. Hemostatic mechanisms include vasoconstriction of the wounded vessels to reduce blood flow as well as formation of a platelet plug and coagulation of the blood to reduce blood loss through the damaged vessel wall [1].

Primary hemostasis

The primary hemostasis includes vasoconstriction and platelet plug formation. Platelets are cell fragments derived from the cytoplasm of megakaryocytes [2] with a life-span of up to 10 days in circulation [3]. Platelets contain mitochondria, α- and dense granules but no nucleus. The α granules contain proteins needed for platelet adhesion and repair of the vessel wall, such as the membrane-bound protein P-selectin (CD62p) and the soluble proteins von Willebrand factor (vWF) and fibrinogen needed for platelet-platelet and platelet-endothelial interactions [4]. They also contain prothrombin and the coagulation factors V, IX and XIII. Dense granule content includes adenosine diphosphate (ADP), calcium ions, serotonin and the membrane-bound protein CD63. Upon platelet activation, the membrane-bound proteins will be expressed on the platelet surface membrane while soluble mediators will be secreted into the extracellular environment [4]. Platelets can become activated by different stimuli as they express a number of different receptors on their surface (Figure 1). A damaged vessel wall exposes collagen/vWF complexes which circulating platelets can bind to by first rolling and then adhering to the endothelial surface. When the platelet has bound and become activated by collagen, the granule content is released which will recruit more platelets to the damaged area in order to form a platelet plug. Release of thrombin, ADP and thromboxane A_2 (TXA_2) will activate the recruited platelets. For bridging between platelets, the activated fibrinogen receptor glycoprotein IIb/IIIa (GP IIb/IIIa) enables binding of fibrinogen, fibrin and vWF and allows for platelet-platelet adhesion. By adhering to each other, the platelets will form a platelet plug to seal the wounded area and reduce blood loss [5].

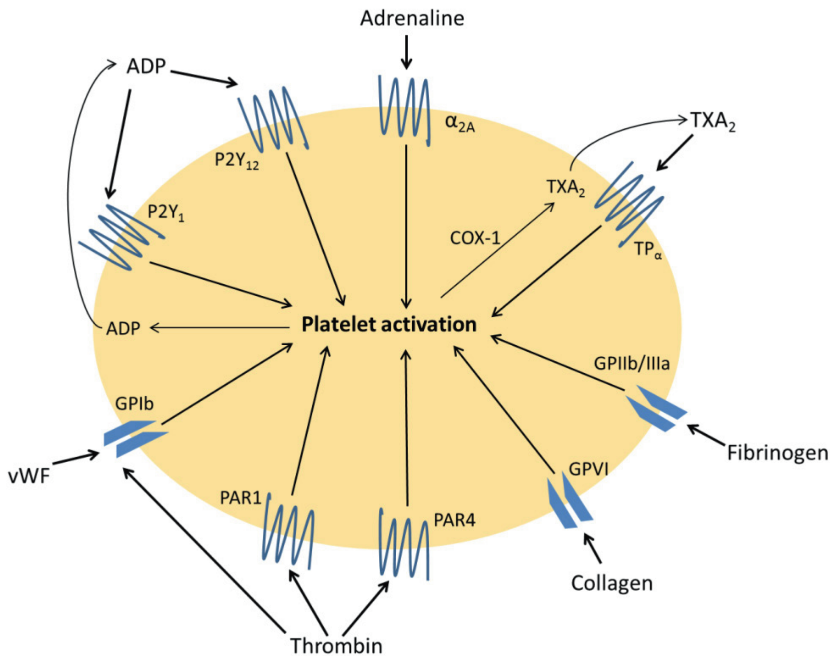


Figure 1. Schematic illustration of a platelet with a number of receptors and activation pathways. ADP: adenosine diphosphate; COX-1: cyclooxygenase-1; GP: glycoprotein; PAR: protease-activated receptor; TP: thromboxane receptor; TXA_2: thromboxane A_2; vWF: von Willebrand factor.

Secondary hemostasis

To stabilize the formed platelet plug, the secondary hemostasis occurs which is the formation of a stabilized fibrin-crosslinked clot (Figure 2). This process depends on coagulation factors that will activate each other forming a cascade (Figure 3).

In the initiation phase, released tissue factor from, for instance, damaged endothelial cells activates factor VII in the blood. This complex further activates factor IX and X. Factor V can both be released by platelets and be bound to the platelet membrane. The activated factor X will form a complex with factor V on the platelet membrane and is together with calcium ions known as the prothrombin activator. Activated factor X in this complex will convert prothrombin to thrombin [1, 6].

In the amplification phase, the generated thrombin from the initiation phase will activate platelets and also activate factors V, VIII and XI [6]. Activated platelets expose a negatively charged procoagulant surface on which complexes of coagulation factors can form rapidly. This localizes the coagulation to the site of injury. Activated factor IX, formed in the initiation phase, forms a complex with activated factor VIII on the membrane and activated factor X binds activated factor V which results in a fast and large thrombin generation [6]. Thrombin can cleave fibrinogen to fibrin and activate factor XIII which can then crosslink the fibrin. The fibrin will stabilize the original platelet plug to form a stabilized clot together with adhesive proteins to keep the clot attached to the damaged area (Figure 2) [1].

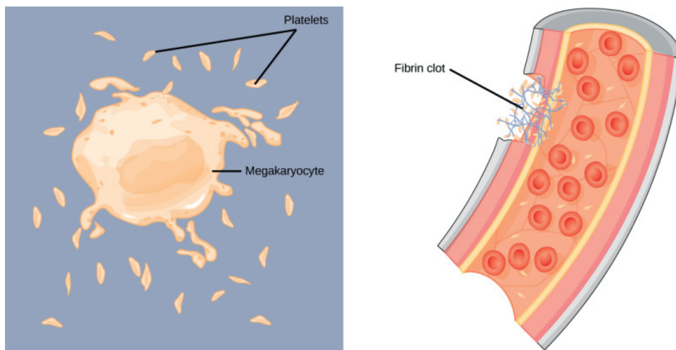


Figure 2. Schematic illustration of platelets derived from cytoplasm of megakaryocyte (left) and platelets entrapped in a fibrin mesh located to the injured area of a blood vessel (right). © 1999-2018, Rice University. OpenStax CNX. https://cnx.org/contents/GFy_h8cu@10.53:_XipwKIy@4/ Components-of-the-Blood Public domain.

Different mechanisms for controlling the clot formation exist. These restrict clotting from occurring when not needed as well as dissolve the clot when the site of injury is repaired. There are also soluble factors that can bind components of the coagulation to inactivate them. For instance, heparin/antithrombin III binds to thrombin and inhibits its action. Endothelial cells also exhibit anticoagulant properties. The intact endothelium expresses heparan sulfate and secrete soluble factors such as prostaglandin I_2 and nitric oxid to prevent platelet adhesion and aggregation [1, 7]. Thrombomodulin is expressed on the membrane of endothelial cells and forms a complex with thrombin to inhibit its action. In addition, this complex activates protein C which inactivates coagulation factors V and VIII (Figure 3). To dissolve the clot when the injured area has been repaired, fibrinolysis will occur. Fibrinolysis is the cleavage of the fibrin threads and occurs when endothelial cells secrete tissue plasminogen activator which will catalyze the formation

of plasmin from plasminogen localized in the clot. Plasmin will cleave the fibrin threads to dissolve the clot [1].

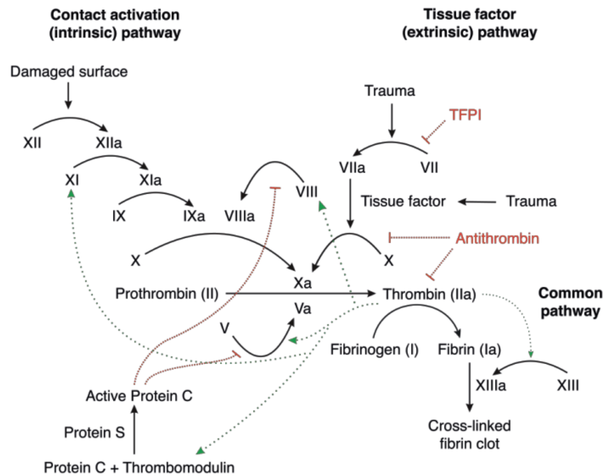


Figure 3. Illustration of the classic version of the coagulation cascade. TFPI: Tissue factor pathway inhibitor. https://commons.wikimedia.org/wiki/File:Coagulation_full.svg Public domain.

1.2 Coronary artery bypass grafting

In 1896, Ludwig Rehn performed the first successful surgery on the heart when he sutured a 1.5 cm stab wound [8]. At the end of World War II, many operations removing foreign objects from in or near the heart of soldiers were performed [9]. In the 1950s, strategies to interrupt the blood flow to the heart were introduced in order to perform surgery in the heart. John Gibbon developed a mechanical heart and lung machine which was an extracorporeal circuit that maintained circulation and oxygenation of the patient’s blood [10]. In 1953, he performed the first successful open heart surgery with total cardiopulmonary bypass (CPB) in a 18-year old patient with a large atrial septal defect [10]. The heart and lung machine was further developed and solutions for priming the extracorporeal system were introduced [11]. After the introduction of coronary angiography for the visualization of arteries and possible blockage, techniques for revascularization in patients with coronary artery disease were developed. In the late 1960s, coronary artery bypass grafting (CABG) which bypasses the blockage using saphenous vein was first introduced [11] (Figure 4).

In Sweden, 5,809 cardiac operations were performed in 2017 and 3,285 operations were CABG alone or combined with other cardiac interventions. The thirty-day mortality rate was 1.8% [12]. In a population-based study from Sweden, the mortality rate in patients that had survived the first 30 days after CABG alone (in the time-period 2002–2006) was approximately 1% in patients aged 18–54 years and 2% in patients that were 55 years or older [13]. In addition, the older patient group had a lower mortality risk than the general population (age- and gender-matched) [13].

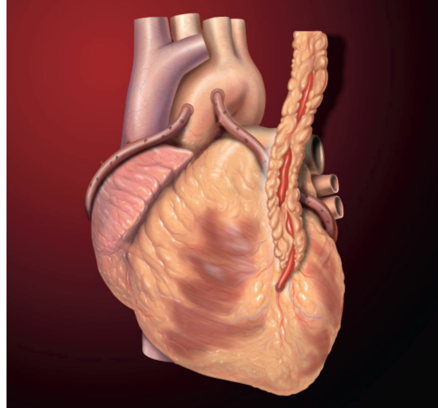


Figure 4. Schematic illustration of a heart and coronary artery bypass grafts done by Patrick J. Lynch, medical illustrator; C. Carl Jaffe, MD, cardiologist. https://commons.wikimedia.org/wiki/File:Heart_saphenous_coronary_grafts.jpg Public domain.

Bleeding in cardiac surgery

During cardiac surgery, there is a risk of bleeding complications which is associated with high morbidity and mortality rates [14]. In a study on adult cardiac surgery patients, severe bleeding was associated with an eight-fold increase in perioperative deaths, after adjustment for other factors influencing mortality [14]. Furthermore, CABG patients operated after an episode of acute coronary syndrome (ACS) and suffering from severe bleeding had a 14 times higher unadjusted 30-day mortality compared to patients without bleeding complications (9.9% vs 0.7%) [15]. In approximately 5% of all cardiac operations, the patient needs to undergo reoperation due to bleeding [12] which increases the risk of perioperative renal failure, stroke and mortality significantly [16, 17]. The postoperative mortality rate was doubled in patients undergoing reoperation due to bleeding after adjustment for other factors associated with death [16]. There are different factors that may contribute to the bleeding including hemodilution by the priming solutions in the CPB circuit, hypothermia, use of high dose heparin, and the use of CPB, which impairs platelet function, activates the coagulation cascade, and increases inflammation and fibrinolysis as the blood becomes exposed to foreign surfaces [18, 19]. Another factor that affects bleeding is ongoing or recently discontinued treatment with anticoagulant or antiplatelet medication.

1.3 Antiplatelet medication

Antiplatelet medication is used to prevent thrombotic events in patients with ACS by inhibition of platelet activation and aggregation pathways. Dual antiplatelet therapy (DAPT) with acetylsalicylic acid (ASA) and an ADP-receptor ($P2Y_{12}$) antagonist such as clopidogrel or ticagrelor is more effective in reducing thrombotic events than monotherapy with ASA only, but also increases the risk of perioperative bleeding complications [20-22].

Acetylsalicylic acid

ASA (Figure 5) was first marketed in 1899 as aspirin and used to relieve pain, fever and inflammation. It was later in the 1980s discovered that ASA also had an effect on platelets [23]. ASA irreversibly inhibits cyclooxygenase-1 (COX-1) and the inhibition will therefore last for the whole life-span of the platelet. COX-1 is needed for conversion of arachidonic acid (AA) to prostaglandin H_2 to ultimately produce TXA_2 (Figure 6) [24-26]. TXA_2 induces platelet aggregation and blockade of this production will result in irreversible inhibition of platelet aggregation [23].

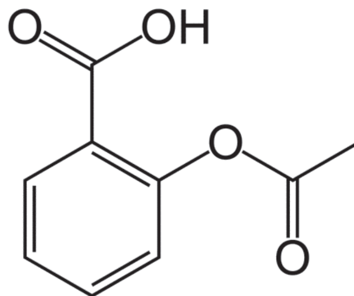


Figure 5. Molecular structure of acetyl-salicylic acid. https://commons.wikimedia.org/wiki/File:Aspirin-skeletal.svg Public domain.

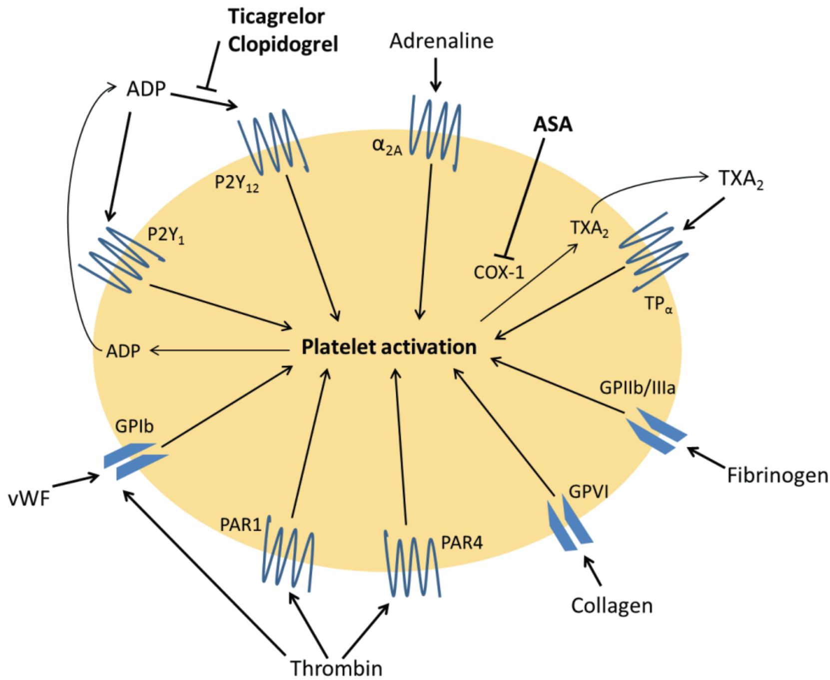


Figure 6. Schematic illustration of a platelet with a number of receptors, activation pathways and targets of the antiplatelet agents acetylsalicylic acid, ticagrelor and clopidogrel. ADP: adenosine diphosphate; ASA: acetylsalicylic acid; COX-1: cyclooxygenase-1; GP: glycoprotein; PAR: protease-activated receptor; TP: thromboxane receptor; TXA_2: thromboxane A_2; vWF: von Willebrand factor.

$P2Y_{12}$-receptor antagonists

ADP can induce platelet aggregation by binding to the G-protein coupled receptors $P2Y_1$ [27] and $P2Y_{12}$ [28, 29]. The co-activation of both the $P2Y_1$- and the $P2Y_{12}$-mediated pathway is required for a normal ADP-induced aggregation [30-32]. $P2Y_{12}$-receptor antagonists such as clopidogrel and ticagrelor bind to the $P2Y_{12}$-receptor and inhibit ADP-induced aggregation. The antagonists have different mechanisms of action [33]. Clopidogrel is a thienopyridine and a pro-drug that needs to be metabolized in the liver to become active (Figure 7). The active metabolite binds irreversibly to the $P2Y_{12}$-receptor which modifies the ADP-binding site [34] and thus inhibits ADP-induced platelet aggregation (Figure 6). As clopidogrel inhibition is irreversible, it will last for the whole life-span of the platelet. The hepatic metabolism of clopidogrel is dependent on the cytochrome P450 and genetic variation in the cytochrome P450 isozymes results in a varied response to

clopidogrel treatment [35-37]. The occurrence of non-responders has previously been reported to be between 15–30% [38].

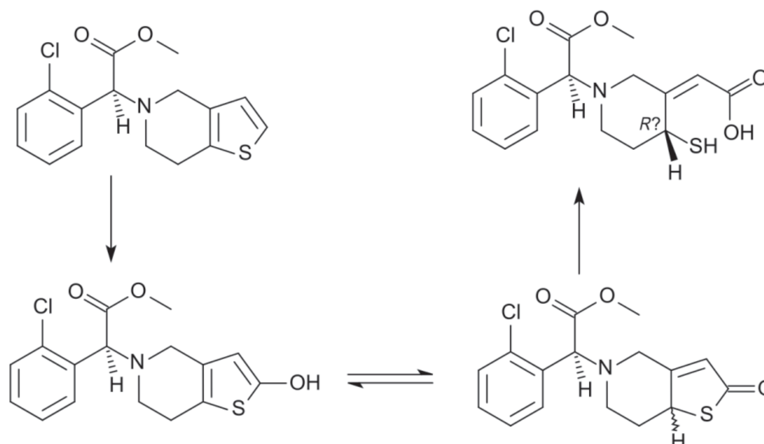


Figure 7. Conversion of clopidogrel (upper left) to its active metabolite (upper right). https://commons.wikimedia.org/wiki/File:Clopidogrel_activation.svg Public domain.

Ticagrelor (Figure 8) is a $P2Y_{12}$-receptor antagonist that binds to the receptor non-competitively with ADP [39]. It binds reversibly and locks the receptor in an inactive state [39] and thus inhibits ADP-induced aggregation (Figure 6). It has a faster onset than clopidogrel in stable coronary artery disease patients [40] as it is active in its native form as well as produces an active metabolite [39]. It also has a faster offset in these patients [40] with a plasma half-life of 6–13 h [33].

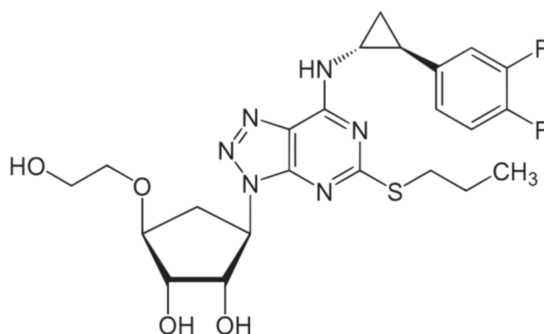


Figure 8. Molecular structure of ticagrelor. https://en. wikipedia.org/wiki/File:Ticagrelor.svg Public domain.

There are currently no commercially available antidotes to the $P2Y_{12}$-receptor antagonists and the treatment is therefore discontinued 3–5 days before non-emergent cardiac and non-cardiac surgery [41] while ASA treatment is continued. However, in a significant portion of ACS patients, the $P2Y_{12}$-receptor antagonists cannot be discontinued before surgery due to e.g. ongoing myocardial ischemia, alarming angiographic findings or recent coronary stenting. In a recent national study from Sweden, 40% of ACS patients undergoing CABG were operated after a shorter $P2Y_{12}$-receptor antagonist discontinuation period than recommended [15]. This means that the patients underwent surgery with ongoing potent platelet inhibition. For these patients, there is a need for a strategy to reduce bleeding complications.

1.4 Platelet concentrates

Today, transfusions of different blood products such as red blood cells, platelets, plasma, and coagulation factor concentrates are used to improve hemostasis in patients with ongoing bleeding. Transfusion of platelet concentrates is used to treat bleeding complications if it is suspected that low platelet count or platelet dysfunction contributes to the bleeding. Platelet concentrates can be prepared using different methods. Apheresis platelet concentrates are prepared using an apheresis device which separates platelets, together with some plasma, from the other blood components which are returned to the donor. As only platelets are retrieved from the donor and not whole blood, more platelets can be collected compared to whole blood donations which are restricted to the amount of red blood cells one can safely collect from an individual. Thus a sufficient amount of platelets are collected from a single donation to yield at least one platelet unit. Buffy-coat platelet concentrates are prepared from whole blood donations in a two-step process. The blood components are separated using centrifugation and a blood expander platform. After removal of red blood cells and plasma, the resulting buffy-coat can be used for platelet concentrate preparation. Buffy-coats from multiple donors and storage medium are pooled together in order to yield one platelet unit after a second centrifugation step. Interim platelet unit (IPU) concentrates are also prepared from whole blood donations but only require one platform for centrifugation and separation [42]. Using a separation platform, an IPU containing platelets in plasma and the blood components are separated. IPUs from multiple donors are pooled together with storage medium to yield one platelet unit.

The platelets can be stored in different media such as plasma and/or different platelet additive solutions (PAS) which, for instance, provide buffering

substances to maintain the pH [43, 44]. The aim is to keep the platelets in an inactivated state as well as to maintain the platelets' ability to aggregate in response to agonists. The platelet concentrates are stored in a platelet incubator at room temperature with horizontal agitation until use or for a maximum of 7 days. However, storage at room temperature influences the platelet quality negatively, a process referred to as platelet storage lesion [45, 46]. This includes changes in platelet morphology and an impaired platelet function. Current routine assays used in transfusion medicine for quality assessment of platelet concentrates include visual inspection as well as measuring platelet concentration, pH, glucose and lactate levels [46-48]. The platelets' ability to aggregate in response to agonists has also been shown to attenuate over time in stored platelet concentrates [45] and platelet activation increases and can thus be used to monitor platelet storage lesion [46].

1.5 New potential treatment strategy

Platelet transfusion can restore AA-induced aggregation inhibited by ASA [49] while it only has a small or no effect on the ADP-dependent pathway inhibited by $P2Y_{12}$-receptor antagonists [49-51]. In previous studies, the perioperative bleeding risk in patients undergoing cardiac surgery with $P2Y_{12}$-receptor antagonist treatment is associated to the their preoperative ADP-dependent platelet aggregation response [52-54]. A potential strategy to reduce bleeding complications in these patients could be one that improves ADP-dependent platelet aggregation.

Adrenergic agents

Adrenaline and noradrenaline (Figure 9) are catecholamines released from two main sources in the human body. Adrenaline is synthesized and released by chromaffin cells in the adrenal medulla while noradrenaline is mainly released from sympathetic nerve endings and to some extent from the adrenal medulla [55]. Noradrenaline can be converted to adrenaline by the enzyme phenylethanolamine-N-methyltransferase in the adrenal medulla [55]. These catecholamines are involved in the body's "fight or flight" response (response to stressful or fearful situations) and are released from the adrenal medulla upon acetylcholine stimulation of the nicotinic acetylcholine receptors located on the chromaffin cell membrane [56]. After their release, adrenaline and noradrenaline bind to adrenergic receptors on effector cells. The adrenergic receptors are G-protein coupled receptors and are divided into α-receptors and β-receptors. Adrenaline and noradrenaline bind to α-receptors causing vasoconstriction but can also bind to β-receptors which causes vasodilation [57-59]. The release of these catecholamines increases cardiac

output as activation of adrenergic receptors in the myocardium results in an increased heart rate and myocardial contractility [60-62]. Due to these effects, adrenaline and noradrenaline are commonly used in cardiac surgery to increase blood pressure during and after CPB.

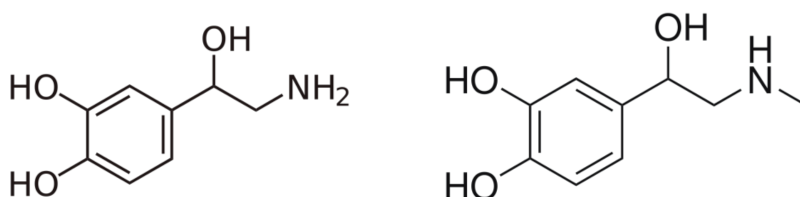


Figure 9. Molecular structures of noradrenaline (left) and adrenaline (right). https://commons.wikimedia.org/wiki/File:Noradrenaline2.svg https://sv.m.wikipedia.org/wiki/Fil:Adrenaline.svg Public domain.

Effects of adrenergic agents on platelets

Platelets express both α- and β-receptors [63]. In studies on untreated human platelets, supplementation with adrenaline or noradrenaline caused mild aggregation by activation of α-receptors [63-65]. The results also indicated that adrenaline was more potent than noradrenaline in inducing platelet aggregation [63-65]. These catecholamines also potentiate ADP-induced aggregation of human platelets without antiplatelet agents present [64]. In addition, adrenaline has been shown to potentiate ADP-induced aggregation in blood samples from clopidogrel-treated patients [66] and in platelet-rich plasma from healthy subjects where the $P2Y_{12}$-receptor antagonists prasugrel, cangrelor, ticagrelor or the active metabolite of clopidogrel had been added *in vitro* [67]. The potentiation may be due to the molecular mechanisms of these substances affecting platelet signaling pathways. ADP induces platelet activation and aggregation by binding to the G-protein coupled receptors $P2Y_1$ [27] and $P2Y_{12}$ [28, 29]. The binding of ADP to $P2Y_1$ activates the G-protein G_q which results in the activation of phospholipase C and consequent increase in cytosolic level of calcium ions, initiating the platelet activation and aggregation response (Figure 10) [27]. When ADP binds to $P2Y_{12}$, the G-protein G_i inhibits adenylyl cyclase and activates phosphoinositide 3-kinase (PI3K) which activates downstream effector molecules (Figure 10) [68-70]. The inhibition of adenylyl cyclase results in a reduced level of cyclic adenosine monophosphate (cAMP) and although an increased level of cAMP inhibits platelet activation, the reduced level does not directly affect platelet aggregation [71-74]. Activation of PI3K and subsequent activation of

downstream effector molecules result in granule secretion [74]. The co-activation of both the $P2Y_1$- and $P2Y_{12}$-mediated pathway is required for a normal ADP-induced activation and aggregation [30-32]. $P2Y_{12}$-receptor antagonists such as ticagrelor act by inhibiting the ADP-induced activation of $P2Y_{12}$. Adrenaline and noradrenaline can bind to α-receptors [63-65]. Adrenaline binds to the α_{2A}-receptor which is coupled to the G-protein G_z (Figure 10). Binding of adrenaline results in inhibition of adenylyl cyclase [31, 75] and activation of PI3K [68] thus mimicking the $P2Y_{12}$-mediated pathway (Figure 10) [72]. Therefore, adrenaline may potentiate ADP-induced aggregation in the presence of $P2Y_{12}$-receptor antagonists as responses from both signaling pathways can be achieved.

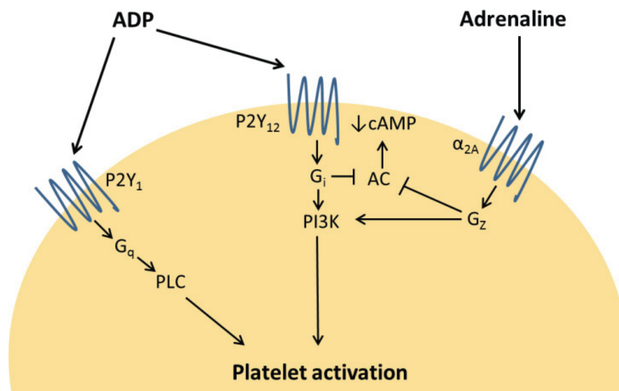


Figure 10. Schematic illustration of intracellular signaling in response to binding of ADP and adrenaline to their respective G-protein coupled receptors $P2Y_1$, $P2Y_{12}$ and α_{2A}. AC: adenylyl cyclase; ADP: adenosine diphosphate; cAMP: cyclic adenosine monophosphate; PI3K: phosphoinositide 3-kinase; PLC: phospholipase C.

Infusion of adrenaline (0.07 µg/kg/min) has previously improved platelet aggregation in healthy volunteers without antiplatelet therapy [76]. Infusion of noradrenaline (0.03 and 0.14 µg/kg/min) has also increased platelet aggregation in untreated healthy volunteers [77]. In another study on both healthy volunteers and hypertensive patients, the ADP-induced aggregation increased after infusion of noradrenaline (0.1 µg/kg/min) [78]. No previous studies assessing the effect of adrenaline or noradrenaline infusion on platelet function in subjects with ongoing antiplatelet therapy have been conducted. Infusion of adrenaline or noradrenaline to increase platelet activation and aggregation could be a potential strategy for prevention of bleeding complications in cardiac surgery patients with ongoing or recently

discontinued antiplatelet therapy. In two previous randomized trials, perioperative infusion of low dose adrenaline (0.05 μg/kg/min) in combination with the antifibrinolytic agent tranexamic acid during total hip arthroplasty reduced early postoperative total blood loss compared to placebo groups receiving saline solution [79, 80].

1.6 Platelet function testing

Various methods that target different phases of platelet function can be used to assess the function.

Activation

One phase of platelet function is platelet activation. This can be studied with flow cytometry using various fluorescently-labeled antibodies or reagents binding to different platelet activation markers. Flow cytometry allows for the simultaneous analysis of various activation-dependent changes [81]. In this method, anticoagulated whole blood or platelet suspension is diluted and added to fluorescently-labeled antibodies or reagents. Markers expressed on the surface membrane of activated platelets include the granula proteins P-selectin [82, 83] and CD63 [84] as well as activated fibrinogen receptor GP IIb/IIIa (which PAC-1 binds to) [85] and the phospholipid phosphatidylserine [86, 87]. To distinguish platelets from other cells in whole blood, an antibody binding to a platelet surface marker such as CD61 or glycoprotein Ib (GPIb, also referred to as CD42b) can be used. Antibodies binding to platelet surface markers will bind to both resting and activated platelets. Agonists such as ADP, thrombin receptor-activating peptide-6 (TRAP) and collagen may be added to study platelet reactivity [81].

In this method, the sample is placed in the flow cytometry instrument and follows a liquid stream into the apparatus (Figure 11). Droplets are formed by a vibrating nozzle. The droplets go past one or multiple laser beams which will excite the fluorophores and the emitted fluorescence and light-scatter are recorded by detectors. Platelets can be identified by their light-scatter properties and by the platelet surface marker. The median fluorescence intensity or the percentage of platelets expressing the activation marker can be used to quantify platelet activation [81].

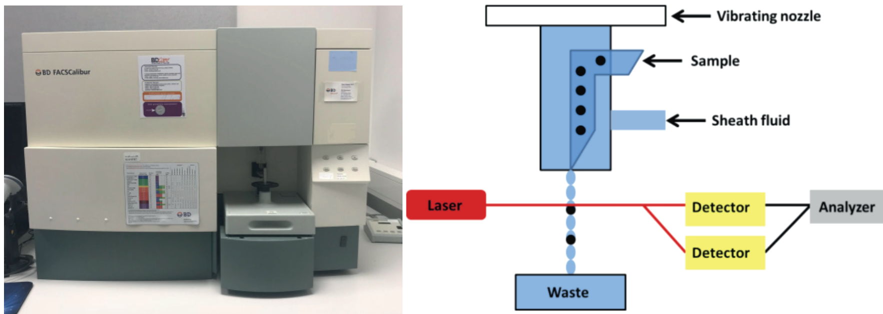


Figure 11. A flow cytometer (left) and the principle of flow cytometry (right).

Aggregation

Another phase of platelet function is platelet aggregation. Light-transmission aggregometry (LTA) is considered the gold standard for studying platelet function *in vitro*. It was first published by Born and O'Brien in 1962 [88, 89]. Anticoagulated whole blood is centrifuged and the resulting platelet-rich plasma and platelet-poor plasma can be utilized in this method. Low shear platelet aggregation is analyzed by measuring the change in optical density after agonists, such as ADP, adrenaline, AA and collagen have been added to platelet-rich plasma. When the platelets aggregate, the sample become less turbid resulting in a diminished light absorbance and increased light transmission. The platelet-poor plasma is used as a blank to set the limit for no aggregation. Disadvantages with LTA include it being time-consuming, requiring a large volume of blood and sample preparation [90, 91].

Point-of-care (POC) tests for measuring platelet function have been developed for a simpler, faster and more standardized analysis. One example is the VerifyNow® system (Accumetrics, San Diego, CA, USA), which is a fully-automated whole blood analysis that measures platelet aggregation. In this method, the binding of platelets to fibrinogen-coated polystyrene beads in response to ADP and prostaglandin E1, AA or TRAP is detected for monitoring antiplatelet therapy. When the platelets bind to the beads, the light transmission increases which will be detected in the instrument. The results are expressed in reaction units based on the rate and degree of aggregation [92].

Another POC method is whole blood impedance aggregometry such as the commercially available Multiplate® (Roche Diagnostics, Basel, Switzerland) (Figure 12). In this method, anticoagulated blood is added to test cells at 37°C. The Multiplate aggregometer has five channels which enable parallel

testing using different agonists such as ADP, AA, collagen and TRAP. The test cell contains a magnetic stirrer and two sets of electrodes (Figure 12). After the agonists have been added, the platelets aggregate on the electrodes which will cause the impedance between the electrodes to increase. This is measured for 6 minutes. As each test cell contains two sets of electrodes, the results will be presented as two aggregation curves. The area under the aggregation curve (AUC) (mean value of the two curves), measured in aggregation units (U), is used to quantify platelet aggregation. Multiplate has been used in previous studies which showed that the bleeding risk in cardiac surgery patients with ongoing or recently discontinued treatment with a $P2Y_{12}$-receptor antagonist correlates to the residual ADP-dependent platelet aggregation [52-54].

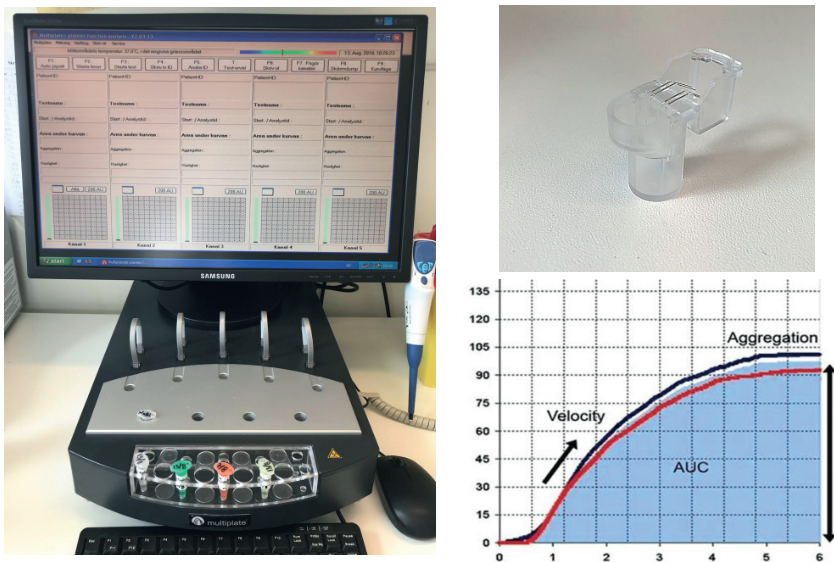


Figure 12. The Multiplate® analyzer (left), a test cell with two electrode pairs (upper right) and an example of a resulting aggregation curve (lower right). AUC: Area under curve.

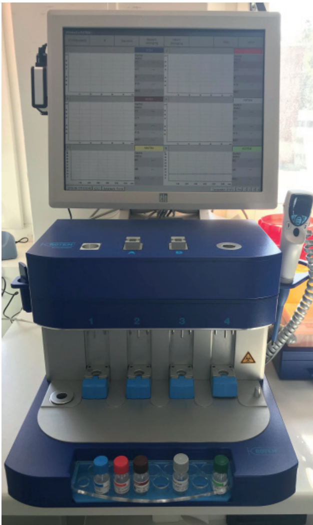
1.7 Clot formation testing

Different methods of monitoring clot formation exist. Two standard screening methods are prothrombin time (PT) test and activated partial thromboplastin time (APTT). The PT test utilizes thromboplastin (containing tissue factor and phospholipids) to extrinsically activate clot formation and is sensitive for abnormalities of factors V, VII, and X, prothrombin and fibrinogen [93]. Citrated platelet-poor plasma is incubated with thromboplastin at 37°C and calcium is then added. The time it takes to formation of fibrin filaments is the PT [93]. To standardize PT, the international normalized ratio (INR) can be calculated by dividing the PT for the patient sample with the PT for normal plasma. To study intrinsic activation of clot formation, the APTT test can be used. In this test, partial thromboplastin (phospholipids) in combination with an activator of the contact pathway, such as silica or ellagic acid, is used to intrinsically activate clot formation. The APTT test is sensitive for abnormalities of factors V, VIII, IX, X, XI, XII, prothrombin and fibrinogen [93]. Citrated platelet-poor plasma is incubated with partial thromboplastin and an activator at 37°C and then calcium is added. The time it takes to formation of fibrin filaments is the APTT [93]. A drawback with the PT and APTT tests is that it requires plasma preparation from whole blood.

POC tests for assessing clot formation have been developed for a more standardized analysis without the need for sample preparation. These include thromboelastography (TEG®; Haemonetics Corporation, Braintree, MA, USA) and thromboelastometry (ROTEM®; Pentapharm GmbH, Munich, Germany) [94, 95]. Both TEG and ROTEM are viscoelastic methods. In TEG, anticoagulated whole blood is added to a test cup at 37°C and a pin that is coupled to a computer through a torsion wire is suspended into the cup. The test cup oscillates and as a clot starts to form, the pin will become restrained thus creating tension in the wire which will be mechanically detected and processed by the computer [94, 95]. From the resulting graph, multiple parameters are obtained. These include reaction time (s), which is the time it takes from the start of the test to the formation of a clot with an amplitude of 2 mm, and maximum amplitude (mm) which reflect the clot strength.

ROTEM is a modified version of TEG. In ROTEM, anticoagulated blood is added to a test cup and activators of clot formation are added at 37°C. The sample cup is then placed so that an oscillating pin is suspended into the cup. As a clot starts to form, the pin becomes increasingly limited in its movement. The device optically detects the change in movement yielding a

graph from which multiple parameters are obtained [94]. These include clotting time (s) (equivalent to reaction time), clot formation time (s) and maximum clot firmness (mm) (equivalent to maximum amplitude) (Figure 13).



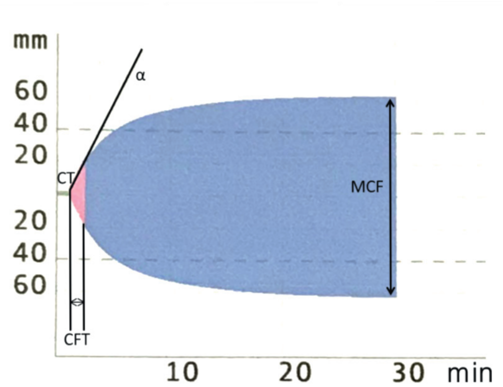


Figure 13. The ROTEM® delta instrument (left) and an example of a resulting graph (right). CT: clotting time; CFT: clot formation time; MCF: maximum clot firmness.

2 AIMS

The overall aim of this thesis was to identify and evaluate current and new potential methods to prevent and reduce perioperative bleeding complications in patients with ongoing antiplatelet therapy.

The specific aims were

1. To compare the new interim platelet unit (IPU) concentrates with the more established buffy-coat and apheresis platelet concentrates in terms of platelet storage lesion markers (**Study I**)

2. To investigate the *in vitro* effects of adrenaline supplementation, alone or combined with platelet concentrate, on platelet aggregation and activation in blood samples from patients with ongoing DAPT with ASA and ticagrelor (**Study II**)

3. To investigate the effect of adrenaline infusion on platelet aggregation, activation and clot formation in ticagrelor-treated healthy volunteers (**Study III**)

4. To investigate the effect of intraoperative noradrenaline infusion on platelet aggregation and clot formation in patients undergoing coronary artery bypass grafting (**Study IV**)

3 METHODS

3.1 Participants

In Study I, platelet concentrates from blood donors at Sahlgrenska University Hospital were evaluated. All donors signed the health questionnaire, which specifies that blood components may be used for research and quality assurance purposes, before donation. This study was part of the internal evaluation and regarded as quality assurance for the introduction of a new type of platelet concentrate at the hospital. Studies II–IV were approved by the Regional Research Ethics Committee in Gothenburg, Sweden, and were conducted according to the Declaration of Helsinki. Studies III and IV were approved by the Swedish Medical Products Agency and Study IV was also approved by Sahlgrenska Radiation Safety Committee. All participants gave written informed consent.

In Study II, blood samples from in-hospital ACS patients on ongoing DAPT with ASA and ticagrelor were included. Exclusion criteria were known bleeding disorder, renal or liver disease. In Study III, only healthy men were included to avoid the effect of the hormone cycle on platelet function [96]. The following exclusion criteria were applied: chronic or mental disease, age above 40 years, former intracranial bleeding, abnormalities in electrocardiography (ECG), chronic medication, and any occasional intake of non-steroidal anti-inflammatory drugs, acetylsalicylic acid or any other substances that may interact with ticagrelor, adrenaline or metoprolol less than one week before the investigational visit. Additional exclusion criteria were known intolerance or contraindication to ticagrelor, adrenaline or metoprolol or known drug abuse of any kind. In Study IV, patients planned for elective CABG during May 2017-January 2018 were asked to partake in the study when the required research means were available. The following exclusion criteria were applied: hypersensitivity to ^{125}I-Albumine, contraindication for radioactive contrast, age below 40 years, diabetes mellitus, previous stroke, untreated hypertension, pregnancy, breast-feeding, a known carotid artery stenosis, and/or left ventricular systolic ejection fraction of 45% or less. The characteristics of all the study participants are given in Table 1.

Table 1. Characteristics of participants in Studies II–IV. Median and interquartile range or number.

	Study II	Study III	Study IV	
	Adrenaline *in vitro*	Adrenaline *in vivo*	Control	Noradrenaline
n	40	10	12	12
Female/male gender	7/33	0/10	3/9	2/10
Age (years)	64 (54–73)	24 (19–30)	67 (64–69)	64 (55–69)
Height (m)	1.79 (1.71–1.82)	1.82 (1.76–1.93)	1.71 (1.69–1.76)	1.81 (1.74–1.87)
Weight (kg)	85 (76–95)	83 (74–103)	80 (77–82)	85 (74–91)
BMI (kg/m^2)	27 (25–29)	24 (23–30)	28 (25–29)	25 (24–27)
Diabetes mellitus	4 (10%)	0	0	0
Hemoglobin (g/L)	138 (131–156)	145 (141–151)	146 (139–152)	143 (135–148)
Platelet concentration (× 10^9/L)	210 (183–262)	235 (222–249)	204 (190–241)	256 (232–306)

3.2 Study procedure

Study I

Platelet concentrates prepared by the regional blood bank at Sahlgrenska University Hospital, according to local routines in agreement with European guidelines [48], were evaluated. The new IPU concentrates (n=10) were compared to buffy-coat (n=10) and apheresis platelet concentrates (n=10) during the 7-day storage period (Figure 14). All platelet concentrates were leukocyte-reduced (<1 × 10^6 leukocytes per unit) and gamma-irradiated (25 Gray). After preparation, the platelet concentrates were stored in a platelet incubator at 22°C with horizontal agitation until use.

IPU platelet concentrates were prepared from whole blood donations. Whole blood (anticoagulated with citrate, phosphate and dextrose) was separated using the processing program 3C in the Reveos system (Terumo BCT Europe) yielding one unit of red blood cells, one unit of plasma and one interim platelet unit (IPU) as well as a waste pack with leukocytes. The resulting IPU contained approximately 30 mL plasma and was incubated overnight (22°C) with horizontal agitation. Four IPUs were then pooled together with PAS (SSP+) to a target count of approximately 250 × 10^9

platelets/unit. The plasma carryover in the IPU concentrates was approximately 40%.

Buffy-coat platelet concentrates were also prepared from whole blood donations. Whole blood (anticoagulated with citrate, phosphate and dextrose) was centrifuged and separated into its components using a blood expander platform. After the resulting buffy-coat had been incubated overnight (22°C) with horizontal agitation, four buffy-coats were pooled together with PAS (SSP) giving rise to one unit. For the elimination of residual red blood cells, the pooled unit was further processed using a blood component processing device (TACSI). The plasma carryover in the buffy-coat platelet concentrates was approximately 20%.

Apheresis platelet concentrates were collected according to standard protocols (Trima Accel; Terumo BCT Europe) from donors with a platelet concentration of ≥230 × 10^9/L before donation. The target platelet concentration was 1,600 × 10^9/L and the whole blood to citrate ratio was 10:1. The resulting platelet concentrates consisted of platelets in autologous plasma and were subjected to a 2-hour rest period before further handling.

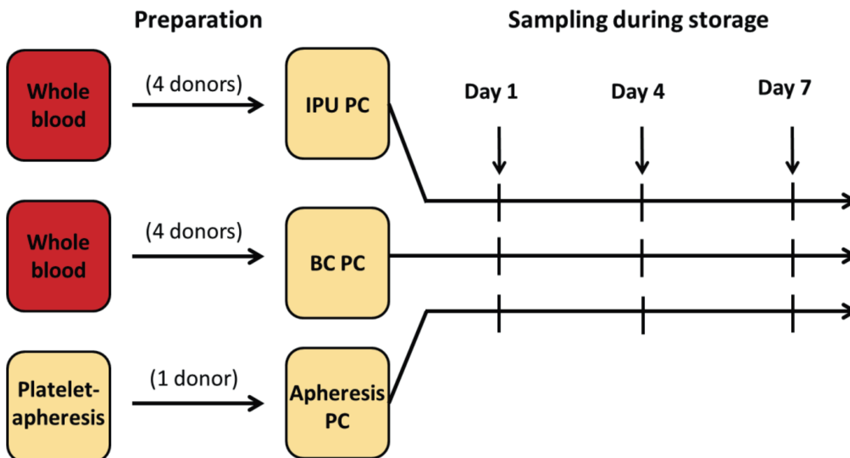


Figure 14. Interim platelet unit (IPU), buffy-coat (BC) and apheresis platelet concentrates (PC) were prepared and then sampled on day 1, 4 and 7 after donation (Study I).

On days 1, 4 and 7 after donation, metabolic parameters (pH, lactate and glucose) were measured in all platelet concentrates and platelet activation was evaluated with flow cytometry. In addition, agonist-induced platelet aggregation was evaluated using impedance aggregometry.

Study II

In this study, blood samples from patients on DAPT with ASA and ticagrelor were collected for platelet aggregation and activation analysis. The blood samples were supplemented with adrenaline, apheresis platelet concentrates, and/or ADP. First, the effect of five plasma concentrations of adrenaline in the blood sample (6 nM, 27 nM, 82 nM, 153 nM and 770 nM) on ADP- and AA-induced aggregation was evaluated with impedance aggregometry (n=10) (Figure 15).

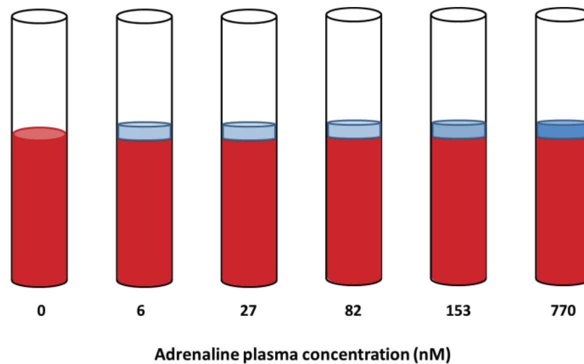


Figure 15. Adrenaline supplementation to blood samples. Concentrations are the resulting plasma concentrations of adrenaline in the blood sample (Study II).

Next, adrenaline (770 nM) was added alone or in combination with apheresis platelet concentrate to investigate the effect on ADP- and AA-induced aggregation (n=10) (Figure 16). The apheresis platelet concentrates were prepared according to the methods section for Study I and stored for a median of 1 (range 1–3) day. The addition of 120×10^6 platelets to a 1 mL blood sample was evaluated which approximately corresponds to an increase in platelet count achieved by transfusion of three apheresis units to a 70 kg patient.

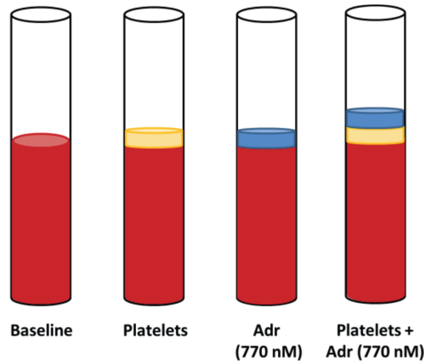


Figure 16. Supplementation with platelet concentrate and adrenaline (Adr) to blood samples (Study II).

Lastly, adrenaline (770 nM) was added alone or combined with ADP to evaluate their effect and possible potentiation of platelet aggregation (n=10) and activation (n=10) using flow cytometry (Figure 17).

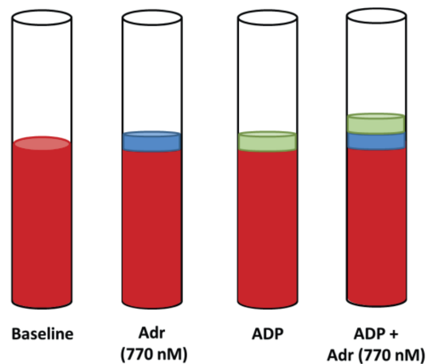


Figure 17. Supplementation with adrenaline (Adr) and ADP to blood samples (Study II).

Study III

This study was a proof-of-concept study performed on healthy volunteers (n=10). During a screening visit, information about medical history was collected and a physical examination was performed which included heart and lung auscultation, height, weight, blood pressure and heart rate registrations and electrocardiography (ECG). Furthermore, laboratory testing was conducted and included hemoglobin and platelet concentration,

electrolytes, liver function tests and a urine drug test. If the participant was still qualified for study participation, the investigational visit was conducted within 21 days.

During the investigational visit, the study participants were treated with ticagrelor (2x90 mg) and an increasing infusion of adrenaline (0.01, 0.05, 0.10, 0.15 µg/kg/min) was administered according to Figure 18. Lastly, the β-blocker metoprolol (5 mg) was intravenously given in combination with the highest dose of adrenaline. Registrations of blood pressure, heart rate and perceived dyspnea (Borg scale 1–10) and blood samples were collected at seven time-points (Figure 18) with a maximum deviation of 3 minutes accepted at each time-point. Impedance aggregometry was used to assess platelet aggregation while flow cytometry was used to measure platelet activation. Clot formation was evaluated with thromboelastometry. If the participant's systolic blood pressure increased to over 180 mmHg or the heart rate increased to over 120 beats/min, the adrenaline infusion would be terminated according to the study protocol.

Phone follow-up calls to the participants were conducted 1 and 3 days after the investigational visit to record any potential adverse events after their visit.

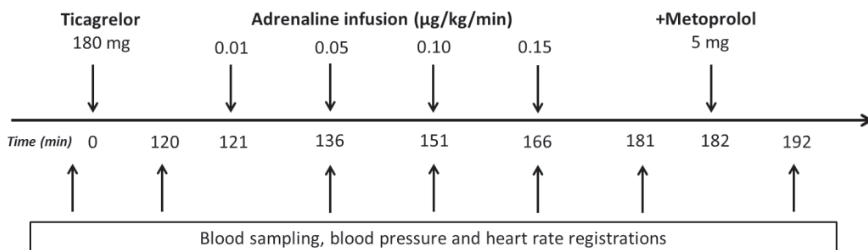


Figure 18. Flow chart of treatment during the investigational visit (Study III).

Study IV

Study IV was a randomized controlled trial where 24 CABG patients were randomized to either maintenance of pre-anesthesia mean arterial pressure (MAP) by noradrenaline infusion until 50 minutes after anesthesia induction (before start of CPB) or standard treatment (noradrenaline infusion only if MAP below 60 mmHg and/or as a complement to the Trendelenburg position) (Figure 19). Block randomization with sealed envelopes was used to guarantee that the distribution of the treatment (maintenance of MAP or standard treatment) was equal within the gender group. Registrations of MAP and blood samples were collected 10 minutes before anesthesia induction and 50 minutes after induction. Platelet aggregation was assessed with impedance aggregometry and clot formation was assessed with thromboelastometry. Study IV was a predefined substudy to a trial investigating plasma volume changes in relation to blood pressure.

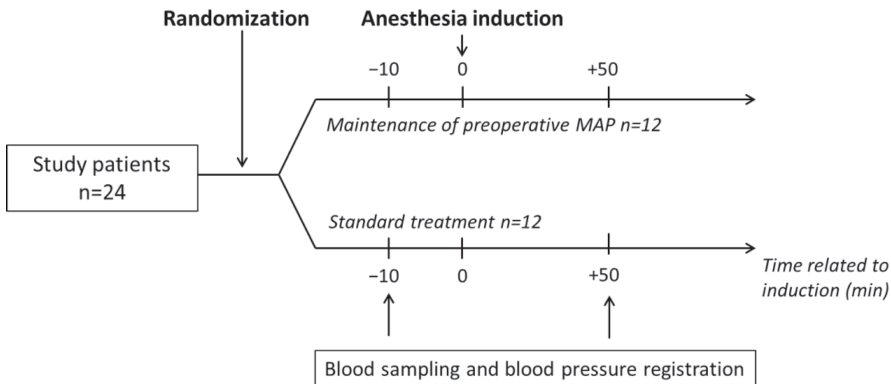


Figure 19. Flow chart of the randomization and treatment (Study IV). MAP: mean arterial pressure.

3.3 Analyses

Flow cytometry

In Studies I–III, flow cytometry (FACSCalibur flow cytometer) was used to assess platelet activation. In all three studies, 10,000 positive platelet events were analyzed and the percentages of platelets expressing the platelet activation markers were reported. To set the limit between positively and negatively stained platelets, background control samples were used which show the nonspecific binding of the antibodies. The limit is set to obtain approximately 1 to 2% positive platelets in the background control samples [97].

In Study I, the unstimulated (spontaneous) activation during storage of platelet concentrates was evaluated by measurement of expression of the platelet granule proteins P-selectin and CD63 and exposure of phosphatidylserine. Antibodies and reagents for the platelet activation markers and prepared platelet concentrate were added to tubes. After 15 minutes of incubation and subsequent dilution, flow cytometry was performed within 30 minutes.

In Studies II and III, both spontaneous and agonist-induced platelet activation was evaluated by measuring expression of P-selectin and binding of PAC-1 (binds to activated fibrinogen receptor GP IIb/IIIa). Whole blood was collected in hirudin tubes (0.15 mg/L). Duplicate samples containing either no agonist (unstimulated), adrenaline alone (plasma concentration 770 nM), ADP alone (6.5 μM) or a combination of adrenaline and ADP were prepared in Study II. In Study III, duplicate samples containing either no agonist, ADP (6.5 μM) or TRAP (32 μM, positive control) were prepared. Antibodies binding to the activation markers and whole blood were added to tubes. After 10 minutes of incubation and subsequent dilution, flow cytometry was performed within an hour in Study II and within 30 minutes after blood collection in Study III.

Impedance aggregometry

Impedance aggregometry (Multiplate®; Roche Diagnostics, Basel, Switzerland) was used to study platelet aggregation in all four studies. Whole blood was collected in hirudin tubes (0.15 mg/L). In this method, 300 μL of anticoagulated whole blood is added to 300 μL NaCl (9 mg/mL) in each test cell and allowed to incubate for 3 minutes at 37°C. Agonists are thereafter added to initiate aggregation and the impedance between two pairs of electrodes is measured for 6 minutes. The two resulting aggregation curves

should not differ more than 20%. The AUC (U) was reported. In Study I, platelet concentrate was added instead of whole blood. Specifically, 150 µL platelet concentrate was added to 450 µL phosphate-buffered saline (PBS) for apheresis concentrates. For the IPU and buffy-coat platelet concentrates, 150 µL of the PBS was replaced with 150 µL of allogeneic plasma of blood group AB.

In Study I, the ADP, ASPI, TRAP and COL tests were used. The ADP test assesses $P2Y_{12}$-receptor dependent aggregation using ADP as an agonist (final concentration 6.5 µM) while the ASPI test evaluates cyclooxygenase-dependent aggregation (i.e. ASA-sensitive) using AA as an agonist (final concentration 0.5 mM). Further the TRAP test detects PAR1-receptor dependent aggregation (sensitive to GP IIb/IIIa antagonists) using TRAP-6 as an agonist (final concentration 32 µM) while collagen is used in the COL test to evaluate cyclooxygenase-dependent aggregation (final concentration 3.2 mg/L). In Study II, the ADP high-sensitivity (ADP HS) and ASPI tests were used while in Studies III and IV, ADP HS, ASPI and TRAP tests were used. The ADP HS test evaluates $P2Y_{12}$-receptor dependent aggregation with higher sensitivity than the ADP test by the use of ADP (final concentration 6.3 µM) in combination with prostaglandin E1 (final concentration 9.4 nM). The reference ranges provided by the manufacturer for the ADP, ADP HS, ASPI, TRAP and COL tests in blood samples from healthy individuals are 57–113 U, 43–100 U, 71–115 U, 84–128 U, and 72–125 U, respectively when using double wall hirudin-anticoagulated blood tubes.

Thromboelastometry

Clot formation was assessed using thromboelastometry (ROTEM®; Pentapharm GmbH, Munich, Germany) in Studies III and IV. Blood samples were collected in citrated tubes (0.109 M citrate). In this method, 300 µL of anticoagulated whole blood is added to a test cup and then an activator is added at 37°C. The EXTEM test was used in Study III while the EXTEM, FIBTEM, INTEM and HEPTEM tests were used in Study IV. The EXTEM and FIBTEM tests evaluate the extrinsically activated clot formation using tissue factor as an activator. In the FIBTEM test, cytochalasin D is also added to inhibit the platelet contribution to clot formation. In the INTEM test, clot formation is intrinsically activated by addition of ellagic acid and partial thromboplastin phospholipid. As the INTEM test is sensitive for heparin, the HEPTEM test can be used to eliminate the effect of heparin by the addition of heparinase together with the same activators as in the INTEM test. The clotting time, clot formation time and maximum clot firmness were reported. The clotting time is the time from the start of the test until the clot reaches an

amplitude of 2 mm while the clot formation time is the time it takes from an amplitude of 2 mm until 20 mm is reached. The maximum clot firmness is the maximum amplitude that is reached during the test reflecting clot strength.

3.4 Statistics

Data are presented as median with interquartile range (25th–75th percentiles).

Study I

IPU platelet concentrates were compared with buffy-coat concentrates and with apheresis concentrates using the Mann-Whitney U test at each sampling time-point. The tests were conducted at a significance level of 0.05 and performed with SPSS Statistics version 22 (IBM Corporation, Armonk, NY, USA).

Study II

Comparisons of continuous data before and after addition of the substances were performed using the Friedman test for repeated measures. The tests were conducted at a significance level of 0.05. In order to find the pairs of samples that differed significantly, the Wilcoxon signed-rank test (matched pairs) was used with Bonferroni-Holm correction [98]. The statistical analyses were performed with SPSS Statistics version 22 (IBM Corporation, Armonk, NY, USA).

Study III

Comparisons of continuous data between the seven different blood sampling time-points were conducted with the Friedman test. The tests were conducted at a significance level of 0.05. In order to find the pairs of samples that differed significantly, Wilcoxon signed-rank test was used. The sample size was based on preliminary results from Study II where a mean difference in ADP-induced aggregation after adrenaline supplementation (153 nM) was 8.6 U with a standard deviation of the difference of 8.7 U. A sample size of 8 study participants was needed to achieve 80% power to detect such a difference with a two-sided test at a significance level of 0.05. A sample size of 10 study participants was chosen to account for any discontinued participation. The statistical analyses were performed with SPSS Statistics version 22 (IBM Corporation, Armonk, NY, USA).

Study IV

Within each randomization group, values at 10 minutes before and 50 minutes after anesthesia induction were compared using Wilcoxon signed-rank test. To compare continuous data between the two randomization groups, the Mann-Whitney U test was used. Two secondary analyses were performed. In one analysis, the patients were allocated according to if they had ongoing noradrenaline infusion at 50 minutes after induction or not. In the other analysis, one patient was excluded due to not having ongoing ASA-treatment. Furthermore, regression models with natural cubic splines were used to evaluate the effect of noradrenaline infusion rate on the changes in platelet aggregation between the two time-points. Piecewise linear functions were used to simplify the splines. The difference in Least Squares Means (ΔLSM) with 95% Confidence Intervals (CI) is presented for each 0.01 μg/kg/min increase in noradrenaline infusion rate. The tests were conducted at a significance level of 0.05. No separate power analysis was conducted for this substudy. Statistical analyses were performed with SPSS Statistics version 22 (IBM Corporation, Armonk, NY, USA) and SAS Software version 9.4 (SAS Institute Inc., Cary, NC, USA).

4 RESULTS

4.1 Comparison of stored platelet concentrates (Study I)

Platelet concentration and metabolic parameters

Comparing IPU and buffy-coat platelet concentrates, the platelet concentration did not significantly differ while the rates of lactate production and glucose consumption were significantly lower in IPU concentrates ($p=0.003$ and $p=0.022$ respectively on day 4, and $p=0.002$ and $p<0.001$ respectively on day 7) (Table 2). Furthermore, a higher pH was observed in IPU concentrates ($p=0.002$ on day 1, and $p<0.001$ on days 4 and 7).

*Table 2. Platelet concentration and in vitro metabolic parameters of interim platelet unit concentrates (IPU PCs), buffy-coat PCs, and apheresis PCs during storage (n=10 for each). Values on day 1 were used as reference points for the calculation of lactate production and glucose consumption. Median and interquartile range. ***p<0.001, **p<0.01 compared to IPU PCs at the same time-point of sampling. PLT: platelets.*

Days after donation	Day 1	Day 4	Day 7
Platelet concentration ($\times 10^9$/L)			
IPU PCs	804 (758–858)	772 (749–834)	729 (711–826)
Buffy-coat PCs	893 (864–965)	854 (833–913)	856 (809–911)
Apheresis PCs	1,872 (1,701–1,971)***	1,860 (1,731–1,908)***	1,841 (1,735–1,884)***
Lactate production rate (mmol/10^{12} PLT/day)			
IPU PCs		1.0 (0.8–1.1)	1.2 (1.1–1.5)
Buffy-coat PCs		1.3 (1.2–1.4)**	1.7 (1.6–1.7)**
Apheresis PCs		1.1 (1.0–1.2)	1.2 (1.1–1.2)
Glucose consumption rate (mmol/10^{12} PLT/day)			
IPU PCs		0.4 (0.4–0.5)	0.5 (0.4–0.6)
Buffy-coat PCs		0.6 (0.5–0.7)*	0.8 (0.8–0.9)***
Apheresis PCs		0.5 (0.4–0.7)	0.6 (0.6–0.8)**
pH			
IPU PCs	7.30 (7.27–7.30)	7.43 (7.39–7.43)	7.39 (7.33–7.41)
Buffy-coat PCs	7.23 (7.22–7.27)**	7.30 (7.29–7.32)***	6.98 (6.94–7.06)***
Apheresis PCs	7.45 (7.40–7.48)***	7.42 (7.38–7.46)	7.21 (7.11–7.27)**

Comparing IPU and apheresis platelet concentrates, a lower platelet concentration was observed in IPU concentrates (p<0.001 for all) while the lactate production rate did not significantly differ (Table 2). A lower glucose consumption rate was observed in IPU concentrates at the end of the storage period (p=0.002 on day 7). A lower pH was observed in IPU concentrates in the beginning of storage (p<0.001 on day 1) while it was higher in IPU concentrates at the end of the storage period (p=0.002 on day 7).

Platelet activation

There were significant differences in the percentage of platelets expressing platelet activation markers between the different types of platelet concentrates during storage (Table 3).

*Table 3. Percentage of platelets expressing activation markers P-selectin, CD63, and phosphatidylserine in interim platelet unit concentrates (IPU PCs), buffy-coat PCs, and apheresis PCs during storage (n=10 for each). Median and interquartile range. ***p<0.001, **p<0.01, *p<0.05 in comparison to IPU PCs at the same time-point.*

Days after donation	Day 1	Day 4	Day 7
P-selectin (%)			
IPU PCs	22.2 (18.6–25.3)	18.2 (16.4–21.2)	25.8 (25.0–29.4)
Buffy-coat PCs	16.8 (15.4–19.3)*	53.1 (46.2–57.0)***	62.6 (59.2–65.9)***
Apheresis PCs	5.0 (3.0–14.9)*	11.8 (6.9–16.0)*	19.5 (13.9–24.9)*
CD63 (%)			
IPU PCs	15.4 (13.2–18.0)	18.7 (13.6–20.0)	20.7 (16.8–21.9)
Buffy-coat PCs	8.3 (6.1–10.1)**	29.0 (25.6–35.4)***	31.5 (26.3–39.3)**
Apheresis PCs	11.0 (4.5–24.4)	14.0 (7.0–19.1)	15.3 (8.9–19.2)*
Phosphatidylserine (%)			
IPU PCs	2.7 (2.6–4.0)	5.1 (4.2–5.9)	7.4 (5.6–8.4)
Buffy-coat PCs	1.0 (0.9–1.5)*	4.2 (3.1–4.4)*	8.9 (8.2–10.9)*
Apheresis PCs	1.5 (1.2–1.8)**	2.8 (2.3–3.1)***	4.1 (3.2–7.0)*

Comparing IPU and buffy-coat platelet concentrates, a significantly higher percentage of platelets expressed the activation markers P-selectin (p=0.023), CD63 (p=0.001), and phosphatidylserine (p=0.028) in IPU concentrates in the beginning of the storage period (Table 2). However at the later time-points, a lower percentage of platelets expressed P-selectin (p<0.001 for both) and CD63 (p<0.001 and p=0.001, respectively) in IPU concentrates. The percentage of platelets expressing phosphatidylserine was still higher on day 4 in IPU concentrates (p=0.045) but lower at the end of the storage period in IPU concentrates (p=0.034 on day 7).

Comparing IPU and apheresis platelet concentrates, more platelets expressed P-selectin ($p=0.028$ on day 1, and $p=0.023$ on days 4 and 7) and phosphatidylserine ($p=0.001$ on day 1, $p<0.001$ on day 4, and $p=0.016$ on day 7) in IPU concentrates (Table 3). Also, a higher percentage of platelets expressed CD63 in IPU concentrates at the end of the storage period ($p=0.034$ on day 7).

Platelet aggregation

There were significant differences in agonist-induced platelet aggregation between the different types of platelet concentrates during storage (Table 4).

*Table 4. Adenosine diphosphate (ADP)-, arachidonic acid (AA)-, collagen (COL)- and thrombin receptor-activating peptide-6 (TRAP)-induced platelet aggregation in interim platelet unit concentrates (IPU PCs), buffy-coat PCs, and apheresis PCs during storage (n=10 for each). Median and interquartile range. ***p<0.001, **p<0.01, *p<0.05 in comparison to IPU PCs at the same time-point. ND, not detectable*

Days after donation	Day 1	Day 4	Day 7
ADP (units)			
IPU PCs	ND	ND	ND
Buffy-coat PCs	2 (0–4)**	ND	ND
Apheresis PCs	14 (10–39)***	1 (0–4)**	0 (0–0)
AA (units)			
IPU PCs	21 (9–36)	18 (11–32)	22 (10–38)
Buffy-coat PCs	65 (48–81)**	35 (26–42)	0 (0–0)**
Apheresis PCs	82 (74–89)***	76 (71–87)***	68 (62–79)**
COL (units)			
IPU PCs	12 (10–13)	7 (5–9)	6 (5–7)
Buffy-coat PCs	15 (12–16)	10 (6–10)	2 (2–3)**
Apheresis PCs	29 (24–45)**	11 (6–15)	3 (1–7)
TRAP (units)			
IPU PCs	74 (67–77)	65 (57–71)	65 (61–67)
Buffy-coat PCs	67 (59–72)	57 (54–61)	42 (41–49)**
Apheresis PCs	75 (70–88)	72 (69–79)*	69 (62–75)

Comparing IPU and buffy-coat platelet concentrates, a lower ADP-induced aggregation ($p=0.002$) and AA-induced aggregation ($p=0.007$) in IPU concentrates were observed in the beginning of the storage period (Table 4). However towards the end of storage, higher AA-, collagen-, and TRAP-induced aggregation were observed in IPU concentrates ($p<0.001$ for all on day 7) (Table 4).

Comparing IPU and apheresis platelet concentrates, the ability of platelets to aggregate was significantly lower in IPU concentrates during storage (Table 4). In the beginning of the storage period, lower ADP-, AA-, and collagen-induced aggregation were observed in IPU concentrates ($p<0.001$, $p<0.001$, and $p=0.001$ respectively on day 1) (Table 4). On day 4 after donation, lower ADP-, AA-, and TRAP-induced aggregation were observed in IPU concentrates ($p=0.005$, $p<0.001$, and $p=0.034$). The AA-induced aggregation was still lower in IPU concentrates at the end of the storage period ($p=0.001$ on day 7).

4.2 Supplementation with adrenaline, platelets and/or ADP (Study II)

Effect of adrenaline on platelet aggregation

Supplementation with adrenaline at resulting plasma concentrations of 153 nM and 770 nM significantly increased ADP- (Figure 20A) and AA-induced aggregation compared to samples with no added adrenaline (n=10) (p=0.009 and p=0.007 for ADP, p=0.012 and p=0.007 for AA, respectively). Aggregation did not significantly change after adrenaline supplementation at lower concentrations.

Pooling the results from all aggregation trials (ADP: n=30, AA: n=20), the ADP-induced aggregation improved by 80 (51–165) % after addition of the highest concentration of adrenaline (p<0.001) (Figure 20B). Adrenaline supplementation increased AA-induced aggregation by 60 (18–133) % with the highest concentration (p<0.001).

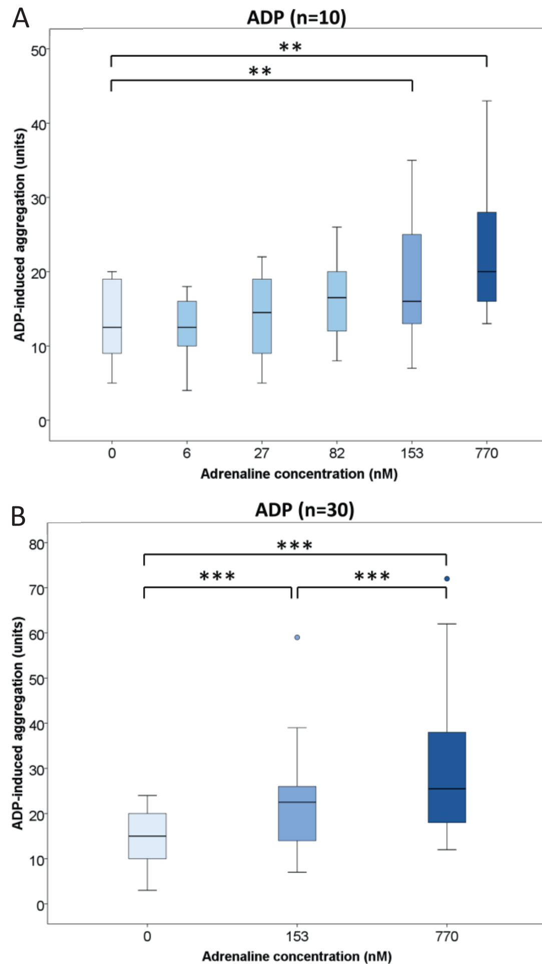


*Figure 20. Effect of adrenaline supplementation (resulting plasma concentration) on ADP-induced aggregation in blood samples from patients on dual antiplatelet therapy with acetylsalicylic acid and ticagrelor (n=10) (A) and pooled data from all aggregation experiments (n=30) (B). Median and interquartile range. Outliers are presented as dots.***p<0.001, **p<0.01.*

Effect of adrenaline alone or combined with platelets on platelet aggregation

Supplementation with apheresis platelet concentrate did not increase ADP-induced aggregation significantly (p=0.57) while it significantly increased AA-induced aggregation (p=0.007). Compared to samples supplemented with platelet concentrate, ADP-induced aggregation was higher while AA-induced aggregation was lower after adrenaline (770 nM) supplementation alone (p=0.005 and p=0.007, respectively). The combination of adrenaline and platelet concentrate increased both ADP- and AA-induced aggregation more than platelet concentrate alone (p=0.005 and p=0.007, respectively).

Effect of adrenaline alone or combined with ADP on platelet aggregation and activation

Supplementation with adrenaline alone (770 nM) increased platelet aggregation and activation compared to samples with no added agonist (Figure 21A and 21B). Supplementation with ADP alone increased aggregation and activation more than adrenaline alone. The combination of adrenaline and ADP increased aggregation more than either substance alone. The combination also resulted in the highest activation response.

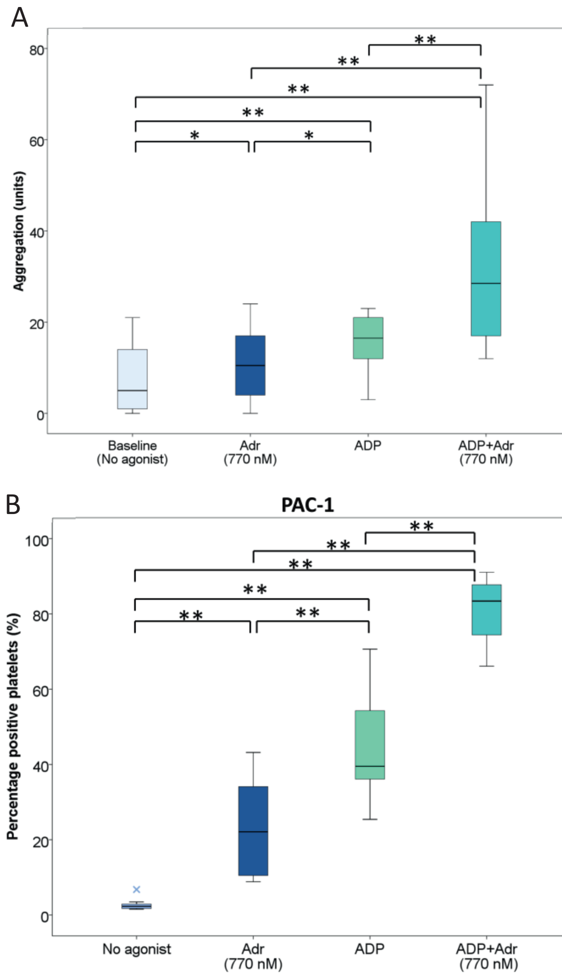


*Figure 21. Effects of adrenaline (Adr) (resulting plasma concentration 770 nM) and/or ADP on platelet aggregation (A) (n=10) and activation (PAC-1 binding to activated fibrinogen receptor) (B) (n=10) in blood samples from patients on dual antiplatelet therapy with acetylsalicylic acid and ticagrelor. Median and interquartile range. Extreme points are indicated by the symbol ×. **p<0.01, *p<0.05.*

4.3 Adrenaline infusion in ticagrelor-treated subjects (Study III)

Infusion of adrenaline after ticagrelor administration increased the systolic blood pressure at infusion rates of 0.05–0.15 µg/kg/min from 137 (136−141) mmHg to 160 (147−175) mmHg (p=0.007) at 0.10 µg/kg/min and heart rate at all infusion rates from 61 (54−68) beats/min to 86 (72−87) beats/min (p=0.015) at 0.15 µg/kg/min. In addition, adrenaline plasma concentration increased at all infusion rates from 0.25 (0.20–0.40) to 14.28 (14.13–16.82) nM (p=0.008) at 0.15 µg/kg/min. In contrast, infusion of adrenaline decreased the diastolic blood pressure and MAP at all infusion rates. Injection of metoprolol concomitantly with adrenaline infusion did not significantly change blood pressure or heart rate compared to the same infusion rate of adrenaline alone.

Effect on platelet aggregation and activation

After ticagrelor administration, the ADP- (Figure 22A), AA-, and TRAP-induced aggregation decreased (p=0.005, p=0.005, and p=0.007, respectively). Following infusion of adrenaline, the ADP-induced aggregation improved without obvious dose-response; from median 17 (14–31) U to 25 (21–34) U (p=0.012) at 0.10 µg/kg/min (Figure 22A). Seven study participants had an ADP-induced aggregation of <22 U after ticagrelor administration. In six of these, the ADP-induced aggregation increased to >22 U with infusion of adrenaline. AA- and TRAP-induced aggregation improved with adrenaline infusion rates of 0.05–0.15 µg/kg/min. Injection of metoprolol concomitantly with adrenaline infusion did not significantly influence the ADP- or TRAP-induced aggregation in the presence of ticagrelor, compared to the same infusion rate of adrenaline alone (p=0.18 and p=0.68 respectively) while AA-induced aggregation significantly increased with the combination (p=0.011).

Ticagrelor administration significantly decreased ADP-induced PAC-1 binding (p=0.012) (Figure 22B) and P-selectin expression (p=0.017) compared to baseline. Adrenaline infusion significantly increased both ADP-induced PAC-1 binding (Figure 22B) and P-selectin expression. Injection of metoprolol concomitantly with adrenaline infusion significantly reduced ADP-induced PAC-1 binding compared to the same infusion rate of adrenaline alone (p=0.028) (Figure 22B) while the combination did not significantly change P-selectin expression (p=0.24).

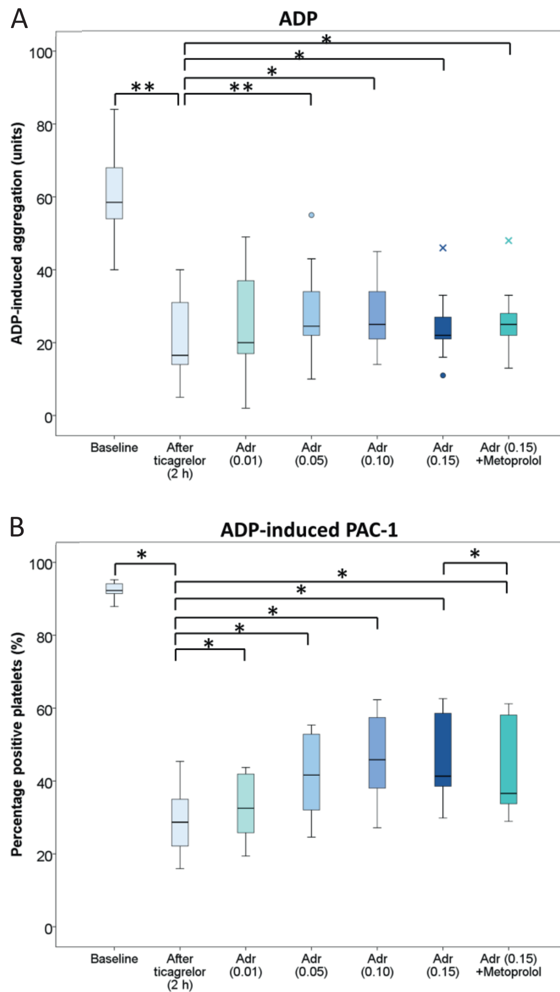


*Figure 22. The effect of ticagrelor, adrenaline (Adr) infusion (0.01, 0.05, 0.10, and 0.15 µg/kg/min) and metoprolol (5 mg) on ADP-induced platelet aggregation (A) (n=10) and activation (PAC-1 binding to activated fibrinogen receptor) (B) (n=8) in healthy volunteers. Median and interquartile range. Outliers are presented as dots and extreme points are indicated by the symbol ×. **p<0.01, *p<0.05.*

Effect on clot formation

Ticagrelor administration did not significantly influence clot formation parameters (clotting time: Friedman test p=0.26, clot formation time: p=0.33, maximum clot firmness: p=0.076). Infusion of adrenaline did not significantly influence clotting time (Friedman test p=0.26) while the clot formation time reduced from 97 (89−110) s to 83 (76−90) s (p=0.008) at 0.15 µg/kg/min and maximum clot firmness increased from 59 (57−60) mm to 62 (61−64) mm (p=0.007) at 0.15 µg/kg/min. Injection of metoprolol combined with adrenaline infusion did not significantly change clot formation time or maximum clot firmness compared to the same infusion rate of adrenaline alone (p=0.18 and p=0.58, respectively).

4.4 Noradrenaline infusion in CABG patients (Study IV)

Comparing the two randomization groups (noradrenaline and control), there were no significant differences 10 minutes before anesthesia in MAP, hemoglobin concentration, hematocrit, fibrinogen concentration, platelet aggregation, or clot formation.

Dose of noradrenaline

Patients in the noradrenaline group received a noradrenaline dose of median 0.09 (range 0–26) µg/kg/min at 50 minutes after anesthesia induction. One patient without noradrenaline infusion at 50 minutes received noradrenaline up to 40 minutes after anesthesia induction. Four patients in the control group had a noradrenaline infusion at a rate of 0.03–0.12 µg/kg/min at 50 minutes after anesthesia induction.

Effect on mean arterial pressure

MAP did not significantly change between the two time-points in the noradrenaline group (p=0.31) while it significantly decreased in the control group (p=0.002). There was a significant difference in the change in MAP between the two groups; 3 (−3–9) mmHg in the noradrenaline group compared to −35 (−40–[−27]) mmHg in the control group (p<0.001).

Effect on platelet aggregation

In the noradrenaline group, ADP-induced aggregation significantly increased from 71 (53–94) U at 10 minutes before anesthesia to 87 (70–103) U (p=0.023) at 50 minutes after anesthesia induction. In contrast, ADP-induced aggregation significantly decreased between the two time-points, from 85 (67–90) U to 72 (64–80) U (p=0.028), in the control group. The change in ADP-induced aggregation significantly differed between the groups (p=0.002) (Figure 23). The AA- and TRAP-induced aggregation did not significantly change in either group between the two time-points (p=0.27 and p=0.12, respectively for noradrenaline; p=0.12 and p=0.61, respectively for control). However, the change in AA-induced aggregation was significantly different between the groups (p=0.046) (Figure 23). There was no significant difference in the change in TRAP-induced aggregation between the groups (p=0.12) (Figure 23).

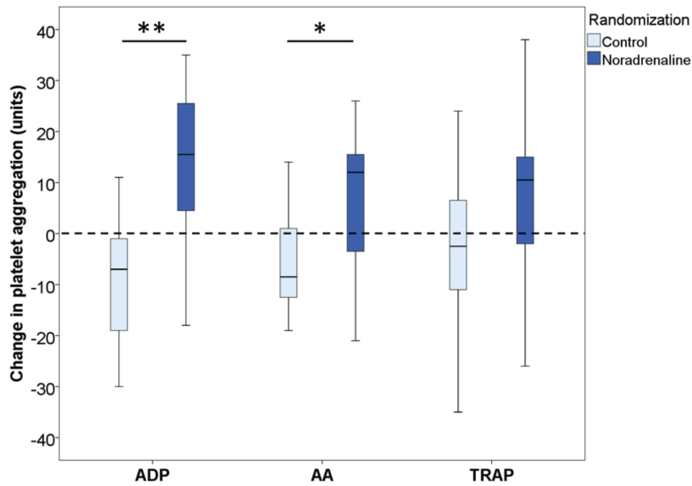


*Figure 23. Change in ADP-, arachidonic acid (AA)-, and TRAP-induced aggregation between samples collected at 10 minutes before and 50 minutes after anesthesia induction in cardiac surgery patients randomized to either maintenance of pre-induction mean arterial pressure by noradrenaline infusion (n=12) or standard treatment (control) (n=12). Median and interquartile range. **p<0.01, *p<0.05.*

The effect of an increase of 0.01 µg/kg/min in noradrenaline infusion rate (up to 0.13 µg/kg/min) on the changes in ADP- and AA-induced aggregation between the two time-points were significant; ΔLSM 2.68 (95% CI 1.06–4.30) (p=0.003) for ADP (Figure 24A) and ΔLSM 1.79 (95% CI 0.80–2.78) (p=0.001) for AA (Figure 24B). Thus the mean effect of each 0.01 increase in noradrenaline infusion rate on the change in ADP- and AA-induced aggregation was +2.68 U and +1.79 U, respectively. When the infusion rate was >0.13 µg/kg/min, there was no significant effect. Furthermore, no significant effect of noradrenaline infusion rate on the change in TRAP-induced aggregation was observed.

Effect on clot formation

A significant increase in INTEM maximum clot firmness in the noradrenaline group (p=0.009) was observed while no significant difference was observed in the control group (p=0.89). Also, a significant difference in the change in INTEM maximum clot firmness between the groups was observed (p=0.008). There was no significant change in FIBTEM maximum clot firmness in the noradrenaline group between the two time-points (p=0.12) while it significantly decreased in the control group (p=0.047). No other significant differences in clot formation parameters were observed.

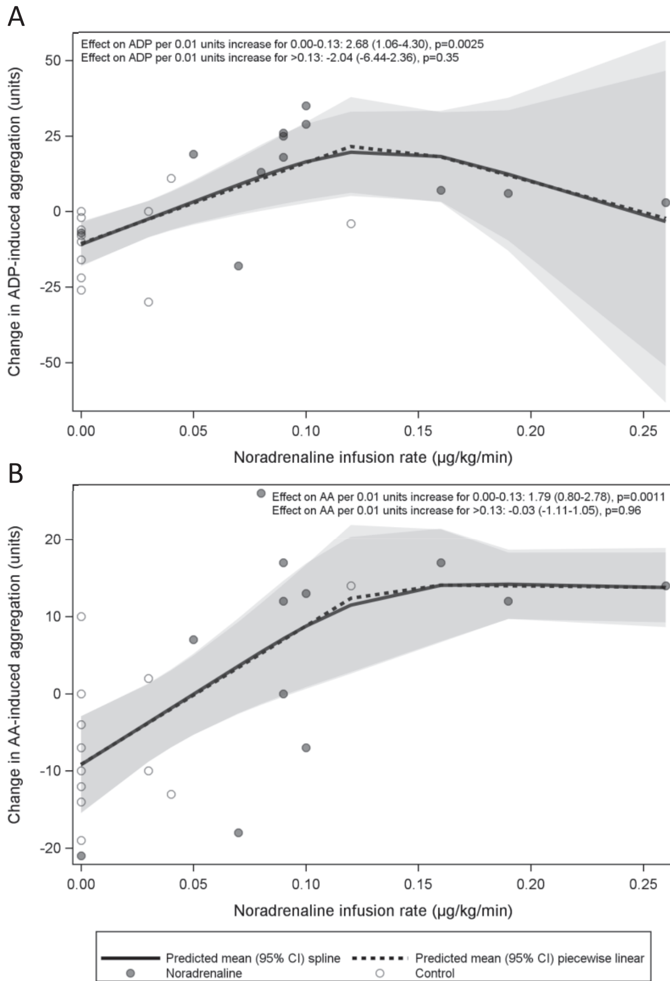


Figure 24. Effect of the infusion rate of noradrenaline on the change in ADP- (A) and arachidonic acid (AA)- (B) induced platelet aggregation between the two sampling time-points in cardiac surgery patients randomized to maintenance of pre-induction mean arterial pressure by noradrenaline infusion (black circles) (n=12) or standard treatment (control) (white circles) (n=12). Regression models with natural cubic splines (black lines) were used. The spline functions were simplified into piecewise linear functions (dotted lines) with a breaking point set at 0.13 µg/kg/min. Difference in Least Squares Means (ΔLSM) with 95% Confidence Intervals (CI) are presented per 0.01 µg/kg/min increase in the infusion rate.

5 DISCUSSION

The main findings in this thesis were; IPU concentrates maintained a better platelet function compared to buffy-coat platelet concentrates during storage while apheresis platelet concentrates had a better maintained platelet function throughout the storage period (Study I); Adrenaline improved both ADP-induced platelet aggregation and activation in the presence of ticagrelor *in vitro* (Study II) and *in vivo* (Study III) while supplementation with apheresis platelet concentrate did not enhance ADP-induced platelet aggregation *in vitro* (Study II); Adrenaline infusion also improved clot formation in the presence of ticagrelor *in vivo* (Study III) while intraoperative infusion of noradrenaline improved ADP-induced platelet aggregation and clot formation in cardiac surgery patients (Study IV).

Platelet concentrates during storage (Study I)

There were significant differences in platelet storage lesion markers between IPU, buffy-coat and apheresis platelet concentrates during storage. This may be explained by the differences in collection and preparation method, storage medium, and plasma carryover which are known to affect the storage lesion of platelet concentrates [99]. The different number of centrifugation steps required for the preparation of IPU and apheresis concentrates (single step), and buffy-coat platelet concentrates (two steps) may affect platelet activation and aggregation. Furthermore, IPU and buffy-coat platelet concentrates were stored in the platelet additive solutions SSP+ and SSP, respectively. The additional magnesium and potassium only present in SSP+ can reduce platelet activation [100, 101] while the additional phosphate acts as a buffer. Lastly, the plasma carryover (40% in IPU, 20% in buffy-coat, and 100% in apheresis platelet concentrates) may affect platelet activation. Using plasma as storage medium has previously been shown to cause a lower activation of buffy-coat platelets compared to using SSP [102]. However, it is beneficial to have a lower plasma carryover as transfusions of plasma may cause adverse transfusion reactions [103].

Platelets in apheresis platelet concentrates had a better platelet function compared to platelets in IPU concentrates during storage. To our knowledge, no previous studies have compared the new IPU concentrates to apheresis platelet concentrates. Comparisons between IPU concentrates and buffy-coat concentrates have previously been conducted. In a study by Johnson and colleagues, IPU concentrates (Reveos system prototype) were compared to buffy-coat platelet concentrates (Optipress system) [104]. The results of that

study are not in line with the results of our study. Johnson and colleagues found; no significant difference in pH and glucose while lactate concentration was significantly different, that more platelets in IPU concentrates were activated throughout the storage period, and that ADP-induced aggregation was higher while collagen-induced aggregation was lower in IPU platelet concentrates. The differences in results of metabolic parameters and platelet activation between the studies may be explained by the fact that the storage medium differed between our IPU and buffy-coat platelet concentrates (SSP+ and SSP, respectively) while SSP+ was used for both types of preparations in the other study. The discrepancy in aggregation results between the two studies may be due to differences in aggregometry methods, agonist concentrations, buffy-coat collection methods and storage medium.

Platelet storage lesion is associated with the storage temperature. The platelet concentrates at our hospital are stored at 22°C. Cold-storage (2–6°C) of platelet concentrates results in a lower metabolic rate and a better maintained platelet aggregation response compared to platelet concentrates stored at room temperature [105, 106]. However *in vivo*, transfusion of cold-stored platelets resulted in quicker clearance of the transfused platelets from the circulation compared to platelets stored at room temperature [107]. Cold-stored platelets may be of use in patients with ongoing bleeding as a rapidly improved platelet function to stop bleeding might be more important than the need for platelets with a longer circulation time.

Effects of adrenaline on platelet function (Studies II and III)

Supplementation with adrenaline to blood samples from patients on DAPT with ASA and ticagrelor, and infusion of adrenaline to ticagrelor-treated healthy volunteers increased ADP-induced platelet activation and aggregation, and AA-induced aggregation (Studies II and III). The ADP-induced aggregation reached median levels of approximately 25 U both *in vitro* (resulting adrenaline plasma concentration 770 nM) and *in vivo* (9.95 nM at 0.10 µg/kg/min). Thus in both studies, adrenaline improved ADP-induced platelet aggregation to reach above a previously suggested cut-off level of 22 U for when CABG may be performed without an increased bleeding risk [52, 53]. This suggests that adrenaline infusion could potentially be used to prevent perioperative bleeding complications in patients with ongoing or recently discontinued treatment with ASA and ticagrelor.

A potentiating effect of adrenaline on ADP-induced aggregation has previously been observed in blood samples from clopidogrel-treated patients

[66]. Also, a study on platelet-rich plasma from healthy subjects where ticagrelor was added *in vitro* showed that ADP-induced aggregation improved after adrenaline supplementation (1 μM) [67]. The *in vivo* effect of adrenaline on platelet function has also been studied. Infusion of adrenaline (0.07 μg/kg/min) was shown to improve platelet aggregation in healthy volunteers without antiplatelet therapy in two studies [76, 108] while there was no difference in ADP-induced aggregation after infusion of adrenaline (0.06 μg/kg/min) or saline only in another study [109]. The difference in methods used for analyzing platelet aggregation may be a reason for the conflicting results. To our knowledge, no studies have investigated platelet function after adrenaline supplementation to blood samples from ticagrelor-treated ACS patients or during adrenaline infusion in ticagrelor-treated subjects.

As is evident in Studies II and III, a higher adrenaline plasma concentration was required for potentiation of ADP-induced aggregation *in vitro* (153 and 770 nM) compared to measured plasma concentrations after infusion *in vivo* (14 nM for the highest rate). Also, the effect of adrenaline supplementation was concentration-dependent *in vitro* (Study II) while no evident dose-dependent effect of adrenaline was observed *in vivo* (Study III). Adrenaline infusion at rates of 0.05–0.15 μg/kg/min improved ADP-induced aggregation to nearly the same degree. A possible explanation could be that the α_{2A}-receptors become desensitized with a continuous infusion. The affinity of adrenaline for α_{2A}-receptors has previously been shown to decrease after a 2-hour infusion of adrenaline (0.05 μg/kg/min) [110].

The potentiation of ADP-induced aggregation by adrenaline may be explained by its effect on platelet signaling pathways. Adrenaline can bind to platelet α_{2A}-receptors which activates a pathway that mimics the $P2Y_{12}$-mediated pathway [31, 68, 72] which ticagrelor inhibits. An alternative explanation to the improvement in platelet aggregation observed *in vivo* (Study III) is that adrenaline infusion increases the platelet concentration. In a previous study, adrenaline infusion decreased the splenic platelet pool size and increased venous platelet concentration [111]. We did not measure platelet concentration during the infusion of adrenaline and can thus not with certainty determine the cause of the improved platelet aggregation. At the screening visit, the platelet concentration was in the normal range for all participants (ranged between 180–307 × 10^9/L). Only a platelet concentration of ≤100 × 10^9/L has previously been shown to influence impedance aggregometry results [112].

Adrenaline is used to increase blood pressure during and after CPB if patients undergoing cardiac surgery become hypotensive. As adrenaline increases heart rate and systolic blood pressure, an agent that can counteract the increase may be needed if adrenaline is used as a platelet function-enhancing agent in patients on DAPT. For this purpose, we investigated the effect of metoprolol (β_1-selective blocker) administration during adrenaline infusion. The results indicate that metoprolol does not negatively influence adrenaline's effect on platelet function (except lower ADP-induced PAC-1 binding). Thus a β_1-selective blocker may be used in combination with adrenaline to prevent an increased blood pressure without impairing adrenaline's effect on platelets.

In Study II, supplementation with apheresis platelet concentrate alone did not significantly improve ADP-induced aggregation while it improved AA-induced aggregation. This is in agreement with previous studies [49-51]. This shows that platelet transfusion may be used to target the ASA-induced platelet inhibition in patients on DAPT.

Adrenaline infusion also improved clot formation (Study III). Although the inhibiting effect of ticagrelor cannot be identified using only viscoelastic coagulations tests, a positive effect of adrenaline infusion was still observed (shortening of clot formation time and increased maximum clot firmness). This is most probably due to the effect of adrenaline on platelets, but also an increase of platelet concentration [111] and/or effects on vWF and factor VIII coagulant activity [113] may contribute. A positive effect of adrenaline infusion on coagulation parameters has previously been observed. In a randomized trial, perioperative infusion of low dose adrenaline (0.05 μg/kg/min) in combination with the anifibrinolytic agent tranexamic acid during total hip arthroplasty improved early postoperative coagulation parameters compared to a placebo group [79].

Effect of intraoperative noradrenaline infusion (Study IV)

Noradrenaline infusion resulted in improvements in ADP- and AA-induced aggregation that were significantly different from the changes observed in the control group. In addition, INTEM maximum clot firmness increased in the noradrenaline group. This strongly indicates that intraoperative noradrenaline infusion improves platelet aggregation and clot stability.

Noradrenaline is the adrenergic agent used for maintenance of blood pressure during cardiac surgery at our institution. No previous studies have assessed platelet function during noradrenaline infusion in patients undergoing

surgery. However, in a previous study on healthy volunteers, noradrenaline infusion (0.03 and 0.14 µg/kg/min) improved platelet aggregation [77]. An improvement was also observed in another study on healthy volunteers and hypertensive patients after noradrenaline infusion (0.10 µg/kg/min) [78]. In the present study, noradrenaline infusion improved ADP-induced aggregation and the effect of the infusion rate on the changes in ADP- and AA-induced aggregation was significant up to 0.13 µg/kg/min. Higher infusion rates did not significantly affect the change in platelet aggregation which corresponds to the results from Study III where there was no obvious dose-dependency for ADP-induced aggregation over an adrenaline infusion rate of 0.05 µg/kg/min. The improvement observed after noradrenaline infusion could be because noradrenaline also binds to α-receptors on platelets and binding can result in aggregation, but it is not as potent as adrenaline [63-65]. Another explanation for the improvement is that the platelet concentration may increase during noradrenaline infusion [77]. We only measured platelet concentration preoperatively and can therefore not with certainty determine if the improvement is due the effect of noradrenaline on platelet aggregation or on platelet concentration. However as previously discussed, only a platelet concentration of $\leq 100 \times 10^9$/L has previously been shown to influence impedance aggregometry results [112]. All patients had a platelet concentration within the normal range preoperatively (ranged between 163–388 $\times 10^9$/L) and it is unlikely that the platelet concentration was $\leq 100 \times 10^9$/L when the second measurement was conducted since the CPB had not yet been started.

In a prospective randomized trial of patients undergoing open radical cystectomy, noradrenaline infusion (first 0.033 µg/kg/min then maintenance of MAP 60–100 mmHg) in combination with a restrictive fluid regimen resulted in lower perioperative blood loss and red blood cell transfusion requirement compared to a control group [114]. In a study on cardiac surgery patients, noradrenaline infusion resulted in a higher hemoglobin concentration upon admission to the intensive care unit and lower frequency of perioperative blood transfusions compared to a historical control group [115]. However, the mechanism behind the effect of noradrenaline on hemostasis was not explored in these studies. Our results indicate that noradrenaline's effect on platelet aggregation may contribute.

Limitations and strengths of the studies

There are some limitations of the studies. In Study I, we did not control for the differences in storage medium and plasma carryover between the three types of platelet concentrates. Thus, we cannot distinguish between the

effects of the different factors on platelet storage lesion. The aggregation analysis was performed with the same volume of platelet concentrate regardless of the platelet concentration as the concentration is not considered when giving the product to the patient. It may have affected the aggregation results although we only observed significant correlations between platelet concentration and TRAP-induced aggregation. Furthermore, impedance aggregometry is not yet established as a method for evaluating platelet function in platelet concentrates. It has however been used in previous studies [45, 116, 117].

In Study II, we only observed significant effects on platelet aggregation at the two highest adrenaline plasma concentrations tested. These plasma concentrations cannot be achieved *in vivo* by adrenaline infusion at doses that are clinically relevant [118]. Furthermore, the *in vitro* supplementation of adrenaline to blood samples does not take into account the effect of the vascular endothelium and blood flow on primary hemostasis. In Study III, no women were included to avoid effect of the hormone cycle on platelet function [96]. This is a limitation as the findings may not apply to the female population. Furthermore, ticagrelor, which is almost solely given in combination with ASA, was in our study given as monotherapy to avoid a further increased bleeding risk for the healthy volunteer. Lastly, we did not have a control group receiving placebo to determine the effect of other variables in the study setup.

Study IV was a substudy of a randomized trial with another objective and was thus not powered for evaluation of hemostatic variables, postoperative bleeding, transfusions or outcomes. In addition, the sample size was small. Lastly, a limitation with both Studies III and IV was that platelet concentration was not measured during or after adrenaline or noradrenaline infusion.

Strengths of Study I include the study design which enabled us to compare the actual platelet products given to patients at our hospital. In addition, we were able to follow each individual platelet concentrate for 7 days. We also used multiple methods of assessing platelet storage lesion.

Strengths of Study II include that blood samples with no added agents (baseline) were used for the comparisons. In this way, the patients were their own control. In addition, both platelet activation and aggregation were evaluated yielding a more comprehensive picture of the effect of adrenaline on platelet function.

Strengths of Study III include the healthy volunteers being their own control. In addition, platelet activation and aggregation, and clot formation were studied yielding information about the effect of adrenaline on different aspects of hemostasis. We also evaluated five infusion rates of adrenaline as well as the combination of adrenaline and metoprolol under controlled conditions.

Strengths of Study IV include the surgical setting and the randomization aspect which allowed for comparisons between a treatment group and a control group of cardiac surgery patients. In addition, both platelet aggregation and clot formation were evaluated.

6 CONCLUSIONS

1. IPU, buffy-coat and apheresis platelet concentrates significantly differed in platelet storage lesion markers. The new IPU concentrates are at least comparable to buffy-coat platelet concentrates in terms of quality markers during storage.

2. Adrenaline supplementation to blood samples from patients treated with ASA and ticagrelor improved ADP- and AA-induced platelet aggregation and ADP-induced platelet activation.

3. Supplementation with apheresis platelet concentrate to blood samples from patients treated with ASA and ticagrelor improved AA-induced platelet aggregation while the combination of adrenaline and platelet concentrate improved both ADP- and AA-induced aggregation.

4. Adrenaline infusion at clinically relevant doses improved platelet aggregation, activation and clot formation in ticagrelor-treated healthy volunteers.

5. Intraoperative infusion of clinically relevant doses of noradrenaline in ASA-treated cardiac surgery patients improved platelet aggregation and clot formation.

7 FUTURE PERSPECTIVES

Treatment or prevention strategies of perioperative bleeding during cardiac surgery, especially in patients with ongoing or recently discontinued treatment with a $P2Y_{12}$ antagonist, are needed. Current strategies include transfusion of platelet concentrates. We have compared platelet storage lesion in the newly introduced IPU concentrates with buffy-coat and apheresis platelet concentrates prepared according to local routines. The preparation of platelet concentrates differs between hospitals. Studies investigating the optimal preparation and storage conditions need to be conducted. With our study design, we could not distinguish the effect of the different factors on platelet storage lesion. To investigate the effect of the collection methods, platelet concentrates prepared with the same storage medium and plasma carryover need to be evaluated. Furthermore, the *in vivo* effect of transfusion of the different platelet concentrates on hemostasis should be investigated.

As a possible new prevention strategy, adrenergic agents were tested in the next studies. We found that adrenaline improved platelet aggregation and activation in the presence of ticagrelor both *in vitro* and *in vivo* as well as clot formation *in vivo*. Furthermore, we found that noradrenaline infusion improved platelet aggregation and clot formation in ASA-treated cardiac surgery patients. To confirm our findings and to evaluate if the effects are sufficient to improve perioperative hemostasis, larger studies investigating the effect of adrenergic agents in cardiac surgery patients with ongoing or recently discontinued DAPT should be conducted. Such a study can also investigate if an optimal dose of adrenaline can be determined for the enhancement of ADP-induced platelet reactivity.

Adrenaline supplementation and infusion improved ADP-induced platelet aggregation in ticagrelor-treated samples however; the aggregation did not reach the normal levels observed before antiplatelet therapy is initiated. For the purpose of restoring ADP-induced aggregation, an antidote to ticagrelor has been developed and is currently evaluated. The antidote was shown to restore ADP-induced aggregation in ticagrelor-treated human platelet-rich plasma and mice [119]. It has also more recently been tested in pigs treated with ASA and supra-therapeutic level of ticagrelor during ongoing bleeding [120]. The antidote cleared free ticagrelor and its active metabolite in plasma within 5 minutes and gradually restored ADP-induced platelet aggregation. However, it did not significantly reduce blood loss. Studies in humans will reveal if the antidote may be used in the future for prevention and treatment of bleeding complications in ticagrelor-treated patients.

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